NON-LYSOSOMAL PROTEIN DEGRADING SYSTEMS IN CHICKEN SKELETAL MUSCLE

Submitted in fulfilment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

in the

FACULTY OF MEDICINE (MEDICAL BIOCHEMISTRY)
UNIVERSITY OF CAPE TOWN

by

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February 1990

CERTIFICATE OF SUPERVISOR

In terms of Paragraph GP8 of "General rules for the degree of PhD", I, as supervisor of the candidate, Jane Elizabeth Arnold, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.
(See reference Arnold, J.E. & Gevers, W. (1990))

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ABSTRACT

In an attempt to understand the roles played by the ubiquitin-dependent and calpain pathways in protein degradation in chicken skeletal muscles, biochemical studies were conducted on components of these two systems as well as their potential endogenous and exogenous substrates.

ATP- and ubiquitin-dependent breakdown of endogenous proteins (measured by tyrosine release) or exogenous proteins (measured by the appearance of trichloroacetic acid-soluble radiolabel after incubation with ^{125}I-lysozyme) took place in muscle extracts; the specific activities of these processes were significantly lower than those detected in rabbit reticulocytes. Conjugation of ubiquitin to a subset of endogenous proteins was detected by incubating muscle extracts (fraction II: depleted of ubiquitin by DEAE-cellulose chromatography) with ^{125}I-ubiquitin and Mg^{2+}-ATP, followed by analysis of the radiolabelled conjugates by one-dimensional polyacrylamide gel electrophoresis in the presence of SDS, and autoradiography. Discrete conjugates were formed with apparent molecular weights between 30 -100 000, as well as a large number of undifferentiated entities of higher molecular weights. Conjugation of ubiquitin to the exogenous protein lysozyme was detected only when fresh, as opposed to previously frozen fraction II preparations were assayed: three bands were obtained, as opposed to the six ubiquitin conjugates formed by reticulocyte extracts. The muscle system catalyzed the ubiquitination of partially purified myofibrillar proteins, principally myosin and possibly actin. Fractionation of the ubiquitin-activating enzymes into E1 and E2 on the one hand, and E3 on the other, permitted mixing experiments to be conducted by means of conjugation assays, and confirmed the low content of E3 in muscle as opposed to reticulocytes.

Fraction II from muscle displayed ubiquitin conjugate-degrading activity but again this was less active than in reticulocytes. A number of other proteolytic activities, independent of ubiquitin, were also present. Isopeptidases, active on ^{125}I-ubiquitin conjugates were strongly inhibited by sulphydryl alkylating agents such as N-ethylmaleimide.
The overall picture of the ubiquitin pathway in muscle is one where many proteins may be converted into long-lived conjugates but not in all cases requiring the action of E3; some E3-dependent protein degradation undoubtably does occur in this physiologically basal system.
Formation of a ubiquitin conjugate of the ubiquitin-activating enzyme (E1) and some of the ubiquitin carrier proteins (E2's) was detected during incubations of ^{125}I-ubiquitin and ATP lasting 2 hr or longer. Because treatment of such systems with NaOH, even at early times during the incubations, greatly enhanced the appearance of the same entities, the phenomenon appeared to be one of auto-, rather than E3-mediated ubiquitination. The bonds involved had properties compatible with their being peptidic in nature, and their formation occurred from ubiquitin thiolesters bound to E1 and E2. The protease inhibitor and alkylating agent, TLCK, when pre-incubated with fraction II for 2 hr before the addition of ^{125}I-ubiquitin and ATP, greatly enhanced the subsequent auto-ubiquitination of E1 in the absence of NaOH treatment, and caused the inhibition of its adenylate-forming and thiolester-transferring activities: thus ubiquitin transfer to E2's and further to other acceptors was markedly impaired. Such an inactivation of E1 by TLCK may, in a manner analogous to that described in the thermolabile ts85 mutants (Finley et al., 1984), be the basis of the action of this agent to block the cell cycle in late G_2 or early M phase (Schnebli & Haemmerli, 1974). TLCK-induced inactivating auto-ubiquitination of E1 may be an important tool for the study of ubiquitin-dependent processes which (apart from possible intrinsic

protease activity), all appear to require the activity of this enzyme.

The number of calpain species existing in chicken skeletal muscle is controversial with only one (Ishiura et al., 1978) or three (Wolfe et al., 1985) species having been reported. When extracts of chicken skeletal muscle were applied to a DEAE-cellulose column and the bound protein eluted in a linear salt gradient, two calpain activities, separated from their endogenous inhibitors (calpastatins), were detected. The first eluting activity, "calpain I", was active at low Ca^{2+} concentrations, was heat-labile and had a lower apparent molecular weight on gel filtration when compared with the later eluting activity which appeared to be a typical calpain II species. "Calpain I" was not an autolytic product of calpain II but appeared to be derived from a more heat-stable calpain I species.

A proportion (up to 14%) of the calpains in crude muscle extracts was bound to membrane fractions in the presence of Ca^{2+}; this could be removed by EGTA treatment. In addition, membrane-bound fractions examined by gel filtration contained calpain forms of an apparent molecular weight lower than that of calpain which had not been membrane-associated. Membrane binding of the calpains (especially of calpain II), may be important in physiological activation.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks and appreciation to the following:

Professor Wieland Gevers for his excellent guidance as a supervisor and for his creativity and enthusiasm at all stages of this project.

Professor Deneys van der Westhuyzen for critical and insightful discussions of this work.

Dr. H.F. Deutsch for the gift of purified ubiquitin.

Drs. D.E. Goll and W.C. Kleese for their gifts of poly-clonal and monoclonal anti-calpain antibodies.

All my friends in the Department of Medical Biochemistry for providing a pleasant working environment.

Arlene Smith and Malcolm Estment for providing a peaceful environment in which to work as well as their invaluable assistance in the printing of this thesis.

My housemates, Denver Hendricks for his friendship and support, Felicity Ellemore for her patience and Geoff Smith and Marina Reid for their understanding.

My parents for their encouragement and support over the years.

Sarah, my sister, and John for their willingness to help and offer advice.

The South African Medical Research Council and the University of Cape Town for financial support.

ABBREVIATIONS

ATP	adenosine 5'-triphosphate
2,3 BPG	2,3 biphospho-D-glyceric acid
BSA	bovine serum albumin
°C	degree Celsius
CM	carboxymethyl
cpm	counts per minute
CTP	cytidine 5'-triphosphate
DEAE	diethylaminoethyl
DTT	dithiothreitol
E1	ubiquitin-activating enzyme
E2	ubiquitin carrier protein
E3	ubiquitin protein ligase
E 64	N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine)
EDL	extensor digitorum longus
EDTA	ethylenediamine tetracetic acid
EGTA	ethyleneglycol-bis-(aminoethyl ether)-N,N,N',N'-tetracetic acid
g	acceleration due to gravity (all values are given for g_{av})
G-3-PDH	glceraldehyde-3-phosphate dehydrogenase
GTP	guanosine 5'-triphosphate
hr	hour
M	molar concentration
min	minutes
Mb	myoglobin
M_r	molecular weight
NEM	N-ethylmaleimide
P_i	inorganic phosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMSF	phenylmethylsulphonyl fluoride
PK	phosphorylase kinase
RNase	ribonuclease

rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
STI	soybean trypsin inhibitor
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl) amino methane
tRNA	transfer ribonucleic acid
TLCK	N-α-tosyl-L-lysine chloromethyl ketone
TPCK	N-α-tosyl-L-phenylalanine chloromethyl ketone
Ub	ubiquitin
UTP	uridine 5'-triphosphate
V_o	void volume
V_t	total volume
v/v	volume per volume
w/v	weight per volume

CONTENTS

CHAPTER 1
INTRODUCTION

1.1 MUSCLE PROTEOLYSIS

The diverse range of movements produced by vertebrates reflects the fundamental importance of skeletal muscle in locomotion; a role which links commands and responses of the nervous system with the coordinated contraction and relaxation of sets of muscles. Physiological modifications of the muscle fibres in accordance with specific locomotory needs, greatly enhances the functional versatility of this tissue. As 40-45% of the body mass of a vertebrate is composed of skeletal muscle (of which 20% represents myofibrillar proteins), skeletal muscle is a vast potential energy resource for a stressed or starving animal. Thus under conditions of starvation, an important metabolic role of skeletal muscle involves the degradation of muscle proteins to form amino acids which can be used inter alia as precursors of the gluconeogenic pathway (Felig, 1975). Precise control of the extent of myofibrillar degradation is obviously essential if locomotion (necessary for the provision of food) is not to be critically affected in these circumstances.

The maintenance of muscle mass is accordingly under intricate hormonal controls (Goldberg et al., 1980; Goldspink, D.F., 1980), upon which are dependent the rates both of protein synthesis and degradation; the muscle weakness and wasting seen in conditions of hormonal imbalance as in diabetes mellitus, Cushing's syndrome and hyperthyroidism emphasizes the critical roles played by these factors. Whilst much is known about the synthesis of muscle proteins (Booth & Morrison, 1986), much less information is available about the detailed mechanism(s) of muscle protein degradation. Although a multiplicity of proteases have been identified in muscle tissue (Pennington, 1977), it is mostly uncertain whether some proteins are recognized by specific proteolytic enzymes, or whether a kind of controlled mayhem prevails.

Three categories of protein pools can be visualized as being substrates of muscle proteinases; firstly, intact, native proteins (myofibrillar and non-myofibrillar), are turned over under basal metabolic conditions, with the proteins of the myofibrils turning over more slowly than soluble proteins of the cytoplasm (Bates & Millward, 1983); some proteolytic system(s) must accordingly be present which is(are) able selectively to recognize and to degrade intact muscle proteins, located in appropriate cellular compartments. Secondly, in the light of the highly organized and stochiometric structure of the myofibrils involving the assembly of actin, myosin, and other molecules, over-production of some of these monomeric or oligomeric proteins is very likely to occur, resulting in their presence in the cytosol from which they have to be removed: this may (but need not) require another system of proteolytic enzymes. Thirdly, muscle proteins are likely to become damaged (denatured or disorganized) under conditions of prolonged or intense exercise and may need to be removed to enable recovery of the muscle cells to occur: the increased levels of N-methylhistidine measured in the urine of untrained individuals undergoing strenuous bouts of exercise suggests that damaged proteins are removed during recovery (Dohm et al., 1985; Kasperek & Snider, 1985); one is inclined to think that a specific system of proteases would be necessary for the degradation of such damaged proteins. Whether, in fact, separate proteolytic systems are involved in the breakdown of these different protein pools or whether one system is capable of degrading all of these proteins (with possibilities of overlap) is at present unresolved.

It is easier to picture mechanisms for the degradation of damaged proteins and superfluous monomers than of proteins emplaced within structures as highly organized as intact myofibrils. Because myofibrils are composed of linear arrays of sarcomeres containing interdigitating thick and thin filaments, it is conceptually possible that units of

assembled or disassembled proteins could be removed proteolytically from the ends of myofibrils without affecting their contractile activity (Fig. 1.1a). Goldspink, G., (1980) has shown that new sarcomeres are added at the ends of the myofibrils; according to the above model, only newly synthesized proteins would thus be degraded. Alternatively, it is possible that individual thick and thin filaments could be removed from the periphery of myofibrils, allowing protein turnover without disrupting the myofibrillar structure (Fig. 1.1b). Unless mixing of myofilaments occurs within the myofibril, however, only a particular population of myofibrillar proteins would ever be degraded. It is thus likely that in order for contractile proteins to be degraded, whole myofibrils (or sarcomeres) must be affected; initial fragmentation of these structures prior to the degradation of the individual proteins may well occur (Fig. 1.1c). This notion has gained support from the observations that a specific protease, activated by Ca^{2+}, is capable of degrading the Z-lines of the myofibrils with resulting disruption of the entire myofibrillar structure (Dayton et al., 1976).

In order to understand how the bulk muscle proteins can be degraded, it is necessary to identify the proteolytic systems involved in the process. All eukaryotic cells contain at least two major proteolytic pathways; one that involves proteases confined within lysosomes or related structures and active under acidic conditions, and another that largely or entirely involves proteases present in the cytosol with its neutral pH (for review, see Pennington, 1977). The relative roles of these two systems in degrading skeletal muscle proteins is controversial and reflects, in part, the complexities involved in measuring protein degradation within this tissue. Whether either system can degrade intact bulk muscle proteins completely to the amino acid level or whether a combination of both pathways is necessary, has also not been clarified.

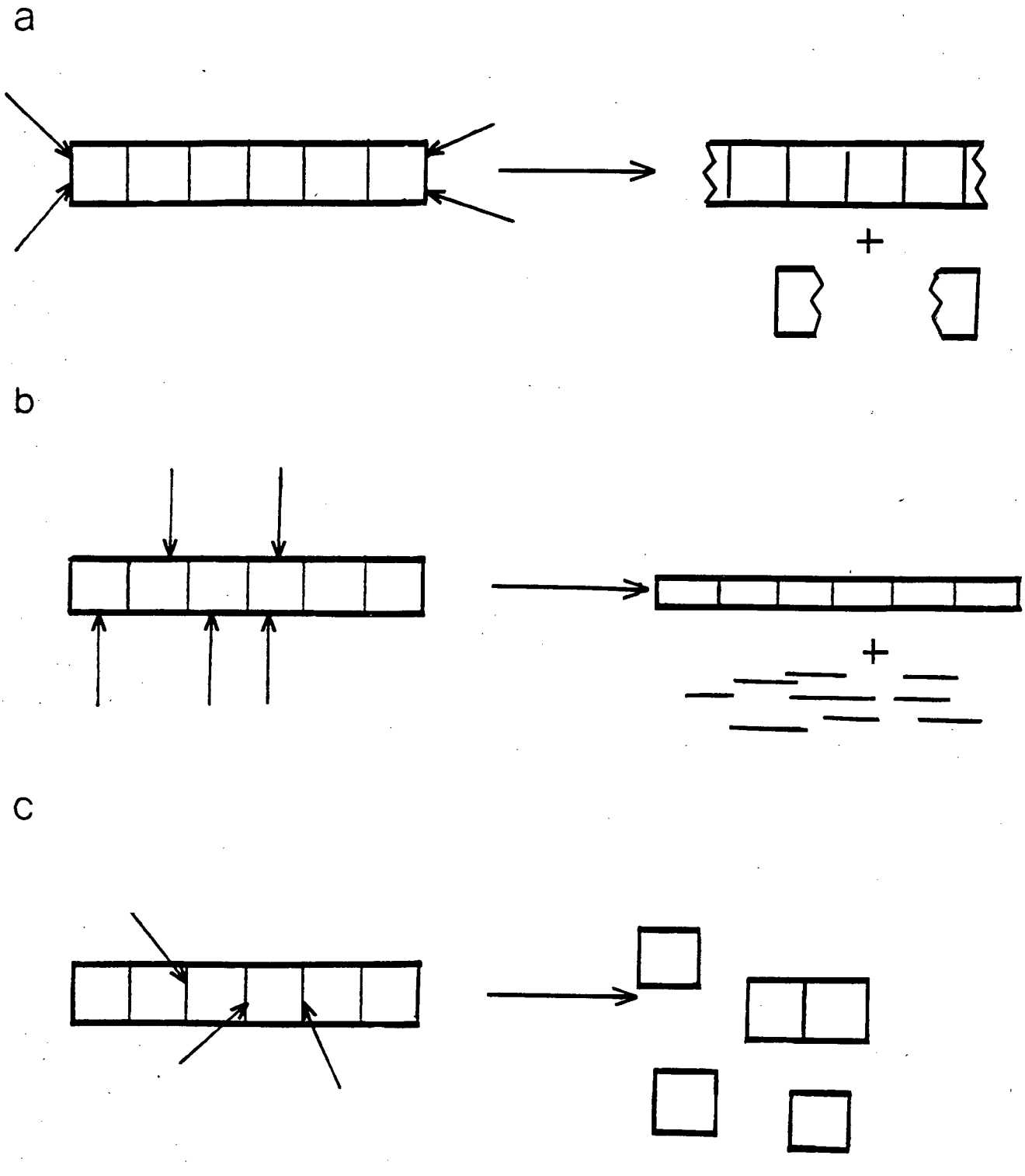


Fig. 1.1 Possible pathways of myofibrillar protein degradation

Proteases (indicated by arrows) may attack at the ends (Fig. 1.1a), the periphary (Fig. 1.1b) or along the length of the myofibril (Fig. 1.1c). See text for details.

The notion that lysosomes are not involved in the basal degradation of myofibrillar proteins has gained support in recent years because of the sparse distribution of lysosomes within normal skeletal muscle (Bird et al., 1980), and the apparent non-selectivity of these organelles in protein degradation. It is difficult to imagine how a whole myofibril could undergo autophagy without prior fragmentation. This viewpoint is reinforced by studies of Lowell et al., (1986), who have compared total muscle protein breakdown under basal conditions (as measured by tyrosine release), to the breakdown specifically of myofibrillar protein (as measured by N-methylhistidine release). Although there is some doubt as to the validity of using N-methylhistidine as a specific indicator of myofibrillar protein degradation (Rennie & Millward, 1983), it was observed that degradation of total muscle protein was sensitive to lysosomal inhibitors, while that of myofibrillar proteins was not. Apart from suggesting that lysosomes are not involved in the turnover of myofibrillar proteins, there was also the implication that the degradation of myofibrillar and non-myofibrillar proteins was catalyzed by independent proteolytic systems. Other workers have similarly observed an insensitivity of basal myofibrillar protein degradation to lysosomal inhibitors (Furuno & Goldberg, 1986; Zeman et al., 1985; Wildenthal & Wakeland, 1985).

It must, however, be noted that purified lysosomal cathepsins can readily degrade myosin and actin, and it remains possible that at least some degradation of these proteins by lysosomes may occur *in vivo* (Schwartz & Bird, 1977; Dufour et al., 1989). The actual presence of myofibrillar proteins within lysosomes has been observed in one study using the technique of labelling intact muscle proteins *in situ*, by injecting a muscle with ^{3}H-NEM and following the fate of the covalently modified radioactive proteins (Gerard & Schneider, 1979). Under these conditions, and in the presence of the lysosomotropic

agent, chloroquine, accumulation of actomyosin and other muscle proteins within lysosomes undoubtably occurred; at the same time an increase in assayable tissue cathepsin B activity was detected while the activity of cytosolic proteases was not altered (Gerard et al., 1988). Caution, however, should be exercised in interpreting these results, as the situation described was somewhat unphysiological, and the "control" (contralateral) muscles may also have been exposed to the injected chemicals during the 48 hr incubation period.

It has become generally accepted that lysosomes are involved in the degradation of myofibrillar structures under conditions of nutritional deprivation when bulk and non-specific digestion of the myofibrillar proteins occurs (Li et al., 1979; Furuno & Goldberg, 1986). This notion, however, has recently been disputed by workers who were unable to detect lysosomal involvement in the degradation of myofibrillar proteins during starvation (Lowell et al., 1986). Interestingly, there is accumulating evidence that oxidation of amino acids derived from muscle proteins represents up to 10 - 18% of the energy consumption during prolonged exercise (Lemon et al., 1983; Houston, 1986); a role for lysosomes in proteolysis has been postulated in these stressed conditions.

Assuming for the time being that lysosomal proteases are not responsible for the basal turnover of myofibrillar proteins, one is left with the search for cytosolic proteases that may be involved in such processes. Recently a ubiquitin-dependent proteolytic system (including a high molecular weight proteinase specific for ubiquitin conjugates) has been identified in rabbit skeletal muscle (Fagan et al., 1987); as this pathway has been firmly established as participating in the degradation of abnormal and short-lived proteins in reticulocytes and some other cell types (for review, see Hershko, 1988), it is possible that it may also be involved in the degradation of damaged or superfluous muscle proteins. A second cytosolic protease identified in rat skeletal muscles is a high molecular weight, multi-

protease has been demonstrated in rat myotubes, however, (Heath et al., 1987; Stauber et al., 1987) and again it remains possible that such a protease could be involved in myofibrillar protein degradation.

A number of aminopeptidases have been identified in muscle tissue, which are likely to be important inter alia in the terminal degradation of partially proteolyzed myofibrillar proteins (Daly et al., 1985; Mantle et al., 1983; Jacobs-Sturm et al., 1982; Gibson et al., 1985).

To date the only cytosolic proteases that have been shown selectively to degrade certain structures of the myofibril are the calcium-activated proteases, known as the calpains (Murachi et al., 1981b); these proteases can remove the Z-lines of myofibrils, causing this structure to lose its three-dimensional stability (a more detailed account of the calpain system is given in Section 1.3). Elevated levels of these proteases have been reported in certain diseased states (Johnson & Hammer, 1988; Otsuka et al., 1985) and they have accordingly been implicated in myofibrillar turnover. Whether in this case, partially degraded myofibrils are further broken down by lysosomes or by another cytosolic system, is not known.

Associated with the question of calpain activity in muscles has been the finding that increased intracellular Ca^{2+} levels, apart from causing myofilament damage (Duncan, 1987) may also be associated with an increase in protein degradation (Zeman et al., 1985). This has been demonstrated by incubating skeletal muscles in the presence of exogenous Ca^{2+}, with and without the Ca^{2+} ionophore, A23187, followed by measurement of the release of protein-derived tyrosine. When the muscles were incubated under passive tension, however, the rates of protein degradation decreased while those of protein synthesis increased (Furuno & Goldberg, 1986; Etlinger et al., 1981; Van der Westhuyzen et al., 1981). Similarly, under conditions of fever (Clowes et al., 1983), sepsis (Ruff & Secrist, 1984), thyroxine excess (Zeman et al., 1986) and hypoxia (Toyo-oka

et al., 1985), there was an increase in the intracellular Ca^{2+} concentration which coincided with an increase in protein degradation. Although it would be simplistic to correlate the increases in overall intracellular Ca^{2+} concentrations with necessary increases in calpain activity, no firm evidence is yet to hand that this is indeed the case. In some cases, it has been shown that calpain specifically is not involved: in fever-induced degradation, although an increase in PGE_2 was initially thought to be responsible for the increase in protein degradation (Rodemann & Goldberg, 1982; Rodemann et al., 1982), an unidentified factor released from macrophages has since been implicated (Goldberg et al., 1988). Other workers have shown that increased protein degradation associated with increased intracellular Ca^{2+} concentrations was insensitive to calpain inhibitors (Toyo-oka, 1985). In particular, Goodman (1987a) found that non-myofibrillar degradation was selectively increased under conditions of elevated Ca^{2+} concentrations; since calpain was thought to be specifically implicated in the degradation of myofibrillar proteins, the increased proteolytic activity seen under these conditions is unlikely to have involved the calpain system.

Although a number of hormonal treatments (Goldberg et al., 1980) and otherwise altered conditions (including abnormal Na^+ fluxes (Goodman, 1987b) and chronic ethanol ingestion (Preedy & Peters, 1989)), are known to affect muscle breakdown, the proteolytic systems involved in such reactions are still obscure. It is likely, however, that cytosolic proteases play an important role in the selective turnover of the bulk myofibrillar and cytosolic proteins, and that both the calpain system and the ubiquitin-dependent pathway may be involved. Because of the selectivity of the calpain system in removing the Z-line of myofibrils and thus destroying the ordered contractile architecture, it might be envisaged as being important in rate-determining steps in the turnover of the myofibrillar

proteins. The ubiquitin-dependent pathway, on the other hand, is generally thought to be involved in the degradation of abnormal and short-lived proteins (Hershko, 1988), although little is known about this system in cells other than reticulocytes. A more detailed discussion of these two potentially important non-lysosomal proteolytic systems follows.

1.2 UBIQUITIN-DEPENDENT PROTEOLYSIS

Although ubiquitin was first identified in 1975 (Goldstein et al.), it was only in 1980 that the molecule was proposed to mark proteins for degradation (Hershko et al., 1980). The model of ubiquitin-dependent protein degradation has since been refined, with greater insight into the structure of this unusual "coenzymic" protein and the conjugation mechanisms involved. The specificity of ubiquitin towards certain protein substrates and the manner in which these are degraded, although less well understood, has also been clarified to an impressive extent in recent times. It has, however, become increasingly apparent that ubiquitin, apart from marking some proteins for degradation, forms stable conjugates with other proteins and this points to the existence of multiple functions for this highly conserved molecule.

1.2.1 Structure and properties of ubiquitin

In order to understand how ubiquitin can be conjugated to many different cellular proteins, it is necessary to consider known structural features of this molecule. Initial work showed ubiquitin to be conjugated to the

substrate proteins through its C-terminal glycine residue (Hershko et al., 1981), forming an isopeptide bond with a lysine residue of the substrate protein. Analysis of the ubiquitin C-terminus showed an essential glycyl-glycine dipeptide to be present (Wilkinson & Audhya, 1981); removal of this by trypsin yielded ubiquitin molecules unable to form conjugates. The primary sequence of the whole molecule has since been determined in a number of organisms; out of the 76 amino acids present (M_r = 8 650), only three amino acid changes are present between yeast (Wilkinson et al., 1986b) and humans (Schlesinger et al., 1975). These changes occur on the surface of the molecule and do not affect known functions of ubiquitin (Vijay-Kumar et al., 1987b). Higher structures of ubiquitin have been resolved both by X-ray crystallography (Vijay-Kumar et al., 1987a) and NMR spectroscopy (Di Stefano & Wand, 1987); both methods show ubiquitin to be a compact, globular, highly H-bonded protein with the essential C-terminal tail protruding from the structure. Due to the high level of secondary structure, ubiquitin is a very stable molecule both to heat and extremes of pH (Lenkinski et al., 1977).

Chemical and genetic modifications of certain amino acid residues in ubiquitin have shown that the C-terminal tail is crucial for interaction with E1 in the initial activation reaction (Duersken-Hughes et al., 1987; Ecker et al., 1987). Modification of surface residues, especially of histidine 68, produces alterations in ubiquitin behaviour, specifically an increase in the rate of substrate protein degradation in the ubiquitin-dependent pathway, whilst the ubiquitin activation reaction is not altered; from this comes the suggestion that a conformational change in ubiquitin may be important in signalling protein degradation (Cox et al., 1986). More direct support for this notion has come from Ecker et al. (1989) who showed that ubiquitin molecules, which have been conformationally restrained by incorporation of disulphide bonds into the molecule, whilst allowing activation, do not support

subsequent substrate protein degradation. Thus the initial concept of conjugation to ubiquitin causing unfolding of protein substrates, making them more susceptible to degradation, has been replaced by the notion that ubiquitin molecules, when conjugated to protein substrates, undergo conformational changes which specific protease(s) recognize as a signal for degradation. One has to assume, however, that only certain substrates allow ubiquitin to undergo these conformational changes and thus to signal degradation.
Apart from conjugation to protein substrates, ubiquitin has also been shown to contain an intrinsic protease activity (Fried et al., 1987) as well as an esterase activity (Matsumoto et al., 1984); these are unexpected findings because of the lack of an active site cleft in the molecule.

1.2.2 Ubiquitin genes

Two types of ubiquitin genes have been identified in organisms ranging from yeast to man (Ozkaynak et al., 1987; Ohmachi et al., 1989; Gausing & Barkordittar et al., 1986; Dworkin-Rastl et al., 1984; Lee et al., 1988; Wiborg et al., 1985; Lund et al., 1985). One gene encodes a poly-ubiquitin precursor protein in which coding units for ubiquitin are joined together in a tandem array; the number of repeating units vary from five in yeast (Ozaynak et al., 1984) to at least forty in trypanosomes (Kirchhoff et al., 1988; Swindle et al., 1988). The poly-ubiquitin gene has been shown to be essential for cellular survival under stressed conditions (Finley et al., 1987). The other gene type encodes a single ubiquitin fused to conserved basic protein(s) known as carboxyl extension protein(s) (CEP). These gene products are apparently also involved in the maintenance of normal ubiquitin levels in the organism (Finley et al., 1987). Carboxyl extension protein has

recently been identified as the ribosomal protein S27a (Redman & Rechsteiner, 1989) and the incorporation of this protein into ribosomes is enhanced by the presence of ubiquitin, suggesting an additional role of ubiquitin as a chaperone-like activity (Finley et al., 1989).
Ubiquitin can be readily cleaved from carboxyl extension protein(s) (Monia et al., 1989; Redman & Rechsteiner, 1988); specific endoproteases have recently been identified which cleave either the poly-ubiquitin gene product into monomeric units, or the ubiquitin fusion proteins into ubiquitin and carboxyl extension protein(s) (Jonnalagadda et al., 1989). An enzyme with the last-mentioned kind of activity has been partially purified from reticulocytes (Butt et al., 1989).

