SOME OBSERVATIONS ON THE COFACTOR REQUIREMENT FOR PARTIALLY PURIFIED DNA LIGASE FROM *ESCHERICHIA COLI*

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1. Introduction

Polynucleotide ligase (DNA ligase) catalyses the synthesis of a phosphodiester bond by esterification of the 5′-phosphoryl group to the 3′-hydroxyl group of DNA chains which have been properly aligned in a double helical structure. Although the DNA substrate specificity is the same for all ligases studied, the cofactor requirement for the reaction does vary depending on the source of the enzyme. *Escherichia coli* ligase specifically requires NAD^+ whereas the specificity of T4 bacteriophage-induced and mammalian ligases is for ATP [1].

In the course of investigating mammalian cell ligase we decided to check a simple ligase assay system using enzyme prepared from *E. coli*. This paper reports our findings that partially purified ligase from *E. coli* C64† catalysed the covalent closure of "Hershey" circles [2] of λ DNA only in the presence of ATP and that NAD^+ was not required for the reaction. The requirement for ATP was lost during purification of the ligase, and after the final stage of purification NAD^+ could be shown to increase the amount of covalently closed product. The possibilities of exceptionally high levels of ligase-AMP in the bacterial cell, a protective effect of ATP on the DNA substrate and a "modification" of the ligase during purification are discussed.

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† *E. coli* C64 (Hfr, met^-, endo I^-, T_1^R, λ^S, $azide^R$) is an endonuclease I^- strain and was a gift of Dr. R. Hayward, Department of Molecular Biology, University of Edinburgh.

2. Materials and methods

2.1. *Preparation of ligase*

Ligase was prepared from *E. coli* C64 grown to late log phase at 30° in an orbital incubator using M9 medium containing 0.2% casamino acids (Difco). The cells were harvested by centrifugation, washed once with 50 mM potassium phosphate, pH 7.6 and stored at −20° until required. Ligase was extracted and purified from the cells by the method of Olivera [3].

2.2. *Preparation of bacteriophage* λ

Bacteriophage λ was prepared by thermal induction of *E. coli* W1485 (λ $cI_{857}S_7$SuII). This strain was a kind gift from Dr. K. Murray, Department of Molecular Biology, University of Edinburgh. Cells were incubated, with shaking, at 30° in 150 ml amounts of 2.5% nutrient broth (Oxoid No. 2) containing 0.1% yeast extract (Oxoid) until the absorbance of the culture at 600 nm was between 0.5 and 0.6. The culture was then heated at 45° for 15 min, and uridine added to a final concentration of 0.5 mg/ml followed by 200 μl of [6-^{3}H]thymidine (1 mCi/ml; Amersham). After incubation for 60 min at 38°, 150 μl more of [6-^{3}H]thymidine was added to each 150 ml culture. Incubation was continued at 38° for 3 to 4 hr more before harvesting the cells by centrifugation. Bacteriophage λ S_7 is lysozyme negative, therefore the phage is still within the cell.

2.3. *Purification of bacteriophage* λ

The cell pellet from approx. 600 ml of culture was resuspended in 20 ml of 20 mM Tris-HCl, 5 mM $MgSO_4$, pH 8.0 (bacteriophage buffer). Cells were lysed by shaking with a few drops of chloroform and the host

DNA and RNA digested at 37° by the addition of 50 μg/ml DNAase (Deoxyribonuclease 1, DN-100, Sigma) and 50 μg/ml RNAase (Ribonuclease-A, Sigma). Bacterial debris was removed by centrifugation at 9,000 *g* (MSE 25) and 4° for 15 min, the supernatant re-centrifuged at 45,000 *g* for 4 hr and the precipitate resuspended by overnight contact with bacteriophage buffer.

The low and high speed centrifugation was repeated and the final high speed precipitate, resuspended in 2 ml of bacteriophage buffer, was layered on top of successive 5 ml layers of CsCl of density, 1.7, 1.6, 1.5 and 1.4. The phage were then centrifuged for 2 hr at 45,000 *g* and 20° in the Spinco SW 25.1 rotor. The gradient was fractionated and the bacteriophage fractions, located on the basis of their radioactivity, were pooled and dialysed overnight at 4° against 3 1 of bacteriophage buffer.

