SUBUNITS OF DNA POLYMERASE III HOLOENZYME OF *Escherichia coli*

by

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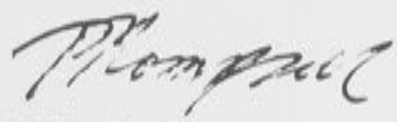
The work described herein is the author's own work, unless otherwise stated, and was carried out within the Research School of Chemistry, Australian National University, from February 1988 - February 1992. None of the material has been submitted in support of an application for any other degree.



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A copy of this thesis will almost certainly find a resting place on my Mum's coffee table. Therefore I would like to thank her for supporting me during a good part of this nine year vacation from reality. This thesis is the product of that support.

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ABBREVIATIONS

A.N.U.	Australian National University
A_λ	absorbance at wavelength λ
AAS	atomic absorption spectroscopy
ATPγS	thiotriphosphate analogue of deoxyadenosine triphosphate
bla	beta lactamase gene
bp	base pairs
BSA	bovine serum albumin
°C	degrees Celcius
Ci	Curie
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
dNMP	deoxyribonucleic acid monophosphate
dsDNA	double-stranded DNA
DTT	dithiothreitol
E. coli	*Escherichia coli*
ε_{CH}	a chymotryptic fragment of the ε subunit
ε_{DP}	a degradation product of the ε subunit
EDTA	ethylenediamine tetraacetic acid
ε_M	molar absorbtivity
ESR	electron spin resonance
FPLC	fast protein liquid chromatography
g	gravity
GuHCl	guanidine hydrochloride
kb	kilobase pairs
k_{cat}	catalytic constant or turnover number
k_D	binding constant for a molecule
kDa	kilodaltons
k_I	binding constant for an inhibitor
K_M	Michaelis constant
µl	microlitre
LBT	Luria broth supplemented with thymine (25 µg/ml)

M. Wt.	molecular weight
nts	nucleotides
ori	*E. coli* origin of replication
P_L	major leftward promoter of bacteriophage λ
*p*np-TMP	thymidine 5´- monophosphate *p*-nitrophenyl ester
P_R	major rightward promoter of bacteriophage λ
RBS	ribosome binding site
RE STOP	solution used to inhibit restriction endonuclease and ε activity
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
ssDNA	single-stranded DNA
TE	Tris-EDTA buffer
TEMED	N,N,N´,N´-tetramethylethylenediamine
Tris.HCl	tris(hydroxymethyl)aminomethane hydrochloride
U	unit
UV	ultraviolet
V_{max}	maximum velocity

THE LISTENERS

By Walter de la Mare

"Is there anybody there?" said the Traveller,
    Knocking on the moonlit door;
And his horse in the silence champed the grasses
    Of the forest's ferny floor:
And a bird flew up out of the turret,
    Above the Traveller's head:
And he smote upon the door again a second time;
    "Is there anybody there?" he said.
But no one descended to the Traveller;
    No head from the leaf-fringed sill
Leaned over and looked into his grey eyes,
    Where he stood perplexed and still.
But only a host of phantom listeners
    That dwelt in the lone house then
Stood listening in the quiet of the moonlight
    To that voice from the world of men:
Stood thronging the faint moonbeams on the dark stair,
    That goes down to the empty hall,
Hearkening in an air stirred and shaken
    By the lonely Traveller's call.
And he felt in his heart their strangeness,
    Their stillness answering his cry,
While his horse moved, cropping the dark turf,
    'Neath the starred and leafy sky;
For he suddenly smote on the door, even
    Louder, and lifted his head:
"Tell them I came, and no one answered,
    That I kept my word," he said.
Never the least stir made the listeners,
    Though every word he spake
Fell echoing through the shadowiness of the still house
    From the one man left awake:
Ay, they heard his foot upon the stirrup,
    And the sound of iron on stone,
And how the silence surged softly backward,

When the plunging hoofs were gone.

ABSTRACT

A series of plasmid vectors was developed that allow the high-level expression of proteins in *E. coli*. These vectors all bear the strong bacteriophage λ promoters P_RP_L, arranged in tandem so that both promote transcription into genes inserted into or between unique restriction sites. Transcription is controlled by the product of the λ *c*I857 gene which is present in each vector. Transcription from the bacteriophage λ promoters is repressed at 30 °C and active at 42 °C. These expression vectors (pPT144 and pPT150) also contain a ribosome-binding site (RBS) perfectly complementary to the 3′ end of *E. coli* 16-S rRNA, which enables substitution for a natural RBS in cases where it is translationally limiting.

One of the bacteriophage λ promoter vectors (pPT150) was used to obtain the high-level expression of the *dnaX* gene, which encodes the γ and τ subunits of DNA polymerase III holoenzyme. The γ and τ subunits comprise approximately 15% of total cell protein in strains harbouring the *dnaX* plasmid (pPT153) after 2.5 h at 42 °C. The γ subunit was purified from the overproducing strain in a three-step procedure including selective precipitation with ammonium sulfate, and ion-exchange chromatography. Polyclonal antibodies were raised to purified γ subunit; this antibody recognises both the γ and τ subunits of DNA polymerase III holoenzyme.

The proofreading subunit (ε) of holoenzyme was purified to homogeneity from an overproducing strain. The ε subunit is purified on the basis of its insolubility. Approximately 95% of overexpressed ε remains in the cell pellet following lysis. ε is solubilised with guanidine hydrochloride. Following gel filtration to remove the denaturant, the ε subunit is further purified by ion-exchange chromatography on DEAE-Sephacel and Blue Gel A. ε subunit purified in this manner is as active in assays of proofreading activity as that reported by another group.

The ε subunit has a broad substrate specificity. In addition to its ability to remove incorrectly paired nucleotides at a 3′-OH end of dsDNA, ε will readily hydrolyse single-stranded DNA. Indeed, under conditions of assays used, the activity of the ε subunit on ssDNA is > 100-fold greater than on a mispaired terminus. RNA is also degraded, but less efficiently. The activity of the ε subunit on a chromophoric analogue of TMP (*p*np-TMP) is ≈ 500-times that on a mispaired terminus.

As with other exonucleases, the ε subunit has an absolute requirement for divalent metal ion for activity. Mn^{2+} or Mg^{2+} gave rise to an enzyme with the highest activity. However, proofreading activity in the ε subunit was detected with a wide variety of divalent metal ions - consistent with their role as Lewis acids. Unlike the

holoenzyme and subassemblies, ε is stimulated by concentrations of NaCl up to 500 mM. The activity of the ε subunit was inhibited to some degree by all 4 deoxynucleoside monophosphates, the products of the reaction it catalyses. TMP was a competitive inhibitor of activity.

ε can also hydrolyse a chromophoric analogue of thymidine 5′-monophophate, thymidine 5′- monophosphate *p*-nitrophenyl ester, to *p*-nitrophenol and thymidine 5′-monophosphate. The formation of *p*-nitrophenol was measured continuously in a spectrophotometer. Hydrolysis of substrate obeyed Michaelis-Menten kinetics. Investigations of functional ternary complexes of enzyme, metal, and substrate by kinetic measurements and ESR spectroscopy suggest that the binding of enzyme, metal and substrate is random, with binding of metal ion first. The ESR data suggests that a further metal ion is involved in the hydrolysis of substrate. It binds more tightly ($K_d \approx$ 30 μM), and is not detected in the kinetic studies.

Lastly, peptide mapping experiments have suggested a two domain model of ε structure: an N-terminal exonuclease active site domain, and a C-terminal domain that may associate with the polymerase (α) subunit of holoenzyme.

CHAPTER 1

GENERAL INTRODUCTION

1.0 INTRODUCTION

1.1 AN OVERVIEW of DNA REPLICATION in *ESCHERICHIA COLI*

The study of cellular processes has historically been carried out in single cell organisms, typically *Escherichia coli*. Much of the knowledge outlined in this chapter was pioneered by Arthur Kornberg and colleagues who were the first people to isolate and characterise DNA polymerases in living organisms. The particularly incisive studies have been those using *in vitro* replication reactions reconstituted with purified and well-characterised DNA templates, proteins and enzymes. Replication of the *E. coli* chromosome can be divided into a number of distinct phases which are described below.

1.1.1 *Initiation of Replication at OriC*

Initiation of replication of the *E. coli* chromosome occurs at a unique site in the chromosome called *oriC*. This 245-bp region (Oka *et al.*, 1980) has an extremely important role in the replication of a bacterial chromosome. It can only be deleted from wild-type strains when there is a secondary origin, provided by an integrated plasmid or bacteriophage.

Initiation begins by the cooperative binding of dnaA protein to its several binding sites in the origin, primarily to four 9-bp 'dnaA boxes' (Bramhill and Kornberg, 1988). This binding results in localised melting of the DNA which can be detected by sensitivity to endonuclease P1 (Bramhill and Kornberg, 1988). The association of the dnaB protein (a helicase) with the *oriC*-dnaA protein complex represents the first step in the assembly of the replication enzymes involved in DNA synthesis. DnaB is delivered to the dnaA-*oriC* complex by the dnaC protein (Wahle *et al.*, 1989b). DnaC is essential for assembly of the replication machinery, but is not stably maintained in the complex (Wahle *et al.*, 1989b). The interaction of the dnaB-dnaC complex with the origin complex leads to the ATP-dependent release of the dnaC protein (Wahle *et al.*, 1989b), which enables expression of the normal function of dnaB as a helicase.

Melting of duplex DNA by DNA helicases is necessary for the progress of a replication fork. Melting of the DNA duplex proceeds bidirectionally from *oriC* as

dnaB helicase molecules migrate from the *oriC*-dnaA complex to provide a single-stranded template for the priming and replication enzymes (Baker *et al.*, 1987). The topoisomerase, DNA gyrase provides the swivel that allows the melted parental strands to become unlinked (van der Ende *et al.*, 1985). Single-stranded DNA binding protein (SSB) binds to newly unwound single strands, preventing them from reannealing (van der Ende *et al.*, 1985).

1.1.2 *Structure of the Replication Fork*

Chromosomal replication proceeds bidirectionally from *oriC*, involving two growing points or replication forks (Fig. 1.1). Replication is semidiscontinuous: it is continuous on one (the leading) strand, and discontinuous on the other (lagging) strand. The short fragments of the lagging strand are in the range of 1000 to 2000 nucleotides long and are called Okazaki fragments after their discoverer (Okazaki and Okazaki, 1969). These nascent fragments are later joined to produce a contiguous lagging strand.

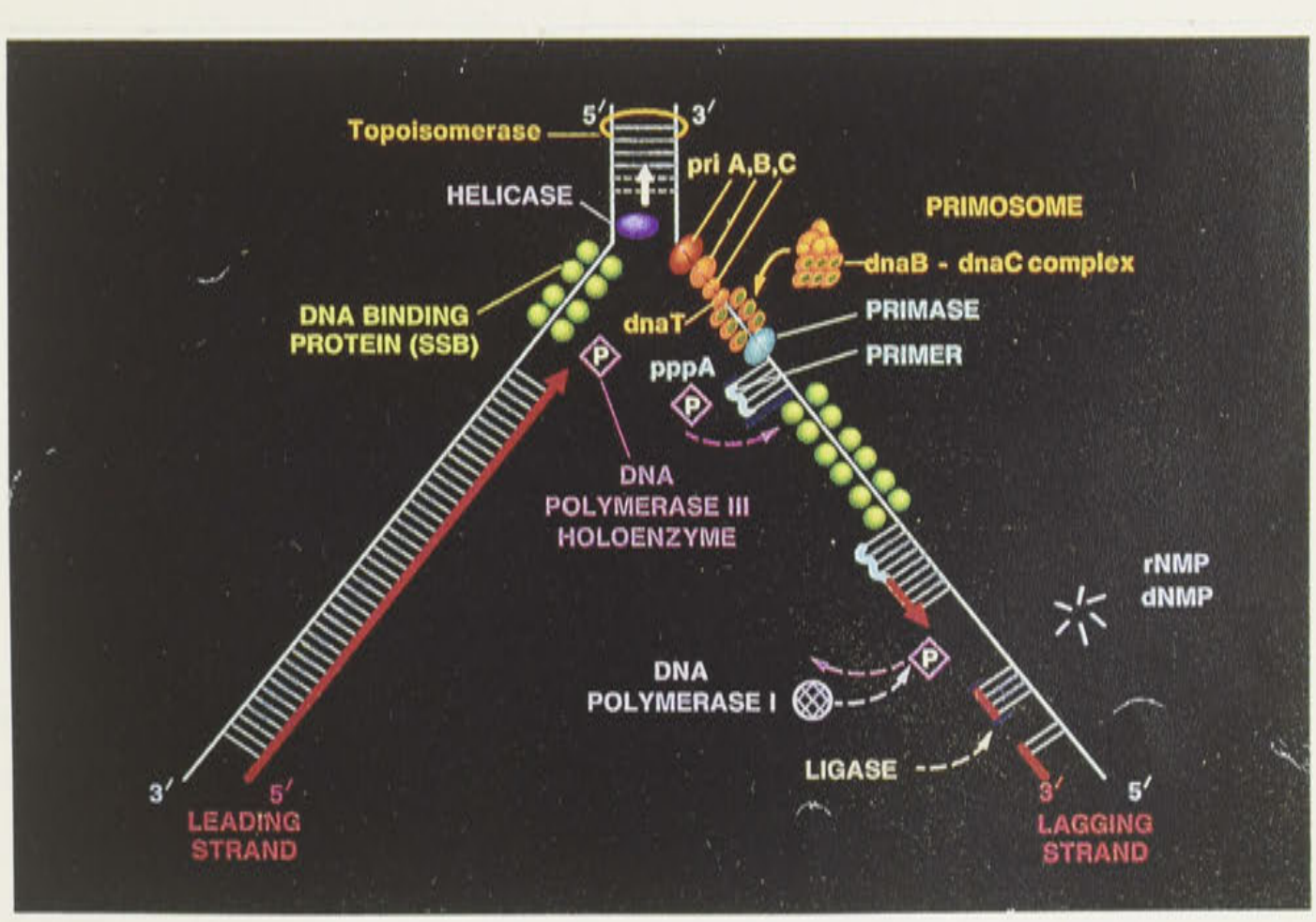

Figure 1.1: A model for the synthesis of DNA in *E. coli* at a replication fork (Reproduced from Kornberg and Baker, 1991).

1.1.3 *The Primosome*

The major replicative polymerase of *E. coli* is the DNA polymerase III holoenzyme (Section 1.2). Unlike RNA polymerases, DNA polymerases cannot start polynucleotide chains, but can only extend RNA or DNA chains that have a 3′-OH terminus paired to a template strand: a primer/template. Accordingly, the specialist priming enzyme, primase, through its interaction with dnaB lays down a short RNA transcript from which the pol III holoenzyme can begin replication of the template DNA. The dnaB-primase interaction generates the mobile priming apparatus needed for the multiple primings of discontinuous-strand synthesis.

E. coli primase often acts in concert with other replication proteins which together form what is known as a primosome. Assembly of the primosome used in replication of ϕX174 ssDNA starts by recognition of a primosome assembly site (*pas*) on ssDNA by the priA protein (Arai and Kornberg, 1981a; Zipursky and Marians, 1981). The priB and priC proteins are also involved in the process (Shlomai *et al.*, 1981). DnaB and dnaC join the complex as a preformed B-C complex, with the help of dnaT (protein i) (Shlomai *et al.*, 1981). DnaC is then released back into solution. The primosome is completed by the arrival of primase.

1.1.3 *Extension of Primer Templates*

DNA polymerase III holoenzyme extends primed template strands of both the leading and lagging strands (McHenry, 1988). DNA polymerase I (Section 1.2), replaces RNA primers with DNA (nick translation) to enable the short lagging-strand fragments to be joined by DNA ligase. The replication fork continues to advance aided by unwinding by dnaB, perhaps assisted by other helicases, and movement of the primosome (LeBowitz and McMacken, 1986) until the entire chromosome is duplicated.

1.1.4 *Termination*

The cycle of DNA replication ends when the replication forks converge and fuse on the opposite side of the chromosome in a region called the terminus (Kuempel *et*

al., 1989, 1990). Termination of replication of the *E. coli* chromosome occurs within a large region (≈ 350 kb) flanked on either side by polar terminator sites (*ter* sites) at its left and right hand limits (Kuempel *et al.*, 1989). Each flanking region contains two terminator sites of approximately 20 bp that are in the same orientation and separated by > 50 kb of DNA (Kobayashi *et al.*, 1989; François *et al.*, 1989). These two pairs of terminators have been purported to function as a replication fork trap (Hill *et al.*, 1987; Hill and Marians, 1990). Function of the *ter* sites is dependent on the presence of the DNA-binding protein Ter (the *tus* gene product; Hill *et al.*, 1989), which specifically binds the terminator sites.

1.2 THE DNA POLYMERASES of *ESCHERICHIA COLI*

Escherichia coli has three enzymes capable of synthesizing DNA, which were designated DNA polymerase I, DNA polymerase II and DNA polymerase III in the order they were discovered. Although the three polymerases share several properties (Table 1.2), each has a separate role to perform in the cell.

1.2.1 *DNA Polymerase I*

DNA polymerase I (pol I) was first isolated by the Kornberg laboratory in 1956 (Kornberg *et al.*, 1956) and is the best characterised of the three *E. coli* DNA polymerases. The product of the *polA* gene, the enzyme is a single polypeptide chain of 928 residues with a molecular weight of approximately 103,000 (Joyce *et al.*, 1982). Pol I has numerous functions. The principal, discrete activities of the enzyme are polymerisation (5′ → 3′) (Kornberg *et al.*, 1956) and two exonucleolytic activities, one which degrades in the 3′ → 5′ (Lehman and Richardson, 1964; Brutlag and Kornberg, 1972), the other in the 5′ → 3′ direction (Klett *et al.*, 1968). The former activity is used for exonucleolytic proofreading: the removal of incorrectly paired nucleotides at the 3′-OH terminus of a growing strand. The other (5′ → 3′) exonuclease activity degrades duplex DNA from a 5′ end releasing mono- or oligonucleotides. It is probably employed in the removal of thymine dimers, and of RNA primers on the discontinuous lagging strand (Kornberg and Baker, 1991). Pol I is the only replication enzyme known to date to possess 5′ → 3′ exonuclease activity. Simultaneous polymerisation and 3′→ 5′ exonuclease activity allows pol I to promote

	pol I	pol II	pol III[1]
Functions			
Polymerisation: 5′ → 3′	+	+	+
Exonuclease: 3′ → 5′	+	+	+
Exonuclease: 5′ → 3′	+	-	-
Pyrophosphorolysis and PP_i exchange	+		+
Template primer			
Intact duplex	-	-	-
Primed single-strands	+	-	-
stimulation by SSB[2]	-	+	-
Nicked duplex	+	-	-
Duplex with gaps or protruding single-stranded 5′ends of:			
< 100 nucleotides	+	+	+
> 100 nucleotides	+	-	-
Activity			
K_M for dNTP's	low	low	high
Stimulation by β subunit	-	+	+
Inhibition by 2′-deoxy analogues	-	+	+
Inhibition by sulfhydryl blocking agents	-	+	+
General			
Size (kDa)	103	90	167.5
Molecules/cell, estimated	400		10-20
Structural gene	*polA*	*polB*	*polC*[4]
Conditional lethal mutant	yes	no	yes

+, possesses the property; -, does not.

[1]The core complex, consisting of the α, ε and θ subunits.

[2]SSB = single-stranded DNA binding protein.

[4]Also known as the *dnaE*, the gene for the α subunit.

Table 1.1: Properties of polymerases I, II and the core of polymerase III of *E. coli* (Adapted from Kornberg and Baker, 1991).

replication at a nick, unaided by other proteins (Kornberg and Baker, 1991). The physiological role of DNA polymerase I in *E. coli* is in repair of damaged DNA, and in gap-filling and removal of RNA primers during chromosomal replication (Kunkel, 1988).

Pol I is a mildly processive enzyme: it is able to incorporate approximately 20 residues into a gapped and nicked calf thymus DNA template before dissociating (Fay *et al.*, 1982). These findings are consistent with Pol I having a repair function in the cell (Kunkel, 1988).

Pol I is readily cleaved by subtilisin to a small N-terminal fragment of 35 kDa which contains the 5′ → 3′ exonuclease activity (Setlow *et al.*, 1972), and a large C-terminal fragment of 68 kDa (the Klenow fragment) which contains the polymerase and 3′ → 5′ (proofreading) exonuclease activities (Brutlag *et al.*, 1969). A modified *polA* gene encoding just the Klenow fragment was cloned and used to generate a strain (Joyce and Grindley, 1983) from which the enzyme could be purified in quantities required for studies of structure and function.

The structure of the Klenow fragment has been elucidated by X-ray crystallography (Ollis *et al.*, 1985; Joyce and Steitz, 1987; Freemont *et al.*, 1988). The protein has two distinct domains: a polymerase and a 3′→ 5′ exonuclease. These studies indicated that a binding cleft for dsDNA is located within the polymerase domain, while the proofreading exonuclease domain contains a ssDNA binding region (Joyce and Steitz, 1987). The polymerase and 3′ → 5′ exonuclease active sites are believed to be ≈ 25 - 30 Å apart (Joyce and Steitz, 1987).

This finding has an important implication for the mechanism of proofreading, which necessarily involves movement of the primer terminus from the polymerase to the 3′→ 5′ exonuclease active sites. In order for the 3′-OH primer terminus to traverse the distance between the two active sites, the DNA would have to slide about 8 bp through the enzyme. At the same time the terminal 3 to 4 base pairs of the primer terminus strand would have to separate (Joyce and Steitz, 1987). From experiments done with a synthetic cross-linked primer/template, Cowart *et al.* (1989) have also suggested that at least 5 bp must melt out before excision can take place by the Klenow enzyme.

The large fragment of pol I provides an excellent model system for the study of the molecular basis of template-directed DNA synthesis. As the polymerase and proofreading exonuclease sites reside on the same polypeptide, it is clear that the two sites must cooperate in some fashion during the synthesis of a DNA strand. This raises the question of how a DNA substrate is shuttled from one site to the other.

Joyce (1989) proposed DNA can move between sites by either an intra- or an intermolecular route, depending on the type of substrate. The intramolecular route may be used with single-stranded DNA templates, but excision of nucleotides from a mispaired terminus occurs predominantly by the second pathway. There is nothing in the structure of the protein or its mechanism that dictates which route may be used. Rather, the use of the intra- or the intermolecular pathway is determined by the competition between the polymerase or exonuclease reaction and DNA dissociation (Joyce, 1989). However, critics of this model claim it is unlikely to provide much specificity (Echols and Goodman, 1991). This is because 4 bp must be unwound in the melting step to accomodate the strucural constraints of the two sites. Echols and Goodman (1991) claim the major determinants of editing specificity for pol I are kinetic constraints delaying chain elongation from a mispaired base. Kinetic analysis of the pol I reaction has indicated specificity resides in two steps that allow the exonuclease more time to work: delayed pyrophosphate release after a misinsertion; and delayed base addition to a mismatched 3′ terminus (Kuchta *et al.*, 1988).

Elucidation of the three-dimensional structure of the Klenow enzyme has greatly facilitated studies to determine the contribution made by various amino acids in the 3′ → 5′ exonuclease active site. A cluster of acidic amino acid residues (Asp^{355}, Asp^{424} and Asp^{501}) seems to be involved in anchoring two divalent metal ions that are essential for exonuclease activity (Derbyshire *et al.*, 1991). Another carboxylate (Glu^{357}) appears to have a separate, but as yet unknown, role in catalysis, while a second group of residues (Leu^{361}, Phe^{437} and Tyr^{497}) located around the terminal base and ribose positions (of the substrate) plays a secondary role, ensuring correct positioning of the substrate in the active site (Derbyshire *et al.*, 1991).

The pH dependence of exonuclease activity is consistent with a mechanism in which nucleophilic attack on the terminal phosphodiester bond is initiated by a hydroxide ion coordinated to one of the enzyme-bound metal ions (Derbyshire *et al.*, 1991).

Metal binding by both the enzyme and the substrates is essential in the pol I polymerase and exonuclease reactions (Englund *et al.*, 1969; Springate *et al.*, 1973): there is binding of a divalent cation (Mg^{2+}, Mn^{2+}, or Zn^{2+}) at two separate sites in the 3′ → 5′ exonuclease domain (Beese and Steitz, 1991), and binding of one or more divalent cations (e.g. Mg^{2+}) in the polymerase domain. A recent report used kinetic and direct binding studies to show that three divalent cations bind cooperatively to activate the 3′→ 5′ exonuclease site in pol I (Han *et al.*, 1991). The authors suggest that the role of the third metal ion may be indirect, adjusting the protein structure and thereby facilitating the correct orientation of the substrate at the active site. Only

metal-bound nucleotides bind to the polymerase site (Slater *et al.*, 1972; Sloan *et al.*, 1975).

The wealth of knowledge accumulated on the structure and function of DNA polymerase I allows it to serve as a model for other enzymes with similar activities, in particular, the proofreading activity of pol III.

1.2.3 *DNA Polymerase II*

DNA polymerase II was first identified in *polA* mutant strains (Kornberg and Gefter, 1970; Moses and Richardson, 1970a). The *polB* gene encoding DNA polymerase II maps at a position (2 minutes; Campbell *et al.*, 1974) distinct from *polA*, *dnaE*, or any of the known structural genes for DNA polymerase III holoenzyme auxiliary subunits. *polB* mutants do not exhibit a clear biological phenotype (Hughes *et al.*, 1991), indicating that DNA polymerase II is not essential for cell viability. Hence, the physiological role of DNA polymerase II remains unknown. In *polA* - *polC* double mutants, a modest level of *polB*-dependent repair synthesis is observed, suggestive of a possible role in DNA repair (Tait *et al.*, 1974). Hughes *et al.* (1991) showed pol II activity to be markedly stimulated by pol III auxiliary subunits. This finding suggests that DNA polymerase II may replace DNA polymerase III under specific physiological conditions, perhaps during part of the SOS-induced error-prone replication reaction.

The structural gene for DNA polymerase II has been cloned and its complete nucleotide sequence determined (Hughes *et al.*, 1991). The *polB* gene encodes an 88 kDa polypeptide with some homology to the eukaryotic replicative DNA polymerase α (Chen *et al.*, 1990).

A *polB* mutation has been identified as an allele of the damage-inducible gene *dinA* (Bonner *et al.*, 1990) and synthesis of pol II is induced in the SOS response. The *polB* reading frame is preceded by a consensus *lex*A binding site, which is consistent with the suggestion that the enzyme is under SOS control (Chen *et al.*, 1990). Further, DNA polymerase II activity can be induced 7-fold by nalidixic acid, an inducer of the SOS response (Bonner *et al.*, 1988).

Although pol II was the second of the polymerases to be discovered, studies of its structure and function have lagged behind that of DNA polymerases I and III. The recent availability of DNA polymerase II in large quantities will accelerate these

investigations.

1.2.4 *DNA Polymerase III Holoenzyme*

DNA polymerase III holoenzyme ("holoenzyme") is a complex multisubunit enzyme responsible for most of the synthesis of the *E. coli* chromosome (McHenry, 1988). It was originally isolated by Thomas Kornberg and Gefter (1970) on the basis of its requirement for the replication of single-stranded phage DNA (ssDNA) templates in the presence of the *E. coli* priming appartatus (Wickner *et al.*, 1973; Wickner and Kornberg, 1974).

1.2.4.1 Subunit Structure and Properties

Holoenzyme, purified 7500-fold from log-phase *E. coli* contains at least 10 components (Table 1.2) detectable at approximately stoichiometric levels on SDS-polyacrylamide gels (McHenry, 1988). Of these 10 components, 5 have been shown to be *bona fide* subunits[1]; the functions of the other five remain in question (O'Donnell, 1987; Maki and Kornberg, 1988b; Maki and Kornberg, 1988c). Only the α, ε, γ, δ and β subunits are required for reconstitution of a fully active processive enzyme (Studwell and O'Donnell, 1990). Current theory of holoenzyme function is based on research compiled over the last 10 years (eg., Maki and Kornberg, 1988c, 1988d; Studwell and O'Donnell, 1990).

DNA polymerase III has several functional sub-assemblies (Table 1.3). The catalytic core (Pol III) contains the subunits (α, ε and θ) responsible for polymerase and exonuclease (proofreading) activity. This subassembly can be purified intact, and the subunits are not easily dissociated. The remaining components are termed the auxiliary subunits. On the addition of the τ subunit, Pol III dimerises to form Pol III´. Pol III* contains all but the β subunit . These functional assemblies provide us with useful models from which to develop a better understanding of roles of subunits in holoenzyme structure and function.

The core DNA polymerase was initially detected using a gap-filling assay on

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[1]A bona fide holoenzyme subunit is defined here as a protein that complements a deficiency in a mutant defective in some DNA replication activity.

nuclease-activated DNA (Gefter *et al.*, 1971). Even though it is as active as the holoenzyme in these assays, it cannot replicate single-stranded phage DNA without the addition of certain of the auxiliary subunits. This enzyme contains the products of two essential replication genes (*dnaE* and *dnaQ*) and, thus, is required for replication. The

Gene	Product	Size (kDa)	Proposed function
dnaE	α	132	Polymerase
dnaQ	ε	27	Proofreading
holE	θ	10	Unknown[2]
dnaX	γ	52	Processivity
dnaX	τ	71	Dimerisation of core
dnaN	β	37	Clamps holoenzyme to primed terminus
holA	δ	35	Processivity
holB	δ′	33	Processivity
holC	χ	15	Unknown
holD	ψ	12	Unknown

Table 1.2: The components of DNA polymerase III holoenzyme of *E. coli* (Adapted from Kornberg and Baker, 1991).

θ subunit is not required for the assembly from separate subunits of a processive holoenzyme (Studwell and O'Donnell, 1990). Under most conditions, DNA polymerase III appears to exist as a monomer of approximately 165 kDa. However, it can be isolated as a dimer using a new purification protocol (Maki and Kornberg, 1988d).

DNA polymerase III′ contains the core DNA polymerase plus the τ auxiliary subunit of holoenzyme. This assembly can replicate long single-stranded stretches of

[2]The observation that the χ, ψ and θ subunits do not appear to be crucial for rapid and processive replication suggests they have other functions; for example, signalling between holoenzyme halves, or interactions with the primosomal proteins (O'Donnell and Studwell, 1990).

Polymerase form	Subunit composition	Aggregation state
Holoenzyme	$[\alpha,\varepsilon,\theta,\tau,\gamma,\delta,\delta',\chi,\psi,\beta_2]_2$	Dimer
Pol III*	$[\alpha,\varepsilon,\theta,\tau,\gamma,\delta,\delta',\chi,\psi]_2$	Dimer
Pol III´	$[\alpha,\varepsilon,\theta,\tau]_2$	Dimer
Pol III (core)	$\alpha,\varepsilon,\theta$	Monomer

Table 1.3: The functional assemblies of DNA polymerase III (McHenry, 1988). The dimers may be asymmetric (see the text).

DNA in the presence of physiological concentrations of spermidine (McHenry, 1988). Sedimentation velocity and gel filtration studies have shown pol III´ to be dimeric (McHenry, 1982).

DNA polymerase III* is usually only seen following chromatography of holoenzyme on phosphocellulose which removes the β subunit. This subassembly is processive in replication assays, and it appears to be an asymmetric dimer (Section 1.2.4.3; O'Donnell and Studwell, 1990).

The γ complex (γ δ δ´ χ and ψ) was originally recognised as an activity able to be purified from crude extracts, that was necessary to attain the high processivity of the holoenzyme (Maki and Kornberg, 1988b). The activity is 4-fold higher in cells with plasmids directing the high level synthesis of the τ and γ subunits (Maki and Kornberg, 1988b). From analysis of SDS-PAGE profiles, the complex has a subunit stoichiometry of 2 γ to one each of the others (Maki and Kornberg, 1988b). The γ subunit alone is unable to restore processive synthesis to the core polymerase and β (Maki and Kornberg, 1988b).

Pol III holoenzyme contains all 10 of the subunits listed in Table 1.2. It has been reported to possess a catalytic efficiency of > 500 nucleotides/sec/molecule, is highly processive (> 10^5 nucleotides), duplicates a length of ssDNA to the last nucleotide, and then diffuses over duplex DNA in search of a new primer terminus (O'Donnell, 1987). It is thought to function as an asymmetric dimer (Section 1.3.4).

1.2.4.2 Stages in the DNA Polymerase III Holoenzyme-Catalysed Reaction.

Unlike simpler DNA polymerases, the DNA polymerase III holoenzyme-catalysed reaction has several distinct reaction stages. The ability to isolate intermediates has increased our understanding of this complex enzyme.

Upon addition of the DNA polymerase III holoenzyme to primed ssDNA in the presence of ATP, a tight stable initiation complex forms, accompanied by hydrolysis of ATP to ADP and orthophosphate (Burgers and Kornberg, 1982; Johanson and McHenry, 1984). Location of a primer terminus by the holoenzyme occurs extremely rapidly ($k_{on} > 10^8\ M^{-1}\ s^{-1}$; O'Donnell, 1987). The formation of the initiation complex is inhibited by antibody to the β subunit (Johanson and McHenry, 1984). The initiation complex is resistant to this antibody after formation (ie., processive synthesis occurs). The initiation complex can be isolated by gel filtration. It contains all of the components required for initiation complex formation since the complex can recycle (Burgers and Kornberg, 1983). This indicates β is contained within the complex, but that it either becomes buried within the complex, or undergoes a conformational change so that it is no longer accessible to antibody.

Formation of the initiation complex was investigated by reconstitution of holoenzyme from purified subunits (core, β, τ, γ and δ) onto a primer/template scaffold (O'Donnell, 1987). The experiments showed that assembly of an initiation complex can be divided into two distinct phases. In the first and lengthy phase, β, γ and δ (or γ and δ') form an ATP-activated pre-initiation complex which is tightly bound to the RNA primer/template (O'Donnell, 1987). In the second rapid phase, pol III core and τ locate and tightly bind to the preinitiation complex. The fully assembled holoenzyme is stably bound to the primer terminus and in the presence of all four dNTP's and Mg^{2+}, rapidly replicates the entire template strand.

The time required for cycling between primed templates has been observed to be approximately 10 s (O'Donnell, 1987), but this is much longer than expected for cycling between nascent primer-templates *in vivo* (Section 1.2.4.3; Chandler *et al.*, 1975). This cycling is increased if transfer occurs between primer termini along the same DNA strand, but the directional preference observed is in the direction of fragment synthesis, opposite to fork movement. The formation of a preinitiation complex (β, τ, δ) can be the rate-limiting step in holoenzyme recycling (O'Donnell, 1987). The cell may solve the recycling problem by preforming complexes in advance of polymerase transfer. Alternatively, the holoenzyme might function as a dimer.

1.2.4.3 The Asymmetric Dimer Hypothesis

The speed of advancement of a replication fork *in vivo* is approximately 1 kb/s at 37 °C (Chandler *et al.*, 1975). Hence, the lagging strand must be primed and extended every 1 or 2 seconds. Since there are only 10 - 20 copies of pol III holoenzyme in the cell, and as many as 8 replication forks in rapidly growing cells, the polymerase on the lagging strand must repeatedly cycle from a completed Okazaki fragment to a new RNA primer back at the replication fork (O'Donnell, 1987). The observed cycling time (10 s, O'Donnell, 1987) is too long to account for the anticipated high frequency of nascent chain initiations at the replicating fork of the *E. coli* chromosome (Chandler *et al.*, 1975; O'Donnell, 1987).

Since the holoenzyme contains all of the subunits (core, γ, δ, τ, δ′ and β) found to be essential for processive replication (O'Donnell, 1987; Studwell and O'Donnell, 1990), it may also have the ability to form two preinitiation complex clamps simultaneously at two primer termini (O'Donnell and Studwell, 1990). Two preinitiation complexes within one holoenzyme may mediate its rapid cycling to multiple primers on the lagging strand. This also provides support for the hypothesis of holoenzyme as a dimeric polymerase (see below) (O'Donnell and Studwell, 1990). However, a recent study by the same authors casts some doubt on this proposal (see below).

The very high rate of chromosomal replication could be explained if holoenzyme functioned as a dimer enabling concurrent replication of leading and lagging strands at a replication fork. Early evidence for such a model arose from gel filtration and ultracentrifugation studies which provided molecular weights of functional assemblies consistent with dimeric forms (McHenry, 1982). Additional support for this model came by observation of functional differences between two holoenzyme populations in solution. The ATP analogue, ATPγS can substitute for ATP in the formation of the initiation complex (Johanson and McHenry, 1984). However, the maximum amount of initiation complex formed with this analogue was only half that obtained with ATP. Further, if maximal complex is formed in the presence of ATP and subsequently purified, one-half is dissociated on the addition of ATPγS. Johanson and McHenry (1984) proposed that this functional heterogeniety arose from a difference between two halves of a dimeric holoenzyme.

The model also predicts that the two halves of the holoenzyme are in communication so as to coordinate leading and lagging strand synthesis. This notion was tested in binding experiments using analogues of ATP which demonstrated strong

positive cooperativity for both ATP and the analogue (McHenry, 1985). The finding indicated that the two halves of holoenzyme are in communication. Further data was obtained from inhibition studies which showed that low concentrations of the inhibitor

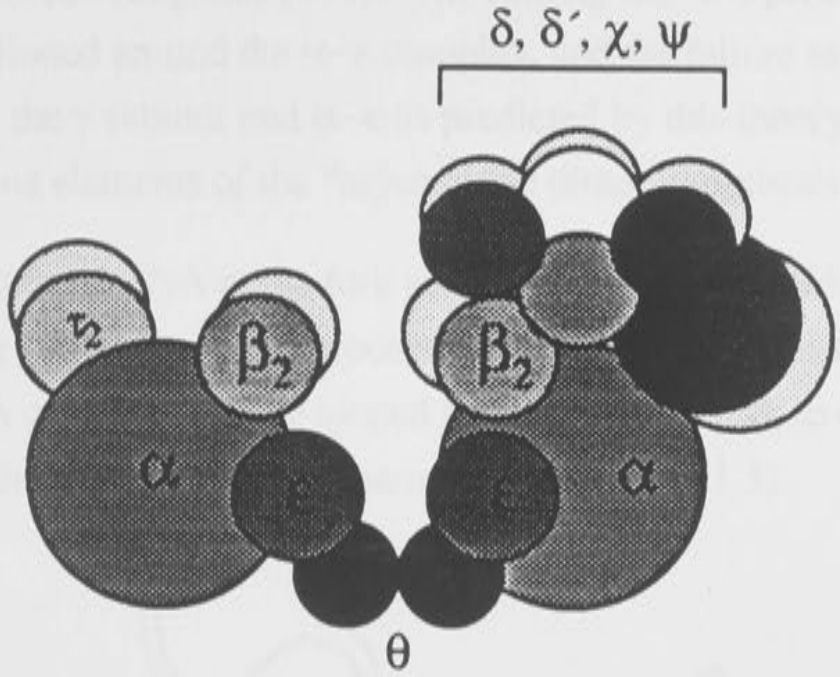

Figure 1.2: Model for the asymmetric dimer structure of DNA polymerase III holoenzyme. However a recent report suggests that dimerisation of pol III takes place around the τ subunits (see below). Adapted from Kornberg (1988).

ATPγS actually stimulated initiation complex formation, suggesting that the binding of the analogue to one-half must increase the affinity of the other for ATP (McHenry *et al.*, 1987).

Studies of holoenzyme reconstitution from its subunits were used to determine which of the halves was responsible for replication of the leading and lagging strands. It was shown that when τ was present (with all the other subunits), a processive polymerase was formed that contained two τ subunits per monomeric core, and very little γ complex (Maki and Kornberg, 1988a). When τ was absent, a processive holoenzyme was still formed but tended to dissociate into its subassemblies (Maki and Kornberg, 1988c). This suggested that one half of the holoenzyme has both the τ subunits and is responsible for leading strand synthesis, while the other (γ) half is more unstable and carries out lagging strand replication (Fig. 1.2 and Fig. 1.3). This proposal was consistent with the requirement that the lagging strand polymerase be able to dissociate readily from each Okazaki fragment and reassociate with a new primer terminus in a fork while the leading strand polymerase may never need to do so. However, it has been recently reported that the τ subunit was able to dimerise

complexes of the α and ε subunits to form a structure containing two molecules of each subunit (Studwell-Vaughan and O'Donnell, M., 1991). The α subunits in this structure bind to the C-terminal region of τ; no interaction between the ε and τ subunits was observed (Studwell-Vaughan, 1991). The finding that τ is probably symmetrically positioned around the α–ε complex, and the failure to detect an interaction between the γ subunit and α–ε as predicted by this theory, may cause a re-evaluation of some elements of the "asymmetric dimer hypothesis".

Since the strands of DNA at the fork are antiparallel, and DNA synthesis on the lagging and leading strand templates proceeds in opposite directions (Fig. 1.1), it is necessary that DNA on one strand be looped back across the fork to enable the halves of the asymmetric dimer to move in the same direction (Fig. 1.3).

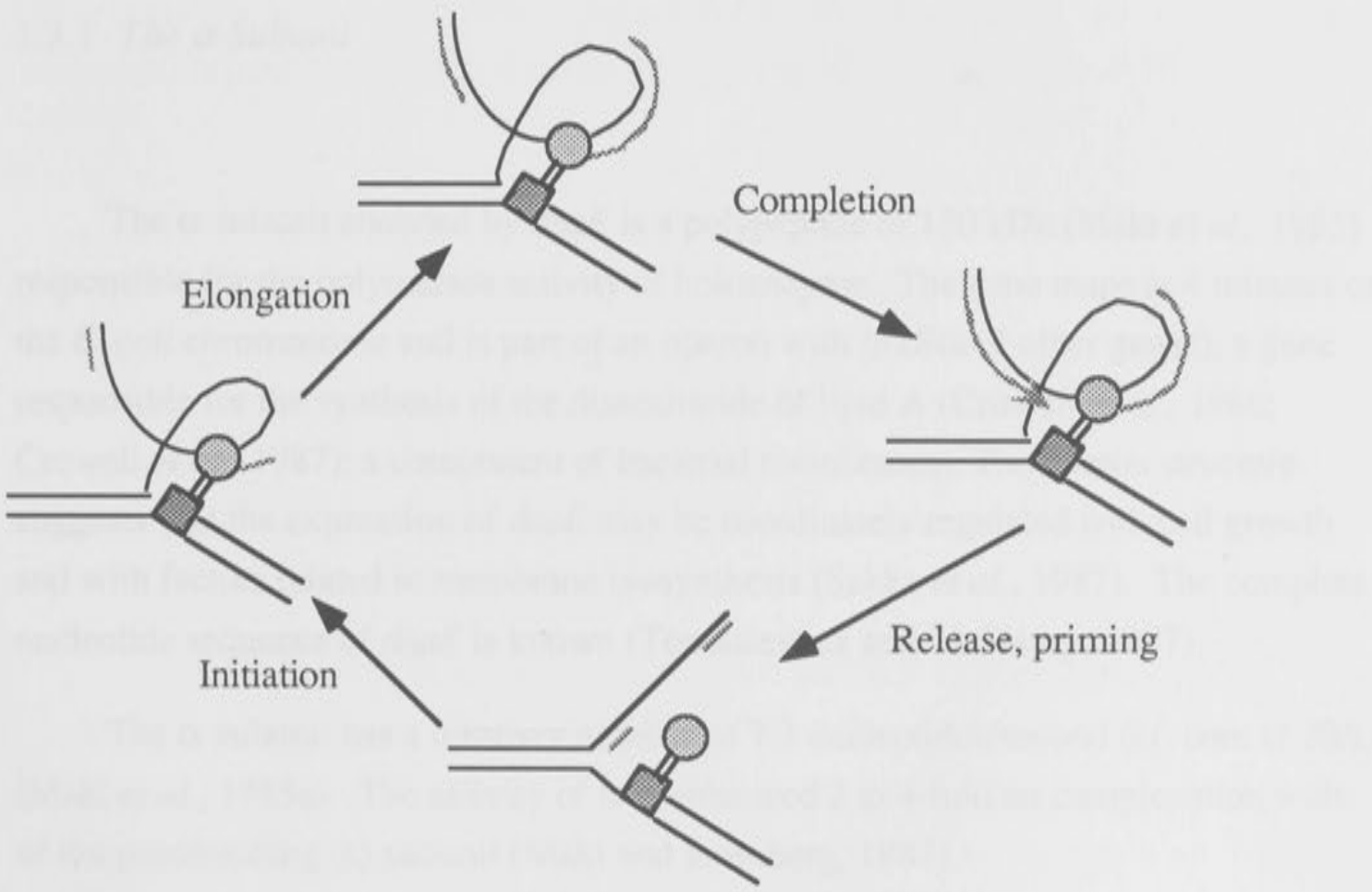

Figure 1.3: Cycling of an asymmetric dimeric polymerase at a replication fork. The leading strand polymerase (square) remains continuously clamped to the template, while the lagging strand polymerase (circle) can rapidly recycle, mediated in part by its looser template interactions. In this model, the dissociated lagging strand polymerase must recycle to the next primer synthesised at the replication fork.

Adapted from McHenry (1991).

Given the dynamic nature of holoenzyme indicated by its cycling reaction it seems likely that it at least partially assembles and reassembles during replication of the lagging strand. It may even be possible that several different functional forms of holoenzyme are involved in chromosomal replication, along with others that participate in DNA repair and recombinational processes.

Clearly, many more detailed physical and biochemical studies on holoenzyme structure and function are needed to formulate a comprehensive view of its behaviour at a replication fork.

1.3 THE SUBUNITS OF DNA POLYMERASE III HOLOENZYME

1.3.1 *The α Subunit*

The α subunit encoded by *dnaE* is a polypeptide of 130 kDa (Maki *et al.*, 1985) responsible for the polymerase activity of holoenzyme. The gene maps at 4 minutes on the *E. coli* chromosome and is part of an operon with *lpxB*(and other genes), a gene responsible for the synthesis of the disaccharide of lipid A (Crowell *et al.*, 1986; Crowell *et al.*, 1987): a component of bacterial membranes. The operon structure suggests that the expression of *dnaE* may be coordinately regulated with cell growth and with factors related to membrane biosynthesis (Sakka *et al.*, 1987). The complete nucleotide sequence of *dnaE* is known (Tomasiewicz and McHenry, 1987).

The α subunit has a turnover number of 7.7 nucleotides/second (cf. core at 20/s) (Maki *et al.*, 1985a). The activity of α is enhanced 2 to 4-fold on complexation with of the proofreading (ε) subunit (Maki and Kornberg, 1987).

1.3.2 *The β Subunit*

1.3.2.1 Historical Background

The β subunit (41kDa) of holoenzyme was identified as the factor that complemented the activity of a thermolabile mutant *dnaN59* (Burgers *et al.*, 1981).

dnaN is present in an operon structure with *dnaA* (Ohmori *et al.*, 1984). The enzyme was first purified 10,000-fold to homogeniety from wild-type *E. coli* HMS83 (Johanson and McHenry, 1980). The *dnaN* gene was first cloned by Blanar *et al.* (1984). This enabled the construction of an overproducing strain from which the β subunit was purified to homogeniety (Johanson *et al.*, 1986).

1.3.2.2 Properties and Physiological Roles

β subunit from *E. coli* HMS83 was shown to have native and denatured molecular weights of 72 kDa and 37 kDa, respectively (Johanson and McHenry, 1980). Thus, β would appear to normally exist as a dimer when in a state free of the other holoenzyme subunits. This conclusion was supported by the native molecular weight calculated from sedimentation equilibrium studies (Johanson and McHenry, 1980).

Polyclonal antibody directed against the β subunit was found to block reactions catalysed by holoenzyme, but not the core polymerase (Johanson and McHenry, 1980). Furthermore, the antibody inhibited only the formation of an initiation complex; the elongation complex was unaffected by the presence of antibody (Johanson and McHenry, 1980). Johanson and McHenry (1982) went on to report that this was due to the immersion of β within the initiation complex; β was present as a part of holoenzyme at all stages in the reaction.

A large excess of the β subunit can bypass the ATP requirement for processive synthesis by holoenzyme (Crute *et al.*, 1983). This β-mediated reaction can be distinguished from the ATP-mediated one by its salt sensitivity and the lack of a stable initiation complex formation between pol III´ and primed DNA (Crute *et al.*, 1983). In the absence of ATP, saturating levels of the β subunit cause a 7-fold increase in synthetic activity (Crute *et al.*, 1983).

The involvement of the β subunit in increasing the processivity of the holoenzyme was demonstrated by the observation that two populations of DNA products were generated by a pol III* - β complex. In the absence of the β subunit, only short products were synthesised; in the presence of β, much longer products were generated (LaDuca *et al.*, 1986). These findings also supported observations made by Johanson and McHenry (1980 and 1982) that the β subunit interacts functionally with the core polymerase. The role of β in holoenzyme function was confirmed in a reconstitution experiment that showed that the addition of β to pol III* resulted in a

fully processive replicative complex (Lasken and Kornberg, 1987). Further, β was necessary for cycling from one primed DNA template to another.

1.3.3 *Other Holoenzyme Subunits*

The γ, τ and ε subunits of holoenzyme will be discussed in Chapters 3 and 4, respectively.

Little is known about the χ, δ, δ′, θ and ψ subunits due to the lack of known structural genes for these proteins. However, two recent reports indicate that genes encoding all of these subunits are now known (McHenry, 1991; Studwell-Vaughan and O'Donnell, 1991). The gene for δ is reported to be located immediately downstream of *rlpB* at 16 min (Takase *et al.*, 1987), and the structural gene for ψ is between *xerB* and *valS* at 97 min (Stirling *et al.*, 1989). All 5 of these holoenzyme subunits have distinct genes and map to unique locations on the *E. coli* chromosome (McHenry, 1991; Studwell-Vaughan and O'Donnell, 1991). The θ subunit forms part of the core polymerase. It is thought to confer thermostability on this complex (Maki and Kornberg, 1987). The χ,δ,δ′ and ψ subunits confer processivity on pol III′, although δ alone is sufficient with assays currently available (Maki and Kornberg, 1988b; O'Donnell and Studwell, 1990).

1.4 OTHER DNA POLYMERASES WITH EXONUCLEASE ACTIVITY

1.4.1 *T7 DNA Polymerase*

Upon infection of *E. coli*, bacteriophage T7 directs production of its own DNA polymerase. Purification of the polymerase activity revealed a complex consisting of two polypeptides in a 1:1 stoichiometry: the 80 kDa gene 5 protein encoded by the phage (Modrich and Richardson, 1975), and the 12 kDa thioredoxin specified by the *trx*A gene of *E. coli* (Mark and Richardson, 1976). The two proteins form a complex with a K_D of 5 nM. The gene 5 protein alone has only low processivity; the binding of thioredoxin confers high processivity to the enzyme.

In addition to catalysing the polymerisation of nucleotides, T7 DNA polymerase

has an extremely active (at least 1000 times that of the Klenow enzyme), Mg^{2+} ion-dependent 3′ → 5′ exonuclease activity active on both ss- and dsDNA (Tabor and Richardson, 1989; Cowart *et al.*, 1989). This activity resides wholly within the gene 5 protein. Thioredoxin increases the exonuclease activity on dsDNA several hundred-fold, while it has little effect on the activity on ssDNA (Tabor and Richardson, 1989). Several peptide regions of the T7 gene 5 protein show strong amino acid homology to DNA polymerase I (Ollis *et al.*, 1985).

1.4.2 *T4 DNA polymerase*

T4 DNA polymerase (104 kDa) functions as a holoenzyme composed of a polymerase core and three distictive phage-encoded accessory proteins. The polymerase has an extremely active 3′ → 5′ exonuclease, similar to that of pol I but 200 times more active (Bessman *et al.*, 1974). The primary structure of T4 polymerase has been determined and the enzyme is more similar to animal virus polymerases and human polymerase α than to *E. coli* pol I or T7 DNA polymerase (Spicer *et al.*, 1988). The fidelity of the T4 polymerase is high. Mutator and antimutator mutations reside in the gene coding for the exonuclease function, as does the error-prone mutagenic repair observed after UV damage (Bessman *et al.*, 1974; Bedinger and Alberts, 1983). The 3′ → 5′ exonuclease degrades ssDNA to the terminal dinucleotide and this property has made it a useful reagent in the laborotory.

1.4.3 *Eukaryotic and Plant DNA Polymerases*

(for a review, see Bambara and Jessee, 1991)

The polymerase and exonuclease activities of both DNA polymerases δ and ε purified from calf thymus (125 kDa) and mouse cells (178 kDa), respectively, are contained within a single polypeptide. They digest ssDNA and dsDNA substrates in a 3′ → 5′ direction. Single-stranded DNA is hydrolysed more rapidly, suggesting that dsDNA may be acted upon only during transient melting. Mispaired 3′-terminal nucleotides are a preferred substrate of DNA polymerase ε and it is thought to be responsible for the synthesis of the leading strand at a replication fork (Bambara and Jessee, 1991).

The exonuclease activity of both DNA polymerases is inhibited by dNMP's, presumably by competitive binding at the active site.

The fidelity of these enzymes is very high. Calf DNA polymerase ε was found to produce less than one error per 10^6 nucleotides using the M13mp2*lacZ*α nonsense codon reversion assay (Kunkel *et al.*, 1987). However, lack of a standard assay has precluded meaningful comparison with other eukaryotic DNA polymerases.

A chloroplast DNA polymerase has been purified from pea (McKown and tewari, 1984) and spinach (Keim and Mosbaugh, 1991). The exonuclease components of these polymerases have been characterised and exhibit: (i) an absolute requirement for divalent metal ion (Mg^{2+} or Mn^{2+}), (ii) stimulation by NaCl or KCl, and, (iii) a broad alkaline pH optimum. The polymerases have a native molecular weight ranging from 87,000 to 110,000 and are inhibitited by *N*-ethylaleimide (Keim and Mosbaugh, 1991). The preference for mispaired 3′-OH termini by these enzymes suggested they may have a role in controlling fidelity of replication (McKown and Tewari, 1984; Keim and Mosbaugh, 1991).

1.5 AIMS AND OBJECTIVES

Elucidation of the structure, and physico-chemical properties of holoenzyme has been slow due to its very low cellular abundance: 10 to 20 molecules per cell. Use of recombinant DNA technology has made it possible to clone the individual subunit genes and obtain their products in milligram quantities.

This project set out to investigate the structure and function of three of the subunits of DNA polymerase III holoenzyme, focussing in particular on the proofreading exonuclease ε. The broad aims of the project were to:

1) Develop vectors for the high-level expression of proteins in *E. coli* (Chapter 2).

2) Clone known holoenzyme genes for *dnaX* (Chapter 3) and *dnaQ* (Chapter 4) into these vectors with a view to the high level expression of their gene products.

3) Purify overexpressed gene products (Chapters 3 and 4, respectively).

4) Investigate the properties of overexpressed ε (Chapter 5).

5) Examine the mechanism of the phosphodiesterase activity of ε using kinetic methods (Chapter 6), and

6) Develop a simple structural model for the ε subunit (Chapter 7).

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CHAPTER 2

CONSTRUCTION OF BACTERIOPHAGE λ PROMOTER VECTORS FOR THE HIGH-LEVEL EXPRESSION OF PROTEINS IN *Escherichia coli*

2.0 INTRODUCTION

The elucidation of the structure and functions of many of the enzymes involved in the replication of bacterial DNA has been hampered by their very low cellular abundance: approximately 20 copies/cell for DNA polymerase III holoenzyme (McHenry, 1988). This suggests that the synthesis of many DNA replication proteins is tightly regulated. Therefore, the design of vectors for the high-level expression of such proteins requires some understanding of the molecular mechanisms by which gene expression is controlled.

The expression of genes may be controlled at several levels. At the transcriptional level, control is mediated by the strength of the promoter, i.e., how frequently RNA polymerase is able to bind and initiate transcription. The major rightward and leftward promoters of bacteriophage λ (P_R and P_L) are reported to provide high levels of mRNA synthesis (Reznikoff and McClure, 1986). Transcription from these promoters is controlled by the λ cI repressor, product of the *c*I gene. If a temperature-sensitive allele of *c*I is used, (e.g. *cI857*) transcription will be repressed at 30 °C because the repressor protein is functional. At 42 °C, inactivation of the thermolabile repressor occurs allowing RNA polymerase access to the P_R and P_L promoters and high-level transcription occurs.

Control of gene expression can also be exercised at the level of translation initiation. Several factors have to be taken into consideration when assessing the potential yield of a protein. The first is the initiation codon. Most *E. coli* genes begin with AUG, approximately 8% start with GUG, and less than 1% use UUG (Stormo, 1986). If the initiation rate depends on the equilibrium constant for association of the initiator methionyl-tRNA with the initiation codon, a decrease in the rate of initiation should be observed with GUG (Ikemura, 1981). It was shown that by altering an AUG initiation codon to GUG, the overall synthesis of a protein in several systems was decreased by 25 to 40% (O'Neill *et al.*, 1986). It seems fairly clear that AUG is preferred over GUG.

The second factor affecting protein synthesis is the Shine-Dalgarno sequence (Shine and Dalgarno, 1974), which comprises a polypurine tract with the consensus sequence AGGAG. The Shine-Dalgarno sequence or ribosome-binding site (RBS) is usually located between 5 and 9 nucleotides upstream of the initiating codon (see Fig. 7.1). The importance of the RBS in translation initiation has been demonstrated from experiments in which even small changes in its sequence have resulted in decreases of > 5-fold in protein synthesis (Singer *et al.*, 1981; Munson *et al.*, 1984). The 13

nucleotides at the 3´end of 16 S rRNA to which the mRNA binds are:

$$5´\text{-GAUCACCUCCUUA}_{OH}\text{-}3´$$

These are predicted to be not involved in intramolecular base pairing (Noller, 1984) and are therefore available for base-pairing with complementary sequences located on an mRNA (see Fig. 7.1). There is good correlation between the degree of complementarity of the RBS present in genes with the sequence presented above, and the level of protein synthesis (Stormo, 1986).

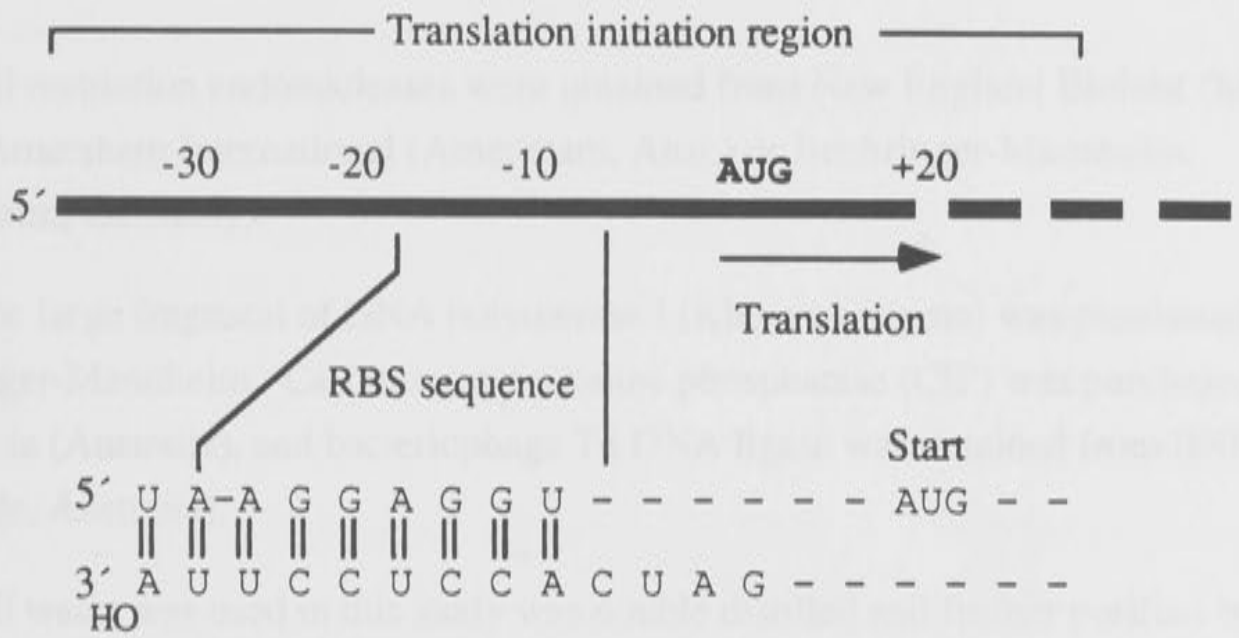

Figure 7.1: The structure of the translation initiation region.

Adapted from McCarthy and Gualerzi, 1990

The third factor affecting protein synthesis at the level of translation is the spacing between the RBS and the initiation codon. An analysis of data derived from experiments in which the RBS to ATG spacing has been altered suggests that the yield of protein synthesis for spacings greater than 4, and up to about 13 vary only 3-fold, with a spacing of 9 being optimal (Shepard *et al.*, 1982; Thomas *et al.*, 1982; Wood *et al.*, 1984). Spacings of less than 5 have been reported to result in very poor yields. This is due to the finding that the minimum distance between the RBS and ATG required for the simultaneous association of the 16 S rRNA and a tRNA is > 4 bp (Stormo, 1986).

This chapter describes the construction of a series of vectors that address some of the parameters affecting the expression of gene products in *E. coli*. These vectors will be used in this project for the high-level expression of holoenzyme subunits.

2.2 MATERIALS AND METHODS

2.2.1 *Enzymes*

All restriction endonucleases were obtained from New England Biolabs (Mass. USA), Amersham International (Amersham, Aust.) or Boehringer-Mannheim (Mannheim, Germany).

The large fragment of DNA polymerase I (Klenow enzyme) was purchased from Boehringer-Mannheim. Calf intestinal alkaline phosphatase (CIP) was purchased from Pharmacia (Australia), and bacteriophage T4 DNA ligase was obtained from BRESA (Adelaide, Australia).

All water was used in this study was double distilled and further purified by filtration through a MilliQ system (Millipore, U.S.A.).

2.2.2 *Radionuclides*

[α–^{32}P]dATP (3000 Ci/mmol) was purchased from Amersham International.

2.2.3 *Bacterial Strains and Plasmids*

The bacterial strains and plasmids used throughout this study are described in Table 2.1.

2.2.4 *Growth Media*

E. coli strains were grown in LB medium (Luria and Burrous, 1957) supplemented with 25 µg/ml thymine (LBT). Media for strains hosting plasmids pCE30 and its derivatives contained ampicillin (50 µg/ml). One strain, *E. coli* JM1 (pRK248-*c*Its/pNS360) (Chapter 4), required tetracycline (10 µg/ml) in addition to ampicillin (50 µg/ml) to stably maintain plasmids. Strains containing pCE30 and its derivatives were grown at 30 °C, while other strains carrying plasmids were grown at 37 °C. For large scale preparations of plasmid DNA, strains were grown in minimal 56 medium (Monod *et al.*, 1951) containing 1 mM magnesium sulfate and supplemented with casein hydrolysate (20 µg/ml) and trace salts (Gibson *et al.*, 1977), 40 mM glucose, 1 µg/ml vitamin B1 and the appropriate antibiotic.

	Genotype	Reference
Strains		
AN1459	*sup*E44*thr-1ilv leu rec*A *srl*A::Tn10	Elvin *et al.* (1986)
AN2666	*sup*E44 *thi* Δ(*lac-pro*AB) *rec*A *srl*A::Tn10	Yanisch-Perron *et al.* (1985)
K37	*HfrC supD*32 *relA*1 *pit*-10 *spoT*1 *tonA*22 *ompF*627 *phoA*4 λ⁻	Hines and Ray (1980)
Plasmids		
pCE30	pUC9 (λ *c*I857 P_RP_L)	Elvin *et al.*, 1990
pCE44	ptac85 (*dnaA*⁺)	C.M. Elvin[a] (unpublished)

Table 2.1: Bacterial strains and plasmids. [a]See Section 2.3.

2.2.5 *Plasmid Preparations*

2.2.5.1 Plasmid Extraction by Alkaline Lysis

Plasmid DNA was prepared on a small-scale for the purpose of analysis essentially as described (Silhavy *et al.*, 1984) from cells grown on LBT plates containing the appropriate antibiotic. Plasmid DNA was prepared by this procedure on a medium-scale from 25 ml bacterial cultures with one modification: following clarification of the suspension (10,000 x *g*; 30 min, 4 °C), the supernatant was treated with repeated phenol-chloroform extractions (Sambrook *et al.*, 1989) to remove protein prior to precipitation with ethanol.

2.2.5.2 Purification of Plasmid DNA in CsCl Gradients

Large-scale isolations of highly purified DNA were prepared by two successive bandings through CsCl density gradients from cells grown in minimal media, amplified with spectinomycin, and lysed with Triton X-100 using a modification of the procedure by Davis *et al.* (1980). All procedures were carried out at 0 °C unless otherwise indicated.

An overnight culture of the *E. coli* strain harbouring a plasmid was used to inoculate 1 litre of 56-minimal medium containing ampicillin. The culture was aerated until $A_{595} = 0.5$. Spectinomycin (300 mg) was added, and aeration continued at 30 °C for a further 16 h. The cells were chilled and harvested by centrifugation (8,000 x *g*, 20 min). The cell pellet was resuspended with 6.25 ml of ice-cold 50 mM Tris.HCl pH 8.0, 25% (w/v) sucrose and stored at -70 °C until use.

The cell suspension was thawed, then diluted to 7.5 ml with resuspension buffer containing lysozyme (10 mg/ml) and swirled for 5 min. To this 1.25 ml of 500 mM EDTA (pH 8.5) was added and the suspension was swirled for a further 5 min. Following the method of Katz *et al.* (1973), cell lysis was performed with the addition of Triton X-100 solution (10 ml; 0.1% (v/v) Triton X-100, 50 mM Tris.HCl pH 8.0, 62.5 mM EDTA). After 10 min, the lysate was centrifuged (48,000 x *g*, 1 h).

The density of the lysis supernatant was adjusted by dilution to 25 ml with H_2O and the addition of 24.38 g CsCl. Insoluble material was removed by centrifugation

(13,000 x *g*, 1 h) and the supernatant was decanted through a tissue filter into a Sorvall T-865 polyallomer tube. The tube was filled with the addition of 2.55 ml of a 10 mg/ml solution of ethidium bromide and TE (10 mM Tris.HCl pH 7.4, 1 mM EDTA) to which had been added 0.975 g/ml CsCl. The plasmid DNA was isolated in the density gradient produced by centrifugation (125,000 x *g*, 40 h). The plasmid DNA band, located using a longwave UV lamp, was collected from tubes using an 18-gauge hypodermic needle and syringe as described (Sambrook *et al.*, 1989). Plasmid DNA was transferred to a T-1270 polyallomer tube and topped up with 0.88 ml of 10 mg/ml ethidium bromide solution and buoyant CsCl in TE. This was centrifuged (125,000 x *g*, 40 h); the plasmid band was isolated as before. Ethidium bromide was removed by repeated extraction with an equal volume of propan-2-ol saturated with 5 M NaCl in TE. The plasmid solution was dialysed against 2 changes of TE (1 litre) over 24 h.

2.2.5.3 Quantification of DNA

The concentration of dsDNA was determined spectrophotometrically assuming a solution with A_{260} = 1.0 contains 50 µg of DNA per ml. Plasmid DNA was routinely stored in TE at -70 °C.

2.2.6 *Restriction Endonuclease Digestion of DNA*

In the simplest case, digestions were carried out at 37 °C for 1 h. Activity was quenched by the addition of 0.5 volumes of restriction endonuclease stop mix (50 mM EDTA, 17% (v/v) glycerol, 0.07% (w/v) bromophenol blue, pH 8.5) (RE STOP) at 0 °C.

Buffers supplied by the manufacturer were used wherever possible. Where two or more restriction enzymes were used, digestion was carried out with the enzyme requiring the lower salt concentration first. Subsequently, the salt concentration was elevated prior to digestion with the second restriction enzyme.

With partial restriction endonuclease digestions, it was first necessary to establish optimal conditions for cleavage at only one restriction site. A progressive series of two-fold dilutions of enzyme were made in equivalent volumes of buffer containing the plasmid DNA. Following 30 min treatment at 37 °C, the reaction was

quenched by the addition of an equal volume of RE STOP at 0 °C. Partially digested DNA's together with a digest of the same plasmid linearised with a suitable restriction endonuclease, were electrophoresed on agarose gels (Section 2.2.16). From analysis of fragment patterns on the agarose gel, the optimum amount of enzyme for generation of the maximum amount of the appropriate linear species in a scaled-up reaction could be determined.

2.2.7 *DNA End-filling with the Klenow Enzyme*

Recessed 3´-termini created by restriction endonuclease digestion were filled-in using the large fragment of DNA polymerase I as described (Sambrook *et al.*, 1989).

2.2.8 *5´- Dephosphorylation of dsDNA*

To preclude intramolecular recircularisation of the vector DNA in subsequent ligation reactions, the 5´-phosphate groups of the vector produced by restriction endonuclease digestion were removed. This was achieved by adding calf intestinal alkaline phosphatase to the reaction mixture containing DNA and restriction endonuclease essentially as described (Sambrook *et al.*, 1989).