1.2.3 Ubiquitin conjugation

The mechanism of ubiquitin conjugation has been extensively described in reticulocytes and three separate factors involved in ubiquitin conjugation are separable by ubiquitin-affinity columns (Hershko et al., 1983). Ubiquitin molecules are activated in the presence of ATP by the ubiquitin-activating enzyme, E1, to form bound ubiquitin adenylates with the release of PP_i (see Fig. 1.2). The activated ubiquitin moieties are then transferred in each case to presumably adjacent thiol groups on E1 with the release of AMP, after which a second ubiquitin adenylate is formed to give the doubly charged, predominant form of the enzyme (Ciechanover et al., 1982; Haas et al., 1982). Transfer of ubiquitin from its thiolester form bound to E1, readily occurs to any one of a number of ubiquitin carrier proteins, E2's, with the maintenance of a thiolester type of linkage (Pickart & Rose, 1985a). Although it would be feasible for the ubiquitin molecule to be transferred between E2 molecules, no evidence for this has been forthcoming. Ubiquitin molecules can finally be

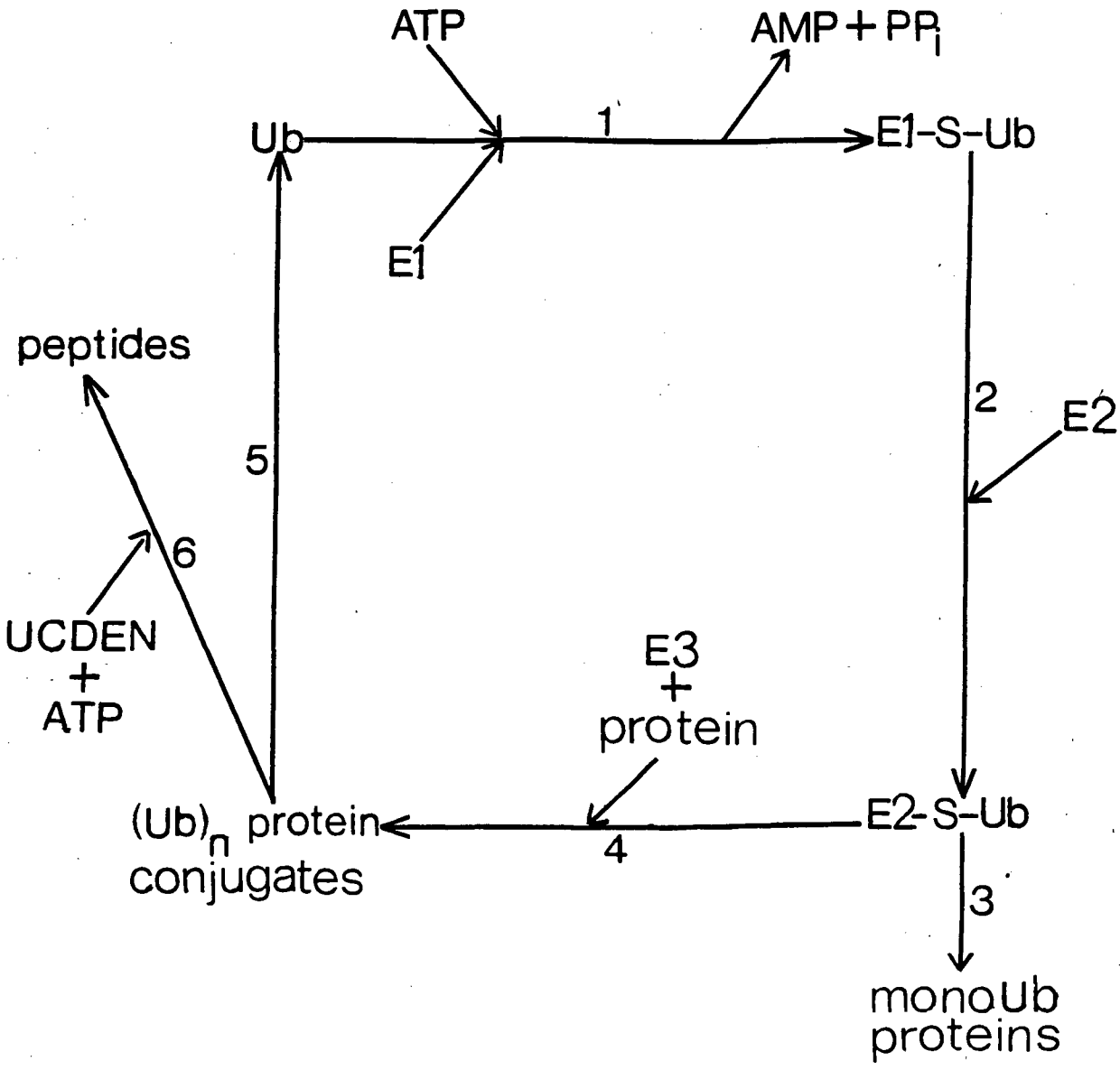


Fig. 1.2 The ubiquitin-dependent pathway of protein degradation

(1). Activation of ubiquitin by E1. (2). Transfer of ubiquitin to E2. (3). Formation of stable mono-ubiquitinated protein conjugates. (4). Formation of multi-ubiquitinated protein conjugates. (5). Removal of ubiquitin by isopeptidases. (6). ATP-dependent degradation of the multi-ubiquitinated protein to peptides by the ubiquitin conjugate-degrading enzyme.

transferred either to certain protein targets or to protein substrates which are pre-bound to molecules of ubiquitin protein ligase, usually called E3; such protein substrates are available for degradation (see Fig. 1.2).

1.2.3.1 Ubiquitin activating enzyme

E1 consists of a polypeptide chain with an M_r value of 105 000 (Ciechanover et al., 1982), which probably exists *in vivo* as a dimer joined by weak subunit interactions, although monomeric forms have been detected (Ciechanover et al., 1982; Hatfield & Vierstra, 1989). Multiple forms of E1 have been identified in plants (Hatfield & Vierstra, 1989) but this has not been verified in other tissues.

1.2.3.2 Ubiquitin carrier proteins

Five carrier proteins of low molecular weight have been identified in rabbit reticulocytes; these have M_r values of 32 000, 24 000, 20 000, 17 000 and 14 000, respectively (Pickart & Rose, 1985a) and have been termed $E2_{32K}$, $E2_{24K}$, $E2_{20K}$, $E2_{17K}$, and $E2_{14K}$. More recently, an arsenite-sensitive, high molecular weight species has been identified with an M_r value of 230 000 and this has been termed $E2_{230K}$ (Klemperer et al., 1989). Dimers and tetramers of some of these species have been reported which are involved in weak subunit interactions (Haas & Bright, 1988). Although one ubiquitin molecule normally binds to each E2 monomer, the $E2_{17K}$ and $E2_{20K}$ species seem to be able to bind two ubiquitin molecules per monomer (Haas & Bright, 1988). Of the E2 species, only the $E2_{14K}$ form is known to be involved in the transfer of ubiquitin to substrate proteins bound to E3 destined for degradation (Pickart & Rose, 1985a). In contrast, the $E2_{32K}$, $E2_{20K}$, $E2_{14K}$ and $E2_{230K}$ species are all able to ubiquitinate

histones in an E3-independent manner (Haas et al., 1988; Pickart & Vella, 1988b). The role of the $E2_{17K}$ and $E2_{24K}$ species is unknown, although it is possible that these may be degradation products of the $E2_{20K}$ and $E2_{32K}$ species, respectively (Haas & Bright, 1988).
The gene structure of the E2's has recently been examined and in yeast the $E2_{20K}$ and $E2_{32K}$ species have beeen shown to be the products of the RAD6 gene and CDC34 gene, respectively (Jentsch et al., 1987; Goebl et al., 1988). The former of these genes was previously known to be involved in DNA repair and the latter is essential for the progression of the cell through the S/G_1 boundary of the cell cycle suggesting that ubiquitination of certain proteins is necessary for the continuation of the cell cycle.

1.2.3.3 Ubiquitin protein ligase

Binding of protein substrates to E3 and their subsequent ubiquitination by activated ubiquitin species is the least understood conjugation reaction. It is, however, one of the most important steps, as due to the substrate specificity of E3, selectivity is determined as to the proteins which become ubiquitinated (for review, see Ciechanover & Schwartz, 1989). E3 has an M_r of 250 000; a subunit with an M_r value of 180 000 is an essential component (Hershko et al., 1986). Different E3 activities have been separated which show distinct but overlapping substrate specificities (Lee et al., 1986); no purified forms have been characterized, however. A major contribution to the understanding of the E3 mechanism has come from work by Reiss et al. (1988) who have identified three distinct protein-binding sites on E3; one for proteins with a basic N-terminus, another for proteins with a hydrophobic or bulky N-terminus and a further site for proteins that do not interact at their N-termini. More recently, it has been

shown that E2 can form a complex with E3 and that ubiquitinated proteins are more tightly bound to E3 than to non-ubiquitinated proteins (Reiss et al., 1989). This suggests a mechanism in which transfer of activated ubiquitin from E2 to a protein substrate bound to E3 is facilitated; once initially ubiquitinated, the modified substrate may be subject to the processive addition of multiple ubiquitin molecules.

The finding of specific binding sites on E3 for free N-termini explains the previous observations of proteins with blocked α-NH_2 groups at their N-termini being resistant to degradation in ubiquitin-dependent systems (Hershko et al., 1984a; Tanaka et al., 1983; Katznelson & Kulka, 1983; Breslow et al., 1986). Although Hershko et al. (1984a) proposed that the ubiquitination of free α-NH_2 groups might be an important signal for ubiquitin degradation, this notion has since been dispelled by the above work and by that of Butt et al. (1988) who have shown yeast metallothionein molecules, which are ubiquitinated at the α-NH_2 terminus are as stable as the non-ubiquitinated proteins.

Mayer et al. (1989b) used anti-E1 antibodies to show that proteins with acetylated N-terminal residues can be degraded in reticulocyte lysates by a ubiquitin-dependent pathway but not by a crude fraction of DEAE-cellulose-bound proteins (fraction II) to which ubiquitin had been added; this suggests that a factor, removed by the chromatographic step, is a separate protein ligase that specifically recognizes these structural feature(s) of the protein. As suggested by Lee et al. (1986), it is thus likely that multiple E3 species do indeed exist.

1.2.3.4 Effects of tRNA

Early studies showed tRNA to be necessary for the conjugation of certain proteins to ubiquitin (Ciechanover

et al., 1985). More recently, it has been established that only proteins with an acidic N-terminus are involved in this reaction; specifically, arginine residues are transferred to the N-termini of such substrate proteins enabling them to become better substrates for E3 (Ferber & Ciechenover, 1987). This transferase has been purified from rabbit reticulocytes: it has an M_r of 360 000 and is composed of several molecules of arginyl-tRNA synthetase and arginyl-tRNA-protein transferase (Ciechanover et al., 1988).

In the case of extracts of both cardiac and skeletal muscle, formation of ubiquitin conjugates to exogenous and endogenous proteins has been observed (Gehrke & Jennissen, 1987; Fagan et al., 1987), indicating that an active conjugation pathway exists in these tissues. No individual components of this pathway, however, have been identified to date; whilst it is assumed that a similar mechanism involving E1, E2 and E3 proteins must be present, the precise nature of these muscle components is not known.

1.2.4 Model for ubiquitin-dependent degradation

Although the ubiquitin-dependent proteolytic pathway has been extensively studied in reticulocytes, some other tissues have also been shown to degrade certain proteins in a similar manner. In rabbit skeletal muscle and liver, ubiquitin-dependent degradation of both endogenous and exogenous proteins has been described (Fagan et al., 1987) and this is true also of eye lens tissue (Jahngen et al., 1986) and of plants and yeast (Vierstra and Sullivan, 1988). In other tissues, however, conjugates have been identified which are not degraded, including conjugates found in rabbit cardiac muscle and stable actin conjugates of muscle (Ball et al., 1988; Atida & Kulka, 1982; Gehrke & Jennissen, 1987). Thus the pertinent question of why some conjugated proteins are degraded and others are not, has

still to be answered. A major advance came from the work of Chau et al. (1989) who showed that the addition of a multi-ubiquitin chain of up to 20 ubiquitins, (joined by isopeptide linkages between their individual C-termini and lysine 48 residues on the preceeding member of the chain) acts as a signal for protein degradation in a model system involving an "engineered" protein substrate studied in reticulocyte and yeast extracts. Bachmair & Varshavsky (1989) have since incorporated these findings into a general model of protein breakdown for short-lived proteins. Two determinants of a substrate protein are necessary in order for it to be degraded; the one is a destabilizing N-terminal amino acid (in accordance with the N-End Rule) and the other is a mobile region of the substrate protein containing a lysine residue. The proposed mechanism is that a protein with a destabilizing N-terminal amino acid is able to reversibly bind to E3; the off-reaction is slowed by the attachment of the "mobile" lysine residue to a separate site on E3. Ubiquitin thiolester linked to an E2 molecule (already bound to E3) is then conjugated to the lysine residue and a multi-ubiquitin chain is formed by the processive addition of activated ubiquitin molecules; it is this chain that acts as the protease-recognition site.

The model developed by Varshavsky's laboratory is able to explain the stability of mono-ubiquitinated proteins compared with the instability of multi-ubiquitinated species, as well as being compatible with the more recent data on the E3 binding sites (Reiss et al., 1989) and the conformational changes that ubiquitin undergoes when conjugated to the substrate protein (Ecker et al., 1989).

There are, however, some processes in this model which require further clarification; the least understood mechanism is how the multi-ubiquitin chain is processively added to the substrate protein. Although the initial ubiquitination of the lysine residue of the protein substrate is readily explained by the findings of

ubiquitinated conjugates being more tightly bound to E3 and that of E2 carrying activated ubiquitin (E2-Ub) being able to bind to E3 (Reiss et al., 1989), it is not yet possible to understand how the rest of the chain is formed. In particular, a major problem concerns how each ubiquitin moiety, carried by a ubiquitin carrier protein, is delivered specifically to the lysine 48 residue of the preceeding ubiquitin molecule to form the specific isopeptide linkage required for the formation of the multi-ubiquitin chain. It is possible, for example, that the $E2_{14K}$ species is specifically able to recognize the multi-ubiquitin chain, but on the other hand, it may be necessary for this E2-Ub species first to bind to E3, after which isopeptide bond formation to the preceeding ubiquitin could occur. This latter possibility can be visualized as occurring on two adjacent binding sites and after isopeptide bond formation, the E3 molecule could move, in a mechanism analogous to that of protein translation on the ribosome, so that the ubiquitin binding site would be free for further binding of an E2-Ub species and subsequent isopeptide bond formation to the growing multi-ubiquitin chain. Further unexplained aspects require resolution: how the length of the multi-ubiquitin chain is controlled, and how such a complex, once formed, is released from E3. Presumably, steric hindrances resulting from the length of the multi-ubiquitin chain could prevent further ubiquitin addition but release of the complex from E3 is a problem because Reiss et al. (1989) have demonstrated ubiquitinated proteins to be more tightly bound to E3. It is indeed possible that the E3 species is not released from the ubiquitinated substrate and this possibility is supported by the work of Hough et al. (1986) who suggested that other proteins apart from ubiquitin and the substrate are involved in the formation of high molecular weight conjugates.

1.2.5 Degradable ubiquitin conjugates

According to the model of Bachmair & Varshavsky (1989), only those proteins that are multiply ubiquitinated will be degraded. To support this notion, these authors demonstrated that the short-lived forms of β-galactosidase in their particular system were multi-ubiquitinated, whilst the long-lived forms were not (Bachmair et al., 1986). Other proteins degradable by the ubiquitin-dependent pathway have also been shown to be short-lived. Specifically, lysozyme has been considered a model substrate which forms rapidly degraded, high molecular weight conjugates (Hershko et al., 1984b) in processes which entail the formation of multi-ubiquitinated structures (Hershko & Heller, 1985). It has not been clarified, however, whether one specific lysine residue is multi-ubiquitinated or whether one or more of the other five are involved in this reaction. Similarly, Shanklin et al. (1987) have shown that the plant protein, phytochrome, becomes multi-ubiquitinated and degraded after red light treatment. In this case also it is not known whether the protein is ubiquitinated at more than one site but a recent possible conjugation site near to the C-terminal domain of phytochrome has been identified (Shanklin et al., 1989).
A number of other proteins degraded by the ubiquitin-dependent pathway have been identified in the past, although the question of their possible multi-ubiquitination was not addressed. Abnormal proteins were some of the first proteins to be recognized as degradable substrates of the ubiquitin-dependent pathway (Speiser & Etlinger, 1982) and Hershko et al. (1982) were among the first workers to demonstrate that abnormal proteins were in fact degraded faster in a ubiquitin-dependent manner. This was conclusively demonstrated by Ciechanover et al. (1984) in the thermolabile mutant, ts85, in which the bulk of the abnormal and short-lived proteins were degraded in a process requiring the function of intact E1 molecules. The

short-lived α-globin chains produced in excess in β-thalassemic patients are similarly degraded (Shaeffer et al., 1988). The fact that abnormal proteins are good substrates for ubiquitin-dependent degradation is likely to be due to their being partially unfolded; this is in keeping with the model of Bachmair & Varshavsky (1989) in that the partially unfolded proteins would contain more mobile lysine residues than their stable counterparts and thus be better E3 substrates. Some workers, however, have shown that unfolded but not abnormal proteins are not preferred substrates for ubiquitin-dependent degradation (Evans & Wilkinson, 1985; Rote & Rechsteiner, 1986), and this is at variance with the Bachmair & Varshavsky model. It is possible, however, that these proteins each containing the second determinant of a "mobile" lysine residue, do not contain the appropriate N-terminus.

Another discrepancy concerning substrate degradability arises from the use of abnormal globin as a ubiquitin-dependent substrate; whilst Chin et al. (1982) found that abnormal globin was a good substrate for ubiquitin-dependent proteolysis and that the amount of ubiquitinated hemoglobin was proportional to the degradation rate of hemoglobin, Fagan et al. (1986) have reported that this substrate, despite forming ubiquitin conjugates, is not degraded in a ubiquitin-dependent manner but rather by an ATP-independent mechanism. The use of abnormal globin as a substrate for the ubiquitin-dependent pathway is thus questionable.

Skeletal muscle contains many proteins that can readily become damaged by a variety of mechanisms; this would especially be the case after periods of prolonged muscle exertion or from chemical modifications including oxygen-derived free radicals, etc. The fact that red muscle proteins are degraded more rapidly than those of white muscle has been postulated to be due to it being more exposed to oxidative stresses; interestingly, Riley et al. (1988) have found the rat soleus muscle (red oxidative

fibres) to contain twice as much ubiquitin as the EDL muscle (white glycolytic), consistent with a role for ubiquitin in the degradation of oxidatively modified proteins. In fact, Hershko et al. (1986) have shown proteins containing oxidized methionine residues to be better substrates for ubiquitin-dependent degradation. On the other hand, muscle proteins damaged by free radical attack were degraded faster than control proteins but this was not dependent on ubiquitin; instead, an ATP-independent proteolytic system appeared to be more important (Davies, 1986).

It has become clear that not all protein substrates degraded by the ubiquitin system are multi-ubiquitinated. Calmodulin is considered to be one of the classic substrates for ubiquitin-dependent degradation; the non-mammalian form is ubiquitinated at lysine 119 and is degradable, whilst the mammalian form containing a methylated lysine 119 is not conjugated to ubiquitin and is more stable (Gregori et al., 1985; Gregori et al., 1987). The ubiquitinated and degradable form of calmodulin contains only one ubiquitin molecule per calmodulin molecule, however, and this fact is discordant with the model of multi-ubiquitinated proteins postulated by Bachmair & Varshavsky. Ziehenhagen et al. (1988) have now shown, however, using the methylated mammalian form of calmodulin, that both reticulocytes and cardiac muscle extracts can modify this protein with up to three ubiquitins per calmodulin molecule, in a process that requires Ca^{2+} as well as ATP and Mg^{2+} (Ziegenhagen & Jennissen, 1988; Jennissen & Laub, 1988). This suggests that calmodulin may undergo conformational changes in the presence of calcium and may be able to bind to E3.

1.2.6 Stable ubiquitin conjugates

Evidence is accumulating which suggests that populations of stable mono-ubiquitinated proteins exist in living cells. The most widely known of these are the histone-ubiquitin conjugates where 10% of the intracellular ubiquitin is conjugated to H2A and H2B (Carlson & Rechsteiner, 1987). The ϵ-amino groups at lysine 119 in H2A molecules (Goldknopf & Busch, 1978) and those at lysine 120 in H2B molecules (Thorne et al., 1987) have been shown to be the ubiquitinated residues. Polyubiquitinated histones have also been identified (Nickel & Davie, 1989) and these appear to be formed by the $E2_{20K}$ and $E2_{32K}$ species (Pickart & Vella, 1988b; Haas et al., 1988). Other non-histone nuclear proteins have recently been identified as ubiquitin conjugates but whether they are similarly conjugated in an E3-independent mechanism is not known (Blanchard & Billett, 1988). The role of ubiquitinated histones is unclear, but they have been shown to disappear during metaphase of the cell cycle (Mueller et al., 1985; Raboy et al., 1986) and it is thought possible that they may play a role in the transcription of active areas of the gene.

A puzzling aspect of the E3-independent ubiquitin conjugation of proteins, is how the specificity of ubiquitin conjugation to specific residues is preserved; specifically in the case of H2A and H2B, lysines 119 and 120 are always ubiquitinated, respectively, but how this specificity occurs in the absence of a protein ligase is mystifying. That more than one carrier protein is involved in this process and with very different M_r values (230 000 compared to 14 000) suggests that ubiquitination is not just due to the steric accessibility of the lysine residues on the histones, but indicates that some form of recognition between E2-Ub and histones must occur. Whether the other mono-ubiquitinated proteins are as specifically ubiquitinated is not known as no such intensive studies have been undertaken.

Recently, ubiquitin has been found to be present in a number of cell surface receptors, specifically lymphocyte homing receptors (Siegelman et al., 1986; St John et al., 1986), platelet-derived growth factor receptors (Yarden et al., 1986), growth hormone receptors (Leung et al., 1987) and proteins on the surfaces of rat cerebral cortex synaptosomes (Meyer et al., 1986). The finding of Raboy et al. (1986) of high molecular weight ubiquitin conjugates in the membrane-bound fraction of hepatoma cell homogenates suggests that ubiquitin has further roles to play on cell surfaces. How these ubiquitin residues reach an extracellular location, however, and the functions they perform once there, are questions which are still unanswered.

Another ubiquitin conjugate not destined for degradation is that of the outer mitochondrial membrane protein, monoamine oxidase B; the formation of a ubiquitin conjugate has been shown to be necessary for the incorporation of this protein into the outer mitochondrial membrane (Zhaung & McCauley, 1989).

Certain structural proteins are known to form stable ubiquitin conjugates; in particular, actin molecules of Drosophila flight muscle are ubiquitinated proteins which form part of the active actin unit (Ball et al., 1987). Similarly, both the microtubule network of cultured cells (Murti et al., 1988; Shaw & Chau, 1988) as well as intermediate filament proteins are ubiquitinated (Ohta et al., 1988). Ubiquitin is also associated with intermediate filaments in non-membrane-bound inclusions in certain neurodegenerative diseases and diseased tissues (Lowe et al., 1988; Manetto et al., 1989); specifically this is the case in the neurofibrillary tangles (NFT) of Alzheimers' disease (Mori et al., 1987; Perry et al., 1987; Cole & Timiras, 1987; Brion et al., 1989; Manetto et al., 1988), Lewy bodies of Parkinsons disease (Bancher et al., 1989; Kuzuhara et al., 1988), Pick bodies of Pick's disease (Love et al., 1988) as well as Mallory bodies of alcohol-damaged

hepatocytes (Ohta et al., 1988). The role of ubiquitin in these inclusions is not known; they may represent abnormal proteins that are ubiquitinated or they may reflect a defect in the degrading pathway. On the other hand, it is possible that their presence is simply due to the sequestration of normally ubiquitinated intermediate filaments and microtubules, which become predominant protein species in the inclusions.

1.2.7 Ubiquitin pools and their distribution

The concentrations of free and bound forms of ubiquitin in cell systems can be investigated either by microinjection of ^{125}I-ubiquitin into whole cells (where feasible) or by immunological quantitation of free and conjugated ubiquitin molecules in extracts. Using these methods, in a variety of cell types, an average of 50% of total ubiquitin has been found to be present in conjugated forms, with an upper limit of 80% in reticulocytes (Haas & Bright, 1985) and a lower limit of 30% in mouse fibroblasts (Haas et al., 1987). As regards whole tissues, eye lens and muscle have been most extensively studied: while 25% of the intracellular ubiquitin was conjugated in skeletal muscle (Riley et al., 1988), only 5% was conjugated in lens tissue cores (Jahngen et al., 1986). A similar distribution of ubiquitin among conjugates was established in HeLa cells where 40% of the ^{125}I-ubiquitin was conjugated to cytosolic proteins, 10% to histones, 15% was tied up in thiolester linkages and 35% was free (Carlson & Rechsteiner, 1987).
In skeletal muscle, ubiquitin conjugates seem to be widely distributed over the myofibrils, mitochondria and intermyofibrillar regions, although an enrichment in respect to ubiquitin conjugates was detected in the Z-line region (Riley et al., 1988). A similar wide distribution of ubiquitin has been demonstrated in hepatoma cells; not only was ubiquitin present in the cytoplasm, nucleus and microvilli but it was also found in vacuoles and lysosomes

reports concerning the degradation of accumulated conjugates exist; Parag et al. (1987) and Bond et al. (1988) have shown that moderate heat-shock caused a transient increase in protein degradation, while Carlson & Rechsteiner (1987) surprisingly showed a rather small decrease in proteolysis after heat-shock. This latter result has been suggested to be a result of over-heating, rather than an effect of the heat-shock response (Pratt et al., 1989).

A model for ubiquitin in the heat shock response has been proposed by Munro & Pelham (1985). Under normal conditions, a heat-shock transcription factor is thought to be present in an inactive ubiquitinated state, although the ubiquitin moiety is continually removed and replaced. Upon heat-shock, due to the free ubiquitin pools decreasing, a non-ubiquitinated active form of the fraction predominates and this promotes the transcription of heat-shock genes. Obviously, the identification of such a ubiquitinated factor is essential for the verification of this model.

Although skeletal muscle tissue can regularly rise transiently to temperatures of 41°C during certain forms of exercise (Nadel, 1983) no role of the heat-shock response or the ubiquitin system in this tissue has been proven.

1.2.9 Age-related changes of ubiquitin

The maturation of reticulocytes into erythrocytes is the best-studied system for showing changes in the ubiquitin-dependent pathway with increasing age of an organism or cell type in an age-dependent manner. Specifically, a decrease in ATP- and ubiquitin-dependent degradation of proteins has been extensively reported (Speiser & Etlinger, 1982; Fagan et al., 1986). This decrease is partially due to a drop in E3 levels (Raviv et al., 1987) as well as to a fall in E2 species, specifically the $E2_{20K}$ and $E2_{14K}$ species (Pickart & Vella, 1988a). Concomitant with the

decrease in ubiquitin-dependent degradation is a decrease in the activity of hexokinase, an enzyme, which has been shown to be degraded in a ubiquitin-dependent manner (Magnani et al., 1986). These changes in reticulocytes probably reflect changes in this type of cell's functional role rather than a general age-related changes in the ubiquitin-dependent pathway. Similarly, the process of spermatogenesis has been shown to proceed with a decrease in the concentrations of free ubiquitin and ubiquitin conjugates, in an age-related manner, going from spermatids to mature spermatozoa (Agell & Mezquita, 1988).
Lens tissue also undergoes a decrease in the levels of ubiquitin conjugates and degradation with time, possibly due to a progressively more inefficient conjugation pathway coupled to an inefficient degradation pathway (Jahngen et al., 1986). Such a decrease in ubiquitin-dependent proteolysis can possibly lead in the older tissues to the accumulation of abnormal proteins which may be related to the formation of cataracts.
In skeletal muscle, although characteristic physiological changes occur during aging which includes the loss of muscle fibres and an increased variation of the size of the muscle fibre (Shafiq et al., 1978), it is not known if these are related to altered rates of protein degradation and specifically to changed levels or activities of the components of the ubiquitin-dependent pathway.

1.2.10 Isopeptidases and hydrolases

Regeneration of functional ubiquitin from degradable protein substrates, stable conjugates or non-protein adducts is an essential process for the maintenance of free ubiquitin levels. Three types of disassembling activities have been identified to date: one that removes ubiquitin from non-protein substrates and is termed ubiquitin-carboxyl terminal hydrolase (Pickart & Rose, 1985b),

another that disassembles ubiquitin-protein conjugates joined by isopeptide bonds and is known as an "isopeptidase" (Matsui et al., 1982) and thirdly, an activity that cleaves the peptide bond between poly-ubiquitin and ubiquitin fusion-protein gene products, which is termed (α-NH-ubiquitin)protein endoprotease (Jonnalagadda et al., 1989).

Ubiquitin-carboxyl terminal hydrolases are the best-studied entities and are involved in the regeneration of ubiquitin from small ubiquitin derivatives, either ubiquitin amides and thiolesters that have formed during the conjugation reaction, or ubiquitin peptides that are the probable end-products of ubiquitin-dependent proteolysis (Pickart & Rose, 1985b). One such activity has been identified in reticulocytes and calf thymus with an M_r of 30 000 (Rose & Warms, 1983; Mayer & Wilkinson, 1989); a detailed enzyme mechanism has been elucidated in this case (Pickart & Rose, 1986).

An isopeptidase activity specific for histones is clearly important when considering the de-ubiquitination of histones that occurs during metaphase: a 38 000 M_r species has been identified that removes ubiquitin from histone H2A, is inhibited by thiol inhibitors and metal ions (Matsui et al., 1982) and only cleaves ε-amino bonds (Kanda et al., 1986).

Isopeptidase activity on cytosolic protein substrates have been described with M_r values of 400 000, 300 000 and 100 000 (cited in Hershko & Rose, 1987) and 200 000 (Hough et al., 1986), indicating that more than one species exists. A specific substrate has been synthesized for the assay of isopeptidase activity, namely ubiquitin ethyl ester (Wilkinson et al., 1986a) with which four disassembling activities have recently been identified in calf thymus (Mayer & Wilkinson, 1989). These have been further characterized by the use of an affinity column specific for ubiquitin-binding proteins (Duerksen-Hughes et al., 1989).

Although post-translational processing of the ubiquitin fusion gene product is an essential activity for the formation of monomeric ubiquitin, not much about these (α-NH-ubiquitin)protein endoproteases is known. Recently multiple forms of this activity have been found with M_r values between 150 000 and 200 000; these are sensitive to thiol inhibitors (Jonnalagadda et al., 1989).
Due to the importance of isopeptidases and hydrolases in the maintenance of free ubiquitin pools, it is likely that skeletal muscle contains ispoeptidase activities similar but possibly not identical to those previously mentioned; to date no such activities have been described in this tissue.