2.4. *Extraction of λ DNA and preparation of Hershey circles*

An equal volume of redistilled phenol, saturated with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 was added to the bacteriophage, previously made 5 mM with EDTA, and the mixture swirled gently at 4° for 15 min. The layers were then separated by low speed centrifugation, the aqueous phase re-extracted with phenol and dialysed for 3 days at 4° against five 1 1 changes of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Hershey circles were prepared by heating λ DNA (5 μg/ml) in 2 M NaCl, 3 mM EDTA, pH 7.6 at 78° for 15 min followed by quick cooling in ice. The DNA was then heated at 55° for 4 hr and allowed to cool slowly to room temperature. Finally the DNA was dialysed overnight at 4° against 2 1 of 10 mM Tris-HCl, 1 mM EDTA, pH 7.6. 60 to 70% conversion of linear DNA to Hershey circle DNA was obtained as determined by centrifugation through 5–20% neutral sucrose gradients.

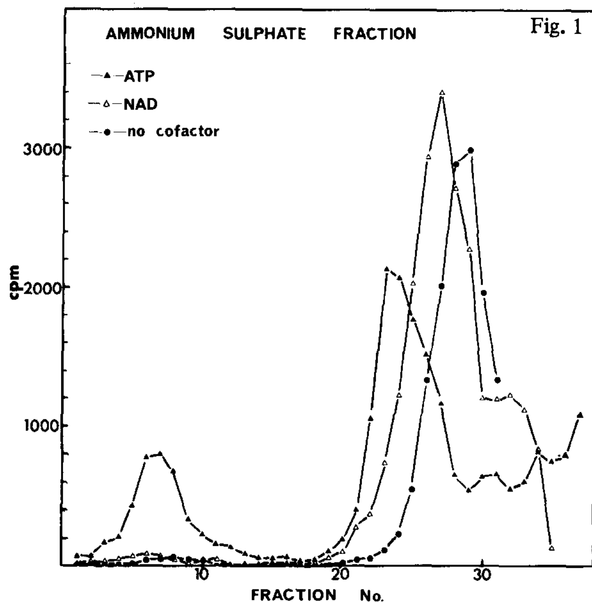
2.5. *Assay of DNA ligase*

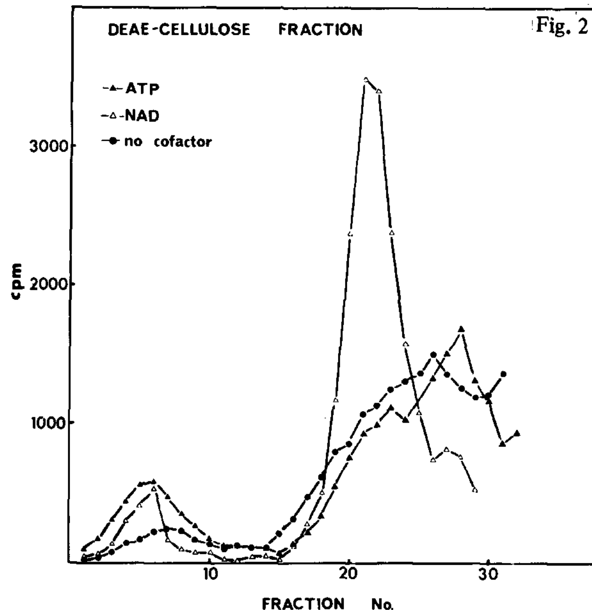
Each ligase assay mixture (0.31 ml) contained: Tris-HCl, pH 7.5 (50 mM); $MgCl_2$ (13 mM); dithiothreitol (6 mM); *E. coli* tRNA (15 μg); Hershey circle DNA (0.01 mM in terms of nucleotide); ATP (Sigma, 0.5 mM) or NAD^+ (Sigma, 0.03 mM); and enzyme. After 20 min at 30° the incubation tubes were chilled and the reaction stopped by the addition of 70 μl of 10% sucrose containing 1.15 M NaOH, 0.31 M EDTA, 1.85 M NaCl. The reaction mixture was then layered on a 4.4 ml 5–20% alkaline sucrose gradient (0.3 M NaOH, 0.7 M NaCl, 0.001 M EDTA), and centrifuged for 60 min at 36,000 rpm (106,000 *g*) and 12° in the SW 50 rotor of the Spinco L2. The gradients were fractionated by collecting 6-drop fractions directly onto glass fibre filters (Whatman GF/C). The discs were washed with 5% trichloroacetic acid, ethanol, and counted in an Intertechnique liquid scintillation counter using a toluene-based scintillator.