2.2.9 *Ligation of DNA Fragments*

Ligations were carried out with T4 DNA ligase in buffer prepared as described (Sambrook *et al.*, 1989). For the ligation of insert DNA fragments of <100 bp to the vector fragment, an insert to vector ratio of 10:1 was used to maximise cloning efficiency. Otherwise, concentrations of insert and vector were adjusted according to that recommended by Legerski and Robberson (1985).

2.2.9.1 Ligation of Cohesive Termini

DNA fragments with cohesive termini were ligated using buffer described by Sambrook *et al.* (1989). Reactions were routinely carried out at 14 °C overnight in volumes of 20 - 50 µl.

2.2.9.2 Ligation of Blunt-ended Termini

Ligation of DNA fragments with blunt-ended termini was performed in the presence of $[Co(NH_3)_6]Cl_3$ and spermidine (Rusche and Howard-Flanders, 1985) using blunt-end ligation buffer (Sambrook *et al.*, 1989). The DNA was often dephosphorylated prior to ligation to maximise intermolecular ligation between vector and the DNA insert. Reactions were routinely carried out at 30 °C for 2 h.

2.2.10 *Bal 31 Exonuclease Digestion of DNA*

Nuclease Bal31 progressively degrades both strands of a fragment of dsDNA from both ends (Gray *et al.*, 1975). To establish conditions for the removal of the required number of nucleotides, it was always necessary to conduct a trial digest.

This was performed in a 50 µl reaction volume containing Bal31 buffer (Sambrook *et al.*, 1989) and a known quantity of purified linear DNA (≈ 5 µg). After prewarming the reaction mixture (25 °C, 10 mins), a precise amount of Bal31 was added to the reaction and mixed in thoroughly. Treatment was continued at 25 °C; 5 µl samples were removed at 1 min intervals and transferred to separate tubes containing 0.2 M EGTA. The range of fragment sizes produced by Bal31 digestion at each time point was determined from agarose gel electrophoresis by reference of the mobilities to a set of size standards. On the basis of this information, the reaction was scaled up to obtain a maximum yield of fragments of the desired size.

2.2.11 *Labelling the 3′-Terminus of DNA*

DNA was digested with the appropriate restriction endonuclease to create a linear molecule with 5′-protruding ends. These ends were then radioactively labelled with [α–^{32}P]dAMP using the Klenow enzyme (see 2.2.7).

A mixture (24 µl) was prepared containing 4 µM each of dGTP, dCTP, TTP; 5 µCi of [α–^{32}P]dATP and 2U of Klenow enzyme in buffer (Sambrook *et al.*, 1989). A portion of this (6 µl) was added to plasmid DNA (≈ 0.5 µg) that had been digested with the appropriate restriction endonuclease. Reactions were carried out at 30 °C for 30 mins. The reaction was terminated by the addition of unlabelled dATP to 100 µM and an equal volume of RE STOP at 0 °C.

The sizes of small fragments of interest could be determined following polyacrylamide gel electrophoresis and autoradiography by reference to mobilities of suitably labelled size standards (such as *Sau*3A-digested pCE30).

2.2.12 *Preparation of Competent Cells*

Cells of *E. coli* were made competent for transformation with plasmid DNA using $CaCl_2$ by the procedure of Morrison (1979). Cells could be stored at -70 °C for some months in 15% (w/v) glycerol prior to use.

2.2.13 *Transformation of Competent E. coli*

Transformation of *E. coli* was carried out with plasmid DNA as described (Morrison, 1979), with one modification: after addition of the plasmid DNA, the cells were briefly heated at 30 °C prior to incubation in broth. Cells were subsequently plated out on LBT agar containing the appropriate antibiotic.

2.2.14 *Oligonucleotide Synthesis*

Synthetic oligodeoxynucleotides used as primers in dideoxy sequencing reactions, or as linkers required for some vector constructions, were prepared by the ANU Biomolecular Resource Facility using an Applied Biosystems 380B DNA Synthesizer. Oligonucleotides were supplied by the Facility in solutions containing ammonia and were deprotected by treatment at 56 °C for 12 h before drying *in vacuo*. After resuspension in H_2O, re-evaporation and resuspension in TE, oligonucleotides were stored at -20 °C until use. Quantification of oligonucleotides was made by dividing the A_{260} of a solution of the oligonucleotide by the sum of the extinction coefficients (the ε_{260}) of component nucleotides.

2.2.15 *Dideoxy Sequencing of Double-Stranded DNA*

Sequencing of purified dsDNA was performed using the Sanger dideoxy-mediated chain termination reaction (Sanger *et al.*, 1977). The procedure followed was essentially that of Tabor and Richardson (1987) using T7 DNA polymerase and with [α–^{32}P]dATP. The primer used for the sequencing of pCE30 derivatives was: 5′-GTTGGGTAACGCCAGGG-3′ (Elvin, *et al.*, 1990). Electrophoresis of reaction products, preparation of gels for autoradiography, and autoradiography of dried gels was carried out as described in Section 2.2.18.

2.2.16 *Agarose Gel Electrophoresis*

For both preparative and analytical agarose gel electrophoresis, gels were cast in a Davis system horizontal submarine apparatus (Sambrook *et al.*, 1989) using a toothed comb to form wells for loading of samples. Concentrations of agarose ranged from 0.7% to 1.2% (w/v) for 200 ml gels (147 mm x 136 mm x 10 mm), and 35 ml 'minigels' (75 mm x 50 mm x 10 mm). Gels were made up in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) buffer containing 0.5 µg/ml ethidium bromide. Samples were mixed with a 1/4 volume of loading buffer (0.25% (v/v) bromophenol blue, 0.25% (v/v) xylene cyanol, 30% glycerol), loaded and electrophoresed in TBE buffer containing 0.5 µg/ml ethidium bromide.

2.2.17 Visualisation of DNA

DNA fragments in agarose or polyacrylamide gels were visualised under UV light using a short-wave transilluminator (UV transilluminator Model TS-15, Ultraviolet Products). Photography of gels placed on the transilluminator was carried out using a model MP-4 Land Camera (Polaroid) with Polaroid 4 x 5 type 57/high speed film.

2.2.18 *Isolation of DNA Fragments*

Two methods were used for the recovery of DNA fragments from gels. Generally, electroelution from agarose gels was used to isolate fragments generated by restriction endonuclease digestion, while electroelution from polyacrylamide gels was employed for the purification of oligonucleotides where necessary (Section 5.1.5.1.1).

2.2.18.1 Electroelution from Agarose Gels

DNA products from restriction endonuclease, nuclease Bal31, Klenow end-filling and dephosphorylation reactions were separated by agarose gel electrophoresis (200 ml gels). Fragments of DNA were isolated by electroelution onto NA45 membrane (Schleicher and Schuell, Keene, USA). The DNA was eluted from the membrane by heating at 70 °C for 1 h in a solution (400 µl) of 1 M NaCl containing 50 mM arginine. The membrane was removed and the DNA was precipitated from aqueous solution with 2 volumes of ethanol (-70 °C, 1 h), followed by centrifugation in a microcentrifuge. The pellet was washed with 70% ethanol, dried briefly *in vacuo*, resuspended in TE, and stored at -20 °C.

2.2.19 *Electrophoresis of Radiolabelled Fragments of DNA*

Separation and analysis of radiolabelled DNA fragments was carried out on 5% polyacrylamide gels (19:1 acrylamide:bisacrylamide) polymerised in a glass mould

with 0.052% (w/v) ammonium persulfate and 0.1% (v/v) TEMED in TBE buffer. Denaturing gels also contained 8 M urea. Samples[1] were loaded on to slab gels (500 mm x 380 mm x 0.4 mm) mounted in a Bio-Rad Sequi-gen DNA sequencing apparatus containing TBE buffer. Gels were pre-electrophoresed at 50 W for 15 min; sequencing gels required up to 30 min. Electrophoresis was performed at 50W using a Bio-Rad Model 3000 Xi electrophoresis power supply for the required time.

Following electrophoresis, gels were transferred to Whatman 3MM filter paper, encased with Gladwrap, and dried in a Bio-Rad Model 483 slab drier *in vacuo* at 80 °C for 1 h.

2.2.20 *Autoradiography*

Dried polyacrylamide gels containing radiolabelled fragments of DNA were autoradiographed with Kodak AR50 X-Omat film in a light-proof cassette at room temperature for 4 to 18 h. Films were developed in a Kodak X-Omatic X-ray film developer.

2.2.21 *Induction of Cells Containing Bacteriophage λ Promoter Vectors*

Recombinant strains were grown as described in Section 2.2.4 overnight at 30 °C in an orbital shaking waterbath (Paton Scientific, Adelaide). A portion (1 ml) of the overnight culture was diluted to 25 ml with fresh broth containing ampicillin and grown at 30 °C in a shaking water bath. At a cell density measured as $A_{595} \approx 0.5$, the cells were transferred to a shaking water bath at 42 °C and growth was continued for 2.5 h. A sample of cells (1 ml) was removed just before the temperature shift and after treatment at 42 °C. The cells were harvested in a MSE Microcentaur microfuge and stored at -20 °C until use.

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[1]Products from sequencing reactions were heated at 80 °C for 2 min prior to loading.

2.2.22 *Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis*

Electrophoresis of proteins was carried out under denaturing conditions using sodium dodecyl sulphate (SDS) as described by Laemmli (1970). Slab gels (1.5 mm x 200 mm x 150 mm) were formed in vertical glass moulds and consisted of a stacking gel and a resolving gel. Depending on the size of the proteins, the resolving gels contained 9 - 15% (w/v) acrylamide (38:1 acrylamide:bisacrylamide), 375 mM Tris.HCl pH 8.8, 0.1% (w/v) SDS, and were polymerised with 0.033% (w/v) ammonium persulphate and 0.033% (v/v) TEMED. Stacking gels contained 4.5% acrylamide, 125 mM Tris.HCl pH 6.8, 0.1% SDS, and were polymerised with 0.08% (w/v) ammonium persulphate and 0.08% (v/v) TEMED. Sample wells were formed by use of a toothed comb in the stacking gel. The electrophoresis buffer contained 51 mM Tris, 384 mM glycine and 0.1% (w/v) SDS. Prior to loading, protein samples were denatured in an equal volume of loading buffer (300 mM Tris, 15% (w/v) glycerol, 0.6% bromophenol blue, 50 mM dithiothreitol (DTT, Cleland's reagent), 1% (w/v) SDS) by heating at 100 °C for 5 min (whole cells) or 75 °C for 5 min (homogeneous protein solutions).

Gels were electrophoresed at 65 V until samples had migrated to the interface between the stacking and resolving gels. Electrophoresis was then continued at 150 V for the appropriate time.

2.2.23 *Staining of Protein Gels with Coomassie Blue.*

Gels were fixed and stained with a solution containing 0.3% Coomassie blue R250, 40% (v/v) methanol, 10% (v/v) glacial acetic acid for 45 min at room temperature with constant agitation. Destaining was carried out in destain (10% (v/v) ethanol, 10% (v/v) glacial acetic acid) at room temperature for up to 48 h with constant agitation.

2.2.24 *Photography of Coomassie-stained Gels*

Gels were photographed under visible light using a Model MP-4 Land Camera (Polaroid) and Polaroid 4 x 5 type 55/high speed film. Film was developed according

to the manufacturer's instructions.

2.3 RESULTS

Plasmid pPT144 was constructed to serve as a precursor for other expression vectors. One of these was plasmid pPT150, described below (Section 2.3.3). The precursor plasmids were pCE30 (Elvin *et al.*, 1990) and the *dnaA*$^+$ plasmid pCE44 (a kind gift of Dr C. Elvin). pCE44 contains Nts. 233-1787 of the published sequence of *dnaA* (Sakokibona *et al.*, 1984), followed by Nts. 654-1338 of pBR322 (Sutcliffe, 1979) inserted at the end-filled *Nco*I site of ptac-85 (Marsh, 1986) in the orientation required for expression of *dnaA* from the *tac* promoter of the vector. *NcoI* sites were regenerated at both ends of the insert during cloning. Immediately preceeding the *Nco*I site upstream of *dnaA* is a *Bam*HI site derived from the vector (the sequence in this region is - GGATCCATGGTG, where the GTG codon is the natural initiation codon of *dnaA*). Immediately downstream of the other *Nco*I site is a vector-derived *Sal*I site.

2.3.1 *Construction of vector pPT144*

Plasmid pPT144 which carries the bacteriophage λ promoters P_R and P_L in tandem, was constructed as outlined in Figure 2.2a. A 2.2 kb *Bam*HI/*Sal*I fragment containing the *dnaA* gene from pCE44 was isolated, and end-filled with the Klenow enzyme. Phosphorylated *Bam*HI linkers (Fig. 2.2b) were blunt-end ligated to the filled-in *dnaA*$^+$ fragment. Excess linkers that contained a RBS were removed with *Bam*HI. The fragment was purified (Section 2.2.18.1) and then ligated to pCE30 that had been linearised with *Bam*HI and its 5´ phosphate groups removed with calf alkaline intestinal phosphatase (Section 2.2.8). The *dnaA* gene was now downstream from the λ promoters and its translation was controlled by the RBS derived from the linkers (Fig. 2.2b). *E. coli* strain AN1459 was transformed with part of the ligation mixture and plated on to LBT agar containing ampicillin. Since an excess of the *dnaA* gene product is known to affect cell viability, transformants were then screened by their ability to grow normally at 30 °C, but failure to grow at 42 °C (temperature sensitivity) and their resistance to ampicillin. Plasmid DNA was prepared from 36 such colonies (Section 2.2.5.1) and were checked by the criteria of:

a) correctly-sized plasmids (6.3 kb, indicating which plasmids had the insert), and

Figure 2.2a: Construction of pPT144. Briefly, a 2.2 kb *Bam*HI/*Sal*I fragment containing the *dnaA* gene isolated from plasmid pCE44 (a kind gift from Dr. C. Elvin), and the fragment end-filled with the Klenow enzyme. Linkers (Fig. 2.2b) were ligated to the *dnaA* fragment; excess linker was separated from the *dnaA*$^+$ fragment by *Bam*HI digestion, followed by agarose gel electrophoresis and purification of the large fragment by electroelution onto NA45 membrane. This fragment was then ligated to pCE30 (Elvin *et al.*, 1990) that had been linearised with *Bam*HI and dephosphorylated. *E. coli* strain AN1459 was transformed with part of the ligation mixture.

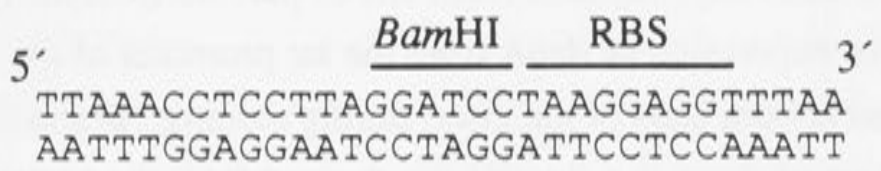
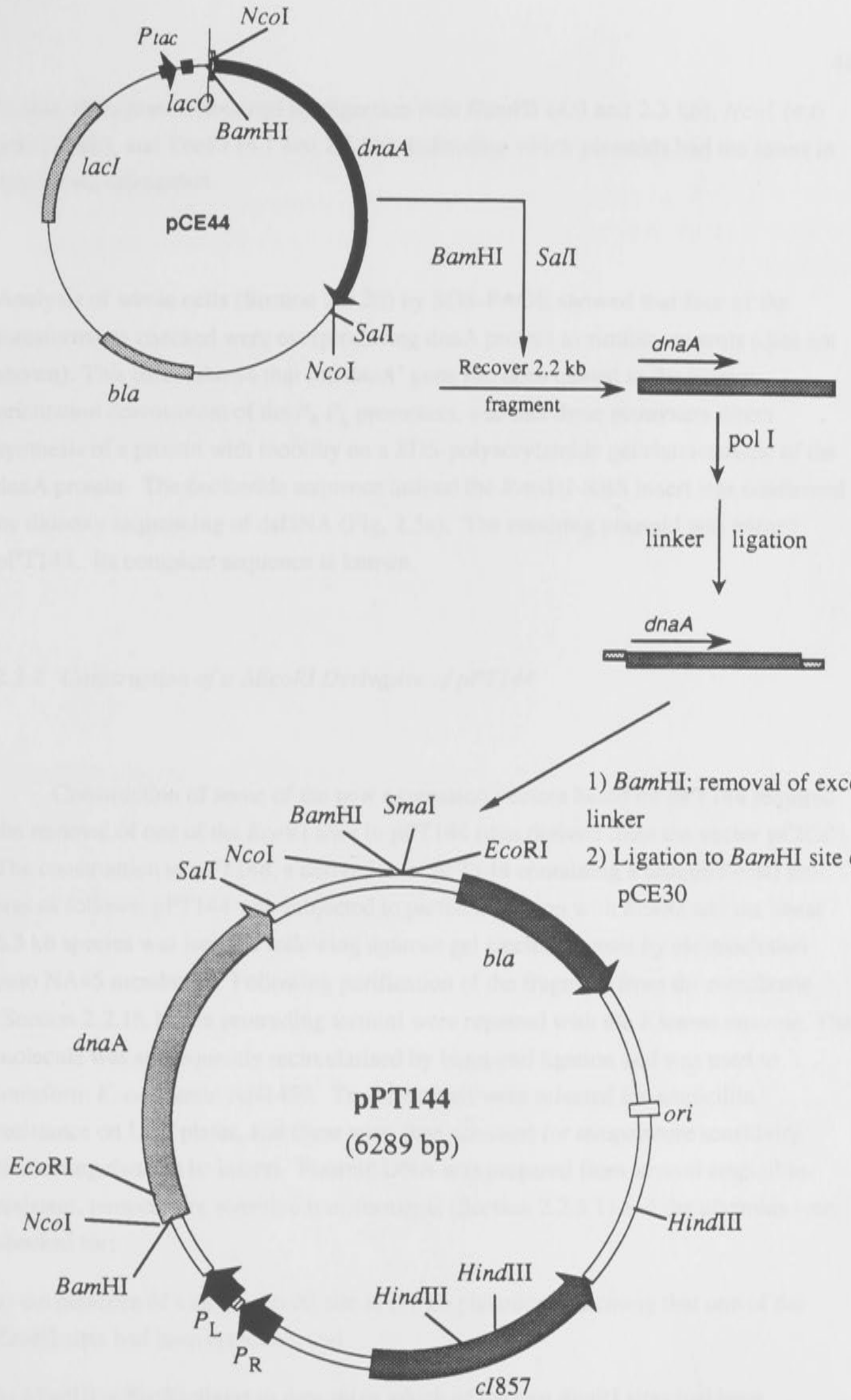


Figure 2.2b: Self-complementary 32 bp *Bam*HI-RBS linker used in the construction of pPT144. The symmetry of the adaptor means that it is functional in either orientation.


NcoI
Ptac
lacO
BamHI
dnaA
lacI
pCE44
BamHI
SalI
SalI
NcoI
bla
Recover 2.2 kb
fragment
dnaA
pol I
linker
ligation
dnaA
1) BamHI; removal of excess linker
2) Ligation to BamHI site of pCE30
BamHI
SmaI
NcoI
EcoRI
SalI
bla
dnaA
pPT144
(6289 bp)
ori
EcoRI
NcoI
HindIII
BamHI
HindIII
HindIII
PL
PR
cI857

b) size of fragments obtained by digestion with *Bam*HI (4.0 and 2.3 kb), *Nco*I (4.0 and 2.3 kb), and *Eco*RI (4.1 and 2.2 kb), indicating which plasmids had the insert in the correct orientation.

Analysis of whole cells (Section 2.2.20) by SDS-PAGE showed that four of the transformants checked were overproducing dnaA protein to similar amounts (data not shown). This result shows that the *dnaA*$^+$ gene has been cloned in the correct orientation downstream of the P_R P_L promoters, and that these promoters direct synthesis of a protein with mobility on a SDS-polyacrylamide gel characteristic of the dnaA protein. The nucleotide sequence around the *Bam*HI-RBS insert was confirmed by dideoxy sequencing of dsDNA (Fig. 2.3a). The resulting plasmid was named pPT144. Its complete sequence is known.

2.3.2 *Construction of a ΔEcoRI Derivative of pPT144*

Construction of some of the new expression vectors based on pPT144 required the removal of one of the *Eco*RI sites in pPT144 (that derived from the vector pCE30). The construction of pPT148, a derivative of pPT144 containing a unique *Eco*RI site, was as follows: pPT144 was subjected to partial digestion with *Eco*RI and the linear 6.3 kb species was isolated following agarose gel electrophoresis by electroelution onto NA45 membrane. Following purification of the fragment from the membrane (Section 2.2.18.1), the protruding termini were repaired with the Klenow enzyme. The molecule was subsequently recircularised by blunt-end ligation and was used to transform *E. coli* strain AN1459. Transformants were selected for ampicillin resistance on LBT plates, and these were then screened for temperature sensitivity (indicating *dnaA* to be intact). Plasmid DNA was prepared from several ampicillin-resistant, temperature sensitive transformants (Section 2.2.5.1) and the plasmids were checked for:

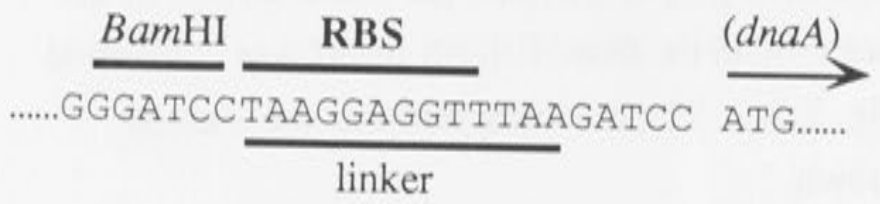
a) the presence of a single *Eco*RI site in 6.3 kb plasmids, indicating that one of the *Eco*RI sites had been removed, and

b) *Hin*dIII + *Eco*RI digest to determine which of the two *Eco*RI sites had been removed.

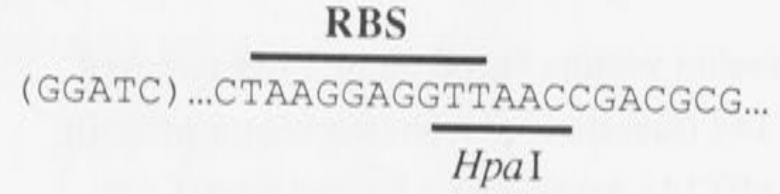
One plasmid which had a single *Eco*RI site at the desired location was called pPT148. The complete sequence of pPT148 is known.

Figure 2.3: Dideoxy sequencing of dsDNA. Preparation of DNA, primers and the sequencing reactions were performed as described (Sections 2.2.5.2, 2.2.14, 2.2.15). The sequence reads from the autoradiographs between the lines marked, from left to right: G A T C.

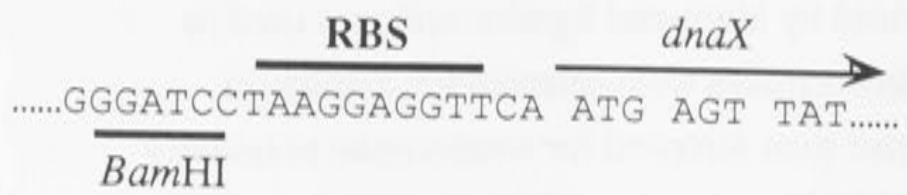
1) Plasmid pPT148 (5′ → 3′)

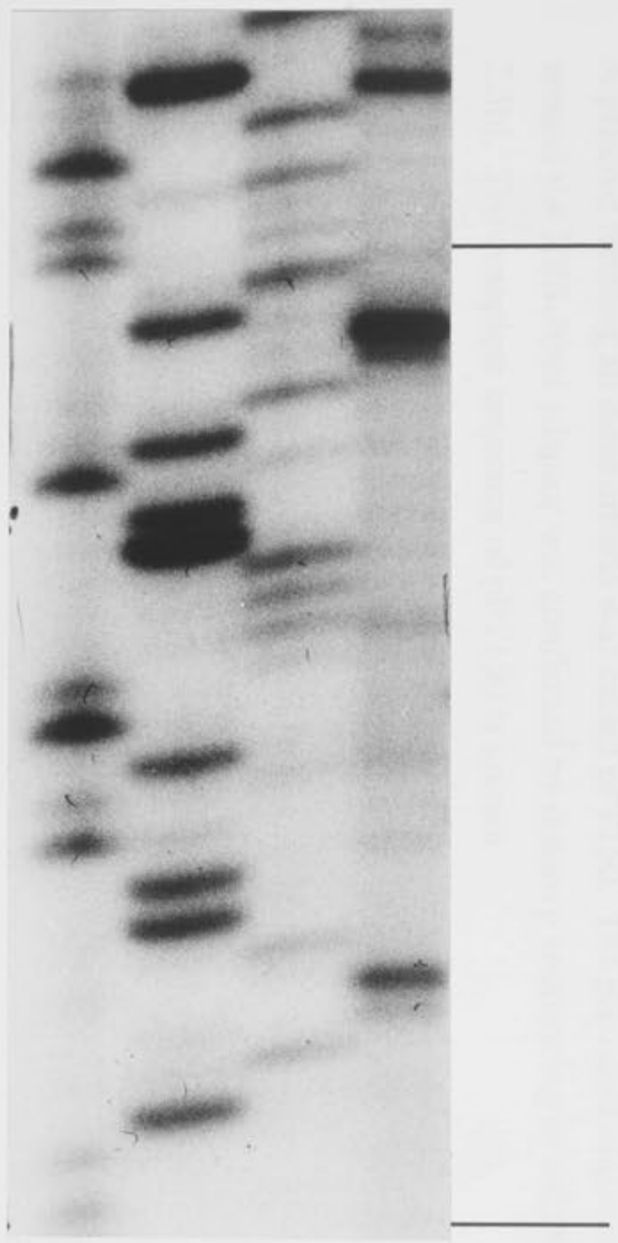


2) Plasmid pPT150 (5′ → 3′)

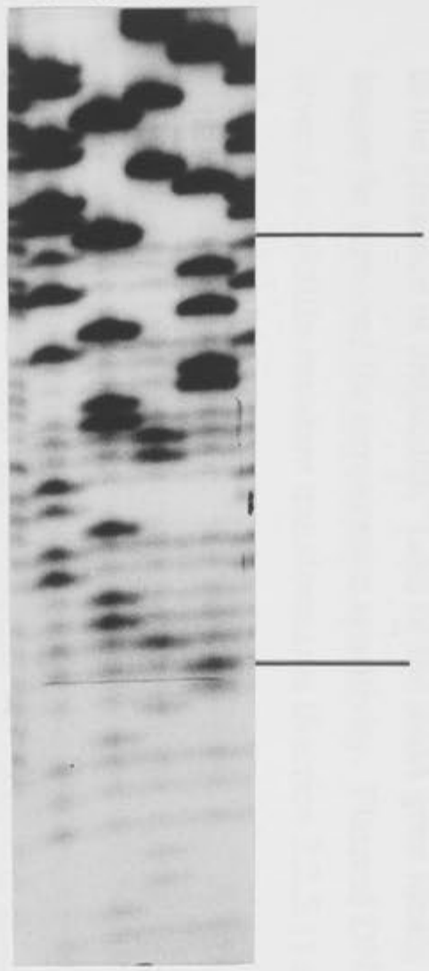


3) Plasmid pPT153 (5′ → 3′) (see Chapter 3)

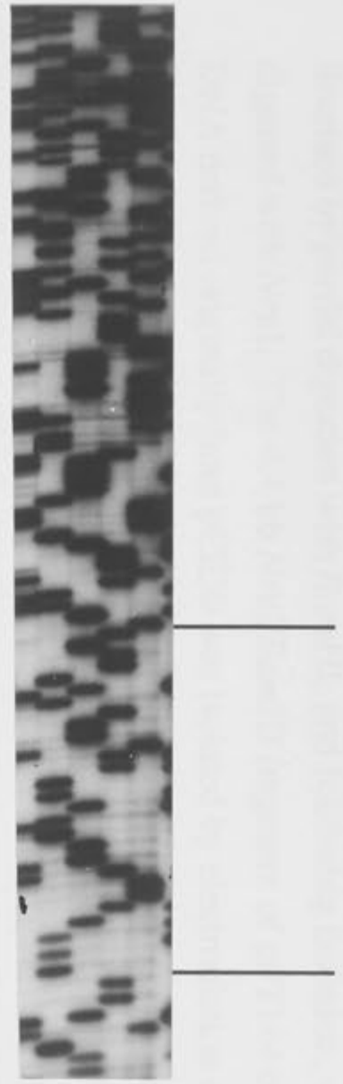




1)



2)



3)

2.3.3 *Construction of Vector pPT150*

The construction of pPT150 is outlined in Figure 2.4. Plasmid pPT144 was linerised by partial digestion with *Bam*HI, and following isolation, was further digested with *Nru*I. The 4.4 kb *Nru*I/*Bam*HI fragment of pPT144 containing all of the DNA derived originally from pCE30 was isolated by electroelution onto NA45 membrane following agarose gel electrophoresis. An unphosphorylated adaptor molecule containing a RBS perfectly complementary to the 3´ end of *E. coli* 16S rRNA, *Bam*HI-compatible 3´ overhangs, and a *Hpa*I site was ligated to this fragment. Following ligation, the linkered fragment was heated at 65 °C for 15 min and cooled slowly at room temperature to promote reannealing of the two overhanging ends. Plasmid DNA was subjected to agarose gel electrophoresis and isolated by electroelution onto NA45 membrane. *E. coli* strain AN1459 was transformed with a portion of the isolated DNA and colonies were initially selected by their ability to grow in the presence of ampicillin. Loss of the *dnaA* gene meant transformants could no longer be screened for temperature sensitivity. Plasmid DNA was prepared from several ampicillin-resistant transformants (Section 2.2.5.1) and checked by the criteria of:

a) plasmid size (≈ 4.4 kb), indicating loss of the fragment containing the *dnaA* gene, and

b) the presence of a *Hpa*I site, indicating the successful attachment of the adaptor molecule, and

c) two *Bam*HI sites.

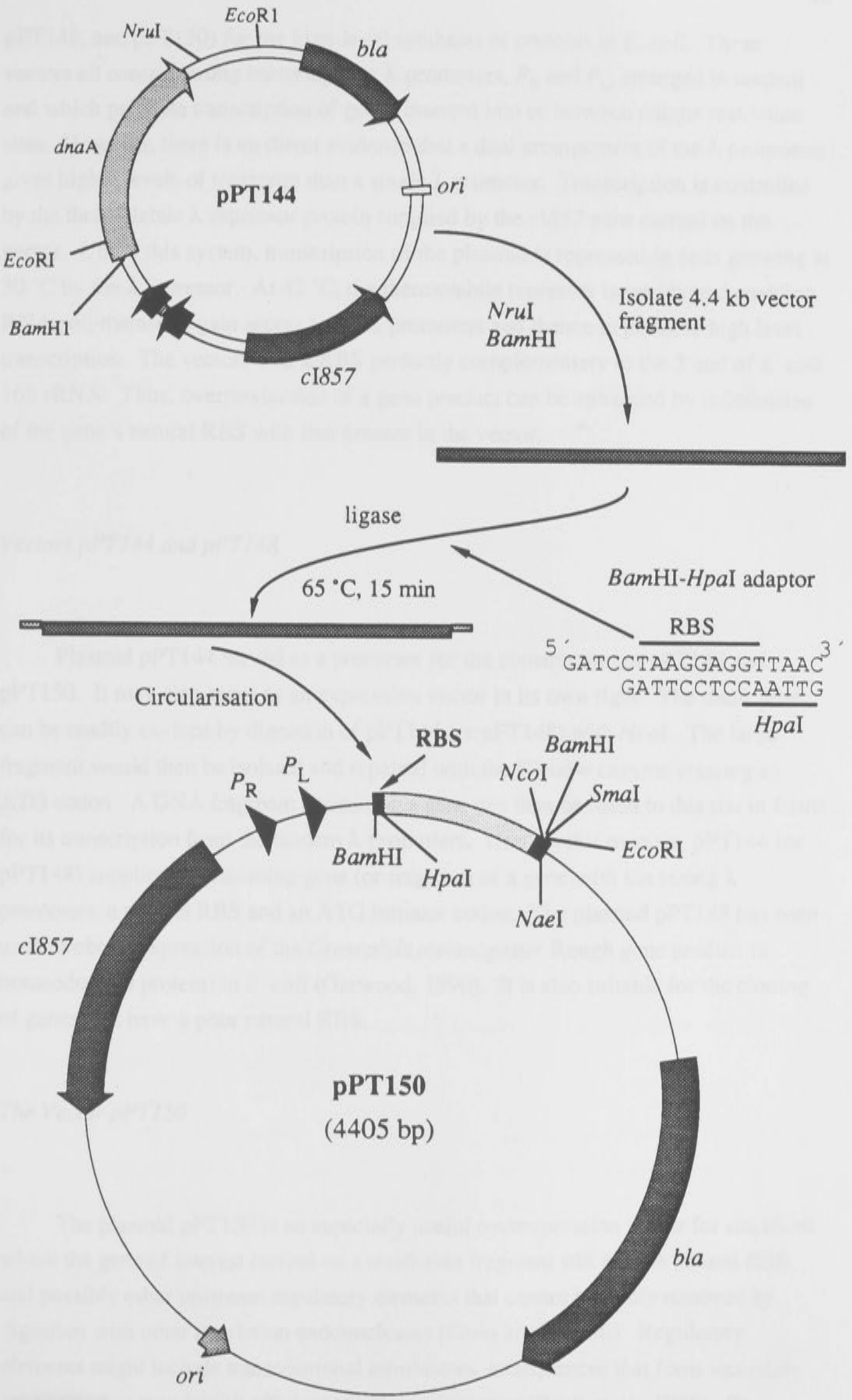
A plasmid meeting all these criteria was called pPT150. The predicted sequence around the RBS-*Hpa*I adaptor was confirmed by dideoxy sequencing of dsDNA (Fig. 2.3b). The complete sequence of pPT150 is known.

2.4 DISCUSSION

The λ Promoter Vectors

This chapter describes the construction of three expression vectors (pPT144,

Figure 2.4: Construction of pPT150. Briefly, a 4.4 kb *Nru*I/*Bam*HI fragment of pPT144 was isolated by electroelution onto NA45 membrane following agarose gel electrophoresis. An adaptor molecule containing a RBS perfectly complementary to the 3´ end of *E. coli* 16SrRNA and a unique *Hpa*I site was inserted between the ends of this fragment during its recircularisation *in vivo*.


EcoR1
NruI
bla
dnaA
pPT144
ori
EcoRI
BamH1
cI857
NruI
BamHI
Isolate 4.4 kb vector fragment
ligase
65 °C, 15 min
BamHI-HpaI adaptor
RBS
5´ GATCCTAAGGAGGTTAAC 3´
GATTCCTCCAATTG
HpaI
Circularisation
RBS
BamHI
NcoI
SmaI
EcoRI
PR
PL
BamHI
HpaI
NaeI
cI857
pPT150
(4405 bp)
bla
ori

pPT148, and pPT150) for the high-level synthesis of proteins in *E. coli*. These vectors all contain strong bacteriophage λ promoters, P_R and P_L, arranged in tandem and which promote transcription of genes inserted into or between unique restriction sites. However, there is no direct evidence that a dual arrangement of the λ promoters gives higher levels of transcript than a single λ promoter. Transcription is controlled by the thermolabile λ repressor protein supplied by the *cI857* gene carried on the vector. Under this system, transcription of the plasmid is repressed in cells growing at 30 °C by the λ repressor. At 42 °C, the thermolabile repressor is inactivated enabling RNA polymerase to gain access to the λ promoters and thence to promote high level transcription. The vectors bear a RBS perfectly complementary to the 3´end of *E. coli* 16S rRNA. Thus, overproduction of a gene product can be enhanced by substitution of the gene's natural RBS with that present in the vector.

Vectors pPT144 and pPT148

Plasmid pPT144 served as a precursor for the construction of pPT148 and pPT150. It may also serve as an expression vector in its own right. The *dnaA* gene can be readily excised by digestion of pPT144 (or pPT148) with *Nco*I. The large fragment would then be isolated and repaired with the Klenow enzyme creating an ATG codon. A DNA fragment containing a gene can then be fused to this site in frame for its transcription from the tandem λ promoters. Used in this manner, pPT144 (or pPT148) supplies the incoming gene (or fragment of a gene)with the strong λ promoters, a perfect RBS and an ATG initiator codon. The plasmid pPT148 has been used to obtain expression of the *Drosophila melanogaster* Rough gene product (a homeodomain protein) in *E. coli* (Garwood, 1990). It is also suitable for the cloning of genes that have a poor natural RBS.

The Vector pPT150

The plasmid pPT150 is an especially useful overexpression vector for situations where the gene of interest carried on a restriction fragment still has the natural RBS, and possibly other upstream regulatory elements that cannot be easily removed by digestion with other restriction endonucleases (Elvin *et al.*, 1990). Regulatory elements might include transcriptional terminators, or sequences that form secondary structures that may inhibit efficient translation initiation (Buell *et al.*, 1985). To

facilitate the removal of such upstream DNA, the vector contains a segment of DNA between the *Hpa*I site and a cluster of restriction sites (Fig. 2.4). Use of pPT150 first involves insertion of the gene, with up to 350 bp of DNA upstream from its start codon, into one of these sites (e.g. *Sma*I) in the orientation necessary for its transcription from the λ promoters. The isolated plasmid is then linearised with, for example, *Nco*I. The linearised plasmid is then treated with nuclease Bal31 to remove DNA to the region just upstream from the start codon. The product is then cleaved at the unique *Hpa*I site and the large fragment is recircularised by blunt-end ligation. Figure 3.1 shows a graphical representation of how pPT153 may be used in this way.

Plasmid pPT150 has been used to obtain the high level overproduction of the *dnaX* (see Ch. 3) and the *dnaT* replication gene products (Stamford, 1991).

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CHAPTER 3

OVERPRODUCTION AND PARTIAL PURIFICATION OF THE *dnaX* GENE PRODUCTS

3.0 INTRODUCTION.

3.1.1 *Historical Background*

The *dnaX* gene, formerly *dnaZX*, directs the synthesis of the γ and τ subunits of DNA polymerase III holoenzyme. The names *dnaZ* and *dnaX* were initially assigned as two separate loci because of the ability of separate plasmids from the Clarke-Carbon collection (Clarke and Carbon, 1976) to complement each mutation separately (Walker *et al.*, 1979). The *dnaZX* region was first cloned and its gene products identified by Mullin *et al.* (1983) as being polypeptides of 75 kDa and 56 kDa. Transposon Tn5 mutagenesis of the *dnaZX* plasmid abolished complementation of both mutants (Mullin *et al.*, 1983; Flower and McHenry, 1986). These studies provided the first indication that the *dnaZX* products are derived from a single gene. The 75 kDa polypeptide was identified as the τ subunit of holoenzyme (McHenry, 1981; Mullin *et al.*, 1983) and the 56 kDa polypeptide as the γ subunit (Hubscher and Kornberg, 1980). The γ subunit complements mutations in the initial part of *dnaX*, whereas the τ subunit can complement mutations in the region further downstream (Hubscher and Kornberg, 1980; Maki and Kornberg, 1988a). Additionally, the N-terminal amino acid sequence of both purified subunits agree with those predicted from the nucleotide sequence, and antibodies raised to either polypeptide are cross-reactive (Maki and Kornberg, 1988a). Ergo, the two polypeptides are the product of a single open reading frame (ORF).

Since both subunits are derived from a single gene, there was considerable interest in establishing how the γ subunit was generated. Several theories were propounded: proteolytic cleavage of τ to γ, proteolytic cleavage of a τ–τ dimer to generate a γ–τ heterodimer, and lastly, that τ acted as a protease in its own cleavage (McHenry, 1988). Eventually, a flurry of publications demonstrated that a (-1) frameshift during translation allows the use of a UGA stop codon located within the τ reading frame to terminate translation of the γ polypeptide with an efficiency of approximately 40% (Tsuchihashi and Kornberg, 1990; Flower and McHenry, 1990; Blinkowa and Walker, 1990). Examination of the mRNA for *dnaX* revealed a stem-and-loop structure immediately downstream of a stretch of 6 adenine residues. The UGA stop codon is located in a -1 frame between the adenines and the hairpin structure (Tsuchihashi and Kornberg, 1990; Flower and McHenry, 1990; Blinkowa and Walker, 1990). The hairpin and the so-called 'slippery' adenines allow the tRNA in both the aminoacyl and peptidyl sites of the ribosome to pair with at least 2 of the 3 nucleotides after the slippage that generates a -1 frameshift (See Appendix 2;

Tsuchihashi and Kornberg, 1990).

3.1.2 *Properties and Physiological Roles*

A detailed discussion of the roles of the τ and γ subunits in replication as a component of holoenzyme has been given in Chapter 1 (Sections 1.2.4.2 and 1.2.4.3).

The τ subunit (71 kDa) is a ssDNA-dependent ATPase (Lee and Walker, 1987). Unlike other DNA dependent ATPases, homopolymers are poor effectors (Tsuchihashi and Kornberg, 1989), suggesting that some secondary structure in the DNA may be essential for ATPase activity. It has been proposed that the recognition by τ (as part of the holoenzyme) of such a structural feature in a template may contribute to the several known functions of τ. These include participation in formation of initiation complexes and increasing the processivity of the holoenzyme, processes known to require ATP hydrolysis (Kornberg and Baker, 1991). τ was believed to cause Pol III to dimerise, and promote stable binding of holoenzyme to a primed template (Maki and Kornberg, 1988c). It has been reported that τ is not important for either the transfer of β onto primed DNA, or reconstitution of processive synthesis from an α–ε complex (O'Donnell and Studwell, 1990; Studwell and O'Donnell, 1990). However, a recent report by the same authors found that τ was able to dimerise complexes of the α and ε subunits to form a structure containing two molecules of each subunit (Studwell-Vaughan and O'Donnell, 1991). The α subunits bind to the C-terminal region of the τ dimer; there appeared to be no interaction betweem the ε and τ subunits (Studwell-Vaughan and O'Donnell, 1991). The finding that τ is probably symmetrically positioned around the α–ε complex, and an interaction between the γ subunit and α–ε as predicted by such a theory, may cause a re-evaluation of the "asymmetric dimer hypothesis". The organisation of subunits within the DNA polymerase III holoenzyme will no doubt be a target for much further study.

The γ subunit, in a complex with 4 other polypeptides (δ, δ′, χ, and ψ) known as the 'γ complex', participates in the ATP-dependent formation of the initiation complex (see Section 1.2.4.2). It is essential for processive synthesis of DNA by holoenzyme (Maki and Kornberg, 1988c). γ has no ATPase activity.

3.1.3 *Aims*

For studies of holoenzyme structure and function, it was desirable to construct a high-level overproducer of τ and γ using the bacteriophage λ promoter vectors described previously (Section 2.4). Both these subunits could be purified in large amounts from this overproducer. Thus, this chapter describes the use of plasmid pPT150 to facilitate the high-level expression and subsequent purification of the *dnaX* gene products in high yield. As a further step to studies of structure and function, polyclonal antibody was raised to the γ subunit.

3.1 MATERIALS AND METHODS

Many of the protocols for the manipulation of DNA have been described in Chapter 2, and will be cited here as necessary.

3.1.1 *Resins*

Heparin agarose was prepared as described (Farooqui, 1980) using Sepharose 4B (Pharmacia).

3.1.2 *Reagents*

Freund's incomplete adjuvant was purchased from the Commonwealth Serum Laboratories (Melbourne, Australia). Secondary antibody (goat anti-rabbit alkaline phosphatase (IgG)), nitro blue tetrazolium (NBT), and 5-bromo-4-chloro-3-indolyl (BCIP) were purchased from Sigma Chemical Company. Bovine serum albumin (BSA) (98% pure) and pre-stained molecular weight markers were purchased from BioRad.

3.1.2 *Determination of Protein Concentraion*

The concentration of protein was determined by the method of Bradford (1976) using BSA as standard.

3.1.3 Determination of NaCl Concentration

The concentration of NaCl in fractions collected from chromatography was determined by measurement of the conductivity. The concentration of NaCl was determined from the data by reference to standard solutions of NaCl in the approriate buffer.

3.1.4 *Overproduction of the dnaX Gene Products*

The construction of pPT153 which carries the *dnaX* gene downstream of the bacteriophage λ promoters P_R and P_L is outlined in Figure 3.1. A 2.1 kb *Sma*I-*Pvu*II fragment containing the *dnaX* gene from pJH16 (a kind gift from Dr A .Kornberg, Stanford University; Mullin *et al.*, 1983) was isolated (Section 2.2.18.1) and inserted into the *Sma*I site of 5´-dephosphorylated pPT150 by blunt-end ligation. This step placed the *dnaX* gene downstream of the λ promoters P_RP_L. A portion of the ligation mixture was used to transform *E. coli* AN1459. Colonies were initially selected by their resistance to ampicillin, and screened for temperature sensitivity. Transformants harbouring recombinant plasmid (pPT151) showed low expression of the τ and γ subunits (data not shown). To improve expression, it was necessary to fuse the vector RBS to the *dnaX* initiation codon. To this end, plasmid DNA prepared (Section 2.2.5.2) from one transformant was linearised with *Sma*I, and then treated with Bal31 (Section 2.2.10). Subsequent treatment with *Hpa*I (see Section 2.4) generated a large fragment that was subjected to agarose gel electrophoresis and isolated by electroelution onto NA45 membrane. The isolated large fragment was repaired with the Klenow enzyme, reclosed by ligation and part of the ligation mixture used to transform *E. coli* strain AN1459. Transformants were plated on to LBT agar containing ampicillin.

Purification of the γ Subunit of DNA Polymerase III Holoenzyme.

3.1.5 *Bulk fermentation of AN1459/pPT153*

Forty litres of LB containing 25 μg/ml thymine and 50 μg/ml ampicillin was inoculated with an overnight culture of AN1459/pPT153. When the optical density of the culture at 595nm reached 0.5, the temperature of incubation was rapidly shifted from 30 °C to 42 °C to induce the overproduction of γ and τ. After 3 h, the cells were chilled, and harvested at 4 °C by centrifugation in a Sharples centrifuge. The cell paste (87.5 g) was thoroughly resuspended with 88 ml of 20 mM Tris.HCl pH 7.5, 10% sucrose, frozen in liquid nitrogen, and stored at -70 °C.

3.1.6 *Cell Lysis*

Cells (5 g) were thawed overnight at 4 °C and resuspended to a cell density of A_{595} = 25 in lysis buffer (50 mM Tris.HCl pH 7.6, 10% sucrose, 167 mM NaCl, 25 mM spermidine.3HCl, 13.33 mM $MgCl_2$, 3.33 mM DTT). Cells were lysed by dropwise addition of 3 ml of 20 mg/ml eggwhite lysozyme and vigorously stirred for 60 min at 4 °C. The suspension was transferred to SS34 centrifuge tubes, briefly heated at 30 °C for 2 min to complete the lysis, and centrifuged for 45 min at 15,500 x *g*. The supernatant containing γ and τ was called **Fraction I** (35 ml).

3.1.7 *Ammonium Sulfate Precipitation*

Protein was precipitated from Fraction I by the addition of 0.25 g/ml powdered $(NH_4)_2SO_4$. The suspension was allowed to stand for 60 min at 4 °C, after which the precipitated protein was harvested by centrifugation (26,400 x *g*, 60 min). The pellet was redissolved with 25 ml of buffer T (50 mM Tris.HCl pH 7.6, 5 mM EDTA, 5 mM DTT, 10% (w/v) glycerol) and dialysed for 48 h against two changes of buffer T + 25 mM NaCl (2 litres). The solution was centrifuged again (26,400 x *g*, 30 min) to remove insoluble material and the supernatant was called **Fraction II** (25 ml).

3.1.8 *Heparin Agarose Chromatography*

Fraction II was loaded at a flow rate of 50 ml/h onto a column of Heparin Agarose (28 mm x 120 mm) that had been equilibrated with buffer T + 25 mM NaCl (250 ml). The column was washed at a flow rate of 50 ml/h with 100 ml buffer T + 25 mM NaCl; 6 ml fractions were collected. Bound protein was eluted with a linear gradient of 25 to 350 mM NaCl in buffer T (300 ml) at a flow rate of 50 ml/h; 6 ml fractions were collected. The homogeneity of the preparation was assessed by SDS-PAGE. Pooled fractions containing the γ subunit (35 - 42) were called **Fraction III** (48 ml). Fractions were assayed for protein (Section 3.1.2) and NaCl.

3.1.9 *Polyclonal Antisera to the γ Subunit*

3.1.9.1 Generation of Polyclonal Antisera in Rabbits

γ subunit (750 µg) prepared as described above was dialysed overnight *versus* 50 mM HEPES pH 7 (1 litre) at 4 °C. A portion of this (250 µl, 300 µg) was added to an equal volume of Freund's incomplete adjuvant and mixed thoroughly to obtain an emulsion. This was injected intradermally into a female New Zealand white rabbit at multiple sites at the peripheral lymph nodes so as to maximally stimulate the immune response. Ten days after the initial immunisation, the rabbit was boosted with another 0.6 ml of adjuvant-antigen suspension containing 300 µg γ subunit. The rabbit was bled from the lateral ear vein 7, 14, and 21 days later. Serum was treated at 37 °C for 60 min to induce clot formation. The clot was 'rung' from the sides of the collection tube which was allowed to stand overnight at 4 °C. The clot was then removed by centrifugation. Antibody titres were determined by measurement of the inhibition of holoenzyme activity in a replication assay. Sera was stored at -20 °C until use.

3.1.9.2 Titration of Anti-γ Ig

The assay is based on the ability of holoenzyme to replicate the single-stranded genome of bacteriophage G4 to the double-stranded form (Maki and Kornberg,

1988d).

A reaction mixture was prepared that contained assay buffer (20 mM Tris.HCl pH 8.0, 4% (v/v) glycerol, 80 µg/ml BSA, 8 mM DTT), 8 mM Mg^{2+}, 600 ng SSB, 500 ng primase, 16 ng β, 2.1 mM ATP, 50 µM tritiated dNMP's and R199G4ori DNA. Portions (18.5 µl) were dispensed to chilled glass assay tubes at 0 °C. Serial dilutions of the anti-γ Ig (collected 14-days after immunisation) were made in the assay buffer in separate tubes at 0 °C. DNA polymerase III* (10 U[1]) was added to each dilution of serum and left at 0 °C for 10 min. The assay was started by the addition of 18.5 µl of the assay mixture; the reaction was carried out at 30 °C for 10 min. The reaction was terminated by the adddition of 100 µl of ice-cold 'Reaction Stop' (10 mM EDTA, 0.2 mg/ml herring sperm DNA) and cooling at 0 °C. The DNA was then precipitated by the addition of 1 ml of 10% (v/v) trichloroacetic acid containing 100 mM sodium pyrophosphate on ice and cooling at 0 °C for at least 10 min. The contents of the glass tubes were filtered through Whatman GF/C glass fibre filters. The filters were washed in 1 M HCl, 100 mM sodium pyrophosphate, dehydrated with ethanol, dried, and residual radioactivity determined in a Beckman LS 6000IC liquid scintillation counter. By reference to a standard, the amount of label incorporated into the replicated DNA could be determined. From this data, the highest dilution of antiserum at which holoenzyme activity was completely inhibited was determined.

3.1.10 *Western Blotting*

3.1.10.1 Electrophoretic Separation of Antigens

Antigens to be blotted were first separated by SDS-PAGE. The gel and nitrocellulose membrane (BioRad), cut to the dimensions of the gel, were equilibrated in blotting buffer (48 mM Tris, 39 mM glycine, 0.04% (w/v) SDS, and 20% (v/v) methanol) for 30 min prior to blotting. A blotting 'sandwich' was prepared as described (Sambrook *et al.*, 1989), and electrophoretic transfer was undertaken at 10 V for 18 h at 4 °C in a Bio-Rad blotting apparatus. The efficiency of transfer was gauged by the intensity of the pre-stained molecular weight markers on the membrane and by Coomassie blue staining of the gel following transfer.

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[1]A unit is the amount of enzyme catalysing the incorporation of 1 pmol nucleotide in 1 min at 30 °C.

3.1.10.2 Detection of Bound Antigens Using the Enzyme-Coupled Antibody System

In this enzyme-coupled system the substrate, BCIP/NBT, is converted *in situ* to a deep blue compound by alkaline phosphatase conjugated to a secondary antibody (Sambrook *et al.*, 1989).

The membrane was washed in 100 ml of TBS (20 mM Tris, 500 mM NaCl) with gentle agitation at room temperature. Non-specific binding sites on the membrane were blocked by washing the membrane in 100 ml of TTBS (20 mM Tris, 500 mM NaCl, 0.2% (v/v) Tween 20) containing 3% (w/v) gelatin for 1 h at room temperature. Excess blocking solution was removed by washing the membrane in 100 ml of TTBS. The membrane was allowed to stand for 2.5 h at 4 °C in a solution containing the primary antibody (rabbit anti-γ subunit) that had been diluted 1/1500 in TTBS containing 1% (w/v) gelatin. The membrane was washed twice in TTBS, and then treated for 2 h in a solution containing the secondary antibody (goat anti-rabbit alkaline phosphatase-(IgG)) diluted 1/2000 in TTBS. Excess secondary antibody was removed by washing 3 times in TTBS. The colour reaction was carried out as described (Sambrook *et al.*, 1989). When the blue colour had developed sufficiently, the reaction was terminated by immersion of the membrane in 5% (v/v) acetic acid.

3.2 RESULTS

3.2.1 *Overproduction of the dnaX Gene Products*

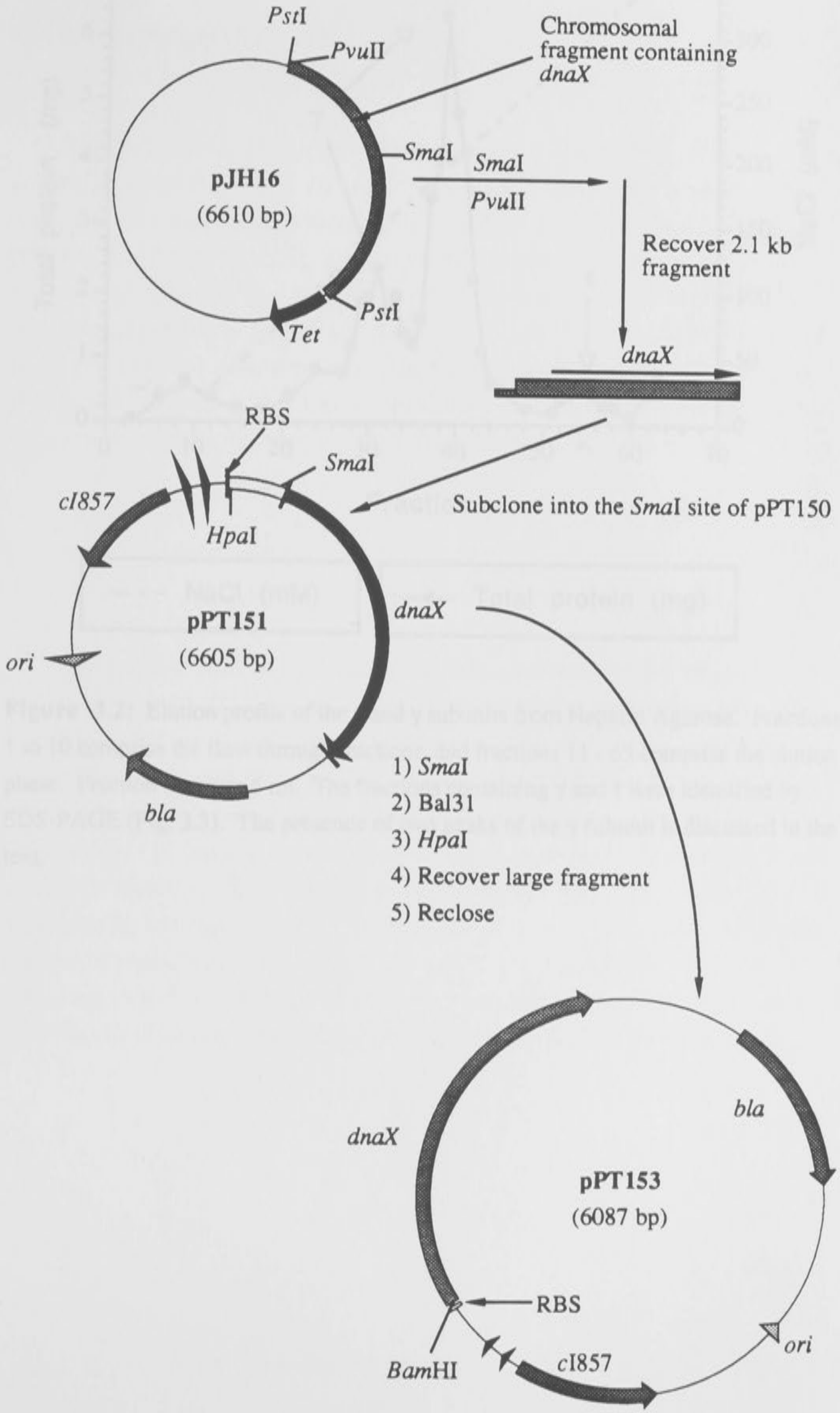
3.2.1.1 Construction of pPT151

Transformants were initially selected on the basis of ampicillin resistance and screened for temperature sensitivity. Plasmid DNA was prepared (Section 2.2.5.1) from selected transformants and checked on the criteria of:

a) the presence of correctly-sized plasmids (6.5 kb) from a *Hpa*I digest, indicating that the vector contained the *dnaX* insert, and

b) correct orientation of the insert downstream from the λ promoters from a *Hpa*I-*Sma*I double digest (Fig. 3.1).

Figure 3.1: Overproduction of the *dnaX* gene products. Briefly, a 2.1 kb *Sma*I-*Pvu*II fragment containing the *dnaX* gene from pJH16 was isolated and inserted into the *Sma*I site of 5´-dephosphorylated pPT150 by blunt-end ligation. Ampicillin resistant transformants of *E. coli* AN1459 were selected on LB plates at 30 °C. Transformants harbouring recombinant plasmid showed low expression of the τ and γ subunits (data not shown). Plasmid DNA prepared (Section 2.2.5.2) from one transformant was linearised with *Sma*I, and then treated with sufficient Bal31 to remove around 130 bp from each end (Section 2.2.10). Treatment of the Bal31-digested fragment with *Hpa*I (Section 2.4) generated a large fragment that was isolated by electroelution onto NA45 membrane following agarose gel electrophoresis. The purified DNA fragment was repaired, reclosed and *E. coli* strain AN1459 was transformed with part of the ligation mixture.


PstI
PvuII
Chromosomal fragment containing dnaX
SmaI
pJH16
(6610 bp)
SmaI
PvuII
Recover 2.1 kb fragment
PstI
Tet
dnaX
RBS
SmaI
cI857
HpaI
Subclone into the SmaI site of pPT150
dnaX
pPT151
(6605 bp)
ori
bla
1) SmaI
2) Bal31
3) HpaI
4) Recover large fragment
5) Reclose
bla
dnaX
pPT153
(6087 bp)
RBS
ori
BamHI
cI857

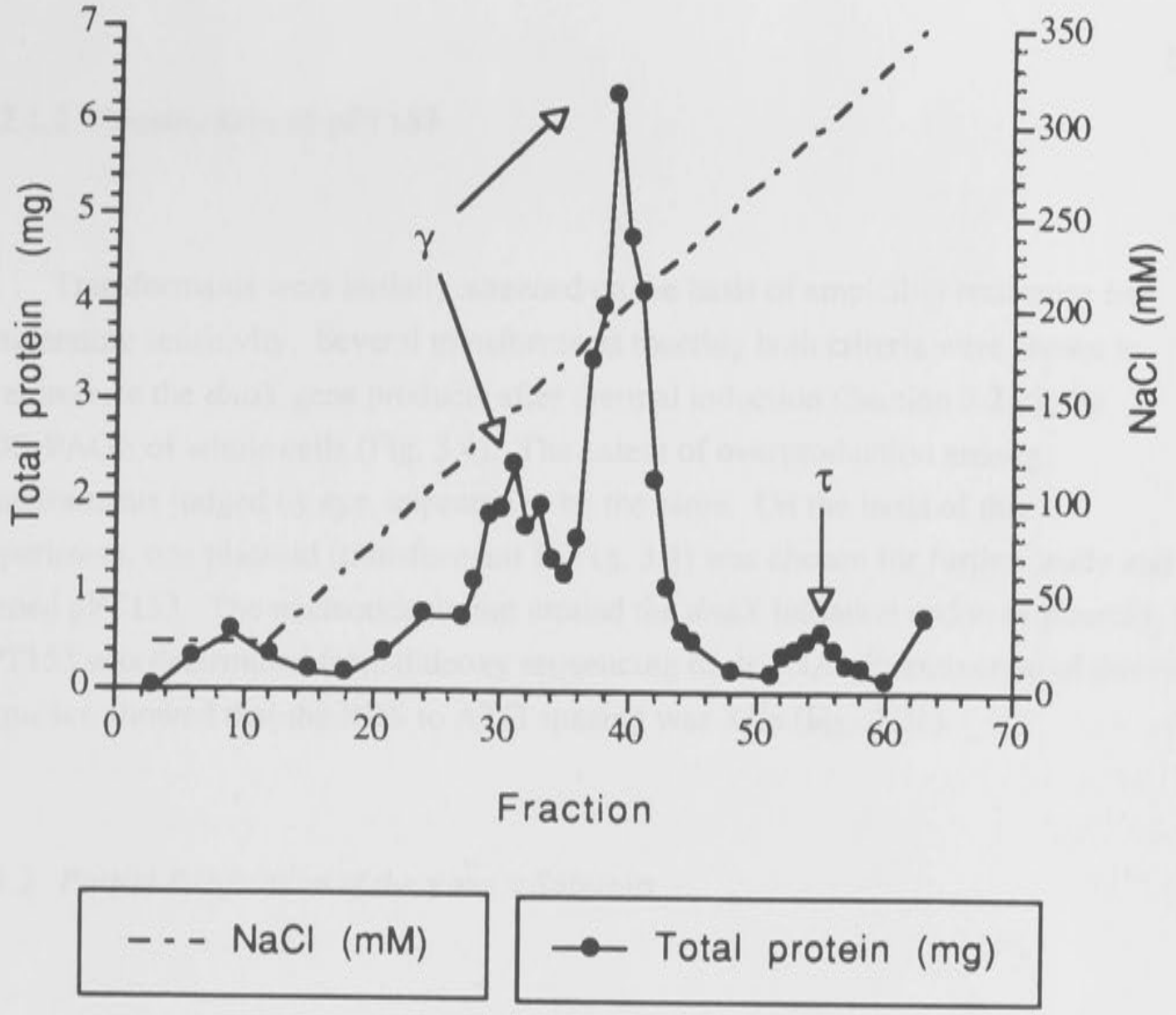


Figure 3.2: Elution profile of the τ and γ subunits from Heparin Agarose. Fractions 1 to 10 comprise the flow through fractions, and fractions 11 - 63 comprise the elution phase. Fraction size was 6 ml. The fractions containing γ and τ were identified by SDS-PAGE (Fig. 3.3). The presence of two peaks of the γ subunit is discussed in the text.

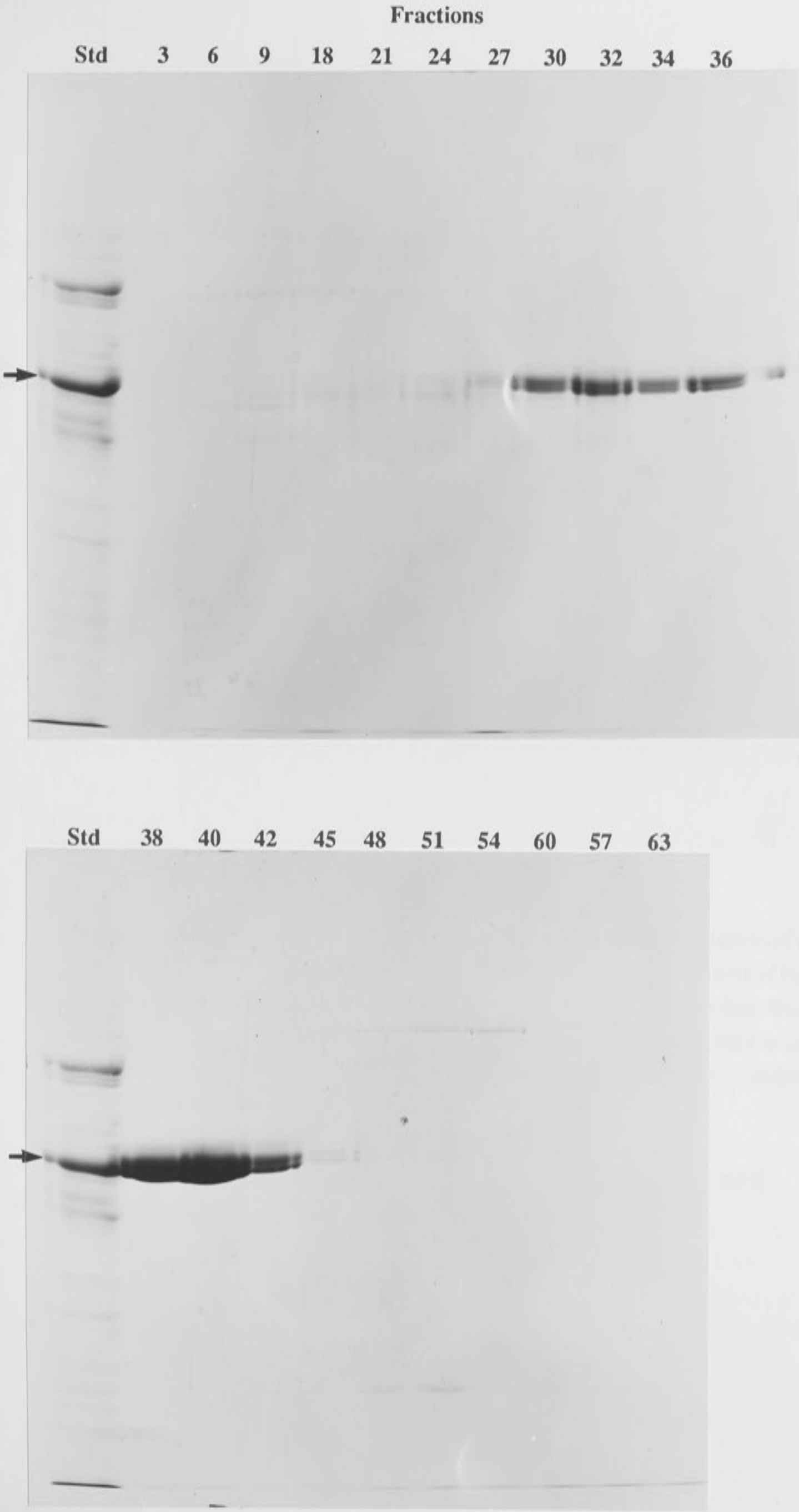
3.2.1.2 Construction of pPT153

Transformants were initially screened on the basis of ampicillin resistance and temperature sensitivity. Several transformants meeting both criteria were shown to overproduce the *dnaX* gene products after thermal induction (Section 2.2.21) by SDS-PAGE of whole cells (Fig. 3.4). The extent of overproduction among transformants judged by eye, appeared to be the same. On the basis of this experiment, one plasmid (transformant E, Fig. 3.4) was chosen for further study and named pPT153. The nucleotide region around the *dnaX* initiation codon of plasmid pPT153 was determined from dideoxy sequencing of dsDNA. Examination of this sequence showed that the RBS to ATG spacing was 3 bp (Fig. 2.3c).

3.2.2 *Partial Purification of the γ and τ Subunits*

The γ subunit of DNA polymerase III holoenzyme was purified (Fig. 3.5; Table 3.1) from AN1459/pPT153 in a procedure utilising selective precipitation of proteins from a crude cell lysate with $(NH_4)_2SO_4$ followed by chromatography on heparin agarose (Figs. 3.2 and 3.3, Table 3.1). Under some conditions of SDS-PAGE the γ subunit appeared as a pair of bands. This phenomenon was also observed by Maki and Kornberg (1988a) who showed from N-terminal amino acid sequencing that both bands were identical. There were two peaks of γ protein (Fig. 3.2). Several milligrams began to elute at a NaCl concentration of approximately 145 mM, with the peak of the second γ fraction eluting at 200 mM NaCl. It is possible that γ was interacting with other proteins. The τ subunit requires further purification; nearly all of the τ loaded did not bind to Heparin Agarose, eluting in the load flow-through.

Figure 3.3: Chromatography of the γ and τ subunits on Heparin Agarose. The chromatography was performed as described (Section 3.1.8). A portion of each fraction (25 µl) was subjected to SDS-PAGE (10% gel) (Section 2.2.22) and the proteins were visualised by staining with Coomassie Brilliant Blue. The marker is whole cells of AN1459/pPT153 induced for 2.5 h at 42 °C. The γ and τ subunits are indicated on the gels by arrows. The appearance of bands as doublets is likely an artifact of conditions used for denaturation.