1.2.11 Ubiquitin conjugate-degrading enzyme

A high molecular weight protease that preferentially degrades ubiquitin-lysozyme conjugates rather than free lysozyme, in an ATP-dependent manner, has been described in reticulocytes (Hough et al., 1987; Waxman et al., 1987). This protease sediments at 26S on a glycerol gradient and has been estimated to have an M_r of 1 500 000, with a number of subunits, determined by SDS-PAGE, having M_r values of 34 000 -110 000 (Hough et al., 1987). Recently, this protease was found to be composed of three factors; two heat-labile components, CF-1 and CF-2 which have M_r values of 600 000 and 250 000, respectively, and a heat-stable component, CF-3, which has an M_r of 650 000 (Ganoth et al., 1988). All three factors, as well as ATP and Mg^{2+}, are required for the formation of the active protease, suggesting that at least one role of ATP in ubiquitin-dependent protein degradation is the formation of this high molecular weight complex.
Similar high molecular weight activities have been described in rabbit skeletal muscle (Fagan et al., 1987; Matthews et al., 1989) and baby hamster kidney (BHK) cells

(McGuire et al., 1988). These activities have a complex inhibition profile, being sensitive to the protease inhibitors, chymostatin and leupeptin, as well as hemin and thiol reagents (Hough et al., 1987). Recently, the reticulocyte protease has been shown to be inhibited by polyamines, in particular by spermine, although the site of action is not known (Wajnberg & Fagan, 1989).

1.2.12 Ubiquitin-independent proteases

1.2.12.1 Proteasomes

A major proteolytic activity that co-purifies through many of the chromatographic steps with the ubiquitin conjugate-degrading enzyme, has been identified as the proteasome (Hough et al., 1987; Waxman et al., 1987; Fagan et al., 1987). This activity was first isolated in lens tissue (Wilk & Orlowski, 1983) and since then has been well characterized in a number of tissues including rat skeletal muscle (Dahlmann et al., 1985), rat liver (Tanaka et al., 1986; Rivett, 1989) and fish muscle (Folco et al., 1988; Hase et al., 1980). Proteasomes are found throughout eukaryotes (Tanaka et al., 1988; Falkenburg & Kloetzel, 1989) and have recently been identified in archaebacteria (Dahlmann et al., 1989a) indicating a more widespread distribution than ubiquitin. This protease was recently identified as being identical to the 19S ribonucleoprotein particles known as prosomes (Falkenburg et al., 1988; Arrigo et al., 1988) and has been shown to have rRNA degrading activity (Tsukahara et al., 1989). Thus although this protease has a widespread distribution, as yet it has no defined function.

The proteasome has an approximate M_r value of 600 000 and is composed of four to ten subunits with M_r values between 24 000 and 32 000. Upon electron microscopy this protease is composed of four stacked rings which give their

characteristic cylinder-like shape (Baumeister et al, 1988).

Whilst much is known about the structure and distribution of this protease, its role in the cell is undefined. One of its characteristics is the multi-catalytic activity identified by Wilk & Orlowski (1983). Originally this protease was thought to be ATP-independent but recent work has shown it in fact to have a detectable ATP-dependence (Driscoll et al., 1989; Tanaka & Ichihara, 1988; McGuire & DeMartino, 1989). Of great interest is the observation that the proteasome is identical to the component CF-3, of the ubiquitin conjugate-degrading enzyme (Eytan et al., 1989) and this is further supported by the observation of immunological cross-reactivity between the two species (Matthews et al., 1989). Thus the exact relationship between these two proteases awaits further clarification.

A similar, but not identical multi-catalytic protease has been described in BHK cells, which is present at low levels under normal growth conditions. In the presence of protease inhibitors, however, the levels of this protease rise to 8% of the total protein of the crude cellular extract and is accordingly termed PABI (protease accumulated by inhibitors) (Tsuji & Kurachi, 1989). Whether this protease is present in muscle tissue is not known, but it would be difficult to detect in crude extracts due to its low levels and also to the fact that its catalytic activities are similar to that of the proteasome.

1.2.12.2 ATP-stimulated proteases

Although ATP-dependent proteases are well-defined and well-characterized in E. Coli (Menon & Goldberg, 1987; Woo et al., 1989; Katayama et al., 1988) and mitochondria (Watabe & Kimura, 1985), in eukaryote cytoplasm the situation is more confusing. An ATP-stimulated activity has been described in erythroleukemia cells (Waxman et al., 1985;

Rieder et al., 1985) which seems to be identical to the ATP-stimulated proteasome activity as judged by immunological cross-reactivity (Tsukahara et al., 1988a). More recently, an ATP-requiring protease has been partially purified from chicken skeletal muscle, which although similar to the activity from erythroleukemia cells, differs from that of the multi-catalytic protease (Fagan & Waxman, 1989).

Another high molecular weight ATP-stimulated protease has been described in cardiac muscle (DeMartino, 1983; Gehrke & Jennissen, 1987) and skeletal muscle (Dahlmann et al., 1983; Ismail & Gevers, 1983), but the role of this protease and its relationship to the ATP-stimulated proteasome are still unresolved.

Thus within the cytosol, various proteases have been identified and partially characterized. Although the relationships between these high molecular weight proteases are ill defined, it is possible that some of these species are components of the ubiquitin conjugate-degrading enzyme. One hopes that further developments in this field will clarify the rather confusing situation that presently exists.

1.3 THE CALPAIN SYSTEM

Despite the fact that the calpain proteolytic pathway operates in the same cellular environment, at the same pH and in contact with the same pool of potential substrates as the ubiquitin-dependent pathway, no correlation or mechanistic inter-relationship between the two proteolytic systems has been described, suggesting that they represent two independent pathways with different cellular functions.

The calpain system, although perhaps not as complex as the ubiquitin-dependent pathway, is distributed throughout many eukaryote systems including insects (Pinter & Friedrich, 1988), fish (Toyohara & Makinodan, 1989), crustaceans (Mykles & Skinner, 1986), reptiles (Kleese et al., 1987), birds (Ishiura et al., 1978) and mammals (Pontremoli & Melloni, 1986). Despite recent advances in the elucidation of the structure of calpains, the role(s) in vivo of these proteases as well as the question of their activity at intracellular Ca^{2+} concentrations are still unresolved. Studies of the action of calpains in different cell types suggests generally that these proteases, rather than degrading bulk proteins non-specifically to the level of amino acids, are instead involved in the selective and limited cleavage of certain substrate proteins. This in turn causes major and irreversible functional alterations in cells; a role far removed from the breakdown of abnormal or short-lived proteins apparently characteristic of the ubiquitin-dependent pathway. It is feasible, however, that some of the partially degraded calpain substrates could be degraded by the ubiquitin-dependent system.

The calpain system has been shown to be composed of at least two forms of the protease calpain together with an endogenous inhibitor, calpastatin (for review, see Murachi et al., 1981b); in some instances, a calpain activator has also been described, although this entity has not been purified to homogeneity (DeMartino & Blumenthal, 1982; Pontremoli et al., 1988a; Takeyama et al., 1986). Varying levels of calpain and calpastatin occur in different cell types, presumably a reflection of the role(s) of the calpain pathway in particular cell types; in skeletal muscle, the level of the calpains (under V_{max} conditions) is approximately equivalent to that of the calpastatins (Murachi et al., 1981a; Kawashima et al., 1988). The calpains are readily separable from calpastatin under Ca^{2+}-free conditions by either DEAE-cellulose, gel filtration or hydrophobic interaction chromatography.

Historically, the two main calpain forms were separated from each other by DEAE-cellulose chromatography and these species were called calpain I and calpain II, repectively in their order of elution off the column with a linear salt gradient (Murachi et al., 1981b). The former activity is active at μM Ca^{2+} concentrations whilst the latter is only active at mM concentrations of this ion; although calpain I can thus be envisioned as being active at physiological intracellular Ca^{2+} concentrations, the problem of how calpain II is ever active within living cells is still unsolved. Thus an understanding of the entire calpain system encompasses not only the structure and functions of the calpain species but also how the intracellular Ca^{2+} concentration is regulated.

1.3.1 Structure of the calpains

The mechanism of action of the calpains depends on the molecular structure of the two species of enzyme and this has provided an intriguing study in terms of the evolution of this molecule. The calpain I and calpain II species are heterodimers, each having two subunits with M_r values of 80 000 and 30 000, respectively. Recently, advances in the structural characterization of these proteins have been made due to the isolation of cDNA clones of both calpain species from different tissues. The M_r = 30 000 subunit is identical in both calpains I and II, being derived from the same gene (Kawasaki et al., 1986; Kikuchi et al., 1984; Emori et al., 1986a). In both rabbit and porcine tissues, this protein has been shown to be composed of a C-terminal region containing a calmodulin-like Ca^{2+} binding site involving four E-F hand structures and an N-terminal domain which is predominately composed of hydrophobic amino acids (Minami et al., 1988; Emori et al., 1986a; Sakihama et al., 1985). This subunit, although not containing any proteolytic active site, is important in the Ca^{2+}-dependent

subunit association with the M_r = 80 000 subunit, which results in a heterodimer having an increased proteolytic activity compared with the monomeric forms (Imajoh et al., 1987; Hatanaka et al., 1985).

The M_r = 80 000 subunits of calpain I and II have been shown to be coded by separate genes (Emori et al., 1986b), although homology of a part of the domain structure is conserved. This polypeptide is composed of four domains of which the second (II) has an amino acid sequence similar to that of the active site of cysteine proteases, whilst the fourth (IV) contains a calmodulin-like Ca^{2+} binding region; domains I and III as yet have no known function (Ohno et al., 1984; Emori et al., 1986c; Aoki et al., 1986; Imajoh et al., 1988). Such a structure containing two unrelated protein segments suggests that during evolution, fusion of a cysteine proteinase with a calmodulin gene might have occurred, resulting in a uniquely regulated protease.

In muscles, from various species, two forms of calpain have also been described which are in keeping with the general calpain structure (Dayton et al., 1981) although recently Sorimachi et al. (1989) have described a higher molecular weight species in rat and humans which is exclusive to skeletal muscle. In chicken smooth (Hathaway et al., 1982) and skeletal muscle (Ishiura et al., 1978), however, only one form of calpain was initially identified, with a Ca^{2+} sensitivity lower than that characteristic of calpain II. This led to the suggestion that there may only be one form of calpain in the chicken, distinct from mammalian species where two calpain forms are present. This notion has, however, been disputed recently by other workers (Murakami et al., 1988; Wolfe et al., 1985).

1.3.2 Calpastatin structure

Calpastatins, like the calpains, are intracellular proteins occurring in many different cell types. They are

characteristically heat-stable and inhibit calpains by forming a complex with them in the presence of Ca^{2+} i.e. not by sequestering Ca^{2+} from the environment (Murachi et al., 1981b). Although the inhibitory action is believed to be specific for calpains, Murakami & Etlinger (1986) found that calpastatin inhibited a high molecular weight protease from human erythrocytes (similar to the proteasome), in a Ca^{2+}-independent manner. This suggests that the calpastatins may be able to inhibit more than one major cytosolic protease and that an inter-relationship between these two pathways may exist. Extracellular inhibitors of calpains have also been shown to exist; these include the kininogens (Schmaier et al., 1986; Ishiguro et al., 1987) and α_2-macroglobulin (Crawford, 1987; Sasaki et al., 1983). Thus the activities of calpains in an extracellular environment (probably arising from tissue damage) can be kept to a minimum.

That maintenance of correct calpastatin levels may be important has been suggested in hypertensive rats and humans, where the defective function can be speculatively explained by a decrease in steady-state calpastatin levels and hence an increase in the level of uncontrolled calpain activity (Pontremoli et al., 1987; Pontremoli et al., 1988b).

Calpastatins were first isolated by Guroff (1964) in brain tissue. Subsequently, calpastatins with very many different M_r values have been reported. Although the native M_r by gel filtration has been estimated to be approximately 280 000, and to be composed of more than one subunit (Murachi et al., 1981b; Melloni et al., 1984), the reported monomeric M_r values range from 170 000 (Lepley et al., 1985) to 34 000 (Yamoto et al., 1983; Takahashi-Nakamura et al., 1981). Calpastatin is, however, readily destroyed both by the action of proteases and during various purification steps, including heating; it is likely that this fact is responsible for the confusion concerning the true M_r value of this protein (Otsuka & Goll, 1987; Lepley et al., 1985).

Analysis of the cDNA structure of porcine and rabbit calpastatin has shown it to be composed of approximately 700 amino acids, giving an M_r of 70 000 (Takano et al., 1988; Emori et al., 1988). The monomer migrates anomalously, however, on SDS-PAGE with an apparent M_r value of 110 000; a phenomenon believed to be due to its high negative charge and not to post-translational modification (Takano et al., 1988).

The main characteristic of calpastatin is the presence of repeptitive domains, each composed of 140 amino acids, which are the functional units involved in calpain inhibition (Emori et al., 1987; Maki et al., 1987); in rabbit liver, four such domains have been identified, while in erythrocytes only three such structures are present (Maki et al., 1988). Within each repetitive domain, there is a highly homologous region of appproximately 30 amino acids which is believed to be the actual site of calpain interactions (Emori et al., 1988) and this is flanked by regions of α-helix which are important in stabilizing the tertiary structure of the inhibitory domain (Maki et al., 1988). Of interest is the finding that this homologous region involved in calpain binding shares a sequence of six amino acids similar to the Glu^{34}-Gly-Ile-Pro-Pro-Asp^{39} sequence of ubiquitin (Maki et al., 1988); whether this is of any functional significance is not known.

The structure of the repetitive functional domains of calpastatin explains why calpastatin fragments with M_r values as little as 17 000 can still retain inhibitory activity (DeMartino et al., 1988) as well as why from two (DeMartino et al., 1984; Takahashi-Nakamura et al., 1981) to ten (Otsuka & Goll, 1987) calpain molecules have been reported to be inhibited by one calpastatin molecule. It is likely that one functional domain of calpastatin can bind one calpain molecule, although definite proof of this is still needed.

Although calpastatin has usually been considered to be cytosolic in cellular location, certain reports have

suggested that a small percentage may also be membrane-associated, in particular with those of the sarcolemma and the sarcoplasmic reticulum (Mellgren, 1988; Mellgren et al., 1987). Interestingly, immunocytochemical studies on calpastatin distribution in skeletal muscles has shown this molecule to be associated not only with the Z-line of relaxed myofibrils but also with the sarcolemma (Lane et al., 1985). Recently, Murachi (1989) found that calpastatin can be phosphorylated and in this state is preferentially associated with the cell membrane; thus the membrane association of calpastatin may be physiologically important.

A central problem in the interaction of calpains and calpastatin is still largely unresolved; whilst Ca^{2+} is required for calpain activation, it is also necessary for calpain-calpastatin interaction. This problem has been specifically addressed by Otsuka & Goll (1987) who reported that the Ca^{2+} concentration required for calpastatin association with calpain was 0.53 mM Ca^{2+}, whilst calpain II is only half-maximally activated at 0.93 mM. This raises again the question as to how calpain II could ever be active in living cells. Presumably compartmentalization, or membrane binding of either or both of these activities, could be involved; apart from calpastatin possibly being able to bind to membranes, Gopalakrishna & Barsky (1986) have shown that a certain percentage of the calpains can be loosely associated with the cell membrane in a Ca^{2+}-independent manner.

In the case of chicken breast muscle, a calpastatin species has also been purified (Ishiura et al., 1982) with an M_r of 68 000; in the light of the model of calpastatin structure, it is likely to contain only two or three functional domains. Whether this particular form occurs naturally or is an artifact of the isolation procedure has not been defined.

1.3.3 Autolysis of calpain

As already mentioned elsewhere, the high Ca^{2+} requirements of calpain II are difficult to reconcile with the low physiological intracellular Ca^{2+} concentrations, which are normally in the submicromolar range. It is accordingly of considerable interest that calpain II, as well as calpain I, can undergo autolysis in the presence of Ca^{2+}, forming species of lower molecular weight that require lower Ca^{2+} concentrations for activity (Suzuki et al., 1981a; Dayton, 1982). In chicken gizzard (smooth) muscle, calpain II is autolyzed from a form requiring 150 μM Ca^{2+}, to a species requiring only 5 μM Ca^{2+} for half maximal activity (Hathaway et al., 1982).

During autolysis of calpain, the M_r = 80 000 and 30 000 subunits are converted into entities with M_r = 76 000 and 18 000, respectively; the N-terminal regions of both subunits being removed (Imajoh et al., 1986a). The M_r = 76 000 form is able to undergo further autolysis by removal of domain III, to yield a form with an M_r of 54 000 which has decreased proteolytic activity (Crawford et al., 1987). Although autolysis results in the formation of forms more sensitive to Ca^{2+}, a non-physiological concentration of this ion is still required to bring about autolysis (Hathaway et al., 1982). The Ca^{2+} requirement for autolysis can apparently be lowered in the presence of calpain substrates (Pontremoli et al., 1984a; Pontremoli et al., 1984b) and also in the presence of phospholipids (Coolican & Hathaway, 1984; Imajoh et al., 1986b; Pontremoli et al., 1985). This has led these workers to propose a model of calpain activation in which, after a small increase in the intracellular Ca^{2+} concentration, calpain binds to the cell membrane through the N-terminus of the 30 000 subunit (Imajoh et al., 1986a). Autolysis of calpain by both inter- and intra-molecular mechanisms (Inomata et al., 1988) is then thought to occur with the cleavage of the N-terminal regions of both the M_r = 80 000 and 30 000 subunits to

yield a free species of calpain, which is active at μM Ca^{2+} concentrations (for reviews, see Pontremoli & Melloni, 1986; Suzuki et al., 1987; Mellgren, 1987). This model has been supported by the observation of lag periods in the measurement of calpain activities (Coolican et al., 1986; DeMartino et al., 1986; Inomata et al., 1986) which is compatible with the notion of calpain existing in a pro-enzyme form, activated by membrane binding. This model, however, has been criticized by Cong et al. (1989) who have shown calpain I to be active at calcium concentrations lower than those required for autolysis, indicating that the enzyme may not require activation by these means. The Ca^{2+} concentration dependence of calpain II autolysis, on the other hand, parallels that of proteolysis, which again suggests that autolysis of calpain II may accompany its activity at low substrate concentrations. Furthermore, the finding that autolysed calpains still have a lower Ca^{2+} requirement for calpastatin interactions than for activation (Kapprell & Goll, 1989), suggests that many of the autolysed species will be inhibited by calpastatin, *in vivo*. Autolysis can thus occur *in vitro*, but its role *in vivo* is still dubious.

The events associated with calpain activation under various physiological conditions might fruitfully be re-examined in the light of recent models of intracellular Ca^{2+} regulation such as those proposed by Rasmussen (1989). It is thought that the notion of transient rises and falls in Ca^{2+} levels usually thought of in relation to the second messenger concept, (Carafoli, 1987), may be replaced (or extended) by changes in the rates of Ca^{2+} cycling between various pools of the ion, through membranes; for example, such events may be associated with prolonged responses such as smooth muscle contraction of blood vessels. An increase in Ca^{2+} influx, followed by an increase in Ca^{2+} efflux, may lead to an elevated Ca^{2+} concentration in a restricted location close to the cell membrane. Calpain species which are located in such a submembrane region could thus be

activated without having to undergo autolysis, although autolysis may occur as a terminating event.

1.3.4 Physiological functions of calpain

1.3.4.1 Calpains in muscle tissue

One of the first postulated physiological roles of the calpains was related to the ability of these enzymes to remove the Z-lines from myofibrils (Dayton, 1976; Reddy et al., 1975; Azanza et al., 1979); α-actinin, a component of the Z-line, was in fact found to be very susceptible to calpain degradation (Otsuka et al., 1988; Suzuki et al., 1978; Kulesza-Lipka & Jukubiec-Puka, 1985). This led to the notion that specific degradation of α-actinin by calpain might lead to the disruption of Z-lines and a breakdown of the myofibrils. This role of calpains could be envisaged as being more involved in the selective cleavage of a particular protein, which then gave rise, in a cascade, to further alterations catalyzed by other entities. This idea has subsequently been supported by other studies including those of Haeberle et al. (1985), who showed that treatment of skinned smooth muscles with calpain caused a loss of contractile activity, whereas the interaction between myosin and actin was not affected and the content of these two proteins was not altered; this is consistent with the idea that calpain was involved in the disruption of cytoskeletal structure(s) and not in the degradation of actin and myosin. Other myofibrillar proteins have, however, been found to be degraded by calpain, including the troponins, tropomyosin and myosin heavy chains (Hara et al., 1983; Azanza et al., 1979; Pemrick & Grebenau, 1984; Ishiura et al., 1979). Interestingly, Riley et al. (1987) have shown ubiquitin conjugates to be associated in rat skeletal muscle, with the Z-lines and these workers have suggested that α-actinin, due to its short half-life, may

be the ubiquitinated protein. Thus in this tissue it is possible that the ubiquitin-dependent and calpain pathways are inter-related.

The importance of the calpains in myofibrillar turnover has been supported by the findings that dystrophic muscles (Johnson & Hammer, 1988; Neerunjun & Dubowitz, 1979), denervated muscle (Hussain et al., 1978) as well as vitamin E-deficient muscles (Otsuka et al., 1985; Dayton et al., 1979) all have increased calpain activities; in particular, vitamin E-deficient muscles show losses of Z-lines, similar to the picture given by normal muscles after treatment with calpain. In cardiac muscle, certain stresses, especially starvation are associated with an increase in calpain activity and this has been thought to be related to increased catecholamine levels occurring as part of the stress response (Tolnai & von Althen 1987).

Apart from protein turnover, other functions of calpains in muscle have been suggested; in particular, the re-organization of neuromuscular junctions in developing muscle requires calpain activity, but the mechanism for this is obscure (Connold et al., 1986). The distribution of calpains in muscle tissue sections is indicative of some of the further roles of this protease; thus, structures including collagen fibrils, basal lamina, sarcolemma and neuromuscular junctions have been found to be associated with calpain (Badalememte et al., 1987).

1.3.4.2 Calpains and other structural proteins

Much evidence has accumulated over the last few years that is consistent with the involvememt of calpains in the transmission of cellular signals through the cytoskeleton, causing changes in cell shape upon specific stimuli, such as those encountered in platelet aggregation. That the calpains are able to degrade intermediate filament proteins, (IF), in particular a specific constituent, known

as vimentin (Nelson & Traub, 1982; Fischer et al., 1986; Traub et al., 1988) and that a calpain species has been purified closely assocated with these structures (Nelson & Traub, 1981; Ishizaki et al., 1985), is consistent with this role. The intermediate filaments of neuronal tissue (neurofilaments) are also excellent calpain substrates (especially in an unphosphorylated state (Pant, 1988)) whilst the nuclear intermediate filament structures, nuclear lamins, are not readily degraded by the calpains (Traub et al., 1988). These facts are suggestive of calpains being involved in causing changes in the cytoskeleton.

Apart from intermediate filaments, calpains can also partially cleave a class of proteins associated with the membrane-bound filamentous network. These structures are essentially composed of actin filaments bound to the cell membrane by specific proteins amongst which spectrin, actin-binding protein, and talin have been identified (Fox et al., 1987). Selective cleavage of these proteins by calpain (Fox et al., 1985; Onji et al., 1987; Collier & Wang, 1982; Beckerle et al., 1987) may be important in the disruption of the submembrane network and in cellular shape changes.

A particular specialized role for calpain has been proposed in the limited cleavage of brain spectrin (fodrin); an association has been found between the disruption of the fodrin network at the post-synaptic membrane by calpain and the appearance of glutamate receptors which are believed to be involved in long-term storage of memory (Lynch & Baudry, 1984). This has thus led these workers to suggest a role of calpain in memory storage (Lynch & Baudry, 1984; Lynch & Baudry, 1987; Siman et al., 1984). In addition, fodrin degradation by calpain is increased when calmodulin is associated with fodrin (Seubert et al., 1987; Harris et al., 1988); the fact that this association occurs only in the brain and not in the case of erythrocyte spectrin,

suggests that the phenomenon may be related to the specialized roles of calpain in the brain.

Associated with the actions of calpains in disrupting the submembrane cytoskeleton are studies showing calpains to be important in cell fusion. Interesting work at an immunofluorescent level in myoblasts has shown calpain II to relocate from a random, dispersed distribution in non-fusing cells to one that is peripheral and membrane-associated in fusion-competent cells; calpain I, on the other hand, maintained a general cytoplasmic distribution (Schollmeyer, 1986b; Schollmeyer, 1986a). This suggests that calpain II may cause the disassembly of cytoskeletal protein-membrane linkages. The inhibition of cell fusion by calpastatin is again indicative of the role of the calpains in this process (Glaser & Kosower, 1986).

Other components of the cytoskeleton have been shown to be subject to calpain-mediated degradation, in particular the microtubule proteins, tubulin and microtubule-associated protein (MAP) (Klein et al., 1981; Billger et al., 1988; Sato et al., 1986). The degradation of microtubules may be a component of the actions of calpain II in mitosis, since Schollmeyer (1988) showed that calpain II is associated with mitotic chromosomes. The observation that histones are degraded by calpains adds yet another item to the list of calpain actions being involved in relation to chromosomes (Sakai et al., 1987).

1.3.4.3 Activation of cellular enzymes and receptor proteins

Apart from the maintenance of cell structure, calpains have also been associated with the proteolytic activation of certain cellular enzymes; in fact, the first action of calpain to be described was that of the activation of phosphorylase kinase (Meyer et al., 1964; Drummond & Duncan 1966). Since then, other enzymes have been shown to be

activated and these include phophorylase phosphatase (Mellgren et al., 1979), tyrosine hydroxylase (Togari et al., 1986), transglutaminase (Ando et al., 1988b), glycogen synthetase (Hiraga & Tsuiki, 1986), Ca^{2+}- ATPase (Wang et al., 1988; Au et al., 1987) as well as platelet factor XIII (Ando et al., 1987). The protein kinases in particular, have been identified as substrates of calpain (Ito et al., 1987; Beer at al., 1984; Kajiwara et al., 1987); the best described of these is protein kinase C which is activated by calpain from a Ca^{2+}-dependent form to a Ca^{2+}-independent form (Kishimoto et al., 1983; Pontremoli et al., 1988a; Melloni et al., 1985).

Calpains also act as activators of receptor proteins such as the platelet-derived growth factor receptor (Ek & Heldin, 1986) and epidermal growth factor receptor (Cohen et al., 1982), as well as intracellular Ah receptors (Poland & Glover, 1988) and steroid receptors. Additionally, the activation of platelet factor V in the course of platelet activation apparently occurs by the action of calpain (Rodgers et al., 1987; Bradford et al., 1988). The physiological role of the calpains, however, in activating surface or extracellular proteins must be questioned when one considers that calpain is essentially an intracellular protein.

1.3.5 Role of calmodulin

Many of the above-mentioned calpain substrates including enzymes, receptors and structural proteins, are known to bind to calmodulin; an event which alters the rate of their degradation by calpain, as in the case of fodrin. Wang et al. (1989) have shown that many of the calmodulin-binding proteins contain a high number of so-called PEST sequences (Rogers et al., 1986); these authors have proposed that calpains recognize the calmodulin-binding protein through these PEST sequences so that the resulting proteolysis of

calmodulin-binding proteins can be controlled by the binding of calmodulin. In a manner analogous to that of the ubiquitin-dependent system, where ubiquitin binding marks proteins for degradation by the ubiquitin conjugate-degrading enzyme, calmodulin may control calpain-dependent proteolysis of calmodulin-binding proteins; further clarification of these ideas is obviously needed.
Due to the different roles presently connected to the ubiquitin-dependent and the calpain systems, it is difficult to envisage a scenario in which the two proteolytic pathways are connected in some functional context. That the two pathways are related has proved difficult to test because of the many different kinds of protein substrates examined; so far it seems that the ubiquitin-dependent system readily ubiquitinates cytosolic proteins, while the majority of the well characterized calpain substrates are membrane-bound or cytoskeletal components.

1.4 Research objectives

While the calpains are thought by some to play a role in the turnover of myofibrillar proteins as described above, the function(s) of the ubiquitin system in this context are very poorly resolved. The aim of this research project was accordingly firstly, to identify the calpain and ubiquitin-dependent systems in chicken skeletal muscle, secondly, to characterize the components of these separate proteolytic pathways and thirdly, to examine the potential endogenous and exogenous substrates of both systems. Such information would help to broaden the understanding of the roles played by the ubiquitin-dependent and calpain pathways in non-lysosomal protein degradation in skeletal muscles.

CHAPTER 2

UBIQUITIN CONJUGATION TO ENDOGENOUS AND EXOGENOUS PROTEINS IN CHICKEN MUSCLE EXTRACTS

2.1 INTRODUCTION

The role of the ubiquitin-dependent pathway of protein degradation in skeletal muscle is not well characterized, despite the importance of skeletal muscle protein as a potential source of amino acids during starvation and in various other states characterized by negative nitrogen balance. The crucial event in the ubiquitin-dependent pathway is the conjugation of ubiquitin to substrate proteins; an event which marks some proteins for subsequent degradation. Detailed studies on this pathway have shown the existence of two types of ubiquitin-protein conjugates. Firstly, conjugates in which a multi-ubiquitin chain of up to 20 ubiquitin residues joined to each other in covalent isopeptide linkages, is linked to a mobile lysine residue of each substrate protein; such conjugates are recognized by the ubiquitin conjugate-degrading protease and are consequently rapidly degraded (Chau et al., 1989; Bachmair & Varshavsky, 1989). Secondly, a variety of mono-ubiquitinated proteins have been identified which are stable entities and are not recognized by the protease responsible for conjugate degradation. These include histones (Pickart & Rose, 1985a; Haas et al., 1988, Pickart & Vella, 1988b), cell-surface receptors (Siegelman et al, 1986; Yarden et al., 1986), and actin (Ball et al., 1987); their specific roles in the cell are largely unknown. Some of the mono-ubiquitinated proteins have been shown to be ubiquitinated via an E3-independent mechanism and this may involve a species of E2 different to that involved in the ubiquitination of proteins destined for degradation.