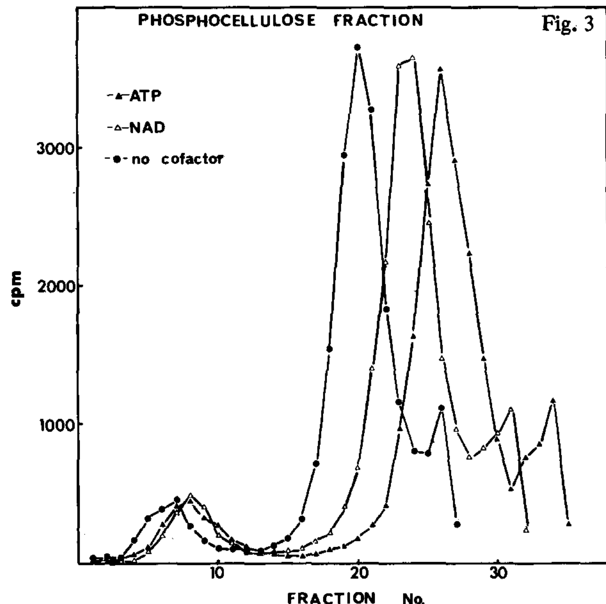
3. Results and discussion

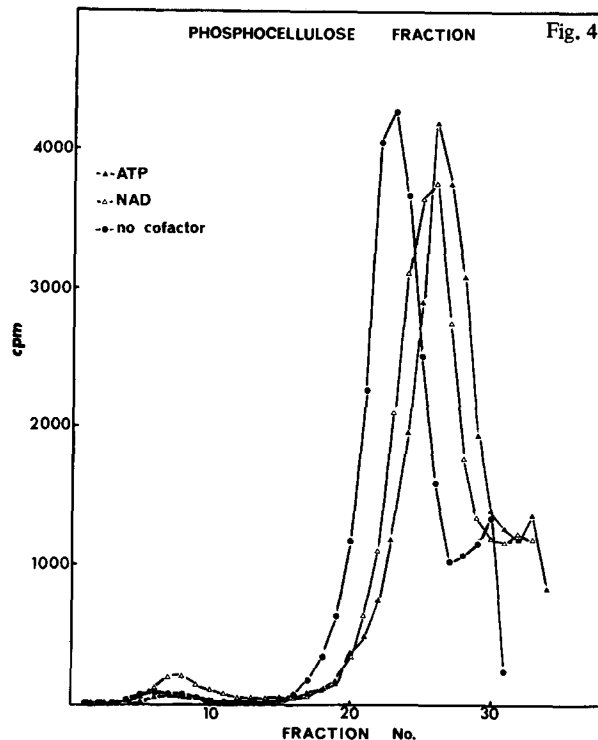
Figs. 1 to 3 show the results of ligase assays, using ATP, or NAD^+ or no cofactor, performed at different stages of ligase purification. Fig. 1 clearly shows that only ATP allows the formation of covalent circles when enzyme purified by ammonium sulphate precipitation is used. The radioactivity in a discrete peak sedimenting approx. 3.8 times faster than the starting material was taken to be the circular DNA product [4]. The use of neutral sucrose gradients containing actinomycin D, as described by Gellert [5] provided further evidence of the covalent nature of our product. Again, with the DEAE-cellulose fraction of the enzyme (fig. 2), ATP is necessary for maximal activity, 20% conversion to covalent circles; while the amount of conversion in the presence of NAD^+ (9.5%) is only slightly greater than that in the absence of any cofactor (8.5%). After the enzyme has been purified by phosphocellulose column chromatography the same amount of product is formed, irrespective of whether ATP, NAD^+ or no cofactor is present (fig. 3). However, when this purified enzyme is stored and re-tested a few days later it is clear (fig. 4) that although the enzyme activity has decreased, NAD^+ now stimulates the reaction. Since covalent closure also takes place in the *absence* of the accepted cofactor (NAD^+) this suggests that the ligase is being isolated in the "charged" form, that is, as a ligase–AMP complex.

These data seem to indicate that the less highly purified enzyme extracts contain some activity which destroys the ability of the Hershey circle DNA to act as substrate. ATP inhibits this activity and allows endogenous ligase–AMP to form a covalent circle. This hypothesis was tested by pre-incubating Hershey circle DNA









Figs. 1–4. The effect of cofactor on the conversion of λ DNA to covalent circles as described in Materials and methods by the extracts indicated below. The direction of sedimentation is from right to left. (▲—▲—▲) Assay containing ATP; (△—△—△) assay containing NAD^+; (●—●—●) assay containing no cofactor. Fig. 1: 500 μg ammonium sulphate fraction. Fig. 2: 140 μg DEAE-cellulose fraction. Fig. 3: 11 μg phosphocellulose fraction. Fig. 4: 11 μg phosphocellulose fraction stored 3 days at −20° before assay.