Fractions
Std
3
6
9
18
21
24
27
30
32
34
36
Std
38
40
42
45
48
51
54
60
57
63

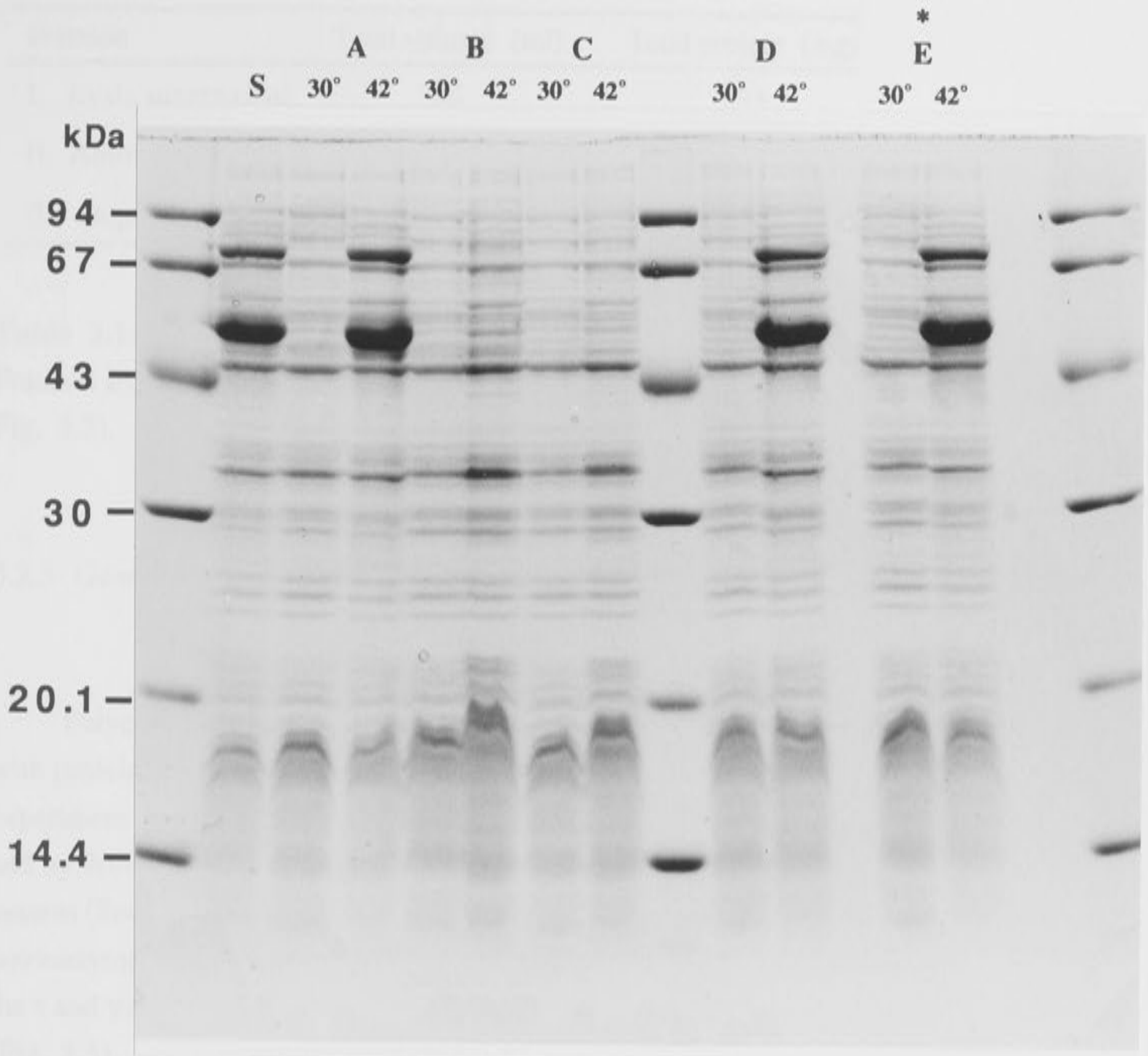


Figure 3.4: Induction of *E. coli* strains AN1459 harbouring derivatives of plasmid pPT151 generated by treatment with nuclease Bal31 so as to remove ≈134 bp of DNA preceeding the *dnaX* start codon. The experiment was performed as described (Section 3.1.4 and Fig. 3.1). Transformants were induced (Section 2.2.21) and the cells subjected to SDS-PAGE (12% gel) (Section 2.2.22). Proteins were visualised by staining with Coomassie Brilliant Blue.

S = marker for γ and τ using AN1459/pPT153 induced for 2.5 h at 42°C

* = AN1459/pPT153, used for subsequent isolation of γ and τ

Fraction	Total volume (ml)	Total protein (mg)
I. Lysis supernatant	35	235
II. Ammonium sulfate	25	85
III. Heparin Agarose	48	27.6

Table 3.1: Partial purification of the γ subunit of DNA polymerase III holoenzyme. Fraction III is a pool of Heparin Agarose fractions 35 - 42 containing the γ subunit (see Fig. 3.2).

3.2.3 *Generation of Antibody to the γ Subunit*

Polyclonal antibody to the γ subunit was raised in a New Zealand white rabbit with protein purified from AN1459/pPT153 (Table 3.1). The success of this experiment was checked by titration of antibody in a replication assay (Section 3.1.9), and by Western blotting of selected antigens using an enzyme-coupled detection system (Section 3.1.10). Antibody to the γ subunit completely inhibited the activity of holoenzyme at a dilution of 1/80 (Table 3.2). As expected, antibody recognised both the τ and γ subunits in holoenzyme as well as purified recombinant γ subunit (Fig. 3.5).

3.3 DISCUSSION

Overproduction of the dnaX Gene Products

The γ and τ subunits of DNA polymerase III holoenzyme have been expressed to high levels in *E. coli* using the bacteriophage λ promoter vector pPT150. Gene trimming techniques were employed to remove the native RBS, and to fuse the vector RBS to within a few bases of the *dnaX* start codon. A sequencing experiment showed that the RBS to *dnaX* ATG spacing was 3 bp (Fig. 2.3c). This would appear to contradict data claiming that RBS to ATG spacings of less than 5 bp result in very poor yields of proteins (Stormo, 1984), (but counting of nucleotides in the spacer region

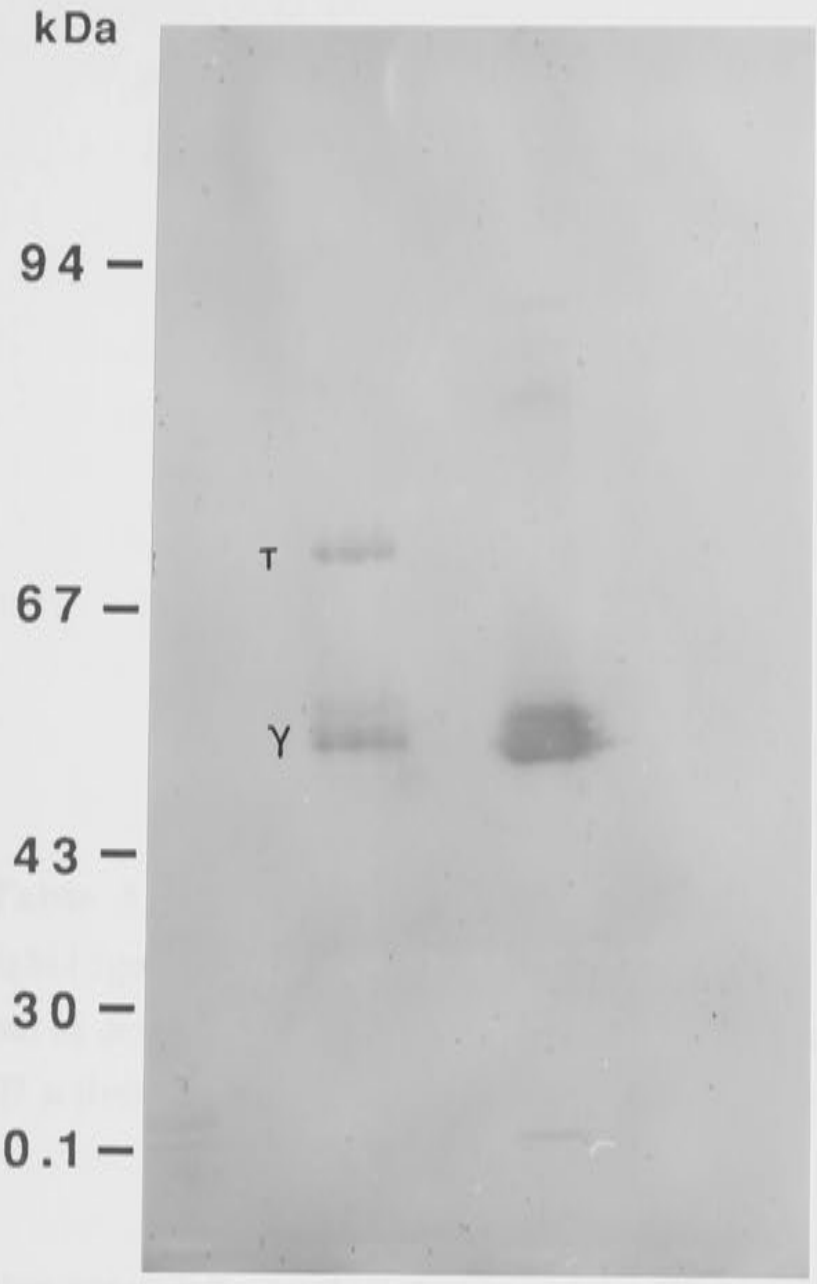


Figure 3.5: A Western blot of the γ and pol III* subunits. The experiment was performed as described (Section 3.1.10) using polyclonal antibody raised to purified γ subunit (Section 3.1.9.1). The positions of the molecular weight markers are superimposed onto the figure.

Dilution of anti-γ Ig	Label incorporated (pmol)
pol III, no serum	226
1/5	<0.02
1/10	<0.02
1/40	<0.03
1/80	4.4 ←
1/160	23
1/320	50
1/640	133

Table 3.2: The inhibition of pol III activity in the replication of ssM13gori to dsM13gori by antibody raised to the γ subunit of holoenzyme. The assay was carried out as described (Section 3.1.9). The arrow indicates the highest dilution at which pol III activity was inhibited.

also depends on one's definition of the RBS). In fact, the level of overproduction attained as described above is at least as good as that obtained by other groups (Maki and Kornberg, 1988a) who used a similar expression system. In the heat-treated strain the τ and γ subunits together comprise about 20% of total cellular protein (Fig. 3.4).

It is interesting to note that the τ and γ subunits are not expressed in equal amounts when synthesised by strains carrying pPT153 (Fig. 3.4). Indeed, the γ subunit appears by eye to be approximately 3 to 4 times more abundant than τ. This phenomenon is also apparent in the overproducing strain constructed in which *dnaX* is transcribed from the bacteriophage λ promoter, P_L (Maki and Kornberg, 1988a). In contrast, non-overproducing cells and cell-free transcription-translation systems express the two subunits in approximately equal amounts (McHenry, 1988). It is conceivable that the translational frameshifting mechanism responsible for the generation of the γ subunit (Tsuchihashi and Kornberg, 1990; Flower and McHenry, 1990; Blinkowa and Walker, 1990) is exaggerated in high-level expression systems.

Partial Purification of the γ and τ Subunits of DNA Polymerase III Holoenzyme

The γ subunit of DNA polymerase III holoenzyme was purified (Fig. 3.6; Table 3.1) in a three-step procedure utilising selective precipitation of proteins followed by ion exchange chromatography. The τ subunit requires further purification. Nearly all of the τ subunit in fraction I applied to the Heparin Agarose column did not bind, and eluted in the flow-through. Since other proteins were present in the flow-through, including some γ subunit, this suggests that too much protein was applied to the column. However, a very small portion of the τ loaded did bind, co-eluting with some of the γ subunit (fractions 26 - 34). Due to the relatively low abundance of τ in the induced cells of AN1459/pPT153, conditions will need to be optimised for its recovery. This would primarily be achieved by ensuring that τ binds to the Heparin Agarose, or another resin used in its place, possibly DEAE-Trisacryl (Maki and Kornberg, 1988a). Alternatively, it is now possible to engineer overproducers of each subunit separately. This could be accomplished using site-directed mutagenesis (see Tsuchihashi and Kornberg, 1990). This would not only simplify their purification, but abolition of expression of the γ subunit should raise the level of expression of τ.

The presence of two species of γ showing different chromatographic properties on Heparin Agarose is curious, especially as this phenomenon has not been reported by other groups (Maki and Konberg, 1988a) who have successfully purified both subunits using a procedure similar to that described above. However, one needs to be circumspect as the experiment was performed only once. It is possible that a portion of the γ subunit is interacting with another protein. A contaminating band (≈ 68 kDa) is visible in the γ fraction that elutes in fractions 26 - 34 from heparin agarose (Fig. 3.2), whereas the second γ fraction (35 - 42) is judged to be electrophoretically pure. The variability in resolution could also be due to batch variation in the Heparin Agarose used in this study. Binding to this resin is a function of the amount of heparin conjugated to the sepharose. Gel filtration, sedimentation studies and activity assays will be necessary to establish whether the observation of two γ populations is a *bona fide* phenomemon, or is caused by a technical difficulty.

Assay of biological activity of τ and γ is based on complementation of the deficiency of γ and τ function in a crude enzyme fraction from mutant dnaX^{ts} cells. This assay involves the conversion of ssM13Gori1 to the (ds) replicative form, a process which requires γ and τ (Maki and Kornberg, 1988a). This assay was not used in this study as the study of τ and γ was discontinued when other groups (while this work was in progress) published extensively (Maki and Kornberg, 1988a to 1988d).

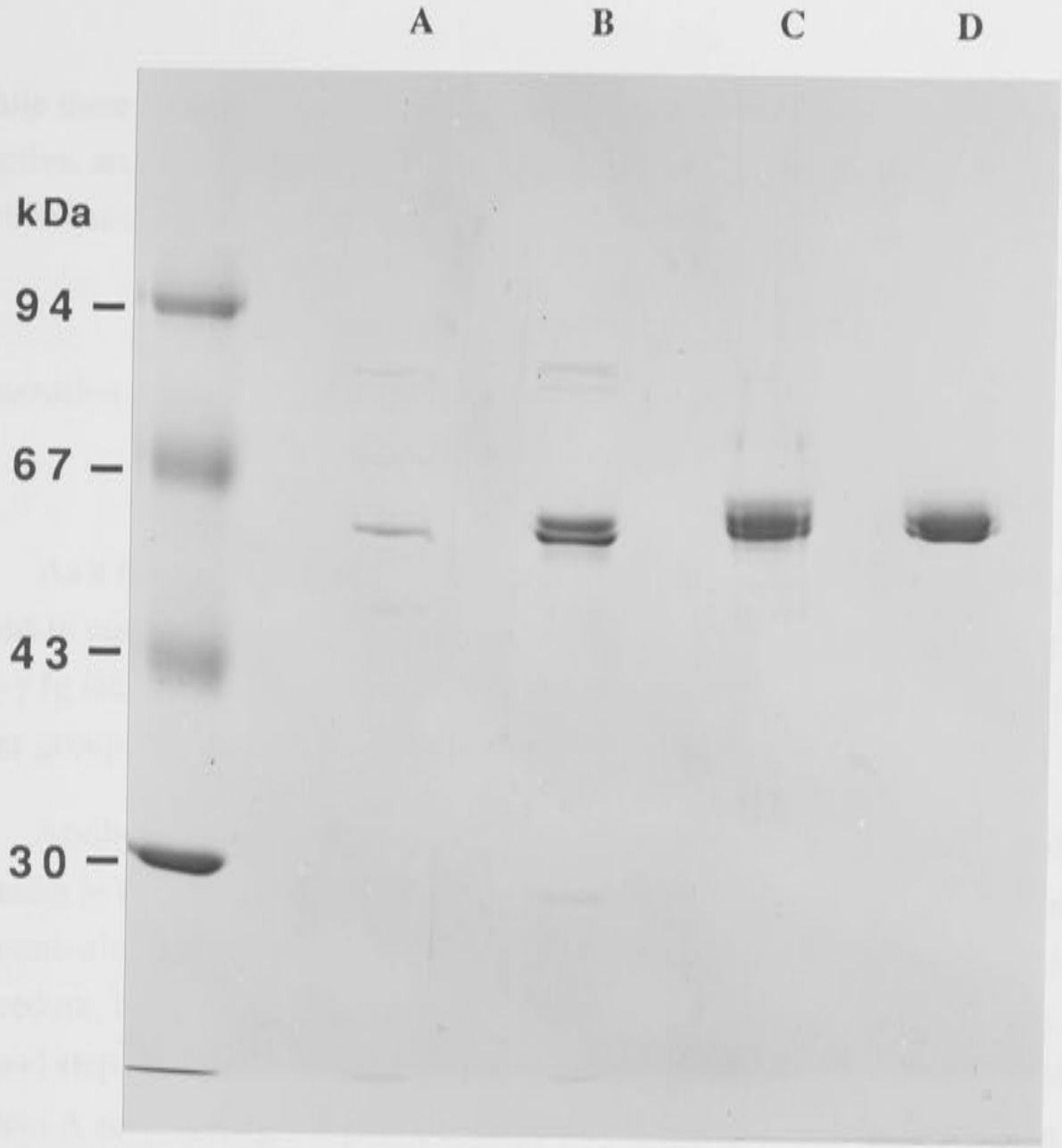


Figure 3.6: Purification of the γ subunit of DNA polymerase III holoenzyme (Sections 3.1.6 to 3.1.8). Approximately 7.5 μg of protein from each stage of the purification protocol was subjected to SDS-PAGE (10% gel); proteins were visualised by staining with Coomassie Brilliant Blue.

A = Lysis supernatant

B = Ammonium sulfate

C = Heparin Agarose, fraction 31 (first γ peak)

D = Heparin Agarose, fraction 39 (second γ peak)

While there is no reason to believe that the τ and γ subunits purified in this study are inactive, an activity assay will need to be developed to verify the activity of γ and τ purified according to Table 3.1.

Generation of Polyclonal Antibodies to the γ and τ Subunits

As a first step to studies of structure and function, polyclonal antibodies were raised to purified γ subunit. Immunoblotting of holoenzyme components showed that anti-γ Ig recognises both the τ and γ subunits (Fig. 3.5), corroborating findings by other groups (Maki and Kornberg, 1988a).

Antibodies raised to the γ (and τ) subunit could be used for the localisation of subunits in the DNA polymerase III holoenzyme complex using the technique of immune-electron microscopy, eg. with 'immuno gold' (Bendayan, 1984). In this procedure, highly purified holoenzyme would be incubated with anti-γ Ig. In the second step, a 'protein A-gold' complex is added to the mixture. The molecules of protein A surrounding the gold particles would interact with the Fc region of the anti-γ Ig. The immunoglobulin-gold complexes and thus, the location of the γ and τ subunits in holoenzyme, could be visualised by transmission electron microscopy. This approach has been used with some success in showing the presence of DNA-binding protein H-NS (also known as protein HU) in the nucleoid (Durrenburger *et al.*, 1991), in localisation of membrane proteins (Shore *et al.*, 1981), and in studies on cellular enzymes (Bendayan and Reddy, 1982; Bendayan *et al.*, 1983).

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2516-2520

CHAPTER 4

OVERPRODUCTION AND PURIFICATION OF THE *dnaQ* GENE PRODUCT.

CONSTRUCTION OF A *dnaE-dnaQ* SYNTHETIC OPERON

4.0 INTRODUCTION

4.1.1 *Replication Fidelity*

Chromosomal replication in *E. coli* is a highly accurate process: mutation rates are around 10^{-9} to 10^{-10} per base replicated (Drake, 1969). This remarkable fidelity factor for DNA replication is thought to be achieved in several ways: i) base selection in the original incorporation of the complementary nucleotide; ii) exonucleolytic editing of newly inserted bases, and iii) postreplicative scanning of the DNA for incorrectly paired nucleotides.

4.1.2 *Historical Background*

As DNA polymerase III (pol III) is responsible for the synthesis of the *E. coli* chromosome, it was not unreasonable to suggest that it was the major determinant of replication fidelity in this organism (Kornberg, 1980).

The identification of *dnaQ* and the knowledge of its role in replication fidelity was a result of work with the *mutD5* and *dnaQ49* mutator mutations[1]. These mutations cause an increase in the general rate of mutations in an organism. Dengen and Cox (1974) reported a very strong mutator phenotype in *E. coli mutD5* strains. The mutation is not conditionally lethal, and phenotypes of *mutD5* were reported to be pleiotropic. The mutator activity of *mutD5* was found to be growth medium-dependent, and *mutD5* cells were sensitive to the mutagen 5-aminoacridine (Dengen and Cox, 1974). The conditional lethal mutant *dnaQ45* was isolated from *E. coli* K12 (Horiuchi *et al.*, 1978). This mutation caused temperature-sensitive growth, defective DNA synthesis and high mutability to cells carrying the mutation (Horiuchi *et al.*, 1978). Such cells also show increased sensitivity to DNA-intercalating reagents or inhibitors of DNA gyrase. The similarity of their phenotypes and their location on the *E. coli* chromosomal map suggested that *mutD5* and *dnaQ45* were mutant alleles of the same gene (Dengen and Cox, 1974; Horiuchi *et al.*, 1978). It was subsequently confirmed with gene mapping and complementation experiments that the *mutD5* mutator and the *dnaQ49* replication mutations occured in a single gene, *dnaQ* (Cox and

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[1]A mutator mutation is one that increases the mutation rate in an organism.

Horner, 1983; Maruyama *et al.*, 1983; Scheuermann *et al.*, 1983).

Cloning of *dnaQ* and identification of its gene product was first reported by Horiuchi *et al.* (1981). A 1.5 kb fragment of *E. coli* chromosomal DNA was cloned into pBR322. This plasmid directed the expression of a 25 kDa protein that was found to complement the activity of *dnaQ45* (Horiuchi *et al.*, 1981). The plasmid was also discovered to co-overproduce RNase H. It was later reported that *dnaQ* and *rnh* are transcribed in opposite directions and that their promoter regions and translational initiation signals overlap (Fig. 4.1; Maki *et al.*, 1983). The *dnaQ* promoter was reported to be 5 times more active than that for *rnh* (Maki *et al.*, 1983). The significance of this finding, if any, is unknown. Scheuermann and Echols (1984) went on to show that the product of the *dnaQ* gene - the ε subunit of pol III - carried the exonuclease activity of holoenzyme. The complete nucleotide sequence of *dnaQ* has been determined (Takano *et al.*, 1986; see Appendix A).

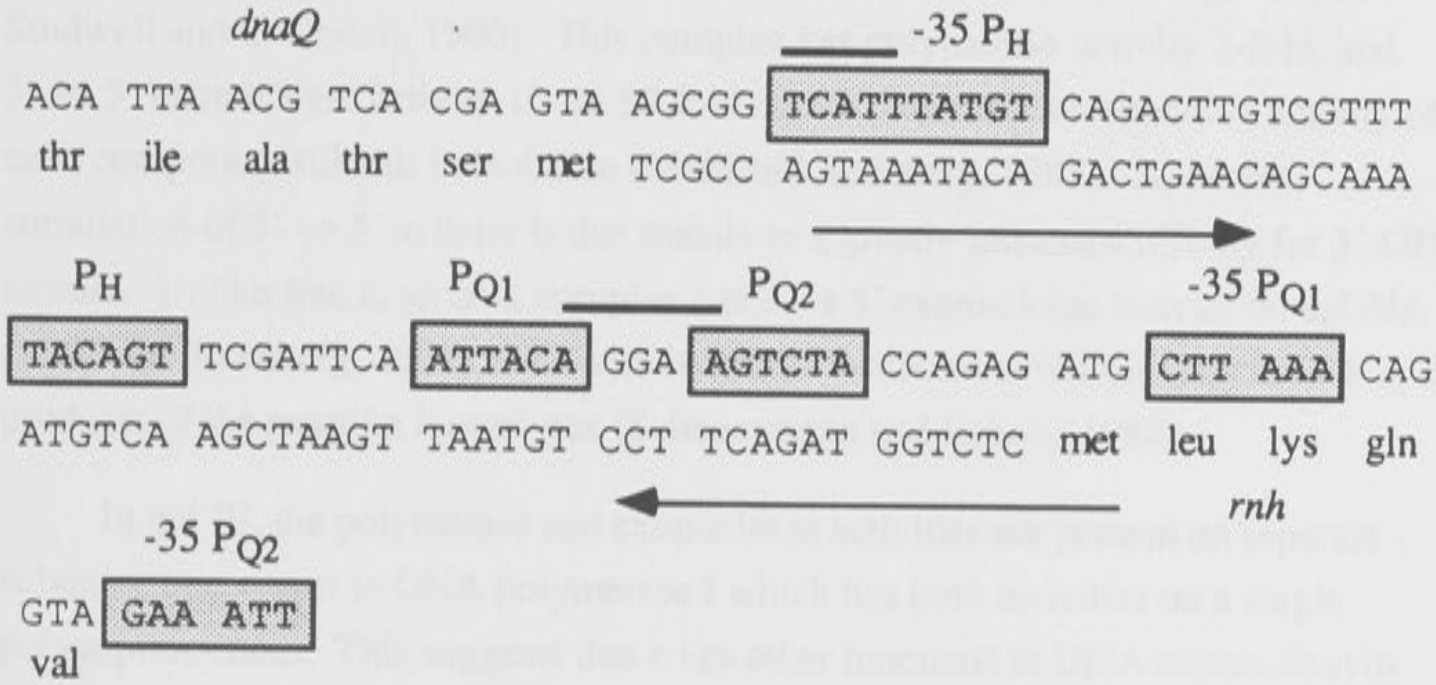


Figure 4.1: Nucleotide sequence of the region between the *dnaQ* and *rnh* genes. The nucleotide sequence of the coding strand of the *dnaQ* gene is given 3′ to 5′ and that of the *rnh* gene is given 5′ to 3′. The -10 and -35 sequences for the *dnaQ* (P_Q1, P_Q2, -35 P_{Q1} and -35 P_{Q2}) and for the *rnh* (P_H, -35 P_H) are shown enclosed in shaded boxes. Bold lines indicate possible RBS. The predicted amino acid sequences are shown below the DNA sequence. The regions of dyad symmetry are marked by arrows. Adapted from Maki *et al*. (1983).

4.1.3 *Properties and Physiological Roles*

The first characterisation of the ε subunit purified from overproducing strains was carried out by Scheuermann and Echols (1984) and Maki and Kornberg (1987). The enzyme possesses 3′ → 5′exonuclease activity specific for single-stranded termini. The ε subunit behaves on a denaturing polyacrylamide gel as a 28 kDa species, and its predicted molecular weight is 26950. ε is normally part of a complex that also includes the α (polymerase) and θ subunits of DNA polymerase III, which together form the core polymerase. In the absence of the α subunit, ε was reported to have only weak affinity for paired and mispaired 3′-hydroxyl termini (Maki and Kornberg, 1987). The ε subunit was reported to be active on ssDNA (Brenowitz *et al.*, 1991) and a range of other substrates such as RNA (Griep *et al.*, 1990). On the addition of α subunit to solutions containing ε, an α–ε complex comprising stoichiometric amounts of both subunits is formed (Maki and Kornberg, 1987; Studwell and O'Donell, 1990). This complex has polymerase activity 2-fold, and 3′ → 5′ exonuclease activity 10- to 80-fold higher than the corresponding activity of each component subunit in isolation (Maki and Kornberg, 1987). Apparent stimulation of 3′ → 5′ activity is due mainly to a greatly increased affinity for 3′-OH termini. Unlike free ε, an α–ε complex has 3′→ 5′ exonuclease activity on dsDNA (Maki and Kornberg, 1987). The activity of the ε subunit is inhibited by dNMP's, the products of the reaction it catalyses (Scheuermann and Echols, 1984).

In pol III, the polymerase and exonuclease activities are present on separate subunits, in contrast to DNA polymerase I which has both activities on a single polypeptide chain. This suggests that ε has other functions in DNA metabolism in addition to its proofreading role in the replication of the *E. coli* chromosome (DiFrancesco *et al.*, 1984; Kunkel, 1988). Ciesla *et al.* (1990) reported that the frequency of UV-induced lesions in the chromosome is reduced 10-fold in cells overproducing the ε subunit. There is some evidence to show that RecA and one or both of the UmuDC proteins interact with the ε subunit during the SOS response (Foster and Sullivan, 1988). Thus, ε may have a separate role in the SOS response.

4.1.4 *Aims*

The *dnaQ* gene product was first overproduced by Scheuermann and Echols (1984) using a plasmid vector in which expression of ε was placed under the control

of the bacteriophage λ promoter, P_L. The level of ε synthesis achieved from this overproducing plasmid was less than 1% of total cellular protein. Examination of the native RBS of *dnaQ* present in the *dnaQ* plasmid constructed by Scheuermann and Echols (1984) revealed poor homology to the consensus RBS sequence, implying that high level expression of ε may have been limited by poor translation initiation. Replacement of the native RBS with one carrying a consensus Shine-Dalgarno sequence brought the level of synthesis of the ε subunit up to approximately 10% of total cell protein (Scheuermann and Echols, 1984).

One approach to studies of the properties of the α–ε complex would normally involves reconstitution from subunits purified from separate overproducing strains. Another approach is to construct a plasmid that directs the synthesis of both gene products simultaneously. This would not only simplify purification, but formation of α–ε complex might more closely resemble that occuring *in vivo*. Co-overproduction of the α and ε subunits may overcome the problem of low solubility of both α and ε when separately overproduced to high levels (Beck, unpublished observations; Scheuermann and Echols, 1984)

This Chapter comprises two bodies of work: the overproduction and purification of the *dnaQ* gene product, and the construction of a synthetic operon containing the *dnaE-dnaQ* genes. Co-overproduction of α and ε was undertaken in the hope the synthesis of a soluble α–ε complex would be less toxic to the cell, and the complex would be more convenient to purify than either subunit separately.

4.1 MATERIALS AND METHODS

DNA manipulations used in this work have been described in Chapter 2 and will be cited where appropriate.

4.1.1 *Resins*

Sephadex G-25 and DEAE-Sephacel were purchased from Pharmacia (Sweden). Blue Gel A was purchased from Amicon (U.S.A.).

4.1.2 *Construction of pPL224*

The plasmid pPL224 was constructed by P.E. Lilley as outlined in Fig. 4.2 (unpublished data), using a DNA fragment containing the *dnaQ* gene present in plasmid pNS360 (a kind gift from Dr. Harrison Echols, Berkeley). This construction placed transcription of *dnaQ* under the control of the tandem λ promoters in vector pND201 (Elvin *et al.*, 1990), and replaced its native RBS with one complementary to the 3´-OH end of 16s rRNA, spaced 11 bp upstream of the initiating ATG codon.

4.1.3 *Construction of a dnaE-dnaQ Synthetic Operon*

The construction of plasmid pPT152 which carries the *dnaE-dnaQ* synthetic operon is outlined in Figure 4.3. A 1.0 kb *Bam*HI-*Eco*RI fragment containing the *dnaQ* gene was isolated following a *Bam*HI partial digest of *Eco*RI-treated pPL224. The fragment was end-filled (Section 2.2.7) and ligated (Section 2.2.9) to phosphorylated 12 bp *Eco*RI linkers. Excess linker was removed by *Eco*RI digestion and the desired fragment isolated by electroelution onto NA45 membrane following agarose gel electrophoresis. The purified *Eco*RI fragment containing the *dnaQ* gene was subsequently inserted into the 5´-dephosphorylated *Eco*RI site of pPL250 (a kind gift from Mrs P. Lilley). This places the *dnaQ* gene downstream of the *dnaE* gene. *E. coli* strain AN1459 was transformed by part of the ligation mix. Ampicillin resistant transformants were selected for screening.

4.1.4 *Purification of the ε Subunit*

4.1.4.1 Large-scale Fermentation of Strain AN1459/pPL224

Forty litres of LB supplemented with thymine and containing ampicillin (50 μg/ml) were inoculated with an overnight culture of AN1459/pPL224. When the cell density of the culture at 595 nm reached ≈ 0.5, the temperature was rapidly shifted from 30 °C to 42 °C to induce the overproduction of ε. After 165 min, the cells were chilled and then harvested in a Sharples centrifuge. The cell paste (44.9 g) was

Figure 4.2: Construction of pPL223 and pPL224 (P. Lilley, unpublished data).

A 1.0 kb *Eco*RI fragment containing the *dnaQ* gene present in plasmid pNS360 (Scheuermann and Echols, 1984) was isolated and inserted into the *Eco*RI site of pND201. Transformants containing pPL223 overproduced the ε subunit to a moderate level following induction at 42 °C. The spacing between the RBS and *dnaQ* initiation codon was shown to be 17 bp by dideoxy sequencing of dsDNA.

Further manipulations reduced the RBS to ATG spacing to 11 bp (pPL224) which was confirmed by dideoxy sequencing.

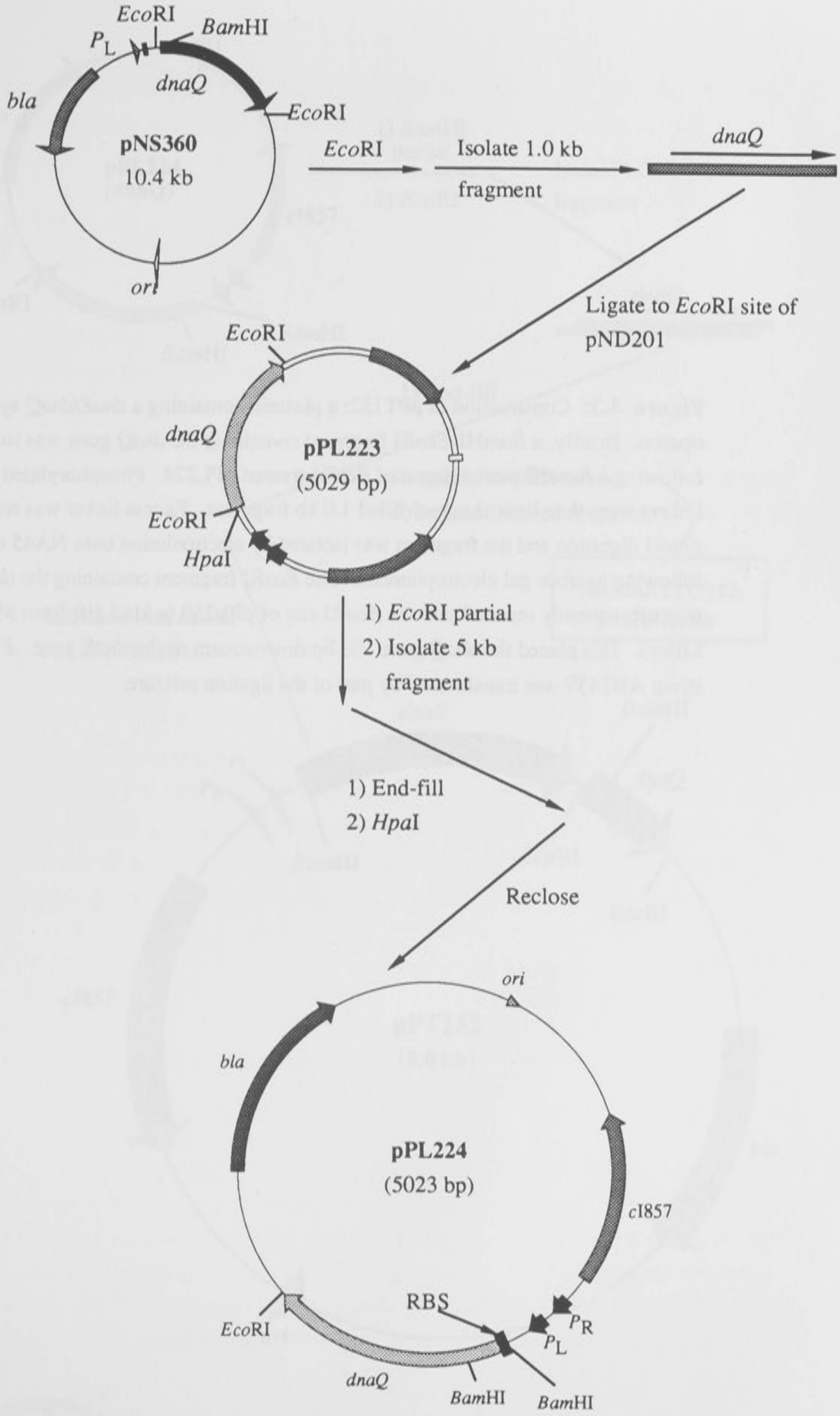
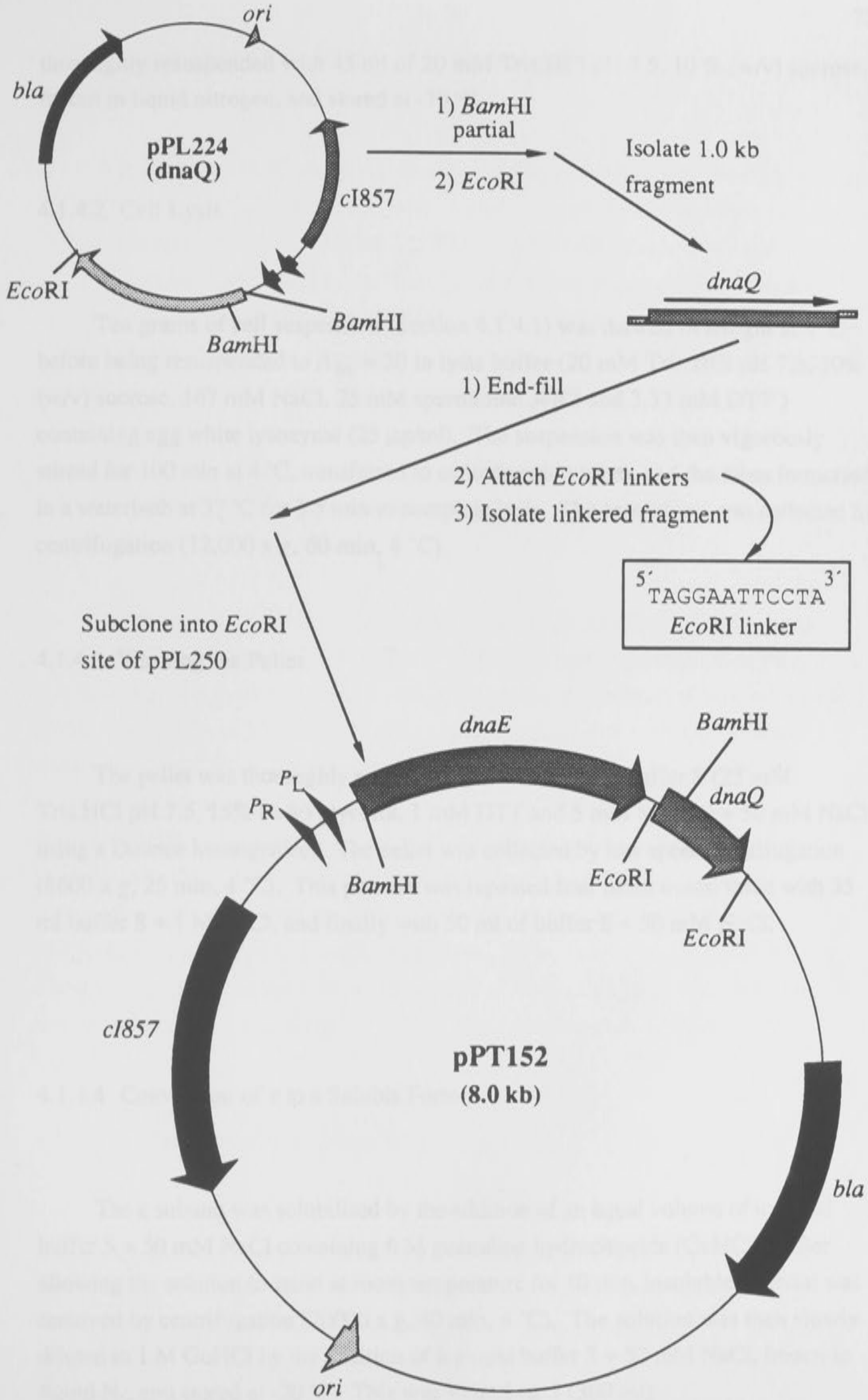

EcoRI
BamHI
P_L
dnaQ
bla
EcoRI
pNS360
10.4 kb
ori
EcoRI
Isolate 1.0 kb
fragment
dnaQ
Ligate to EcoRI site of
pND201
EcoRI
dnaQ
pPL223
(5029 bp)
EcoRI
HpaI
1) EcoRI partial
2) Isolate 5 kb
fragment
1) End-fill
2) HpaI
Reclose
ori
bla
pPL224
(5023 bp)
cI857
RBS
P_R
P_L
EcoRI
dnaQ
BamHI
BamHI

Figure 4.3: Construction of pPT152: a plasmid containing a *dnaE/dnaQ* synthetic operon. Briefly, a *Bam*HI-*Eco*RI fragment containing the *dnaQ* gene was isolated following a *Bam*HI partial digest of *Eco*RI-treated pPL224. Phosphorylated *Eco*RI linkers were then ligated to end-filled 1.0 kb fragment. Excess linker was removed by *Eco*RI digestion and the fragment was isolated by electroelution onto NA45 membrane following agarose gel electrophoresis. The *Eco*RI fragment containing the *dnaQ* gene was subsequently inserted into the *Eco*RI site of pPL250 (a kind gift from Mrs P. Lilley). This placed the *dnaQ* gene 103 bp downstream of the *dnaE* gene. *E. coli* strain AN1459 was transformed by part of the ligation mixture.


ori
bla
pPL224
(dnaQ)
cI857
EcoRI
BamHI
BamHI
1) BamHI
partial
2) EcoRI
Isolate 1.0 kb
fragment
dnaQ
1) End-fill
2) Attach EcoRI linkers
3) Isolate linkered fragment
5′ TAGGAATTCCTA 3′
EcoRI linker
Subclone into EcoRI
site of pPL250
dnaE
BamHI
PL
PR
dnaQ
BamHI
EcoRI
EcoRI
cI857
pPT152
(8.0 kb)
bla
ori

thoroughly resuspended with 45 ml of 20 mM Tris.HCl pH 7.5, 10 % (w/v) sucrose, frozen in liquid nitrogen, and stored at -70 °C.

4.1.4.2 Cell Lysis

Ten grams of cell suspension (Section 4.1.4.1) was thawed overnight at 4 °C before being resuspended to A_{595} = 20 in lysis buffer (20 mM Tris.HCl pH 7.5, 10% (w/v) sucrose, 167 mM NaCl, 25 mM spermidine.3HCl and 3.33 mM DTT) containing egg white lysozyme (25 µg/ml). The suspension was then vigorously stirred for 100 min at 4 °C, transferred to centrifugation tubes, and the tubes immersed in a waterbath at 37 °C for 3.5 min to complete lysis. The insoluble ε was collected by centrifugation (12,000 x *g*, 60 min, 4 °C).

4.1.4.3 Washing the Pellet

The pellet was thoroughly resuspended in ≈ 120 ml of buffer S (25 mM Tris.HCl pH 7.5, 15% (w/v) glycerol, 1 mM DTT and 5 mM $MgCl_2$) + 50 mM NaCl using a Dounce homogenizer. The pellet was collected by low speed centrifugation (8600 x *g*, 25 min, 4 °C). This process was repeated four more times: three with 35 ml buffer S + 1 M NaCl, and finally with 50 ml of buffer S + 50 mM NaCl.

4.1.4.4 Conversion of ε to a Soluble Form

The ε subunit was solubilised by the addition of an equal volume of ice-cold buffer S + 50 mM NaCl containing 6 M guanidine hydrochloride (GuHCl). After allowing the solution to stand at room temperature for 10 min, insoluble material was removed by centrifugation (20000 x *g*, 40 min, 4 °C). The solution was then slowly diluted to 1 M GuHCl by the addition of ice-cold buffer S + 50 mM NaCl, frozen in liquid N_2, and stored at -20 °C. This was **Fraction I** (300 ml).

4.1.4.5 Desalting by Chromatography on Sephadex G-25

A column (210 x 90 mm) was packed with Sephadex G-25 and equilibrated with buffer S + 50 mM NaCl. Fraction I was carefully applied to the top of the bed, and the column was washed with buffer S + 50 mM NaCl. The eluant was collected into 100 - 300 ml beakers chilled in ice. The A_{280} and concentration of NaCl (Section 3.1.3) of each fraction was measured (Table 4.1). The three fractions containing the highest amounts of protein were pooled, and stored at -70 °C. This was **Fraction II** (300 ml).

4.1.4.6 Chromatography on DEAE-Sephacel

A column (115 x 30 mm) was packed with DEAE Sephacel and equilibrated with buffer S + 50 mM NaCl. Fraction II (300 ml, 60 mg) was applied to the column at a flow rate of ≈ 1 ml/min using a peristaltic pump. The resin was then washed with buffer S + 50 mM NaCl (130 ml). Bound protein was subsequently eluted with a linear gradient of 50 to 150 mM NaCl in buffer S (390 ml) at a flow rate of 0.9 ml/min; 4.85 ml fractions were collected. Elution of protein from the column was monitored with a Pharmacia UV-I monitor. At the end of the gradient a steeper gradient to buffer S + 1 M NaCl (100 ml) was applied. Aliquots (200 µl) taken from column fractions that corresponded to peaks of A_{280} were assayed for enzyme activity and protein concentration. Peak fractions (44 - 53) were analysed by SDS-PAGE. Those fractions containing 50% of the activity of the fraction with the maximum activity were pooled and stored at -70 °C. This was **Fraction III** (48.5 ml).

4.1.4.7 Chromatography on Blue Gel A

A column (45 x 30 mm) was packed with Blue Gel A and equilibrated with buffer S + 50 mM NaCl (300 ml). Fraction III was applied to the column using a peristaltic pump at 15 ml per h. The column was washed with buffer S + 270 mM NaCl (60 ml). The ε subunit was eluted with buffer S + 0.5 M NaCl (80 ml). Fractions containing 50% of the activity of the fraction containing the maximum activity were pooled, dialysed overnight against buffer S + 50 mM NaCl (2 litres, 4

°C), and then stored at -70 °C. This was **Fraction IV** (28.5 ml).

4.1.5 *Storage of Purified ε Subunit*

Purified overexpressed ε subunit (Blue Gel A fraction) was stored in 250 µl aliquots in buffer S containing 50 mM NaCl at -70 °C. The ε subunit showed no appreciable loss of activity after 12 months, however, aliquots were always discarded after having been brought to 0 °C for the second time.

4.1.6 *Assay of 3′→5′ Exonuclease and DNA Polymerase Activities*

The activity of the ε subunit could be measured in two ways: by removal of radiolabelled nucleotides from a mispaired terminus ('copolymer' or 'hook' assay), and by the hydrolysis of thymidine 5′-monophosphate *p*-nitrophenyl ester (*p*np-TMP) to *p*-nitrophenol and TMP (colourimetric assay).

4.1.6.1 The Copolymer Assay[2]

A reaction mixture was prepared that contained assay buffer (50 mM Tris.HCl pH 7.5, 4% (w/v) sucrose, 10 mM $MgCl_2$, 8 mM DTT, 50 µg/ml BSA) and ^{3}H-labelled copolymer (40 nM 3′-OH ends). Portions of the assay mixture (23 - 24.5 µl) were dispensed to chilled glass tubes. Reactions were started by the addition of 0.5 - 2 µl of enzyme to the glass tube containing the assay components. After treatment at 30 °C for various times (5 - 15 min) the enzyme was inactivated by rapid cooling and then the addition of 10 µl of 0.5 M EDTA. The reaction mix was spotted onto Whatman DE81 paper which binds DNA strongly and mononucleotides weakly. Nucleotide released from the copolymer by the exonuclease activity of ε was removed by gently washing the filter disks in 0.3 M ammonium formate pH 7.9. The papers were then dehydrated in 95% ethanol, dried, and residual radioactivity was determined in a Beckman LS 6000IC liquid scintillation counter. By reference to a standard, the amount of (dC) label remaining in the substrate, was determined. A unit of activity is

[2]A detailed description of the assay is provided in Chapter 5.

the amount of enzyme that catalyses the release of 1 pmol of nucleotide per minute at 30 °C.

4.1.6.2 The Colourimetric Assay[3]

The assay was started by the addition of enzyme (5µl) to a 1.0 ml glass cuvette containing 1 ml of 50 mM Tris.HCl pH 8.0, 750 µM $MnCl_2$, 150 mM NaCl and 6.2 mM *p*np-TMP. Assay components were dispensed into the glass cuvettes using Hamilton syringes. They were allowed to warm to room temperature, and subsequently placed in the cuvette chamber where the contents were thoroughly mixed with a glass rod. Enzyme was added and the solution mixed again. The temperature of the cuvette chamber was kept at 25 ±0.25 °C. The formation of *p*-nitrophenol was measured continuously at 420 nm in a spectrophotometer. The data were processed as described in Section 6.2.4.1. A unit of activity is the amount of enzyme that catalyses the formation of 1 pmol *p*-nitrophenol per min at 25 °C.

4.1.6.3 Gap-filling Assay

Polymerase activity was determined by the ability of the α subunit to incorporate tritiated dNTP's into nicked calf thymus DNA (Maki and Kornberg, 1985a). One unit of activity is defined as the amount of enzyme that catalyses the incorporation of 1 pmol of nucleotide per min at 30 °C.

4.1.7 *Determination of Protein Concentration*

The concentration of protein was determined either by the method of Bradford (1976) using bovine serum albumin as standard, or by measuring the absorbance of solutions containing ε at 280_{nm}, using an ε_{280} for the ε subunit of 12,090 M^{-1} cm^{-1}, estimated as described by Gill and von Hippel (1989).

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[3]A detailed description of this assay is provided in Chapter 6.

4.2 RESULTS

4.2.1 *Construction of a Strain that Overproduces the ε Subunit*

Cells harbouring pPL224 (Section 4.1.2) overproduced the ε subunit to a high level as judged by SDS-PAGE of whole cells following induction over a period of time (Fig. 4.5). The cells grew poorly at 30 °C suggesting that there may be leakage from the λ promoters at the non-permissive temperature.

4.2.2 *Construction of a dnaE-dnaQ Synthetic Operon*

The construction of plasmid pPT152 was described in Section 4.2.2 and Figure 4.3. Transformants were initially selected on the basis of their resistance to ampicillin, and temperature sensitivity. Plasmid DNA prepared from several clones was checked on the criteria of:

a) size (8.0 kb), indicating the presence of insert, and

b) a *Bam*HI digest to confirm presence of *dnaQ* and the proper orientation of the *dnaQ* insert for transcription from $P_R P_L$.

One transformant was shown to be overproducing the α subunit to a moderate level (≈ 2% total cell protein), and the ε subunit to < 1% of total cell protein as judged by SDS-PAGE of whole cells (Fig. 4.6). The strain harbouring pPT152 grew very poorly at both 30 °C and 42 °C. The integrity of the 5′ region of *dnaQ* was determined by dideoxy sequencing of dsDNA and confirmed the prediction that 103 bp separated the *dnaE* termination codon and the *dnaQ* start site (data not shown).

Exonuclease and polymerase activities in clarified crude lysates were measured as described (Sections 4.1.5.2 and 4.1.5.3). The polymerase specific activity was approximately 1.6×10^2 U/mg (cf. 5000 U/mg, fraction I; Maki and Kornberg, 1985b). The proofreading specific activity was approximately 56 U/mg. This data is consistent with observations of SDS-PAGE profiles of whole cells that show ε is overproduced to a lesser extent than α.

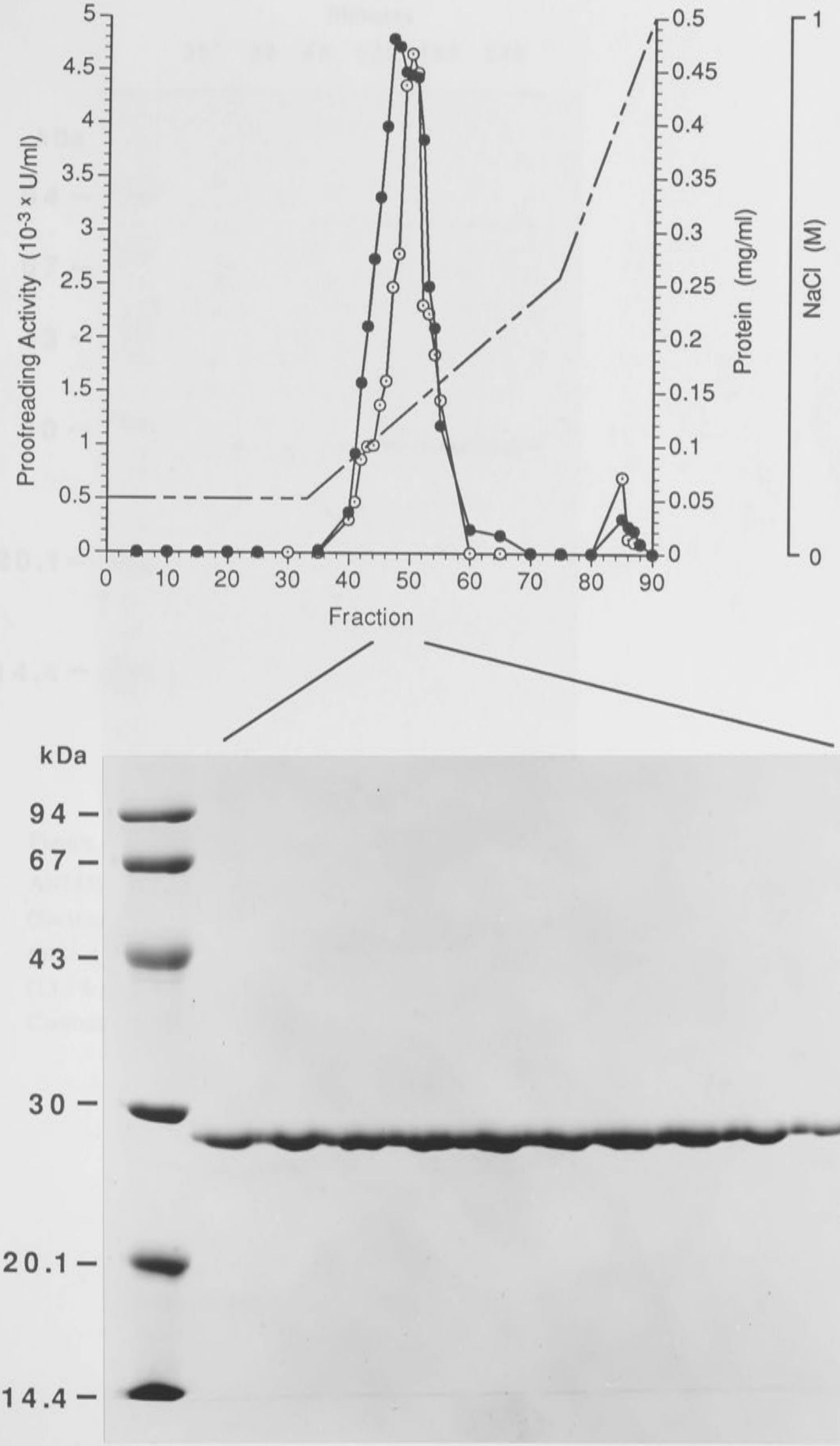
4.2.3 *The ε Subunit is Purified on the Basis of its Insolubility*

The specific activity of (soluble) ε in a crude lysate of induced cells of AN1459/pPL224 was approximately 1% of that of purified enzyme as determined from activity assays using *p*np-TMP (Section 4.1.5.2). The ε subunit cannot be detected in the soluble fraction of induced cells following SDS-PAGE and staining with Coomassie Brilliant blue (Scheuermann and Echols, 1984). ε was purified by a procedure modified from that reported by Scheuermann and Echols (1984).

4.2.4 *Purification of the ε Subunit*

Following resolubilisation with GuHCl, the solution containing the ε subunit was desalted by chromatography on Sephadex G-25. It was necessary to remove the GuHCl at this stage as it would have interfered with subsequent purification steps. Use of dialysis was contemplated, but this was considered time consuming and expensive given the volumes involved. The desalted ε fraction was then further purified by ion exchange chromatography on DEAE Sephacel (Fig. 4.4), and dye-ligand affinity chromatography using Blue Gel A. Although the ε subunit was judged to be electrophoretically pure following chromatography on DEAE-Sephacel, further purification was undertaken as Scheuermann and Echols (1984) reported a two-fold increase in specific activity following chromatography on Blue Gel A. Purification tables are presented below (Tables 4.1 to 4.3). The activity and protein concentration of Fraction I were not determined as GuHCl interfered with assays of both activity and protein.

Figure 4.4: Elution profile of the ε subunit on DEAE-Sephacel. Chromatography was performed as described in the text (Section 4.1.4.6). ● = [protein]; ⊙ = activity; the dashed line indicates the concentration of NaCl. Fractions 44 to 53 were pooled. A portion of these fractions (35 µl) was subjected to SDS-PAGE (13.5% gel) and the proteins were visualised by staining with Coomassie Brilliant Blue.


Proofreading Activity (10-3 x U/ml)
Protein (mg/ml)
NaCl (M)
Fraction
0
10
20
30
40
50
60
70
80
90
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0.5
0
1
kDa
94
67
43
30
20.1
14.4

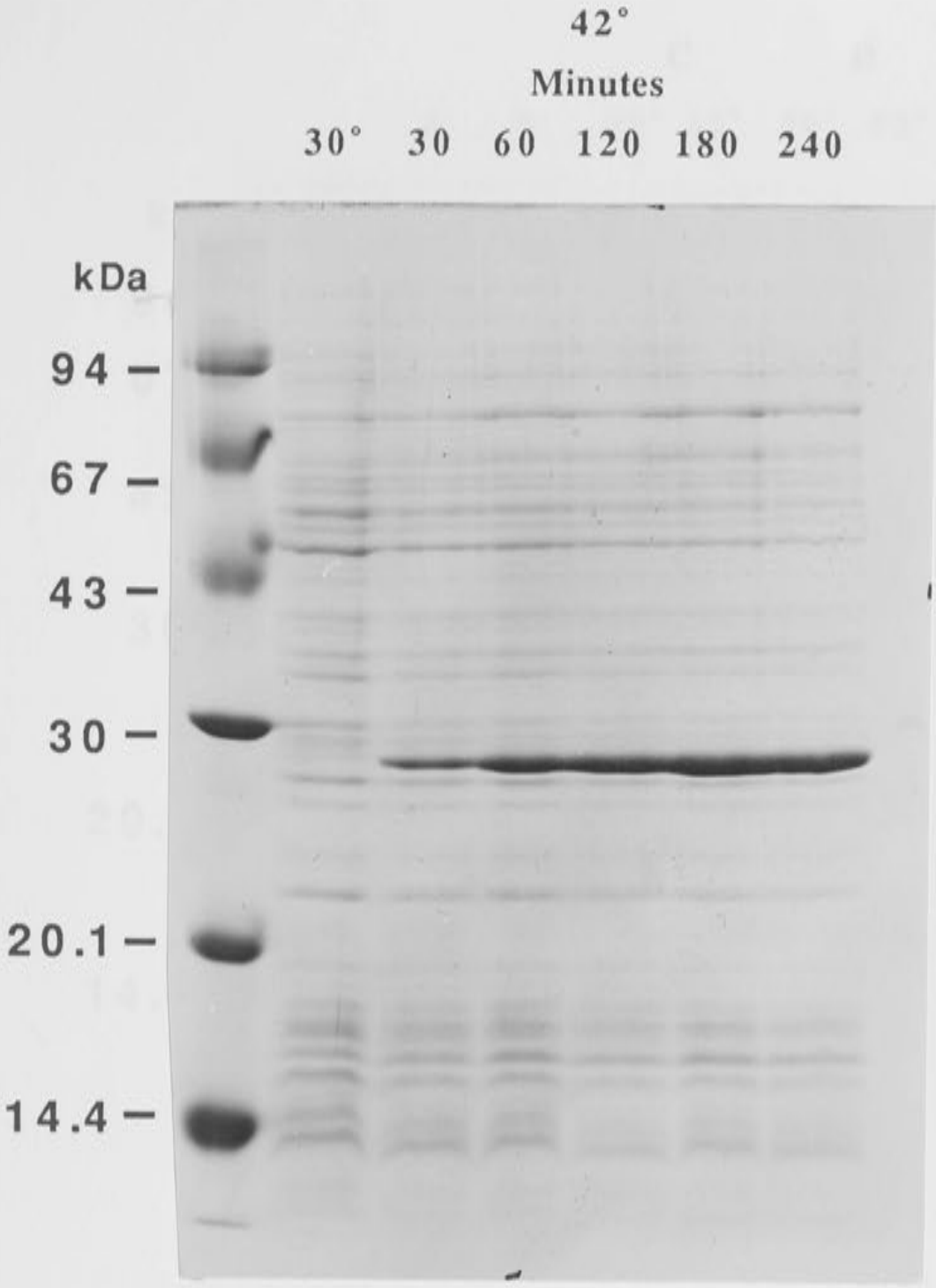


Figure 4.5: Time course of the thermal induction of the ε subunit in strain AN1459/pPL224. The experiment was performed essentially as described (Section 2.2.21). A sample of the culture was taken at 30° C immediately before shifting the temperature to 42° C. Whole cells were subjected to SDS-PAGE (13.5% gel) (Section 2.2.22); proteins were visualised by staining with Coomassie Brilliant Blue.

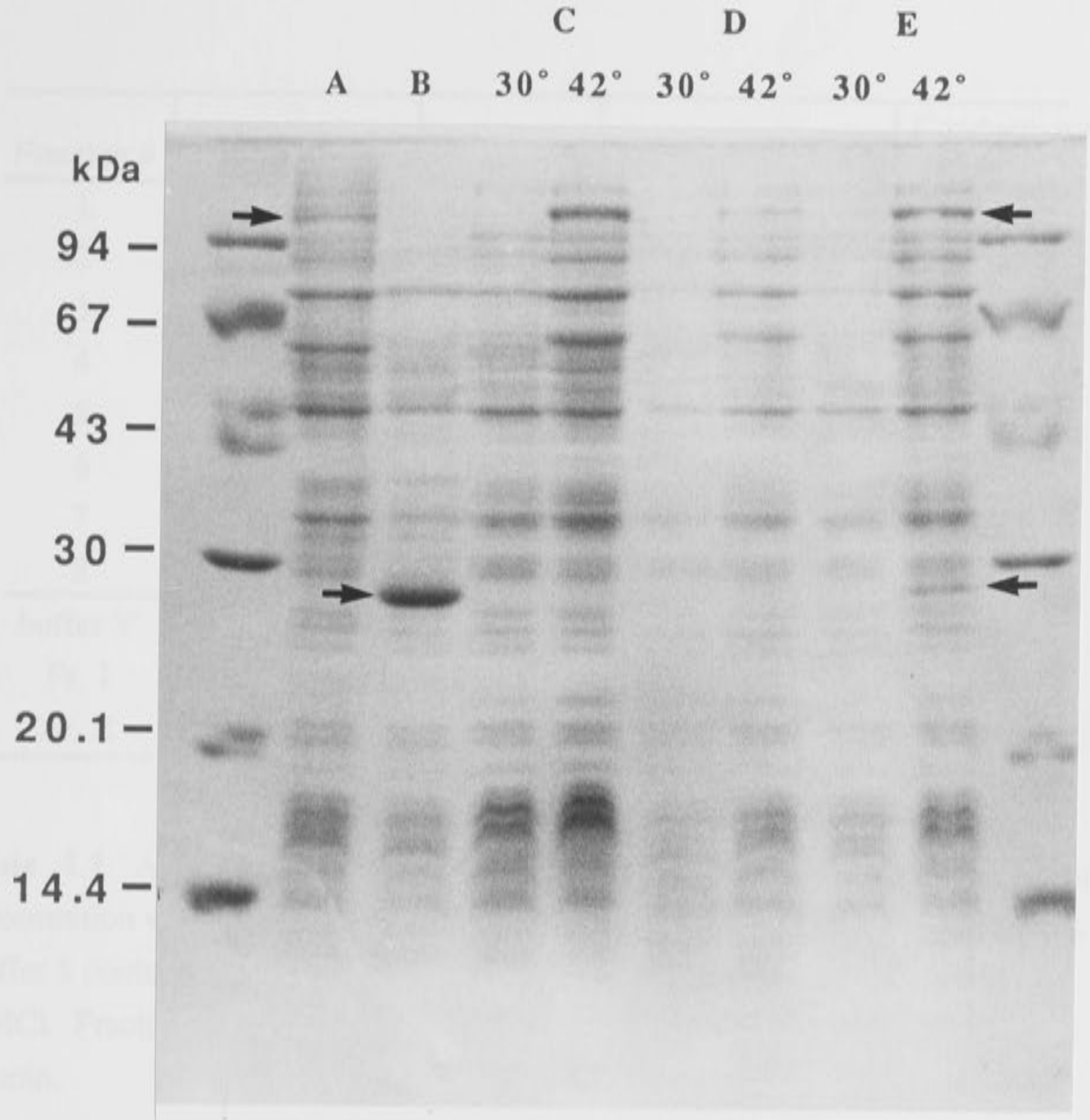


Figure 4.6: Induction of *E. coli* strain AN1459 harbouring plasmids thought to be directing the overproduction of the α and ε subunits of DNA polymerase III holoenzyme. The inductions were performed as described (2.5 h at 42° C; Section 2.2.21). Cells were subjected to SDS-PAGE (12.5% gel) and proteins were visualised by staining with Coomassie Brilliant Blue. The α and ε subunits in the whole cell extracts of transformant #30, and in lanes A and B respectively, are indicated.

A = induced cells of AN1459/pPL250 (α overproducer)

B = induced cells of AN1459/pPL224 (ε overproducer)

C = transformant #13

D = transformant #15

E = transformant #30

Fraction #	Volume (ml)	A_{280}	Total protein (mg)	Conductivity µSi/cm
1	200	0.044		0.09
2	250	0.035		0.09
3	103	0.056		0.08
4	103	0.317	32.7	0.1
5	100	0.178	17.8	0.09
6	100	0.148	14.8	0.1
7	100	0.077		0.09
8	100	0.049		0.14
buffer S*			0.000	0.1
Fr. I	300	0.348	104	2.32
Fr. II	300	0.22	66	0.1

Table 4.1: Analysis of fractions from Sephadex G-25 chromatography. Protein concentration was determined by assuming an A_{280} = 1 is equivalent to 1.0 mg/ml. *Buffer S contained 50 mM NaCl. Fraction I was ε subunit resolubilised with GuHCl. Fraction II represents a pool of fractions 4 to 6 from the Sephadex G-25 column.

Fraction	Total protein mg	Total activity units	Specific activity (U/mg)	Recovery %
I. Cell pellet	N.D	N.D.	-	-
II. Gel filtration	60	1.13×10^8	1.89×10^6	100
III. DEAE-Sephacel	21	7.84×10^7	3.73×10^6	69
IV. Blue Gel A	17.7	8.43×10^7	4.76×10^6	75

Table 4.2: Purification of the ε subunit monitored by assay of hydrolysis of *p*np-TMP. One unit of activity is defined in this table as the amount of enzyme catalysing the formation of 1 pmol of *p*-nitrophenol at 25 °C in 1 min (Section 4.1.5.2). The concentration of protein was determined by the method of Bradford (1976) using BSA as standard.

Fraction	Total activity units	Total protein mg	Specific activity (U/mg)	Recovery %
II. Gel filtration	1.6×10^5	60	2.63×10^3	100
III. DEAE Sephacel	1.35×10^5	21	6.51×10^3	84
IV. Blue Gel A	1.3×10^5	17.65	7.26×10^3	81

Table 4.3: Purification of the ε subunit as monitored using the proofreading assay (copolymer). One unit of activity is defined in this table as the amount of enzyme catalysing the removal of 1 pmol of nucleotide from a mispaired 3′-OH terminus per min at 30 °C (Section 4.1.5.1). The concentration of protein was determined by the method of Bradford (1976) using BSA as standard.

The homogeneity of the ε subunit at each stage of the purification protocol is shown in Figure 4.7.

4.2.4.1 Comparisons with the Scheuermann and Echols (1984) Preparation

Although a similar purification protocol for the ε subunit was developed by Scheuermann and Echols (1984), several differences were noted between the purification of ε described above and that reported by them. This raised the possibility that the enzyme prepared in this study and that by Scheuermann and Echols (1984) were different. Firstly, the ε subunit would not bind to DEAE-Sephacel or Blue Gel A in the absence of Mg^{2+}. Moreover, the specific activities of the preparations at all stages were always 8 to 10-fold less than those quoted by Scheuermann and Echols (1984). A specific activity of 53,000 U/mg for the DEAE-Sephacel fraction, and 110,000 U/mg for the Blue Gel A pool was claimed by the authors.