Although some work has been done on skeletal and cardiac muscle systems (Fagan et al., 1987; Gehrke & Jennissen, 1987; Riley et al., 1988), the characteristics of the system are not well defined. It was accordingly thought to be of interest to examine chicken skeletal muscle, a tissue in which the ubiquitin pathway had not previously been

studied. The main aim would be to investigate whether mono- or multi-ubiquitinated conjugates involving endogenous or exogenous proteins can be formed and thus indicate whether ubiquitin-dependent degradation of some proteins occurs in skeletal muscles.

2.2 METHODS

2.2.1 Preparation of muscle fraction II

Skeletal muscle (10 g) from exsanguinated chickens was homogenized for 1 min at 0°C, using an Ultra-turrax homogenizer (0.75 maximum setting), in 20 mM Tris-HCl, 1 mM DTT, 1% glycerol (v/v), 1 mM EDTA, 1 mM EGTA, all at pH 7.4. The homogenate was centrifuged at 30 000 g for 30 min and the supernatant applied in a cold room to a DEAE-cellulose column (1 x 10 cm), which had previously been equilibrated with 10 mM Tris-HCl, 0.1 mM DTT, 1% glycerol (v/v), 1 mM EDTA, 1 mM EGTA, pH 7.4. The column was washed until the A_{280} readings had returned to baseline, after which the bound proteins were eluted with column buffer containing 0.5 M NaCl. This material, (fraction II), was dialyzed overnight against a buffer containing 20 mM Tris-HCl, 0.5 mM DTT, 1 mM magnesium acetate, 0.1 mM EDTA, 20 mM KCl, 20% glycerol (v/v), all at pH 7.4.

Fraction IIA and fraction IIB were subsequently prepared by addition of solid ammonium sulphate to 38% saturation (235 g/l), incubation at 0°C for 30 min, and collection of the resulting precipitate by centrifugation at 20 000 rpm for 20 min. The precipitate was suspended in a small volume of buffer containing 20 mM Tris-HCl, 0.5 mM DTT, 1 mM magnesium acetate, 0.1 mM EDTA, pH 7.4, and then dialyzed overnight (fraction IIA). To the supernatant, solid ammonium sulphate was added to 80% saturation (285 g/l) and the resulting precipitate was then spun down and dialyzed as above: fraction IIB.

2.2.2 Induction and collection of rabbit reticulocytes

Reticulocytosis was induced in adult rabbits according to the method of Rabinowitz & Fisher (1964) by daily subcutaneous injections of 0.8 ml of 2.5% (w/v) neutralized phenylhydrazine, for 5 days. On day 7, a blood sample was taken from the posterior marginal ear vein and stained for reticulocytes with New Methylene Blue followed by May-Grunwald stain. Of the stained red blood cells, 60% were estimated to be reticulocytes.
Rabbits were anaesthetised with a subcutaneous injection of ketamine (25 mg /3 kg body weight) followed by inhalation of diethyl ether. 90 ml of blood was collected by heart puncture into an equal volume of 2% citrate and 0.2% glucose.

2.2.3 Preparation of reticulocyte fraction II

Reticulocyte fraction II was prepared generally according to the method of Ciechanover et al. (1978). The reticulocytes, were spun at 2 000 rpm for 10 min in a bench-top centrifuge (Beckman Model TJ 6), at 4°C. The packed cells were washed 3 times with phosphate-buffered saline (PBS) and depleted of ATP by incubations at 37°C for 2 hr in the presence of 20 mM 2-deoxy-glucose and 0.2 mM dinitrophenol. The cells were spun down and lysed in 1.6 volumes (v/v) of 1 mM DTT. Cell membranes and debris were removed by centrifugation at 20 000 rpm for 30 min; the resulting supernatant was termed reticulocyte lysate.
Fraction II was prepared from the reticulocyte lysate as described for the chicken system: the lysate was applied to a DEAE-cellulose column and the bound fraction II eluted with high salt and then dialyzed overnight. Fraction IIA and fraction IIB were also prepared as described for the muscle system.

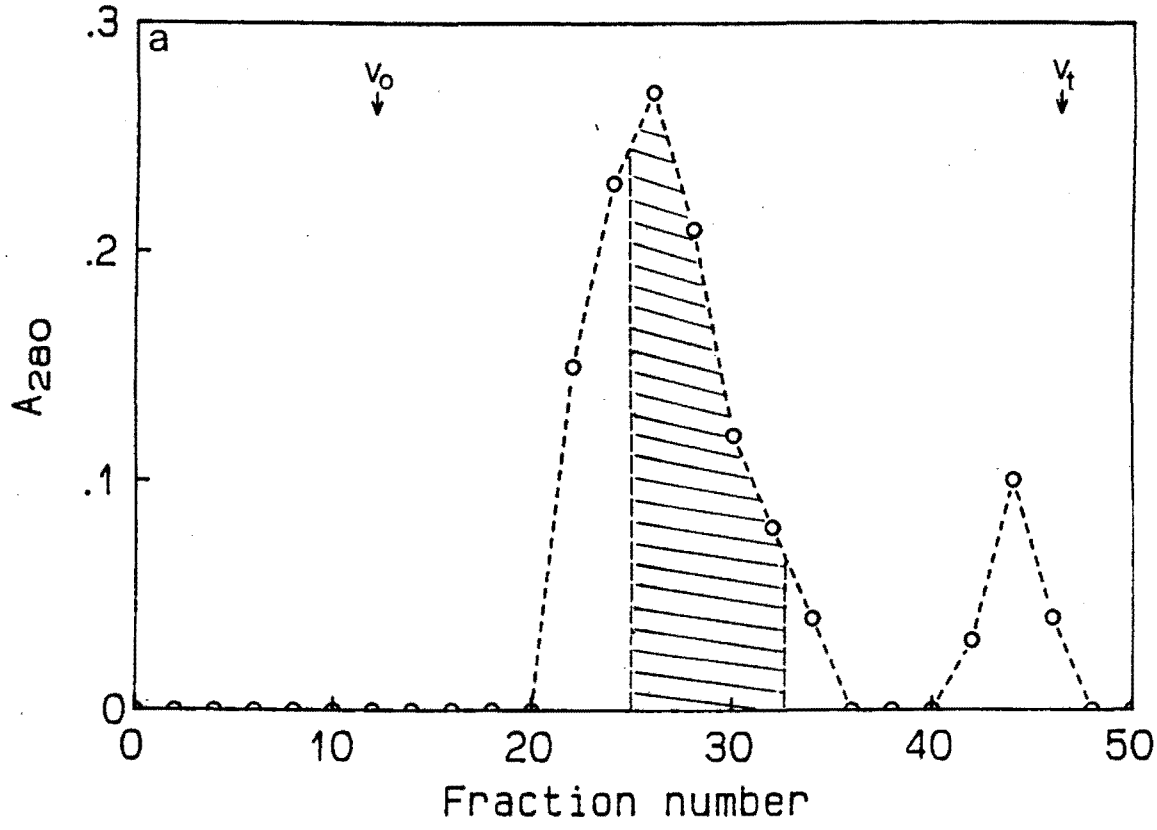
2.2.4 Preparation of chicken skeletal myofibrils

Myofibrils were purified according to the method of Solaro et al. (1971). Chicken breast muscle was homogenized in a buffer containing 0.3 M sucrose and 10 mM imidazole-HCl at pH 7.0, using an Ultra-turrax homogenizer at 0.75 maximum setting. The homogenate was spun in the cold at 17 300 g for 20 min and the pellet was then taken up in Buffer A (60 mM KCl, 2 mM $MgCl_2$, 30 mM imidazole-HCl, pH 7.0). The pellet was re-homogenized as before and spun at 750 g for 15 min, followed by 2 washes in Buffer A. The pellet, containing the myofibrils, was treated with 1% Triton X-100 in Buffer A, and the myofibrils subsequently washed free of detergent by three cycles with Buffer A. The myofibrils were finally either dissolved in 0.6 M KCl (solubilized myofibrils) or resuspended in Buffer A (suspended myofibrils). The myofibrils were judged pure on the basis of characteristic bands detected by Coomassie staining of proteins separated by 5-20% gradient SDS-PAGE.

2.2.5 Purification of ubiquitin from chicken skeletal muscle

Ubiquitin was purified from chicken skeletal muscle essentially according to the method of Haas & Wilkinson (1985). Exsanguinated chicken breast muscle (156 g) was homogenized in a Waring blender at maximum setting for two 1 min bursts in a buffer containing 50 mM Tris-HCl, 0.5 mM DTT, 1% glycerol (v/v), 1 mM EDTA, 1 mM EGTA, 50 μM chymostatin, 50 μM E-64 and 0.5 mM TLCK, all at pH 7.5. The protease inhibitors were included to prevent cleavage of the carboxyl-terminal glycine di-peptide of ubiquitin by a cathepsin-like trypsin protease which has been shown to be active in liver extracts (Haas et al., 1985) and is possibly present in muscle tissue. The homogenate was spun

for 30 min at 10 000 rpm to remove nuclear and myofibrillar material. The resulting supernatant containing 10 560 mg protein (450 ml) was diluted with 200 ml of distilled water and heated to 90°C with continuous stirring. 98% of the cytosolic protein, as judged by Lowry protein, was denatured and could be removed by centrifugation. The resulting heat-inactivated supernatant (261 mg protein) was applied to a DEAE-cellulose column (2.5 x 15 cm; flow rate of 20 ml/hr), which had previously been equilibrated in column buffer; 25 mM Tris-HCl, 0.2 mM DTT, 0.5 mM EDTA, 0.5 mM EGTA, all at pH 7.4. The column was washed with one bed volume of column buffer and all the unbound material (195 mg protein), containing ubiquitin (fraction I) was collected. A 45% - 85% ammonium sulphate fraction, in which ubiquitin and most of the other fraction I proteins were precipitated, was prepared. The centrifuged precipitate was suspended in 25 mM ammonium acetate, pH 7.5 and dialyzed against this buffer overnight. The resulting clarified concentrated material (18 mg protein) was applied to a CM-cellulose column (1.6 x 20 cm) equilibrated in 20 mM ammonium acetate, pH 4.5 at room temperature. The bound protein was eluted with 50 mM ammonium acetate, pH 5.5; SDS-PAGE analysis of these fractions showed ubiquitin to be present but contaminated by an M_r = 20 000 protein. Appropriate fractions of this column were pooled, concentrated to 1 ml by lyophilization (4 mg protein) and dialyzed against 25 mM Tris-HCl, pH 7.0. In order to separate ubiquitin from the contaminating protein, 300 μl of the concentrated sample (4 mg/ml) was applied to a Bio-Gel P-10 column (0.9 x 37 cm), equilibrated in 50 mM Tris-HCl, pH 7.5 and the A_{280} profile monitored (Fig. 2.1a). SDS-PAGE analysis of the column fractions identified ubiquitin essentially free of the contaminating protein eluting between fractions 25-32; most of the contaminant eluted in the V_o region of the column (Fig. 2.1b).



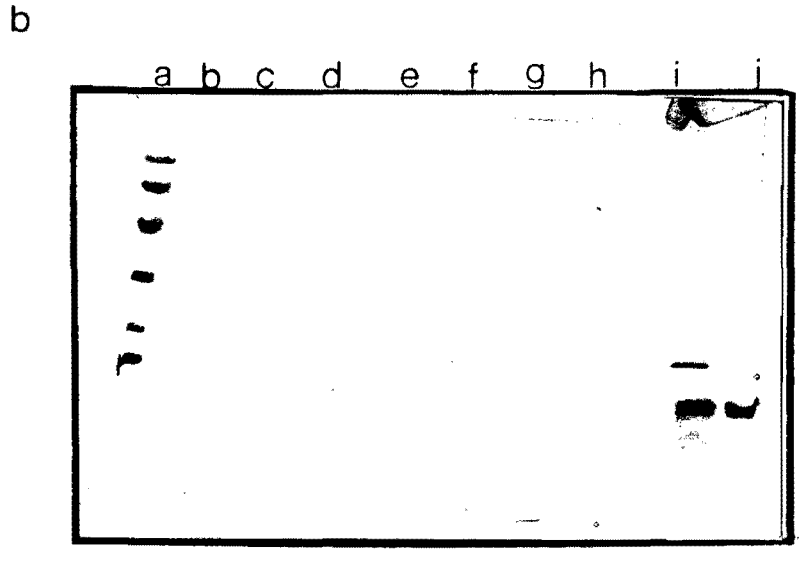


Fig. 2.1 (legend overleaf)

Fig. 2.1 Purification of ubiquitin

Fig. 2.1a Gel filtration of partially purified ubiquitin

300 µl of a partially purified ubiquitin fraction (4 mg/ml) which had previously been subjected to DEAE-cellulose chromatography, heat treatment and CM-cellulose chromatography, as described in section 2.2.5, was applied to a 0.9 x 37 cm Bio-Gel P-10 column at a flow rate of 3 ml/hr previously equilibrated in 50 mM Tris-HCl (pH 7.5). The A_{280} profile was monitored (O) and fractions of 0.5 ml were collected. The V_o and V_t values were determined by calibrating the column with blue dextran (V_o) and ATP (V_t).

Fig. 2.1b SDS-PAGE of ubiquitin samples

The purity of ubiquitin from the above column fractions was analyzed by SDS-PAGE on 5-20% gradient gels. Samples were treated with 1% SDS and 5% 2-mercaptoethanol, heated for 2 min at 90°C and electrophoresed as described in section 2.2.11. **Lane a:** molecular weight markers; myosin heavy chain (M_r = 200 000), phosphorlase b (M_r = 94 000), bovine serum albumin (M_r = 68 000), ovalbumin (M_r = 43 000), carbonic anhydrase (M_r = 30 000), soybean trypsin inhibitor (M_r = 20 100), α-lactalbumin (M_r = 14 400). **Lane b:** fraction 22. **Lane c:** fraction 25. **Lane d:** fraction 28. **Lane e:** fraction 30. **Lane f:** fraction 32. **Lane g:** fraction 35. **Lane h:** fraction 44. **Lane i:** pre-Bio-gel P-10 material. **Lane j:** ubiquitin standard.

2.2.6 Iodinations

Proteins were iodinated according to the Chloramine-T method (Moore et al., 1977). In all cases, 1 mg protein was iodinated with 1 mCi sodium [^{125}I]-iodide and the final material extensively dialyzed against saline-EDTA.

2.2.7 Formation of ^{125}I-ubiquitin conjugates

Samples of fraction II (100-200 μg protein) were incubated with 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) in a mixture (200 μl) also containing 2.5 mM DTT, 2.5 mM magnesium acetate, 5 mM ATP, and 25 mM Tris-HCl, all at pH 7.4. After 2 hr at 37°C, reactions were stopped by the addition of 40 μl of 5% SDS and 5 μl of 2-mercaptoethanol, after which the mixtures were heated to 90°C for 2 min and subjected to 5-20% gradient SDS-PAGE.
When exogenous proteins were being assayed for ubiquitin conjugation, varying quantities (5-500 μg protein) were added to the incubation mixtures and treated as above.

2.2.8 Purification of ^{125}I-ubiquitinated myofibrils

Myofibrils (insoluble or soluble) were ubiquitinated as described in section 2.2.7 and subsequently re-purified to free them of ubiquitinated fraction II proteins. The suspended myofibrils were spun down at 2 000 rpm for 10 min and the pellet repeatedly taken up in distilled water and centrifuged, until no radioactivity could be detected in the supernatant; the pellet then contained only myofibrillar proteins by SDS-PAGE analysis. In the case of the solubilized myofibrils, the ionic strength was lowered from 0.6 M to 0.01 M by the addition of distilled water, after which the myofibrils precipitated out, were washed

free of ubiquitinated cytosolic fraction II proteins and analyzed as described above.

2.2.9 Formation of ^{125}I-protein-ubiquitin conjugates

A sample of 1 μg ^{125}I-protein, usually lysozyme, (1 x 10^5 cpm), was incubated at time intervals ranging from 20 min to 2 hr, at 37°C, with 10 μg ubiquitin, 100-200 μg fraction II protein, 2.5 mM DTT, 2.5 mM magnesium acetate, 5 mM ATP, 25 mM Tris-HCl, pH 7.4, in a final volume of 200 μl. The incubation was stopped as described in section 2.2.7 and the samples were analyzed by 5-20% gradient SDS-PAGE and subsequent autoradiography.

2.2.10 Gel filtration of hemin-induced ^{125}I-lysozyme complexes

500 μl incubates containing 200 μg fraction II, 10 μg ^{125}I-lysozyme (1 x 10^6 cpm), 25 μg ubiquitin, 5 mM ATP, 2.5 mM DTT, 50 μM hemin, 25 mM Tris-HCl, and 2.5 mM magnesium acetate, all at pH 7.4, were incubated at 37°C for 2 hr and the reaction terminated by the addition of NEM (final concentration of 5 mM). Formic acid was added to bring the mixture to a concentration of 5% and the sample was then applied to a 0.9 x 30 cm Sepharose CL-6B column, equilibrated with 5% formic acid. The column was run at a flow rate of 6 ml/hr and fractions of 0.5 ml were collected. The A_{280} profile was monitored and the radioactivity of each fraction was detected by gamma counting.

2.2.11 SDS-PAGE

Samples were applied to 5-20% gradient polyacrylamide gels and subjected to electrophoresis at 30 mA and room temperature (Laemmli, 1970). Free ubiquitin was excised from the gel and ubiquitin conjugates were detected on dried gels by autoradiography for varying periods on Kodak XAR-5 film using Cronex intensifying screens. Detection of radioactivity in specific conjugates was done by excising the relevant Coomassie blue-stained lane areas and counting the ^{125}I-protein in a Packard Crystal II gamma counter.

2.2.12 Protein determinations

Protein concentrations were assayed according to Lowry et al. (1951), using bovine serum albumin as standard.

2.3 RESULTS

2.3.1 Conjugation of ^{125}I-ubiquitin to cytosolic muscle proteins

Incubations of 1 μg ^{125}I-ubiquitin with 100 μg chicken skeletal muscle fraction II, ATP and Mg^{2+} led to the formation of ^{125}I-ubiquitin-protein conjugates (Fig. 2.2, lane a). A number of discrete conjugates were formed with molecular weights between 30 000 and 100 000, with a smear of higher molecular weight entities; a M_r = 19 000 species, most probably di-ubiquitin, was also detected. A similar distribution of ^{125}I-ubiquitin conjugates was seen in the case of rabbit skeletal muscle preparations (Fig. 2.2, lane b) but the patterns were different in the case of rabbit cardiac muscle (Fig. 2.2, lane c) and rabbit reticulocytes (Fig. 2.2, lane d), probably reflecting the distinct protein profiles of different tissues rather than a species variation.

Several experimental parameters were tested to optimize and characterize the conjugation reactions involving endogenous proteins.

i) Role of ATP. A strict ATP-dependence was observed for ubiquitin conjugation to endogenous proteins in that firstly, no conjugates formed in the absence of ATP and Mg^{2+} (Fig. 2.2, lane e) and secondly, the trinucleotides GTP, UTP and CTP did not support this reaction (results not shown). The concentrations of ATP required for half-maximal conjugation, in the presence or absence of an ATP-regenerating system, were 10 μM and 40 μM, respectively (Fig. 2.3), suggesting that a low ATP concentration was sufficient for ubiquitin-protein conjugation. Such a low apparent Km for ATP is in agreement with Ciechanover et al., (1980b) who showed that half-maximal conjugation in the case of rabbit reticulocyte fraction II preparations was attained with less than 0.1 mM ATP.

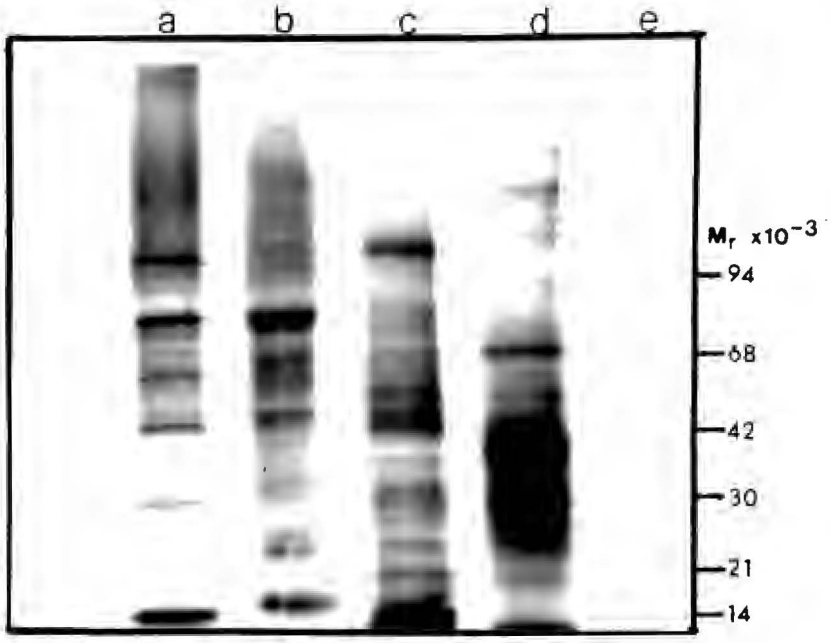


Fig. 2.2 Formation of ^{125}I-ubiquitin conjugates to endogenous proteins

100 µg fraction II protein was incubated with 1 µg ^{125}I-ubiquitin (1 x 10^5 cpm), 2.5 mM DTT, 5 mM ATP, 2.5 mM magnesium acetate, 25 mM Tris-HCl (pH 7.5) in a final volume of 200 µl at 37°C. Samples were treated to 90°C for 2 min in the presence of 1% SDS and 5% 2-mercaptoethanol and subjected to electrophoresis as described (section 2.2.11). The ^{125}I-ubiquitin conjugates were visualized by autoradiography as described in 2.2.11.

Lane a: chicken skeletal muscle fraction II. **Lane b:** rabbit skeletal muscle fraction II. **Lane c:** rabbit cardiac muscle fraction II. **Lane d:** rabbit reticulocyte fraction II. **Lane e:** chicken skeletal muscle fraction II in the absence of ATP.

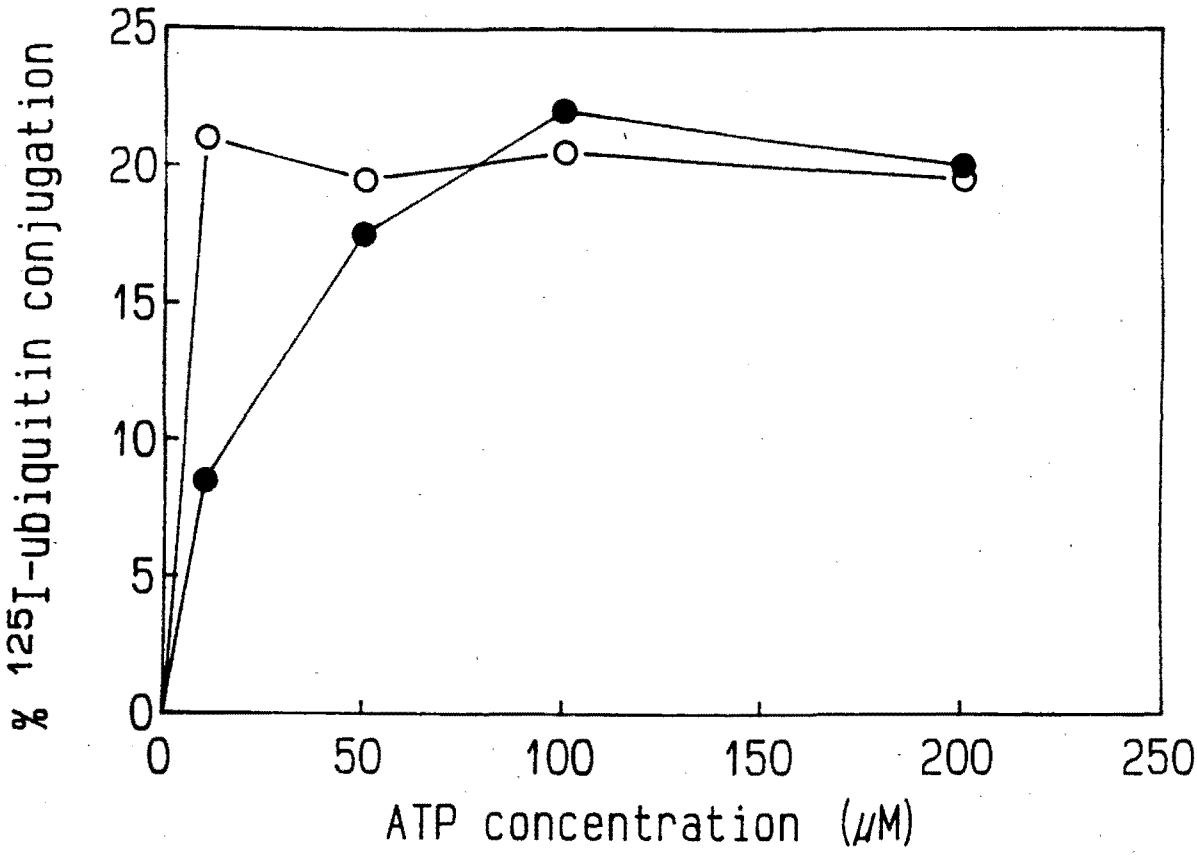


Fig. 2.3 ATP-dependence of ^{125}I-ubiquitin conjugate formation to chicken skeletal muscle fraction II proteins

100 μg chicken skeletal muscle fraction II protein was incubated for 2hr at 37°C in the presence (○) or absence (●) of an ATP-regenerating system (10 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase) in a mixture containing varying concentrations of ATP, 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm), 2.5 mM DTT, 2.5 mM magnesium acetate, 25 mM Tris-HCl (pH 7.4). The reaction was stopped by the addition of 1% SDS and 5% 2-mercaptoethanol and the mixtures analyzed on SDS-PAGE as described in section 2.2.11. The relevant Coomassie blue-stained lane areas of the dried gel were counted in a gamma counter and the percentage of ^{125}I-ubiquitin conjugated to endogenous proteins calculated.

ii) Time course. Under the particular conditions of assay, considerable time was needed to form ^{125}I-ubiquitin conjugates at 37°C in both muscle and reticulocyte preparations (Fig. 2.4); with muscle fraction II, 23% and 31% of the added ^{125}I-ubiquitin became part of conjugates after 2 hr and 4 hr, respectively, compared with the more active rabbit reticulocyte fraction II which had incorporated 25% of ^{125}I-ubiquitin into conjugates after 30 min, and 40% after 2 hr. For routine assays, 2 hr incubations were used in order to allow enough conjugates to form for autoradiographic analysis.

iii) Concentration of fraction II. Increasing the amount of fraction II per assay (and consequently, increasing the concentration of both endogenous substrates and conjugating enzymes) was associated with increasing amounts of ^{125}I-ubiquitin conjugates formed at different ^{125}I-ubiquitin concentrations (Fig. 2.5). For most subsequent assays, 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) and 100 μg fraction II protein were used.

iv) Mg^{2+} and DTT concentrations. The optimal Mg^{2+} concentration for conjugation was 1 mM; at higher concentrations, there was a gradual decrease in conjugate formation (results not shown). DTT concentrations varying from 100 μM to 5 mM did not affect the extent of conjugation (results not shown).

v) tRNA. Ferber & Ciechanover (1987) have shown that tRNA is required for conjugation of ubiquitin to some protein substrates; this appears to involve the addition of a basic amino acid to an acidic N-terminus, allowing the substrate protein then to bind to E3 for ubiquitination. Addition of tRNA, or pre-treatment of fraction II with RNase followed by tRNA addition, did not cause any change in the amount or profile of ^{125}I-ubiquitin conjugates (results not shown). This indicates either that muscle fraction II contained enough endogenous proteins with the correct N-termini for ubiquitin conjugation and/or that

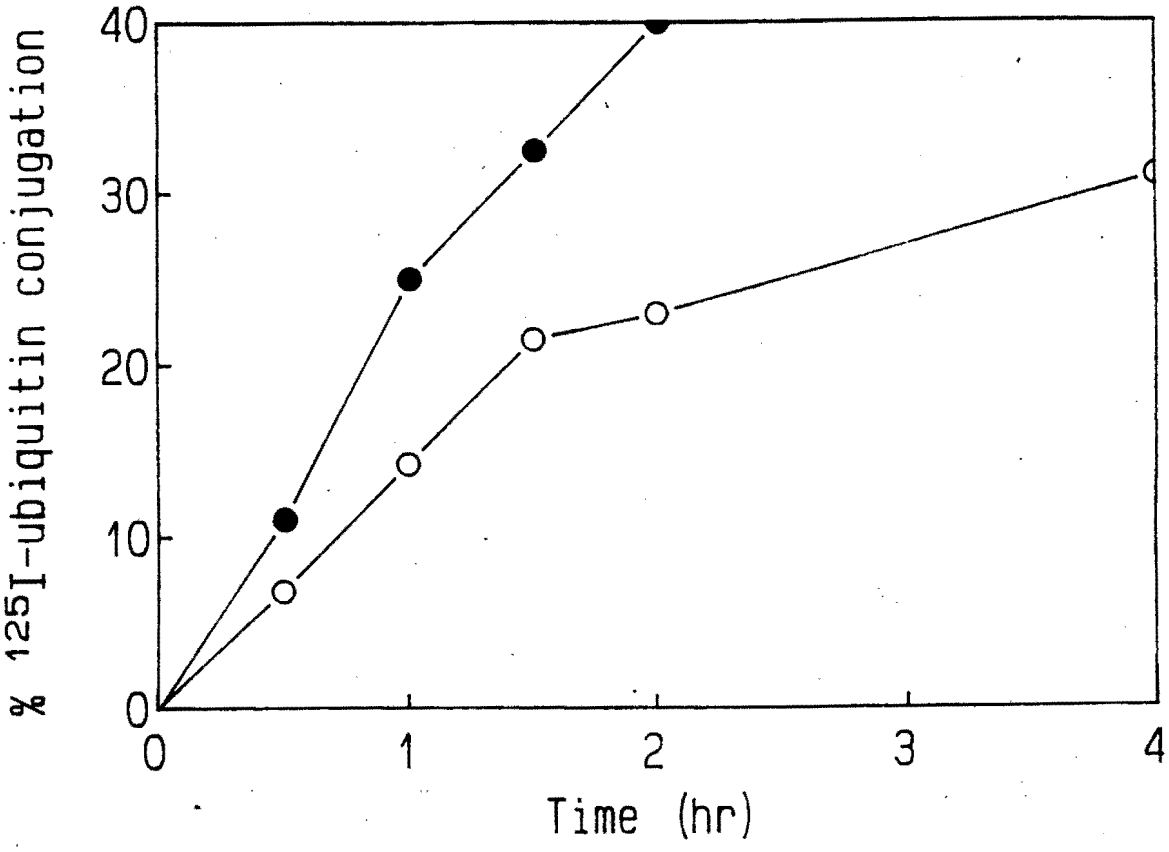


Fig. 2.4 Time course of ^{125}I-ubiquitin conjugate formation in muscle and reticulocyte fraction II extracts

100 μg fraction II protein was incubated with 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm), 2.5 mM DTT, 5 mM ATP, 2.5. mM magnesium acetate, 25 mM Tris-HCl (pH 7.5) in a final volume of 200 μl at 37°C, for the indicated times. The reactions were stopped by the addition of 1 % SDS and 5% 2-mercaptoethanol and the samples analyzed on 5-20% gradient gels and quantitated as previously described (section 2.2.11). Muscle fraction II, (○) reticulocyte fraction II (●).