with the ammonium sulphate fraction in the absence of ATP. On the subsequent addition of ATP only 5% of the covalent product expected was obtained, indicating that the Hershey circle DNA was no longer acting as a substrate for the reaction. After phosphocellulose chromatography the enzyme activity which destroys the substrate activity of the Hershey circle DNA has been removed and consequently, equal activity is given by ATP, NAD^+ or no cofactor. The loss of activity of the phosphocellulose fraction on storage can be explained in terms of the multiple forms of the ligase described by Zimmerman and Oshinsky [6]. The active ligase–AMP complex (Form III) is known to give rise during storage to two other forms (I and II) neither of which shows activity in the routine DNA joining assay. The low level of NAD^+ stimulated activity found after storage could be due to a small amount of free ligase present in this highly purified fraction. This ligase would have been masked in the earlier fractions by the ligase–AMP and not detected in the presence of NAD^+ alone since ATP is necessary to protect the DNA substrate in these early fractions.

The above explanation is dependent on our hypothesis that we are isolating the enzyme in the "charged" form. In support of this, ATP is not bound to the ammonium sulphate enzyme fraction as would be expected if it were being used as cofactor in the reaction. This was shown by incubating the ammonium sulphate fraction with ATP (^{3}H-labelled in the adenine moiety) in the absence of DNA and separating the protein peak from the ATP by Sephadex G-25 column chromatography. Although radioactivity was found associated with the protein peak it was not acid precipitable, and not therefore covalently bound to the protein. Using standard procedures [7, 8] we have been unable to discharge the ligase–AMP complex with either NMN or with PP_i. It is therefore possible that the ligase–AMP complex which we obtain from *E. coli* C64 is present in still another form (Form IV), able to react in the normal joining reaction, but unable to discharge the AMP moiety upon exposure to NMN.

Our findings that we are isolating the enzyme mainly in the "charged" form is very unusual since by extracting the enzyme in the presence of EDTA, formation of ligase–AMP during extraction is normally prevented. The high ligase–AMP level might be due to the strain being endonuclease I^- which in turn could imply that the number of "nicks" in the DNA governs the overall level of charged enzyme within the cell. This clearly may have some physiological significance.

The mechanism by which the Hershey circle DNA is modified so as no longer to act as a substrate in the ligase reaction is currently under investigation. Although some protection was afforded by deoxyribonucleoside triphosphates, inactivation by a phosphatase is unlikely since the addition of 5 mM sodium fluoride (an inhibitor of phosphatase activity) to a ligase assay, in the absence of ATP, did not allow formation of covalent product: sodium fluoride at this concentration did not inhibit ligase activity. Similarly, glucose 6-phosphate did not protect the substrate. We favour the idea that the Hershey circle DNA is inactivated by some "restriction" activity present in the cell extract (for example, see Meselson and Yuan [9].)

These results have been presented as a guide to workers who wish to set up an assay system for DNA ligase. However, some of the findings may be peculiar to the ligase reaction involving the covalent closure of λ DNA since NAD^+ dependence of partially purified ligase has been demonstrated using other assay systems [10, 11]. It should also be noted that earlier workers [4], using a similar assay system to the one reported here, made passing reference to variable stimulation of partially-purified ligase due to the addition of ATP and deoxyribonucleoside triphosphates. These last two observations support our view that the Hershey circle DNA may be inactivated due to some form of restriction activity.

Acknowledgements

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References

[1] C.C. Richardson, Ann. Rev. Biochem. 38 (1969) 795.
[2] A.D. Hershey, E. Burgi and L. Ingraham. Proc. Natl. Acad. Sci. U.S. 49 (1963) 748.
[3] B.M. Olivera, Methods Enzymol. 21 Part D (1971) 310.
[4] M.L. Gefter, A. Becker and J. Hurwitz, Proc. Natl. Acad. Sci. U.S. 58 (1967) 240.
[5] M. Gellert, Proc. Natl. Acad. Sci. U.S. 57 (1967) 148.

[6] S.B. Zimmerman and Carol K. Oshinsky, J. Biol. Chem. 244 (1969) 4689.
[7] J.W. Little, S.B. Zimmerman, Carol K. Oshinsky and M. Geller, Proc. Natl. Acad. Sci. U.S. 58 (1967) 2004.
[8] B. Weiss, Ann Thompson and C.C. Richardson, J. Biol. Chem. 243 (1967) 4556.
[9] M. Meselson and R. Yuan, Nature 217 (1968) 1110.
[10] P. Modrich and I.R. Lehman, J. Biol. Chem. 245 (1970) 3626.
[11] M. Gellert and Minnie L. Bullock, Proc. Natl. Acad. Sci. U.S. 67 (1970) 1580.