In an attempt to resolve the issue, ε subunit was prepared from an overproducing strain[4] (gift of Harrison Echols) strictly as outlined in Scheuermann and Echols (1984) through to the stage at which GuHCl is removed from the solution

[4] *E. coli* JM1 pRK248/*dt*s/pNS360

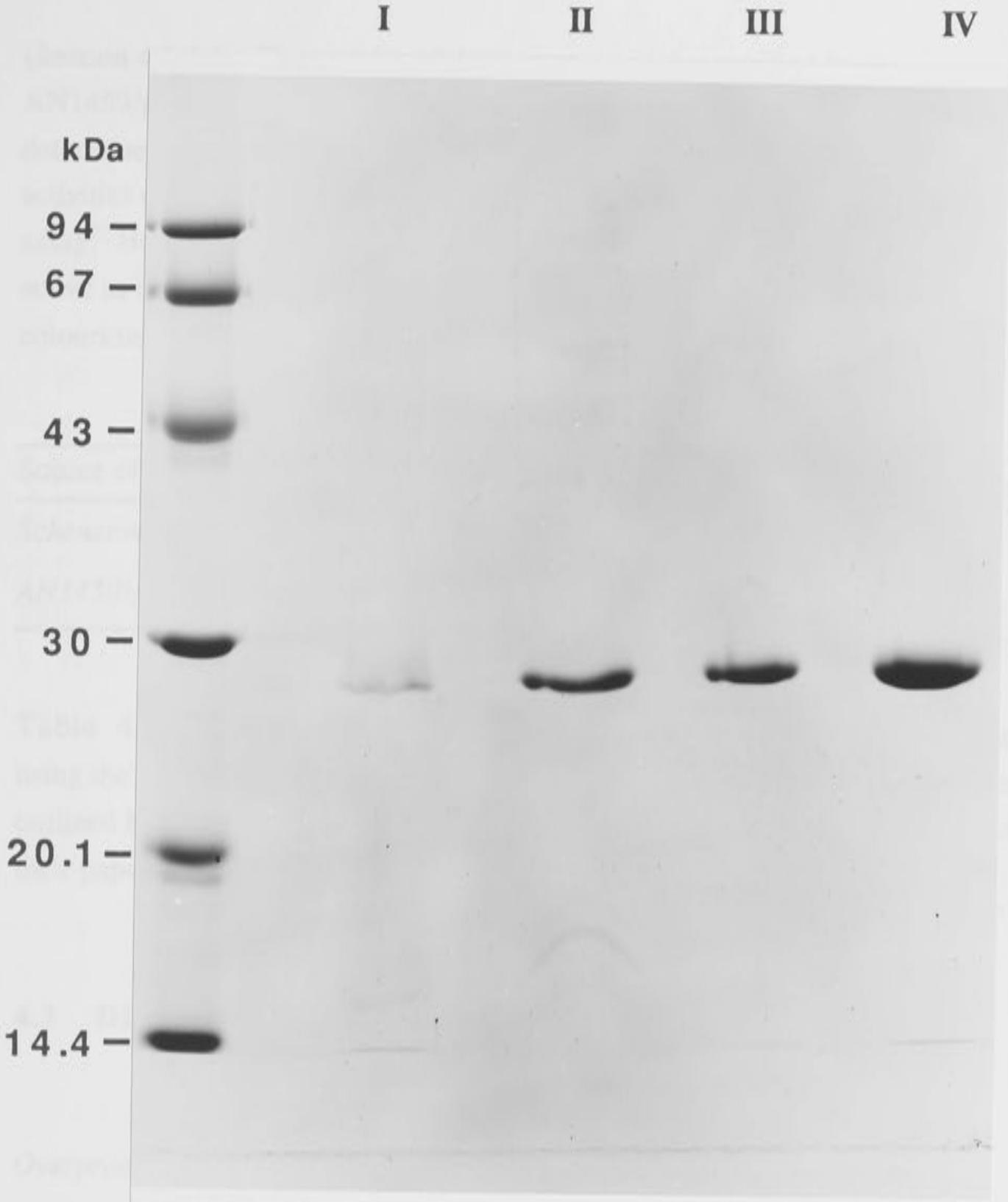


Figure 4.7: Purification of the ε subunit from strain AN1459/pPL224 (Section 4.1.4). ε subunit (3 μg to 10 μg) was subjected to SDS-PAGE (13.5% gel) and visualised with Coomassie Brilliant Blue.

Fraction I: Resolubilised ε (≈3 μg)
Fraction II: Gel filtration (≈5 μg)
Fraction III: DEAE-Sephacel (10 μg)
Fraction IV: Blue Gel A (10 μg)

(Section 4.1.4.5). The activity of the enzyme, and that purified from AN1459/pPL224 to the same stage as described above (Section 4.1.4), was determined using the colourimetric and copolymer assays (Table 4.4). The specific activities of both preparations were essentially identical based on the proofreading assay. However, ε subunit prepared from AN1459/pPL224 was nearly twice as active as that isolated from the Echols and Scheuermann (1984) strain using the colourimetric assay data.

Source of ε subunit	Colourimetric assay	Copolymer assay
Scheuermann and Echols	1.8×10^6 U/mg	2.7×10^3 U/mg
AN1459/pPL224	2.9×10^6 U/mg	2.6×10^3 U/mg

Table 4.4: A comparison of the specific activity of the two ε subunit preparations using the colourimetric and copolymer assays. The ε subunit was purified strictly as outlined by Scheuermann and Echols (1984) using both the overproducer described in their paper, and AN1459/pPL224.

4.3 DISCUSSION

Overproduction of the ε Subunit of DNA Polymerase III Holoenzyme

The ε subunit of DNA polymerase III holoenzyme has been overproduced in *E. coli* using bacteriophage λ promoter vectors. After heat treatment for 4 h at 42 °C, the ε subunit comprised approximately 15% of total cell protein (Fig. 4.5). The level of overproduction is better than that obtained by Scheuermann and Echols (1984) (≈ 10%) on a qualitative level. Cells harbouring pPL224 grew poorly at 30 °C, a temperature at which the λ promoters should be fully repressed due to the activity of the cI857 repressor protein. This suggests that there may be leakage from the λ promoters at 30 °C. Even low levels of *dnaQ* expression may therefore be lethal to *E. coli*cells.

Construction of a dnaQ-dnaE Synthetic Operon

A plasmid, pPT152, was constructed by placing *dnaQ* downstream of the *dnaE* gene resulting in the expression of both genesas a synthetic *dnaE-dnaQ* operon under the control of the bacteriophage λ promoters P_R and P_L. This vector directed the synthesis of a soluble, active complex of the α and ε subunits (Fig. 4.6). Although the two genes are separated by 103 bp, they are likely to be transcriptionally coupled. It was not feasible to place *dnaQ* upstream of *dnaE* due to the presence of a series of potential transcriptional regulatory structures at the 3´ region of *dnaQ*. Potential secondary structures (eg. hairpin loops) in the mRNA could act as premature transcription terminators decreasing the expression of the downstream gene. An extensive series of inverted repeats downstream of *dnaQ* was reported from sequence analysis (Maki *et al.*, 1983).

The level of synthesis of α–ε complex is low as judged from whole cell lysates. Moreover, cells harbouring pPT152 grow very poorly at both 42 °C and 30 °C. Polymerase activity is present in the supernatant of crude lysates of AN1459/pPT152 at 4% of that measured at a comparable stage in the purification of α from cells overproducing the α subunit (Maki and Kornberg, 1985b). Therefore, it would be feasible to purify α–ε complex in quantities greater than that possible from non-overproducing cells (McHenry, 1988). An alternative strategy would be to place each gene under the control of a separate λ promoter; thus, *dnaQ* could be inserted, in a derivative of pCE30, downstream of P_R, *dnaE* downstream of P_L, and *vice versa*. This strategy has been successfully applied to the simultaneous overproduction of the *dnaB* and *dnaC* gene products (N.P.J. Stamford and N.E. Dixon, in preparation). Reducing the intercistronic spacing between *dnaE* and *dnaQ* may enhance expression by ensuring the translational appartus remains bound to the message. However, it is probably easier to reconstitute an active α–ε complex from separately purified subunits. High level overproducers of both the α and ε subunits are available, and purification protocols exist for the recovery of enzymes in high yield (Maki and Kornberg, 1987; Studwell and O'Donnell, 1990).

Purification of Overexpressed ε

The ε subunit has been purified from the overproducing strain in sufficient quantity to allow the studies reported in the next three Chapters. The overproduced

enzyme possesses proofreading activity comparable to that obtained by other groups.

Overproduced ε is almost totally insoluble and remains in the pellet after cell lysis. The insolubility of ε actually simplifies its purification. It facilitates easy separation from the bulk of the soluble cell proteins, and many of the contaminating proteins in the cell pellet are removed by successive washes with buffer containing high concentrations of NaCl. Since ε is normally part of a complex with the α subunit, one might speculate that hydrophobic regions of ε normally buried by this association could be unstable when exposed to solvent in the absence of α. These hydrophobic regions would cause ε to aggregate. The α subunit when expressed from the heat-inducible bacteriophage λ promoter systems is also largely insoluble; when α is expressed under the control of the *tac* promoter at 30 °C, it is soluble (J.L. Beck, unpublished data). The ε subunit may also be more soluble if expressed in a different system.

When using a denaturation-renaturation step in a purification protocol, it is possible that the enzyme may not properly refold on removal of the denaturant. Therefore, it is important to determine whether the denaturated-renaturated protein *in vitro* has the same properties as the corresponding protein *in vivo*. Purified ε subunit would seem to have properties expected of a proofreading enzyme: it possesses 3′→ 5′ exonuclease activity which is substantially more active on mispaired than paired termini (Maki and Kornberg, 1987; Kunkel, 1990). However, ε clearly prefers to be associated with the polymerase subunit of holoenzyme: proofreading activity is reported to be enhanced 16-fold to 80-fold on its binding to α (Maki and Kornberg, 1987).

The recovery of protein after the DEAE-Sephacel step is low. However, the recovery of activity is very good (Tables 4.2 and 4.3). Several milligrams of protein with 3′→ 5′ exonuclease activity elute from the DEAE-Sephacel column by washing with 1 M NaCl, indicating that there may have been more than one species of the ε subunit present (data not shown). However, this does not account for all the protein lost during chromatography on DEAE-Sephacel. It is possible that not all the enzyme refolded properly following removal of the denaturant. Refolding does not appear to be a function of time, as samples dialysed for different times show similar levels of activity (data not shown). The "unfolded" protein may have precipitated irreversibly on the column, or bound so tightly that it might not be removed unless very high salt concentrations (> 1M), or a denaturant, were used. This is an issue that should be investigated, if only to maximise the yield of enzyme. One approach might involve the use of chaperonins, such as the *E. coli* GroE proteins which are reported to help some proteins attain their correct tertiary configuration following translation (Ellis and van

der Vies, 1991). Purified GroE would be added to denatured ε prior to removal of GuHCl (Fraction I). The GroEL-ε complex would be isolated by gel filtration. ε subunit could be recovered free of GroEL by the addition of GroES/MgATP which displaces GroEL from the complex. The effect of the GroE chaperonins would be measured largely by the behaviour of ε on ion exchange chromatography. If the enzyme is correctly folded, the yield of protein should be high compared to the present situation. Whether GroEL exerted a stimulatory effect on the proofreading activity of ε could also be determined. While there appears to be a problem with the recovery of protein, it is clear that the ε subunit purified in this study is as active as that obtained following preparation as described by Scheuermann and Echols (1984) (Table 4.4).

Comparisons with the Scheuermann and Echols (1984) Enzyme

Apparent differences between the behaviour of overexpressed ε subunit purified in this study, and that reported by Scheuermann and Echols (1984) are puzzling. The ε subunit prepared in this study would not bind to DEAE-Sephacel or Blue Gel A in the absence of Mg^{2+} (or presumably, Mn^{2+}). Moreover, the specific activity of enzyme prepared in this study was always 8 to 10-fold less than that quoted by Scheuermann and Echols (1984). However, enzyme purified strictly as they reported from their overproducing strain, was no more active than ε subunit purified from strains harbouring pPL224 using the procedure outlined in Section 4.14. It would be convenient to attribute these differences to variations in assays between laboratories, but the magnitude would seem to argue against this. Regrettably, direct comparisons between preparations of the ε subunit are not possible as purification tables for ε have not been published.

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CHAPTER 5

PROPERTIES OF OVEREXPRESSED EPSILON SUBUNIT

5.0 INTRODUCTION

Initial characterisation of the proofreading exonuclease of DNA polymerase III holoenzyme (the ε subunit) was reported by Scheuermann and Echols (1984) and Maki and Kornberg (1987). However, most studies of the 3′→ 5′ exonuclease activity of the ε subunit have been made with ε present as part of the core polymerase, a reconstituted α–ε complex, or holoenzyme (Maki and Kornberg, 1987; Studwell and O'Donnell, 1990; Griep *et al.*, 1990; Reems *et al.*, 1991; Brenowitz *et al.*, 1991). Use of an α–ε complex will certainly yield information helping describe the function of the ε subunit *in vivo*, but valuable data can be derived from studies on the isolated subunit.

Many basic properties of the ε subunit have yet to be elucidated. It is known that ε will rapidly hydrolyse nucleotides from a mispaired 3′-OH terminus and can degrade fragments of ssDNA. However, there is no data for the relative activities of the ε subunit for these substrates. The role of metal ions in the 3′→ 5′ exonuclease activity of DNA polymerase I is a topic of much research, but such information for the ε subunit has yet to be reported. There have been serveral reports that describe the rates at which ε removes a single misincorporated nucleotide from a 3′-OH terminus (see Section 5.3 and 6.5). It appears that the Klenow fragment requires between 4 and 5 bp of the primer strand to melt out from a duplex DNA for the exonucleolytic removal of nucleotides from the primer terminus (Cowart *et al.*, 1989). Thus, the rate with which ε removes a *series* of nucleotides from a mispaired terminus is worthy of investigation.

This chapter describes the various assays used to characterise the exonuclease activity of ε *in vitro*. These assays utilise radiolabelled oligonucleotide substrates that have a mispaired 3′-OH terminus, and are collectively referred to as the oligonucleotide-based assays. These assays were used to undertake a basic kinetic analysis of proofreading function, and to assess the role of divalent metal ions in the proofreading reaction, and the effect of products on activity. The activity of the ε subunit assayed with other substrates was also determined. Finally, the dynamics of the ε subunit of holoenzyme on a mispair was studied.

5.1 MATERIALS AND METHODS

5.1.1 *Enzymes*

Terminal deoxynucleotidyl transferase and T4 polynucleotide kinase were purchased from Boehringer-Mannheim (Australia). The *E. coli* single-stranded DNA binding protein (SSB) protein was a kind gift of Dr. Philip Hendry.

5.1.2 *Radionucleotides*

[α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]dATP (3000 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol) and [^{3}H]dCTP (9 Ci/mmol) were purchased from Amersham International.

5.1.3 *Oligonucleotides*

Poly $(dA)_{200}$, oligo $(dT)_{16}$ and RNA from phage MS2 were purchased from Boehringer-Mannheim (Australia). Hook DNA's (Section 5.1.5.1) were synthesised by the A.N.U. Biomolecular Resource Facility at the John Curtin School of Medical Research (Section 2.2.1.4). Oligonucleotides described in Section 5.1.10 were prepared and 'purified'by Bresatech (Adelaide). Single-stranded fragments of phage M13 (isolated following digestion with *Hae*III) were a kind gift from Dr. N.E. Dixon.

5.1.4 *Resins*

Chelex 100, a resin for removing metal ions from aqueous solutions, was purchased from BioRad (USA) and activated as instructed by the manufacturer. Sephadex G-100 and Sephadex G-50 (DNA grade) were purchased from Pharmacia (Uppsala).

5.1.5 *Oligonucleotide-Based Assays of 3′→5′ Exonuclease Activity*

5.1.5.1 The 'Hook' DNA Substrate

The hook DNA is a 56 bp oligonucleotide with a 14 bp double-stranded region, a 22 bp single-stranded region, and a 6 bp loop (Fig. 5.1).

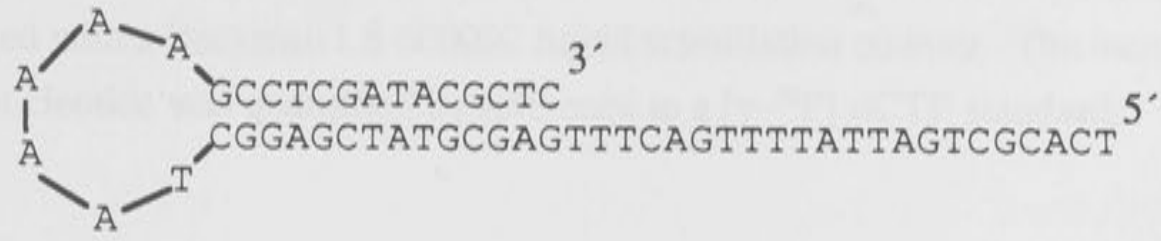


Figure 5.1: The 'hook' DNA: a 56 bp synthetic oligonucleotide.

5.1.5.1.1 Purification of the Hook Oligonucleotide

The hook oligonucleotide was purified by electrophoresis through a 15% polyacrylamide gel. Due to the possibility of intermolecular pairing between complementary regions that would result in the formation of dimers, the oligonucleotide in TE (110 µg) was diluted 1/4 in TE, heated at 80 °C for 10 min, and then cooled rapidly to 0 °C prior to loading. The major band was visualised by UV shadowing, excised, and eluted from the gel slice in an Elutrap apparatus (Schleicher and Schuell). The eluted oligonucleotide was concentrated by ultrafiltration in a Centricon-10 microconcentrator (Amicon). The concentration of the oligonucleotide was determined by measuring the absorbance at A_{260} using a calculated ε_{260} of 5.92 x 10^6 M^{-1} cm^{-1}.

5.1.5.1.2 Labelling the 3′-OH Terminus of the Hook DNA

A 250 µl reaction mixture containing 400 pmol (as 3′-OH ends) purified hook

DNA, 4.2 pmol (12.5 μCi) of [α–^{32}P]dCTP working stock (40 μM), and 450 units of terminal transferase in tailing buffer (140 mM sodium cacodylate, 30 mM Tris.HCl pH 7.6, 100 μM DTT and 1 mM $CoCl_2$) was treated at 25 °C for 3.5 h. A sample was withdrawn after this time so that the number of residues incorporated could be determined. The terminal transferase reaction was quenched by the addition of EDTA to 50 mM. The labelled DNA was purified by filtration through a Sephadex G-100 column previously equilibrated with TE (Section 2.2.5.3) containing 200 mM NaCl. A portion (10 μl) of each fraction collected from the column was precipitated with the addition of 100 μl of 10 mM EDTA, 0.2 mg/ml herring sperm DNA and then 1 ml of 10% (w/v) trichloroacetic acid containing 100 mM sodium pyrophosphate at 0 °C (10 min). The radioactivity present in acid insoluble residues of column fractions was determined with a Beckman LS 6000IC liquid scintillation counter. The incorporation of radionucleotide was quantified by reference to a [γ–^{32}P] dCTP standard.

5.1.5.1.3 Activity Assays with the Hook DNA

Proofreading is defined as the removal of misincorporated nucleotides by an enzyme with 3′→ 5′ exonuclease activity and provides the basis for the assay.

An assay mixture was prepared that contained assay buffer (50 mM Tris.HCl pH 7.5, 4% (w/v) sucrose, 10 mM $MgCl_2$, 8 mM DTT, 50 μg/ml BSA) and labelled hook DNA (20 - 24 nM 3′-OH ends). Portions of the assay mixture (23 - 24.5 μl) were dispensed into chilled glass tubes. Reactions were started by the addition of 0.5 - 2 μl of enzyme to the glass tube containing the assay components to give a final volume of 25 μl. After treatment at 30 °C for various times (5 - 15 min) the enzyme was inactivated by rapid cooling to 0 °C, and the addition of 100 μl of ice-cold 'Reaction Stop' (10 mM EDTA, 0.2 mg/ml herring sperm DNA). The DNA was then precipitated by the addition of 1 ml of 10% (v/v) trichloroacetic acid containing 100 mM sodium pyrophosphate on ice and cooling at 0 °C for at least 10 min. The contents of the glass tubes were filtered through Whatman GF/C glass fibre filters. The filters were washed in 1 M HCl, 100 mM sodium pyrophosphate, dehydrated with ethanol, dried, and residual radioactivity determined in a Beckman LS 6000IC liquid scintillation counter. By reference to a standard, the amount of (dC) label remaining in the substrate, could be determined.

5.1.5.2 The Copolymer

5.1.5.2.1 Structure and Preparation of Substrate

The copolymer substrate is oligo $(dT)_{16}$ tailed with [^{3}H]dCMP which is annealed to poly $(dA)_{200}$ (Fig. 5.2).

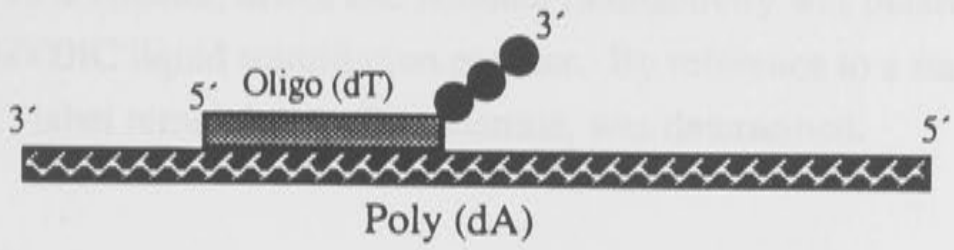


Figure 5.2: The copolymer substrate: [^{3}H$(dC)_6$-$(dT)_{16}$]-$(dA)_{200}$. The black circles represent the [^{3}H]dCMP residues.

An assay mixture (150.5 µl) containing 600 pmol of $(dT)_{16}$, 3 pmol (88 µCi) of [^{3}H]dCTP working stock, 750 µM $CoCl_2$ and 250 units of terminal transferase (in tailing buffer), was treated at 37 °C for 90 min. A sample was withdrawn at the end of this time so that the number of residues incorporated could be determined. The reaction was quenched by the addition of 69 mM EDTA and the reaction moved to ice. The [^{3}H]dCMP-labelled oligo $(dT)_{16}$ was annealed to 1.2 nmol of poly $(dA)_{200}$ (as molecules) in TE, initially at 37 °C for 45 min, and then at room temperature for 10 min. Copolymer was purified by filtration through a Sephadex G-50 column previously equilibrated with 10 mM Tris.HCl pH 7.66, 150 mM NaCl, then stored at -20 °C until use. By reference to the number of counts in a [^{3}H]dCTP standard, it was determined that the oligo $(dT)_{16}$ molecules had an average of 6 dCMP residues incorporated per end.

5.1.5.2.2 Activity Assays with the Copolymer

As with the hook assay, the removal of misincorporated nucleotides by an enzyme with 3′→ 5′ exonuclease activity provides the basis for the assay.

A reaction mixture was prepared that contained assay buffer (50 mM Tris.HCl

pH 7.5, 4% (w/v) sucrose, 10 mM $MgCl_2$, 8 mM DTT, 50 µg/ml BSA) and labelled copolymer (40 nM 3´-OH ends). Portions of the assay mixture (23 - 24.5 µl) were dispensed to chilled glass tubes. Reactions were started by the addition of 0.5 - 2 µl of enzyme to the glass tube containing the assay components. After treatment at 30 °C for various times (5 - 15 min) the enzyme was inactivated by rapid cooling and then the addition of 10 µl of 0.5 M EDTA. The reaction mixture was spotted onto Whatman DE81 paper which binds DNA strongly and mononucleotides weakly. Nucleotide released from the copolymer by the exonuclease activity of ε was removed by gently washing the filter disks in 0.3 M ammonium formate pH 7.9. The papers were then dehydrated in 95% ethanol, dried, and residual radioactivity was determined in a Beckman LS 6000IC liquid scintillation counter. By reference to a standard, the amount of (dC) label remaining in the substrate, was determined.

5.1.6 *Labelling the 3´-OH Terminus of Poly (dA)*

Tailing of the 3´-OH terminus of poly (dA) was achieved with [α-^{32}P]dATP and terminal transferase essentially as described (Section 5.1.5.1.2). The reaction was allowed to proceed for an appropriate time to permit the incorporation of an average of 1.4 labelled (dA) residues per end.

5.1.7 *Involvement of Divalent Metal Ions in Activity*

5.1.7.1 Preparation of Metal Ion-Depleted Enzyme

A portion of pure ε (25 ml) was taken and extensively dialysed (4 days; 4 changes of buffer) at 4 °C against 1 litre of buffer S containing 75 mM NaCl and 10 mM EDTA to remove metal ions. Water used in the preparation of buffers was passed through a 1 litre column of Chelex 100 to remove adventitious metal ions. Plasticware was used wherever possible and was soaked and rinsed in Chelex-treated water containing 25 mM EDTA for 24 h prior to use.

5.1.7.2 Metal Ion Analysis

A portion (10 ml, 8.9 mg) of metal ion-depleted ε and the buffer used in the depletion of metal ions by dialysis was analysed for zinc and manganese. Analyses were carried out using a Varian AA-30/40 atomic absorption spectrophotometer operated by Ms. R. Chao of the ANU Microanalytical Unit. Metal ion standards were prepared in 4% (v/v) nitric acid. For zinc, a spectral line at 213.9 nm with a spectral band pass of 1.0 nm was used. For manganese, a spectral line at 279.5 nm, and a spectral band pass of 0.2 nm was used. Samples were atomized in an air-acetylene flame.

5.1.7.3 Metal Ion Substitution Experiments

The ability of a series of divalent metal ions to promote proofreading activity by metal ion-depleted ε was measured (5.1.5.1.3) using assay buffer that did not contain $MgCl_2$ or DTT.

5.1.8 *Reaction Order for the Removal of Nucleotides from a Mispaired Terminus*

In a first order reaction:

$$A \xrightarrow{k} B$$

with the rate constant k for the conversion of substrate A to product B, the concentration of B at time t, $[B]_t$, is related to the final concentration of B, $[B]_\infty$ (the end point), by

$$[B]_t = [B]_\infty \{1 - \exp(-kt)\}$$

The value of k may be obtained from a semilogarithmic plot of $[B]_\infty - [B]_t$ against t:

$$\ln ([B]_\infty - [B]_t) = \ln [B]_\infty - kt$$

If the plot obtained is linear, the reaction can be said to follow first-order kinetics (Fersht, 1985).

5.1.9 *Activity of the ε Subunit on ssDNA and RNA*

The ability of the ε subunit to degrade ssDNA was ascertained using a gel assay, and by measuring the release of radiolabelled nucleotides from the 3´-OH terminus of poly (dA). In the gel assay, a 1.6 kb *Hae*III fragment of phage M13 DNA was exposed to a range of enzyme concentrations in assay buffer (Section 5.1.5.1.3) over a period of time at 30 °C. Activity was quenched with RE STOP (Section 2.2.6) at 0 °C. Reaction products were separated by agarose gel electrophoresis (Section 2.2.16) in a 1.1% gel. Measurement of the degradation over a period of time of poly (dA) labelled at the 3´-OH terminus with [^{32}P]dAMP was essentially as described (Section 5.1.5.2.2).

The ability of the ε subunit to hydrolyse RNA from phage MS2 was determined in a gel assay. The procedure was essentially as described above.

5.1.10 *Rate of Removal of Nucleotides from a Mispaired Terminus*

5.1.10.1 Preparation of Substrate

Oligonucleotides P1 and P2 (Fig. 5.3) were synthesised and 'purified' by Bresatec (Adelaide).

Duplex DNA were formed from P1 and P2 in TE at room temperature using a slight molar excess of P2, and purified by electrophoresis through a 20% non-denaturing polyacrylamide gel. The major band corresponding to the P1/P2 duplex was visualised through UV shadowing, excised, and eluted from the gel slice in an Elutrap apparatus (Schleicher and Schuell). The oligonucleotide was concentrated by ultrafiltration in a Centricon-3 microfiltrator device (Amicon, USA). The concentration of the oligonucleotide duplex was determined from A_{260} using an ε_{260} of 7.59×10^5 M^{-1} cm^{-1}.

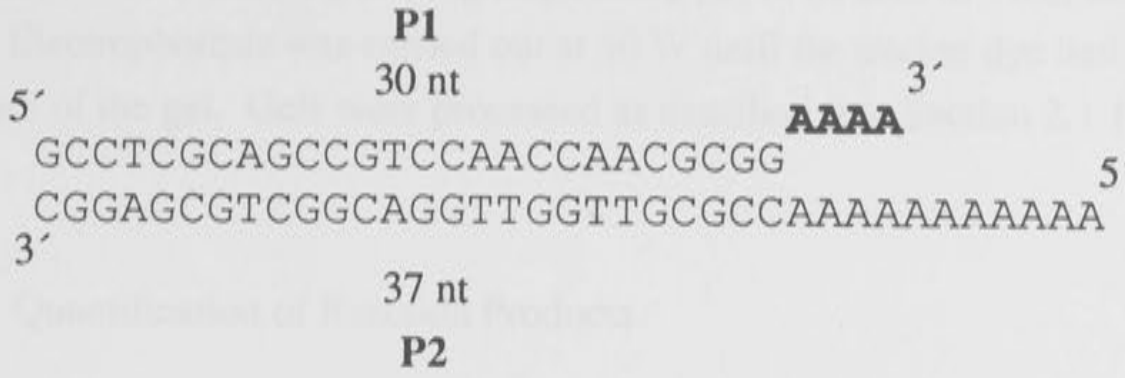


Figure 5.3: The P1/P2 copolymer substrate used to measure the rate of removal of nucleotides from a mispaired terminus by the ε subunit.

Double-stranded oligonucleotide was labelled at the 5′-OH terminus with polynucleotide kinase and [^{32}P]ATP (forward reaction) as described (Sambrook *et al.*, 1989). To ensure even labelling at both termini, DNA was treated with an equimolar amount of dATP prior to the addition of [γ–^{32}P]ATP, for 10 min at room temperature. Labelled DNAs were purified by filtration through Sephadex G-100 previously equilibrated with TE containing 500 mM NaCl. The incorporation of ^{32}P into the copolymer was determined by spotting aliquots (10 µl) onto DE81 papers and scintillation counting (Section 5.1.5.2.2).

5.1.10.2 The Assay

An assay mixture (100µl) that contained assay buffer (50 mM Tris.HCl pH 7.5, 4% (w/v) sucrose, 10 mM $MgCl_2$, 8 mM DTT, 50 µg/ml BSA), P1/P2 copolymer (32.4 nM 3′-OH ends) and 324 nM ε was prepared at 0 °C. Reactions were started by treatment at 30 °C. Aliquots (10 µl) were removed at predetermined time points and placed into tubes containing 5 µl of a modified DNA sequencing loading buffer (90% (v/v) deionised formamide, 30 mM EDTA pH 8, 0.025% (v/v) xylene cyanol, 0.025% (v/v) bromophenol blue) at 0 °C. Samples were then heated at 75 °C for 10 min to ensure the inactivation of ε.

5.1.10.3 Electrophoresis of Reaction Products

A portion (5 µl) of each aliquot was briefly heated at 80 °C immediately before

being loaded onto a 6.5% denaturing sequencing gel (8 M urea in TBE, Section 2.2.19). Electrophoresis was carried out at 50 W until the tracker dye had migrated to the bottom of the gel. Gels were processed as descibed (see Section 2.1.18).

5.1.10.4 Quantification of Reaction Products

Dried gels were trimmed to a size slightly larger than the area containing the ^{32}P-labelled reaction products detected with a hand-held monitor. The gel was then exposed to a Molecular Dynamics Phosphor Screen (20 x 25 cm) for a period of time depending on the intensity of radiation. Typical exposure times ranged from 30 to 90 min. The gel was then removed from the Phosphor Screen 'cassette' and autoradiographed (2.2.19). The screen was then scanned in a Molecular Dynamics Phospho Imager. Data were processed using the Image Quant 3.0 program in which intensity of bands corresponding to fragments of DNA were integrated. This was achieved by defining the region of the scanned image on screen containing a band and then performing the integration. The data used to derive rate constants was the average of three integrations (which differed by no more than 8%).

5.1.10.5 Data Analysis

The total number of counts summed over all bands in each lane that were derived from the primer strand to compensate for differences in the volume of samples loaded. The ratio of the counts in each fragment to the total counts was plotted against time. Assuming first order kinetics under conditions where $[E]_0 > [S]_0$, rate constants for the removal of the first two mispaired nucleotides were determined from the equations below. Theoretical progress curves and calculation of k_1 and k_2 were done using the Apple Macintosh Igor 1.24 program. The equations used to derive k_1 and k_2 were:

$$\underset{\text{4 mispairs}}{A} \xrightarrow{k_1} B \xrightarrow{k_2} C \xrightarrow{k_3} D \xrightarrow{k_4} \underset{\text{0 mispairs}}{E}$$

where,

$$[A] = [A]_o \exp(-k_1 t)$$

$$[B] = ([A]_o k_1/k_2 - k_1)[\exp(-k_1 t) - \exp(-k_2 t)]$$

5.2 RESULTS

5.2.1 *Relationship Between Enzyme Concentration and Activity in the Hook Assay*

The effect of varying the concentration of enzyme on the rate obtained in the hook assay (with Mg^{2+}) was measured. A sigmoidal curve was obtained on plotting the data (Fig. 5.4). The 3 bp mispair is almost completely removed after treatment of the hook substrate at the highest enzyme concentration for 5 min.

5.2.2 *Time Course of Proofreading Activity with the Hook Substrate*

Proofreading activity under conditions where the concentration of ε was less than that of substrate was measured at several different time points (Fig. 5.5). The rate of removal of (dC) nucleotides from a mispaired terminus of the hook substrate by the ε subunit showed a hyperbolic response under these conditions.

5.2.3 *Michaelis-Menten Parameters for the ε Subunit with the Hook DNA*

The rate of removal of (dC) nucleotides from the mispaired terminus of the hook DNA was measured over a range of substrate concentrations (Fig. 5.6). Kinetic parameters were determined from a Lineweaver-Burk plot of the data: the K_M was found to be ≈ 0.5 μM, and k_{cat} was found to be 0.31 ±0.02 min^{-1} (Fig. 5.7). A value for K_M could not be obtained with greater reliability because of difficulty in measuring initial rates in this assay (see Fig. 5.5).

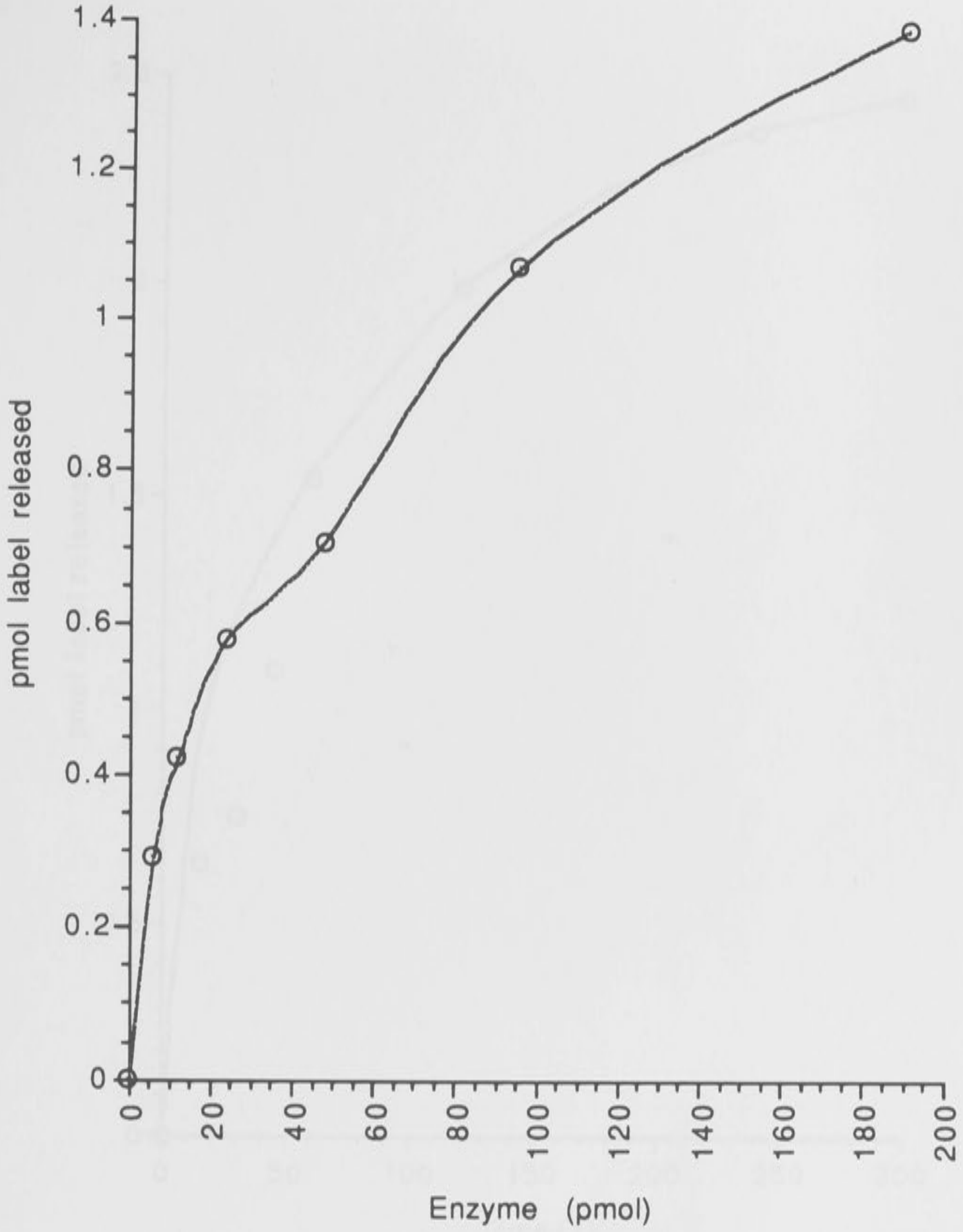


Figure 5.4: Relationship between enzyme concentration and activity in the hook assay. The assay was performed as described in the text. The concentration of 3´-OH termini was 40 nM; the input of label was 3.38 pmol.

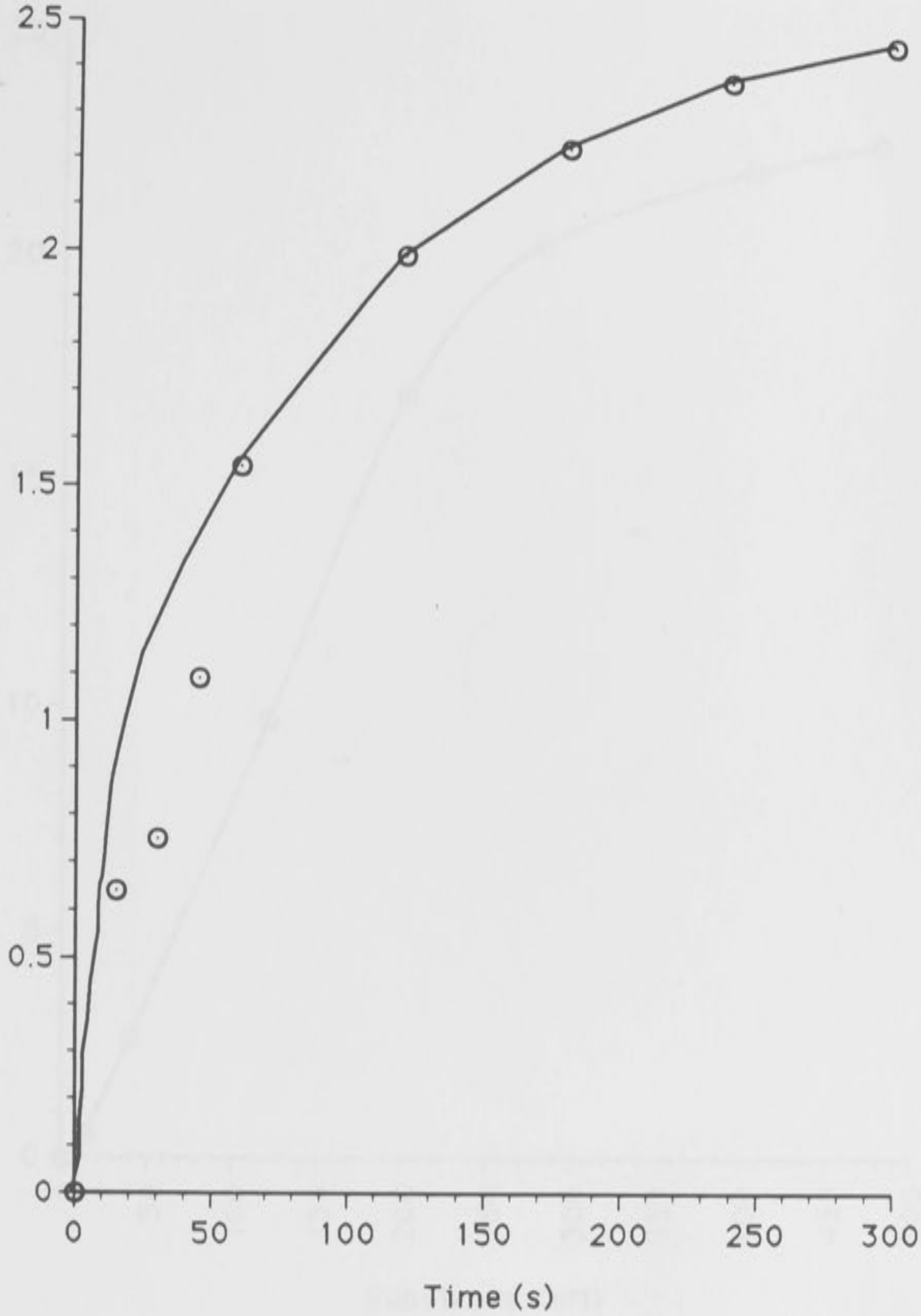


Figure 5.5: Time course of proofreading activity with the ε subunit. The hook DNA substrate (24 nM 3´-OH termini (as (dC)); the input of label was 2.52 pmol) was treated with 20 nM of enzyme at 30 °C. Aliquots (25 µl) were withdrawn at predetermined intervals and processed as described (Section 5.1.5.1.3).

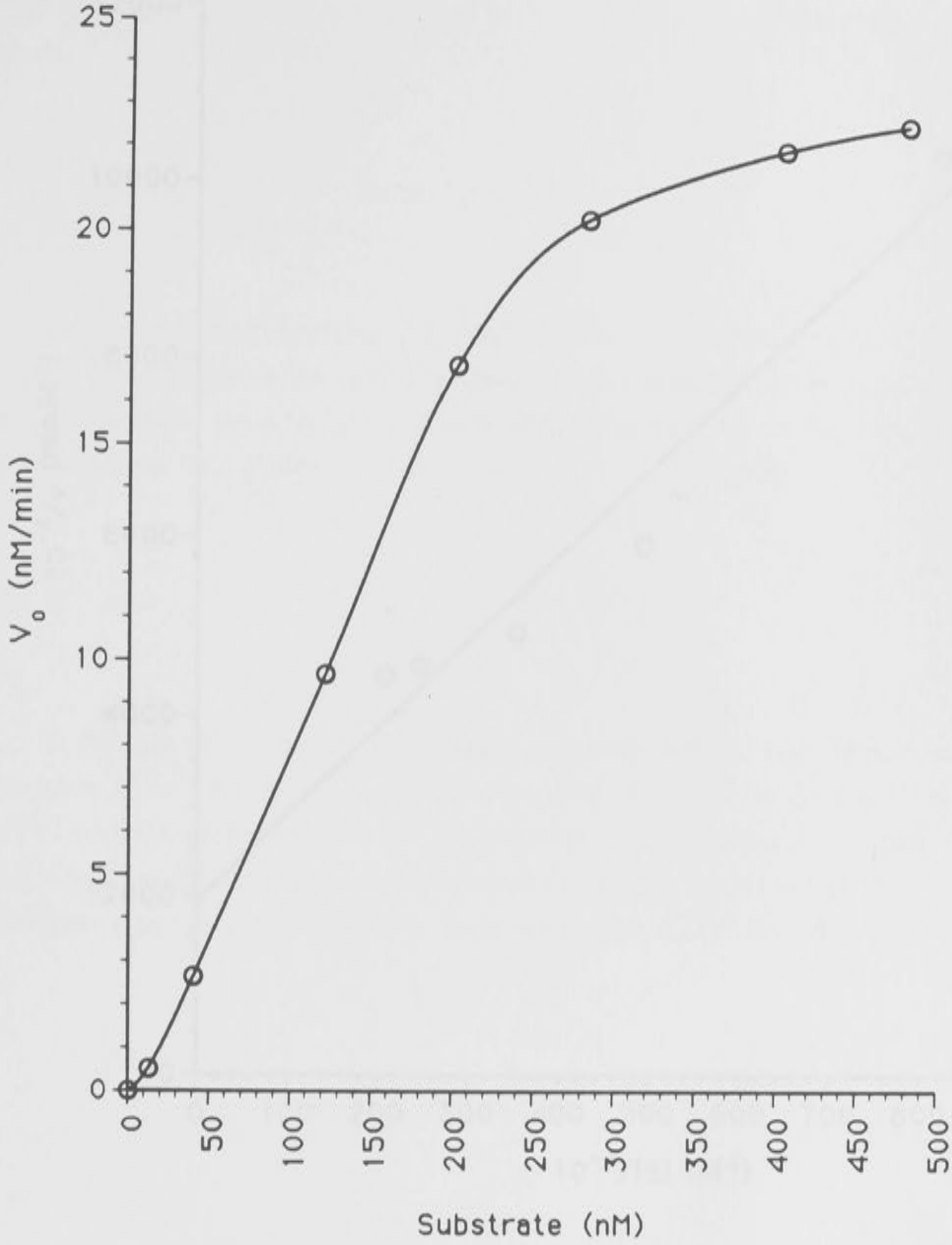


Figure 5.6: Effect of varying substrate concentration on the rate of proofreading activity in the hook assay. The concentration of ε was 169 nM. A control reaction containing no enzyme was included for each concentration of substrate. Reactions were carried out for 10 min at 30 °C and processed as described (Section 5.1.5.1.3).

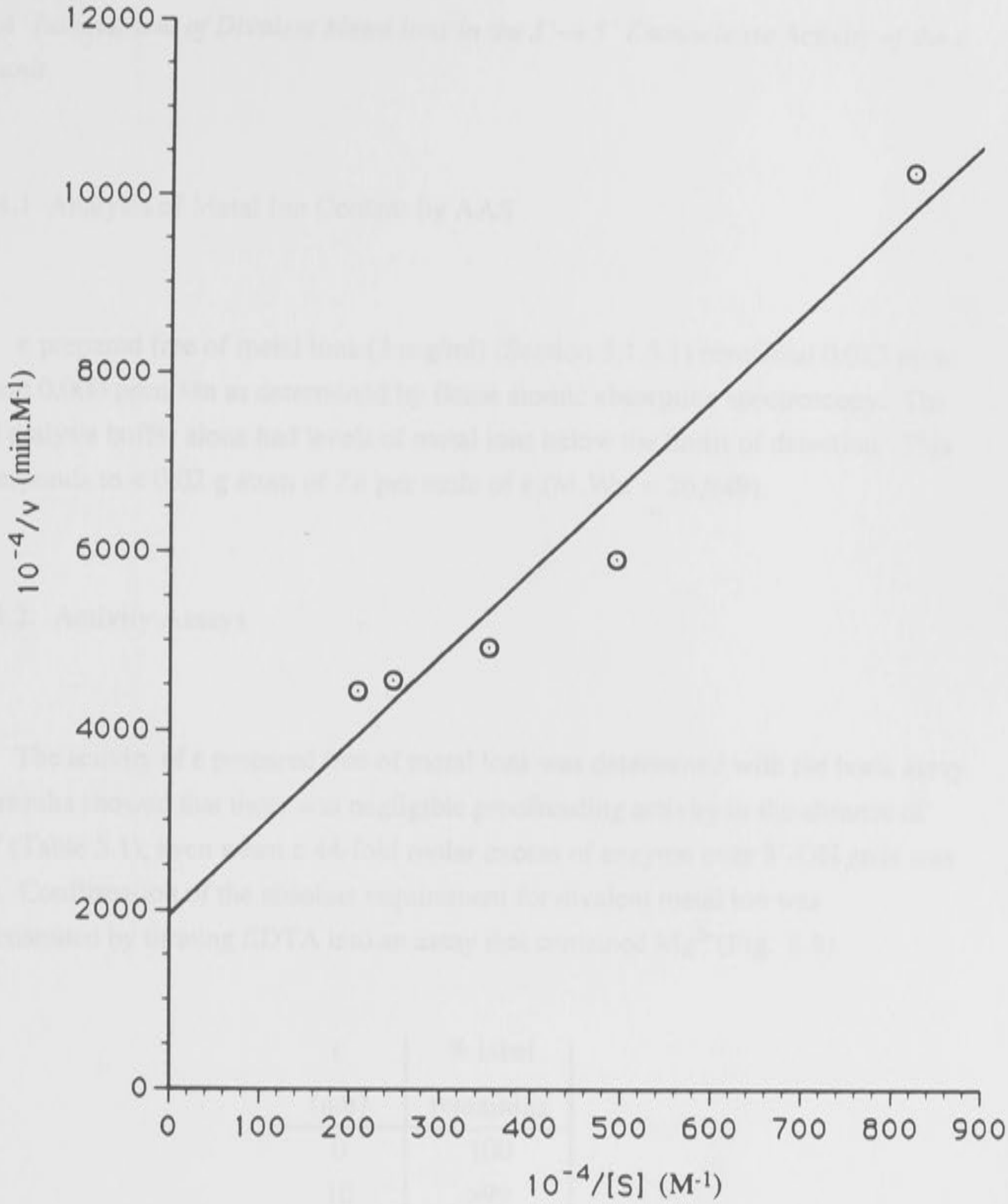


Figure 5.7: A Lineweaver-Burk plot of the data in Figure 5.6. The least-squares line gives K_M = 502 ±95 nM, and a catalytic constant, k_{cat}, of 0.31 ±0.023 min^{-1}.

5.2.4 *Involvement of Divalent Metal Ions in the 3′→5′ Exonuclease Activity of the ε Subunit*

5.2.4.1 Analysis of Metal Ion Content by AAS

ε prepared free of metal ions (3 mg/ml) (Section 5.1.5.1) contained 0.022 ppm Zn and 0.000 ppm Mn as determined by flame atomic absorption spectroscopy. The final dialysis buffer alone had levels of metal ions below the limits of detection. This corresponds to < 0.02 g atom of Zn per mole of ε (M.Wt. = 26,949).

5.2.4.2. Activity Assays

The activity of ε prepared free of metal ions was determined with the hook assay. The results showed that there was negligible proofreading activity in the absence of Mg^{2+} (Table 5.1), even when a 44-fold molar excess of enzyme over 3′-OH ends was used. Confirmation of the absolute requirement for divalent metal ion was demonstrated by titrating EDTA into an assay that contained Mg^{2+} (Fig. 5.8).

ε (nm)	% label remaining
0	100
10	>99
200	99
1164	94

Table 5.1: Proofreading activity of ε dialysed extensively against buffer containing EDTA (Section 5.1.7.1) (hook assay). The assay was performed as described using a mispaired terminus; the concentration of 3′-OH termini (as (dC)) was 26.8 nM.

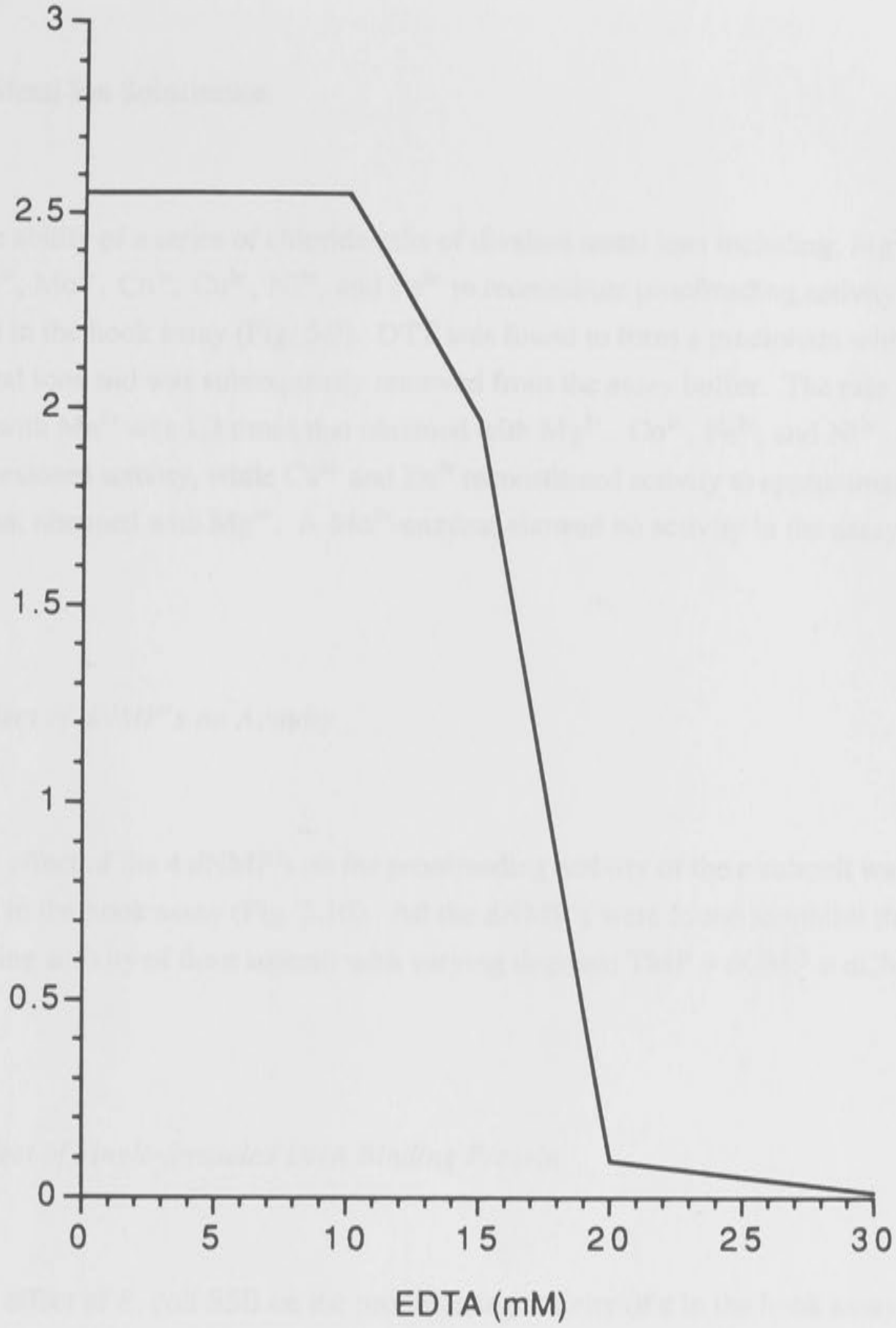


Figure 5.8: Requirement of $MgCl_2$ for exonuclease activity of ε. The assay was performed as described (Section 5.1.5.1.3) with a single modification: EDTA was added to the assay at various concentrations. The concentration of enzyme was 10 nM. The concentration of 3´-OH ends (as (dC)) was 26.8 nM and the input of label was 3.38 pmol. The concentration of Mg^{2+} in the assay was 10 mM.

5.2.4.3 Metal Ion Substitution

The ability of a series of chloride salts of divalent metal ions including, Mg^{2+}, Zn^{2+}, Mn^{2+}, Mo^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, and Fe^{2+} to reconstitute proofreading activity was measured in the hook assay (Fig. 5.9). DTT was found to form a precipitate with some metal ions and was subsequently removed from the assay buffer. The rate obtained with Mn^{2+} was 1.3 times that obtained with Mg^{2+}. Co^{2+}, Fe^{2+}, and Ni^{2+} partially restored activity, while Cu^{2+} and Zn^{2+} reconstituted activity to approximately 10% of that obtained with Mg^{2+}. A Mo^{2+}-enzyme showed no activity in the assay (Fig. 5.9).

5.2.5 *Effect of dNMP's on Activity*

The effect of the 4 dNMP's on the proofreading activity of the ε subunit was measured in the hook assay (Fig. 5.10). All the dNMP's were found to inhibit the proofreading activity of the ε subunit with varying degrees: TMP > dGMP > dCMP > dAMP.

5.2.6 *Effect of Single-Stranded DNA Binding Protein*

The effect of *E. coli* SSB on the proofreading activity of ε in the hook assay was measured (Fig. 5.11). SSB was found to inhibit ε activity measured as the release of radiolabelled (dC). Inhibition was almost complete even at the lowest concentration of SSB. Levels of SSB higher than this produced a marginal increase in inhibition of ε activity.

5.2.7 *Reaction Order*

Data presented in Figure 5.5 obtained under conditions where [ε] ≈ [substrate] were plotted: ln ($[B]_\infty$ - [B]t) *versus* time, as described earlier (Section 5.1.8). The linearity of the plot indicates that the rate of removal of nucleotides from a mispaired

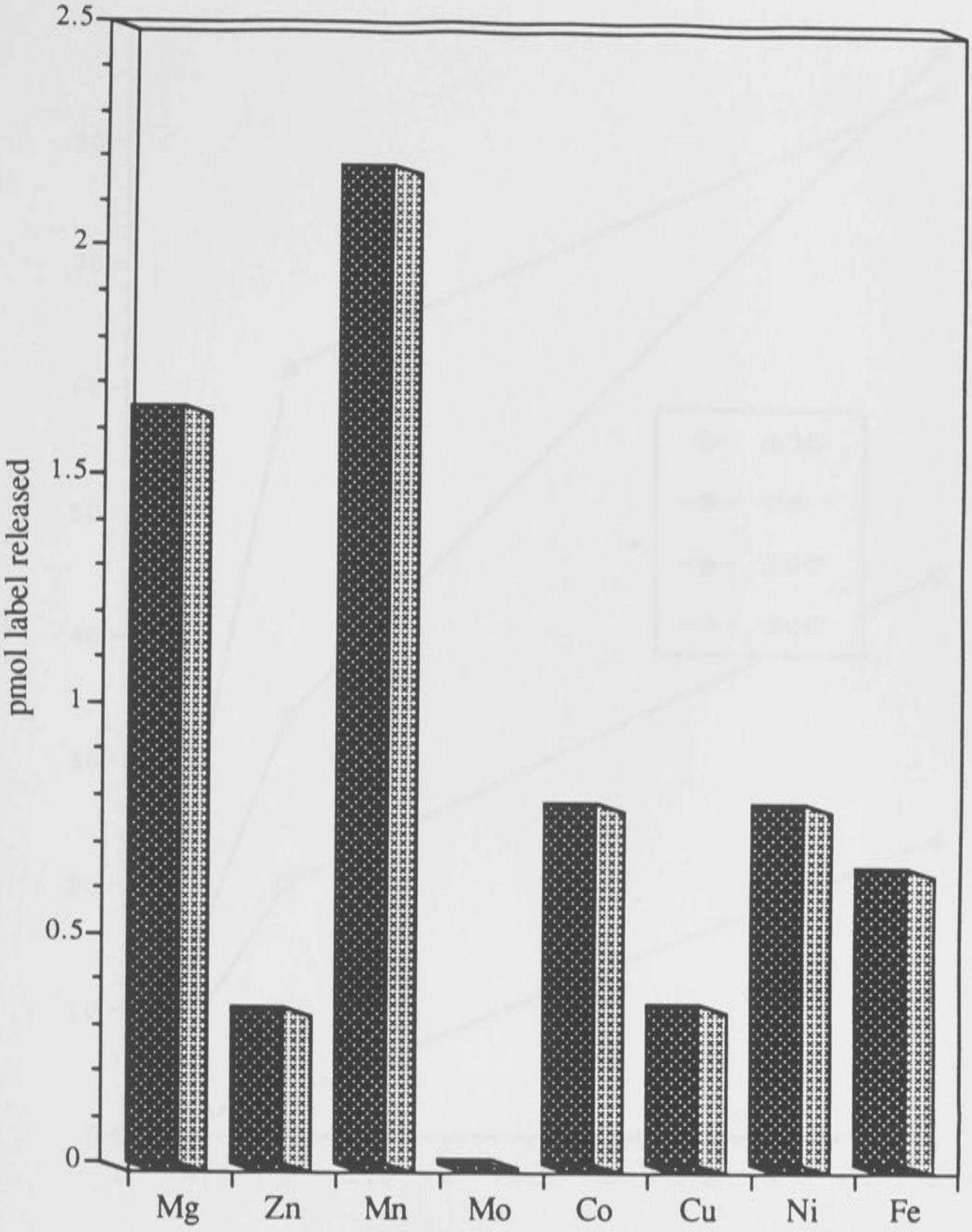


Figure 5.9: Effect of divalent metal ions on proofreading activity. The release of radiolabelled (dC) was measured on addition of various divalent metal ions to metal ion-depleted ε. The concentration of each metal in the assay was 5 mM. Assay buffer was prepared that contained no metal ion. The concentration of enzyme used was 10 nM, and the concentration of 3´-OH ends was 21.6 nM (as (dC)). The input of label was 2.61 pmol.

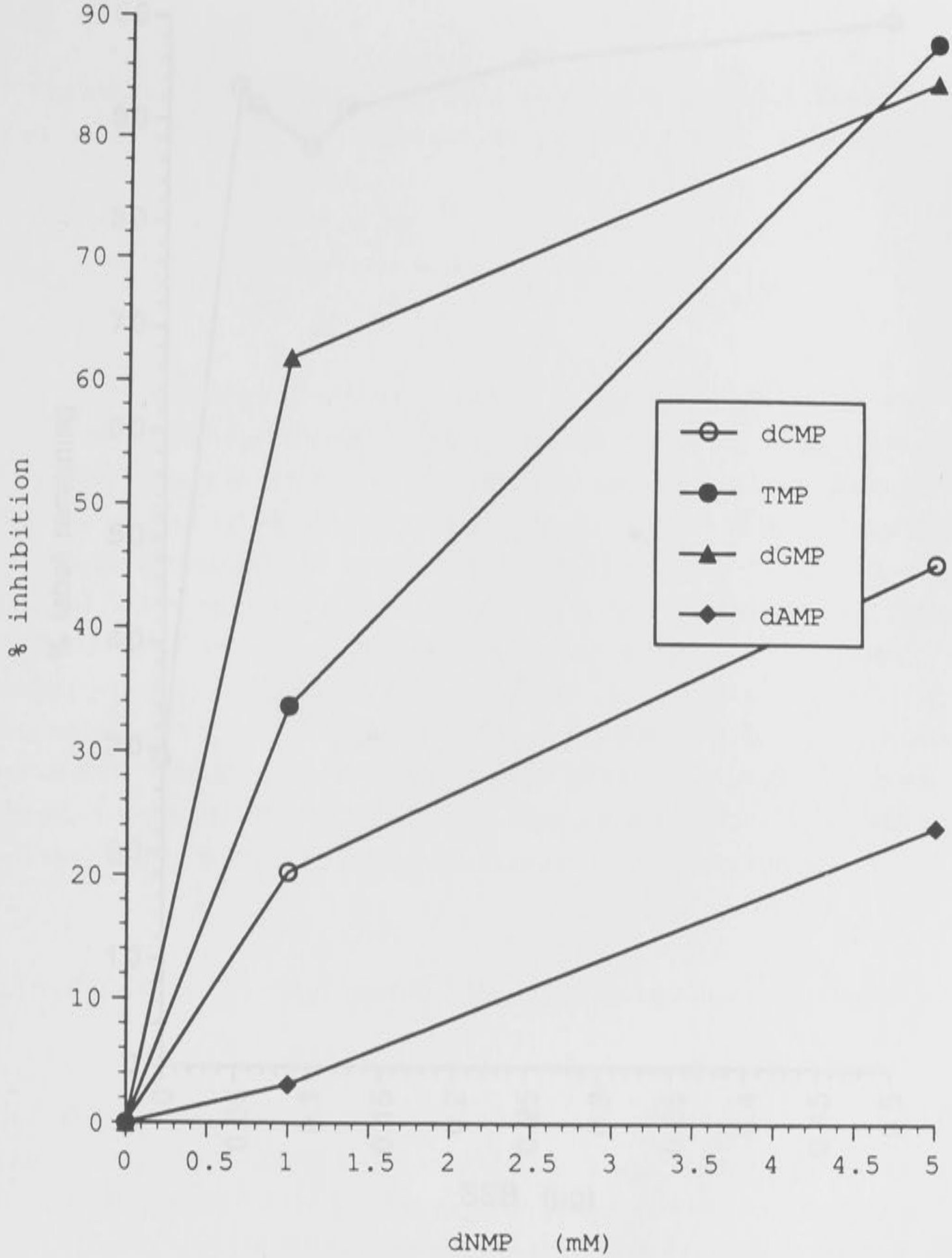


Figure 5.10: Effect of dNMP's on the proofreading activity of the ε subunit. The concentration of enzyme in the assay was 70.4 nM, and the concentration of 3′-OH termini (as (dC)) was 40 nM. The reaction was carried out at 30 °C for 15 min; samples were processed as described (Section 5.1.5.1.3). The values for % inhibition were calculated as 100[1 - (product released in the presence of dNMP/product released in absence of dNMP)]

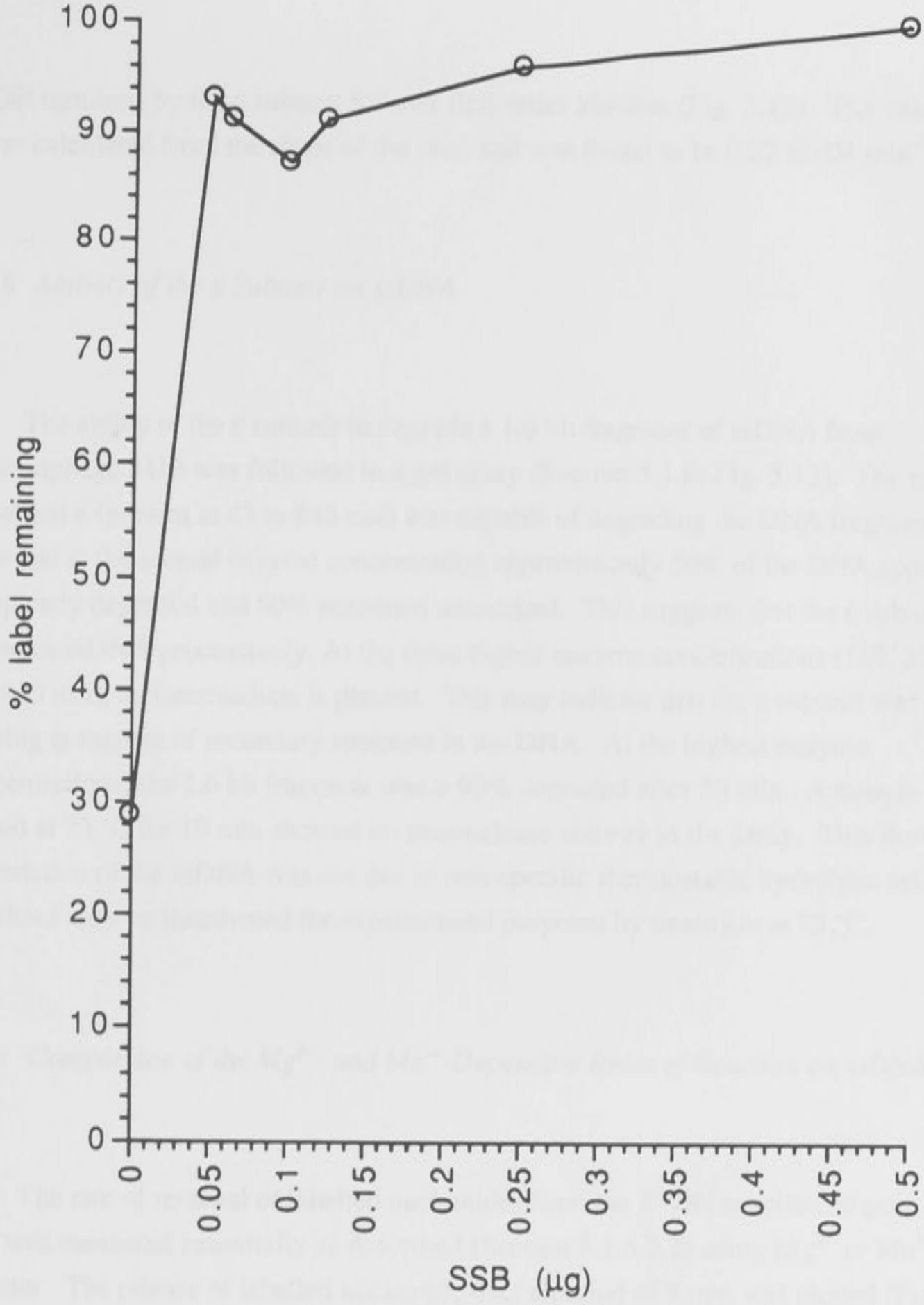


Figure 5.11: Effect of *E. coli* SSB on the activity of the ε subunit in the hook assay. The concentration of the ε subunit was 10 nM, and the concentration of 3´-OH termini was 24 nM (as (dC)). The SSB protein was preincubated with the substrate for 5 min prior to addition of the ε subunit. Otherwise, the assay was performed essentially as described (Section 5.1.5.1.3).