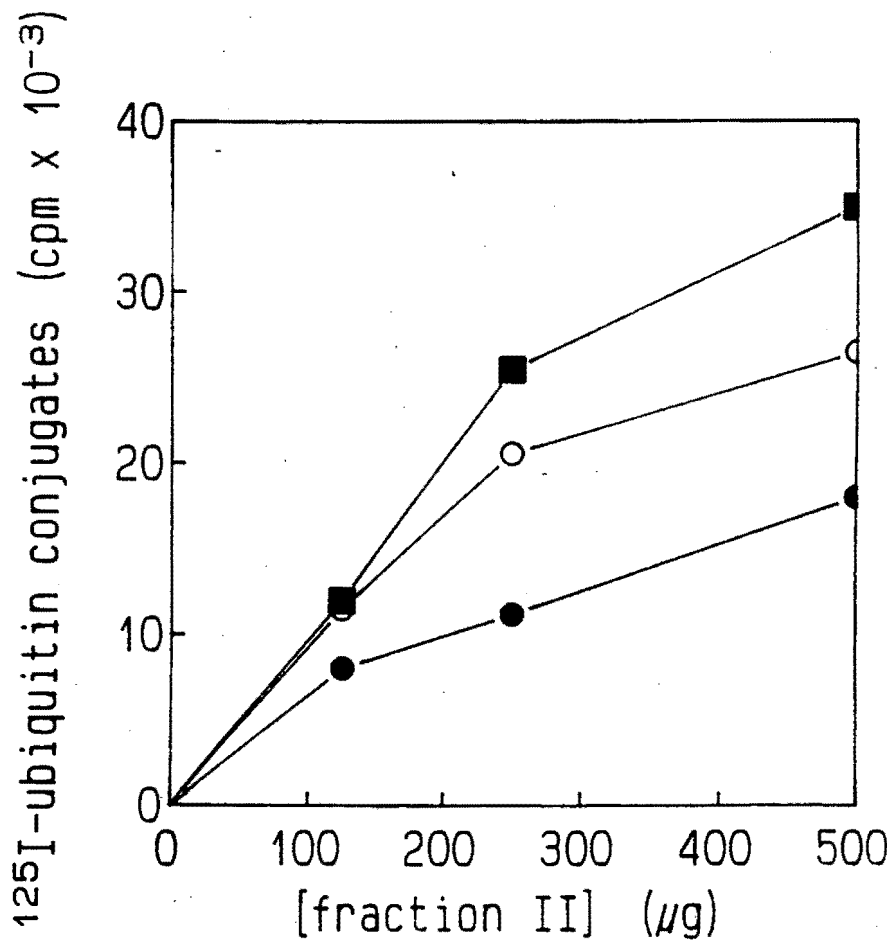


Fig. 2.5 The concentration dependence of ^{125}I-ubiquitin and fraction II on ^{125}I-ubiquitin-conjugate formation

Fraction II protein, at the indicated concentrations, was incubated with varying concentrations of ^{125}I-ubiquitin, 5 mM ATP, 2.5 mM DTT, 2.5 mM magnesium acetate, 25 mM Tris-HCl (pH 7.5) in a final volume of 200 μl for 2 hr at 37°C. The reactions were stopped with 1% SDS and 5% 2-mercaptoethanol and the samples analyzed and quantitated as previously described (section 2.2.11). 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) (●), 2 μg ^{125}I-ubiquitin (2 x 10^5 cpm) (○), 3 μg ^{125}I-ubiquitin (3 x 10^5 cpm) (■).

conjugation to endogenous proteins occurred predominantly via an E3-independent pathway.

vi) Inhibitors. The effects of various compounds on the rate of ^{125}I-ubiquitin conjugation are shown in Table 2.1. Notably, NEM (50 μM) inhibited ^{125}I-ubiquitin conjugation by 100%, a result consistent with the ubiquitin-activation pathway forming ubiquitin-thiolester intermediates. The trypsin inhibitor, TLCK and the chymotrypsin inhibitor, TPCK not only inhibited ^{125}I-ubiquitin conjugation but also caused the appearance of a ubiquitin-conjugate with an M_r value of 115 000: this phenomenon is discussed more extensively in Chapter 3. Various other protease inhibitors did not affect conjugation, suggesting that proteolytic cleavage of substrate proteins and/or activating enzymes was not required for ^{125}I-ubiquitin conjugation to occur. Roche et al. (1987) have previously shown that 2,3 BPG inhibited ATP-dependent proteolysis; lack of an effect of 2,3 BPG on ubiquitin conjugation suggested that this phenomenon is not due to direct inhibition of the conjugation reaction. Addition of 50 μM hemin to the system not only caused a decrease in initial conjugate formation (a result previously described by Haas & Rose (1981)) but also caused a change in the ^{125}I-ubiquitin conjugate distribution to higher molecular weight species (Table 2.1 and Fig. 2.12, lanes c and d) The effects of hemin were concentration-dependent (Fig. 2.6); above 100 μM, hemin markedly inhibited the ^{125}I-ubiquitin conjugation, suggesting that the muscle system was more sensitive to hemin than were other tissues.

viii) Effect of the ubiquitin source on conjugation to endogenous proteins. Red blood cell ubiquitin purified by heating (Sigma, USA) or by non-heating methods (kindly donated by Dr H.F. Deutsch) as well as that purified from chicken skeletal muscle as described in section 2.2.5, were iodinated and their ability to form conjugates to endogenous chicken skeletal muscle proteins measured in each case by incubating ^{125}I-ubiquitin with fraction II

Table 2.1 Effectors of ^{125}I-ubiquitin conjugation

100 μg fraction II protein was incubated for 2 hr with 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) in a mixture (final volume 200 μl) containing 5 mM ATP in the presence of the indicated protease inhibitors. ^{125}I-ubiquitin conjugates were analyzed on SDS-PAGE as described in section 2.2.11.

Effector	% inhibition of ^{125}I-ubiquitin conjugation
None	0
5 mM NEM	100
5 mM 2,3 BPG	0
5 μg tRNA	0
10 μg/ml chymostatin	0
100 μg/ml leupeptin	0
2 mM PMSF	0
50 μg/ml STI	0
100 μM TLCK	80
100 μM TPCK	20
100 μM hemin	50

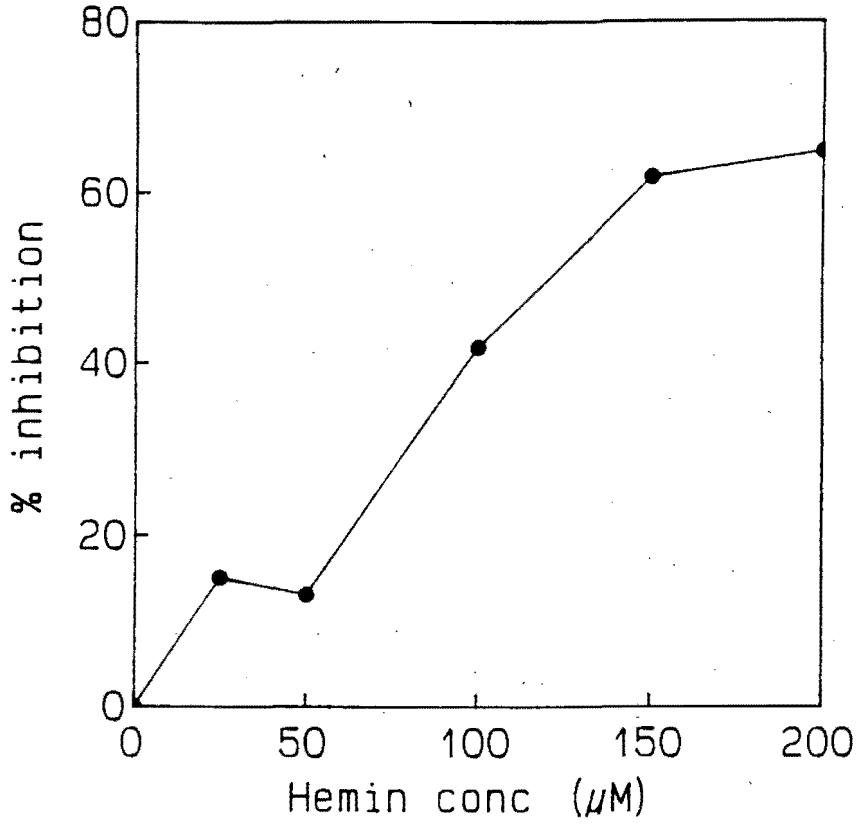


Fig. 2.6 Effect of hemin on ^{125}I-ubiquitin conjugation to endogenous fraction II proteins

100 μg fraction II protein was incubated with 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm), 5 mM ATP, 2.5 mM DTT, 2.5 mM magnesium acetate, 25 mM Tris-HCl (pH 7.5) and varying concentrations of hemin, in a final volume of 200 μl at 37°C for 2 hr. The reaction was stopped by the addition of 1% SDS and 5% 2-mercaptoethanol and the samples analyzed and quantitated as previously described (section 2.2.11). The inhibition (%) of ^{125}I-ubiquitin-conjugate formation was calculated (●).

protein, ATP and Mg^{2+} for 2 hr at 37°C. The conjugates formed were identical irrespective of the ubiquitin source or the method of purification (results not shown), emphasizing the conserved nature of this protein. Associated with the purified chicken skeletal muscle ubiquitin was some low molecular weight material directly below ubiquitin which was assumed to be partially degraded ubiquitin (Fig. 2.1b). Interestingly, during subsequent incubations with fraction II to measure the ability of this purified ubiquitin to form endogenous conjugates, this low molecular weight material disappeared in an ATP-dependent manner, suggesting that ATP-dependent proteases were responsible for its degradation (results not shown).

Although, in general, ^{125}I-ubiquitin conjugation to endogenous proteins has always been found to occur in an ATP-dependent manner, it is usually not known if these conjugates represent many different proteins which are mono-ubiquitinated or a few proteins which are multi-ubiquitinated. Chau et al. (1989) have shown that mono-ubiquitinated proteins tend to be stable entities whilst multiply ubiquitinated proteins are recognized and rapidly removed by the degradation pathway. Thus the extent of conjugation is a function of both the rate of formation and the rate of removal of such populations of endogenous protein substrates. For this reason, addition to such a system of a particular labelled protein, or proteins, as substrates is useful to investigate certain aspects of the ubiquitin pathway(s) in the muscle system.

2.3.2 Ubiquitination of myofibrils

The myofibrillar proteins are not only well characterized but are also plentiful in muscle cells and therefore are potential substrates for ^{125}I-ubiquitin conjugation *in vivo*. Suspended or dissolved myofibrils were prepared from chicken breast muscle and then added to the incubation

mixtures for ubiquitination. The high ionic strength needed to keep myofibrillar proteins in solution did not affect the rate of ^{125}I-ubiquitin conjugation (results not shown). The extent of ^{125}I-ubiquitin conjugation to myofibrillar proteins was less in the case of the dissolved myofibrils than in that of the suspended material (Fig. 2.7, lanes a and b). This may have been due to the activity of the mast cell-derived protease, chymase, which is known to be more active at high ionic strength (Ismail, 1982), which could have led to the breakdown of ^{125}I-ubiquitin-myofibril protein conjugates. Incubations with the potent chymase inhibitor, chymostatin, did not alter the amounts of ^{125}I-ubiquitin-myofibrillar conjugates formed, however, suggesting that the enzyme was not responsible for the observed effects (results not shown). The decrease in ^{125}I-ubiquitin conjugation in the soluble system is thus more likely to have been due to an increase in the soluble protein concentration, expressed per mg soluble protein, resulting in a decrease in the rate of ubiquitin conjugation.

As myofibrils are insoluble at low ionic strength, ^{125}I-ubiquitinated myofibrils were easily purified from cytosolic fraction II by the addition of distilled water; SDS-PAGE analysis of the re-purified myofibrils confirmed that no cytosolic proteins were present. Autoradiography revealed ubiquitination of myosin heavy chains as well as of two other proteins with conjugate molecular weights of 45 000 and 38 000, respectively (Fig. 2.7, lane c); these were possibly ubiquitinated actin and tropomyosin. The total amount of ubiquitin in the myofibrils was low, however, when compared with that of cytosolic proteins, indicating that these plentiful muscle proteins may not be preferred substrates for the ubiquitin conjugation system and may not be involved in ubiquitin-dependent degradation; this conclusion is tentative because the rates of ubiquitination may have been matched by rapid degradation of these modified proteins.

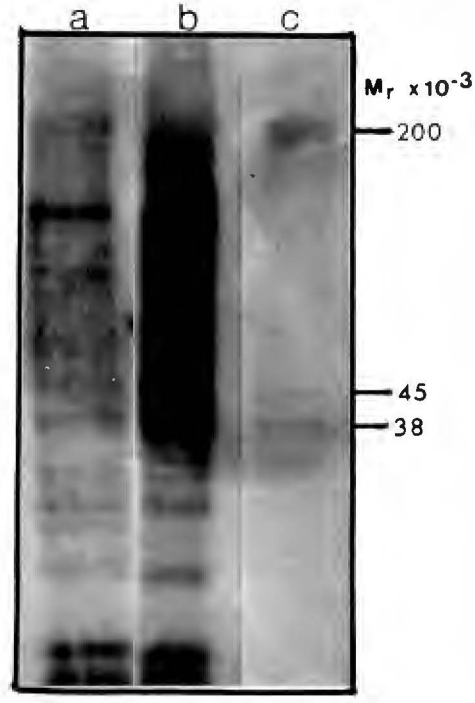


Fig. 2.7 Ubiquitination of myofibrillar proteins

400 μg of solubilized or suspended myofibrils, as prepared in section 2.2.4, were incubated with 100 μg fraction II protein, 2 μg ^{125}I-ubiquitin (2 x 10^5 cpm), 2.5 mM DTT, 2.5 mM magnesium acetate, 5 mM ATP, 25 mM Tris-HCl (pH 7.5) in a final volume of 300 μl for 2 hr at 37°C. The solubilized myofibrils were purified from the endogenous fraction II proteins by decreasing the ionic strength, as described in section 2.2.8. All samples were heat-treated for 2 min at 90°C in the presence of 1% SDS and 5% 2-mercaptoethanol and the ^{125}I-ubiquitin conjugates analyzed on SDS-PAGE as described in section 2.2.11.

Lane a: solubilized myofibrils in the presence of fraction II proteins. **Lane b:** suspended myofibrils in the presence of fraction II proteins. **Lane c:** solubilized myofibrils purified from fraction II proteins.

2.3.3 ^{125}I-ubiquitination of other muscle proteins

Despite the low levels of ubiquitination found in the case of myofibrils, other muscle proteins could be candidates for ubiquitination. Some prevalent muscle proteins including myoglobin (Mb), phosphorylase kinase (PK) and glyceraldehyde 3-phosphate-dehydrogenase (G-3-P-DH) were accordingly investigated, after iodination, as potential substrates for ubiquitination: none of these gave rise to ubiquitin conjugates (results not shown).

Because proteins containing unnatural amino acids are degraded more rapidly than other proteins (Hershko et al., 1982) presumably because they become denatured at 37°C, the possibility was investigated that a subset of denatured proteins in fraction II might be the endogenous substrates for ubiquitination. Denatured fraction II (treated with 2 N NaOH and heated to 60°C for 20 min) was tested as a source of substrates for ubiquitination. Alone, denatured fraction II, as expected, had no ubiquitin-conjugating activity; when incubated in amounts of up to 50 µg protein (but not further) with 100 µg active fraction II and 1 µg ^{125}I-ubiquitin, there was a detectable increase in the general level of ubiquitination (results not shown), but no specific ^{125}I-ubiquitin-protein conjugates were formed (results not shown).

The studies conducted up to this point have left open the possibility that chicken skeletal muscle does not readily form ubiquitin conjugates with proteins other than endogenous cytosolic proteins, or that these proteins are not degraded in a ubiquitin-dependent manner. In order to examine these notions in more detail, conjugation of ubiquitin to purified lysozyme was studied in muscle extracts and compared with that occuring in the reticulocyte system.

2.3.4 Ubiquitination of lysozyme

Many workers have used the egg white protein, lysozyme, as an exogenous substrate for the ubiquitin conjugation pathway (Waxman et al., 1987; Hough & Rechsteiner, 1986; Fagan et al., 1987). Lysozyme contains six lysine residues, all of which appear to be involved in forming ubiquitin conjugates, giving a characteristic ladder arrangement on SDS-PAGE between M_r values of 20 000 and 65 000 (Waxman et al., 1987). In the presence of hemin, high molecular weight lysozyme-ubiquitin conjugates are formed (M_r = 200 000 or greater) and it is these conjugates that are the preferred substrates for ubiquitin-dependent degradation (Hough et al., 1986; Hough et al., 1987).

2.3.4.1 Conjugation of lysozyme and ^{125}I-ubiquitin

^{125}I-ubiquitin-lysozyme conjugates appeared when muscle and reticulocyte fraction II preparations were incubated with ATP, Mg^{2+} and ^{125}I-ubiquitin. The muscle system yielded only three conjugates having M_r values of 44 000, 36 000 and 30 000 (Fig. 2.8, compare lanes a and b), whilst reticulocyte fraction II formed the same three plus two larger conjugates with M_r values of 54 000 and 48 000 (Table 2.2a and Fig. 2.8, compare lanes c and d). These forms are similar, although not identical, to those obtained by Hershko et al. (1981) using reticulocyte fraction II preparations; most obviously, the M_r = 21 000 species reported by these workers was absent from both the muscle and reticulocyte systems described in this study (Table 2.2a), which may possibly have been due to the M_r = 19 000 ^{125}I-ubiquitin contaminant, present in the ubiquitin preparations, masking the M_r = 21 000 species. The fact that the muscle system did not contain the two higher molecular weight ^{125}I-ubiquitin-lysozyme conjugates when compared to those of the reticulocyte system, and that

with 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm), 2.5 mM DTT, 2.5 mM magnesium acetate, 5 mM ATP, 25 mM Tris-HCl (pH 7.5) in a final volume of 200 μl for 1 hr at 37°C. Samples were analyzed on SDS-PAGE as described in section 2.2.11. **Lane b:** as in lane (a), but with 50 μg lysozyme added to the incubation mixture. **Lane c:** as in lane (a) but with 100 μg reticulocyte fraction II instead of muscle fraction II. **Lane d:** as in lane (b) but with 100 μg reticulocyte fraction II instead of muscle fraction II. **Lane e:** as in lane (a) but in the absence of ATP.

Table 2.2 M_r values of ^{125}I-ubiquitin-lysozyme and ^{125}I-lysozyme-ubiquitin conjugates

^{125}I-ubiquitin-lysozyme conjugates and ^{125}I-lysozyme-ubiquitin conjugates were prepared and then analyzed on SDS gels, as described in sections 2.2.7.and 2.2.9, respectively.

Table 2.2a ^{125}I-ubiquitin-lysozyme conjugates

Rabbit reticulocytes	Chicken muscle	Reported*
(M_r x 10^{-3})	(M_r x 10^{-3})	(M_r x 10^{-3})
30	30	21
34	36	34
44	44	38
48		47
54		50

* Hershko et al (1980)

Table 2.2b ^{125}I-lysozyme-ubiquitin conjugates

Rabbit reticulocytes	Chicken muscle	Reported	
(M_r x 10^{-3})	(M_r x 10^{-3})	(M_r x 10^{-3})	
38	39	22†	21‡
42	42	29	27
46	45	38	32
52		42	36
60		46	40
68			

†Hatfield & Vierstra (1989)
‡Waxman et al (1987)

the rate of conjugate formation was much slower in muscle fractions than in those from reticulocytes (results not shown), suggests that muscle has a less active pathway for the conjugation of ubiquitin to exogenous proteins.

2.3.4.2 ^{125}I-lysozyme-ubiquitin conjugates

Reticulocytes readily formed ^{125}I-lysozyme ubiquitin conjugates when incubated at 37°C in the presence of ubiquitin and ATP for 30 min. Molecular weight species with M_r values of 68 000, 60 000, 52 000, 46 000, 42 000 and 38 000 were obtained in a strict ATP- and ubiquitin-dependent manner (Fig. 2.9 lanes a and b). Conjugates with an M_r value below 38 000 were difficult to visualize due to an M_r = 30 000 ^{125}I-lysozyme contaminant. Demonstration of ^{125}I-lysozyme-ubiquitin conjugates in chicken skeletal muscle was hampered by a series of ^{125}I-lysozyme bands which often formed when muscle fraction II was incubated with ^{125}I-lysozyme independently of ATP or ubiquitin (Fig. 2.9, lane c). When, however, fresh extracts of muscle fraction II were used, distinct ^{125}I-lysozyme conjugates appeared with M_r values of 45 000, 42 000 and 39 000 (Table 2.2b, Fig. 2.9, lane d) indicating that, as in the case of ^{125}I-ubiquitin, lysozyme formed the smaller three conjugates in muscle which had molecular weights similar to those found in reticulocytes. Other workers have found conjugates to occur with M_r values between 20 000 and 65 000, while high molecular weight conjugates only appear to occur in the presence of hemin (see Table 2.2b for comparison). In particular, the three largest conjugates found by Hatfield & Vierstra (1989) in plants correspond to the three smaller conjugates formed by extracts from reticulocytes and muscles. ^{125}I-lysozyme conjugates were absent or very faint when frozen muscle fraction II extracts were used (-80°C for 3 months), whereas the reticulocyte fraction II was

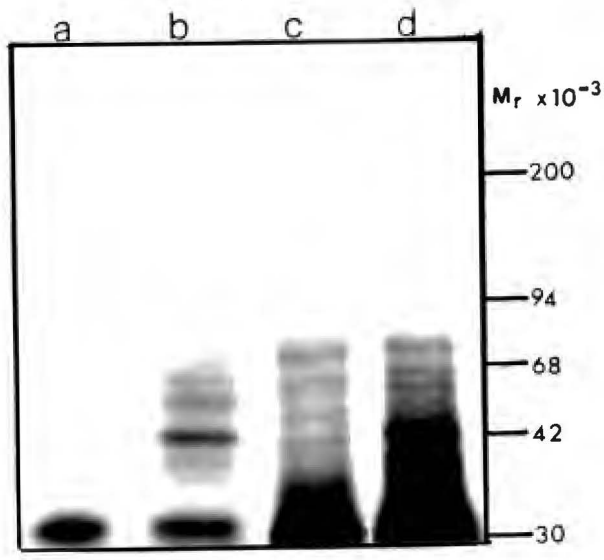


Fig. 2.9 Conjugation of ^{125}I-lysozyme to ubiquitin in chicken muscle and rabbit reticulocyte fraction II extracts

Lane a: 1 μg ^{125}I-lysozyme (1 x 10^5 cpm) was incubated in the presence of 100 μg reticulocyte fraction II, 2.5 mM DTT, 2.5 mM magnesium acetate, 25 mM Tris-HCl (pH 7.5) in a final volume of 200 μl at 37°C for 30 min. The reactions were stopped and analyzed by SDS-PAGE as in section 2.2.11. **Lane b:** as in lane (a) but in the presence of 5 mM ATP and 10 μg ubiquitin. **Lane c:** as in lane (a) but with 100 μg fresh muscle fraction II instead of reticulocyte fraction II. **Lane d:** as in lane (c) but with 5 mM ATP and 10 μg ubiquitin.

quite stable under such conditions (results not shown). This indicates that although muscle fraction II could readily form ^{125}I-ubiquitinated endogenous protein conjugates, the formation of ^{125}I-lysozyme-ubiquitin or ^{125}I-ubiquitin-lysozyme conjugates was less extensive.

2.3.5 E3 status in muscle and reticulocytes

E3 has been reported to be separable from E1 and E2 by ammonium sulphate precipitation, E3 being precipitated in the 0-38% fraction along with the ubiquitin conjugate-degrading enzyme whilst E1 and E2 are precipitated in the 40-80% fraction (Haas & Bright, 1988): these have been termed fraction IIA and fraction IIB, respectively. Fractions IIA and IIB were prepared according to this fractionation method from previously frozen fraction II extracts and were subsequently incubated with ^{125}I-ubiquitin and ATP. This led, in the case of both reticulocytes and muscle preparations, to the formation of ^{125}I-ubiquitin conjugates in the presence of fraction IIB but not fraction IIA (Fig. 2.10, lanes a, b, d and e). This suggests that ubiquitination of endogenous proteins occurred independently of fraction IIA and was thus likely to be independent of E3. When both fraction IIA and fraction IIB were incubated together with ^{125}I-ubiquitin and ATP, a change in the conjugate pattern occurred (Fig. 2.10, lanes c and f). Whether this was due to the participation of E3 in conjugation or to the addition of new protein substrates to be ubiquitinated could not be distinguished.

No ^{125}I-lysozyme-ubiquitin conjugates formed when fraction IIA and fraction IIB were incubated separately in the presence of ATP, but when they were mixed to reconstitute the initial fraction II, typical ^{125}I-lysozyme-ubiquitin conjugates formed in the reticulocyte but not the muscle

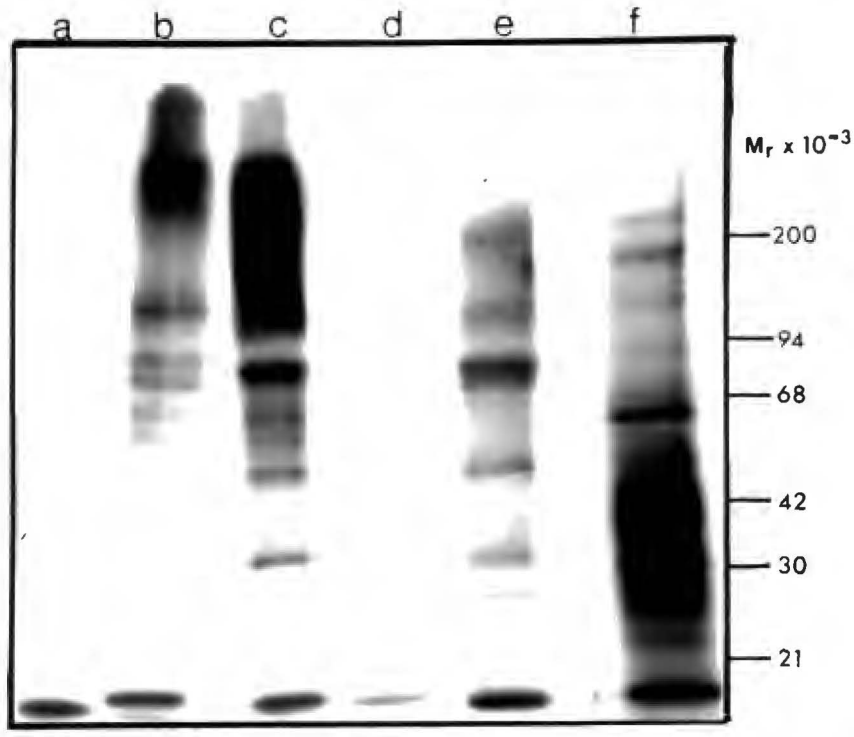


Fig. 2.10 Formation of ^{125}I-ubiquitin conjugates with fraction IIA and IIB

Fraction II protein (as indicated) was incubated with 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm), 2.5 mM DTT, 2.5 mM magnesium acetate, 5 mM ATP, 25 mM Tris-HCl, (pH 7.5) for 1 hr at 37°C in a final volume of 200 μl. The reaction was terminated and samples analyzed on SDS-PAGE as described in section 2.2.11.

Lane a: 50 μg chicken muscle fraction IIA. **Lane b:** 50 μg chicken muscle fraction IIB. **Lane c:** 50 μg chicken muscle fraction IIA and 50 μg muscle fraction IIB. **Lane d:** 50 μg rabbit reticulocyte fraction IIA. **Lane e:** 50 μg rabbit reticulocyte fraction IIB. **Lane f:** 50 μg rabbit reticuloycte fraction IIA and 50 μg rabbit reticulocyte fraction IIB.

system (Fig. 2.11, lanes a and b). The lack of ^{125}I-lysozyme-ubiquitin conjugates in the muscle system was investigated further by incubating reticulocyte fraction IIA with muscle fraction IIB in the presence of ^{125}I-lysozyme and also muscle fraction IIA with reticulocyte fraction IIB. Fig. 2.11 (lanes c and d) show that ^{125}I-lysozyme-ubiquitin conjugates were formed when reticulocyte fraction IIA was used with muscle fraction IIB, but not when muscle fraction IIA was added to reticulocyte fraction IIB. These results again suggests that muscle fraction IIA activity is missing or that it contains rather low levels of the "factor" (E3) necessary for ubiquitination of exogenous protein substrates.