3′-OH terminus by the ε subunit follows first-order kinetics (Fig. 5.12). The value of k was calculated from the slope of the plot, and was found to be 0.82 ±0.04 min^{-1}.

5.2.8 *Activity of the ε Subunit on ssDNA*

The ability of the ε subunit to degrade a 1.6 kb fragment of ssDNA from bacteriophage M13 was followed in a gel assay (Section 5.1.9; Fig. 5.13). The results show that ε (present at 43 to 845 nM) was capable of degrading the DNA fragments. Note that at the second enzyme concentration approximately 50% of the DNA appears completely degraded and 50% remained untouched. This suggests that the ε subunit degrades ssDNA processively. At the three higher enzyme concentrations (169, 338 and 845 nM), an intermediate is present. This may indicate that the ε subunit was pausing at regions of secondary structure in the DNA. At the highest enzyme concentrations, the 1.6 kb fragment was > 90% degraded after 30 min. A sample of ε heated at 75 °C for 10 min showed no exonuclease activity in the assay. This shows degradation of the ssDNA was not due to non-specific thermostable hydrolytic activity, and that ε may be inactivated for experimental purposes by treatment at 75 °C.

5.2.9 *Comparison of the Mg^{2+} - and Mn^{2+}-Dependent Rates of Reaction on ssDNA*

The rate of removal of labelled nucleotides from the 3′-OH terminus of poly (dA) by ε was measured essentially as described (Section 5.1.5.2.2) using Mg^{2+} or Mn^{2+} as cofactor. The release of labelled nucleotide over a period of 8 min was plotted (Fig. 5.14). The Mg^{2+}- and Mn^{2+}-dependent extent of reaction on this substrate are the same within error limits. Due to problems associated with pipetting small volumes in this assay system, identical concentrations of substrate for each sample could not be achieved.

From Figure 5.14, the initial rate (v) of removal of dAMP in the presence of 84.5 nM ε and Mg^{2+} was ≈ 3.1 μM/min. The substrate concentration (as ends) was 1.2 μM. Assuming $K_M \approx 0.5$ μM, as observed for the hook template, then $k_{cat} \approx v/[\varepsilon]$, or ≈ 40 min^{-1}. This is > 100 times that observed for removal of dCMP from a mispaired terminus. ε is clearly an efficient 3′→ 5′ exonuclease on ssDNA than that observed with a mispaired terminus (k_{cat} = 0.31 min^{-1}).

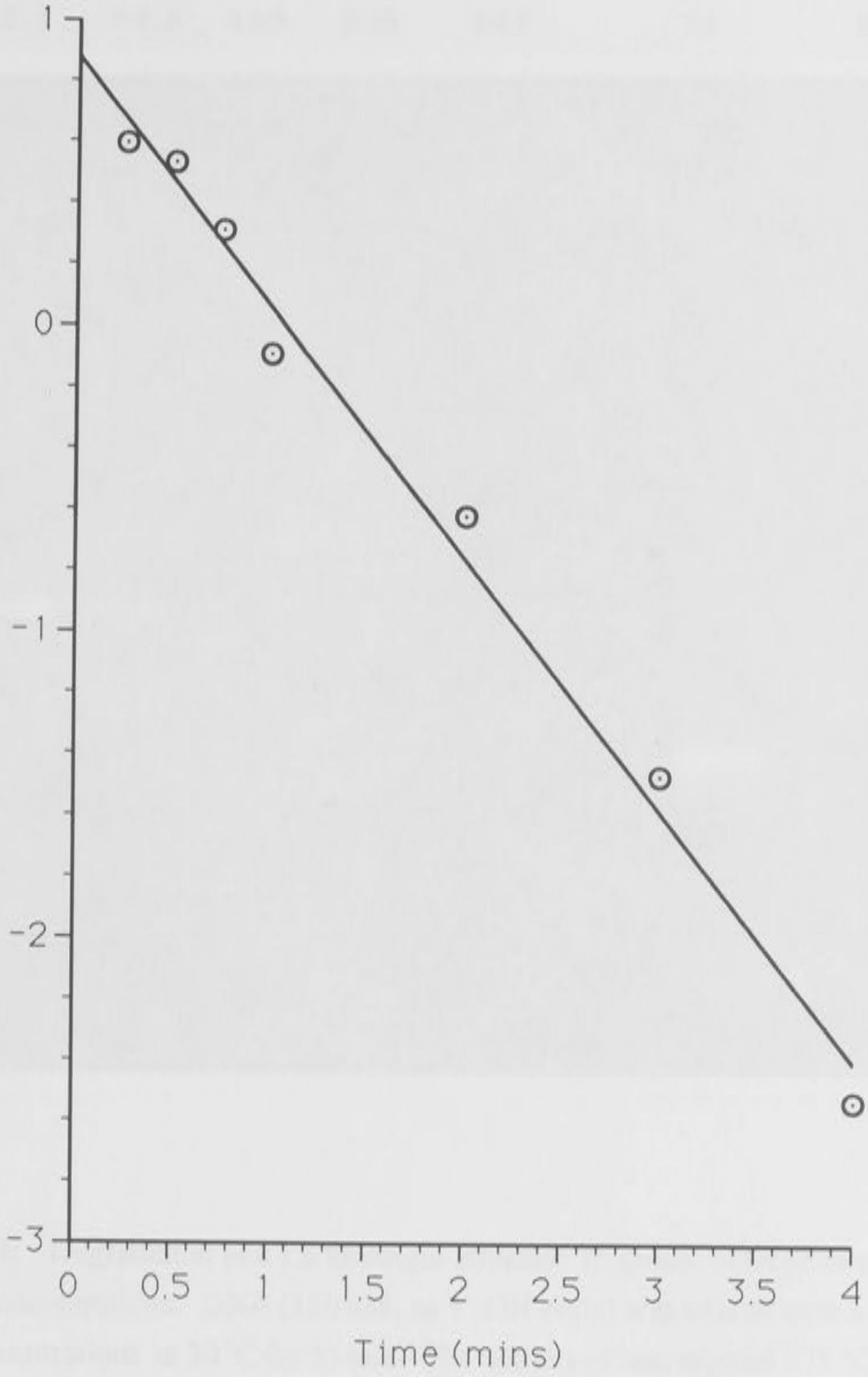


Figure 5.12: A semilogarithmic plot of the data presented in Figure 5.5 to determine the reaction order for the removal of nucleotides from a mispaired terminus of the hook substrate by the ε subunit. The plot was constructed as described in Section 5.1.18.

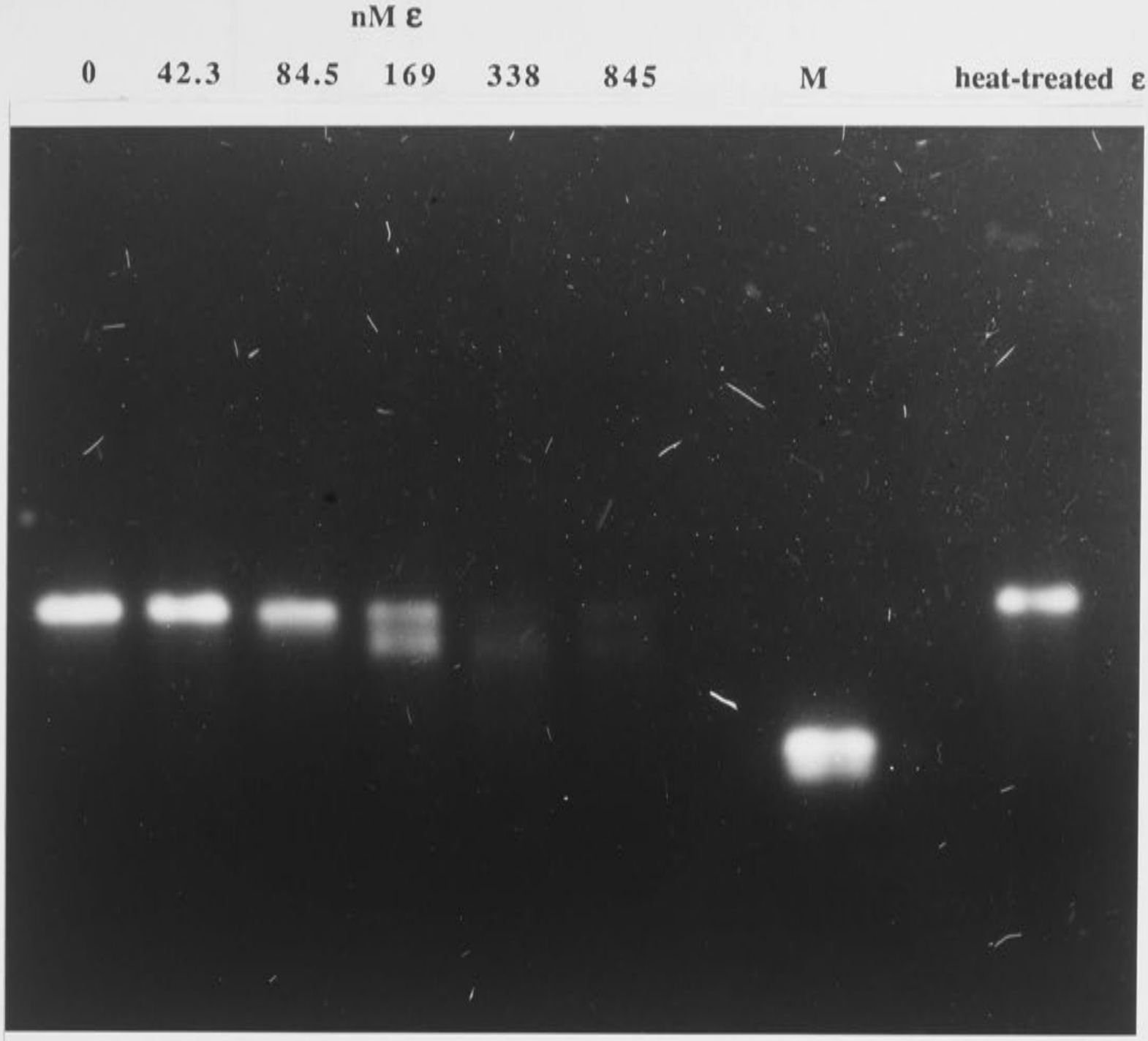


Figure 5.13: Degradation of a 1.6 kb single-stranded fragment of DNA at a range of enzyme concentrations. DNA (110 nM, as 3´-OH ends) was treated with a range of enzyme concentrations at 30 °C for 30 min. The activity of heat-treated (75 °C, 15 min) ε (338 nM) on ssDNA was also assessed. Activity was terminated by the addition of 50 mM EDTA at 0 °C. Reaction products were electrophoresed on a 1.1% agarose gel in the presence of ethidium bromide as described (Section 2.2.16). M = 0.8 kb marker.

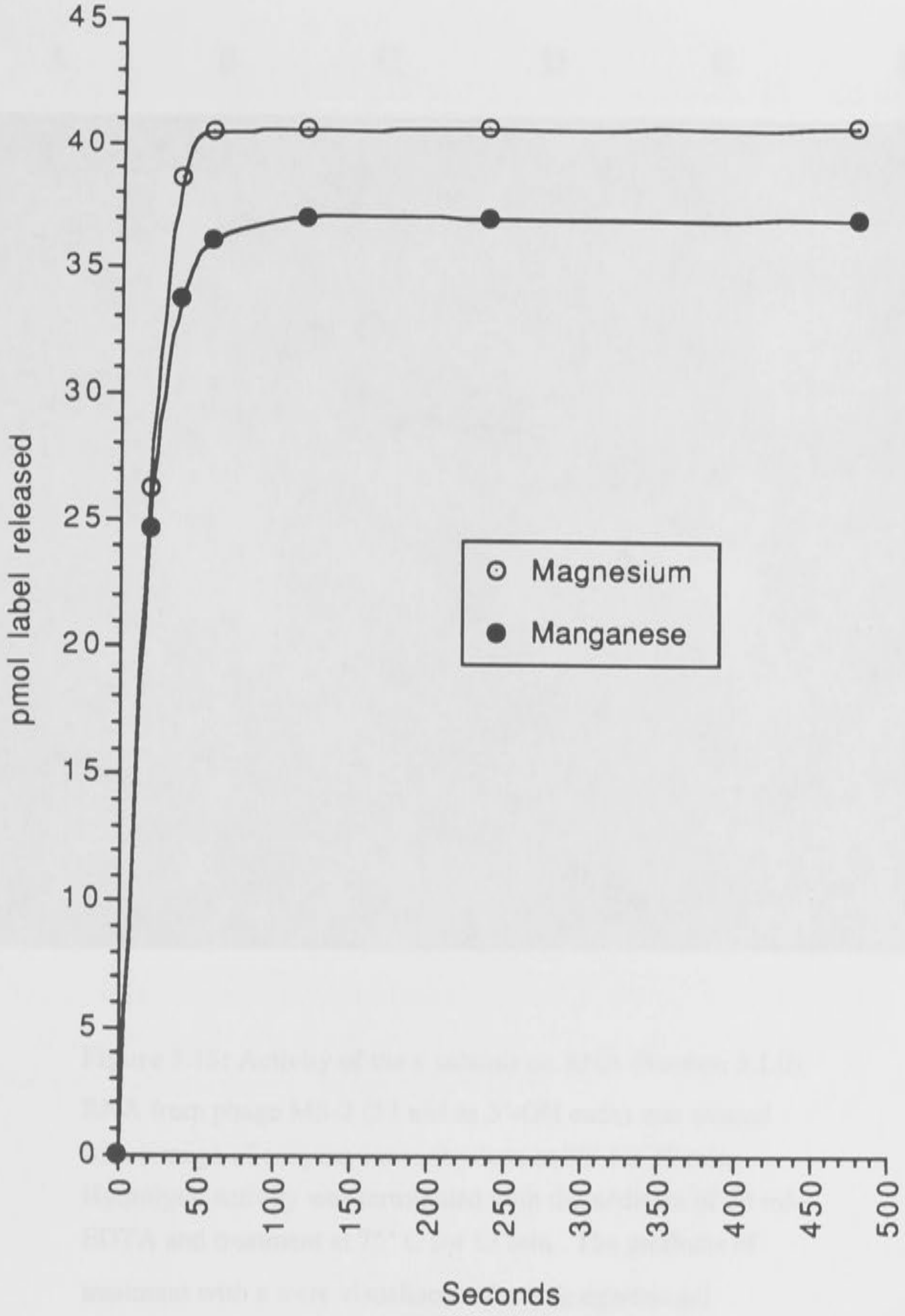


Figure 5.14: Comparison of the Mg^{2+}-and Mn^{2+}-dependent rates of reaction of ε with ssDNA. Labelled poly (dA) was prepared as described (Section 5.1.6) with an average of 1.4 labelled residues per end; the input of label was 41.23 pmol (Mg^{2+}) and 37.2 pmol (Mn^{2+}) in 25 µl. The concentration of the ε subunit was 84.5 nM. Each metal ion was present at a concentration of 10 mM. Samples were processed as described (Section 5.1.5.2.2).

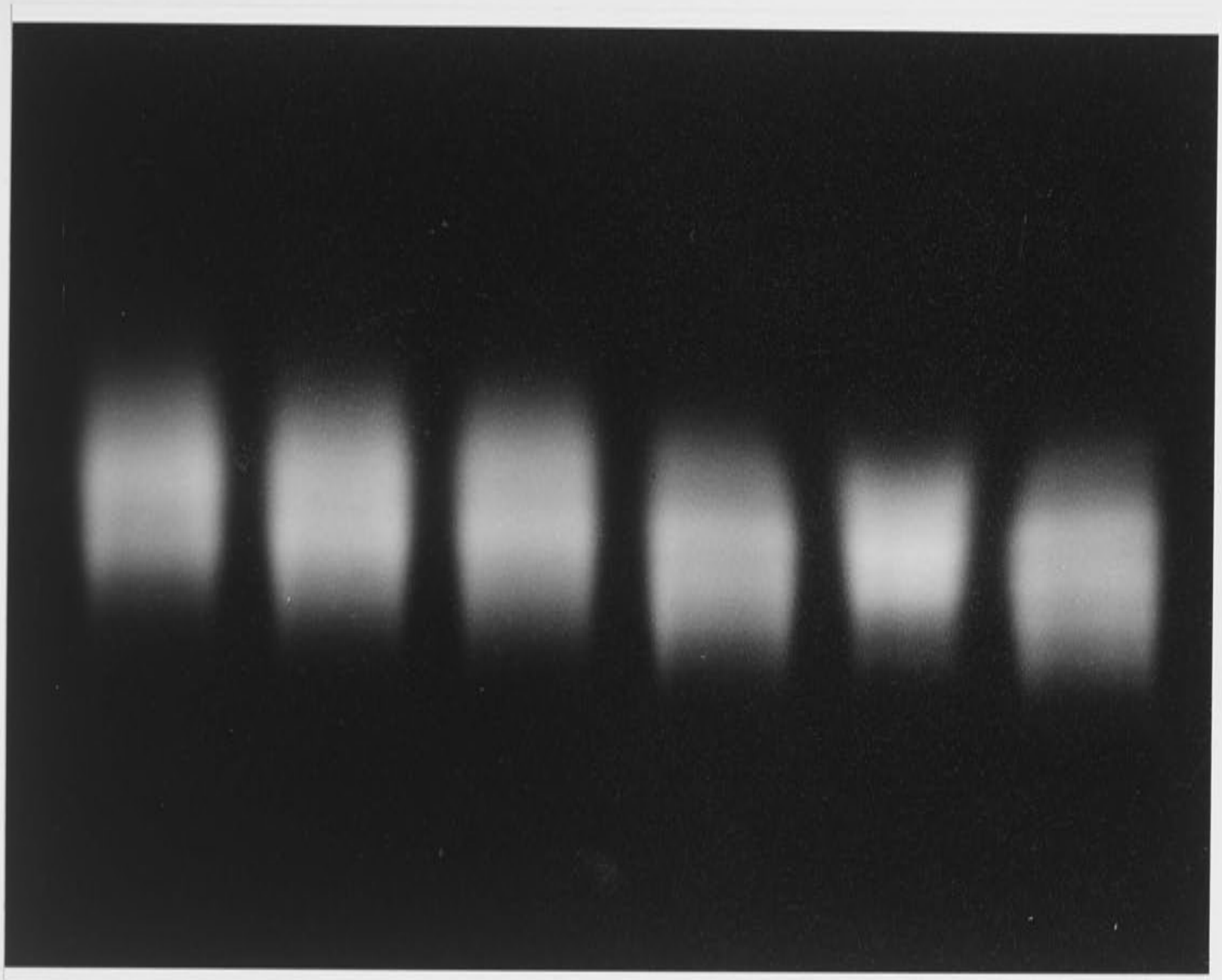


Figure 5.15: Activity of the ε subunit on RNA (Section 5.1.0). RNA from phage MS-2 (53 nM as 3´-OH ends) was treated with a range of enzyme concentrations at 30° for 30 min. Hydrolytic activity was terminated with the addition of 50 mM EDTA and treatment at 75° C for 15 min. The products of treatment with ε were visualised following agarose gel electrophoresis and by staining with ethidium bromide.

A: no ε

B: ε heated at 75° C for 15 min

C: [ε] = 0.573 μM

D: [ε] = 2.87 μM

E: [ε] = 5.73 μM

F: [ε] = 10.32 μM

5.2.10 *Activity of the ε Subunit on RNA*

The ability of the ε subunit to hydrolyse RNA from phage MS2 was assessed in a gel assay (Section 5.1.9). RNA (≈ 53 nM 3´-OH termini) was treated with a range of ε concentrations (0 to 10.32 μM) at 30 °C for 1 h. The reaction products were subjected to agarose gel electrophoresis (Fig. 5.15). The RNA molecule shows signs of having undergone only limited hydrolysis, even at a 195-molar excess of enzyme over substrate. Further, the extent of hydrolysis is approximately equal at the three highest enzyme concentrations. The inability of heat-inactivated enzyme to degrade RNA indicates that the observed RNAase activity in the assay is not due to some non-specific thermolabile contaminating activity.

5.2.11 *Activity of the ε Subunit on a Stretch of Mispaired Nucleotides*

The rate at which the ε subunit hydrolysed a 4 bp (dA) mismatch from the P1/P2 copolymer DNA labelled with ^{32}P at the 5´ terminus of both strands was measured in a gel assay (Fig. 5.16). A 10-fold molar excess of enzyme over substrate (3´-OH ends) was used to ensure pre-steady state kinetics. The rate constants for the removal of the first two mispaired nucleotides under these conditions, k_1 and k_2, were 2.97 min^{-1} and 3.02 min^{-1}, respectively. The higher accumulation of the product of the second step (k_2) relative to others suggests that k_3 and k_4 are smaller than k_1 and k_2.

5.3 DISCUSSION

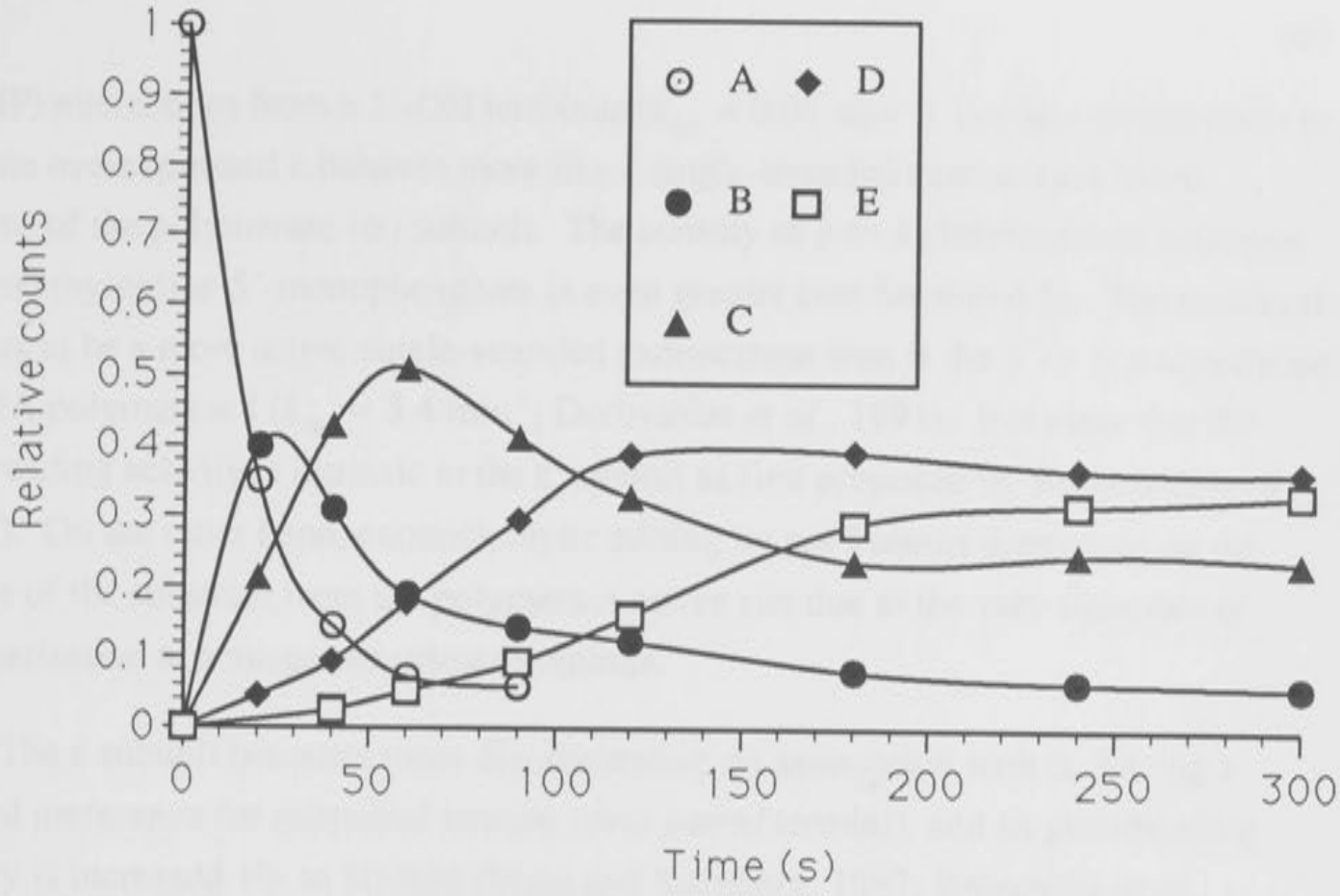
Substrate Specificity

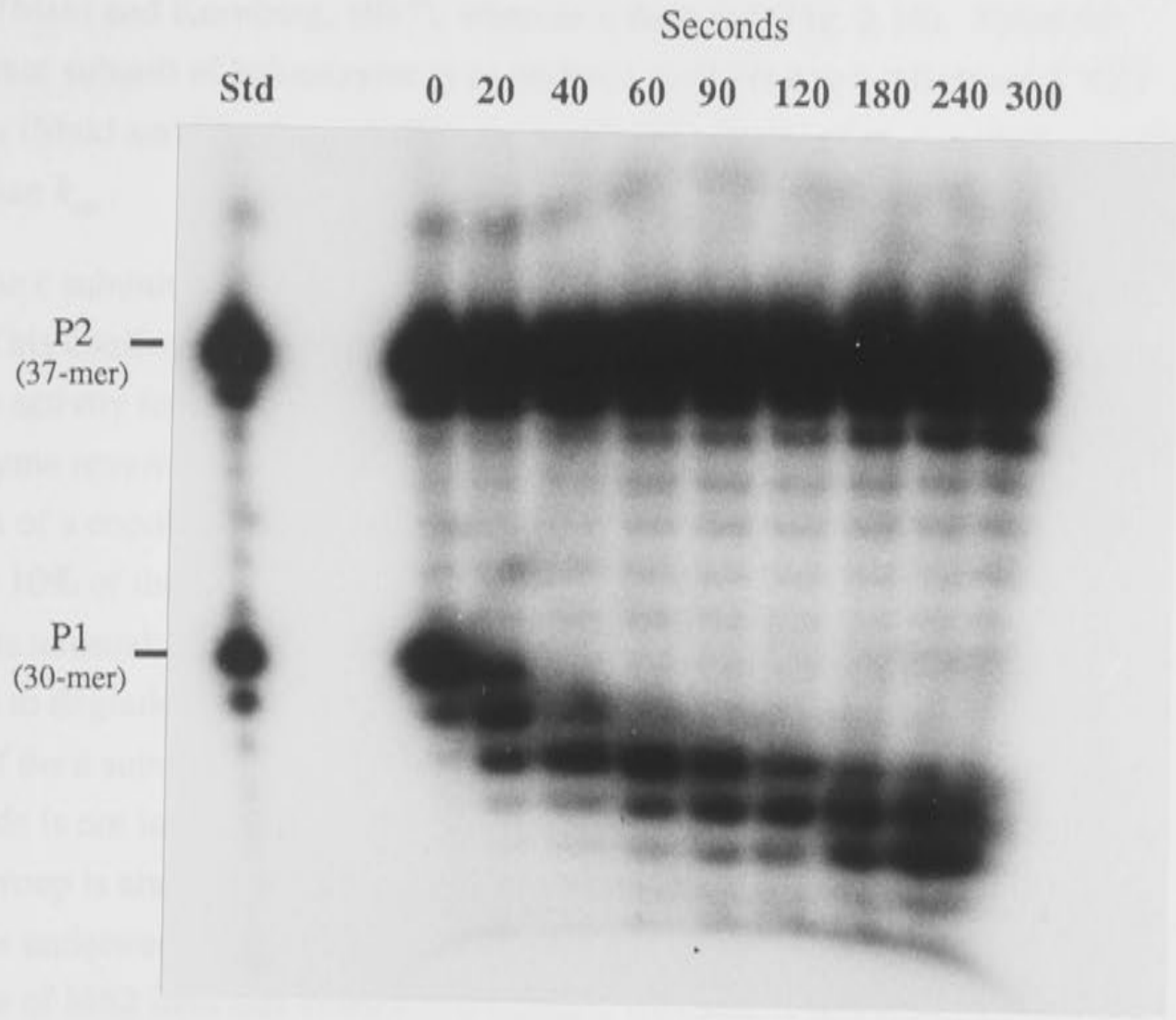
This study has confirmed a recent report (Brenowitz *et al.*, 1991) that the ε subunit of holoenzyme can readily degrade ssDNA (Fig. 5.13) in addition to its ability to remove a mispair from a 3´-OH terminus (Scheuermann and Echols, 1984; Maki and Kornberg, 1987). Indeed, the data presented here would indicate that the 3´→ 5´ ss-exonuclease activity of ε is substantially greater (> 100-fold) than its activity on mispaired 3´-OH termini. Although the ε subunit will remove incorrectly paired

Figure 5.16: Processivity of overexpressed ε subunit. The rate of removal of a 4 bp stretch of mispaired nucleotides from the 3´-OH primer terminus of the P1/P2 copolymer (Fig. 5.3) was determined in a gel assay under pre-steady state kinetics. The concentration of 3´-OH ends was 32.4 nM, and the concentration of ε was 324 nM. The assay was performed as described (Sections 5.1.10.2 to 5.1.10.5).

A . A plot of relative counts versus time. A = 4 mispaired nucleotides at 3´-OH end of primer terminus, B = 3 mispaired nucleotides, C = 2 mispaired nucleotides, 1 = 1 mispaired nucleotide, 0 = all mispairs removed.

B . Autoradiograph of the gel.


A
D
B
E
C
Relative counts
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
50
100
150
200
250
300
Time (s)


Seconds
Std
0 20 40 60 90 120 180 240 300
P2
(37-mer)
P1
(30-mer)

(dCMP) nucleotides from a 3′-OH terminus (k_{cat} = 0.31 min^{-1}), the data would seem to indicate overexpressed ε behaves more like a single-stranded exonuclease in the absence of the polymerase (α) subunit. The activity of ε on a chromophoric analogue of deoxythymidine 5′-monophosphate is even greater (see Section 6.5). The ε subunit appears to be a more active single-stranded exonuclease than is the 3′→ 5′ exonuclease of DNA polymerase I (k_{cat} = 5.4 min^{-1}; Derbyshire *et al.*, 1991). It is clear that the proofreading activity is intrinsic to the ε subunit as first proposed by Brenowitz *et al.* (1991). On the other hand, exonucleolytic editing by pol I seems dependent on the release of the substrate from the polymerase active site due to the very slow rate of polymerisation at a mispaired primer terminus.

The ε subunit becomes more discriminating on association with α, having a marked preference for mispaired termini (over *paired* termini), and its proofreading activity is increased 10- to 80-fold (Maki and Kornberg, 1987; Brenowitz *et al.*, 1991). An α–ε complex will also degrade (base-paired) duplex DNA from 3′-OH termini (Maki and Kornberg, 1987), whereas ε does not (Fig. 5.16). Since the polymerase subunit of holoenzyme is considered to direct ε to a mispaired 3′-OH terminus (Maki and Kornberg, 1987), the increased activity of α–ε is likely an effect on K_M than k_{cat}.

The ε subunit has weak hydrolytic activity on RNA as shown in a gel assay (Fig. 5.15). This confirms a report by Griep *et al.* (1990); the authors claim a more potent RNAase activity for ε as part of the holoenzyme. They measured the rate at which holoenzyme removed a single cordycepin (3′-dAMP) molecule from the 3′-OH terminus of a copolymer very similar to the P1/P2 molecule (Fig. 5.3). The observed rate was 10% of that of a single mispaired dNMP. The RNAase activity of the ε subunit is unusual; among other exonucleases of DNA, only exonuclease III is reported to degrade RNA strands as part of a DNA/RNA hybrid (Weiss, 1981). The ability of the ε subunit to degrade RNA suggests that the 2′-OH group of the 3′-OH nucleotide is not important for recognition. The result with cordecepin suggests the 3′-OH group is also unimportant for substrate recognition by ε. The MS2 RNA molecule underwent only limited hydrolysis (Fig. 5.15). Since the nucleotide sequence of MS2 indicates extensive secondary structure (Fiers *et al.*, 1976), this suggests ε may pause at the first regions of secondary structure in the RNA molecule.

Studies of Proofreading Activity

Functional studies of the proofreading activity of the ε subunit were performed using a specially designed oligonucleotide possessing a hook-like structure (Fig. 5.1) or a dA-dT 'copolymer' (Fig. 5.2). A mispair was created in the hook structure by tailing the 3′-OH terminus with [^{32}P]dCMP, and in the copolymer by tailing with [^{3}H]dCMP. These assays of the proofreading activity of ε using specially designed oligonucleotides are essentially similar to those used by other groups (Scheuermann and Echols, 1984; Maki and Kornberg, 1987).

The effect of varying enzyme concentration on the rate of proofreading activity was measured using the hook oligonucleotide. A plot of the data (Fig. 5.4) shows that dependence on enzyme concentration approximates linearity only when the concentration of substrate exceeds that of enzyme. Such a response is predicted from Michaelis-Menten kinetic law (Segel, 1975). It underlines the fact that if data obtained from these assays is to be used with any confidence, the assay must be performed in the linear region of the curve. In this assay system, that would be at a molar ratio of 3′-OH ends to ε of between 5:1 and 5:7.

A time course of proofreading activity under conditions where $[S]_0 \approx [E]_0$ showed that the removal of nucleotides followed a hyperbolic response (Fig. 5.5). Although the data followed a first-order rate law (Fig. 5.12), the significance of this is unclear for several reasons:

i) Although $[E]_0 \approx [S]_0$, both are substantially lower than the estimated K_M (see below).

ii) Substrate concentration does not vary with time during the bulk of the reaction. The hook was prepared in these studies with an average of 4.3 labelled residues incorporated per end. Consequently, when one residue is removed, *the concentration of 3′-OH ends is unchanged.*

iii) The efficiency with which ε removes successive nucleotides may vary (see below).

The four dNMP's were found to inhibit the proofreading activity of the ε subunit to varying degrees: TMP[1] > dGMP > dCMP > dAMP (Fig. 5.10). The data is in partial agreement with that of Scheuermann and Echols (1984), who reported dGMP to be the most inhibitory, dCMP to be the least; dAMP and TMP gave inhibition intermediate between those obtained with dGMP and dCMP. It is unclear why the

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[1]TMP showed a greater degree of inhibition than dGMP only at the highest concentration used.

degrees of inhibition for the dNMP's differ between the two studies but is likely due to differences in reproducing the assay conditions. Agreement with the Scheuermann and Echols (1984) data was obtained using a colourimetric assay (Section 6.4.3).

The *E. coli* SSB protein was found to inhibit ε activity (Fig. 5.11) when included in the hook assay. The binding of SSB to the single-stranded regions of the hook molecule presumably precludes recognition of the 3´-OH terminus by the ε subunit. It is well known that SSB inhibits the polymerase activity of holoenzyme subassemblies (Fay *et al.*, 1981) presumably by the same mechanism. On the other hand, *E. coli* SSB stimulates the pol I-catalysed hydrolysis of ssDNA (Molineux and Gefter, 1975), and in this way resembles the synergism of the T4 DNA polymerase with gene 32 protein (Bedinger and Alberts, 1983).

The ε subunit displayed a relatively strong affinity for the mispaired terminus of the hook DNA ($K_M \approx 0.5$ μM). This finding is in contrast to that reported by Maki and Kornberg (1987) who obtained a K_M of > 10 μM for their hook DNA, and is similar to that observed by Brenowitz *et al.* (1991) using the core polymerase on a comparable substrate. However, the figures quoted here and in the Maki and Kornberg (1987) paper should be interpreted with caution. Michaelis-Menten parameters were determined in this study using a few substrate concentrations (Fig. 5.9) which do not give a good fit to a straight line when plotted 1/*v versus* 1/S. Maki and Kornberg (1987) omitted to publish their Lineweaver-Burk plot so the degree of linearity of their data cannot be assessed. The issue is complicated by the fact that the number of mispairs at the 3´-OH terminus (hook and copolymer DNA's) was much greater (3 - 6 bp) than that used by other groups (≈ 1 bp). It is conceivable that the affinity for 3´-OH termini by the ε subunit varies with the length of the mispaired region. Several reports have suggested a melting mechanism for the exonucleolytic editing of DNA by proofreading exonucleases (Brutlag and Kornberg, 1972; Bessman and Reha-Krantz, 1977; Brenowitz *et al.*, 1991). In this model, proofreading exonucleases are intrinsically single-stranded nucleases, and substrate recognition is achieved because the mismatched nucleotide is in a single-stranded configuration. It follows that, the longer the mismatch, the greater the degree of melting, and the greater the affinity for the 3´-OH terminus by exonuclease.

The preceding discussion raises some concerns over the reproducibility of data obtained from the oligonucleotide-based assays (by all groups). In addition to batch-to-batch variation with the substrate itself (see above), experimental errors could be significant due to the small assay volumes used (25 μl). Kinetic measurements suffer from the difficulty of employing concentration of substrate greatly exceeding that of enzyme in these assays. Thus, a fundamental requirement for Michaelis-Menten

kinetics cannot be satisfied. There is clearly a need for a better and more reproducible assay of exonuclease activity using a more defined substrate. Such an assay is now available (see Chapter 6).

The Role of Divalent Metal Ion in the 3′→5′ Exonuclease Activity of the ε Subunit

Many enzymes involved in the breakage and formation of phosphate ester bonds require divalent metal ion(s) for activity (Knowles, 1980). The metal ion may bind tightly to specific amino acid residues at the active site; the enzyme is then termed a metalloenzyme. In other cases, the metal is only loosely associated with the enzyme and is referred to as a cofactor.

The ε subunit of DNA polymerase III holoenzyme has an absolute requirement for divalent metal ion. Enzyme dialysed extensively against buffer containing EDTA is inactive in the oligonucleotide-based assays, and the addition of EDTA to reactions containing ε and divalent metal ion abolishes activity. Both Mg^{2+} and Mn^{2+} support exonuclease activity, although the latter (along with Zn^{2+}) was not detected as a component of pure protein by AAS. The failure to detect Mn^{2+} or Zn^{2+} in ε by AAS does not mean that either metal does not play an integral role in enzyme structure. That is, the data does not prove ε is not a metalloenzyme. It would seem to suggest that the metal ion is easily dissociated. It is also important to remember that the purification of ε requires treatment with a denaturant; thus bound metal ion(s) may be lost.

Controversy surrounded the reports that DNA polymerase I was a Zn-metalloenzyme. These findings were based on the detection of stoichiometric amounts of Zn in pol I by AAS (Springate *et al.*, 1973) and also by the inhibition of the *polymerase* activity of pol I by 1,10-phenanthroline (Springate *et al.*, 1973; Slater *et al.*, 1971). This compound inhibits metalloenzymes by two mechanisms (Graham and Sigman, 1984). In the first mechanism, it binds reversibly to available coordination sites of the tightly bound metal ions and blocks access of substrates to catalytically important metal ions. The second and most common mechanism involves the removal of the metal ion upon treatment of enzyme with 1,10-phenanthroline. However, the AAS measurements of Springate *et al.* (1973) could not be repeated (Walton *et al.*, 1982) with enzyme from an overproducing strain. A second report failed to detect inhibition by 1,10-phenanthroline (Graham and Sigman, 1984). The authors further claimed that the results obtained by Springate *et al.* (1973) and by Slater *et al.* (1971) were due to the ability of 1,10-phenanthroline to degrade DNA, presumably as a Cu or

Fe complex in the presence of O_2. This would lead to a failure to detect synthesised DNA products, and give the impression the polymerase was inhibited in the assays used. The controversy was resolved when a crystal structure conclusively showed the presence of two loosely-bound divalent metal ions at the 3′→ 5′ exonuclease active site (Freemont *et al.*, 1988) (not the polymerase active site as reported by Springate *et al.*, 1973). Although pol I is not a metalloenzyme, the experience would indicate that the role of metal ions in enzyme structure and catalysis cannot be ruled out until a crystal structure is known.

The ability of a series of divalent metal ions to reconstitute proofreading activity was assessed (Fig 5.5). A Mn^{2+}-enzyme is 1.3-fold more active than a Mg^{2+}-enzyme in catalysing the removal of nucleotides from mispaired 3′-OH termini; a difference of this magnitude is not likely to be significant. The Mg^{2+}- and Mn^{2+}-enzymes have approximately the same activity on ssDNA. It is not too surprising that either metal ion can be mutually substituted for full activity, as Mn^{2+} and Mg^{2+} have very similar ionic radii. This would be expected if the Lewis acid properties of the metal ion were the essential aspect of catalysis, moderated somewhat by the particular ion, enzyme and substrate (Dixon *et al.*, 1976). Moreover, both metals have been reported to support the 3′→ 5′ exonuclease activity in DNA polymerase I (Lehman and Richardson, 1964), T4 DNA polymerase (Goulian *et al.*, 1968), T7 DNA polymerase (Tabor and Richardson, 1989) and exonuclease III (Weiss, 1981). In the case of pol I and T7 DNA polymerase, Mn^{2+} reduces the discrimination against dideoxynucleotides approximately 1000-fold in the polymerisation reaction (Tabor and Richardson, 1989). There are no reported effects of Mn^{2+} on the exonuclease component of these enzymes. Proofreading activity in the ε subunit was detected with a wide variety of divalent metal ions (Fig. 5.5) - consistent with their role as Lewis acids (Dixon *et al.*, 1976; Williams, 1982).

Derbyshire *et al.* (1988) propose that the two metal ions present at the 3′→ 5′ exonuclease active site of DNA polymerase I play a major role both in substrate binding and catalysis. Mg^{2+}, Mn^{2+}, or Zn^{2+} can fill either site, but the identity of the metal sites has yet to be established. A recent report used kinetic and direct binding studies to show that three divalent cations bind cooperatively to activate the 3′→ 5′ exonuclease site (Han *et al.*, 1991). The authors suggest that the role of the third metal ion may be indirect, adjusting the protein structure and thereby facilitating the correct orientation of the substrate at the active site. Alternatively, the third metal ion may have a role in catalysis but, due to low occupancy in the crystal, may have escaped detection (Han *et al.*, 1991). Beese and Steitz (1991) (Fig. 5.20) have proposed a mechanism in which metal A facilitates the formation of an attacking hydroxide ion whose lone pair electrons are oriented towards the phosphorus by interactions with the

metal ion. The second metal ion is thought to facilitate the leaving of the 3´ hydroxyl group and stabilisation of the 90° O-P-O bond angle between the apical and equatorial oxygen atoms (Fig. 5.20).

In the absence of structural data or detailed kinetic analyses, it is difficult to propose a mechanism of action for the ε subunit. However, Bernad *et al.* (1989) have shown based on amino acid sequence homology, that the 3´→ 5´ exonuclease active site is conserved for both prokaryotic and eukaryotic DNA polymerases. Three segments of *E. coli* DNA polymerase I (Exo I, II and III) containing critical amino acid residues involved in metal binding and catalysis are located in the same N-terminal half and conserved in the same linear arrangement in the ε subunit of DNA polymerase III holoenzyme (Fig. 5.21). The three domains invariably contain carboxylic amino acids. This homology could argue in favour of a similar mechanism for both enzymes, although their metal ion requirements may be slightly dissimilar. Confirmation of this hypothesis will require site-directed mutagenesis experiments of the residues thought to be involved in metal ion binding (Derbyshire *et al.*, 1991). pH-rate data using the colourimetric assay (Ch. 6) would also help identify such residues.

Processivity of the ε Subunit

Enzymes that synthesise and degrade DNA may dissociate after each catalytic event (termed distributive activity), or they may remain bound to the DNA until many cycles of reaction are completed (termed processive activity). If the direction of exonuclease action is known, processivity can be easily determined from the kinetics of release of nucleotides from a DNA strand labelled at the end resistant to hydrolysis. Direction and processivity of exonuclease action can both be determined in the same

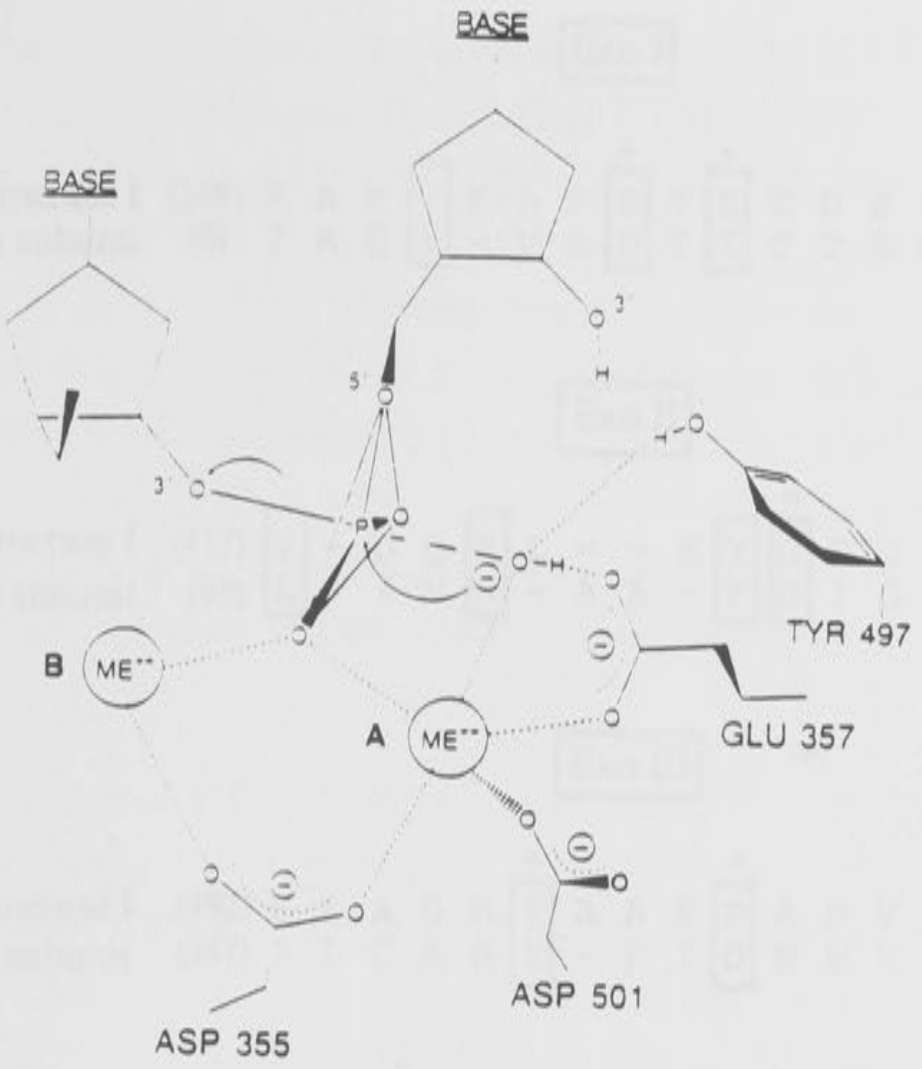

Figure 5.20: Proposed mechanism for the 3′→ 5′ exonuclease activity of DNA polymerase I. The model is described above. Beese and Steitz (1991).

way using a differentially-labelled DNA polymer. Processivity determinations require measurement of single turnovers, hence, an excess of enzyme over substrate is necessary.

The use of gel assays to measure the rate of removal of nucleotides from a primer terminus under pre-steady state kinetics is not new (Reems *et al.*, 1991; Brenowitz *et al.*, 1991; Donlin *et al.*, 1991). However, in all cases, the assays were designed to measure the rate at which the terminal 3′-OH nucleotide is removed. Consequently, these assays tell us very little about how a proofreading enzyme performs over a *stretch* of nucleotides. There is now some evidence to show that proofreading by the Klenow enzyme and T7 DNA polymerase require that the primer terminus melt out by from 2 to 7 nucleotides. Therefore, it would be more informative to determine the activities of a proofreading exonuclease on a substrate that has a series of mispaired nucleotides, such as that in the P1/P2 copolymer.

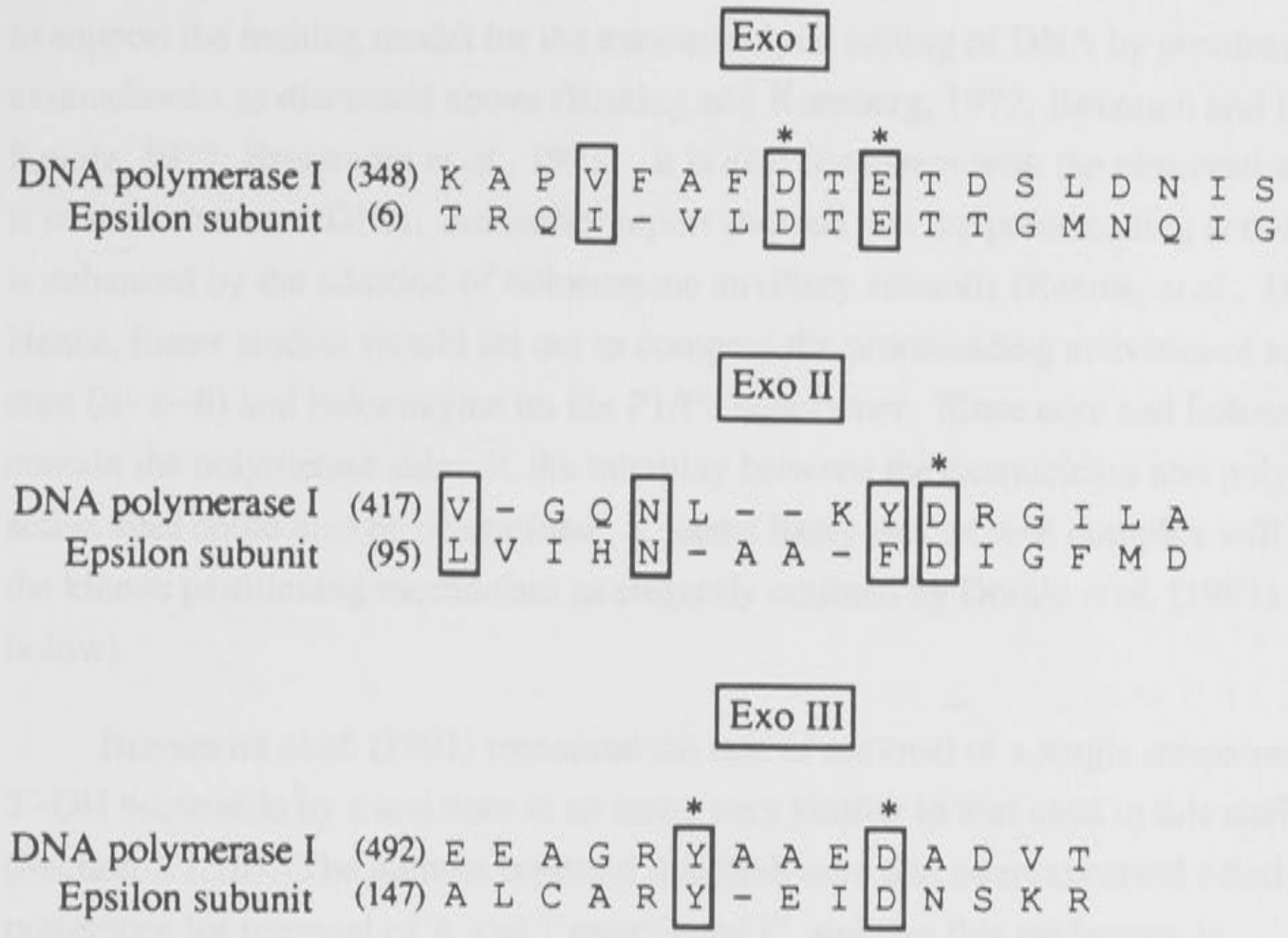

Figure 5.21: Alignment of amino acid residues involved in the 3′→ 5′ exonuclease activity of DNA polymerase I (pol I) and with residues in the ε subunit of DNA polymerase III holoenzyme. The pol I residues involved in metal binding and catalysis are indicated by asterisks. Adapted from Bernad *et al.* (1989).

The rate at which the ε subunit removed a 4 bp (dA) mismatch from a copolymer DNA (Fig. 5.3) labelled with ^{32}P at the 5′ terminus of both strands was measured in a gel assay (Fig. 5.16). The data show that under pre-steady state conditions, the first two mispaired residues are rapidly removed (k_1 = 2.97 min^{-1}, k_2 = 3.02 min^{-1}) while the remaining two mispaired nucleotides are more slowly excised. This is consistent with a distributive mechanism in which the ε subunit rapidly locates and binds a mispaired 3′-OH terminus releasing a nucleotide before dissociating from the DNA. If a processive mechanism were operating, we would not expect to observe any intermediates. The rate constants k_3 and k_4 were not determined due to the complexity of the integrations required to derive the rate equations. In retrospect, four P1 oligonucleotides (see Fig. 5.3) should have been made. One would have a single mispair at the 3′-OH terminus, another with 2 mispairs, and so on. The rate of removal of the first two nucleotides from each of these substrates could then be easily determined using the pre-steady-state gel assays.

The rapidity with which the first two nucleotides are removed would also seem

to support the melting model for the exonucleolytic editing of DNA by proofreading exonucleases as discussed above (Brutlag and Kornberg, 1972; Bessman and Reha-Krantz, 1977; Brenowitz *et al.*, 1991). It is also consistent with the observation that ε is more active on ssDNA. An earlier report showed that the proofreading activity of ε is enhanced by the addition of holoenzyme auxiliary subunits (Reems, *et al.*, 1991). Hence, future studies should set out to compare the proofreading activities of ε, pol III core (α–ε–θ) and holoenzyme on the P1/P2 copolymer. Since core and holoenzyme contain the polymerase subunit, the interplay between the exonuclease and polymerase active sites could also be determined. It seems likely that an α–ε complex will show the kinetic partitioning mechanism as elegantly outlined by Donlin *et al.* (1991) (see below).

Brenowitz *et al.* (1991) measured the rate of removal of a single mispaired 3′-OH nucleotide by ε and core in an assay very similar to that used in this study (Section 5.1.10). The authors reported that both core and overexpressed ε had a preference for removal of A and T over G and C, and that this preference is pronounced with isolated ε subunit. Michaelis-Menten kinetic parameters were determined on a substrate with C.G and T.G mispaired primer termini. While a mispaired 3′-OH nucleotide was found to be removed faster than a correctly paired one, the K_M values for both were approximately the same. The increased V_{max} for removal of the mispaired nucleotide supports the concept that the source of editing specificity is the greater melting capacity of a mispaired 3′-OH terminus. There was a large thermal effect on 3′→ 5′ exonuclease activity for both ε and the core polymerase for correctly paired G.C and A.T and for a G.T mispair (Brenowitz *et al.*, 1991).

Donlin *et al.* (1991) used a series of copolymer substrates very similar to P1/P2, but with 1 to 3 mispairs, to determine the rate at which the T7 DNA polymerase removed the 3′-OH terminal nucleotide of each. Their kinetic data showed that the rate at which T7 DNA polymerase removed the terminal 3′-OH nucleotide increased with increasing number of mispaired 3′-OH mismatches. They further demonstrated that the removal of mispaired nucleotides was facilitated by its faster transfer rate to the exonuclease site relative to the slow rate of polymerisation over a mismatch. Both paired and mispaired DNA bind to the polymerase active site with equal affinities. Removal of mispaired nucleotides was governed by the rate of transfer of the DNA from the polymerase to the 3′→ 5′ exonuclease active site. These observations are all consistent with those made with the Klenow enzyme (see above), and would provide further evidence for a melting model for the specificity of exonucleolytic editing.

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CHAPTER 6

KINETIC ANALYSIS OF THE ε SUBUNIT USING A *p*-NITROPHENYL ESTER OF THYMIDINE 5´-MONOPHOSPHATE

6.0 INTRODUCTION

6.1 *General Introduction*

Assay of 3′→ 5′exonuclease activity by the ε subunit has been based on the removal of radiolabelled nucleotides from a mispaired terminus. These assays have three disadvantages. Firstly, reproducibility is poor, due in part to variability between batches of substrate, and experimental errors tend to be high as the assay volume is small. Secondly, due to the size of the DNA substrates, it is difficult to attain substrate concentrations (ie. 3′-OH ends) greatly exceeding that of enzyme. To satisfy Michaelis-Menten kinetics, the concentration of enzyme must be negligible compared to substrate. Finally, the assay is not continuous. This makes it more difficult to estimate initial rates.

Use of chromophoric substrates show promise as simple and reproducible assays of enzyme activity. Substrates may be precisely defined in chemical terms, and experimental errors are minimised by using a substantially larger assay volume. If release of the chromophore can be monitored by continuous spectrophotometry, this facilitates kinetic studies. In addition, information on substrate specificity can be obtained by varying the substituents on the main chemical group (Cuatrecasas *et al.*, 1969).

Chromophoric analogues of deoxythymidine 5′-monophosphate such as thymidine 5′- monophosphate *p*-nitrophenyl ester (*p*np-TMP) (Fig. 6.1), have been routinely used for several decades in assays of phosphodiesterase activity. Phosphodiesterase I from snake venom and kidney hydrolyses *p*np-TMP to *p*-nitrophenol and TMP (Razzell, 1963). DNA polymerase I is also reported to hydrolyse *p*np-TMP to *p*-nitrophenol and TMP (Lehman and Richardson, 1964).

Staphylococcal nuclease is an extracellular phosphodiesterase of *Staphylococcus aureus* that can hydrolyse either RNA or DNA to release 3′-phosphomononucleotides and dinucleotides (Cunningham *et al.*, 1956; Cuatrecasas *et al.*, 1969). While considerable data on the physicochemical properties of the enzyme had been acquired, studies of catalytic mechanisms and substrate specificity had been hampered by the unavailability of *defined* substrates of low molecular weight. However, a number of *p*-nitrophenyl ester derivatives of deoxythymidine 5′-monophosphate were found to be recognised by staphylococcal nuclease with concomitant release of *p*-nitrophenyl phosphate (Cuatrecasas *et al.*, 1969). In the assay, the accumulation of *p*-nitrophenyl

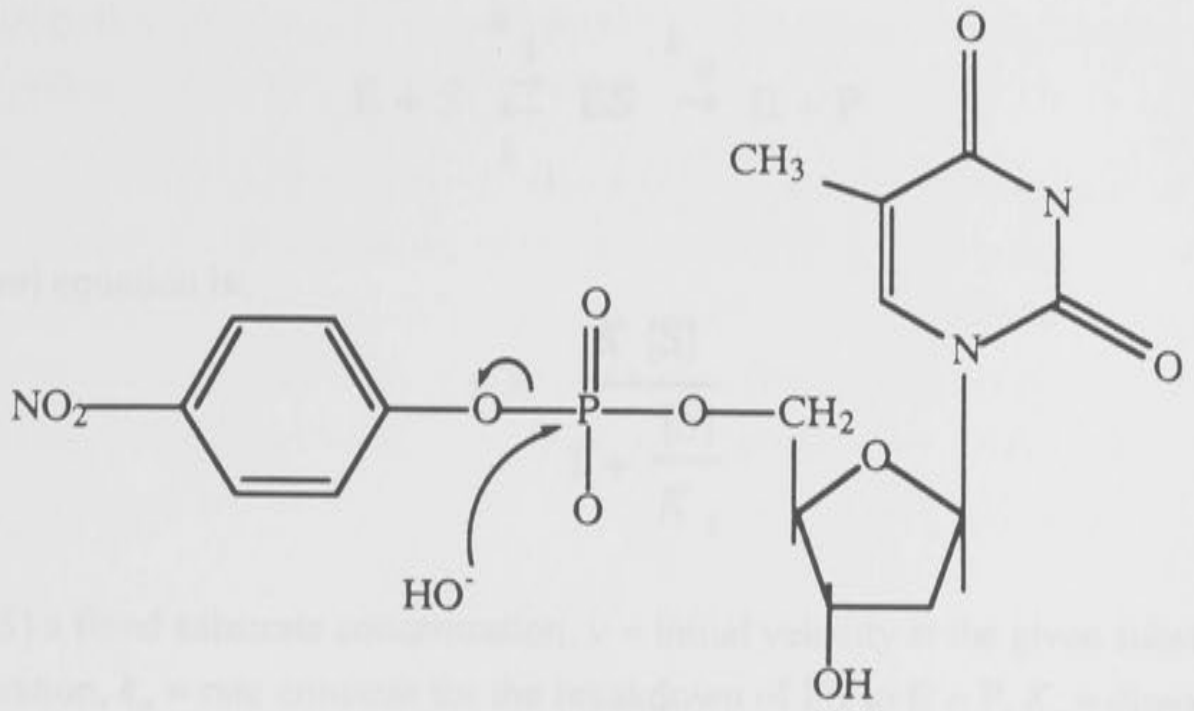


Figure 6.1: Structure of thymidine 5´- monophosphate *p*-nitrophenyl ester (M.Wt.: 443.7 g) showing the overall reaction for enzyme-catalysed hydrolysis of *p*np-TMP.

phosphate was monitored continuously by following the increase in absorbance at any wavelength in the range 310 - 350 nm. The Ca^{2+} ion-dependent hydrolysis of *p*np-TMP was shown to obey Michaelis-Menten kinetics with a K_M of 2.2 mM, and a k_{cat} of 9.1 min^{-1}. Using other *p*-nitrophenyl derivatives of TMP, Cuatrecasas *et al.* (1969) were able to deduce a partial formulation of the mechanism and specificity of the enzyme.

6.1.1 *Fundamental Kinetic theory*

As a great deal of this chapter deals with kinetic studies, it is germane to review some kinetic theory.

Early this century, scientists sought to explain the progress of reactions in terms of known principles of equilibrium and mass action. The first general rate equation for reactions involving enzymes was derived in 1903 by Henri. Henri's equation accounted for the observation that the initial rate of a reaction was directly proportional to the concentration of enzyme, but increased in a nonlinear fashion with increasing substrate concentration up to a limiting maximum rate. The overall reaction was visualised as:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_p} E + P$$

The Henri equation is:

$$v = \frac{K\,[S]}{1 + \dfrac{[S]}{K_s}}$$

where [S] a fixed substrate concentration, v = initial velocity at the given substrate concentration, k_p = rate constant for the breakdown of ES to E + P, K_s = dissociation constant of the ES complex, and K is a constant, characteristic of the particular enzyme preparation = $k_p\,[E]_t/K_s$ where $[E]_t$ is the total concentration of enzyme.

Some years later Michaelis and Menten confirmed Henri's experimental work and presented a slightly modified version of the rate equation:

$$v = \frac{k_p\,[E]_t\,[S]}{K_s + [S]}$$

If $v = k_p\,[ES]$, then $k_p\,[E]_t$ can be taken as V_{max}, the limiting maximal velocity that would be observed when all the enzyme is present as ES. This gives the now well-known "Michaelis-Menten" equation:

$$\frac{v}{V_{max}} = \frac{[S]}{K_s + [S]}$$

For the equation to provide an adequate description of an enzyme-catalysed reaction, conditions will usually be met:

i) The enzyme and substrate react rapidly to form an enzyme-substrate complex,

ii) Only a single substrate and a single enzyme-substrate complex are involved, and the enzyme substrate complex breaks down directly to free enzyme and product,

iii) The rate at which ES dissociates to E + S is much faster than the rate at which ES breaks down to form E + P,

iv) The substrate concentration is very much greater than enzyme concentration, and

v) The overall rate of the reaction is limited by the breakdown of the ES complex to free enzyme and product.

In 1925, Briggs and Haldane derived a general rate equation that did not require the restriction of equilibrium required by the methods of Henri, Michaelis, and Menten. Haldane showed that the ES complex need not be in equilibrium with free enzyme and substrate, but within a very short time after starting the reaction the ES would build-up to a near-constant or "steady state" level. Thus a more realistic sequence of events is:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} E + P$$

The general Michaelis-Menten rate equation is unchanged, with the rate constants are grouped to give the K_M, a dynamic or pseudoequilibrium constant expressing the relationship between the actual steady-state concentrations rather than equilibrium concentrations.

Under usual assay conditions, velocities are measured very early in the reaction before the product concentration has increased to significant levels. Thus, one can calculate the initial velocity for the reaction in each direction from the Michaelis-Menten equation outlined above. Derivations of the Michaelis-Menten rate equation are used in more complex situations, for example, the action of inhibitor molecules and where there are two or more substrates.

6.1.2 *The Role of Metal Ions in Catalysis*

An insight into the mechanism of an enzyme can be obtained by investigating the role played by cofactors such as metal ions in catalysis. Metal ions may play a role in the catalysis, structure and regulation of activity of a protein (Vallee, 1983).

A metal is said to have a catalytic role when it is essential for and directly involved in catalysis by an enzyme. Metals are thought to function as electrophilic catalysts, stabilising the negative charges that are formed (Williams, 1982). For example, divalent metal ions (Mg^{2+}, Mn^{2+} or Zn^{2+}) are implicated in the $3' \rightarrow 5'$ exonuclease reaction of pol I (Derbyshire *et al.*, 1988). A nucleophilic attack on the

phosphorus atom of the terminal nucleotide is postulated to be carried out by a hydroxide ion that is coordinated by one divalent metal, while the expected pentacoordinate transition state and the leaving oxyanion are stabilised by a second divalent metal ion that is 3.9 Å from the first (Beese and Steitz, 1991; see Fig. 5.20).

A metal plays a structural role when it is required solely for the structural stability of the protein. It is necessary for activity only to the extent that the overall conformation of the enzyme affects its action.

A regulatory role is indicated when a metal ion regulates, but is not essential for, enzymatic activity or for the stability of the protein. For example, Zn^{2+} functions as an activator with bovine lens leucine aminopeptidase, and an inhibitor with fructose-1, 6-bisphosphatase (Vallee, 1983).

There is a general expectation that metal-activated enzymes have a simple stoichiometric relationship in their functional ternary complexes among enzyme sites, bound metal, and bound substrate which is often 1:1:1 (Mildvan, 1970). For metal-activated enzymes that form 1:1:1 complexes of enzyme (E), metal (M) and substrate (S), four coordination schemes are possible:

E - S - M

(1)

E - M - S

(3)

M - E - S

(2)

These are substrate bridge complex (1), the metal bridge complexes (simple and cyclic) (2), and the enzyme bridge complex (3) (Mildvan, 1970).

There are a variety of techniques available for distinguishing between the different coordination schemes. These can be placed into two groups: kinetic/binding studies, and physical methods such as electron spin resonance (ESR). Kinetic studies are widely applicable and convenient, but fail to give an unequivocal answer; physical methods are less general, but are capable of providing an unequivocal result. Generally, kinetic and binding studies are carried out first. If the metal ion used is Mn^{2+}, electron spin resonance (ESR), and the enhancement of the proton relaxation rate of water, an NMR technique which measures an effect of bound Mn may be used to confirm the result (Sloan *et al.*, 1975; Serpersu *et al.*, 1986; Weber *et al.*, 1991). Other methods for measuring the binding of metals and other factors include gel

filtration (Cuatrecasas *et al.*, 1967b) and equilibrium dialysis (Englund *et al.*, 1969).

Having established the ε subunit has an absolute requirement for divalent metal ion (Section 5.2.1.2), it was proposed that some information on the interactions of enzyme, metal, and substrate could be initially obtained using a kinetic approach, and subsequently confirmed by ESR spectroscopy.

6.1.3 *Aims and Objectives*

As ε is itself a phosphodiesterase, it was not unreasonable to believe it may also hydrolyse *p*np-TMP to release *p*-nitrophenol and TMP. Hence this body of research had several aims, *viz*:

i) to determine conditions for a reproducible assay of ε activity based on the hydrolysis of *p*np-TMP to *p*-nitrophenol and TMP,

ii) to show that this hydrolysis obeyed Michaelis-Menten kinetics,

iii) to investigate the roles of metal ions,

iv) to measure the effect inhibitors of activity, and

v) to determine whether *p*np-TMP is recognised by other enzymes with proofreading activities.

6.2 MATERIALS AND METHODS

6.2.1 *Materials*

The sodium salt of thymidine 5´- monophosphate *p*-nitrophenyl ester was purchased from Sigma Chemical Company and was reported by the manufacturer to be 97% pure. The substrate (*p*np-TMP) was dissolved in water to give a stock concentration of 225.6 mM. The stock solution was aliquoted and stored at -20 °C. The substrate could be thawed and re-frozen several times without apparent degradation. The four deoxyribonucleoside monophosphates were also purchased from Sigma; solutions (10 mM) were prepared in 50 mM Tris.HCl pH 8. The purified

ε subunit (fraction IV) was as described (Section 4.1.4). The large fragment of DNA polymerase I of *E. coli* (6650 U/mg) and T4 DNA polymerase (> 5000 U/mg) were purchased from Boehringer-Mannheim. T7 DNA polymerase was purchased from Pharmacia. DNA polymerase III* of *E. coli* (5.5×10^5 U/mg) was a kind gift from Dr N.E. Dixon. All other reagents used were of analytical grade.

6.2.2 *Spectroscopic Measurements*

All measurements of enzymatic activity were obtained by measuring absorbance changes continuously with a Cary Model 118 spectrophotometer. The temperature of the cuvette chamber was maintained at 25 ±0.25 °C. The ε_{420} of *p*-nitrophenol was taken to be 13,000 M^{-1} cm^{-1} at pH 8, 5,500 M^{-1} cm^{-1} at pH 6.9 and 14, 200 M^{-1} cm^{-1} at pH 9 (Beck, 1986).

6.2.3 *Standard Assay Conditions* (see also Section 6.3.1)

Assay components (50 mM Tris.HCl pH 8.0, 750 μM $MnCl_2$, 150 mM NaCl, 6.2 mM *p*np-TMP) were dispensed into 1.0 ml glass cuvettes using Hamilton syringes and made up to 1ml with H_2O. They were allowed to warm to room temperature, and subsequently placed in the cuvette chamber where the contents were thoroughly mixed with a glass rod. Enzyme (5 μl) was added and the solution mixed again.

6.2.4 *Kinetics*

6.2.4.1 Data Analysis

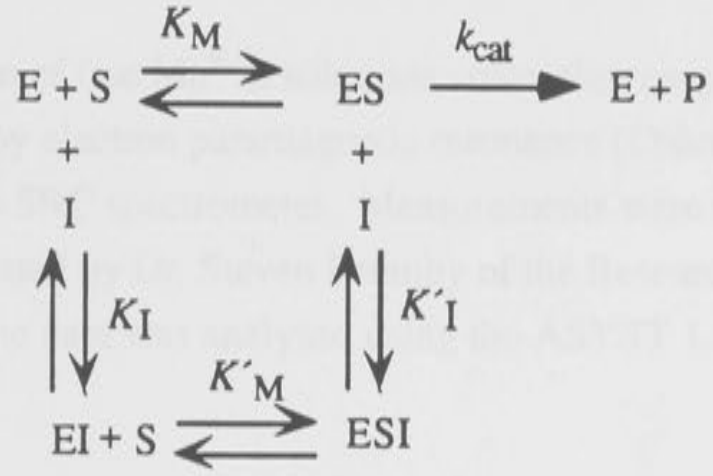
Assays were performed in duplicate, and in all cases velocities were obtained from the early (linear) stages of substrate hydrolysis. The rate expressed as $\Delta A_{420}/\Delta t = v$ was calculated. On dividing this figure by the ε_{420} for *p*-nitrophenol, the rate expressed in molar terms could be obtained. Assays typically were curved toward lower rates. This was almost certainly due to product inhibition by the accumulated

TMP product (see Section 6.4.3, $K_I \approx 17\ \mu M$). Michaelis-Menten parameters were obtained from Hanes-Woolf plots (Segel, 1975). In this plot, the V_{max} is derived from 1/slope, and the K_M is V_{max} multiplied by the y-axis intercept. The catalytic constant, k_{cat}, is calculated by dividing V_{max} by $[E]_t$. In some cases, Lineweaver-Burk plots were used, in which V_{max} is obtained from the y-axis intercept and K_M is V_{max} multiplied by the slope (Fersht, 1985).

6.2.4.2 Inhibitor Studies

Essentially the same procedure was used as described above. The inhibitor was always the last component added before enzyme. The inhibitors used were dAMP, dCMP, dGMP and TMP.

The three types of simple inhibition, competitive, noncompetitive and uncompetitive can be distinguished kinetically (Eq. 6.1) by measuring the rate over a range of substrate concentrations at several different inhibitor concentrations,



Equation 6.1: The equilibria describing competitive inhibition, noncompetitive inhibition, and uncompetitive inhibition (Segel, 1975).

When K'_I and $K'_M = 0$, the inhibition is competitive. When K_I and $K'_M = 0$, the inhibition is termed noncompetitive. When $K_I = K'_I$, the inhibition is uncompetitive, and when $K_I \neq K_I$, the inhibition is termed mixed.

and plotting the data by the method of Lineweaver and Burk (Segel, 1975). Measuring the rates over a range of inhibitor concentrations and at several concentrations of substrate allows the dissociation constant of the EI complexes to be determined (Dixon and Webb, 1964)

6.2.4.3 Dependence on Mn^{2+} Concentration

Assuming equilibria as shown in Eq. 6.2 (see Section 6.4.5.1), then all equilibrium constants may be obtained by performing a single experiment in which the rates are measured at several metal ion concentrations over a range of substrate concentrations. The data may then be plotted in several fashions to derive the rate and equilibrium constants (Segel, 1975; Serpersu *et al.*, 1986). A plot of $1/v$ versus 1/[S] establishes the type of reaction scheme. In an ordered bireactant system, where activator binds before substrate, the family of lines will intersect at the same point on the y-axis. In a random bireactant system, where enzyme can bind either activator or substrate, the family of lines intersects the 1/[S] axis at a point where the concentration of activator is zero. The binding constants may be obtained from replots of the data from the double reciprocal plot.

6.2.4.4 Electron Paramagnetic Resonance Measurements

The concentration of free Mn^{2+} in solutions containing enzyme, substrate, and all three was determined by electron paramagnetic resonance (Cohn and Townsend, 1954) with a Bruker ER2000-SRC spectrometer. Measurements were made at 25 °C. The spectrometer was operated by Dr. Steven Brumby of the Research School of Chemistry, A.N.U. The data was analysed using the ASYST 1.53 program on an IBM computer.

6.3 RESULTS

6.3.1 *Standard Assay Conditions*

Initial experiments were conducted to establish whether the ε subunit could hydrolyse the substrate. The substrate was found to be hydrolysed with concomitant release of *p*-nitrophenol. The change of absorbance at 420 nm over time was not strictly linear, but initial rates could be obtained during the first ≈ 0.6% of substrate hydrolysis (under standard assay conditions). Optimal conditions were then

determined for a reproducible assay of phosphodiesterase activity (Sections 6.3.1.1 to 6.3.1.6).

6.3.1.1 Substrate Stability

Under the conditions described above, there was no appearance of *p*-nitrophenol in the absence of enzyme. On addition of Tris to raise the pH, it was found from measuring the absorbance of the solution that the amount of free *p*-nitrophenol in the substrate was ≈ 0.02%.

6.3.1.2 Background Phosphodiesterase Activity in *E. coli*.

No activity was found in the soluble fraction of a cell lysate containing up to 400 µg of protein from heat-induced AN1459/pCE30 (data not shown). This is a strain harbouring a plasmid similar to pPL224, but it does not direct the synthesis of the ε subunit. This suggests substrate hydrolysis is due solely to the action of the ε subunit.

6.3.1.3 Effect of pH

The rate of reaction was measured at pH 6.9, 8.0 and 9.0, and at two substrate concentrations (Figs. 6.2 and 6.3). Rates were corrected using the appropriate ε_{420} for each pH.

At the lower substrate concentration, the observed rate at pH 8 and pH 9 are the same. The rate at pH 6.9 is lower, possibly due to ionization of important amino acid residues at the active site. At the higher substrate concentration, there is some difference (12%) between the rates observed at pH 8 and pH 9 indicating that maximal rates would be obtained at the higher pH.

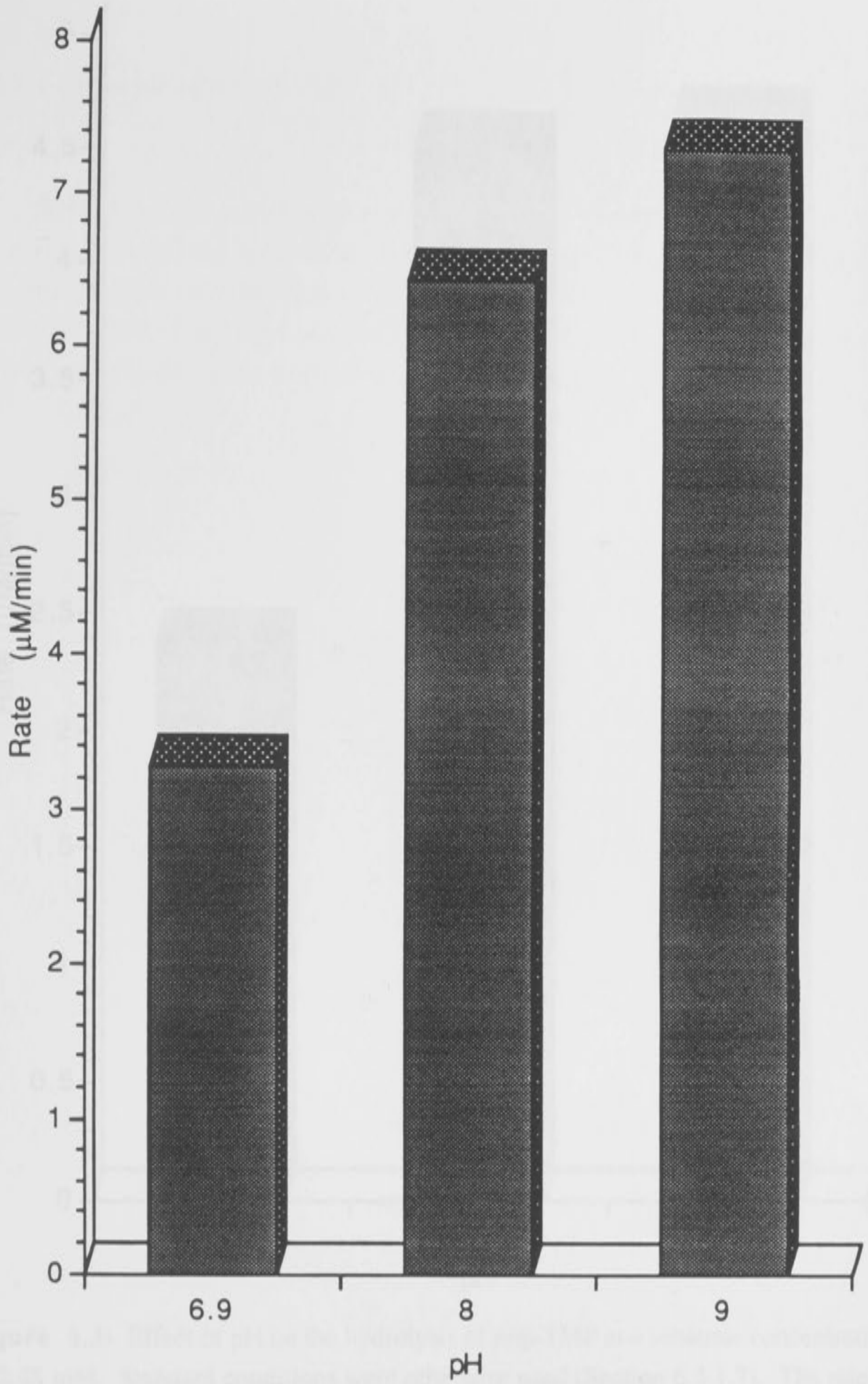


Figure 6.2: Effect of pH on the hydrolysis of *p*np-TMP at a substrate concentration of 6.2 mM. Standard assay conditions were otherwise used (Section 6.3.1.7). The rate was determined using the appropriate ε_{420} of *p*-nitrophenol at each pH. The concentration of enzyme was 107 nM.

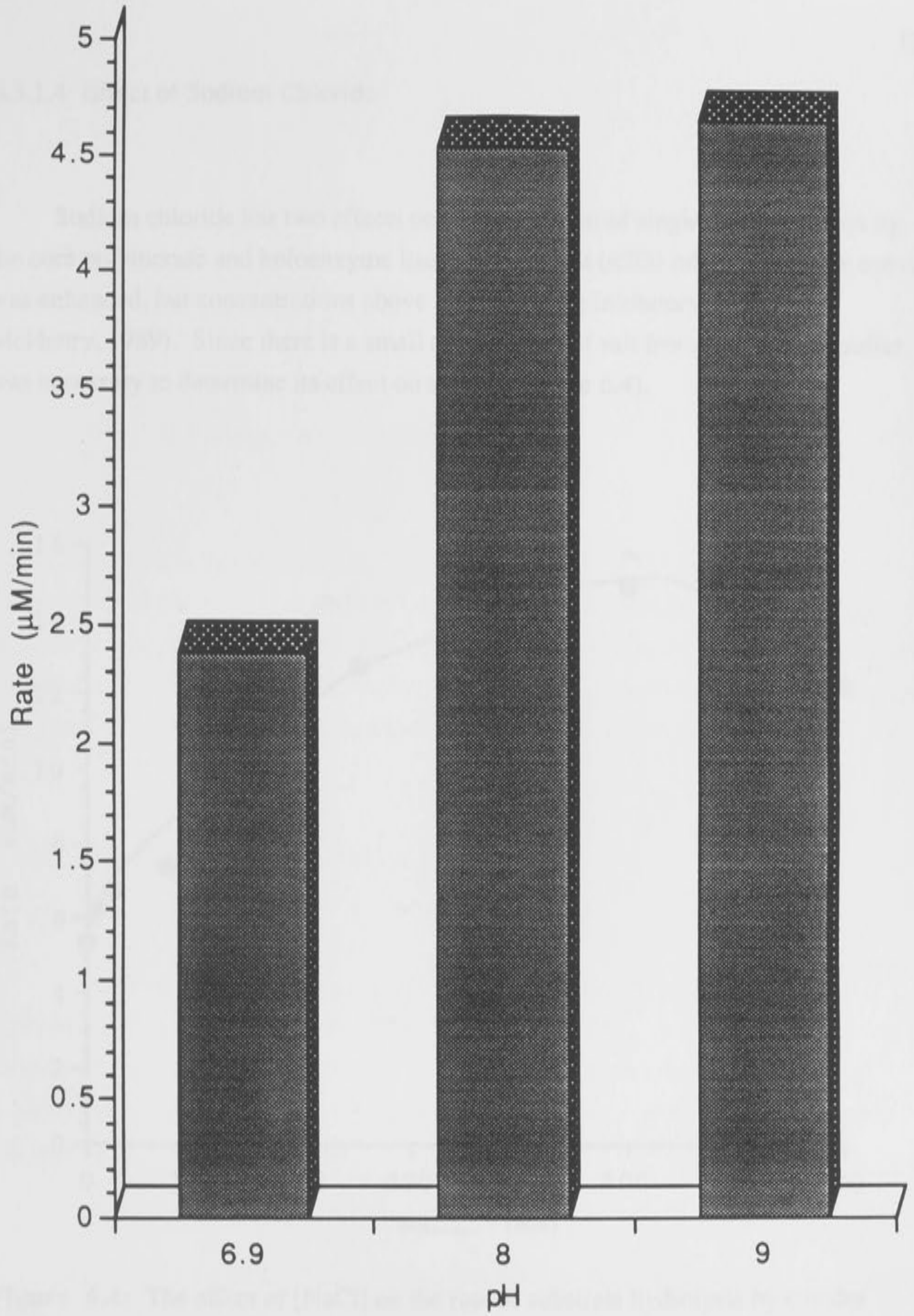


Figure 6.3: Effect of pH on the hydrolysis of *p*np-TMP at a substrate concentration of 2.48 mM. Standard conditions were otherwise used (Section 6.3.1.7). The rate was determined using the appropriate ε_{420} at each pH. The concentration of enzyme was 107 nM.

6.3.1.4 Effect of Sodium Chloride

Sodium chloride has two effects on the replication of single-stranded DNA by the core polymerase and holoenzyme itself. At low salt (<200 mM), replicative activity was enhanced, but concentrations above 200 mM were inhibitory (Griep and McHenry, 1989). Since there is a small contribution of salt from the enzyme buffer, it was necessary to determine its effect on ε itself (Figure 6.4).

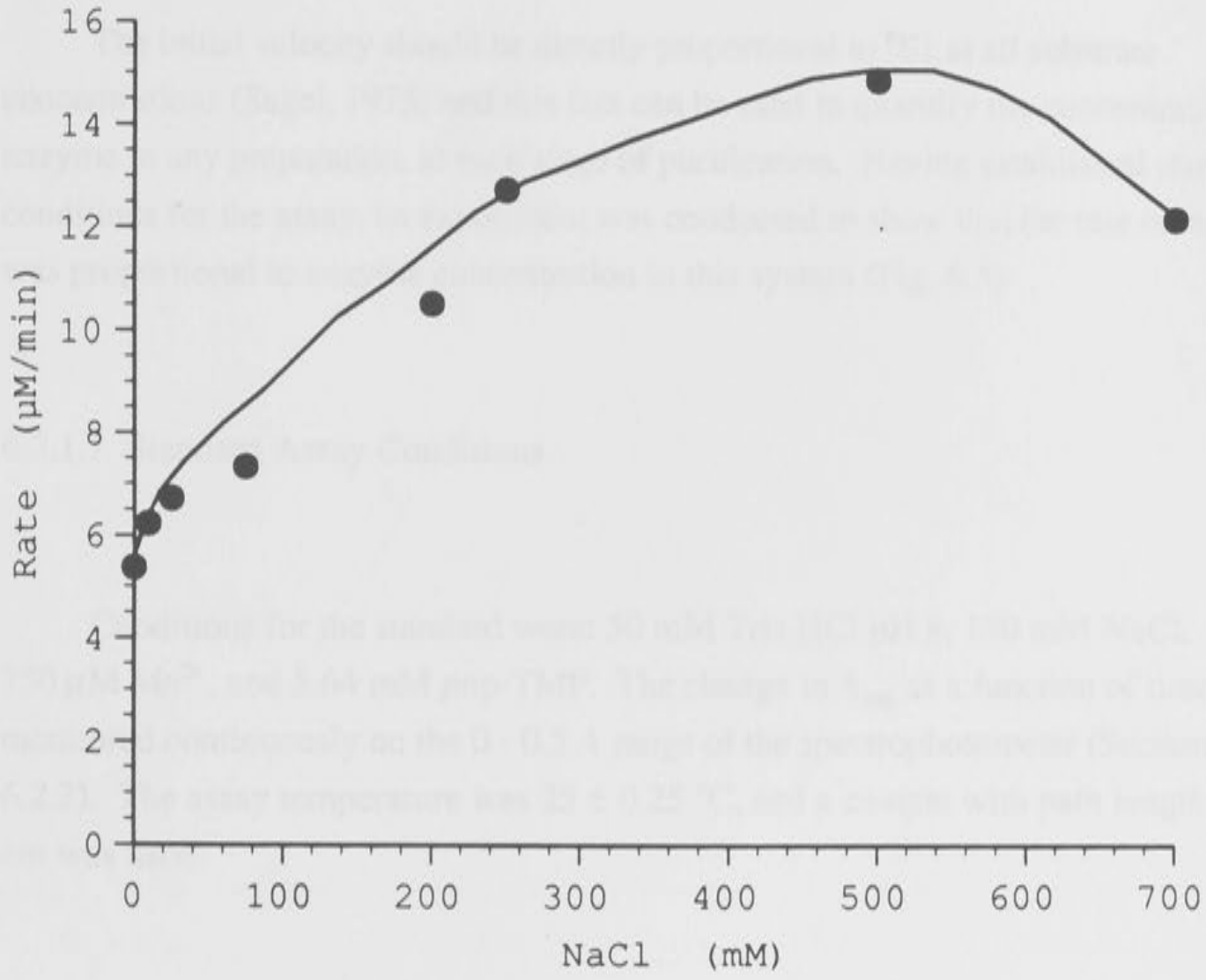


Figure 6.4: The effect of [NaCl] on the rate of substrate hydrolysis by ε under standard assay conditions. $[S]_0$ = 6.2 mM, [metal] = 250 μM, $[\varepsilon]_0$ = 211 nM.

There was a modest stimulatory effect of NaCl on the rate of hydrolysis of *p*np-TMP by ε up to about 500 mM after which no further increase in the rate was observed. Sodium chloride was subsequently included in the assay at a concentration of 150 mM.

6.3.1.5 Effect of Buffers

Hydrolysis of *p*np-TMP under standard assay conditions was measured in 50 mM HEPES pH 8.0 and Tris.HCl pH 8.0, and the rates observed were within experimental error (±5%).

6.3.1.6 Effect of Varying Enzyme Concentration

The initial velocity should be directly proportional to $[E]_t$ at all substrate concentrations (Segel, 1975) and this fact can be used to quantify the concentration of enzyme in any preparation, at each stage of purification. Having established standard conditions for the assay, an experiment was conducted to show that the rate of reaction was proportional to enzyme concentration in this system (Fig. 6.5).

6.3.1.7 Standard Assay Conditions

Conditions for the standard were: 50 mM Tris.HCl pH 8, 150 mM NaCl, 750 μM Mn^{2+}, and 5.64 mM *p*np-TMP. The change in A_{420} as a function of time was monitored continuously on the 0 - 0.5 *A* range of the spectrophotometer (Section 6.2.2). The assay temperature was 25 ± 0.25 °C, and a cuvette with path length of 1.0 cm was used.

6.4 MICHAELIS-MENTEN PARAMETERS FOR SUBSTRATE AND COFACTORS

6.4.1 *Substrate Hydrolysis*

The rate of reaction was determined under otherwise standard conditions at several concentrations of substrate. Figure 6.6 shows a typical *v versus* [S] plot. The data was then analysed in a Hanes-Woolf plot (Fig. 6.7) which showed a linear

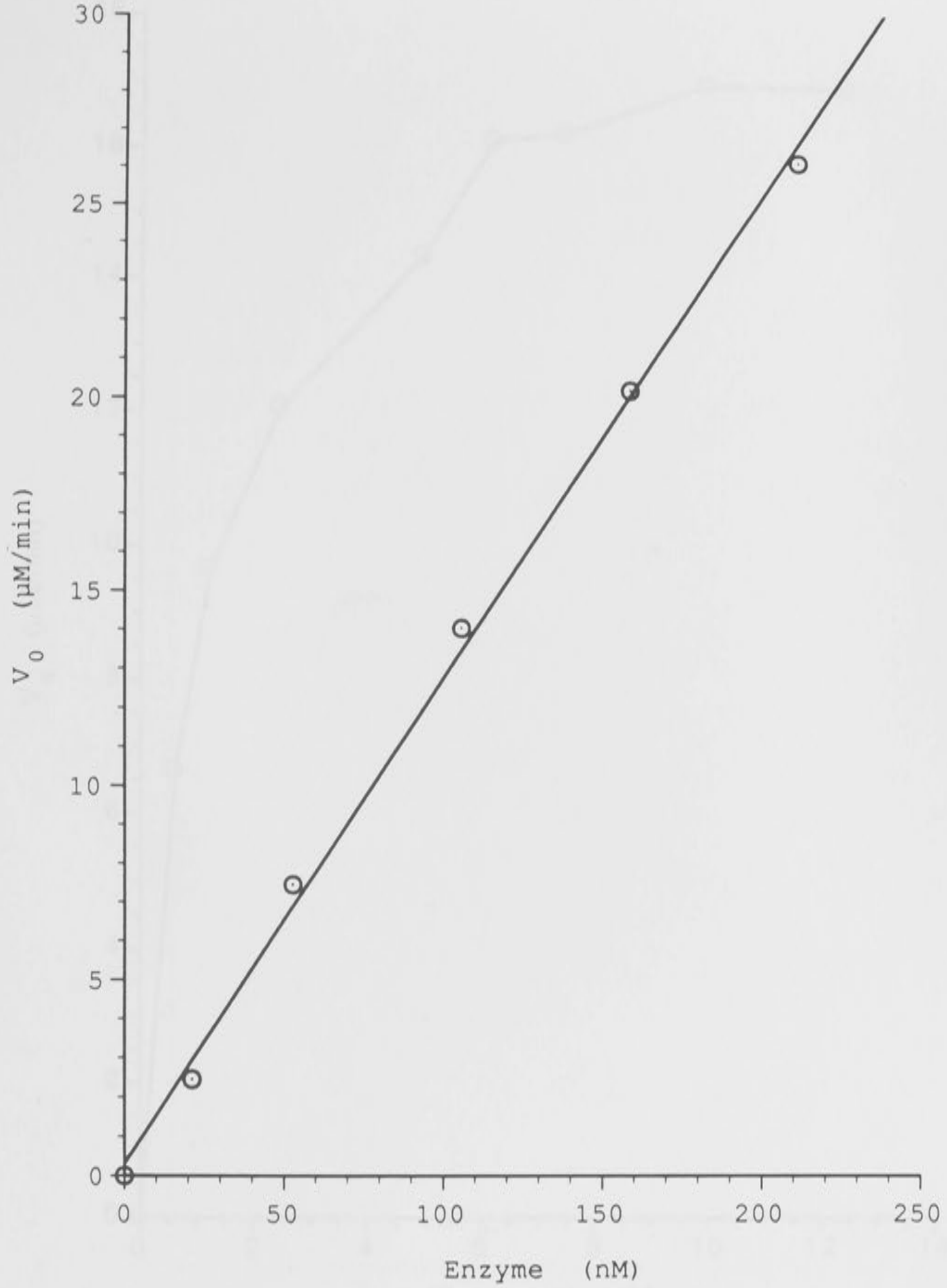


Figure 6.5: Rate of hydrolysis of substrate is proportional to enzyme concentration. The concentration of substrate was 6.2 mM, and the concentration of Mn^{2+} was 400 μM. Standard conditions were otherwise used (Section 6.3.1.7). The enzyme concentrations used were 21, 53, 106, 158 and 211 nM.

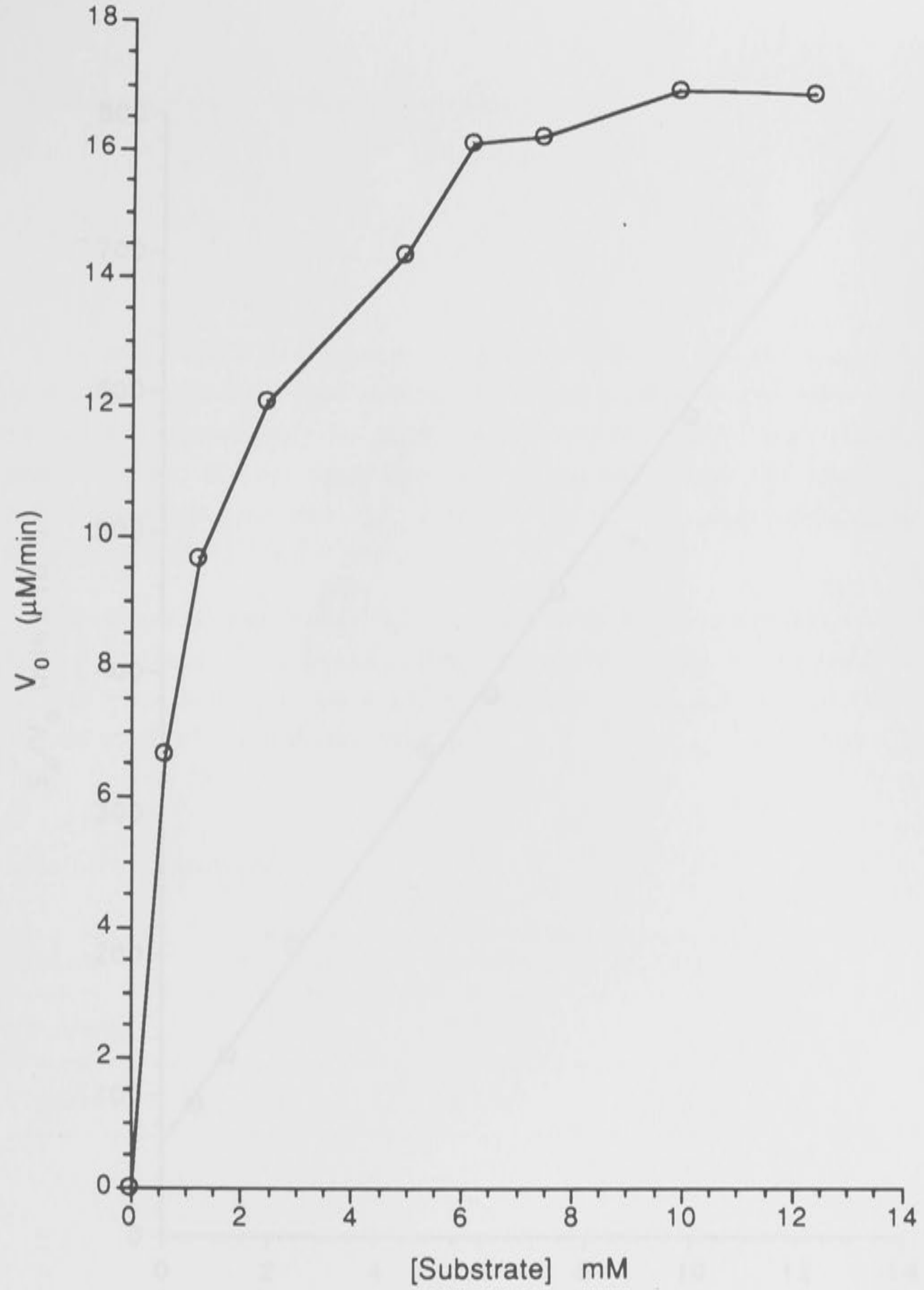


Figure 6.6: Rate versus substrate concentration for the hydrolysis of *p*np-TMP by the ε subunit under standard conditions. The substrate concentrations used were: 0.62, 1.24, 2.48, 4.96, 6.2, 7.44, 9.94 and 12.42 mM. The concentration of enzyme in the experiment was 107 nM.

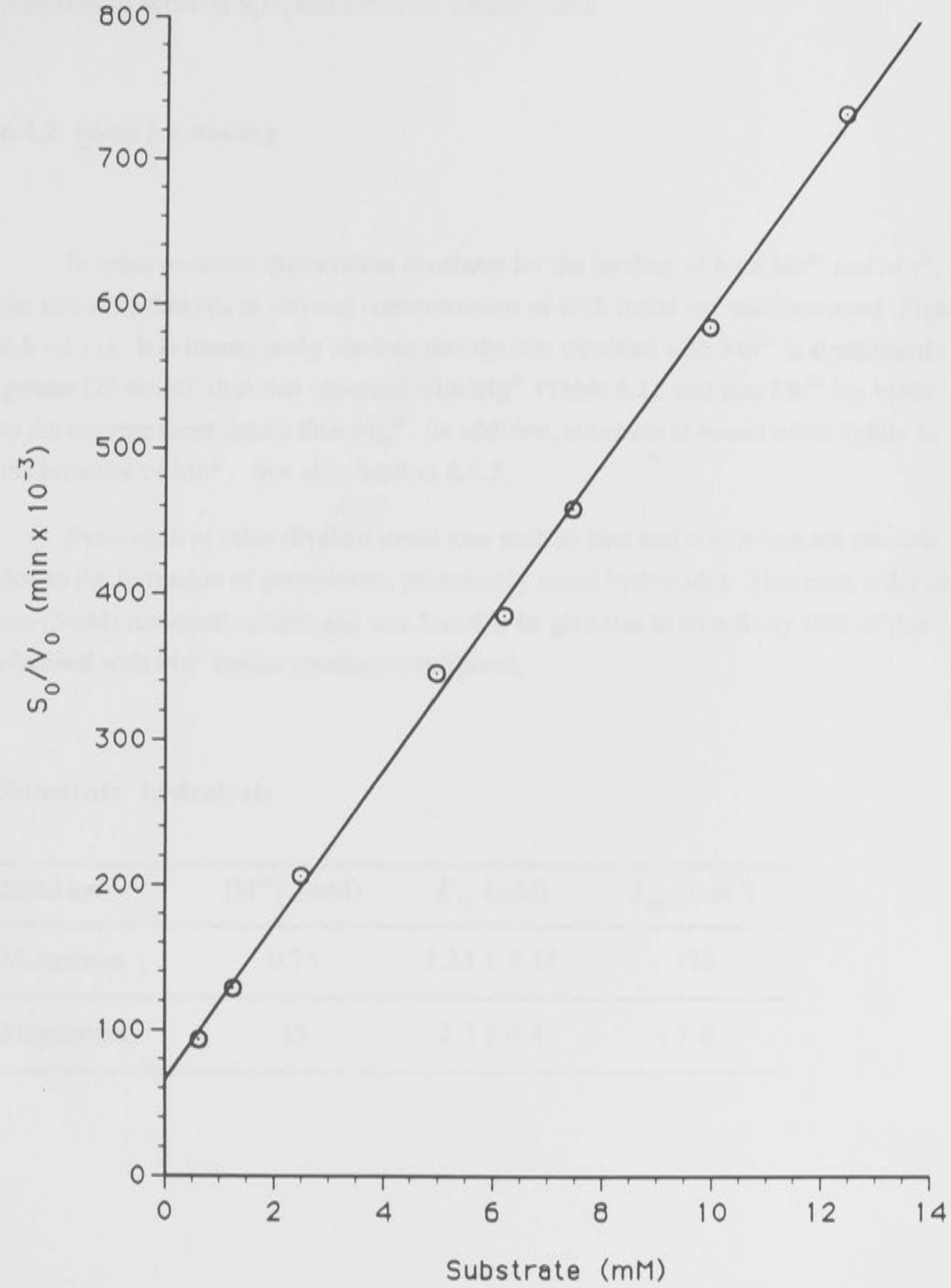


Figure 6.7: Hanes-Woolf plot of the data in Figure 6.6. The K_M was 1.23 ±0.044 mM, and the k_{cat} was 178 ±13 min^{-1}

relationship between S_0/v_0 and substrate concentration.

6.4.2 *Metal Ion Binding*

In order to obtain dissociation constants for the binding of both Mn^{2+} and Mg^{2+}, the rate of hydrolysis at serveral concentrations of each metal ion was measured (Figs. 6.8 - 6.11). It is immediately obvious that the rate obtained with Mn^{2+} is significantly greater (23 times)[1] than that observed with Mg^{2+} (Table 6.1), and that Mn^{2+} ion binds to the enzyme more tightly than Mg^{2+}. In addition, substrate is bound more tightly in the presence of Mn^{2+}. See also Section 6.4.5.

Evaluation of other divalent metal ions such as zinc and cobalt was not possible due to the formation of precipitates, presumably metal hydroxides. However, calcium ion (5mM) remained soluble and was found to be give rise to an activity 10% of that obtained with Mn^{2+} (under standard conditions).

Substrate hydrolysis

Metal ion	$[M^{2+}]$ (mM)	K_M (mM)	k_{cat} (min^{-1})
Manganese	0.75	1.23 ± 0.14	178
Magnesium	15	2.3 ± 0.4	7.8

[1]This figure was derived from (k_{cat} for Mn^{2+} /k_{cat} for Mg^{2+}) .

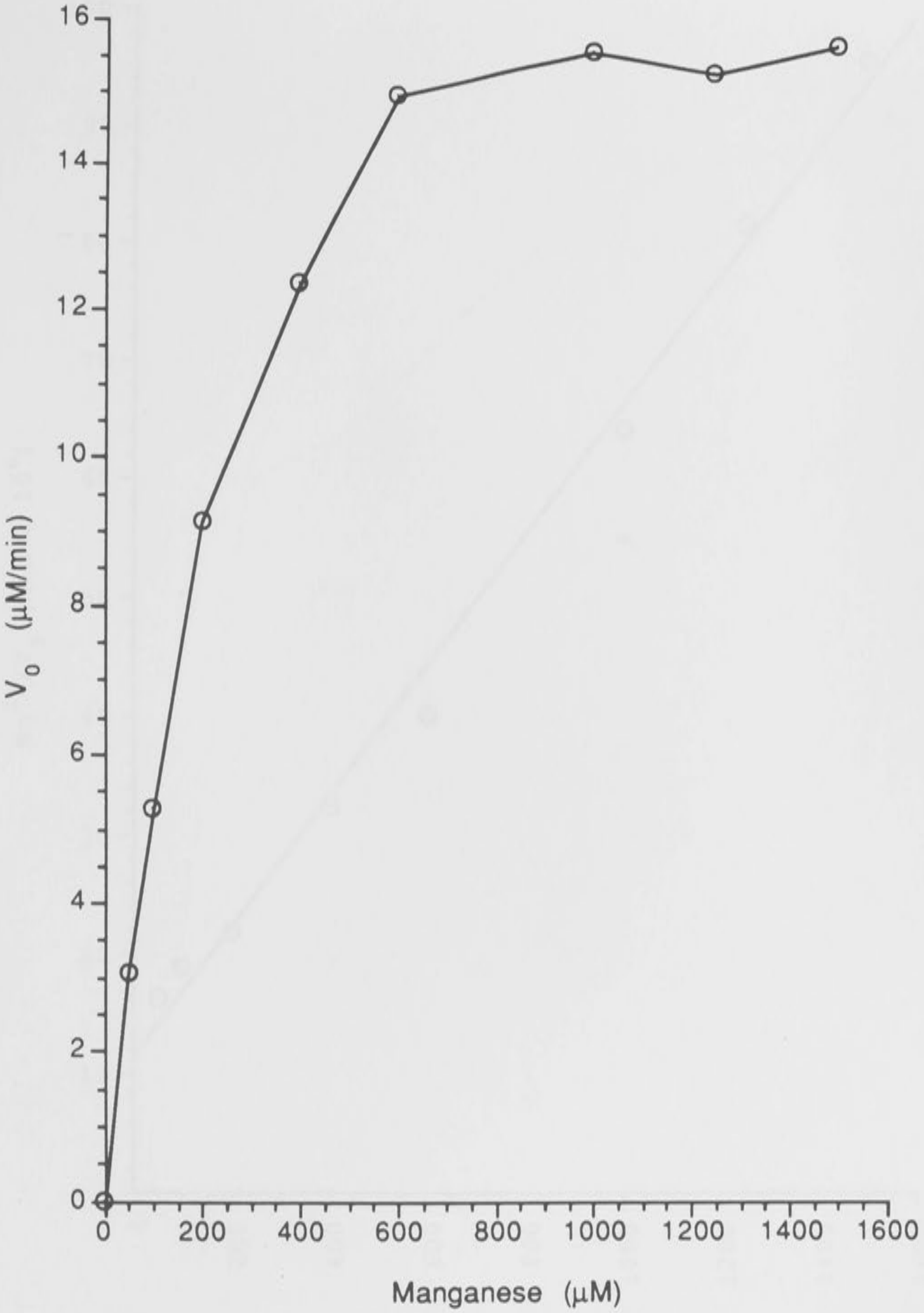


Figure 6.8: Rate of hydrolysis of *p*np-TMP at varying Mn^{2+} concentration under standard conditions. The concentration of ε was 107 nM. The concentrations of Mn^{2+} used were: 50, 100, 200, 400, 600, 1000, 1250 and 1500 μM.

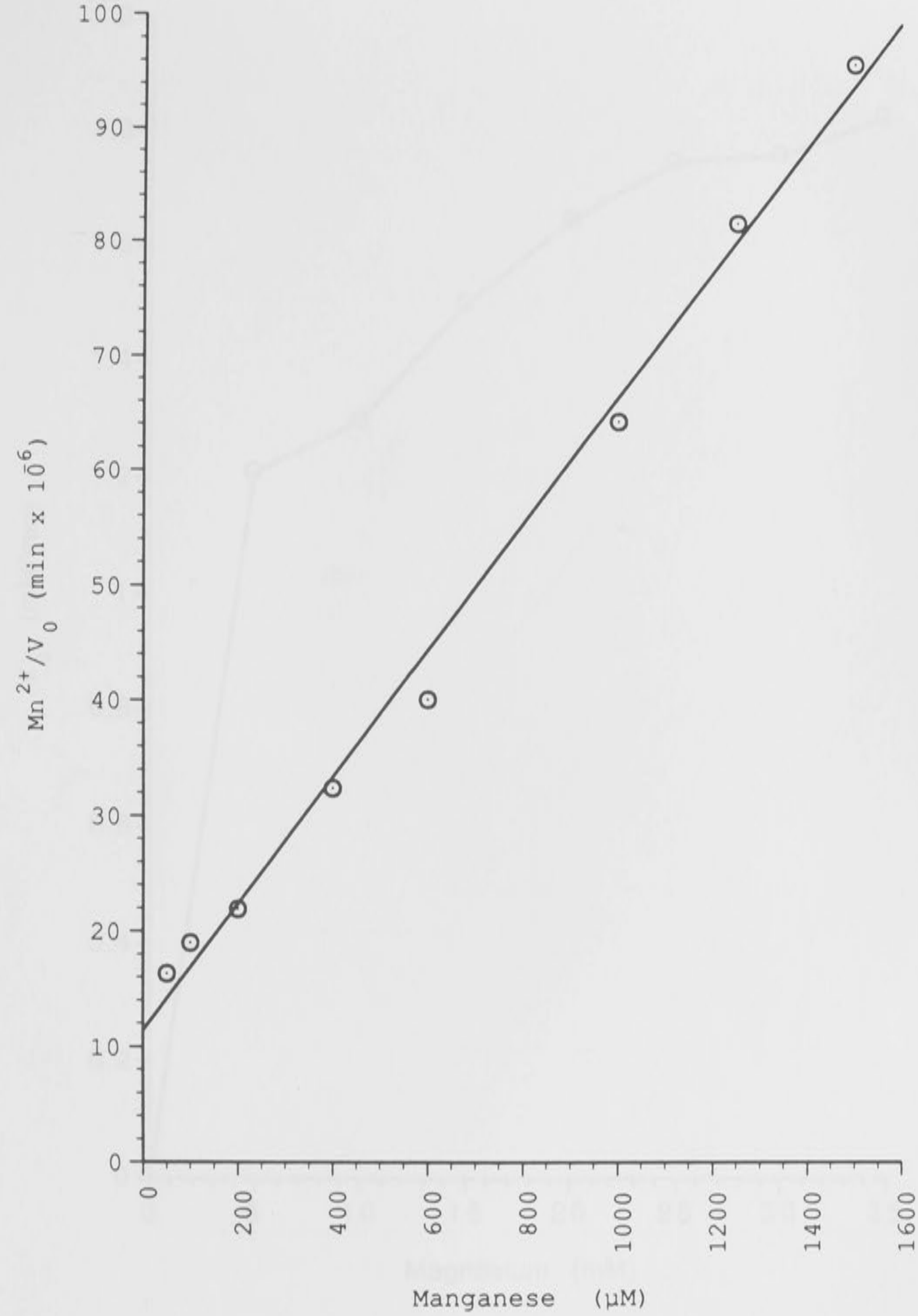


Figure 6.9: Derivation of Michaelis-Menten kinetic parameters for the data in Figure 6.8 using a Hanes-Woolf plot. The K_D (binding constant) for Mn^{2+} was 217 ±35 μM, the k_{cat} was 172 ±14 min^{-1}.

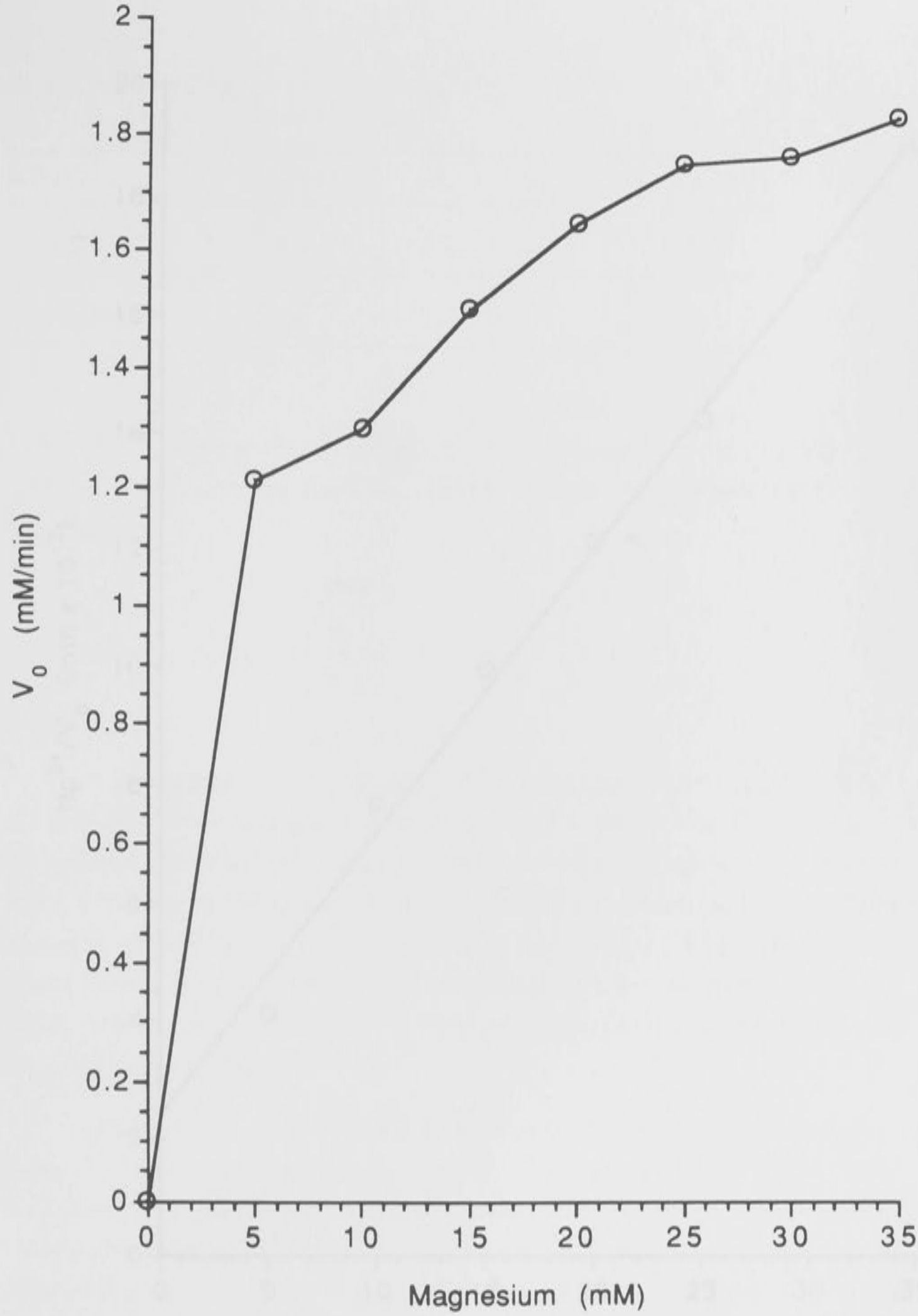


Figure 6.10: Rate of hydrolysis of *p*np-TMP at several concentrations of Mg^{2+} under standard conditions. The concentration of ε was 317 nM. The concentration of Mg^{2+} used were: 5, 10, 15, 20, 25, 30 and 35 mM.

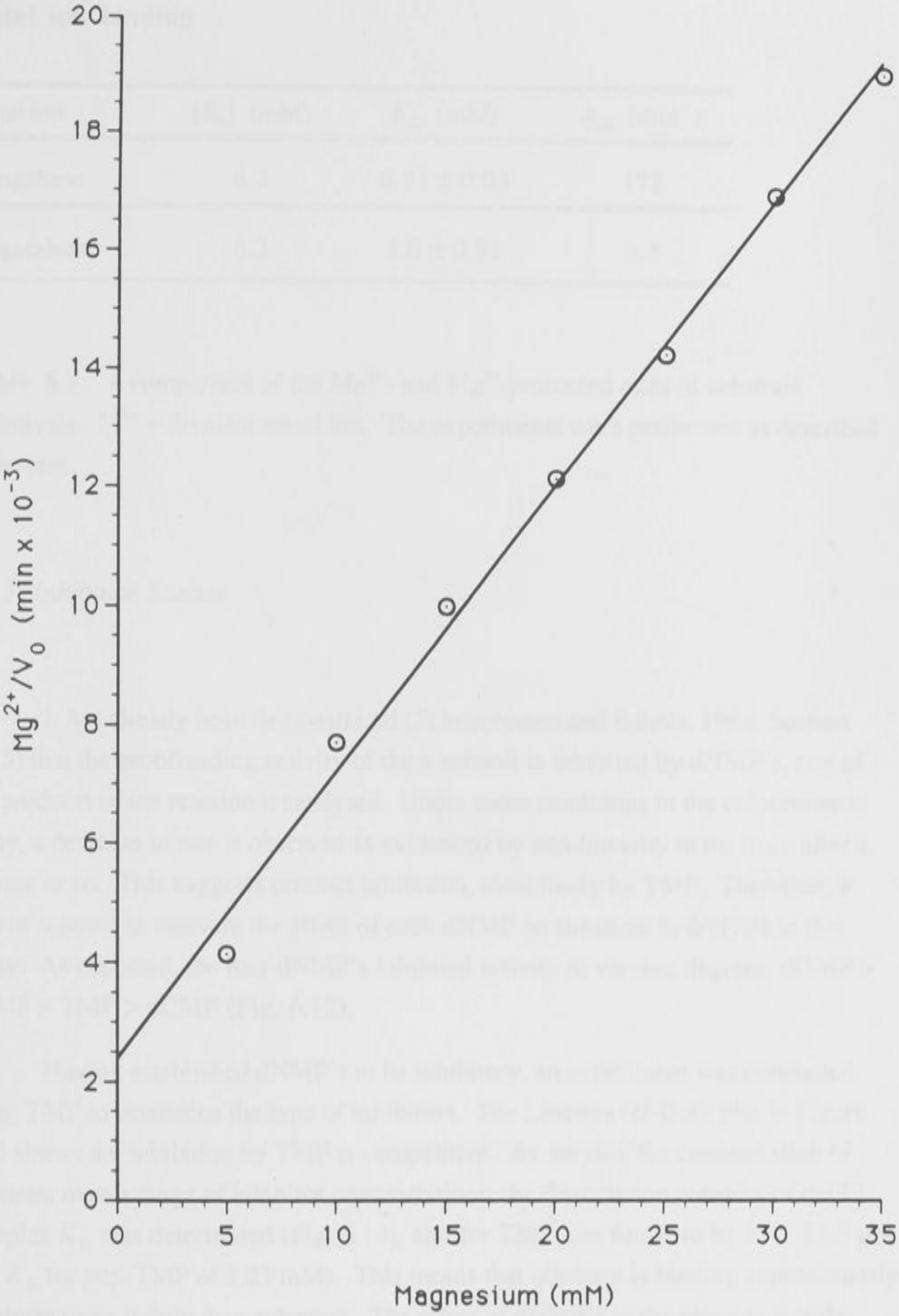


Figure 6.11: Derivation of Michaelis-Menten kinetic parameters for the data in Figure 6.10 using a Hanes-Woolf plot. The K_D for Mg^{2+} was 5 ± 0.91 mM, and the k_{cat} was 9.6 ±0.62 min-1.

Metal ion binding

Metal ion	$[S_0]$ (mM)	K_D (mM)	k_{cat} (min^{-1})
Manganese	6.2	0.21 ± 0.03	172
Magnesium	6.2	5.0 ± 0.91	6.5

Table 6.1: A comparison of the Mn^{2+}- and Mg^{2+}-promoted rates of substrate hydrolysis. M^{2+} = divalent metal ion. The experiments were performed as described in the text.

6.4.3 *Inhibition Studies*

It has already been demonstrated (Scheuermann and Echols, 1984; Section 5.2.5) that the proofreading activity of the ε subunit is inhibited by dNMP's, one of the products of the reaction it catalyses. Under some conditions in the colourimetric assay, a decrease in rate is observed as evidenced by non-linearity in the trace after a minute or so. This suggests product inhibition, most likely by TMP. Therefore, it was of interest to measure the effect of each dNMP on substrate hydrolysis in this assay. As expected, the four dNMP's inhibited activity to varying degrees: dGMP > dAMP > TMP > dCMP (Fig. 6.12).

Having established dNMP's to be inhibitory, an experiment was conducted using TMP to determine the type of inhibition. The Lineweaver-Burk plot in Figure 6.13 shows the inhibition by TMP is competitive. By varying the concentration of substrate over a range of inhibitor concentrations, the dissociation constant of the EI complex K_I, was determined (Fig. 6.14), and for TMP was found to be 17.5 ± 1.5 μM (cf. K_M for *p*np-TMP of 1.23 mM). This means that inhibitor is binding approximately 70-times more tightly than substrate. The effect of dNMP's in the oligonucleotide-based assays appeared to be much less pronounced (Section 5.2.5).

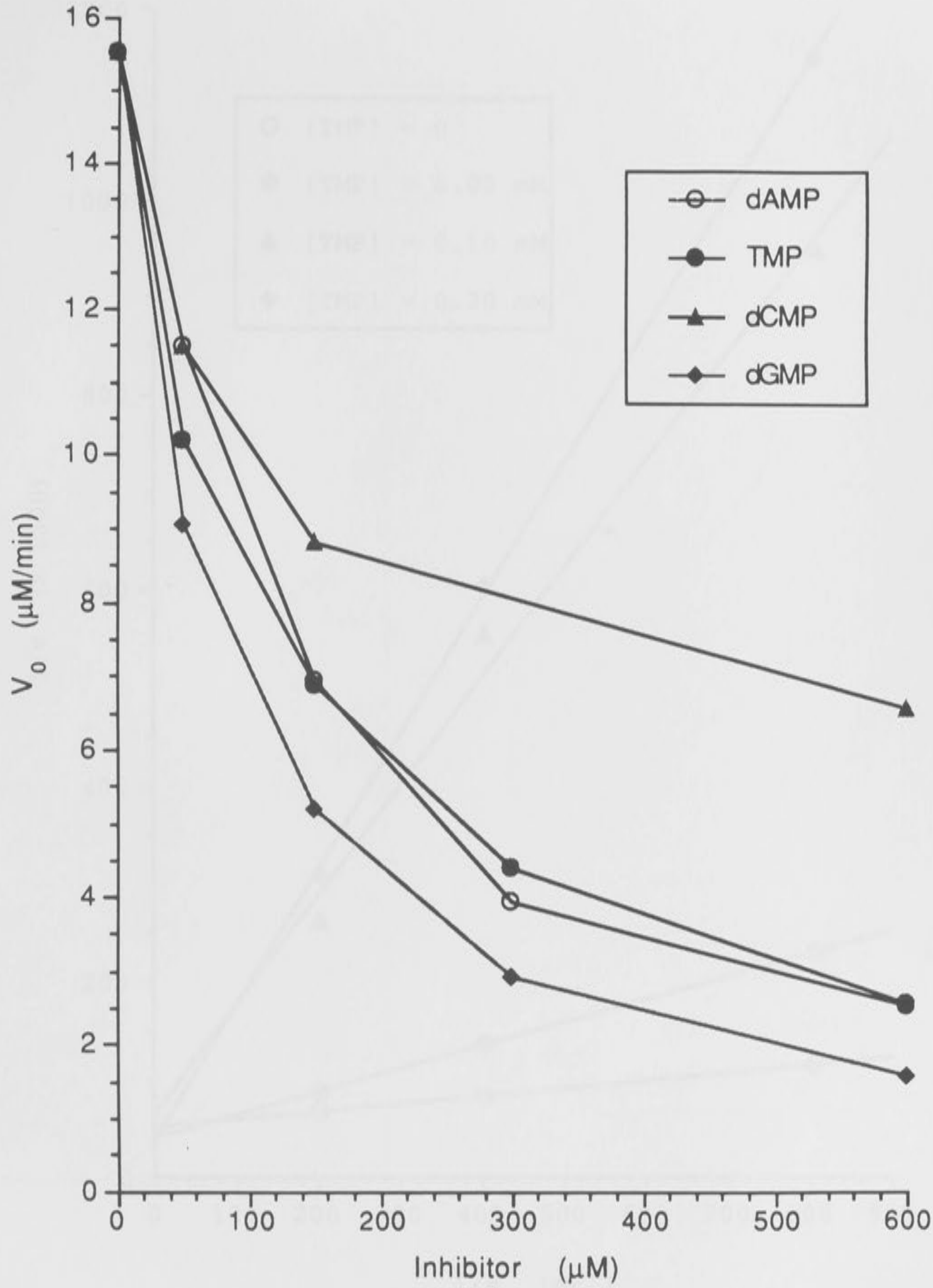


Figure 6.12: Effect of the four dNMP's on the rate of substrate hydrolysis by the ε subunit in the standard assay. The concentration of ε used was 107 nM. The concentrations of the dNMP's used were: 50, 150, 350 and 600 μM.

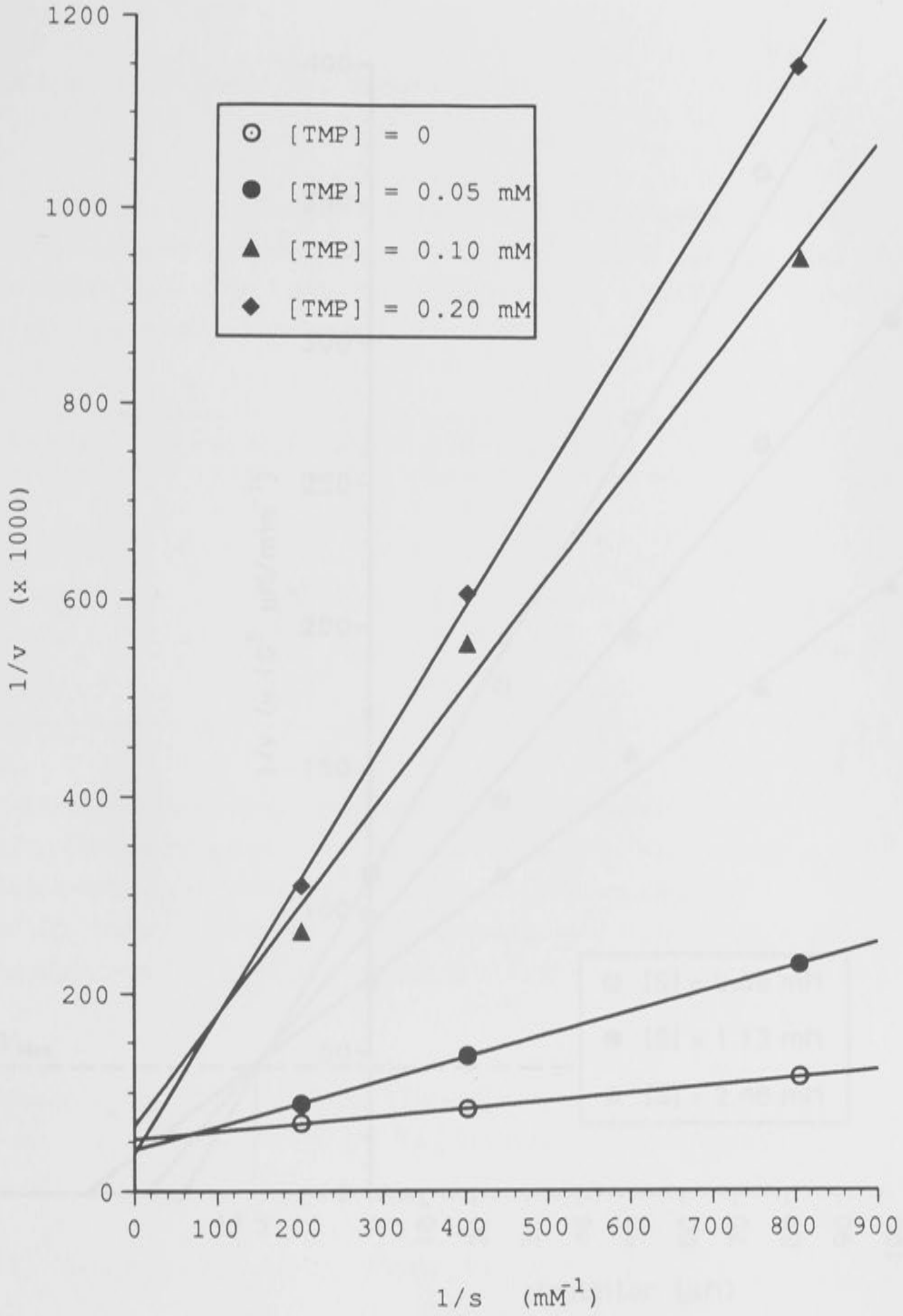


Figure 6.13: Lineweaver-Burk plot illustrating the effect of TMP on substrate K_M and V_{max}. The concentration of enzyme used was 107 nM. The lines were calculated by linear regression.

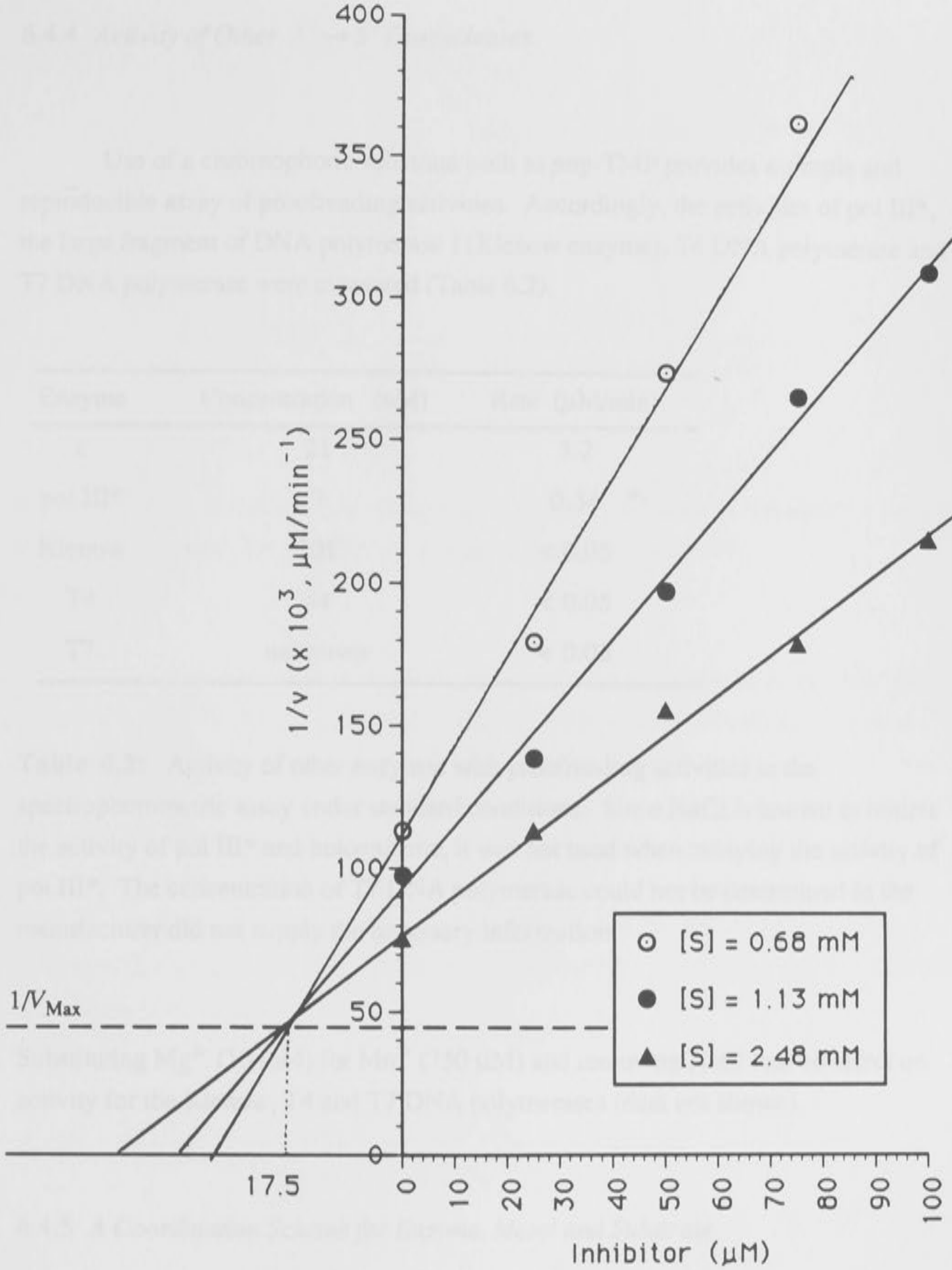


Figure 6.14: Dixon plot for determining the K_I of an inhibitor; for TMP it is 17.5 ±1.5 µM. The concentration of the ε subunit was 107 nM. The concentrations of TMP used were 25, 50, 75 and 100 µM. The value of V_{max} used was 18.77 µM/min.

6.4.4 *Activity of Other 3′ → 5′ Exonucleases.*

Use of a chromophoric substrate such as *p*np-TMP provides a simple and reproducible assay of proofreading activities. Accordingly, the activities of pol III*, the large fragment of DNA polymerase I (Klenow enzyme), T4 DNA polymerase and T7 DNA polymerase were measured (Table 6.2).

Enzyme	Concentration (nM)	Rate (μM/min)
ε	21	3.2
pol III*	9	0.34
Klenow	201	< 0.05
T4	44	< 0.05
T7	unknown	< 0.05

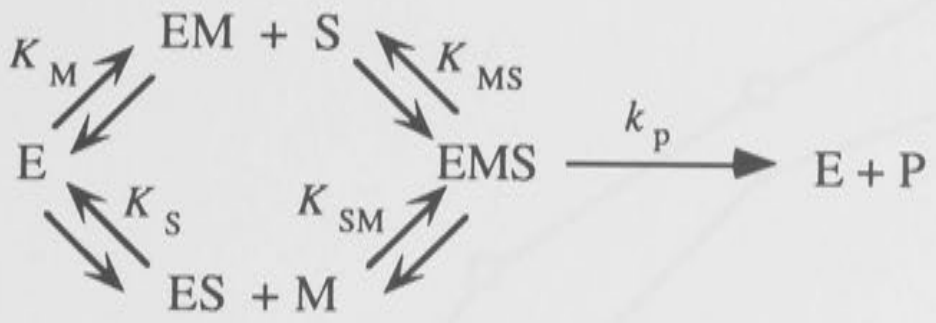
Table 6.2: Activity of other enzymes with proofreading activities in the spectrophotometric assay under standard conditions. Since NaCl is known to inhibit the activity of pol III* and holoenzyme, it was not used when assaying the activity of pol III*. The concentration of T7 DNA polymerase could not be determined as the manufacturer did not supply the necessary information.

Substituting Mg^{2+} (10 mM) for Mn^{2+} (750 μM) and removing NaCl had no effect on activity for the Klenow, T4 and T7 DNA polymerases (data not shown).

6.4.5 *A Coordination Scheme for Enzyme, Metal and Substrate.*

6.4.5.1 Kinetic Experiments

The equilibria shown below describe a rapid equilibrium random bireactant system in which enzyme binds substrate or metal ion cofactor:



$$v = K_p[EMS] \text{ and } \frac{v}{[E]_t} = \frac{K_p[EMS]}{[E] + [EM] + [ES] + [EMS]}$$

Equation 6.2: Equilibria describing a rapid equilibrium random bireactant system in which enzyme binds substrate or metal ion cofactor (Segel, 1975).

To derive these kinetic parameters, an experiment was performed in which the rate of hydrolysis was measured over a range of substrate concentrations at several different Mn^{2+} ion concentration (Fig. 6.15). A double reciprocal plot of initial velocity against the substrate concentration (Fig. 6.16) showed that with the exception of that for the lowest Mn^{2+} concentration, the family of lines intersected at different points on the y-axis. Therefore, the binding of enzyme, metal and substrate is random (Segel, 1975). A replot of the slopes of the lines from Figure 6.16 (Fig. 6.17b), and a double reciprocal plot of apparent V_{max} against the concentration of Mn^{2+} (Fig. 6.17a), solves the system (Fig. 6.18).

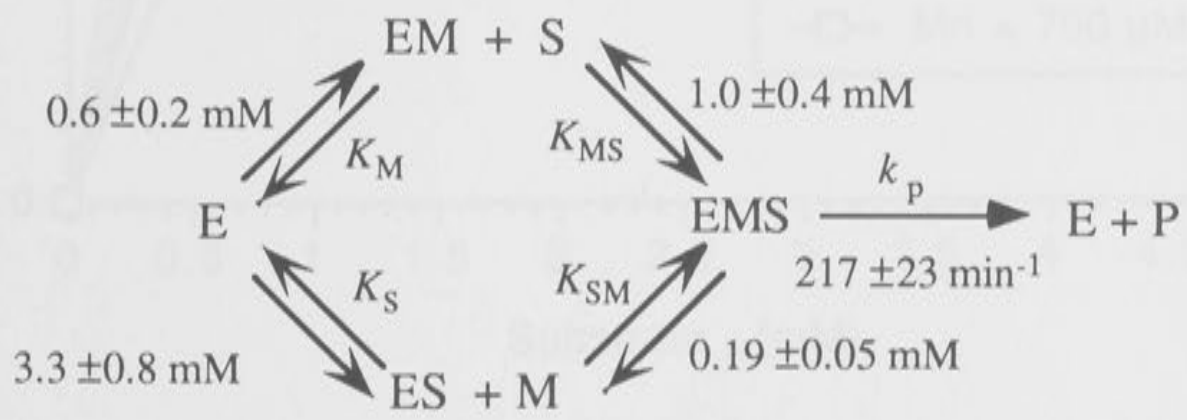


Figure 6.18: Equilibria describing the interaction of the ε subunit with substrate and metal ion.