2.3.6 Presence of ^{125}I-lysozyme bands in chicken muscle fraction II preparations

When muscle fraction II and ^{125}I-lysozyme were incubated at 37°C, with or without ATP and Mg^{2+}, a series of radioactive bands were consistently detected on autoradiography of SDS-PAGE gels (Fig. 2.9 lane c), a phenomenon never seen in the case of reticulocytes. At least five discrete forms were distinguishable from the normally blurred background; these had M_r values of between 88 000 and 33 000. They formed independently of added ubiquitin, indicating either that the muscle extracts contained enough endogenous ubiquitin and ATP to form conjugates or else that the bands were in some way artifacts of the incubation conditions. Pre-incubation of fraction II for 2 hr at 37°C in an effort to deplete it of ATP as well as of ubiquitin thiolesters of E1 and E2, gave no change in the ^{125}I-lysozyme profile. Similarly, use of hydroxylamine-treated fraction II from which all endogenous ubiquitin had been removed (see section 4.3.1), was still associated with the formation of the ^{125}I-lysozyme bands, indicating that they were not

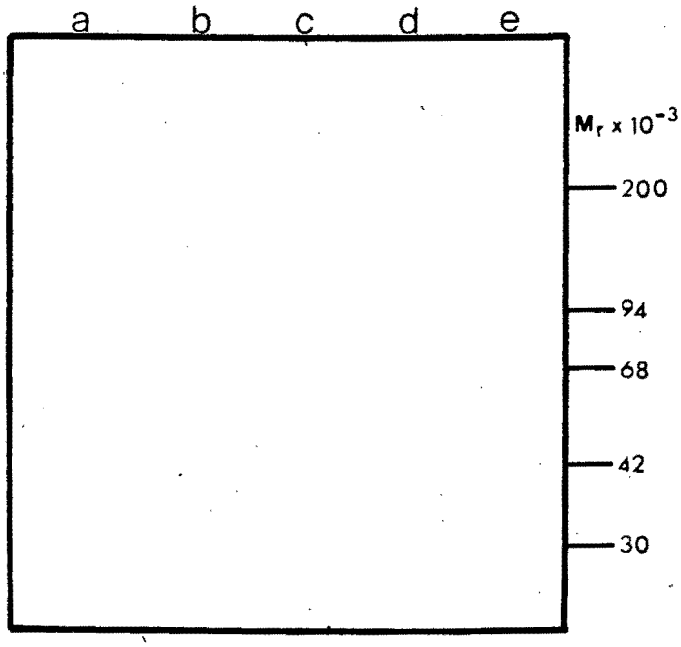


Fig. 2.11 **^{125}I-lysozyme conjugation to ubiquitin with reticulocyte and muscle fractions IIA and IIB**

Fraction II protein, as indicated, was incubated with 10 μg ubiquitin, 1 μg ^{125}I-lysozyme (1 x 10^5 cpm), 2.5 mM DTT, 2.5 mM magnesium acetate, 5 mM ATP, 25 mM Tris-HCl (pH 7.5), for 30 min at 37°C in a final volume of 200 μl. Reactions were terminated by the addition of 1% SDS and 5% 2-mercaptoethanol and the samples analyzed on SDS-PAGE as described in section 2.2.11.

Lane a: 50 μg reticulocyte fraction IIA and 50 μg reticulocyte fraction IIB. **Lane b:** 50 μg muscle fraction IIA and 50 μg muscle fraction IIB. **Lane c:** 50 μg reticulocyte fraction IIA and 50 μg muscle fraction IIB. **Lane d:** 50 μg muscle fraction IIA and 50 μg reticulocyte fraction IIB. **Lane e:** 1 μg ^{125}I-lysozyme (1 x 10^5 cpm) incubated for 30 min at 37°C.

formed in a manner which required endogenous ubiquitin or ATP. The possibility that the bands were degradation products of the high molecular weight ^{125}I-lysozyme forms (M_r = 200 000 and greater) was investigated using a cohort of protease inhibitors (Table 2.3): none of the inhibitors used altered the pattern or the amount of radioactive bands formed, suggesting that proteolytic degradation was not responsible for their formation. Fraction IIB was able to form these ^{125}I-lysozyme bands independently of fraction IIA and they were not altered in their intensity or distribution by increasing the concentration of additional fraction IIA. This indicated that the factor able to form these ^{125}I-lysozyme bands resided in muscle fraction IIB and involved some form of non-specific interaction with ^{125}I-lysozyme.

2.3.7 Effects of hemin on conjugate formation

Previous workers have shown that the addition of hemin to systems in which exogenous proteins are ubiquitinated is associated with an accumulation of high molecular weight ^{125}I-protein-ubiquitin conjugates; an effect attributed to inhibition of conjugate degradation in the face of continued conjugate formation (Hough et al., 1987). These high molecular weight ubiquitin-protein conjugates have M_r values greater than 100 000 and have been reported to be preferred substrates for the ubiquitin conjugate-degrading enzyme (Waxman et al., 1987; Hough et al., 1987).
Rapid degradation of muscle ^{125}I-lysozyme conjugates could have accounted for the lack or low level of such forms detected by analysis of muscle extracts; inclusion of hemin in the incubation mixture might be expected then to prevent this degradation and to allow the accumulation of such conjugates. Autoradiograms of muscle fraction II with hemin in the presence of ATP and Mg^{2+} in fact recorded ^{125}I-lysozyme species with M_r values of 100 000 and above; some

Table 2.3 Effect of protease inhibitors on ^{125}I-lysozyme band formation of muscle fraction II extracts

100 µg fraction II protein was incubated with 1 µg ^{125}I-lysozyme as described in section 2.2.9 with the indicated protease inhibitors. Samples were run on 5-20% gradient SDS gels and the amounts of ^{125}I-lysozyme present in the M_r region between 30 000 and 90 000 were quantitated as described in section 2.2.11.

Inhibitor	% decrease in level of ^{125}I-lysozyme band formation
None	0
5 mM NEM	11
2 mM PMSF	0
5 mM EDTA	2
10 µg/ml chymostatin	0
No DTT	0

of these species were of such high molecular weight that they were retained in the 5% gel stack (Fig. 2.12, lane a). Formation of the high molecular weight ^{125}I-lysozyme protein species was, however, suprisingly also found in the absence of ATP and even of ubiquitin, suggesting that the entities concerned were not true ^{125}I-lysozyme-ubiquitin conjugates (Fig. 2.12, lane b). They were accordingly termed "lysozyme-protein complexes" as opposed to "lysozyme-ubiquitin conjugates", to allow for the fact that they were probably not involved in the ubiquitin-dependent degradation pathway.

When ^{125}I-ubiquitin was added to fraction II preparations, hemin addition also caused the formation of high molecular weight material but to a lesser extent than was the case with ^{125}I-lysozyme (Fig. 2.12, lanes c and d). The reaction was clearly ATP-dependent, suggesting that the high molecular weight ^{125}I-ubiquitin species represented true ^{125}I-ubiquitin-protein conjugates.

In the light of the novel observations with a preparation of a well-characterized substrate of the ubiquitin-dependent protein degradation system, formation of the lysozyme complexes was examined in more detail.

i) Gel filtration of ^{125}I-lysozyme-protein complexes.

In order to estimate the native M_r of the hemin-induced ^{125}I-lysozyme-protein complexes, gel filtration of these samples on Sepharose CL-6B was performed. The elution profiles showed a major ^{125}I-lysozyme peak eluting in the V_o (Fig. 2.13). Comparison of the quantitation of such column runs with normal and hemin-induced ^{125}I-lysozyme-protein complexes demonstrated that there was an approximate 2.5-fold increase in the material eluting in the V_o of hemin-induced ^{125}I-lysozyme-protein complexes (results not shown). This material had an M_r of the order of $1x10^6$, suggesting that hemin caused massive aggregation of ^{125}I-lysozyme in the presence of fraction II. It was unlikely that these high molecular weight forms were "pure" ^{125}I-lysozyme aggregates as firstly, the column was run in

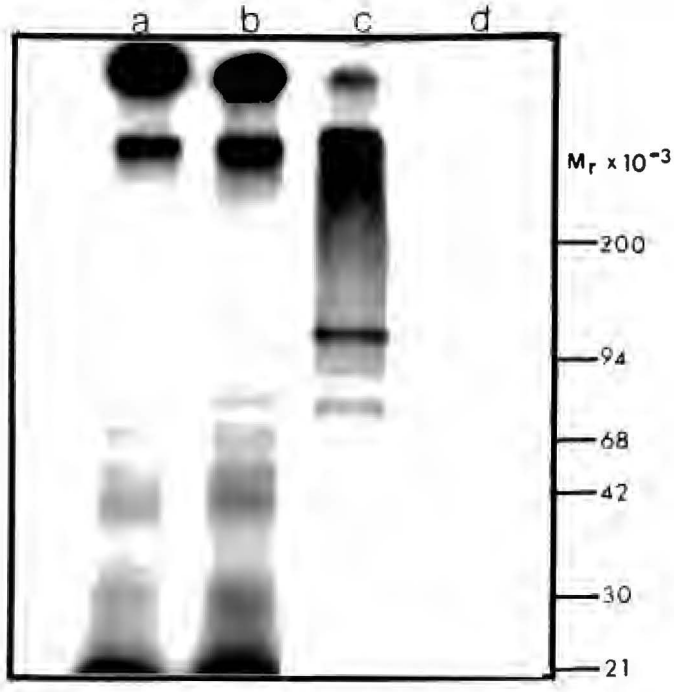


Fig. 2.12 The effect of hemin on ubiquitin conjugation

Lane a: 100 μg muscle fraction II protein was incubated with 1 μg ^{125}I-lysozyme (1 x 10^5 cpm), 10 μg ubiquitin, 2.5 mM DTT, 5 mM ATP, 2.5 mM magnesium acetate, 25 mM Tris-HCl (pH 7.5), 50 μM hemin for 2 hr at 37°C. The reaction was terminated and the samples analyzed as in section 2.2.11. **Lane b:** as in lane (a) but in the absence of 5 mM ATP and 10 μg ubiquitin. **Lane c:** 100 μg muscle fraction II protein was incubated with 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) in 2.5 mM DTT, 2.5 mM magnesium acetate, 5 mM ATP, 25 mM Tris-HCl (pH 7.5), 50 μM hemin for 2 hr at 37°C. The reaction was stopped and the sample analyzed as in section 2.2.11. **Lane d:** as in lane (c) but in the absence of 5 mM ATP.

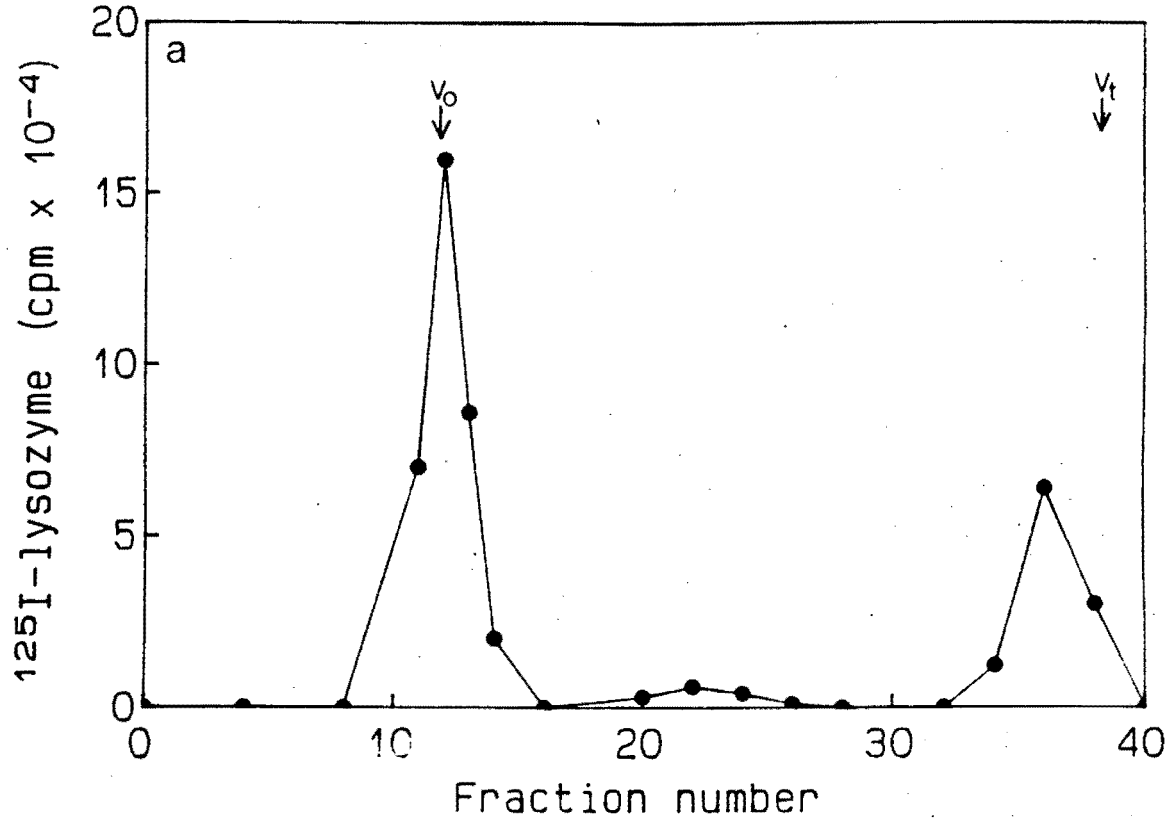


Fig. 2.13 Molecular weight analysis of hemin-induced ^{125}I-lysozyme-protein complexes

Hemin-induced ^{125}I-lysozyme-protein complexes were formed as described in section 2.2.10 and applied to a 0.9 x 30 cm Sepharose CL-6B column equilibrated in 5% formic acid. Samples were run and collected as described in section 2.2.10 and the ^{125}I-lysozyme monitored in a gamma counter (●). The V_o and the V_t values were determined by calibrating the column with a chylomicron sample (V_o) and ATP (V_t).

5% formic acid, a condition known to disrupt ^{125}I-lysozyme aggregates (Hershko et al., 1984b) and secondly, column profiles of ^{125}I-lysozyme incubated in the absence of fraction II and ubiquitin showed only a small V_o fraction (results not shown).

ii) Time- and temperature-dependence. The assay conditions necessary for these complexes to form were investigated over a range of temperatures (0°C - 37°C) and time intervals (10 min - 4 hr). It was shown that the complex formation, particularly of material in the 5% stack was strictly dependent on the time and temperature of the incubation (Fig. 2.14). At 0°C, no complexes were formed, whilst at 20°C complexes were seen after 4 hr and at 37°C, they were seen after 30 min.

iii) Treatment of samples prior to SDS-PAGE. In order to determine the chemical stability of the complexes, variations in the length of incubation at 90°C and the concentration of 2-mercaptoethanol of samples prior to SDS-PAGE were made. An increase in the length of the 90°C incubation showed a decrease in the amounts of the complexes remaining in the 5% gel stack (Fig. 2.15a), but this was independent of the 2-mercaptoethanol concentration (results not shown). Similar experiments with ^{125}I-ubiquitin-protein conjugates showed that these complexes were insensitive to a broad range of 2-mercaptoethanol concentrations (results not shown) and to varying lengths of the heat treatment (Fig. 2.15b), suggesting that different types of bonds were involved in complex formation in the case of the two different ^{125}I-proteins.

iv) Solubility of ^{125}I-lysozyme complexes. When hemin-induced ^{125}I-lysozyme-protein incubates were spun at 2 000 rpm for 10 min after a 2 hr incubation at 37°C, a fine reddish precipitate was collected: this was shown to contain all the ^{125}I-lysozyme label present in the incubates (Fig. 2.16, lanes a- c), indicating that hemin caused ^{125}I-lysozyme (and presumably fraction II) to form insoluble complexes in a time- and temperature-dependent

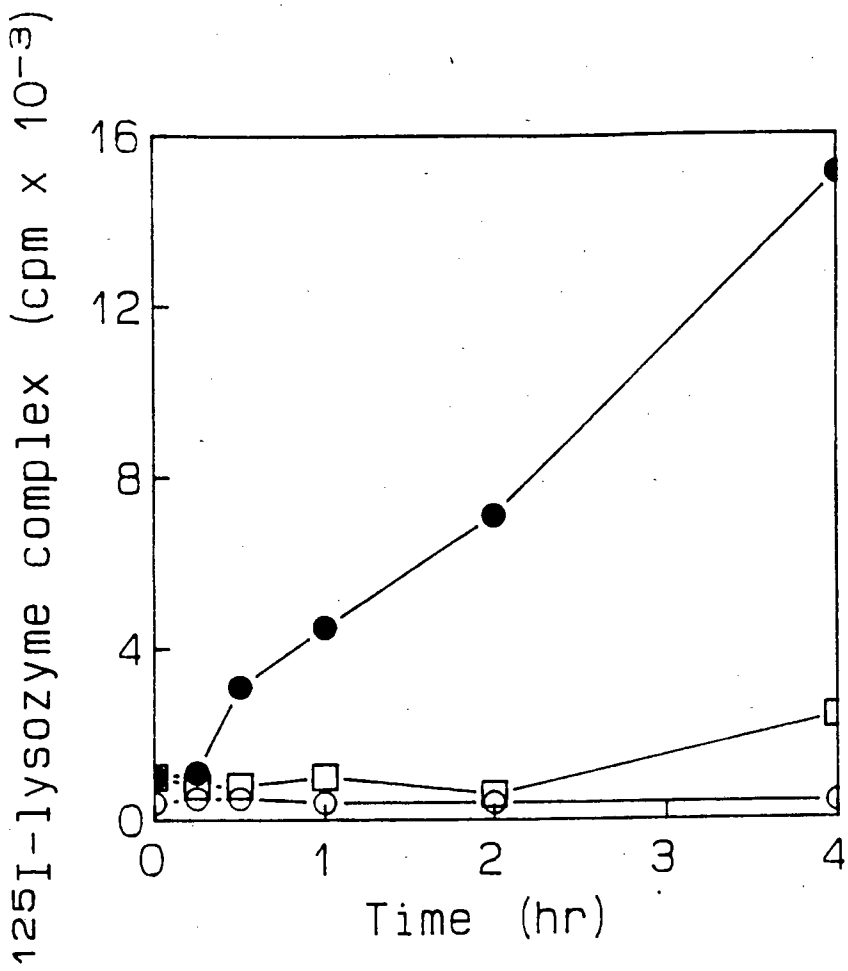


Fig. 2.14 Time- and temperature-dependence on the formation of ^{125}I-lysozyme-protein complexes

100 μg muscle fraction II protein was incubated with 1 μg ^{125}I-lysozyme (1 x 10^5 cpm), 50 μM hemin, 2.5 mM DTT, 5 mM ATP, 2.5 mM magnesium acetate, 25 mM Tris-HCl, 10 μg ubiquitin at 0°C (○), 20°C (□) and 37°C (●) at the indicated times. Samples were treated with 1% SDS and 5% 2-mercaptoethanol and the ^{125}I-lysozyme-protein complexes analyzed as described in section 2.2.11.

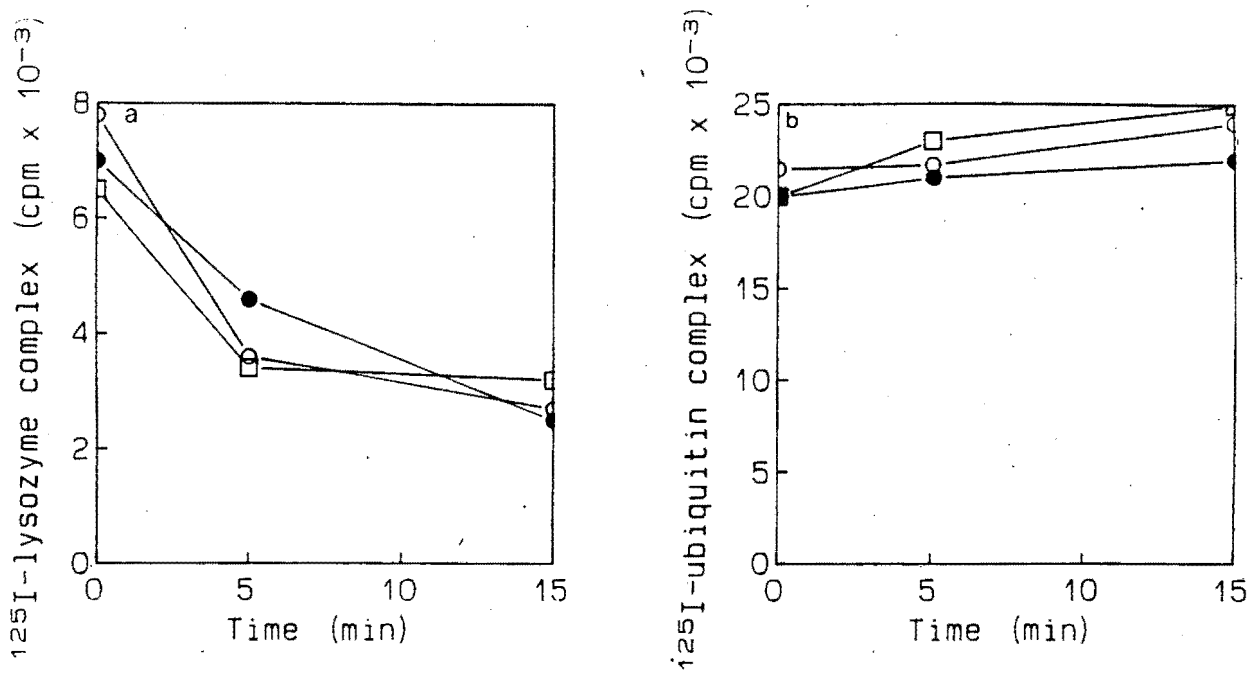


Fig. 2.15 The chemical stability of the hemin-induced protein complexes

Fig. 2.15a Hemin-induced ^{125}I-lysozyme-protein complexes were formed as previously described (Fig. 2. 12, lane (a)) and heated to 90°C in the presence of 1% SDS and 2% (●), 5% (○) and 15% (□) 2-mercaptoethanol for varying lengths of time prior to SDS-PAGE. The amount of complex retained in the gel stack was quantitated as in section 2.2.11

Fig. 2.15b Hemin-induced ^{125}I-ubiquitin-protein conjugates were formed as in Fig. 2.12, lane c, and treated at 90°C for the indicated times, as described above

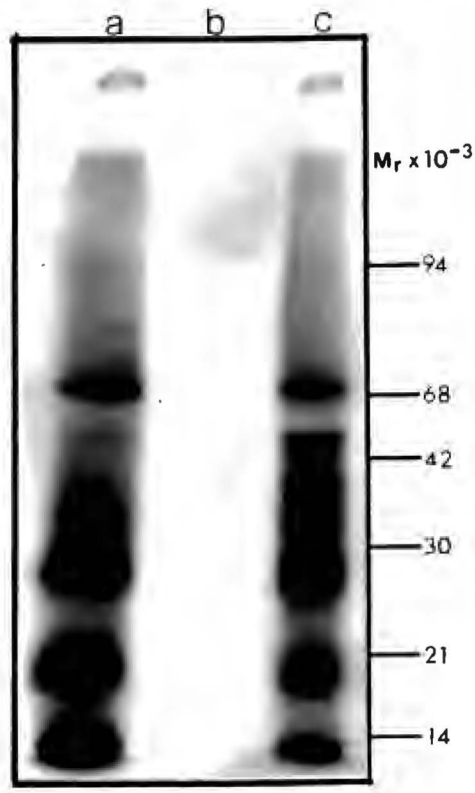


Fig. 2.16 Solubility of hemin-induced protein complexes

Lane a: 100 μg muscle fraction II protein was incubated with 1 μg ^{125}I-lysozyme (1 x 10^5 cpm), 10 μg ubiquitin, 2.5 mM DTT, 5 mM ATP, 2.5 mM magnesium acetate, 50 μM hemin, 25 mM Tris-HCl (pH 7.5) for 2 hr at 37°C. The reaction was stopped by the addition of 1% SDS and 5% 2-mercaptoethanol and the samples analyzed on SDS-PAGE as described (section 2.2.11). **Lane b:** as in lane (a) but after the 2 hr incubation the sample was centrifuged at 2 000 rpm for 10 min and the supernatant applied to the gel. **Lane c :** The insoluble material of the sample prepared in lane (b) was applied to the gel.

manner. A similar experiment with ^{125}I-ubiquitin showed the formation of a slight precipitate after centrifugation, although much of the ^{125}I-ubiquitin was still soluble (results not shown).

v) Essential factors for ^{125}I-lysozyme complexes. Although ATP and ubiquitin were not believed to be important in the hemin-induced ^{125}I-lysozyme-protein complexes, studies were carried out to determine exactly what factors were needed for ^{125}I-lysozyme-protein complexes to form. Surprisingly, only ^{125}I-lysozyme, DTT and hemin were necessary for some degree of complex formation to occur (Fig. 2.17, lanes a-c). Inclusion of fraction II caused these species to attain higher apparent molecular weights on SDS-PAGE analysis, which was independent of the presence of ubiquitin and ATP (Fig. 2.17, lane d). Addition of bovine serum albumin instead of fraction II caused a similar change in molecular weight re-distribution to higher molecular weight forms (Fig. 2.17, lane e). These reactions were partially inhibited by NEM (Fig. 2.17, lane f), suggesting that the disulphide bonds of lysozyme may play a role in the formation of the hemin-induced ^{125}I-lysozyme-protein complexes.

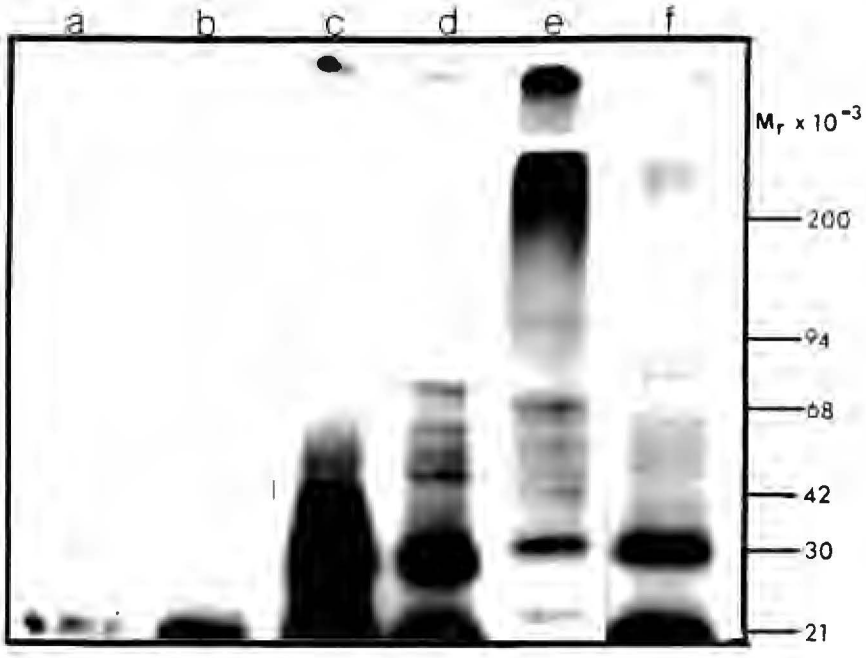


Fig. 2.17 Necessary components for ^{125}I-lysozyme-protein complex formation

Lane a: 1 μg ^{125}I-lysozyme (1 x 10^5 cpm) was incubated alone for 30 min at 37°C. The sample was treated with 1% SDS and 5% 2-mercaptoethanol and heated to 90°C for 2 min prior to electrophoresis as described in section 2.2.11. **Lane b:** 1 μg ^{125}I-lysozyme (1 x 10^5 cpm) was incubated as in lane (a) in the presence of 50 μM hemin. **Lane c:** 1 μg ^{125}I-lysozyme (1 x 10^5 cpm) was incubated as in lane (a) in the presence of 50 μM hemin and 2.5 mM DTT. **Lane d:** as in lane (c) but with the addition of 100 μg muscle fraction II protein. **Lane e:** as in lane (c) but with the addition of 100 μg BSA. **Lane f:** as in lane (e) but with the addition of 5 mM NEM.

2.4 DISCUSSION

A ubiquitin- and ATP-dependent conjugating system involving endogenous cytosolic proteins as substrates, appears to exist in chicken skeletal muscle. Protein conjugating activities have been found in a wide variety of organisms including yeast (Vierstra & Sullivan, 1988), plants (Vierstra, 1987) and vertebrates, including amphibians (Gehrke & Jennissen, 1987), birds (Agell & Mezquita, 1988) and mammals; within the mammals different tissues as wide ranging as reticulocytes (Ciechanover et al., 1980b), eye lens (Jahngen et al., 1986), and muscle (Gehrke & Jennissen, 1987), as well as a range of cell culture lines (for example, Haas & Bright, 1985), have been shown to support ubiquitin conjugation to endogenous proteins. In all cases, where tested, the endogenous ubiquitin conjugates were formed in an ATP-dependent manner and this was NEM-inhibitable, suggesting a common conjugation pathway in all eukaryotes.

^{125}I-ubiquitin conjugates formed by cytosolic fraction II have M_r values ranging from 30 000-100 000 and there are also often high molecular weight forms at M_r = 200 000 and more. The major banded species of conjugates, however, appear to be tissue specific; with respect to the chicken skeletal muscle system, major conjugates occur at M_r = 115 000, 90 000, 64 000, 48 000 and 33 000, compared with the rabbit reticulocyte system which has major conjugates at M_r = 200 000, 80 000, 42 000 and smearing of low molecular weight species. Gehrke & Jennissen (1987) have also shown endogenous ubiquitin conjugates to occur in rat, mouse and frog skeletal muscle, with a conjugate of M_r value of 95 000 being dominant in all these tissues.

The long time (relative to reticulocytes) taken by chicken skeletal muscle extracts to form ^{125}I-ubiquitin conjugates *in vitro* is similar to that demonstrated by Hatfield & Vierstra (1989) in wheat germ extracts. Thus several hours

are needed whilst reticulocytes, oat and yeast extracts form conjugates within minutes (Vierstra & Sullivan, 1988; Ciechanover et al., 1980b). Such short times of conjugate formation must reflect a very active conjugating system but in the case of oats there is also a rapid inactivation of ubiquitin (Vierstra, 1987). The chicken skeletal muscle conjugation system, although not as active as the rabbit reticulocyte system (based on % ^{125}I-ubiquitin conjugation/mg fraction II protein) does not show the features of being rapidly inactivated.

The observation that endogenous ubiquitin conjugates, in both the reticulocyte and muscle systems, can form in the preparation called fraction IIB (40-80% ammonium sulphate fraction containing E1 and E2), but not in fraction IIA (0-38% ammonium sulphate fraction containing E3 and the ubiquitin conjugate-degrading enzyme) can be explained in two ways. Firstly, assuming that no E3 is present in fraction IIB, ^{125}I-ubiquitin conjugation to endogenous proteins could occur via an E3-independent mechanism, in a similar manner to that described for cytochrome c and histone ubiquitination (Pickart & Rose 1985a; Pickart & Vella 1988b; Haas et al., 1988). Such mono-ubiquitinated conjugates are believed to be stable entities, suggesting that conjugates formed in muscle may also be stable. Secondly, Lee et al., (1986) have described multiple forms of ubiquitin-protein ligase (E3), in red blood cells, with different reactivities to different exogenous substrates, of which it is feasible that some are soluble above 40% ammonium sulphate saturation. Thus ubiquitin conjugation could occur via an E3-dependent mechanism but not involving the E3 species generally believed to be important in ubiquitin-dependent protein degradation. Although no experimental support for either of these alternatives is available, the possibility that E3-independent conjugation may play a larger role than was previously envisaged should not be ignored.

Although myosin heavy chains were shown in this study to be ubiquitinated in chicken muscle extracts, generally, ubiquitination of the structural proteins occcurred at a low rate compared with other endogenous proteins, implying that under normal physiological conditions, these proteins would not be ubiquitinated to any great extent (unless immediate degradation of most of such ubiquitinated proteins occurred). That stable ubiquitin-protein conjugates occur in certain muscles has been demonstrated by Ball et al. (1987); the protein arthrin is actually ubiquitinated actin which may form an integral part of the actin cooperative unit. On the other hand, Manetto et al. (1989) have shown that Nemaline bodies of myocytes, which contain predominately actin and α-actinin, do not contain any ubiquitinated species. Other ubiquitin conjugates from muscle have been found by Riley et al. (1988) who have shown the presence of ubiquitin conjugates in the myofibrils, mitochondria and intermyofibrillar regions by immunocytochemistry and by the use of an immunogold probe to detect ubiquitin conjugates. Of particular interest has been the observation that a concentration of immunogold probe occurred at the Z-lines and it has been suggested that α-actinin is a potential substrate for ubiquitination. Such a conjugate in the relevant molecular weight range, however, was not detected in this study, although unmodified α-actinin was in fact identified on Coomassie blue-stained gels. These results, however, do not preclude the possibility that ubiquitination of myofibrils may play a role in damaged or diseased muscle where myofibrils undergo rapid removal; a possibility supported by Riley et al. (1986) who have demonstrated increased ubiquitination in atrophying muscle. To date, however, no ubiquitin-dependent degradation of myofibrillar proteins has been recorded.

The low exogenous protein ubiquitination observed, with lysozyme as a labelled substrate in fresh muscle extracts was not due to to an inefficient assay system, as

reticulocyte fraction II, in this study, was capable of forming ^{125}I-lysozyme-ubiquitin conjugates in an ATP- and ubiquitin-dependent manner, of molecular weights similar to those described in the literature (Waxman et al., 1987; Hatfield & Vierstra, 1989). Thus it is possible that muscle does not contain or has a very low level of some of the conjugating factors; a possibility highlighted by the observation that reticulocyte fraction IIA when incubated with muscle fraction IIB formed ^{125}I-lysozyme-ubiquitin conjugates, but this did not occur when muscle fraction IIA was incubated with reticulocyte fraction IIB. This suggested a deficiency of a factor in fraction IIA, possibly E3. Whether such a deficiency resulted from the low levels of E3 synthesized in muscle tissue in vivo or whether the enzyme was unstable to freezing of fraction II is at present not known.

Lee et al. (1986) have measured E3 activity in rabbit skeletal muscle and have reported that it was comparable to that detected in red blood cells and reticulocytes, even though no ATP-dependent proteolysis could be measured in the muscle and red blood cell system. The method of assaying E3, however, involved incubation with NaOH at the end of the assay, a method that is shown in Chapter 3 to cause auto-ubiquitination of E1 and E2 and would therefore have resulted in an over-estimation of ^{125}I-ubiquitin conjugation to "exogenous" proteins. The only study in which ^{125}I-lysozyme-ubiquitin conjugates were demonstrated in skeletal muscle is that of Fagan et al. (1987) using rabbit extracts. High molecular weight species and an entity of intermediate molecular weight were visible on analysis of crude muscle extracts but were barely visible when fraction II was used. Generally, the appearance of the ^{125}I-lysozyme conjugates was smeared and this could have been due to the addition of hemin during the incubation. Thus ^{125}I-lysozyme conjugates in a typical ladder arrangement as seen in reticulocytes (Waxman et al., 1987)

or wheat germ (Hatfield & Vierstra 1989), have not readily been demonstrated in skeletal muscle.

Inclusion of hemin in both the muscle and the reticulocyte incubates with ^{125}I-lysozyme, was associated with the formation of insoluble ^{125}I-lysozyme-protein complexes, independently of the presence of ATP and ubiquitin. Hemin has been demonstrated to be involved in free radical generation (Ursisi et al., 1981) which in certain cases (as with glutathione-S-transferase), is involved in the aggregation of this protein by non-disulphide covalent bonds (Vincent et al., 1988). It must be mentioned, however, that lysozyme was shown in the same study not to be covalently crosslinked. Apart from causing protein cross-linking, hemin has also been demonstrated to be involved in protein fragmentation (Aft & Mueller, 1984), and causing alterations of protein conformation (Liu et al., 1985), presumably by the generation of free radicals. In the system described in this study the dependence, however, of the ^{125}I-lysozyme-protein complex formation on DTT and partial inhibition by NEM, suggests that disruption of the disulphide bonds of ^{125}I-lysozyme and fraction II proteins by DTT may be more important than free radical generation. Such structures could then unfold and allow the hydrophobic hemin molecules to interact with these proteins and to form insoluble high molecular weight complexes of which some interactions may be covalent. Whatever the mechanism, these ^{125}I-lysozyme-protein complexes are not true ubiquitin conjugates and are not involved in ubiquitin-dependent degradation of proteins.

In conclusion, the chicken skeletal muscle system has a ubiquitin-conjugating activity active on endogenous proteins, and is thus similar to several other tissues. As a result of the heterogeneity of fraction II proteins, one cannot be sure whether the conjugates detected represent mono- or multi-ubiquitinated species. Ubiquitin conjugation to a number of exogenous proteins occurs at rates which are low compared with those observed in reticulocyte extracts,

which may be due to low levels of, or to instability, of E3. Skeletal muscle thus may be able to form stable conjugates with certain endogenous proteins, but the activity of the ubiquitin-dependent pathway of protein degradation is uncertain.

CHAPTER 3

AUTO-UBIQUITINATION OF UBIQUITIN-ACTIVATING ENZYMES FROM CHICKEN BREAST MUSCLE

3.1 INTRODUCTION

In Chapter 2 it was shown that conjugation of ^{125}I-ubiquitin to endogenous proteins takes place in particle-free, ubiquitin-depleted fractions prepared from chicken skeletal muscle. During incubations at pH 7.4, small amounts of stable ubiquitin conjugates formed that had M_r values equivalent to mono-ubiquitinated forms of E1 and several E2 species. The possibility that these conjugates represented auto-modified ubiquitin transferring enzymes was considered. Reversible auto-modification of regulatory proteins is a well-understood process in the case of certain protein kinases, for example tyrosine-specific (receptor) kinases (Yarden & Ullrich, 1988) and calcium, calmodulin-dependent protein kinases (Lickteig et al., 1988). Although the thermolabile mouse mammary carcinoma cell line, ts85, has been reported to contain ubiquitinated E1 molecules (Mayer et al., 1989a; Finley et al., 1984), the mechanisms involved in such a reaction are not known. With this in mind, the ubiquitin conjugation pathway of chicken skeletal muscle has been explored in an attempt to understand the mechanisms and functional consequences of such an auto-modifying reaction.

3.2 METHODS

3.2.1 Muscle and reticulocyte fraction II preparations

Fraction II extracts were obtained for both the muscle and reticulocyte extracts as described in sections 2.2.1 and 2.2.3, respectively.

3.2.2 Ubiquitin-affinity column purification of E1 and E2

Both muscle and reticulocyte fraction II were subjected to ubiquitin-affinity column purification essentially according to the method of Hershko et al. (1983). 20 mg of fraction II protein was applied to a 1 ml ubiquitin-affinity column (10 mg ubiquitin/ml of swollen activated Sepharose 4B) at room temperature in the presence of 5 mM ATP and 5 mM magnesium acetate. The unadsorbed protein was washed from the columnm in 50 mM Tris-HCl, 0.25 M KCl all at pH 7.4. The bound protein was eluted with 50 mM Tris-HCl, 10 mM DTT, pH 7.4. Ovalbumin (0.2 mg/ml) was added to stabilize the conjugating factors and the sample was dialyzed overnight against a buffer containing 25 mM Tris-HCl, 0.5 mM DTT, 0.1 mM EDTA, 1 mM magnesium acetate, 10% (v/v) glycerol, all at pH 7.4.

3.2.3. Formation of ^{125}I-ubiquitin conjugates

^{125}I-ubiquitin conjugates were formed and analyzed on SDS-PAGE as described in sections 2.2.7 and 2.2.11, respectively. ^{125}I-ubiquitin thiolesters were analyzed by treating the incubated samples with 40 μl of 2.5% SDS in the absence of 2-mercaptoethanol. The samples were not heat treated and were run on 5-20% gradient SDS-PAGE at 4°C.

3.2.4. NaOH treatment of incubated fractions

After incubating fraction II with ^{125}I-ubiquitin and ATP as described in section 2.2.7. samples were treated with enough 0.5 M NaOH to bring the final pH to 11.7 (10 μl). The mixtures were incubated for a further 10 min at 37°C and the reaction terminated by addition of SDS and 2-mercaptoethanol. These samples were heat-treated for 2 min at 90°C and electrophoresed at room temperature. Variations in this protocol are mentioned in the text.

3.2.5 Formation of the TLCK-induced ^{125}I-ubiquitin conjugate

100 μg of fraction II protein was pre-incubated for 2 hr at 37°C with 50 μM TLCK, prior to the addition of 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) and 5 mM ATP and incubated for a further 2 hr. Samples were then heated at 90°C in the presence of 5% 2-mercaptoethanol and 1% SDS and run on 5-20% gradient SDS gels.

3.2.6 Assay of the adenylate-forming activity of E1 (ubiquitin-dependent ATP-$^{32}PP_i$ exchanges)

Thiolesters of ubiquitin, present in fraction II after its preparation, were cleaved by treatment with 1 M hydroxylamine at pH 7.0 for 20 min at 37°C, followed by re-chromatography on DEAE-cellulose as described in section 2.2.1. Samples of this material were assayed for adenylate-forming activity by measuring the rate of ubiquitin-dependent transfer of $^{32}PP_i$ to ATP as described by Calendar & Berg (1966): briefly, the samples were incubated, with and without 1 μg ubiquitin, for 20 min at 37°C in the presence of $^{32}PP_i$ (1 x 10^5 cpm), 50 mM NaF, and 2.5 mM ATP, all at pH 7.4 in a final volume of 140 μl. The reaction was

stopped by the addition of 300 µl of 2.5% (w/v) acid-washed activated charcoal in 2% TCA containing 0.1 M tetra-sodium pyrophosphate. Samples were filtered on 2.5 cm Whatman glass fibre discs and the bound ATP was counted by Cerenkov radiation in a Beckman liquid scintillation counter.

3.2.7 Turnover of auto-ubiquitinated proteins

NaOH-treated samples (prepared as described in section 3.2.4.) were neutralized to pH 7.5 using drops of 0.05 M HCl. Aliquots containing $1x10^4$ cpm were incubated for 6 hr at 37°C with 100 µg of fraction II protein, 2.5 mM DTT, with or without 2.5 mM ATP. Protease inhibitors were added as indicated in the text. Samples were heated with 1% SDS and 5% 2-mercaptoethanol and the loss of radioactivity from alkali-induced bands was assessed after SDS-PAGE and autoradiography. TLCK-induced ^{125}I-ubiquitin conjugates as formed in section 3.2.5 were similarly treated but the neutralization step was omitted.

3.3 RESULTS

3.3.1 Ubiquitin conjugates of chicken skeletal muscle extracts; a closer examination

In section 2.3.1 it was shown that ^{125}I-ubiquitin readily formed conjugates with endogenous proteins in an ATP-dependent manner. After incubations of 2 hr or longer, some of the more obvious ^{125}I-ubiquitin conjugates had M_r's of 115 000 and in the 24 000 - 45 000 region (Fig. 3.1, lane a). The possibility that these might be mono-ubiquitinated forms of the ubiquitin-activating enzyme E1 and some of the ubiquitin carrier proteins, E2 was considered. Samples incubated identically but treated to protect the thiolester bond (no heat or 2-mercaptoethanol treatment prior to SDS-PAGE), when analyzed on SDS-PAGE and autoradiography, showed ^{125}I-ubiquitin linked to proteins via a thiolester linkage to occur at the M_r position of 115 000 and in the 24 000- 45 000 region (Fig. 3.1, lane b). This suggested that the isopeptide-linked ^{125}I-ubiquitin conjugates were likely to involve the E1 and some E2 species. In order to investigate the possibility of auto-ubiquitination occurring as a result of nucleophilic attack on the thiolesters by a free amino acid group of these E1 and E2 proteins, samples of fraction II, previously incubated for 3 hr under conjugating conditions with ^{125}I-ubiquitin, were treated with NaOH for 10 min at 37°C (pH 11.7). This resulted in the formation of large amounts of four ubiquitinated proteins with M_r = 115 000, 37 000, 33 000 and 24 000, respectively: these entities survived conditions known to cleave thiolester bonds (Fig. 3.1, lane c). Comparison of these values with those given by the thiolester forms of E1 and E2 after a 30 min incubation at 37°C yielded values of 115 000, 45 000, 37 000, 33 000, 28 000 and 24 000 (Fig. 3.1, lane e), indicating that all of these bands excepting those at the 48 000 and 28 000

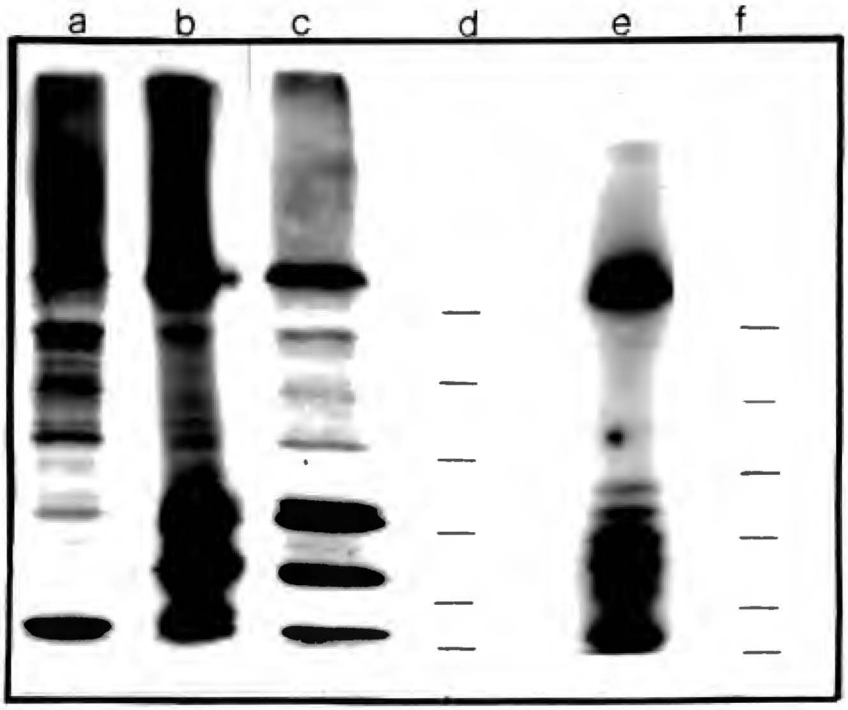


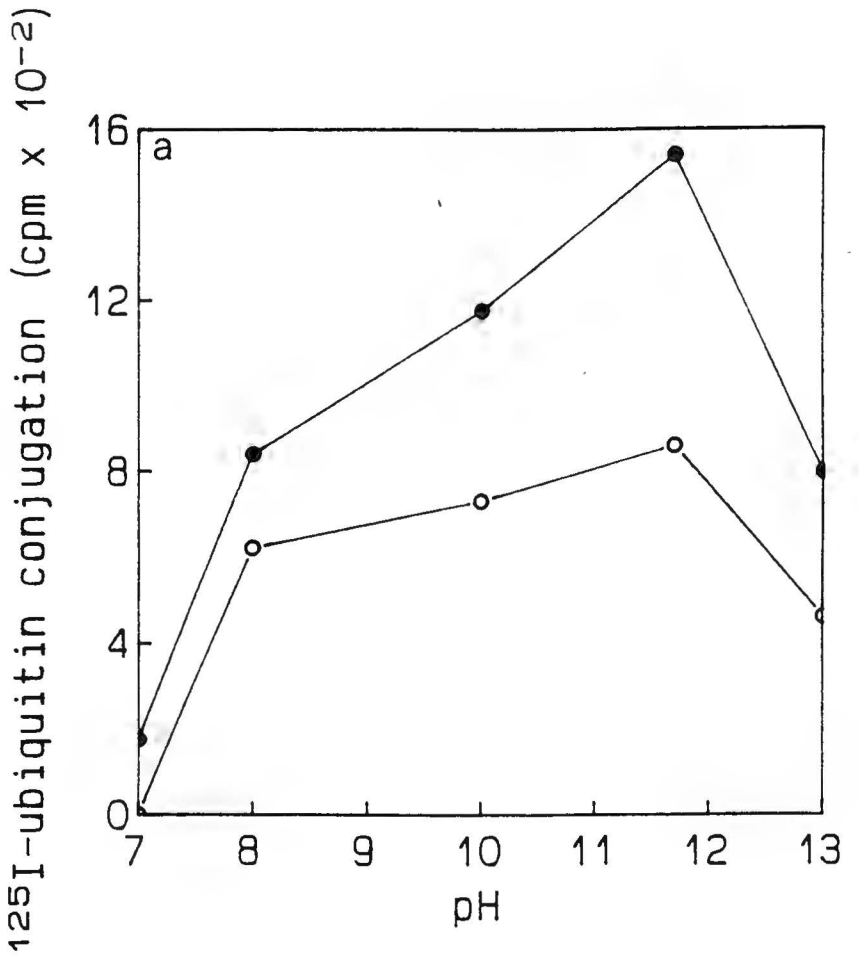
Fig. 3.1 Formation of ubiquitin derivatives of E1, E2 and the endogenous proteins

Lane a: fraction II (100 μg) was incubated at 37°C for 3 hr in the presence of 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) and 5 mM ATP. Samples were heated with 1% SDS and 5% 2-mercaptoethanol at 90°C for 2 min and subjected to SDS-PAGE on a 5-20% gel as described in section 3.2.3. **Lane b:** a separate sample was incubated in the same way but the thiolester bonds were protected by excluding the reducing agent and the heating step and by performing SDS-PAGE at 4°C. **Lane c:** fraction II was incubated as in lane (a) for 3 hr after which NaOH was added to a final pH of 11.7, followed by a further incubation for a further 10 min. Samples were treated as in lane (a). **Lane d:** molecular weight markers for lanes a-c; phosphorlase b (M_r = 94 000), bovine serum albumin (M_r = 68 000), ovalbumin (M_r = 43 000), carbonic anhydrase (M_r = 30 000), soybean trypsin inhibitor (M_r = 20 100), α-lactalbumin (M_r = 14 400). **Lane e:** fraction II was incubated for 30 min and treated as in lane (b). **Lane f:** molecular weight markers for lane (e), as described in lane (d).

positions could be detected by the NaOH treatment. This suggested that the NaOH-induced conjugates were derived directly from the thiolester forms of E1 and some of the E2's, indicative of a mechanism of auto-conjugation to such proteins. The E1 and E2 products formed by NaOH treatment were termed E1-N-Ub and E2-N-Ub as opposed to the thiolester forms, E1-S-Ub and E2-S-Ub.

3.3.2 Optimal conditions for the alkali induction of E1-N-Ub and E2-N-Ub

The presence of ATP and fraction II was essential for the alkali-inducible auto-conjugates of E1 and E2 to form, indicating that anomolous ubiquitin aggregation was not responsible for this phenomenon (results not shown). The length of the incubation in the presence of alkali needed to induce the E1 auto-conjugate was short; maximum conversion occurred in 2 min whilst 10 min was required for the E2 species, whereafter no change in the amounts of alkali-induced bands occurred (results not shown). The optimum pH for the alkali induction was pH 11.7 (Fig. 3.2a) Interestingly, at pH 8.4, although the E1-N-Ub species was only induced to a small extent and those of E2-N-Ub were undetecable, there was the appearance of faint high molecular weight ^{125}I-ubiquitin conjugates (Fig. 3.2b,). These could have been due either to an increase in conjugation at the more alkaline pH, or to a decrease in conjugate breakdown. Lee et al. (1986) likewise found more conjugates present at pH 9.0 and this was attributed to a decrease in the activity of the isopeptidases, which were shown to be more active at neutral pH. Thus these high molecular weight species could represent rapidly turning-over ubiquitin conjugates, which under normal assay conditions do not accumulate to sufficient levels to be detected.



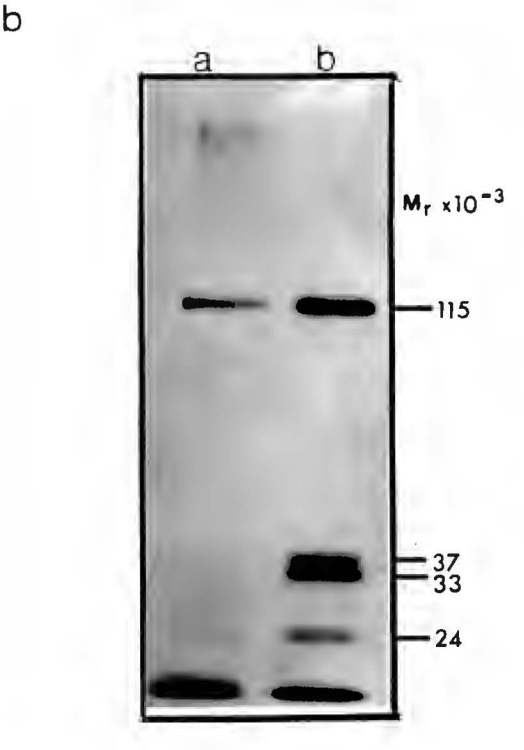


Fig. 3.2 (legend overleaf)

Fig. 3.2 Dependence of auto-ubiquitination of E1 and E2 on pH during post-incubation treatment with NaOH

Fig. 3.2a Quantitation of auto-ubiquitination

Fraction II samples were incubated for 10 min at 37°C with 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) and 5 mM ATP. Various amounts of NaOH were then added to achieve the pH values indicated and the mixtures were incubated for a further 10 min at 37°C. Ubiquitinated E1 (○) and combined E2 (●) species were detected by autoradiography of SDS-PAGE gels and quantitated as described (section 2.2.11).

Fig. 3.2b Autoradiography of auto-ubiquitination

Lane a: sample treated as in Fig. 3.2a and post-incubated with NaOH at pH 8.4. **Lane b:** as in lane (a) but post-incubated at pH 11.7.

In subsequent assays, 25 mM NaOH was added to give a pH of 11.7 and the samples were then post-incubated for 10 min to allow maximum conversion of thiolesters to isopeptide bonds. Under these conditions, the amounts of stable alkali-induced bands were equal (with slight variations in the case of the E2's) to about two-thirds of the thiolesters demonstrably present in the same mixtures before alkali treatment.

3.3.3 Mechanisms of auto-ubiquitination

Various lines of evidence indicated that the alkali-induced E1-N-Ub and E2-N-Ub forms were derived from the thiolester intermediates of E1 and E2.

i) Much shorter periods of incubation were needed for fraction II to become capable of generating alkali-induced E1-N-Ub and E2-N-Ub forms than for the conjugation of other endogenous proteins (Fig. 3.3). Thus after a 10 min incubation at 37°C, the E1-N-Ub and E2-N-Ub species were at a similar level as after 1 hr, whilst no ^{125}I-ubiquitin conjugates had formed. This suggested that these forms represent initial events in the ubiquitin-conjugation pathway, namely the formation of ubiquitin thiolesters to E1 and E2 which are then able to form auto-conjugates by the addition of NaOH.

ii) The necessity of thiolester forms of E1 and E2 being present prior to NaOH treatment was shown in the following ways. Firstly, samples of fraction II were incubated in the presence of ATP and ^{125}I-ubiquitin for 5 min, to enable E1-S-Ub and E2-S-Ub to form; they were then treated with the thiolester-cleaving agent hydroxylamine at pH 7.0 for 20 min at 37°C. Alkali treatment of such fractions did not yield any E1-N-Ub or E2-N-Ub forms when analyzed by SDS-PAGE and autoradiography (Fig. 3.4, lanes a and b). Secondly, pre-treatment of fraction II with NEM at a concentration known to inactivate the thiol sites of E1 and

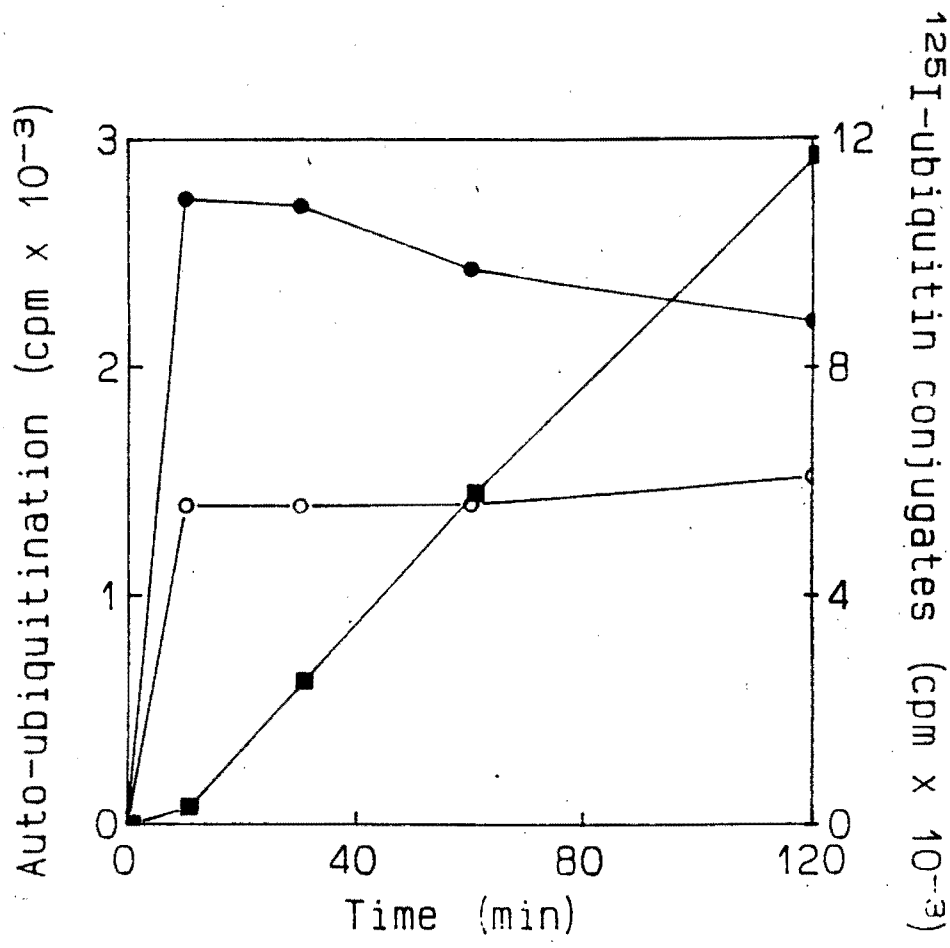


Fig. 3.3 Time course of ^{125}I-ubiquitin conjugate and auto-conjugate formation

100 μg fraction II protein was incubated at 37°C at the indicated times in the presence of 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm), 5 mM ATP, 2.5 mM DTT and 2.5 mM magnesium acetate. Auto-conjugates were formed as described in section 3.2.4 and ubiquitinated E1 (○), combined E2 species (●) and endogenous proteins (■) were analyzed by autoradiography of SDS gels and quantitated as previously described (section 2.2.11).

E2 (Mayer et al, 1989a) prevented the formation of both E1-N-Ub and E2-N-Ub, indicating that the ubiquitin adenylate could not serve as a ubiquitin donor (Fig 3.4 lanes c and d). Thirdly, addition to the incubation mixture of the nucleophile L-lysine, a known ubiquitin acceptor of E2-S-Ub, at the time of NaOH addition, severly depressed the formation of E1-N-Ub and E2-N-Ub (Fig. 3.4, lanes e and f).

iii) The properties of the NaOH-induced E1-N-Ub bonds were those of peptide bonds; they were stable to alkaline conditions and hydroxylamine treatment and resistant to reductive cleavage by 2-mercaptoethanol at 90°C (Table 3.1).