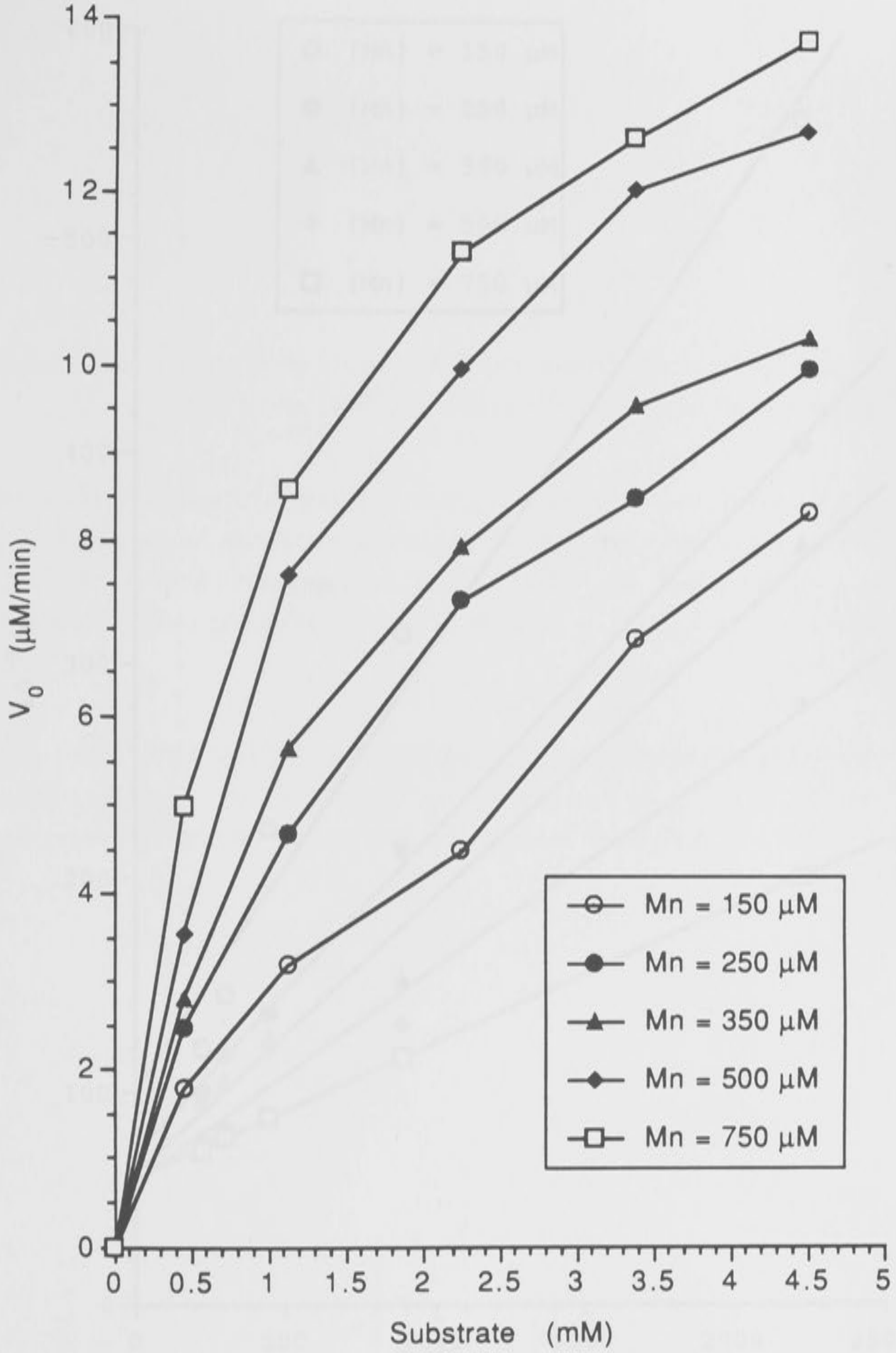


Figure 6.15: Plot of rate *versus* substrate concentration at several concentrations of metal ion. The concentration of ε was 107 nM. The concentrations of substrate were 0.45, 1.13, 2.26, 3.38 and 4.51 mM.

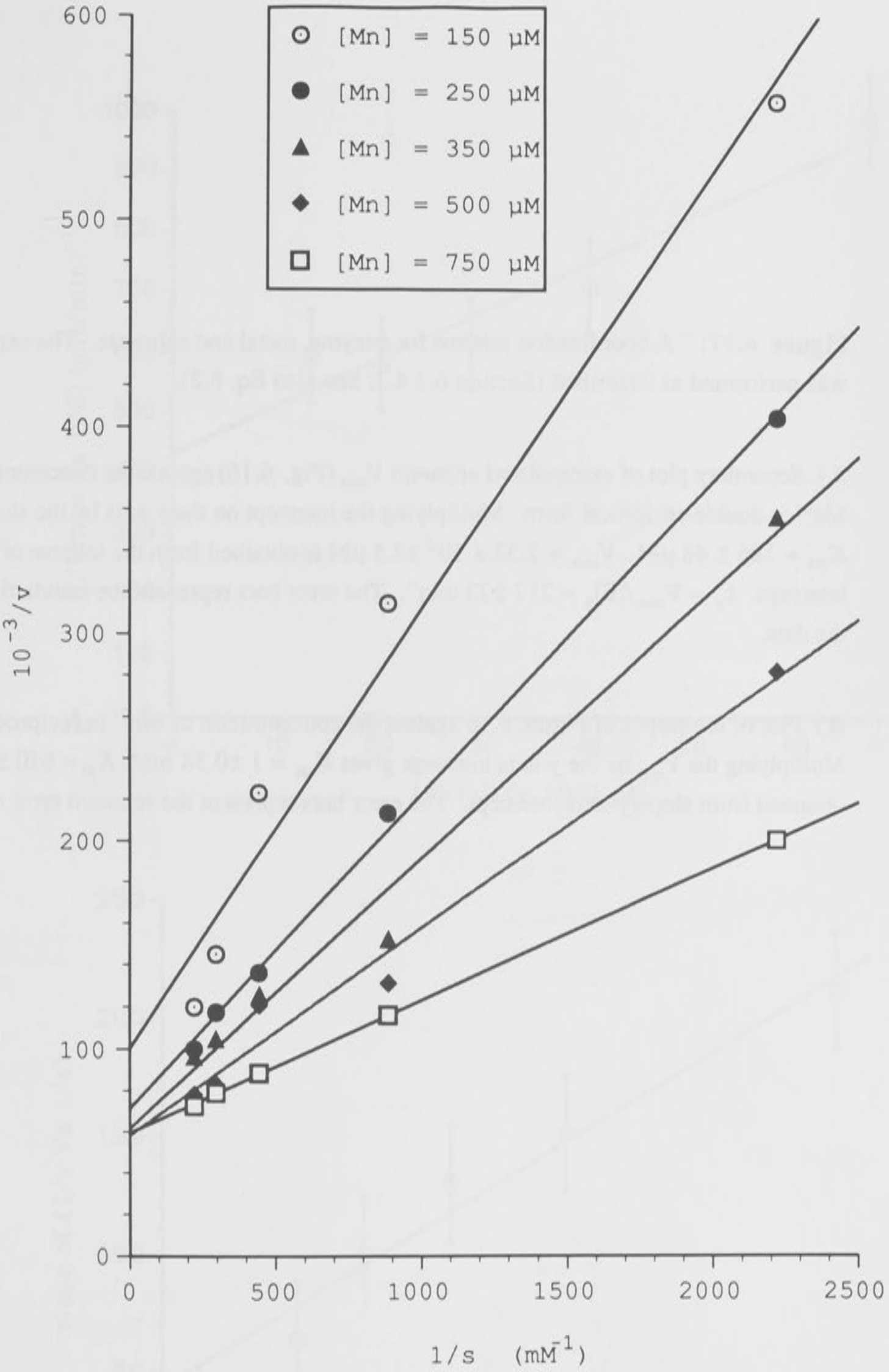
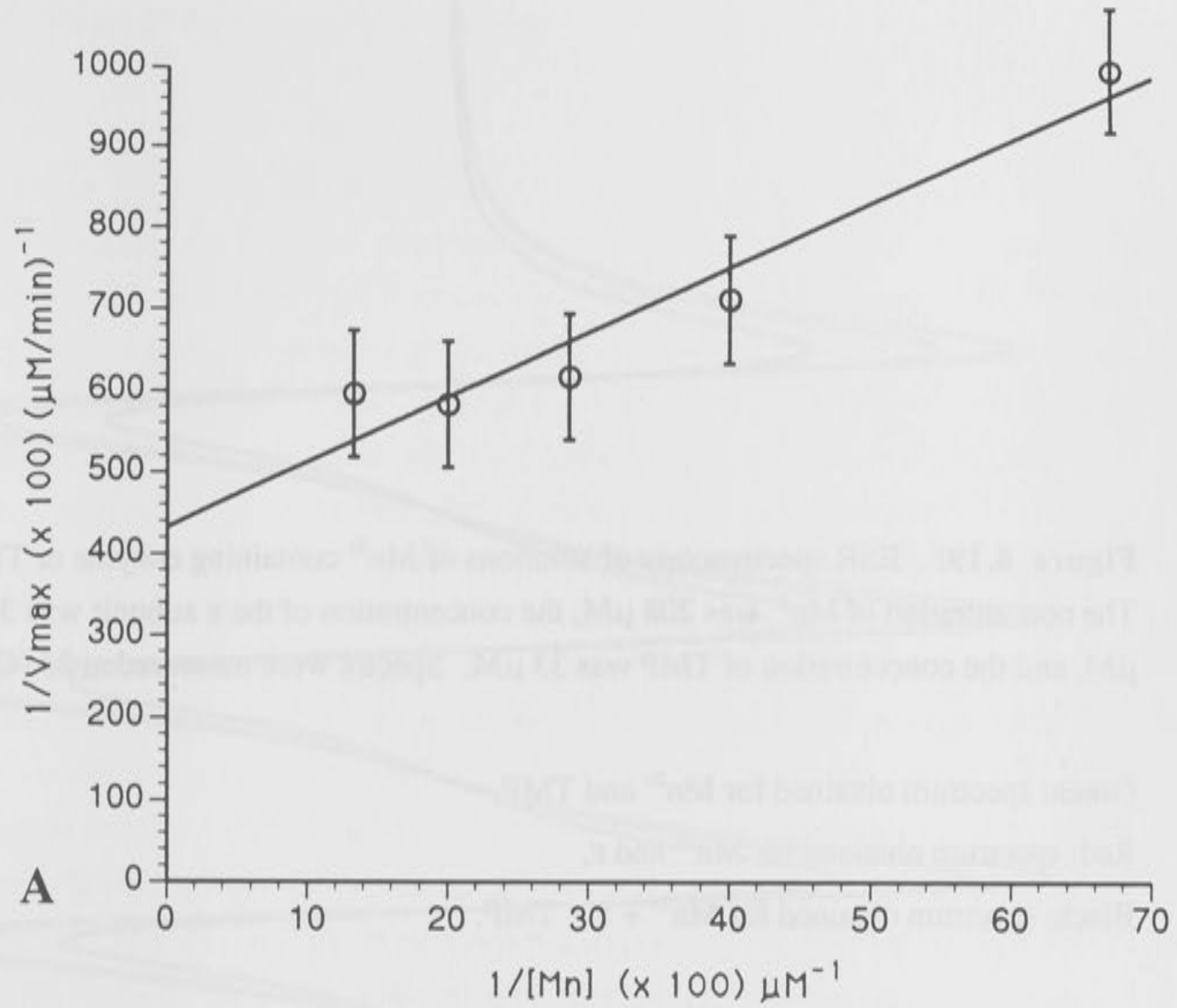


Figure 6.16: Double reciprocal plot of initial velocity against substrate concentration. The y-axis intercept gives the apparent V_{max}. Lines were obtained by linear regression analysis.

Figure 6.17: A coordination scheme for enzyme, metal and substrate. The experiment was performed as described (Section 6.2.4.3; See also Eq. 6.2).

A) Secondary plot of extrapolated apparent V_{max} (Fig. 6.16) against the concentration of Mn^{2+} in double-reciprocal form. Multiplying the intercept on the y-axis by the slope gives $K_{SM} = 186 \pm 48$ µM. $V_{max} = 2.32 \times 10^{-5} \pm 2.5$ µM is obtained from the inverse of the y-axis intercept. $k_p = V_{max}/[E]_0 = 217 \pm 23$ min^{-1}. The error bars represent the standard error of the data.

B) Plot of the slopes of Figure 6.16 against the concentration of Mn^{2+} in reciprocal form. Multiplying the V_{max} by the y-axis intercept gives $K_{MS} = 1 \pm 0.38$ mM. $K_M = 610 \pm 235$ µM is obtained from slope/y-axis intercept. The error bars represent the standard error of the data.


A
1/Vmax (x 100) (µM/min)$^{-1}$
1000
900
800
700
600
500
400
300
200
100
0
0
10
20
30
40
50
60
70
1/[Mn] (x 100) µM^{-1}

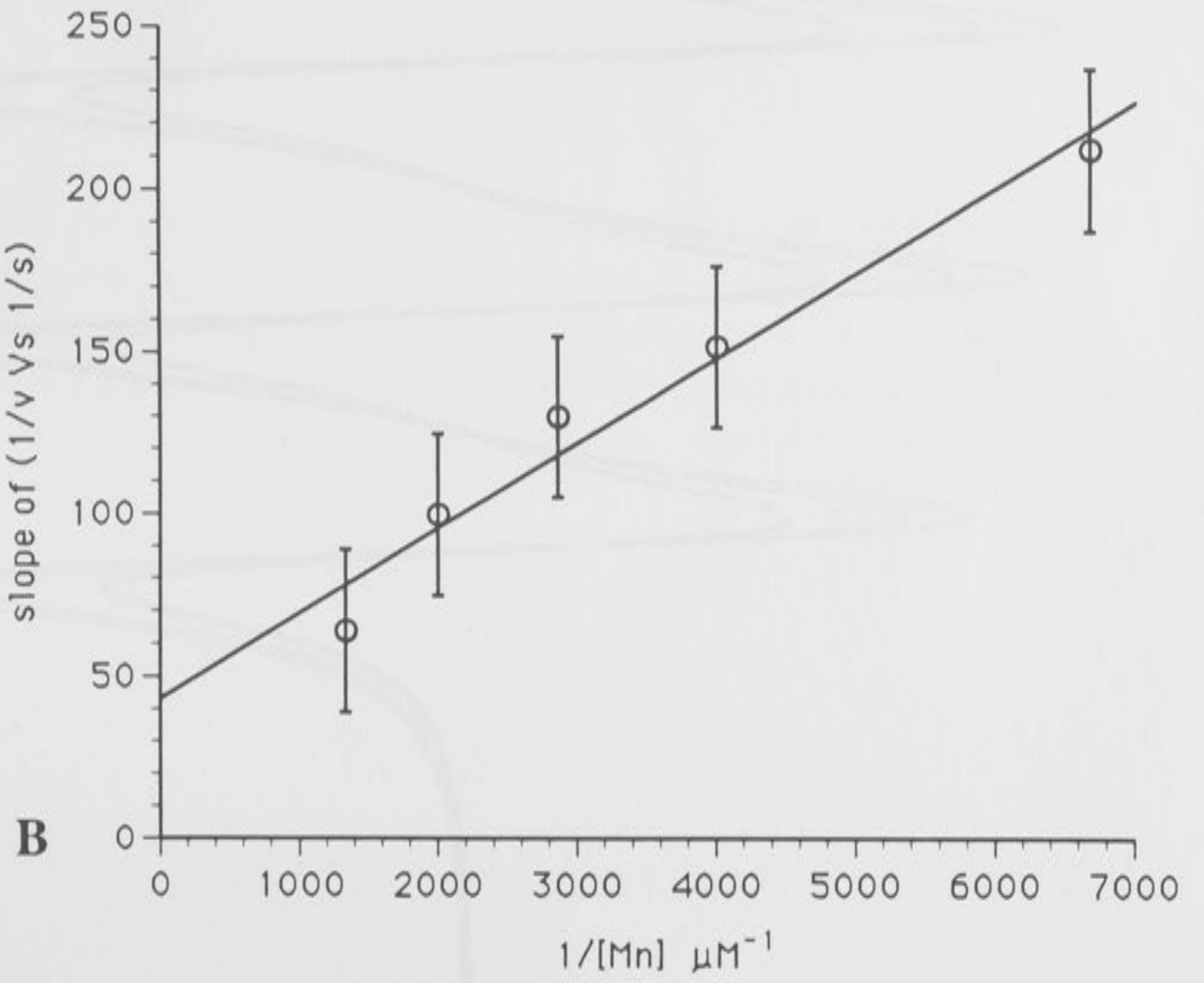
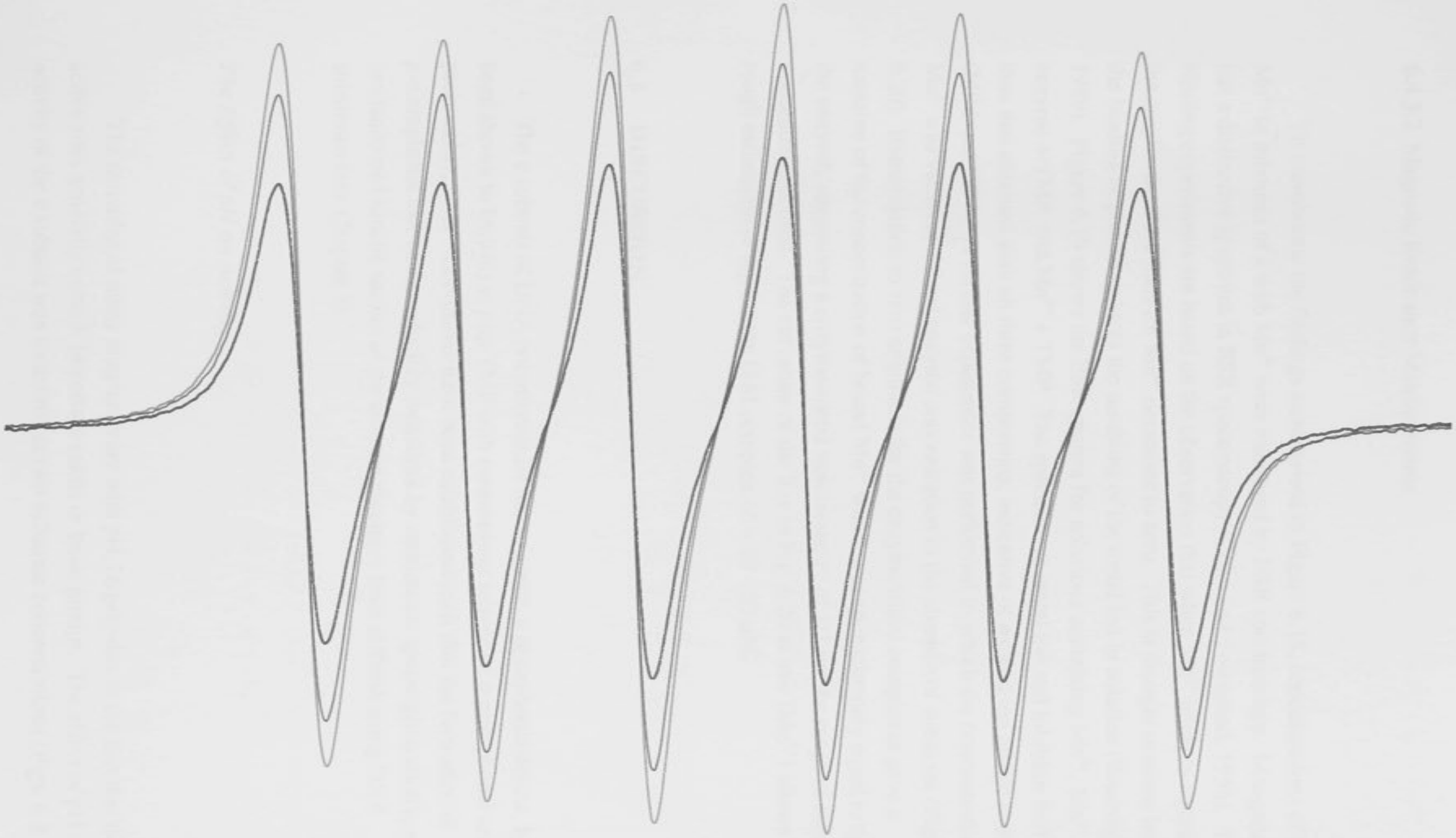

B
slope of (1/v Vs 1/s)
250
200
150
100
50
0
0
1000
2000
3000
4000
5000
6000
7000
1/[Mn] µM^{-1}

Figure 6.19: ESR spectroscopy of solutions of Mn^{2+} containing enzyme or TMP. The concentration of Mn^{2+} was 208 μM, the concentration of the ε subunit was 33.25 μM, and the concentration of TMP was 33 μM. Spectra were measured at 25 °C.

Green: spectrum obtained for Mn^{2+} and TMP,

Red: spectrum obtained for Mn^{2+} and ε,

Black: spectrum obtained for Mn^{2+} + ε + TMP.



6.4.5.2 Magnetic Resonance Measurements

To corroborate the findings summarised in Figure 6.18, concentrations of free Mn^{2+} in mixtures of ε with Mn^{2+} were measured by ESR spectroscopy. Manganese ion has a distinctive spectrum in ESR spectroscopy (Cohn and Townsend, 1956). These binding experiments are based on the observation that when Mn^{2+} binds to a protein, the amplitude of the lines for Mn^{2+} decreases to zero. This is thought to occur because the binding of protein reduces the tumbling of the metal ion in solution (Brudvig, 1989). Figure 6.19 shows the ESR spectra for solutions containing Mn^{2+}, Mn^{2+} + enzyme + TMP, and Mn^{2+} + TMP. The spectrum of metal ion and inhibitor is sharper than that obtained with all three components, indicative of a metal ion bridge complex (Mildvan, 1970). A further experiment was performed in which the concentration of Mn^{2+} was varied at a fixed enzyme concentration in the absence of substrate (Fig. 6.20). Extrapolation to zero amplitude for the enzyme/metal complexes gave a measure of the concentration of bound Mn^{2+} which was approximately equal to that of the enzyme, suggesting a enzyme-metal stoichiometry of 1:1 for the range of metal ion concentrations used. The curvature of the line in Fig. 6.20 at low [Mn^{2+}] allows a rough estimation of K_D for the E-M complex of ≈ 20 - 30 μM.

6.5 DISCUSSION

The ε subunit of DNA polymerase III holoenzyme, a phosphodiesterase, has been shown to hydrolyse *p*np-TMP with concomitant release of *p*-nitrophenol and presumably, TMP. Conditions have been established such that the formation of *p*-nitrophenol can be reproducibly measured by continuous spectrophotometry, which has facilitated kinetic studies of the enzyme that have been difficult using DNA substrates (see Chapter 5).

The Effect of pH on Activity

The activities of many enzymes vary with pH, largely due to the fact that the active sites generally contain important acidic or basic groups. The effect of pH on the activity of the ε subunit was measured at two substrate concentrations (Figs. 6.2 and

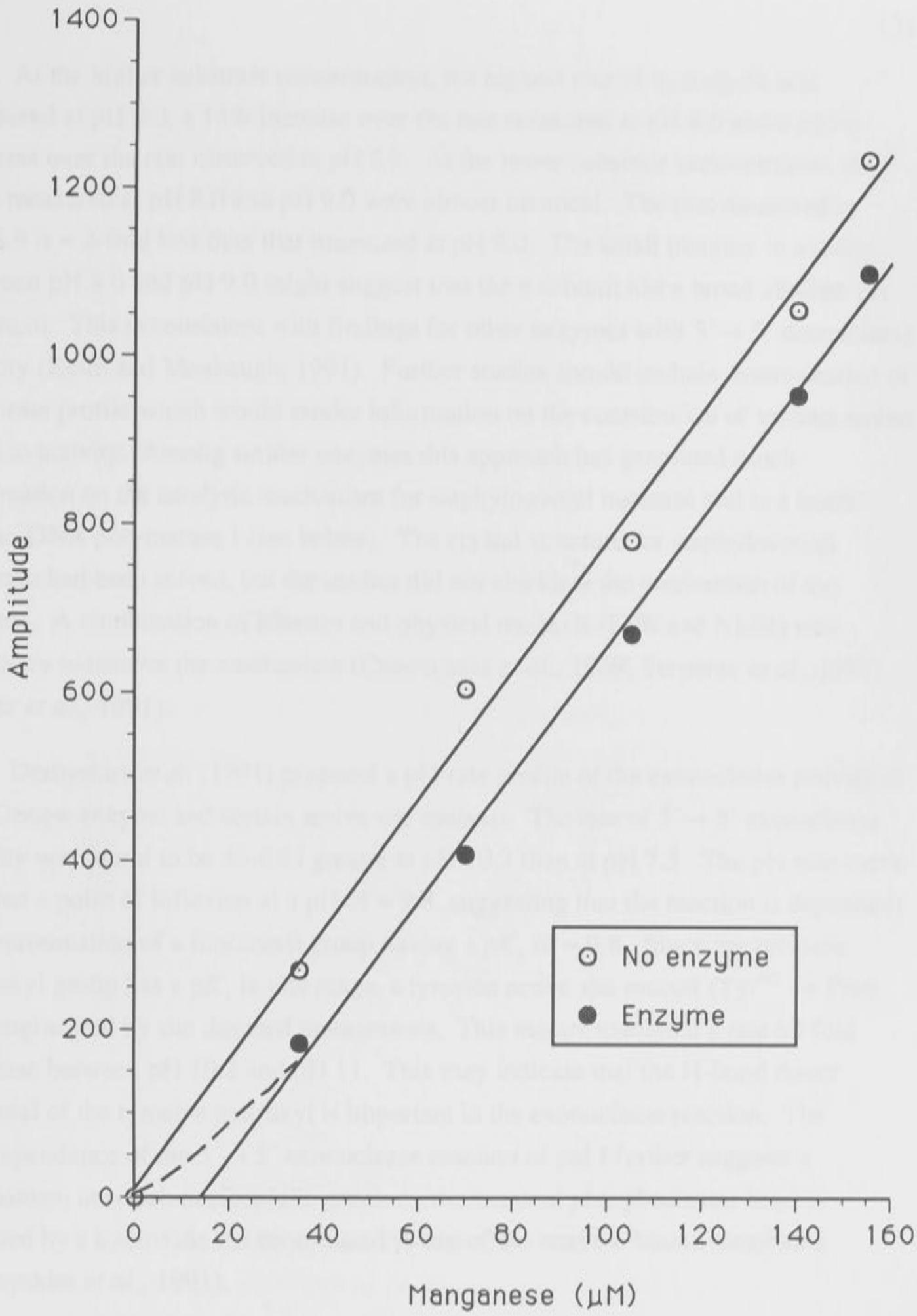


Figure 6. 20: Titration of Mn^{2+} complexes of enzyme and metal using ESR spectroscopy. The concentration of ε was 19.5 μM, the concentration of Mn^{2+} were 35, 70, 105, 140, 155 and 175 μM.

6.3). At the higher substrate concentration, the highest rate of hydrolysis was measured at pH 9.0, a 14% increase over the rate measured at pH 8.0 and a 223% increase over the rate observed at pH 6.9. At the lower substrate concentration, the rates measured at pH 8.0 and pH 9.0 were almost identical. The rate measured at pH 6.9 is ≈ 2-fold less than that measured at pH 9.0. The small increase in activity between pH 8.0 and pH 9.0 might suggest that the ε subunit has a broad alkaline pH optimum. This is consistent with findings for other enzymes with 3′→ 5′ exonuclease activity (Keim and Mosbaugh, 1991). Further studies should include determination of a pH-rate profile which would render information on the contribution of various amino acids to activity. Among similar enzymes this approach has generated much information on the catalytic mechanism for staphylococcal nuclease and to a lesser extent, DNA polymerase I (see below). The crystal structure for staphylococcal nuclease had been solved, but the studies did not elucidate the mechanism of the enzyme. A combination of kinetics and physical methods (EPR and NMR) was necessary to resolve the mechanism (Cuatrecasas *et al.*, 1969; Serpersu *et al.*, 1987; Weber *et al.*, 1991).

Derbyshire *et al.* (1991) prepared a pH-rate profile of the exonuclease activity of the Klenow enzyme and certain active site mutants. The rate of 3′→ 5′ exonuclease activity was found to be 45-fold greater at pH 10.2 than at pH 7.5. The pH-rate curve showed a point of inflexion at a pH of ≈ 9.8, suggesting that the reaction is dependent on deprotonation of a functional group having a pK_a of ≈ 9.8. Since the tyrosine hydroxyl group has a pK_a in this range, a tyrosine active site mutant (Tyr^{497} → Phe) was engineered by site directed mutagenesis. This mutant exhibited a rate 62-fold decrease between pH 10.2 and pH 11. This may indicate that the H-bond donor potential of the tyrosine hydroxyl is important in the exonuclease reaction. The pH-dependence of the 3′→ 5′ exonuclease reaction of pol I further suggests a mechanism in which nucleophilic attack on the terminal phosphodiester bond is initiated by a hydroxide ion coordinated to one of the enzyme-bound metal ions (Derbyshire *et al.*, 1991).

The Effect of NaCl on Activity

The effect of sodium chloride on activity in the assay was established. Activity is stimulated at a concentration of NaCl as little as 3 mM, and up to 3-fold at 500 mM (Figure 6.4). The activity of the ε subunit is inhibited thereafter. The D15 exonuclease from phage T5 has also been reported to be stimulated by concentrations

of NaCl up to 75 mM (Sayers and Eckstein, 1990). DNA polymerase III holoenzyme is completely inhibited by a concentration of NaCl as low as 200 mM (Burgers and Kornberg, 1982), but stimulated between 5 and 100 mM (Griep and McHenry, 1989). In their system, salt was affecting the binding of holoenzyme to template (primed M13Gori ssDNA), and to a lesser degree, elongation. In this assay, it is possible salt may enhance substrate binding, or through ionic interactions stabilise the activated complex.

Michaelis-Menten Kinetics

Substrate hydrolysis was found to obey Michaelis-Menten kinetics thereby allowing the maximum rate of reaction (V_{max}), the catalytic constant (k_{cat}), and the binding constant (K_M) for *p*np-TMP to be determined (Fig. 6.6). The binding of *p*np-TMP to enzyme (K_M = 1.22 mM) is much weaker than that of mispaired termini (≈ 500 nM) in the oligonucleotide-based assays (Sections 5.1.5.1.3 and 5.1.5.2.2). However, it is clear that the ε subunit is at least 500 times more active with *p*np-TMP (k_{cat} = 178 min^{-1}) than a mispaired terminus (k_{cat} = 0.31 min^{-1}), and 4-fold more active than on ssDNA (k_{cat} = 40 min^{-1}). Lehman and Richardson (1964) have reported that DNA polymerase I is able to hydrolyse *p*np-TMP to *p*-nitrophenol. This appears to be the only comparison of *p*np-TMP hydrolysis available between *E. coli* replication proteins. A comparison of the performance of ε and pol I in the colourimetric assay is presented in Table 6.3. In the presence of Mg^{2+}, the rate of hydrolysis of both phosphodiesterases is approximately the same. It would be interesting the measure the effect of Mn^{2+} on the activity of the Klenow enzyme. The inability to measure the hydrolysis of *p*np-TMP by the Klenow enzyme in this study is puzzling, and will be discussed below.

Future experiments could include measuring the activity of ε on other 5′ nucleoside monophosphate *p*-nitrophenyl esters which may provide information on substrate specificity and mechanism. Griep *et al.* (1990) measured the rate of proofreading activity of *holoenzyme* using a copolymer substrate similar to that depicted in Figure 5.3. The 3′-OH terminus of the shorter strand was labelled at the penultimate position with either CMP or dCMP, while at the terminal position either AMP, dAMP, 2′,3′-dideoxyAMP, cordycepin (3′-dAMP), dAMPαS, or 2′,3′-dideoxyAMPαS was incorporated. They reported that placing a ribonucleotide at the penultimate position coupled by a phosphorothioate linkage to a terminal dideoxynucleotide reduced the rate of proofreading activity almost 300,000-fold.

Enzyme	K_M	$V_{max}/[E]_0$
	mM	mol *p*-nitrophenol/min/mol enzyme
ε[a]	1.23	170 (Mn^{2+})
	2.3	7 (Mg^{2+})
pol I[b]	6.0	4 (Mg^{2+})

Table 6.3: A comparison of kinetic constants for the hydrolysis of *p*np-TMP by the ε subunit of holoenzyme and pol I (Lehman and Richardson, 1964). [a]Standard assay conditions (Table 6.1). [b]The conditions for the hydrolysis of *p*np-TMP by pol I were: 70 mM glycine buffer pH 9.2, 1 mM β-mercaptoethanol, 7 mM Mg^{2+}, 37 °C.

If only ribonucleotide and phosphorothioate substitutions were made, proofreading activity was decreased 500-fold. The effect of substituting oxygen in the terminal phosphodiester bond for sulfur was to cause the rate of proofreading activity to decrease even further. The authors claim that this effect is consistent with a change in the rate-limiting step of the exonuclease reaction from a conformational change to the chemical step where the covalent bond is cleaved. Single changes at either the penultimate or 3′-terminal positions with the analogues resulted in only 10-fold decreases in proofreading activity. This data would indicate that the ε subunit has a broad substrate specificity. Cuatrecasas *et al.* (1969) also used this approach in a colourimetric assay to ascertain the substrate specificity of staphylococcal nuclease. They showed that the basic structural unit necessary for recognition for this enzyme was R-pdT-r′, hydrolysis resulting in the release of R-phosphate. Staphylococcal nuclease appears to be essentially unspecific with respect to the R′ substituent. Maximal rates of hydrolysis were achieved with a 3′-OH or 3′-acetyl substitution. The nature of the R′ group makes a very significant contribution to the strength of binding of the substrate to enzyme. There was a 200-fold increase in the affinity of substrate if R′ is a phosphate rather than a hydroxyl or acetyl group, emphasizing the importance of the phosphate substituents (Cuatrecasas *et al.*, 1969). It is likely that the colourimetric assay described in this study will provide a reproducible and simple method for conducting studies to similar those conducted by Griep *et al.* (1990) but with isoalted ε.

The Effect of Metal Ions on Activity

As in the oligonucleotide-based assays, there is an absolute requirement for divalent metal ion (Mg^{2+} or Mn^{2+}) for activity (Figs. 6.7 and 6.9). Whether metal ion is required for substrate binding or catalysis is discussed below. Dissociation constants for the binding of divalent metal ions Mn^{2+} and Mg^{2+} were also determined. The rate of hydrolysis obtained with Mn^{2+} was found to be 23-times higher that obtained with Mg^{2+} (under standard assay conditions); there is only a two-fold increase in activity observed in the oligonucleotide-based assays with Mn^{2+}. A similar finding was obtained with the binding of divalent cations to the $3' \rightarrow 5'$ exonuclease site of the Klenow enzyme. Mn^{2+} was reported to bind 82-times more strongly than Mg^{2+} (Han *et al.*, 1991). The difference in K_M's measured for overexpressed ε suggest that an enzyme-Mn^{2+} complex binds substrate more tightly than an enzyme-Mg^{2+} complex, and that Mn^{2+} itself is bound much more tightly to enzyme than Mg^{2+}. This indicates that metal ion is involved at least in the binding of substrate.

While these experiments go some way toward explaining this phenomenon, modification of metal-binding residues in the ε subunit will be necessary to resolve the issue. This may be possible without a crystal structure of ε since it appears there is a strong degree of homology between the exonuclease active site residues in pol I and ε (Bernad *et al.*, 1989; see Section 5.3 and Fig. 5.21). The colourimetric assay described in this study will then provide a powerful tool for analysing the properties of mutant forms of the enzyme. Using crystallographic data, Derbyshire *et al.*(1991) determined the contribution of each amino acid in the $3' \rightarrow 5'$ exonuclease active site of DNA polymerase I to the reaction by site-directed mutagenesis. They were able to demonstrate that the greatest effects on exonuclease activity resulted from changes to amino acid residues (Asp^{355}, Asp^{424} and Asp^{501}) anchoring two divalent metal ions that are essential for proofreading activity.

Inhibition Studies

Kinetic data obtained from this assay system confirms the ε subunit is inhibited by the products of the reaction it catalyses: dNMP's. TMP was shown to be a competitive inhibitor, with $K_i \approx 17$ μM. Cuatrecasas *et al.*(1969) report that staphylococcal nuclease is inhibited to a lesser extent than ε by a build-up of pdT in the assay ($K_i = 190$ μM), and that the inhibition was competitive. In this study, dGMP

was found to be the most inhibitory, dCMP was the least inhibitory, while dAMP and TMP gave inhibitions intermediate between the two (Fig. 6.12). This finding is in complete agreement with that obtained by Scheuermann and Echols (1984) in their oligonucleotide-based assays. However, this study went further to show that the type of inhibition is competitive (Fig. 6.13). Since TMP is a product of substrate hydrolysis, it was the logical candidate for determination of a binding constant for inhibitor. TMP was found to bind 70-times more tightly to enzyme than *p*np-TMP (Fig. 6.14). This finding explains why a decrease in rate is apparent during the assay and emphasises the need to measure initial rates before the accumulation of TMP becomes inhibitory. Binding constants for the dNMP's were not determined in the oligonucleotide-based assays.

Applicability of the Colourimetric Assay to Other Enzymes with Proofreading Activity

It was hoped that *p*np-TMP could be used for assay of other enzymes with proofreading activities. Derbyshire *et al.*(1991) have generated numerous mutants of the large fragment of DNA polymerase I at both active site and metal ion-binding residues. Kinetic analysis of such mutants is currently based on the removal of nucleotides from the 3′-OH terminus of ssDNA (Derbyshire *et al.*, 1991). A continuous spectrophotometric assay of *p*-nitrophenol formation from the hydrolysis of *p*np-TMP would afford a powerful tool for the assessing the contribution of amino acids to catalysis and substrate binding. However, the Klenow fragment, and the T4 and T7 DNA polymerases showed no activity in this assay at molar concentrations exceeding that of the ε subunit itself (Table 6.2). The inactivity of the Klenow enzyme in the colourimetric assay contradicts findings by Lehman and Richardson (1964) who reported that DNA polymerase I is able to hydrolyse *p*np-TMP to *p*-nitrophenol. A plausible explanation is that the assay was performed in this study at pH 8.0, whereas the pH optimum of pol I is believed to be pH 9.2 (Lehman and Richardson, 1964), and Derbyshire *et al.* (1991) claim a pH optimum of 10.2. However, the Lehman and Richardson (1964) data suggest the exonuclease activity of pol I at pH 8.0 should be at least 20% of that at pH 9.2. Therefore, some substrate hydrolysis should have been measured in this study. It would seem unlikely that the Klenow fragment is itself unreactive in the assay. In the case of the T4 and T7 DNA polymerases, it could be argued that substrate may not be able to gain access to the exonuclease active site, since both enzymes presumably have greater complexity than the ε subunit. This could be tested by isolating the 3′→ 5′ exonuclease domain of each of these enzymes. The exonuclease domain could then be tested for activity in the colourimetric assay. DNA

polymerase III* was nearly half as active (in molar terms) as the ε subunit in the colourimetric assay. This is likely due to the fact that the enzyme preparation was only half pure. Moreover, the assay conditions may not be optimal for the pol III*. The ability of pol III* to hydrolyse *p*np-TMP may suggest that the spatial arrangement of the polymerase and exonuclease active sites is different from that for the T7 and T4 DNA polymerases, and possibly pol I. Further, since pol III* is at least half as active as overexpressed ε in the assay, it is conceivable that the mechanism of action of the 3′→ 5′ exonuclease of the ε subunit is different from that of the other enzymes.

The Role of Metal Ions in Activity

Determination of substrate and cofactor binding to an enzyme can provide important information about the nature of the active site and the mechanism of action of the enzyme. Investigations of functional ternary complexes of enzyme, metal, and substrate by kinetic measurements (Figs. 6.15 to 6.18) and ESR spectroscopy (Fig. 6.19 and 6.20) are reported. The kinetic data is consistent with the binding of enzyme, metal and substrate occurring in random order. This indicates that metal ion can be involved in either the binding of substrate or catalyis, although the scheme indicates that the E-M-S pathway is preferred (Fig. 6.18). ESR spectroscopy of ε, Mn^{2+} and TMP (Fig. 6.19) suggests that the binding order is ordered: E-M-S, based on criteria developed by Albert Mildvan some 20 years ago (Mildvan, 1970). However, Mildvan's criteria have now generally fallen into disrepute. Another ESR experiment would seem to suggest that another metal ion binds to ε in the absence of substrate with a K_D of ≈ 30 μM (Fig 6.20). The measurement of this metal binding is not detected by the kinetic experiments. Only the binding of the second, and more loosely bound Mn^{2+} ion (K_D = 610 μM) is measured under standard assay conditions. It is hypothesised that the first metal ion binds tightly to enzyme giving rise to a still inactive enzyme; at high concentrations of metal ion, we see a second metal ion binding conferring full activity to ε. Binding of the second metal ion is facilitated by prior binding of substrate (Fig. 6.18) as happens with pol I (see below). The involvement of more than one metal ion could explain why the plot of 1/*v* versus 1/S obtained at the lowest concentration of Mn^{2+} was nonlinear in the Lineweaver-Burk analysis (Fig. 6.16).

A combination of kinetic (Englund *et al.*, 1969) and physical methods (NMR) (Slater *et al.*, 1972; Sloan *et al.*, 1975) was used to show that only metal-bound substrates bind to DNA polymerase I. The NMR studies showed that Mn^{2+} in the

ternary Mn^{2+}.TTP.DNA polymerase I complex binds to the γ-phosphorus and possibly also to the α-phosphorus of TTP. Derbyshire *et al.* (1988) reported that the exonuclease active site of pol I has binding sites for two divalent metal ions. Analysis of mutant proteins suggested that one metal ion is involved in substrate binding while the other is involved in catalysis of the exonuclease reaction (Derbyshire *et al.*, 1988). Since only metal-bound substrates bind to enzyme, one of the metals must be delivered to the pol I metal ion binding sites at the exonuclease active site by the substrate.

The association of two or more metal ions with an enzyme is not uncommon. As discussed above, kinetic and genetic studies on the role of divalent cations in pol I activity have shown that at least two metal ions are involved. Two divalent metal ions (Mg^{2+}, Mn^{2+}, Zn^{2+}) are bound at the exonuclease active site, while one or more divalent cations interact with the polymerase active site domain (Beese and Steitz, 1991; Kornberg and Baker, 1991). The acetyl coenzyme A synthetase was also shown to have a double requirement for divalent cations (Webster, 1967). Two weakly bound metal ions (Ni^{2+} or Cu^{2+}) are required for binding substrates to the enzyme and two tightly bound metals (Mg^{2+}, Mn^{2+}, Ca^{2+}) are involved in catalysis.

It seems likely that the kinetic mechanism for the hydrolysis of *p*np-TMP is more complex than anticipated, and may involve the binding of at least two metal ions. It must be emphasised that kinetic experiments alone cannot establish the mechanism, and that physical measurements will be required to corroborate the data (Mildvan, 1970; Cuatrecasas *et al.*, 1967a; Mildvan and Cohn, 1965; Serpersu *et al.*, 1987). The solubility of ε poses a problem in this respect as millimolar concentrations of enzyme are usually required for NMR and ESR analyses. However, the techniques of equilibrium dialysis (Englund *et al.*, 1969) and gel filtration (Cuatrecasas *et al.*, 1967b) do not require high concentrations of enzyme and could be adapted for these studies.

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CHAPTER 7

A STRUCTURAL MODEL FOR THE ε SUBUNIT.

7.0 INTRODUCTION

A successful conclusion to any body of research on an enzyme is elucidating its mechanism of action. The principal investigative route involves obtaining detailed structural information on that enzyme which can then provide a basis for mechanistic studies. There are several methods used to elucidate in detail the structure of a protein, including X-ray crystallography, nuclear magnetic resonance, and predictive techniques.

X-ray Crystallography (Price and Stevens, 1988)

X-ray crystallography is the method of choice for obtaining a three-dimensional structure of a protein. The technique relies on the scattering of electromagnetic radiation of suitable wavelength by electrons belonging to the atoms in a molecule. In the case of a regularly arranged array of atoms, such as is present in a crystal lattice, we can have constructive or destructive interference between the scattered waves. Only constructive interferences will give rise to a detectable signal. X-rays provide suitable radiation to bring about these diffraction effects since their wavelength is comparable with the interatomic distances in a molecule.

The positions and intensities of diffracted rays are measured using photographic film, so that a pattern of spots is obtained. From the pattern of the spots it is possible to deduce certain features such as the overall symmetry of the crystal. However, additional information is required in order to establish the three-dimensional structure of the enzyme. This data is obtained by using heavy atom derivatives of the protein. Electron-dense heavy atoms scatter the X-rays more strongly than the lighter atoms present in proteins (i.e. H, C, N, O and S). Once the phases of the diffracted rays are known, it is then a matter of calculating the three-dimensional map of the electron density of the molecule by a process called Fourier summation. Finally, in order to interpret the electron-density map of an enzyme in terms of atomic structure, it is necessary to know the amino acid sequence of the molecule. One can then trace the path of the chain of α-carbon atoms and amide bonds and locate the various side-chains in the electron density map. When the polypeptide chain has been fitted to the electron density map, the three-dimensional structure of the protein is obtained.

The structure of the Klenow fragment of DNA polymerase I, prepared directly

by cloning (Joyce and Grindley, 1983) has been elucidated by X-ray crystallography (Ollis *et al.*, 1985; Joyce and Steitz, 1987; Freemont *et al.*, 1988). These studies indicated that the polymerase active site is located within a binding cleft for dsDNA, while the proofreading exonuclease active site is associated with a ssDNA binding region (Joyce and Steitz, 1987). The 3′ → 5′ exonuclease and polymerase active sites are believed to be 25-30 Å apart (Joyce and Steitz, 1987). As described earlier (Chapters 1 and 5), the crystallographic data has enabled studies of mechanism of the 3′→ 5′ exonuclease using site-directed mutagenesis (Derbyshire *et al.*, 1991), and interaction between the polymerase and exonuclease active sites (Joyce, 1989) to be undertaken.

By early 1989, over 400 sets of protein coordinates were listed in the *Protein Data Bank Newsletter* (Eisenberg and Hill, 1989). The introduction of more powerful methods for atomic refinement (Eisenberg and Hill, 1989) may see this number increase rapidly. In spite of these advances, growing crystals of sufficient quality remains the major limitation of this technique. In addition, crystal structures render only a static picture of an enzyme (which is dynamic) and might not be the same as that of the enzyme in solution.

Nuclear Magnetic Resonance

In the last decade, nuclear magnetic resonance (NMR) has emerged as a powerful tool for studies of the structure and dynamics of proteins. What makes NMR unique is that it is the only method that can provide high resolution structural information of proteins in solution state (Bax, 1989). Structure determinations provided by two-dimensional NMR correspond very closely with those obtained by X-ray crystallography of the same protein (Wright, 1989; Bax, 1989). However, some differences have been noted between the structure of an enzyme in solution and in the crystal state (Nettlesheim *et al.*, 1988). The substantial differences in helical structure of the human complement protein, observed both at the C and N termini, were believed to be due to crystal packing forces (Nettlesheim *et al.*, 1988). The major limitation of 2D-NMR is that only proteins of up to about 12 kDa in size can be analysed by this method. The advent of three-dimensional NMR methods may make it possible to determine the structures of larger proteins, in the 20 - 30 kDa range (Wright, 1989).

Secondary structure prediction methods are easier to apply. However, the information derived is limited to a topological string of possible secondary structures (helix, sheet, turn and coil configurations).

The methods to elucidate regions of ordered structures from the amino acid sequences in soluble proteins can be classified into three groups: probabilistic, physicochemical, and information theory techniques.

Of the first group, the empirical method of Chou and Fasman (1974, 1978) is perhaps the most well known and is judged to have a 60% level of accuracy (Kabsch and Sander, 1983). This method uses parameters obtained from the analysis of known sequences and structures. Each amino acid is assigned a probability that describes the residue's preference for participation in a helix (P_α), sheet (P_β) or turn (P_c) configuration. Residues with the highest P_α are predicted to form the centre of a helix; residues with low P_α are predicted to terminate the helix. Similarly, residues with the highest P_β values are suggested to initiate β sheet regions, and residues with the lowest P_β values are suggested to terminate β regions (Chou and Fasman, 1978). Thus, a secondary structure can be assembled from these parameters.

The physicochemical methods utilise patterns in the occurrence of polar and non-polar amino acids in secondary structures (Kyte and Doolittle, 1982; Eisenberg *et al.*, 1984). The Kyte and Doolittle method evaluates the hydrophilic and hydrophobic tendencies of a polypeptide chain. Each amino acid is assigned a value reflecting its relative hydrophilicity and hydrophobicity. Plotting these values gives a graphic representation of the hydropathic character of the chain from one end to the other. The authors claim a high degree of agreement between their hydropathic profiles and known structures of proteins (Kyte and Dolittle, 1982). Other physicochemical methods rely on thermodynamic and statistical mechanical theory for determining conformational preferences (Argos and MohanaRao, 1986).

The information approach, typified by the method of Robson and Garnier (1978), is perhaps the most mathematically sophisticated of the procedures. The benefit of this approach is the ability to examine residue interactions in determining the structural types as far as eight residues away in either direction and to weight the validity of the interactive statistics according to the sufficiency of the information and data available (Argos and MohanaRao, 1986). Gibrat *et al.* (1987) re-evaluated the Garnier-Osguthorpe-Robson (GOR) algorithm, and concluded that the existing data

base does not allow evaluation of the parameters required for an exact treatment of the problem. However, they were able to refine the method and claim an increase in the accuracy of prediction by 7% to 63%. The accuracy of this method should increase further as the database of known structures is expanded (Argos and MohanaRao, 1986).

Knowledge of a protein's secondary structure may be useful, but it cannot be a substitute for a tertiary architecture with its attendant implications for active site mechanisms, substrate-binding regions, and external recognition of interactive enzymes or receptors.

Tertiary Structure Prediction

Methods to predict tertiary structure have not, as yet been successful. The methods centre around energy minimisation. These methods are: a starting polypeptide chain model based on a simplified representation of the backbone, or an empirically deduced approximation of the pattern of residues based on known structures, secondary structural predictions, and preferred residue interactions (Argos and MohanaRao, 1986). The methods are not generally applicable and, when partially successful, give information limited to specific structures or folding patterns.

Peptide Mapping

In the absence of crystal or NMR data, dissection of the protein by controlled (limited) enzymatic proteolysis can provide valuable qualitative information on the conformation of the substrate protein, and the location of folded domains and susceptible peptide bonds (eg. hinge regions) (Carrey, 1989; Carrey *et al.*, 1988). It is via this route that a simple structural model for the ε subunit will initially be developed. Ultimately, the elucidation of the three-dimensional structure of ε will depend on a combination of X-ray crystallography, NMR and biochemical characterisation of the wild-type protein and mutant derivatives.

7.1 MATERIALS AND METHODS

7.1.1 *Materials*

Trypsin, chymotrypsin and endoprotease Glu-C (sequencing grades) were purchased from Boehringer-Mannheim. The α subunit of DNA polymerase III holoenzyme (fraction IV (80% pure); prepared as described by Maki and Kornberg, [1985b]) was a kind gift from Mr J. Crowther. Protein size standards for gel filtration: ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa) and bovine serum albumin (67 kDa), were purchased from Pharmacia.

7.1.2 *Limited Proteolysis of the ε Subunit*

7.1.2.1 Preparation of Protease Solutions

Stock solutions (100 μg/ml) of trypsin and chymotrypsin were prepared in 1mM HCl containing 20 mM $CaCl_2$ and these were diluted as necessary in the same buffer. Calcium ion was included in the buffer to minimise autolysis of the proteases (Carrey, 1989). The stock solutions could be stored at -20 °C for several weeks. A stock solution (200 μg/ml) of endo Glu-C was prepared in 500 mM NH_4HCO_3, pH 7.8.

7.1.2.2 Determination of Optimal Conditions for Limited Proteolysis

7.1.2.3 Titration of Proteases

ε subunit (7.5 μg) was treated with a range of concentrations of proteases for 1 h at 37 °C in buffer S (Section 4.1.4.3). Reactions were subjected to SDS-PAGE and the proteins visualised with Coomassie blue (Sections 2.2.22 and 2.2.23).

7.1.2.4 Time Course of Proteolysis

Forty-five micrograms of the ε subunit was treated with an optimal concentration of protease (derived from the experiment above), and aliquots of the reaction were withdrawn at predetermined intervals. Protease activity was quenched by the addition of loading buffer, and heating at 100 °C for 10 min; the quenched reaction mixture was stored in liquid nitrogen until use.

7.1.2.5 Large-Scale Digestion of the ε Subunit with Chymotrypsin

Approximately 563 µg of the ε subunit was treated with 1 µg of chymotrypsin in 1 ml of buffer (25 mM Tris.HCl pH 7.5, 5 mM Mg^{2+}, 1 mM DTT, 25 mM NaCl) at 4 °C for 48 h. The digest was concentrated to 200 µl by ultrafiltration using a Centricon C10 microfiltrator (Amicon).

7.1.2.6 Purification of a Chymotryptic Fragment of the ε Subunit

The chymotryptic digest (563 µg) was applied to a 1 ml Mono-Q FPLC column that had been equilibrated with buffer S (Section 4.1.4.3) containing 25 mM NaCl (10 ml). The column was washed with buffer S containing 25 mM NaCl (2.5 ml). Protein fragments were eluted with a linear gradient of 25 - 200 mM NaCl in buffer S (10 ml) at a flow rate of 0.5 ml/min; 350 µl fractions were collected. Elution of protein from the column was monitored with a Pharmacia UV-M monitor. Fractions corresponding to the major peak of A_{280} were pooled and assayed for protein concentration by the method of Bradford (1976) using BSA as standard. The purified large chymotryptic fragment (ε_{CH}) was used for further studies.

7.1.3 *Serendipitous Isolation of a Degradation Product of the ε Subunit*

A degradation product of the ε subunit (ε_{DP}) was obtained by chance during the course of an ε purification. The procedure follows. ε was purified up to the

denaturation step (Sections 4.1.4.2 to 4.1.4.4). After removal of GuHCl by dialysis, the preparation was further purified by chromatography on DEAE Sephacel. However, the enzyme did not bind to the column due to the absence of divalent metal ion in the buffer. The DEAE Sephacel flow-through was concentrated from 80 ml to 12.6 ml at 4 °C in an Amicon stirred-cell concentrator (Amicon, U.S.A.). The preparation was further purified by chromatography on Sephadex G-100 from which a single peak was obtained (data not shown). A portion of purified enzyme was then dialysed over 4 days at 4 °C against several changes of buffer (2 litres; 25 mM Tris.HCl pH 7.5, 16 mM DTT, 75 mM NaCl, 10 mM EDTA) to remove metal ions (Section 5.1.7.1). The enzyme was finally dialysed overnight at 4 °C into 25 mM Tris.HCl pH 7.5 buffer, containing 16 mM DTT and 75 mM NaCl.

7.1.4 *Determination of the Sizes of Proteins by Gel Filtration*

The molecular weight of overexpressed ε and a chymotryptic fragment (ε_{CH}) was determined by gel filtration on a Superose-6 column using a complete FPLC system at 4 °C. The column was calibrated by using size standards from Pharmacia: ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa) and BSA (67 kDa). Approximately 500 µg of each standard, and 250 µg of ε and ε_{CH} (in buffer S containing 200 mM NaCl) were applied to the column. The progress of chromatography was monitored with a Pharmacia UV monitor at A_{260}. The elution volume, V_e, was determined from peaks of A_{280}. The molecular weight of ε and ε_{CH} was determined from a least squares regression analysis of the data in a plot of V_e *versus* log molecular weight of the protein standards (see Fig. 7.9). Since each FPLC run was identical, it was unnecessary to compute the void volume for determination of M_r.

7.1.5 *Formation of α–ε Complexes*

Complexes of overexpressed α and ε subunits, and of α and ε_{CH} were formed (Studwell and O'Donnell, 1989) and purified (Maki and Kornberg, 1987) as follows. Purified α subunit (119 µg) was added to a 4.5-fold molar excess of purified ε (111 µg) in buffer (20% glycerol (w/v), 25 mM Tris.HCl pH 7.5, 3 mM DTT, 0.5 mM EDTA) thus ensuring that most of the α subunit would be present as an α–ε complex

(Studwell and O'Donnell, 1989). This simplifies their purification by gel filtration. In a second experiment, α subunit (119 μg) was added to a 5-fold molar excess of ε_{CH} (120 μg) in buffer. The proteins were mixed and treated at 15 °C for 30 min. To avoid injecting bubbles into the FPLC system, the reaction mix was made up to 350 μl with buffer. The entire volume was loaded onto a 24 ml FPLC Superose-6 column (using a 200 μl injection loop) that had been equilibrated with the same buffer at 4 °C (50 ml). Proteins were eluted with buffer. The progress of chromatography was monitored at A_{260} with a Pharmacia UV-M monitor. Fractions were assayed for proofreading activity (Section 5.1.20.2).

7.1.6 *Determination of Protein Sizes Using Sedimentation Equilibrium Centrifugation*

Analytical centrifugation of purified proteins was performed in a meniscus-depletion sedimentation equilibrium experiment by Dr Peter Jeffrey of the Protein Chemistry Group, John Curtin School of Medical Research. Overexpressed ε subunit, and ε_{PD} (1 ml) purified as described were dialysed against 25 mM Tris.HCl pH 7.5, 1 mM DTT and 50 mM NaCl (1 litre, 24 h). The speed of 40,000 rpm was considered appropriate for proteins of 20,000 to 30,000 Da. The experiment was performed in a double-sector aluminium-filled cpm cell with sapphire windows at a controlled temperature of 20 °C with the dialysed protein solution (0.1 ml at approximately 1.3 mg/ml) in one sector and its equilibrium dialysate (0.11 ml) in the other using a Beckman Model E ultracentrifuge. After equilibrium had been obtained, a photograph of the pattern of Raeleigh interference fringes was taken using refractometric optics. The fringe patterns were measured using a Nikon microcomparator and the results expressed as *ln* (fringe displacement) *versus* r^2 where r is the distance from the axis of rotation. The weight average molecular weight was calculated from the expression:

$$Mw = \frac{2RT}{(1-\bar{\upsilon}\rho)}\omega^2 \frac{d\ln y}{d(r^2)}$$

where y is the fringe displacement, R is the gas constant, T is the absolute temperature, υ is the partial specific volume of the protein, ρ is the density of the solution, and ω is the angular velocity of rotation

The partial specific volume was assumed to be 0.73 ml/g (for a normal soluble protein), and the density was 1.006 g/ml.

7.1.7 *Protein Sequencing*

The N-terminal amino acid sequence of overexpressed ε, ε_{DP}, and ε_{CH} was determined either by Dr Denis Shaw of the Protein Chemistry Group (John Curtin School of Medical Research, Australian National University), or the Biomolecular Resource Facility (Australian National University) using an Applied Biosystems 477A sequencer, operated under standard conditions.

7.1.8 *Staining of Proteins with Silver*

A more sensitive detection of proteins following SDS-PAGE can be made by staining with silver.

Proteins were detected following SDS-PAGE by staining with silver using the Rapid-Ag Stain kit (ICN).

7.2 RESULTS

7.2.1 *Limited Proteolysis of the ε Subunit with Trypsin, Chymotrypsin and Endo Glu-C.*

The ranges of fragments generated by treatment of the ε subunit (under native conditions) with a range of protease concentrations for a fixed time interval was assessed by subjecting the reaction products to SDS-PAGE (Figs. 7.1 and 7.2). From these results, the desired ratio of substrate to protease was determined. For trypsin it was 900:1, and for chymotrysin it was 225:1. Protease endo Glu-C was not deemed suitable for further analysis due to its limited activity on the ε subunit (see Fig. 7.5). The extent of proteolysis over a period of time was then assessed at the optimal concentration of protease (Figs. 7.3 to 7.4). The activity of endo Glu-C, chymotrypsin and trypsin is summarised in Figure 7.5. Digestion with trypsin gave rise to a number of fragments. The predominant species appeared to be only 1 - 2 kDa smaller than native ε. One tryptic fragment ran at a similar position to ε_{DP}. Digestion with chymotrypsin gave rise to a single band (ε_{CH}) of approximately 24 kDa as judged

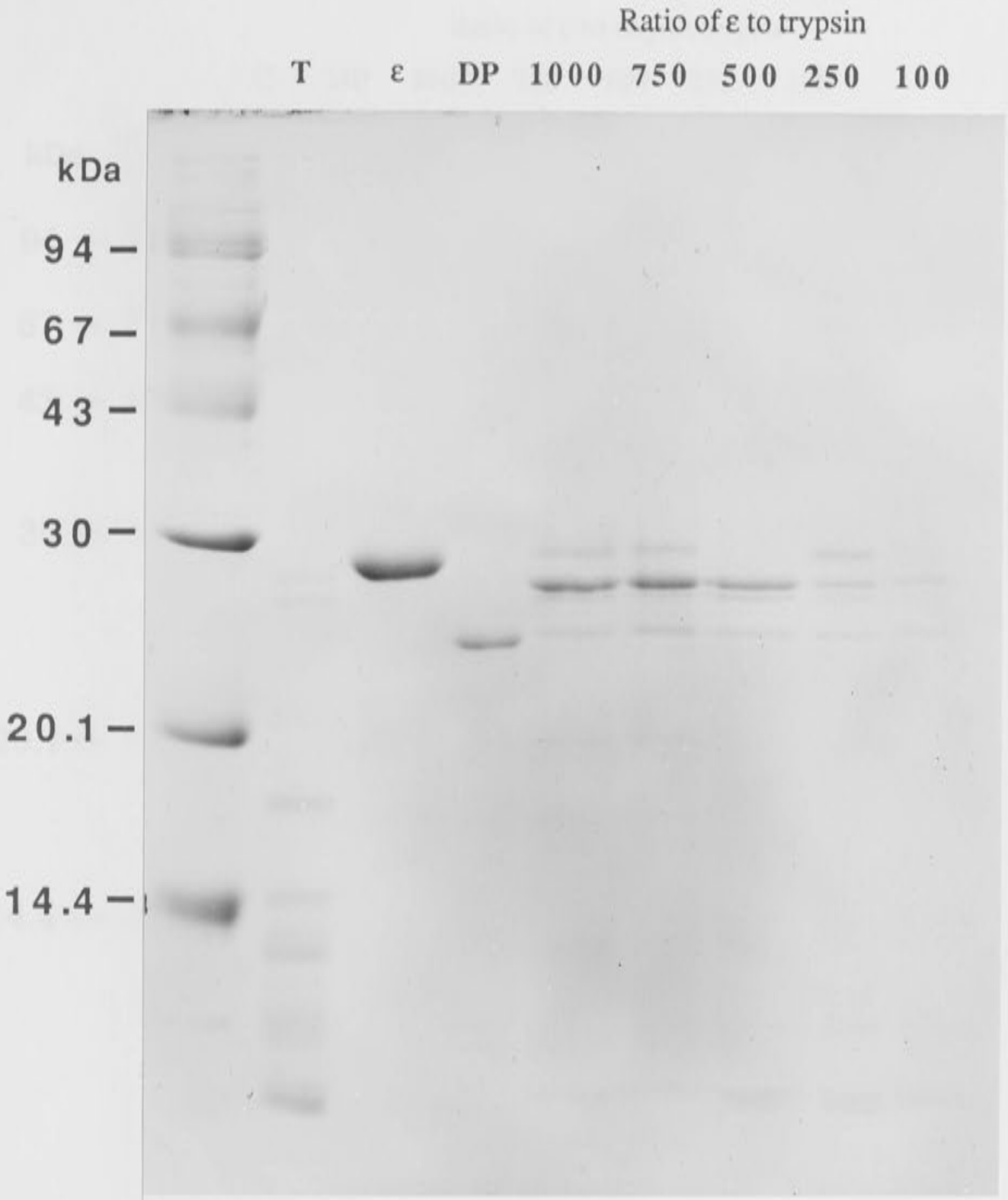

Figure 7.1: Partial proteolysis of the ε subunit with trypsin. ε subunit (7.5 μg) was treated with a range of concentrations of the endoprotease trypsin as described (Section 7.1.2.3). The products of treatment with trypsin were subjected to SDS-PAGE (15% gel) and proteins were visualised by staining with Coomassie Brilliant Blue.

T = trypsin (5 μg)

ε = ε (7.5 μg)

DP = ε_{DP} (5 μg)

1000 = mass ratio of ε:trypsin of 1000:1, and so on.

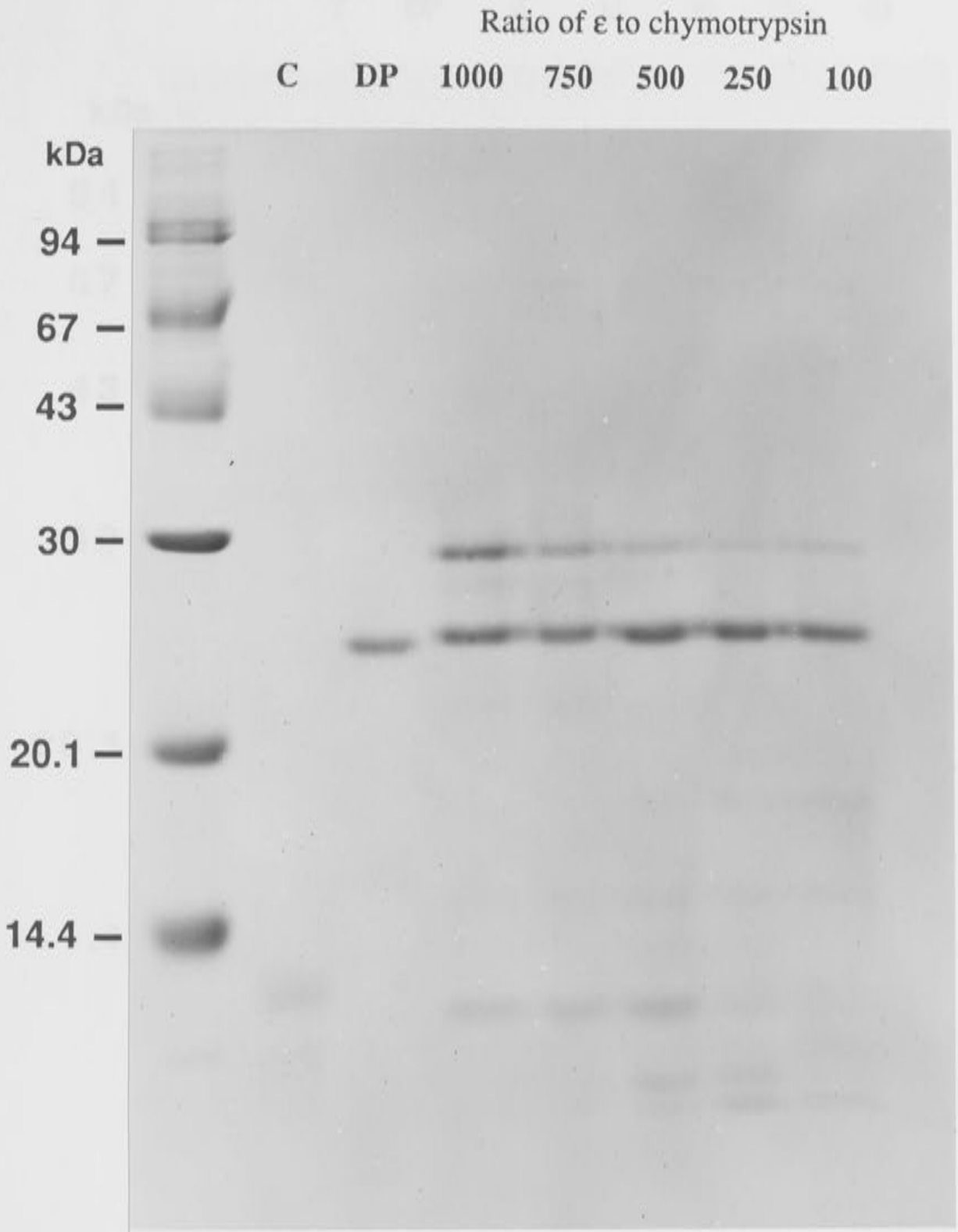

Figure 7.2: Partial proteolysis of the ε subunit with chymotrypsin. ε subunit (7.5 μg) was treated with a range of concentrations of the endoprotease chymotrypsin as described (Section 7.1.2.3). The products of treatment with chymotrypsin were subjected to SDS-PAGE (15% gel) and proteins were visualised by staining with Coomassie Brilliant Blue.

C = chymotrypsin (5 μg)

DP = ε_{DP} (5 μg)

1000 = mass ratio of ε:chymotrypsin of 1000:1, and so on.

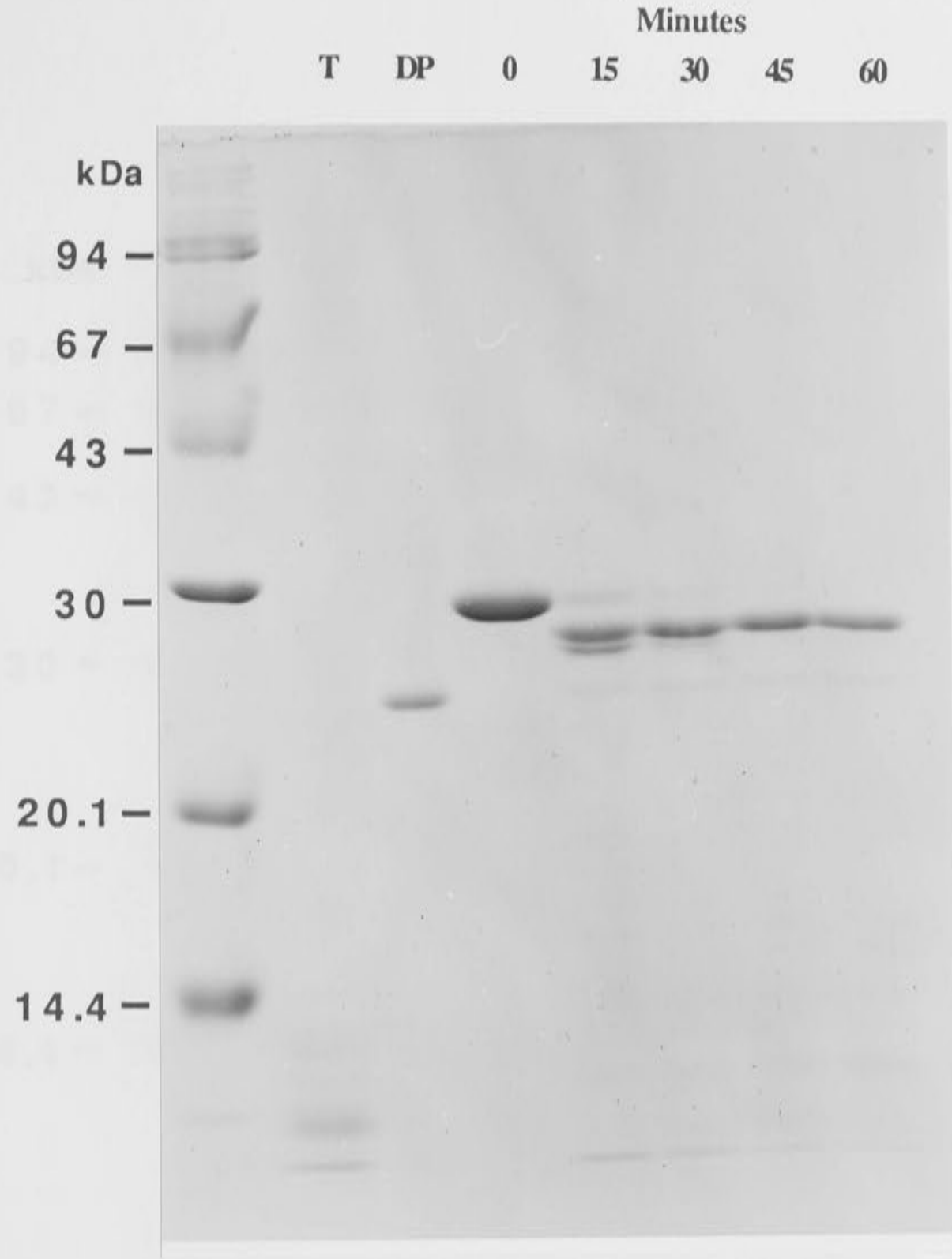

Figure 7.3: Partial proteolysis of the ε subunit with trypsin. ε subunit (7.5 μg) was treated with 50 ng of the endoprotease trypsin at 37° as described for various periods of time (Section 7.1.2.4). The products of treatment with trypsin were subjected to SDS-PAGE (15% gel); proteins were visualised by staining with Coomassie Brilliant Blue.

T = trypsin (5 μg)

DP = ε_{DP} (5 μg)

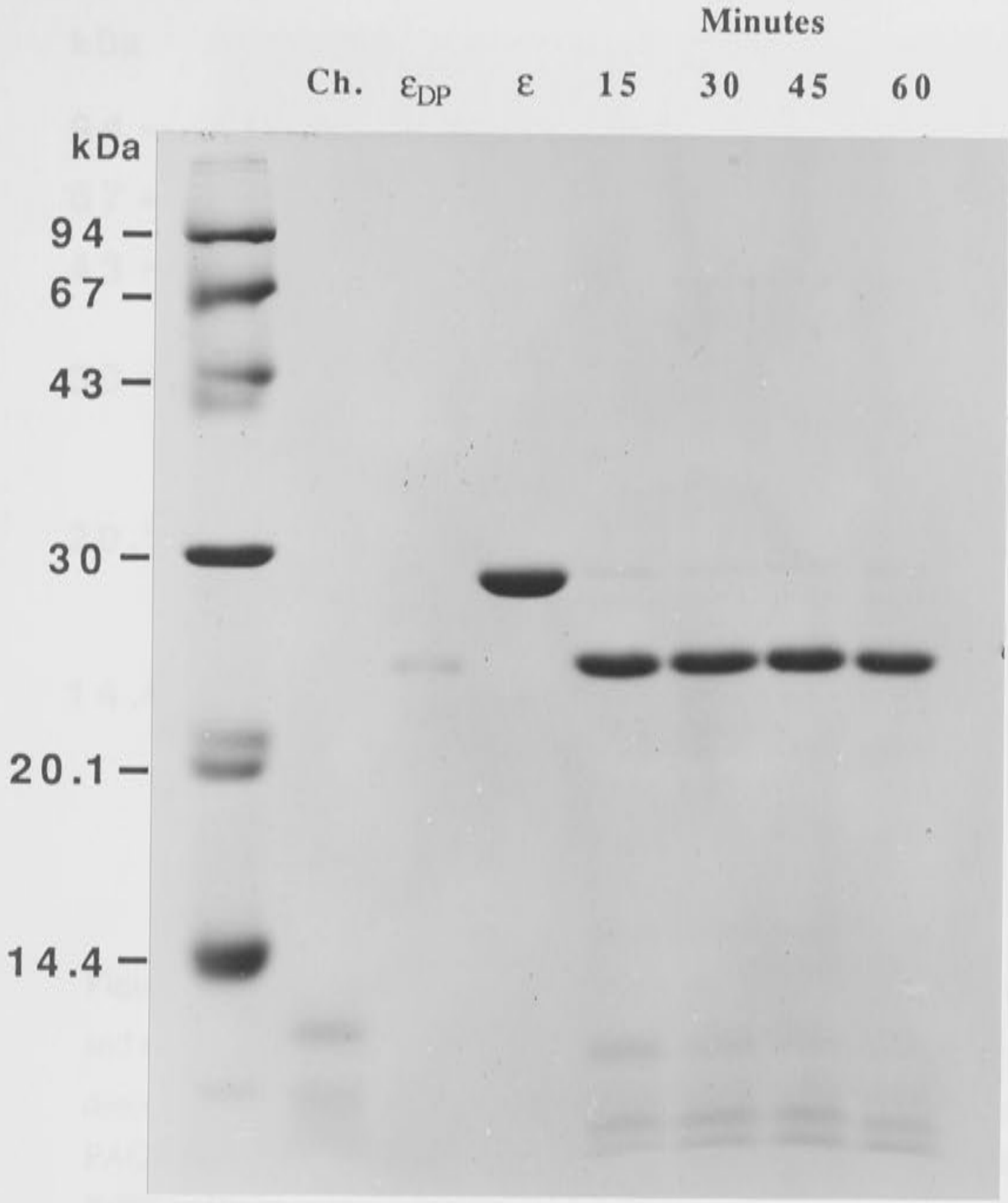

Figure 7.4: Time course of digestion of the ε subunit with chymotrypsin at 37° C. The experiment was performed as described in the text (Section 7.1.2.4). A portion of the digest (corresponding to ≈7.5 μg of protein) at each time point was subjected to SDS-PAGE (15% gel); proteins were visualised by staining with Coomassie Brilliant Blue.

Ch. = chymotrypsin (5 μg)

ε_{DP} = a degradation product of the ε subunit (≈3 μg).

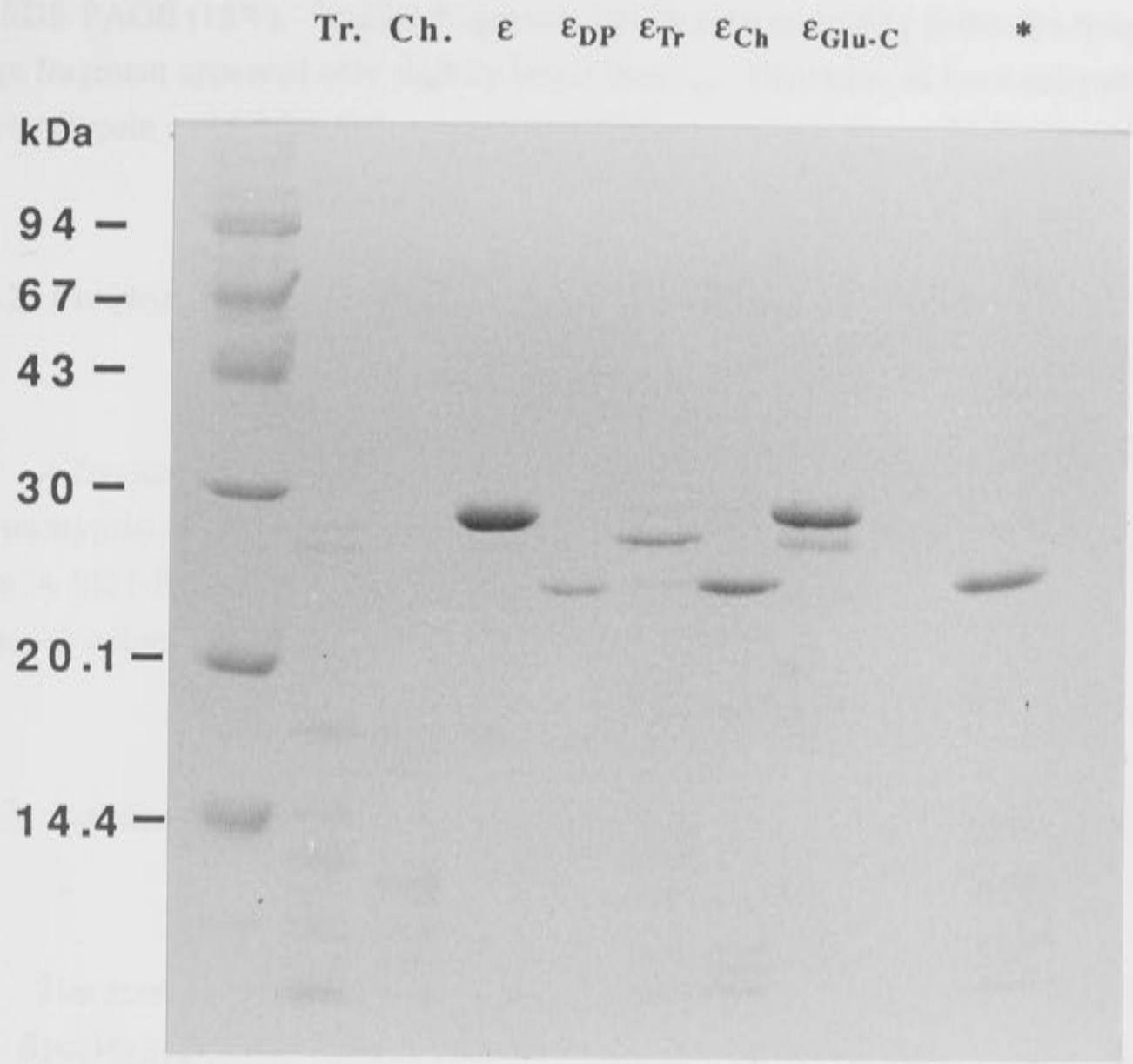

Figure 7.5: Summary of the action of the endo-proteases trypsin, chymotrypsin and endo Glu-C on the ε subunit. Digestion with each protease was performed as described (Section 7.1.2.3). A portion of each digest (7.5 μg) was subjected to SDS-PAGE (15% acrylamide gel); proteins were visualised by staining with Coomassie Brilliant Blue.

Tr. = trypsin as supplied by the manufacturer (5 μg)
Ch. = chymotrypsin as supplied by the manufacturer (5 μg)
ε = ε subunit (5 μg)
ε_{DP} = the adventitious degradation product (≈5 μg)
ε_{Tr} = tryptic digest of ε, 30 min at 37° C
ε_{Ch} = chymotryptic digest of ε, 30 min at 37° C
ε_{Glu-C} = endo Glu-C digest of ε, 30 min at 37° C
* = chymotryptic digest of the ε subunit treated at 4° C for 48 h

by SDS-PAGE (15%). Smaller fragments can be seen migrating at the dye front. The large fragment appeared only slightly larger than ε_{DP}. Digestion of the ε subunit with chymotrypsin at 4 °C for 48 h was equally efficient as digestion at 37 °C for an hour.

7.2.2 *Purification of a Chymotryptic Fragment of the ε Subunit*

A fragment of the ε subunit (ε_{CH}) prepared by limited digestion with chymotrypsin (protease:ε was 1:600, by weight) at 4 °C for 48 h was judged to be pure by SDS-PAGE (Fig. 7.6) after ion-exchange chromatography on a FPLC Mono-Q column.

7.2.3 *Isolation of a Degradation Product of the ε Subunit*

The generation of a degradation product of the ε subunit (ε_{DP}) is shown in Figure 7.7. Species apparently smaller than the ε subunit were first apparent following electrophoresis of a concentrated DEAE Sephacel flow-through fraction. This procedure (Section 7.1.3) resulted in the purification of a single fragment of approximately 24 kDa (as judged by SDS-PAGE on a 13% polyacrylamide gel).

7.2.4 *A Comparison of the Proofreading Activity of Overexpressed ε , ε_{CH} and ε_{DP}*

The proofreading activity of equimolar concentrations of overexpressed ε, ε_{DP} and purified ε_{CH} is shown in Figure 7.8[1]. The degradation product was approximately 1.5 times more active than overexpressed ε subunit, and nearly twice as active as ε_{CH}. However, these differences are not likely to be significant. The activity of ε_{CH} was essentially identical to that of overexpressed ε in the colourimetric assay under standard conditions (Table 7.1).

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[1]Insufficient ε_{DP} remained by this time for inclusion in this comparison.

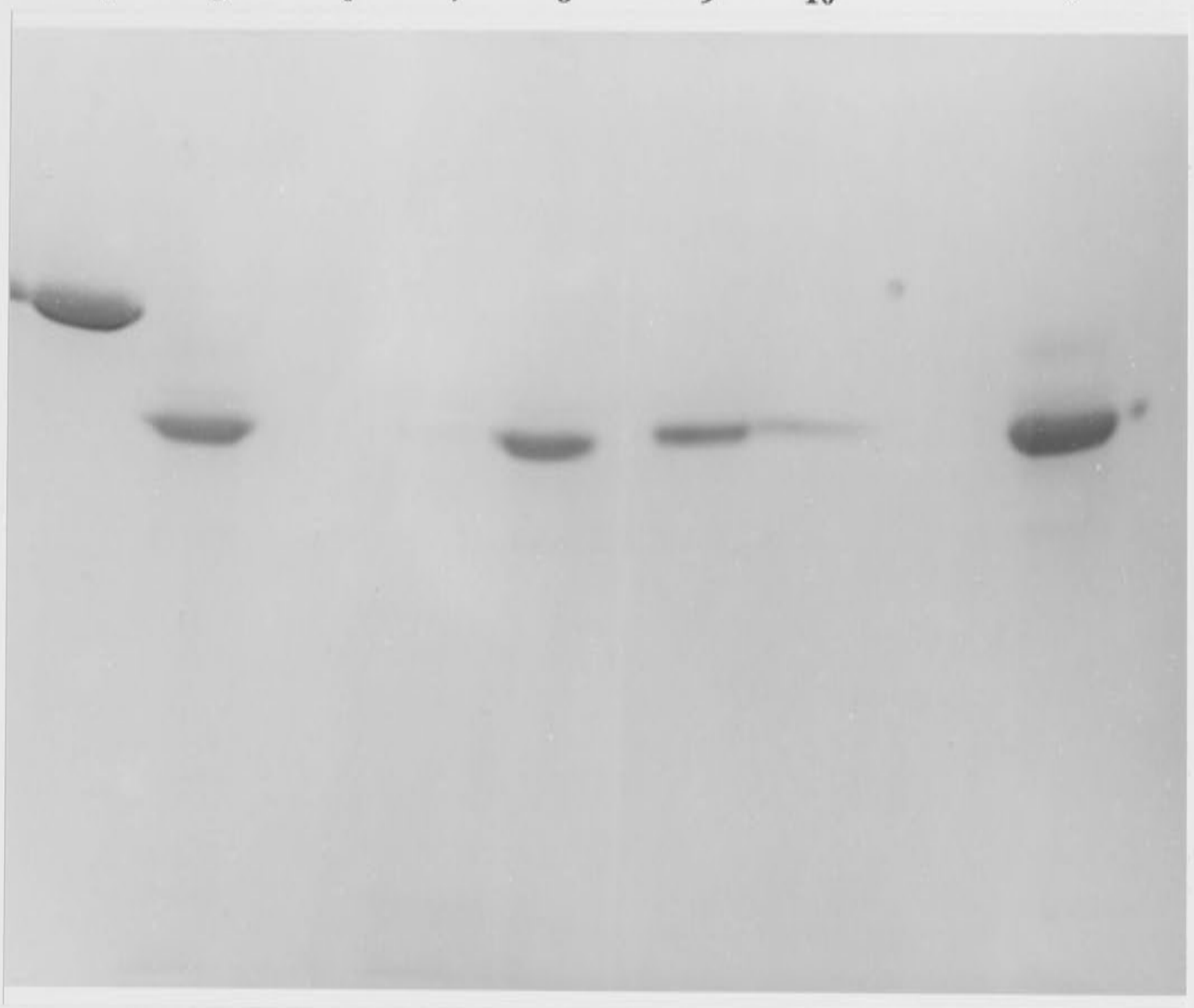


Figure 7.6: Purification of ε_{CH} from an FPLC Mono-Q column.

Chromatography was performed as described (Section 7.1.2.6). A portion of each fraction (30 µl) corresponding to a single peak of protein was subjected to SDS-PAGE (15% gel); proteins were visualised by staining with Coomassie Brilliant Blue.

ε = ε (7.8 µg)

D = chymotryptic digest (≈ 8 µg)

* = A portion (≈ 7.5 µg) of a chymotryptic digest that was not applied to the Mono-Q column, but was instead concentrated by ultrafiltration in a Centricon C3 microfiltrator (Amicon)

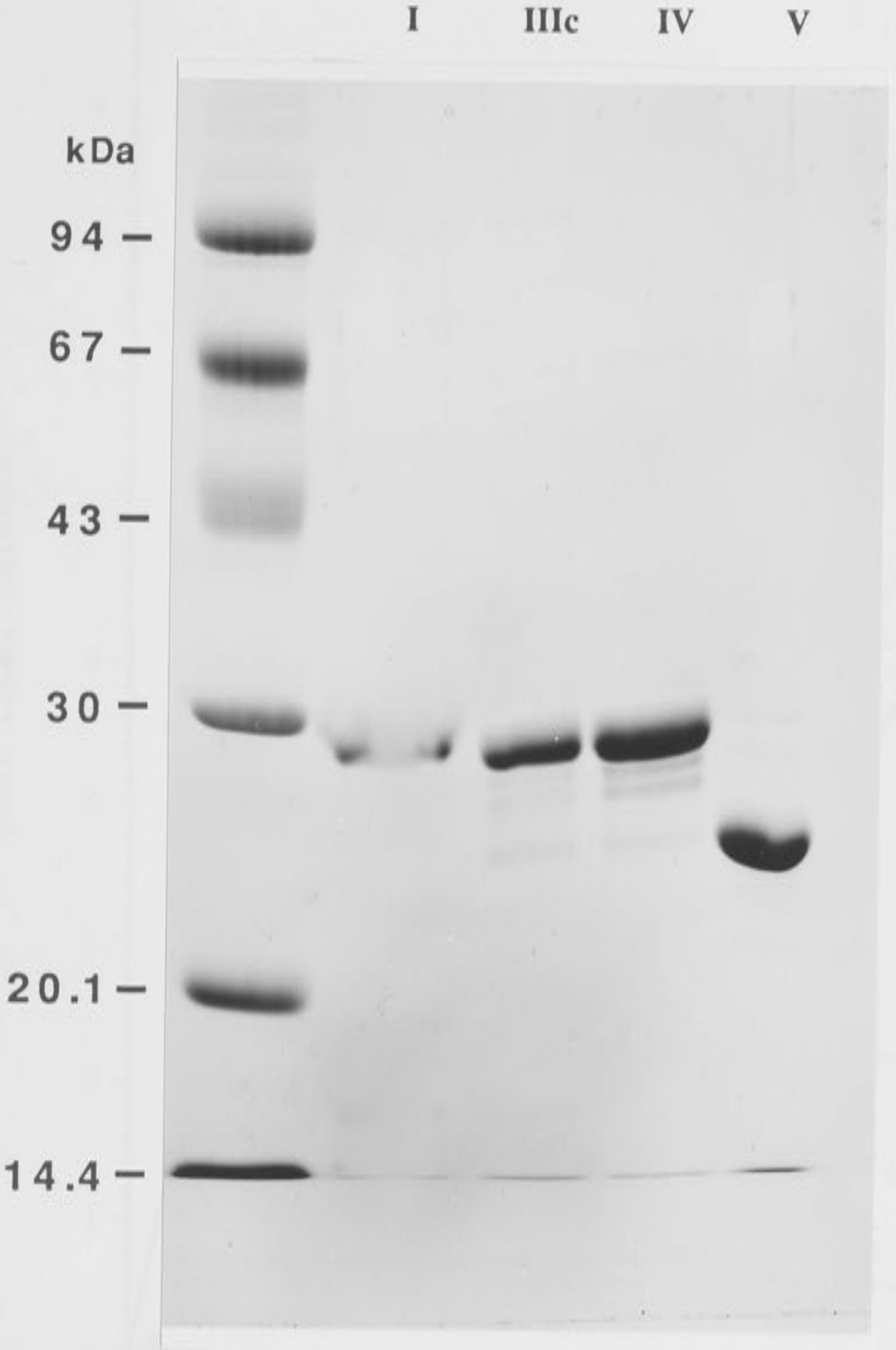


Figure 7.7: Generation of ε_{DP} (Section 7.1.3). ε subunit (≈5 μg) was subjected to SDS-PAGE (13% gel); proteins were visualised by staining with Coomassie Brilliant Blue. Degradation of the ε subunit is first apparent following concentration of a DEAE-Sephacel flow-through (III). Note the low molecular weight material at the dye front.

I = Resolubilised ε
IIIc = concentrated DEAE-Sephacel flow-through
IV = Sephadex G-100
V = ε extensively dialysed with EDTA and concentrated.

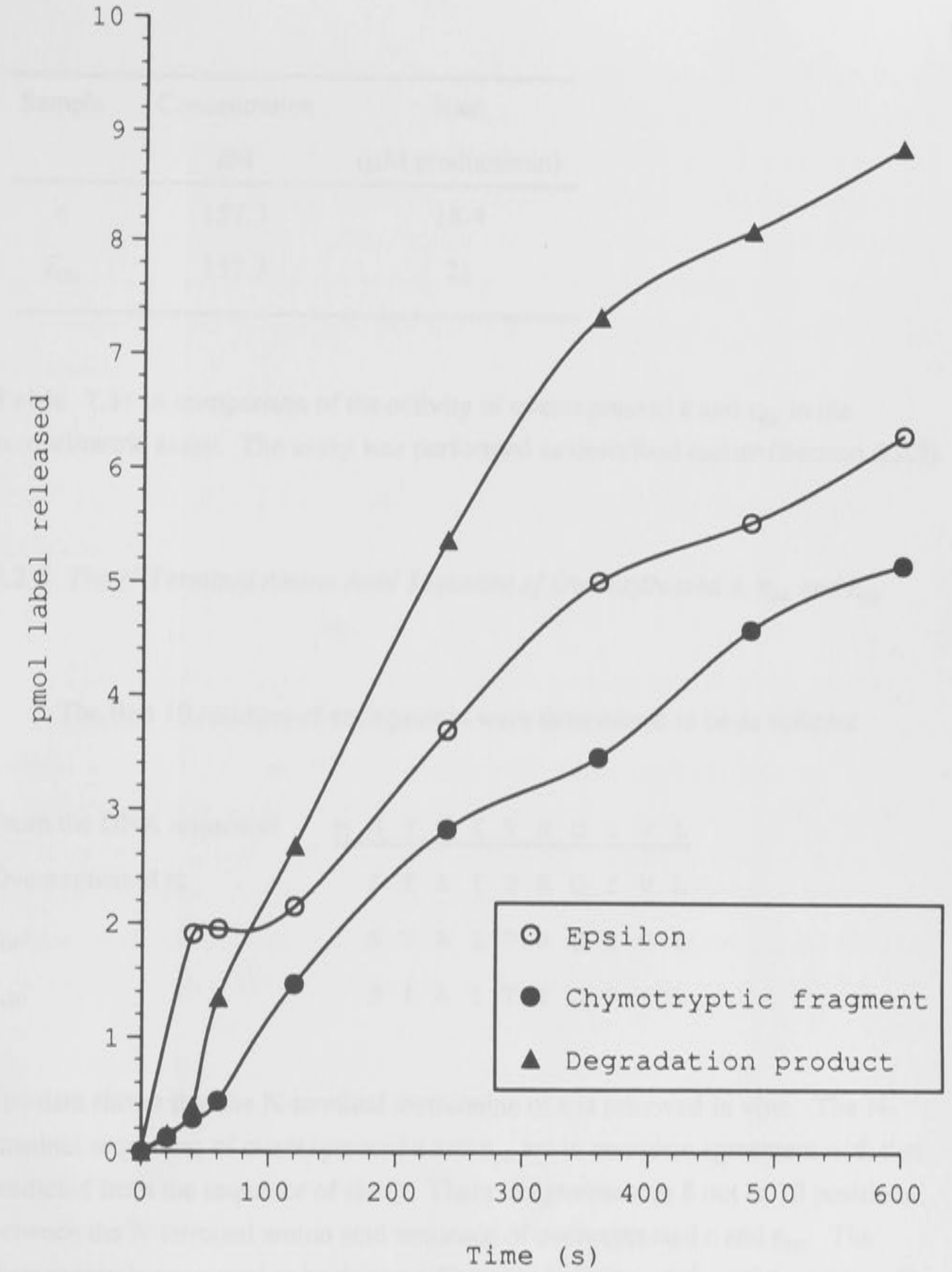


Figure 7.8: A comparison of the proofreading activity of overexpressed ε, ε_{CH} and ε_{DP}. The concentration of substrate (copolymer) was 124 nM (as (dC)); the input of label was 13.99 pmol. The concentration of each enzyme was 100 nM. Assays were performed as described (Section 5.1.5.1.3) with 25 µl aliquots taken at 20, 40, 60, 120, 240, 360, 480 and 600 seconds.

Sample	Concentration nM	Rate (μM product/min)
ε	157.3	18.4
ε_{CH}	157.3	21

Table 7.1: A comparison of the activity of overexpressed ε and ε_{CH} in the colourimetric assay. The assay was performed as described earlier (Section 6.2.3).

7.2.5 *The N-Terminal Amino Acid Sequence of Overexpressed ε, ε_{DP} and ε_{CH}*

The first 10 residues of each protein were determined to be as follows:

From the DNA sequence: M S T A I T R Q I V L

Overexpressed ε: S T A I T R Q I V L

ε_{DP}: S T A I T R Q I V L

ε_{CH}: S I A I T T Q I V L

The data shows that the N-terminal methionine of ε is removed *in vivo*. The N-terminal sequences of overexpressed ε and ε_{DP} are in complete agreement with that predicted from the sequence of *dnaQ*. There is agreement at 8 out of 10 positions between the N-terminal amino acid sequence of overexpressed ε and ε_{CH}. The discrepancy is presumed to be due to differences in interpretation of the sequencing data. Since there is no similar sequence elsewhere within ε (Takano *et al.*, 1986; see Appendix 1), it is likely that all three proteins share the same N-terminus. That is, both ε_{DP} and ε_{CH} are N-terminal fragments.

7.2.6 *Determination of Protein Sizes by Gel Filtration*

The molecular size of overexpressed ε and ε_{CH} was determined in separate experiments by comparing the respective elution volumes of those proteins from a 24

ml FPLC Superose-6 column with the corresponding elution volumes from the same column of proteins of known size (Table 7.2). A plot of elution volume (V_e) versus log molecular weight of the size standards was prepared (Fig. 7.9). The measured elution volumes of ε and ε_{CH} were used to determine the corresponding molecular weights of these proteins. The molecular weights of ε and ε_{CH} were 34,400 and 20,800, respectively. Agreement with predicted molecular weights and values determined by SDS-PAGE indicates that overexpressed ε and ε_{CH} occur in solution as monomers.

7.2.7 *Determination of Protein Molecular Weights by Sedimentation Equilibrium Centrifugation*

The weight-average molecular weights of overexpressed ε and ε_{DP} were determined by sedimentation equilibrium centrifugation. A plot of apparent molecular weight *versus* fringe concentration was linear for both samples (data not shown); the linearity of the plot showed the proteins were homogeneous. The molecular weight of overexpressed ε subunit and ε_{DP} determined in this manner were 30,000 and 20,600, respectively. These figures are within acceptable error limits (± 10%) of the predicted sizes of monomeric species. A summary of the data derived from the gel filtration, analytical centrifugation, and SDS-PAGE experiments is shown in Table 7.3.

Sample	Molecular weight	V_e (ml)
Ribonuclease A	13,700	18.38
Chymotrypsinogen A	25,000	17.5
Ovalbumin	43,000	16.7
BSA	67,000	16.0
ε	26,949	17.0
ε_{CH}	not known	17.8

Table 7.2: The elution volumes of proteins chromatographed on a 24 ml FPLC Superose-6 column. The molecular weight quoted for overexpressed ε was calculated from its amino acid sequence. The N-terminal methionine was included in this determination.

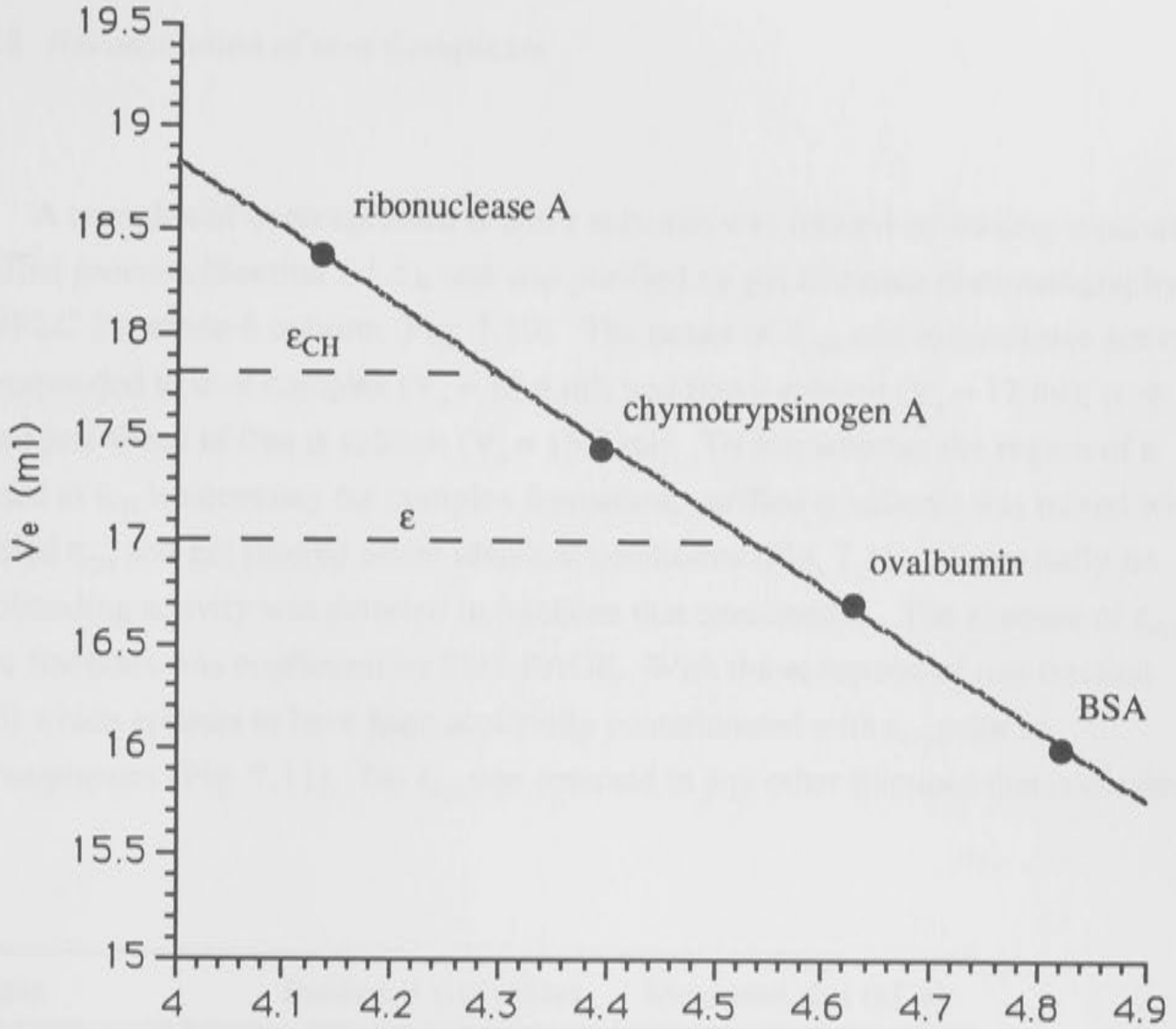


Figure 7.9: Chromatography of proteins on Superose-6. Proteins of known size, ε and ε_{CH} were chromatographed on Superose-6 as described (Section 7.1.4). The molecular weight of ε and ε_{CH} were determined from a least squares regression analysis of the data in this plot.

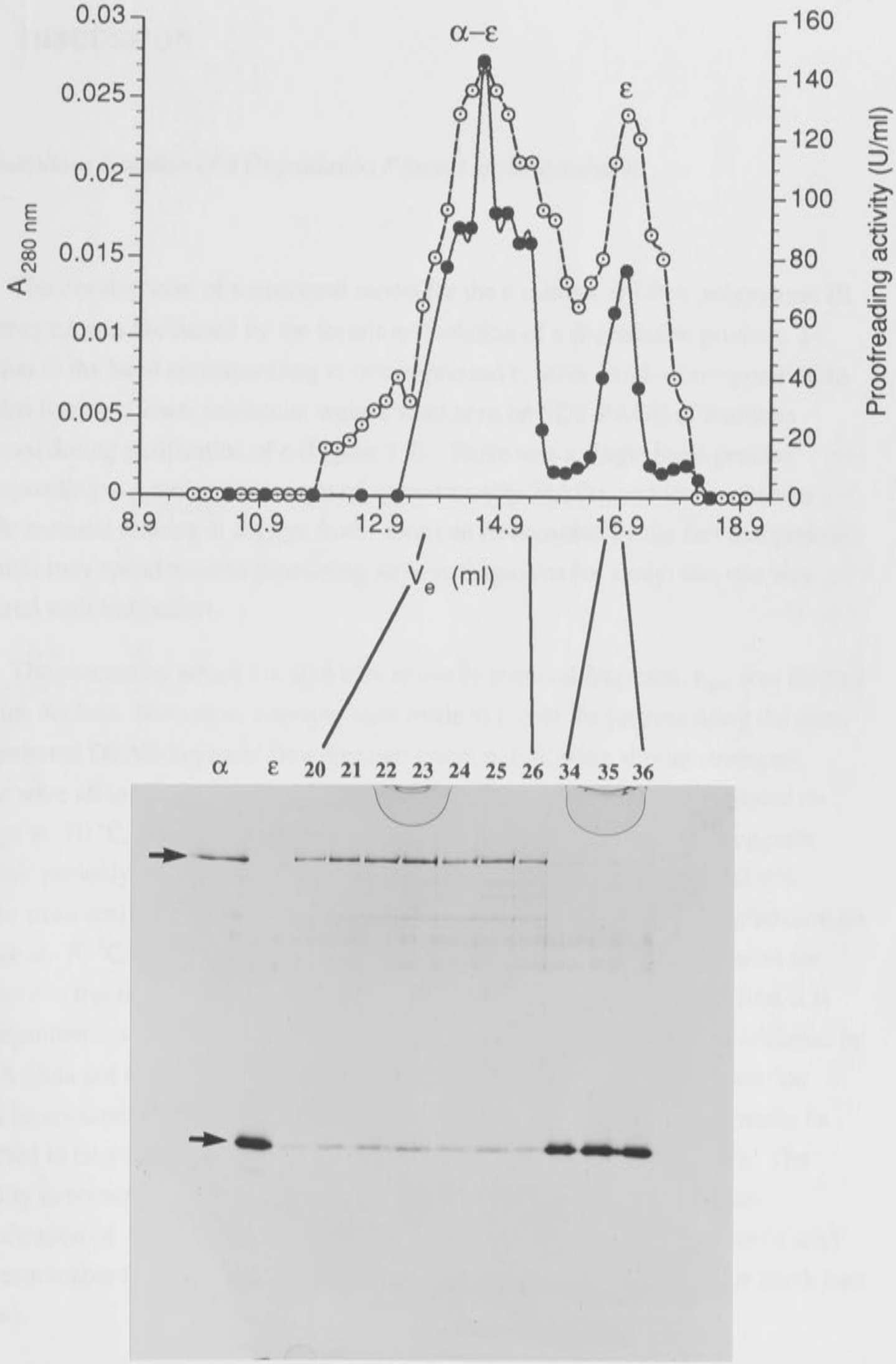
7.2.8 *Reconstitution of α–ε Complexes*

A complex of overexpressed α and ε subunits was formed by mixing separately purified proteins (Section 7.1.5), and was purified by gel filtration chromatography on an FPLC Superose-6 column (Fig. 7.10). The peaks of A_{280} and exonuclease activity corresponded to α–ε complex (V_e = 14.6 ml) and free ε subunit (V_e = 17 ml); α–ε eluted just ahead of free α subunit (V_e = 15.2 ml). To test whether the region of ε deleted in ε_{CH} is necessary for complex formation, purified α subunit was mixed with purified ε_{CH} and gel filtered under identical conditions (Fig. 7.11). Essentially no proofreading activity was detected in fractions that contained α. The absence of ε_{CH} in these fractions was confirmed by SDS-PAGE. With the exception of one fraction (#26) which appears to have been accidently contaminated with ε_{CH} prior to electrophoesis (Fig. 7.11). No ε_{CH} was detected in any other fractions that contained α.

Protein	Predicted size (kDa)	Measured size (kDa)
Overexpressed ε	26.95	34.4[a]
		30[b]
		28[c]
ε_{CH}	20.7	20.8[a]
		24[c]
ε_{DP}	unknown	20.6[b]
		23.5[c]

Table 7.3: Summary of the molecular weight determinations for ε, ε_{CH} and ε_{DP}. [a], from gel filtration, [b], from sedimentation equilibrium centrifugation, [c], from SDS-PAGE. The predicted size is based on the nucleotide sequence. The C-terminal residue of ε_{CH} is believed to be Phe^{187} (see below).

Figure 7.10: Elution profile of α–ε complexes on Superose-6 chromatography. Formation of complexes of subunits with ε in excess and chromatography were as described in the text (Sections 7.1.5). Selected fractions were assayed for proofreading activity (Section 5.1.20.2) and the A_{280}'s were determined from the recorder trace. ● = activity; ⊙ = A_{280}. A portion of selected fractions (20 µl) was subjected to SDS-PAGE (12.5% gel) and the proteins were visualised by staining with silver (Section 7.1.8). The α and ε subunits are indicated by arrows. The proteins used as α and ε standards were purified from overproducing strains as described (Section 7.1.1 and 4.1.4).


α–ε
ε
0.03
0.025
0.02
0.015
0.01
0.005
0
160
140
120
100
80
60
40
20
0
8.9
10.9
12.9
14.9
16.9
18.9
$A_{280\ nm}$
Proofreading activity (U/ml)
V_e (ml)
α ε 20 21 22 23 24 25 26 34 35 36

7.3 DISCUSSION

Serendipitous Isolation of a Degradation Product of the ε Subunit

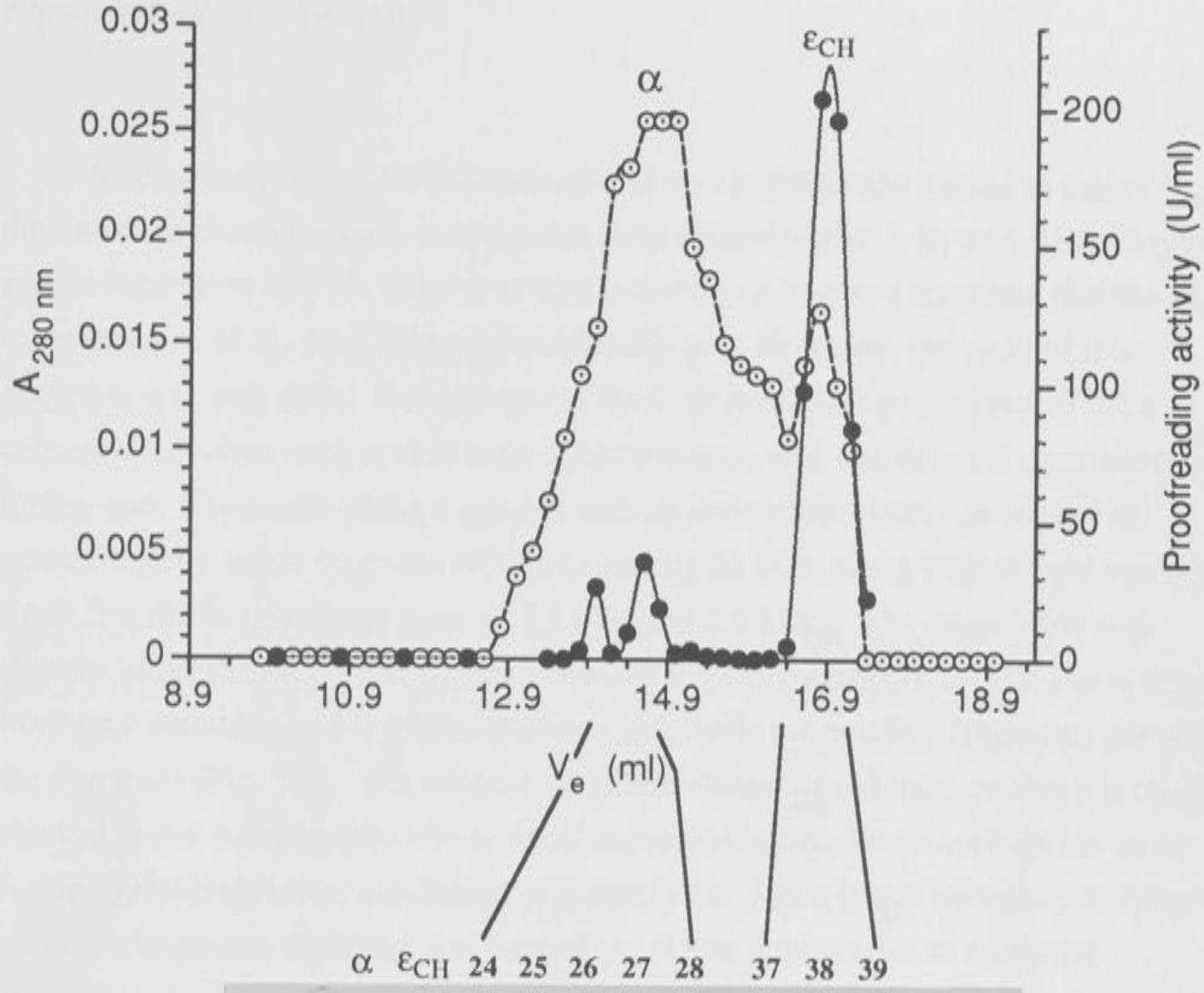
The development of a structural model for the ε subunit of DNA polymerase III holoenzyme was facilitated by the fortuitous isolation of a degradation product. In addition to the band corresponding to overexpressed ε, other bands corresponding to proteins having a lower molecular weight were seen on SDS-PAGE of fractions collected during purification of ε (Figure 7.7). There was a single band present corresponding to a molecular weight of approximately 24 kDa, and low molecular weight material running at the dye front. One can be consoled by the fact that protein chemists may spend months generating suitable fragments for study: this one was prepared with little effort.

The process by which the 20.6 kDa active N-terminal fragment, ε_{DP}, was formed remains unclear. Numerous attempts were made to repeat the process using the same concentrated DEAE-Sephacel flow-through fraction, following similar strategies. These were all unsuccessful, which might indicate that degradation was arrested on storage at -70 °C. The presence of intermediate forms between ε and ε_{DP} suggests enzymic proteolysis as it is reminiscent of the pattern of fragments observed with trypsin treatment of overexpressed ε (Fig. 7.1). The absence of further degradation on storage at -70 °C may also support a proteolytic mechanism, as many proteases are sensitive to freeze-thawing (Carrey, 1989). If a protease is indeed responsible, it is not dependent on metal ion (Zn, Mg, Ca) for activity and, as expected, not inhibited by EDTA (data not shown). However, it is difficult to envisage how the preparation could be contaminated with a protease, and *E. coli* proteases would not normally be expected to target host proteins (although protein turnover occurs in *E. coli*). The inability to repeat the process would seem to rule out an autolytic mechanism. Identification of the C-terminal residue of ε_{DP} would indicate which protease (if any) was responsible for the degradation of ε during purification of this particular batch (see below).

Although the mechanism of formation of ε_{DP} is presently not known, it did possess $3' \rightarrow 5'$ exonuclease activity and hence had most likely retained the configuration found in the exonuclease active site of native ε .

Figure 7.11: Elution profile of α–ε_{CH} complexes on Superose-6 chromatography. Formation of complexes of subunits with ε_{CH} in excess and chromatography were as described in the text (Sections 7.1.5). Selected fractions were assayed for proofreading activity (Section 5.1.20.2) and the A_{280}'s were determined from the recorder trace. ● = activity; ⊙ = A_{280}. A portion of selected fractions (20 µl) was subjected to SDS-PAGE (12.5% gel) and the proteins were visualised by staining with silver (Section 7.1.8). The α and ε_{CH} subunits are indicated by arrows. The protein used as the α standard was purified from an overproducing strain as described (Section 7.1.1). The standard for ε_{CH} was prepared as described (Section 7.2.2), and was not purified following treatment with chymotrypsin.

A profile of the proteins eluting from the Superose-6 column shows that ε_{CH} co-eluted with the α subunit in only 1 fraction (# 26). This is most puzzling. The ε_{CH} cannot be detected in any of the side fractions. The intensity of the band is ≈ 1/3 of ε_{CH} that eluted free of the α subunit (fractions 37 - 39), yet the level of proofreading activity in fraction 26 is approximately 5% of that detected in fraction 38, the peak of free ε_{CH}. One would expect the proofreading activity of an α-ε_{CH} complex to be similar to that observed with α–ε, certainly at the level of the ε_{CH} eluting in fractions 37 - 39. Fraction 27 recorded the highest level of proofreading activity, yet no ε_{CH} can be observed in this sample. These facts are consistent with fraction 26 having become in some way contaminated with ε_{CH} after assaying for proofreading activity.


0.03
0.025
0.02
0.015
0.01
0.005
0
A 280 nm
α
εCH
200
150
100
50
0
Proofreading activity (U/ml)
8.9
10.9
12.9
14.9
16.9
18.9
Ve (ml)
α εCH 24 25 26 27 28 37 38 39

Proteolysis of the ε Subunit

In an attempt to generate a derivative of ε with properties similar to that of ε_{DP}, the ε subunit was separately subjected to mild proteolysis with trypsin, chymotrypsin and endoprotease Glu-C. Digestion with trypsin gave rise to a fragment that ran at the same position as ε_{DP} on a SDS polyacrylamide gel. However, the yield of this fragment was very poor. Endoprotease Glu-C showed little proteolysis of the ε subunit even when used at very high concentrations, and was deemed unsuitable for further use. Treatment of the ε subunit with chymotrypsin clearly generated an extraordinarily stable fragment of approximately 24 kDa, along with at least two other small fragments (predicted sizes of 3.3 kDa and 2.6 kDa). The major band was slightly larger than ε_{DP}. The larger of the two smaller fragments ran just above the dye front on a denaturing 15% polyacrylamide gel, while the smallest fragments ran with the dye front (Fig. 7.5). The absence of intermediates would indicate that ε is rapidly cleaved at two or three sites within the C-terminal region. There appeared to be no further hydrolysis under conditions of proteolysis. Since it was necessary to recover an active fragment, digestion was carried out at low temperature to minimise denaturation. Digestion with chymotrypsin at 4 °C for 48 h was shown to be equally as effective as treatment at 37 °C for an hour. Inspection of the deduced amino acid sequence of ε showed that there were cleavage sites within the C-terminal region that would give rise to a fragment of similar size to ε_{DP} (Fig. 7.13). On the basis of this data, chymotrypsin was judged to be the protease of choice for the generation of a derivative of ε with properties likely to be similar to those of ε_{DP}.

A large-scale digest of the ε subunit with chymotrypsin was carried out at 4 °C for 48 h. The digest was applied to a 1 ml FPLC Mono-Q column from which the 24 kDa fragment was successfully eluted in essentially pure form.

Properties of Fragments of the ε Subunit

The chymotryptic fragment appeared to have a monomeric molecular weight of approximately 24,000 as observed from its migration on a denaturing 15% polyacrylamide gel. The fragment ran just above ε_{DP}, suggesting that ε_{CH} and the ε_{DP} have similar sizes. This was confirmed by a gel filtration experiment, where the chymotryptic fragment eluted in a volume corresponding to a molecular weight of 20,700, and a sedimentation equilibrium experiment which showed ε_{DP} to be

approximately 20.6 kDa. The amino acid sequencing data indicated that ε_{DP} and ε_{CH} share the same N-terminus. Together with a perusal of potential chymotrypsin cleavage sites within the amino acid sequence of the ε subunit (Fig. 7.13), this suggests that ε_{CH} is formed by the chymotryptic cleavage on the C-terminal side of Phe^{187}. Hence, this residue is believed to be the C-terminal amino acid. ε_{CH} will thus be shorter than ε by 56 amino acids and have a predicted molecular weight of 20,571.

Positive identification of the C-terminal amino acid could be made with an accurate molecular weight measurement. The errors associated with the methods of analytical centrifugation and gel filtration range from 10% to 15% (Price and Stevens, 1988). Electrospray mass spectrometry is a technique capable of analysing proteins up to 100,000 molecular mass with unprecedented mass accuracy (0.01%) and resolution (0.1%) (Green, 1991). This capability applies to single proteins, as well as to mixtures of polypeptides. Electrospray mass spectrometry was attempted with overexpressed ε, ε_{CH} and ε_{DP}. However, there has been no success to date due to problems relating to the stability of the proteins in the buffer required for analysis.

Both ε_{DP} and ε_{CH} are about as active as full-size ε judging from assays using radiolabelled oligonucleotide or chromophoric substrates. It is clear from the data plotted in Figure 7.8 that overexpressed ε, ε_{CH} and ε_{DP} do not possess potent proofreading activity. Only ε_{DP} came close (71%) to removing the labelled 6 bp 3′-OH mispair after 10 min at 30 °C. It is unclear why ε_{DP} appeared to be twice as active as ε_{CH} in the proofreading assay. Since overexpressed ε and the two ε fragments share the same N-terminal amino acids, the 3′→ 5′ exonuclease active site must be located in the N-terminal region of the molecule. As a corollary, the N-terminal region must be highly folded[2], while the aromatic residues in the C-terminal region recognised by chymotrypsin must be exposed to solution.

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[2]There are 5 potential chymotrypsin recognition sites in the N-terminal region of ε (ie., the first 80 amino acid's).

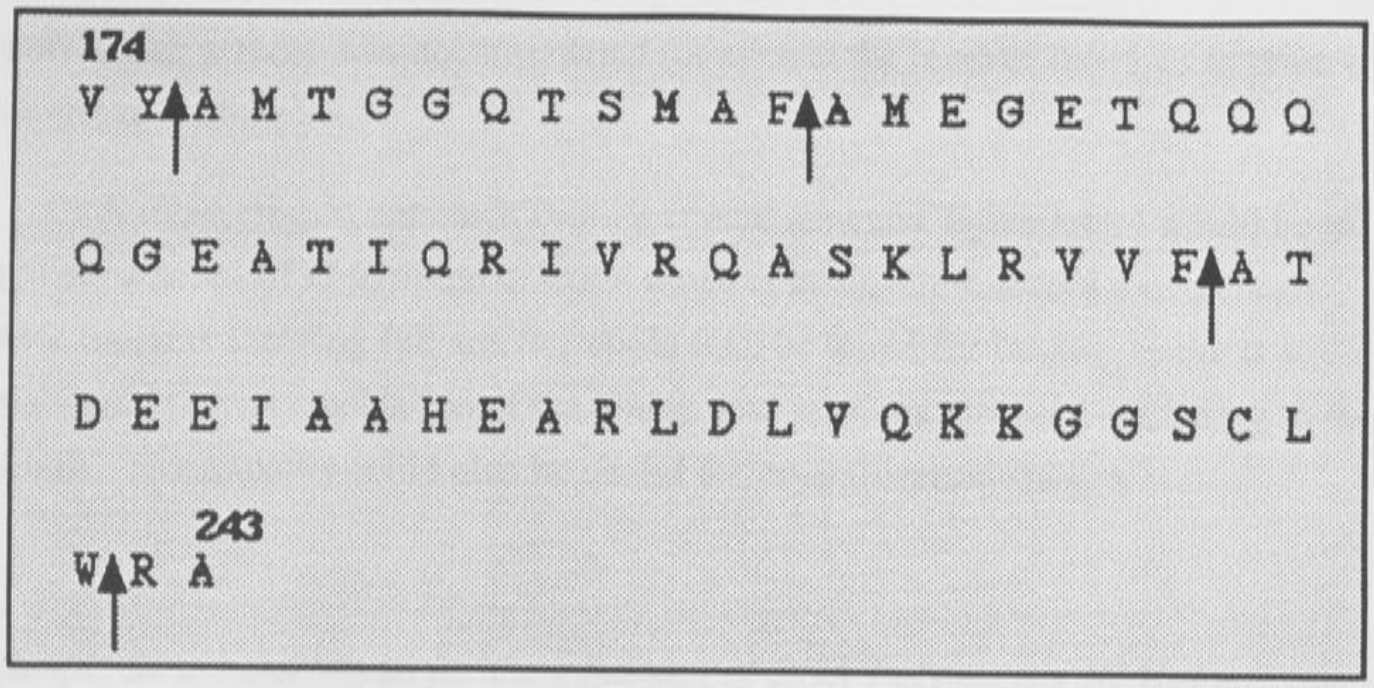

Figure 7.13: Predicted chymotrypsin cleavage sites within the C-terminal region of the ε subunit. The ε subunit is reduced in predicted size after each cleavage as follows: 26,722 (Trp^{241}), 23,985 (Phe^{210}), 20,702 (Phe^{187}) and 19,507 (Tyr^{175}) Daltons.

As the exonuclease active site is located in the N-terminal portion of the ε subunit, one might ask what contribution, if any, the C-terminal region makes to biological function. As the ε subunit is normally tightly associated with the polymerase (α) subunit in the core of DNA polymerase III holoenzyme (Maki and Kornberg, 1987), it is not unreasonable to suggest that the C-terminal region is responsible for this interaction. To test this hypothesis, purified α subunit was mixed with both purified ε subunit and ε_{CH}, which lacks the C-terminal domain following procedures devised by Maki and Kornberg (1987) and Studwell and O'Donnell (1990). A 4.5-fold molar excess of ε was used to ensure that most of the α subunit would be present in the complex which would facilitate its purification from uncomplexed subunits. The formation of complexes of α and ε subunits was tested by assaying for proofreading activity in fractions collected representing peaks of A_{280}. The α subunit formed a complex with overexpressed ε, but appeared not to do so with ε_{CH}. Thus, the C-terminal region of ε is likely necessary for binding to α. Figure 7.10 indicates that most of the α subunit recovered from the Superose-6 column was present as an α–ε complex. It is likely that the subunits are in stoichiometric amounts as reported by other groups (Maki and Kornberg, 1987; Studwell and O'Donnell, 1990). The enhanced proofreading activity of the α–ε complex (≈ 7-fold) (Fig. 7.10) is also consistent with published reports (Maki and Kornberg, 1987). ε_{CH} was detected in just one fraction co-eluting with the α subunit as observed after silver staining of proteins following SDS-PAGE (Fig. 7.11). As discussed in Section 7.2.8, the presence of ε_{CH} in a single fraction may be due to contamination. Certainly,

proofreading activity was not stimulated (or even at the level of free $\varepsilon_{\alpha H}$) as observed with the α–ε complex.

A more elegant approach (until a crystal structure is available) would be to engineer a series of C-terminal deletion mutants using site-directed mutagenesis. Those mutants showing full activity could then be tested for binding to the α subunit. This would permit elucidation of the amino acid residues involved in binding to the α subunit. The mutants could also be useful for crystallisation studies.

A Simple Structural Model for the ε Subunit of DNA Polymerase III Holoenzyme

As a result of studies described in this chapter, a structural model for the ε subunit can now be proposed (Fig. 7.14). The "tadpole" structure is based on:

1) The observation that the N-terminal domain is resistant to limited proteolysis (indicative of a compact structure), whereas the C-terminal domain is susceptible to proteolytic cleavage (residues loosely ordered).

2) Sequencing of the N-terminus of ε_{DP} and $\varepsilon_{\alpha H}$ which lack the proposed C-terminal domain; the fragments retain exonuclease activity indicating that the exonuclease active site is in the N-terminus, and

3) The polymerase subunit (α) of holoenzyme does not recognise $\varepsilon_{\alpha H}$.

The simple model is not intended to be a substitute for a three-dimensional structure of the type obtained from X-ray crystallographic studies. To this end, a quantity of pure ε subunit has been sent to Dr David Ollis (Northwestern University). Since $\varepsilon_{\alpha H}$ contains the exonuclease active site it is itself a prime candidate for crystallography. Unfortunately, the low solubility of overexpressed ε and its size precludes its analysis by 2- or 3-dimensional NMR.

Figure 7.14: A two domain model of ε structure: an N-terminal exonuclease active site domain, and a C-terminal domain that may associate with α, other holoenzyme subunits (eg. τ) or even enzymes involved in the SOS response.

Although the polymerase and proofreading activities of DNA polymerase III holoenzyme reside on separate polypeptides, one might suspect that the structure of an α–ε complex is similar to that determined for the large fragment of DNA polymerase I. Proof of such a hypothesis will not be easy as the structure determination of large proteins is difficult. However, the kinetic and binding studies carried out on the Klenow enzyme can be repeated for the α–ε complex. Such data should enable us to make some predictions of the structure and mechanism of the core polymerase of holoenzyme.

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CONCLUDING STATEMENT

A series of plasmid vectors was developed that allow the high-level expression of proteins in *E. coli*. These vectors all bear the strong bacteriophage λ promoters P_RP_L, arranged in tandem so that both promote transcription into genes inserted into or between unique restriction sites. Transcription is controlled by the product of the λ *c*I857 gene which is present in each vector. These expression vectors (pPT144 and pPT150) also contain a ribosome-binding site (RBS) perfectly complimentary to the 3′ end of *E. coli* 16-S rRNA, which enables substitution for a natural RBS in cases where it is translationally limiting. The plasmid pPT144 can also supply an ATG initiator codon for an incoming gene fragment.

One of the bacteriophage λ promoter vectors (pPT150) was used to obtain the high-level expression of the *dnaX* gene, which encodes the γ and τ subunits of DNA polymerase III holoenzyme. The γ and τ subunits comprise approximately 15% of total cell protein in strains harbouring the *dnaX* plasmid (pPT153) after 2.5 h at 42 °C. The γ subunit was purified to homogeneity from the overproducing strain in sufficient quantity for further study. The τ subunit requires further purification. Polyclonal antibodies were raised to purified γ subunit; this antibody recognise both the γ and τ subunits.

The proofreading subunit (ε) of holoenzyme was also purified to homogeneity from an overproducing strain. The ε subunit has a broad substrate specificity. In addition to its ability to remove incorrectly paired nucleotides at a 3′-OH end, ε will readily hydrolyse single-stranded DNA. Indeed, the activity of the ε subunit on ssDNA is >100-fold greater than on a mispaired terminus. RNA is also degraded, but less efficiently. The activity of the ε subunit on a chromophoric analogue of TMP (*p*np-TMP) is ≈ 500-times that of a mispaired terminus. These data suggests that in the absence of the polymerase subunit of holoenzyme, ε behaves as a single-stranded exonuclease.

As with other exonucleases, the ε subunit has an absolute requirement for divalent metal ion for activity. Mn^{2+} or Mg^{2+} gave rise to an enzyme with the highest activity. However, proofreading activity in the ε subunit was detected with a wide variety of divalent metal ions - consistent with their role as Lewis acids. The marked preference by ε for Mn^{2+} in the colourimetric assay is unclear. The activity of the ε subunit was inhibited to some degree by all 4 deoxynucleoside monophosphates, the products of the reaction it catalyses. TMP was a competitive inhibitor of activity.

ε can also hydrolyse a chromophoric analogue of thymidine 5′-monophophate, thymidine 5′- monophosphate *p*-nitrophenyl ester, to *p*-nitrophenol and thymidine 5′- monophosphate. The formation of *p*-nitrophenol was measured continuously in a spectrophotometer facilitating kinetic studies. Hydrolysis of substrate obeyed

Michaelis-Menten kinetics. Investigations of functional ternary complexes of enzyme, metal, and substrate by kinetic measurements and ESR spectroscopy suggested that the binding of enzyme, metal and substrate is random. Further, ESR data suggests that an additional metal ion is not detected in the kinetic experiments in the hydrolysis of substrate. In spite of certain technical problems relating to the solubility of ε, further studies should include determining the number of Mn^{2+}-binding sites using ESR spectroscopy. Indeed, a combination of the colourimetric assay and ESR will provide powerful tools for characterising active site mutants of the fidelity subunit.

Serendipitous isolation of a degradation product of ε suggested a two domain model of ε structure: an N-terminal exonuclease active site domain, and a C-terminal domain that may associate with the polymerase subunit of holoenzyme. This was subsequently confirmed by peptide mapping experiments. It will be necessary to engineer a series of C-terminal deletion mutants to ascertain which amino acid residues are participating in the association with the polymerase subunit. ε_{CH} and ε are now the target of X-ray crystallography.

APPENDIX

1. Nucleotide and Amino Acid Sequence of *dnaQ*

2. Translational Frameshifting Mechanism Responsible for the Generation of the τ Subunit

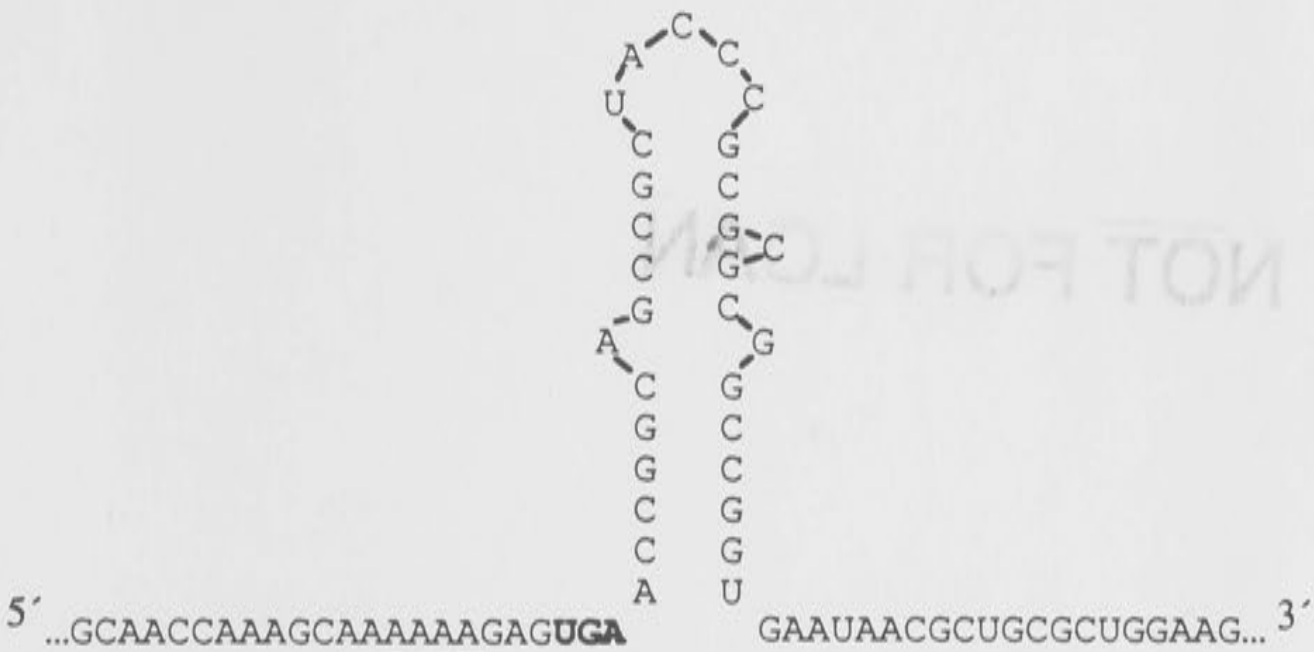
Nucleotide and Amino Acid Sequence for the Epsilon Subunit of DNA Polymerase III Holoenzyme

```
DNA  sequence    732 b.p.   ATGAGCACTGCA ... TGGCGAGCATAA   linear

1    /    1                                  31   /   11
ATG AGC ACT GCA ATT ACA CGC CAG ATC GTT CTC GAT ACC GAA ACC ACC GGT ATG AAC CAG
met ser thr ala ile thr arg gln ile val leu asp thr glu thr thr gly met asn gln
61   /   21                                 91   /   31
ATT GGT GCG CAC TCT GAA GGC CAC AAG ATC ATT GAG ATT GGT GCC GTT GAA GTG GTG AAC
ile gly ala his ser glu gly his lys ile ile glu ile gly ala val glu val val asn
121  /   41                                 151  /   51
CGT CGC CTG ACG GGC AAT AAC TTC CAT GTT TAT CTC AAA CCC GAT CGG CTG GTG GAT CCG
arg arg leu thr gly asn asn phe his val tyr leu lys pro asp arg leu val asp pro
181  /   61                                 211  /   71
GAA GCC TTT GGC GTA CAT GGT ATT GCC GTT GAT TTT TTG CTC GAT AAG CCC ACG TTT GCC
glu ala phe gly val his gly ile ala val asp phe leu leu asp lys pro thr phe ala
241  /   81                                 271  /   91
GAA GTA GCC GTT GAG TTC ATG GAC TAT ATT CGC GGC GCG GAG TTG GTG ATC CAT AAC GCA
glu val ala val glu phe met asp tyr ile arg gly ala glu leu val ile his asn ala
301  /  101                                331  /  111
GCG TTC GAT ATC GGC TTT ATG GAC TAC GAG TTT TCG TTG CTT AAG CGC GAT ATT GCG AAG
ala phe asp ile gly phe met asp tyr glu phe ser leu leu lys arg asp ile ala lys
361  /  121                                391  /  131
ACC AAT ACT TTC TGT AAG GTC ACC GAT AGC CTT GCG GTG GCG AGG AAA ATG TTT CCC GGT
thr asn thr phe cys lys val thr asp ser leu ala val ala arg lys met phe pro gly
421  /  141                                451  /  151
AAG CGC AAC AGC CTC GAT GCG TTA TGT GCT CGC TAC GAA ATA GAT AAC AGT AAA CGA ACG
lys arg asn ser leu asp ala leu cys ala arg tyr glu ile asp asn ser lys arg thr
481  /  161                                511  /  171
CTG CAC GGG GCA TTA CTC GAT GCC CAG ATC CTT GCG GAA GTT TAT CTG GCG ATG ACC GGT
leu his gly ala leu leu asp ala gln ile leu ala glu val tyr leu ala met thr gly
541  /  181                                571  /  191
GGT CAA ACG TCG ATG GCT TTT GCG ATG GAA GGA GAG ACA CAA CAG CAA CAA GGT GAA GCA
gly gln thr ser met ala phe ala met glu gly glu thr gln gln gln gln gly glu ala
601  /  201                                631  /  211
ACA ATT CAG CGC ATT GTA CGT CAG GCA AGT AAG TTA CGC GTT GTT TTT GCG ACA GAT GAA
thr ile gln arg ile val arg gln ala ser lys leu arg val val phe ala thr asp glu
661  /  221                                691  /  231
GAG ATT GCA GCT CAT GAA GCC CGT CTC GAT CTG GTG CAG AAG AAA GGC GGA AGT TGC CTC
glu ile ala ala his glu ala arg leu asp leu val gln lys lys gly gly ser cys leu
721  /  241
TGG CGA GCA TAA
trp arg ala OCH
```

Note : n1 / n2, where n1 is the number of nucleic acid residues at the beginning of a particular line or the middle of a particular line. n2 is the corresponding number of amino acids.

Translational Frameshifting Generates the γ Subunit of DNA Polymerase III Holoenzyme



The mRNA structure of the region of *dnaX* essential for the production of the γ subunit. The bar indicates the stretch of 'slippery' adenines. The UGA codon (in -1 frame is in bold). Adapted from Tsuchihashi and Kornberg, 1990)