The question of the nature of the bonds was further addressed by testing their stability to isopeptidases or ubiquitin conjugate-degrading enzymes present in fraction II. NaOH-treated mixtures were accordingly neutralised and exposed to fresh fraction II; both the E1-N-Ub and E2-N-Ub forms were susceptible to breakdown under these conditions (Table 3.2). These losses were inhibited by NEM (50 mM), $ZnCl_2$ (125 μM) and PMSF (2 mM) which reflects the properties of an isopeptidase recently described by Rose (1988, personal communication). No intermediates were detected and this was also suggestive of isopeptidase activity.

iv) A similar profile of the alkali-induced E1 and E2 forms was seen on fraction II samples that had been eluted at a high thiol concentration from a ubiquitin-affinity column (results not shown) under conditions known to elute E1 and E2 in the rabbit reticulocyte system (Hershko et al., 1983). This supports the notion that the alkali-induced bands represented E1 and E2 species of the ubiquitin pathway.

v) Reticulocyte extracts were also able to form these auto-conjugates, although the E1 band was not as prominent as in the muscle system while some of the E2 species were more prominent (Fig. 3.5). Thus although the auto-ubiquitination reaction was not an exclusive feature of the muscle system

Table 3.1 Stability of auto-ubiquitinated forms of E1, E2 and of ubiquitin-protein conjugates

^{125}I-auto-ubiquitinated E1 and E2 and ^{125}I-ubiquitin conjugates were prepared as described in Fig. 3.1. They were treated as indicated and prepared for SDS-PAGE by heating with 1% SDS and 5% 2-mercaptoethanol at 90°C for 2 min. Residual labelled material was quantitated as described in section 3.2.3.

	Radioactivity remaining (%)		
Treatment	^{125}I-Ub-N-E1	^{125}I-Ub-N-E2	^{125}I-Ub-proteins
None	100	100	100
0.1 M NaOH; 37°C; 60 min	85	94	82
1 M hydroxyl amine pH 7.0; 20 min; 37°C	80	78	68
5% formic acid; 20 min; 37°C	78	82	77

Table 3.2 **Effects of protease inhibitors on the degradation of the ubiquitin-derivatives of E1 and E2 and of ubiquitin-protein conjugates**

Fraction II (500 μg), 5 μg ^{125}I-ubiquitin (5 x 10^5 cpm) and 5 mM ATP were incubated at 37°C for 2 hr, followed by a 10 min incubation in the presence of 25 mM NaOH. The samples were neutralized and aliquots incubated with 50 μg of fresh fraction II, in the presence of the indicated protease inhibitors, for a further 6 hr at 37°C. Loss of ^{125}I-ubiquitin was measured, after SDS-PAGE and autoradiography, as described in section 3.2.6.

	^{125}I-ubiquitin derivative		
	E1	E2	Other proteins
Treatment	% decrease in activity	% decrease in activity	% decrease in activity
Zero time	0	0	0
No inhibitor	54	40	39
+5 mM NEM	0	5	2
+125 μM $ZnCl_2$	17	11	20
+2 mM PMSF	38	27	34
+12.5 μg/ml chymostatin	44	34	40

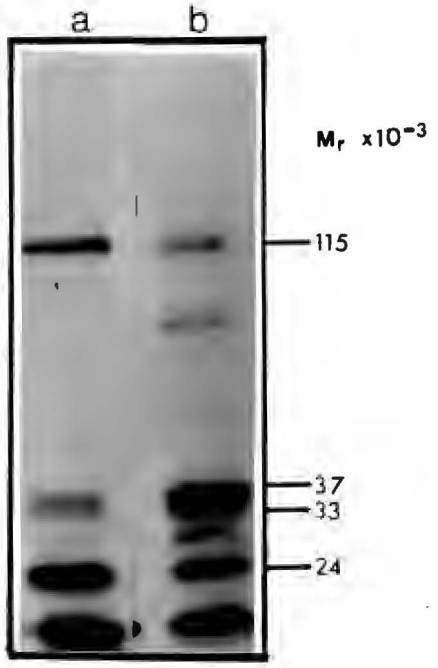


Fig. 3.5 Auto-ubiquitination of reticulocyte fraction II extracts

Lane a: NaOH-induced auto-conjugates of muscle fraction II were formed by incubating fraction II for 10 min at 37°C as described in Fig. 3.4 lane (a). **Lane b:** as in lane (a) but with 100 μg reticulocyte fraction II protein.

the activities varied, possibly indicating different levels of E1 and E2.
These results confirm that the ubiquitin-containing bands appearing after NaOH treatment were derived from the ubiquitin thiolesters of E1 and several but not all of the E2 species. Such auto-conjugation presumably occurred when the pH was increased to a more alkaline level, causing the thiolester linkage between ubiquitin and E1 or E2 to be susceptible to stronger nucleophilic attack by an amino group on the same protein: transacylation to this group would readily occur with the formation of an isopeptide bond, representing E1 and E2 auto-conjugates.

3.3.4 Activity of the adenylate- and thiol-forming sites of E1 after auto-ubiquitination

The fact that the E1-N-Ub band appeared as one of many conjugated proteins after incubations of fraction II and ^{125}I-ubiquitin for several hours at pH 7.5, raised the possibility that enzyme preparations exposed to prior incubations at relatively mild pH with unlabelled ubiquitin and ATP, contained varying numbers of auto-ubiquitinated E1 molecules. This meant that the effects of the modification on enzyme function could be studied. Incubation of fraction II at pH values varying between 7.5 and 9.0 revealed a steady increase in the amounts of E1-N-Ub and E2-N-Ub formed as a percentage of the total thiolester-forming capacity available (Fig. 3.6a). The adenylate- and thiolester-forming activities of such E1 preparations were assayed; Fig. 3.6b shows that the presence of a significant number of auto-ubiquitinated E1 molecules did not affect the ATP-$^{32}PP_i$ exchange reaction, even though the alkaline pH to which these molecules were exposed caused some loss of activity also in the ubiquitin-less controls. This suggests that the covalently bound ubiquitin moiety did not

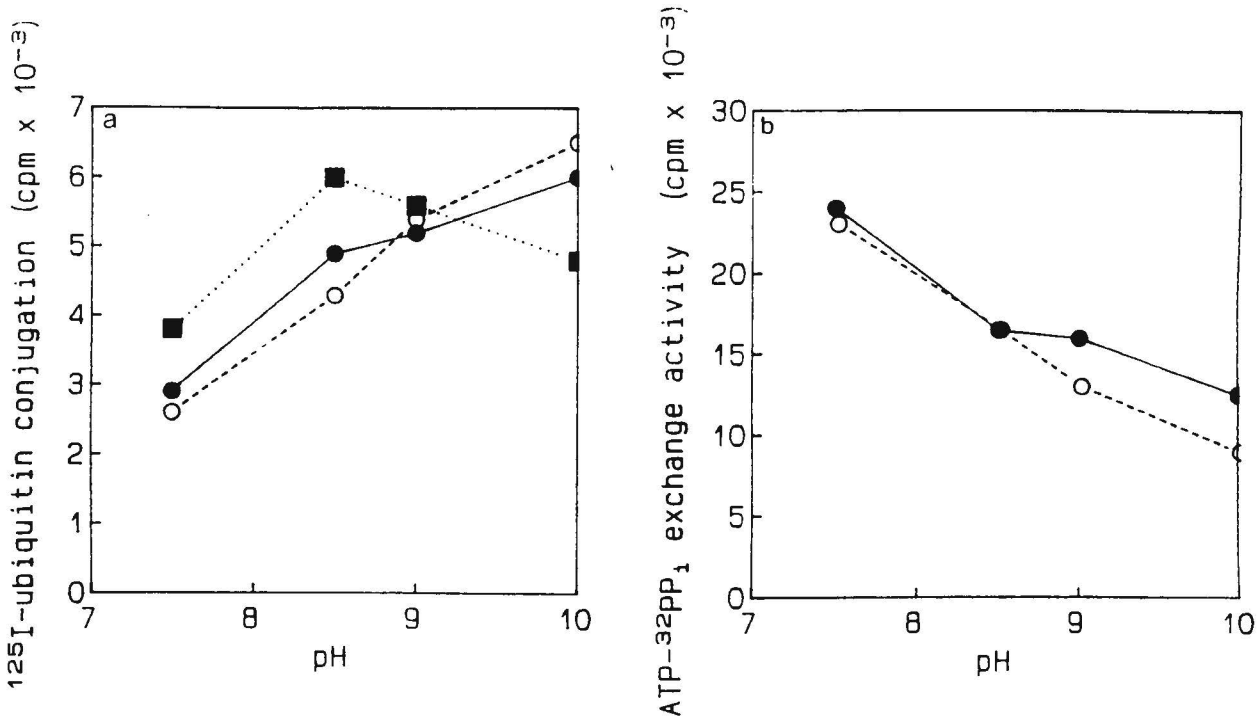


Fig. 3.6 Effects of incubation pH on the formation of ^{125}I-ubiquitin derivatives of E1, E2 and other proteins compared with residual ubiquitin ATP-^{32}PP$_i$ activity

Fig. 3.6a Effect of pH

Fraction II (100 μg) was incubated with 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) and 5 mM ATP for 2 hr at the pH indicated. E1-N-ubiquitin (○), E2-N-ubiquitin (●), and ubiquitin-protein conjugates (■) were quantitated after SDS-PAGE and autoradiography, as descibed in section 3.2.4.

Fig. 3.6b ATP-^{32}PP$_i$ exchange activity

100 μg fraction II protein was pre-incubated for 2 hr in the presence (●) and absence (○) of 1 μg unlabelled ubiquitin and 5 mM ATP. After neutralization of all samples to pH 7.4, ubiquitin-dependent adenylate-forming activity was measured by ATP-^{32}PP$_i$ exchange reactions as described in section 3.2.6.

interfere with the binding of a further ubiquitin molecule to the adenylate site.

In order to determine whether the bound ubiquitin affected the enzyme's ability to form bound ubiquitin thiolesters, thiolester-forming activites of samples similarly incubated in the presence of cold ubiquitin at pH 7.5 and 9.0, were measured after hydroxylamine treatment and removal of free ubiquitin by DEAE-cellulose chromatography. Unfortunately, these procedures were accompanied by a large loss of protein and enzyme activity, but the samples pre-exposed to ubiquitin at the different pH's reproducibly showed a greater loss of activity than those not exposed to ubiquitin (500 cpm and 315 cpm at pH 7.5 in the presence and absence of ubiquitin, respectively, as opposed to 325 cpm and 155 cpm at pH 9.0 in the presence and absence of ubiquitin, respectively), suggesting that the ability to form thiolesters may have been impaired. This preliminary evidence is consistent with the idea that auto-ubiquitination of E1 causes a decrease in the thiolester-forming activity, possibly by sterically hindering ubiquitin transfer from the adenylate to the thiolester site.

3.3.5 Effects of TLCK on ubiquitin conjugation reactions

TLCK is a chloromethyl ketone derived from N-α-tosyl-L-lysine. It is an irreversible inhibitor of trypsin and acts by alkylating a histidine residue in the active site of trypsin (Shaw et al., 1965). It is also able to inhibit thiol proteases by the alkylation of cysteine residues (Barrett, 1973). When fraction II was pre-incubated for 2 hr in the presence of 50 μM TLCK and then incubated with ATP and ^{125}I-ubiquitin, the banding pattern on subsequent SDS-PAGE and autoradiography was dominated by a ubiquitin conjugate at M_r = 115 000 (Fig. 3.7, lanes a and b). This entity was identical in molecular weight to both the E1-N-

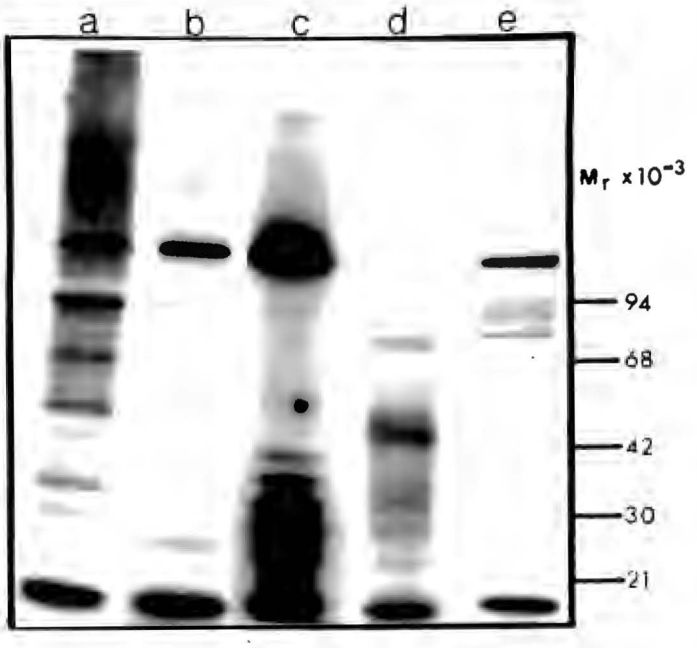


Fig. 3.7 Effects of TLCK on the formation of ubiquitin conjugates and ubiquitin derivatives of E1 and E2

Lane a: 100 μg fraction II protein was incubated for 2 hr as in Fig. 3.1, lane (a). **Lane b:** 100 μg fraction II protein was pre-incubated for 2 hr at 37°C with 50 μM TLCK, prior to the addition of 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) and 5 mM ATP, and incubated for a further 2 hr. Samples were then heated at 90°C in the presence of 2-mercaptoethanol and run on 5-20% gradient SDS gels. **Lane c:** 100 μg fraction II protein was incubated as in Fig. 3.1, lane (b) with the protection of the thiolester bond. **Lane d:** as in lane (a) but with 100 μg reticulocyte fraction II instead of muscle extract. **Lane e:** as in lane (b) but with 100 μg reticulocyte fraction II protein instead of muscle fraction.

Ub and E1-S-Ub forms (Fig. 3.7, lane c), suggesting that TLCK strongly promoted the formation of the auto-ubiquitinated form of E1 even at pH 7.4. No E2-N-Ub forms were observed in this reaction.
A similar pattern was seen in reticulocyte fraction II (Fig. 3.7, lanes d and e). The M_r = 115 000 band, however, was not as marked as in the case of muscle fraction II and some lower molecular weight species were also present. Whether these are ^{125}I-ubiquitin conjugates or breakdown products of E1-N-Ub is not known.

3.3.5.1 Properties of the TLCK-induced ^{125}I-ubiquitin conjugate

The TLCK-induced ^{125}I-ubiquitin conjugate was stable to treatment with 0.1 M NaOH or 1 M hydroxylamine (pH 7.0), indicating, as in the case of NaOH treatment, that an isopeptide bond was formed between E1 and ^{125}I-ubiquitin. This was supported by degradation studies of the TLCK-induced ^{125}I-ubiquitin conjugate incubated with fraction IIA (0-38% ammonium sulphate fraction) which contained both the ubiquitin conjugate-degrading enzyme and isopeptidases. Table 3.3 shows that the TLCK-induced conjugate was susceptible to such degradation in an ATP-independent manner, suggesting that isopeptidases were responsible for the breakdown. This supported the notion that TLCK induced isopeptide bond formation between ubiquitin and E1.
The length of time taken for the TLCK-induced E1-N-Ub species to form was next investigated; a pre-incubation of fraction II with TLCK was essential if the TLCK-induced auto-conjugate was to form. The longer the pre-incubation, the more auto-conjugated E1 was formed in subsequent incubations with ATP and ^{125}I-ubiquitin (Fig. 3.8). Although some auto-conjugation occurred in the incubations after 2 hr or more, these were probably due to the auto-conjugation of E1 and E2's normally seen at pH 7.4 (see

Table 3.3 Susceptibility of the TLCK-induced ^{125}I-ubiquitin conjugate to degradation

Fraction II (500 μg) was pre-incubated with 50 μM TLCK for 2 hr at 37°C, followed by the addition of 5 μg ^{125}I-ubiquitin (5 x 10^5 cpm), 5 mM ATP. The sample was incubated for a further 2 hr at 37°C after which aliquots (corresponding to 1 x 10^4 cpm) were incubated with 50 μg of fresh fraction II under the conditions described. Where indicated, ATP was depleted by incubation with 10 mM glucose and 1 μg hexokinase for 10 min at 37°C. Loss of ^{125}I-ubiquitin from auto-conjugated E1 was measured after SDS-PAGE and autoradiography.

Conditions	E1-N-Ub (cpm)	% decrease in activity
0 hr	812	
2 hr incubation (no fraction II)	746	8
10 hr incubation (no fraction II)	503	38
2 hr incubation (fraction IIA)	310	58
10 hr incubation (fraction IIA)	86	90
10 hr incubation (fraction IIA; no ATP)	94	88

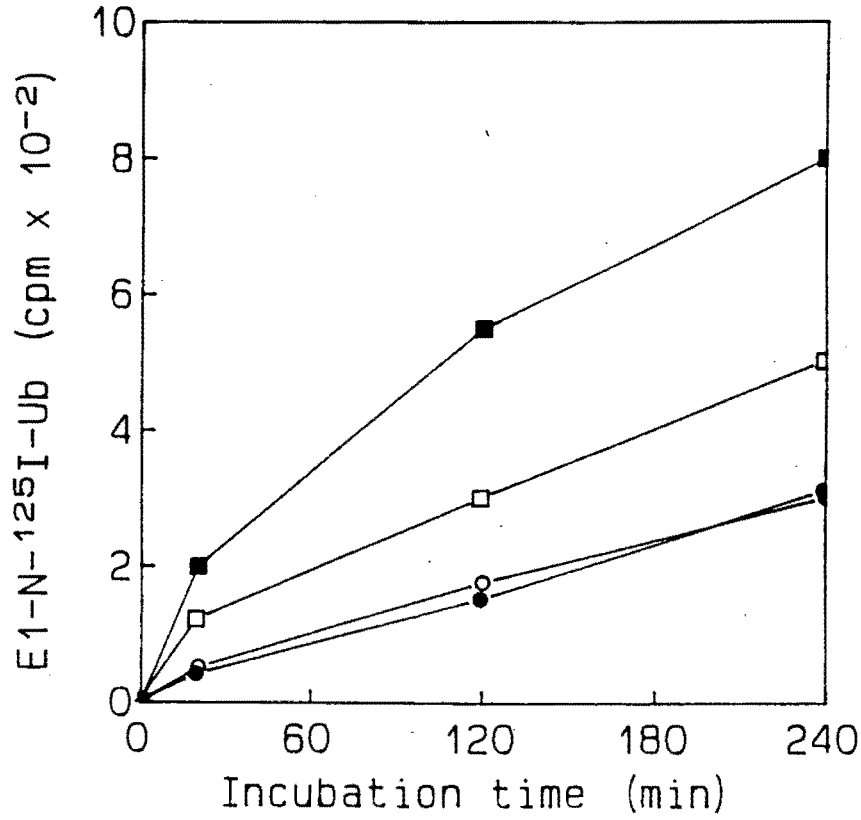


Fig. 3.8 Effect of pre-incubation in the presence of TLCK on the formation of E1-N-Ub

100 μg fraction II protein was pre-incubated at the times indicated at 37°C with 50 μM TLCK, prior to the addition of 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) and 5 mM ATP and incubated further at the indicated times. Samples were heated at 90°C in the presence of 1% SDS and 5% 2-mercaptoethanol and run on a 5-20% gradient SDS gel. The amount of E1 auto-conjugated was quantitated as described in section 3.2.3. No pre-incubation and no TLCK (●), no pre-incubation but assayed in the presence of 50 μM TLCK (○), pre-incubated for 40 min in the presence of 50 μM TLCK (□), pre-incubated for 120 min in the presence of 50 μM TLCK (■).

section 3.3.1). These results imply that the action of TLCK is slow, requiring hours rather than minutes (as in the case of NaOH) to act. For all the incubations unless stated, a 2 hr pre-incubation of fraction II and TLCK, followed by a 2 hr incubation with ATP and ^{125}I-ubiquitin, was used.

The optimal TLCK concentration for E1-N-Ub formation was 50-100 μM (Fig. 3.9). With an increase in the formation of E1-N-Ub there was a marked decrease in the level of ubiquitin conjugates to endogenous proteins, suggesting an impairment of the overall conjugation pathway for other proteins. At concentrations above 100 μM, TLCK presumably became toxic to the conjugation system and no conjugates or E1-N-Ub were formed.

3.3.5.2 Effect of other trypsin protease inhibitors

To see if the effect of TLCK was related to its trypsin protease-inhibiting activity or to other chemical actions, the trypsin inhibitors benzamidine and STI, and the chymotrypsin inhibitor, TPCK were used with the extracts in a manner analogous to TLCK; in each case the E1 auto-conjugation was monitored. Of these inhibitors, only TPCK slightly mimicked the effects of TLCK in that, although ^{125}I-ubiquitin conjugates were still formed, there was a definite increase in the amount of E1-N-Ub that was formed (results not shown); both STI and benzamidine did not alter the conjugation pattern nor induce E1-N-Ub formation. As both TLCK and TPCK inhibit trypsin and chymotrypsin, respectively, by alkylation of an essential histidine residue or thiol site of the protease, it is likely that the induction of E1-N-Ub was due to a similar alkylation of a histidine residue or thiol site on E1.

Addition of NaOH to benzamidine-treated fraction II induced the E1-N-Ub band to the same extent as in the non-treated system (Fig. 3.10, compare lanes e and f with a and b). The

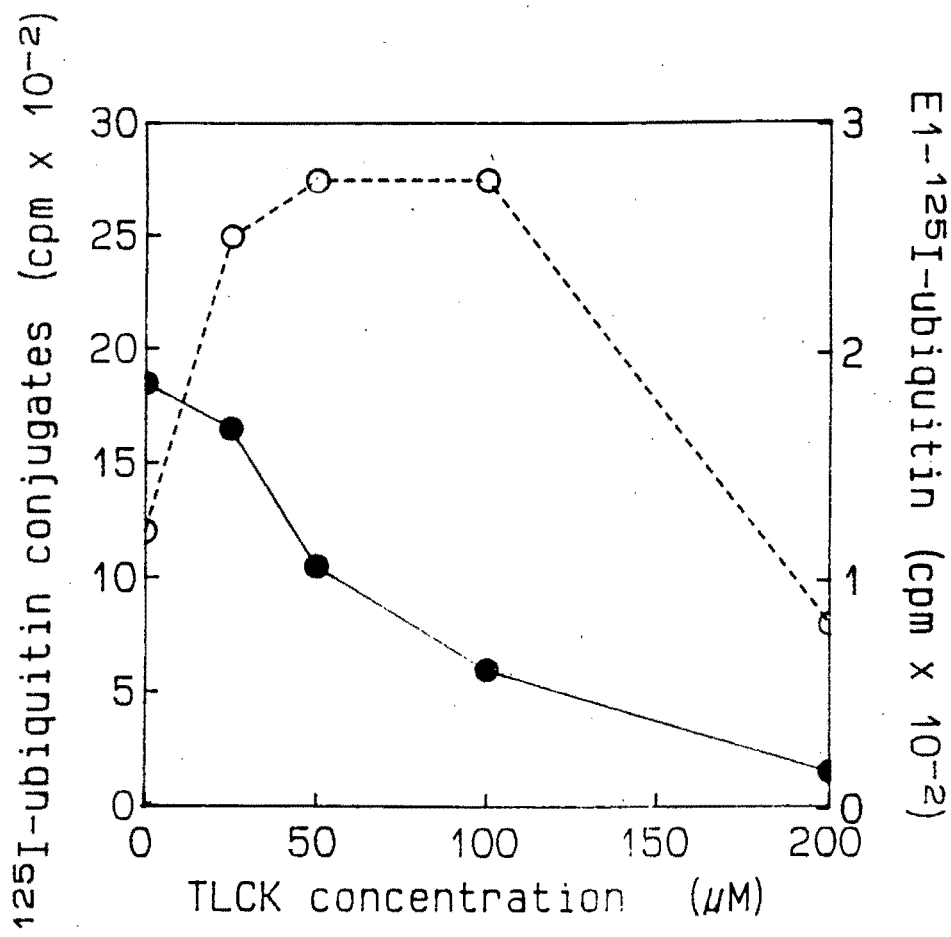


Fig. 3.9 Effect of TLCK concentration at pH 7.4 on the auto-ubiquitination of E1 and the formation of ubiquitin conjugates

100 μg fraction II protein was incubated for 2 hr with TLCK at the indicated concentrations prior to the addition of 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) and 5 mM ATP. Samples were then incubated for a further 2 hr and ^{125}I-ubiquitin conjugates (o) and auto-ubiquitinated E1 (●) quantitated after SDS-PAGE and autoradiography.

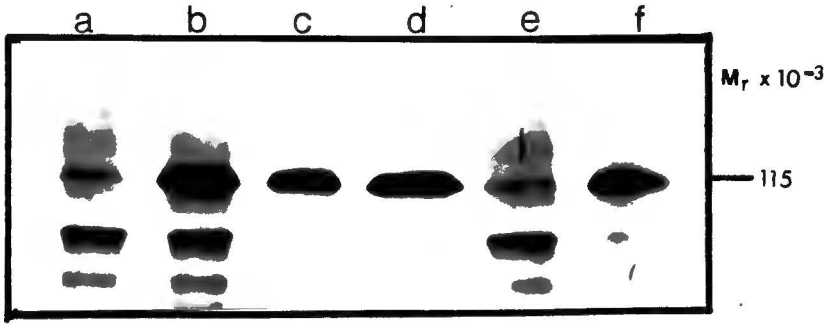


Fig. 3.10 Effect of benzamidine on E1-N-Ub formation

Lane a: fraction II protein (100 μg) was incubated at 37°C for 2 hr in the presence of 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) and 5 mM ATP. Samples were treated with 1% SDS and 5% 2-mercaptoethanol and subjected to SDS-PAGE on a 5-20% gradient gel. **Lane b:** 100 μg fraction II was incubated as in lane (a) after which NaOH was added to a final pH of 11.7, followed by incubation for a further 10 min. **Lane c:** 100 μg fraction II protein was pre-incubated for 2 hr at 37°C with 50 μM TLCK, prior to the addition of 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) and 5 mM ATP and incubated for a further 2 hr. Samples were then treated as in lane (a). **Lane d:** as in lane (c) but treated with NaOH after the incubation, as in lane (b). **Lane e:** as in lane (c) but with 200 μM benzamidine instead of 50 μM TLCK. **Lane f:** as in lane (d) but with 200 μM benzamidine instead of 50 μM TLCK.

fact that benzamidine did not affect E1 auto-conjugation gave further support to the notion that the TLCK induction of E1-N-Ub was independent of a trypsin protease.

3.3.5.3 The effects of NaOH on TLCK-induced auto-conjugates

Alkali treatment of fraction II pre-incubated with TLCK provided information concerning its E2-S-Ub status. At 100 μM TLCK, the amount of ubiquitin associated with the M_r = 37 000 and 33 000 forms of E2 was greatly reduced, indicating that although ubiquitin could be transferred from E1 to these E2's, this occurred at a reduced rate under these conditions (Fig. 3.11). Addition of NaOH to fraction II containing the TLCK-induced E1 auto-conjugates caused an increase in the level of auto-ubiquitination of this species (Table 3.4 and Fig. 3.10, lanes c and d), suggesting that a sub-population of ubiquitin thiolesters present on E1, or a certain population of E1 molecules, may have been unaffected by TLCK.

3.3.5.4 The effects of TLCK on the adenylate site of E1

The activity of the adenylate-forming site of E1 treated with TLCK was next investigated. The ubiquitin-dependent ATP-$^{32}PP_i$ exchange reaction was markedly decreased as the TLCK concentration was increased (Fig 3.12); at TLCK concentrations normally used in the ^{125}I-ubiquitin conjugation assays, a 50% decrease in the adenylate activity occurred. Thus one action of TLCK was to affect the adenylate site, possibly by the alkylation of an essential histidine residue; this decrease in adenylate activity was presumably responsible for the decrease in ^{125}I-ubiquitin conjugates formed.

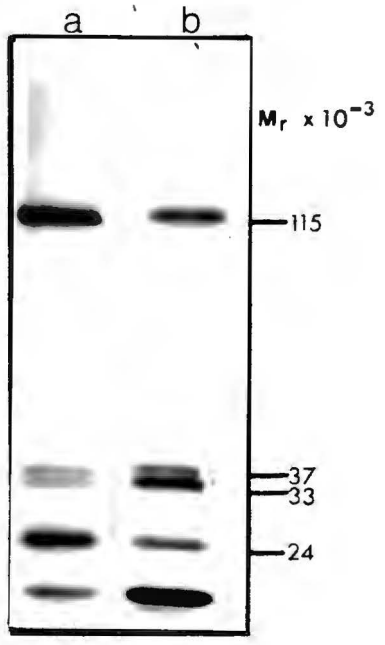


Fig. 3.11 Effect of TLCK on the transfer of ^{125}I-ubiquitin from E1 to E2

Lane a: NaOH-induced auto-conjugates of TLCK-treated fraction II were formed by pre-incubating fraction II (100 μg) for 2 hr at 37°C with 50 μM TLCK, prior to the addition of 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) and 5 mM ATP and incubated for a further 10 min. Samples were then treated with NaOH for 10 min (final pH = 11.7) and heated at 90°C in the presence of 2-mercaptoethanol and run on 5-20% gradient SDS gels. **Lane b:** NaOH-induced auto-conjugates of fraction II were formed as in Fig. 3.4, lane (a) and subjected to SDS-PAGE.

Table 3.4 Effects of alkali treatment on TLCK-induced auto-conjugates

100 μg fraction II protein was incubated for 2 hr with 1 μg ^{125}I-ubiquitin (1 x 10^5 cpm) and ATP for 2 hr, under the indicated conditions and as described in Fig. 3.10, lanes a-d.

Sample	auto-conjugation of E1 (cpm)
Control	800
+ TLCK	1616
+NaOH	2234
+ TLCK + NaOH	2132

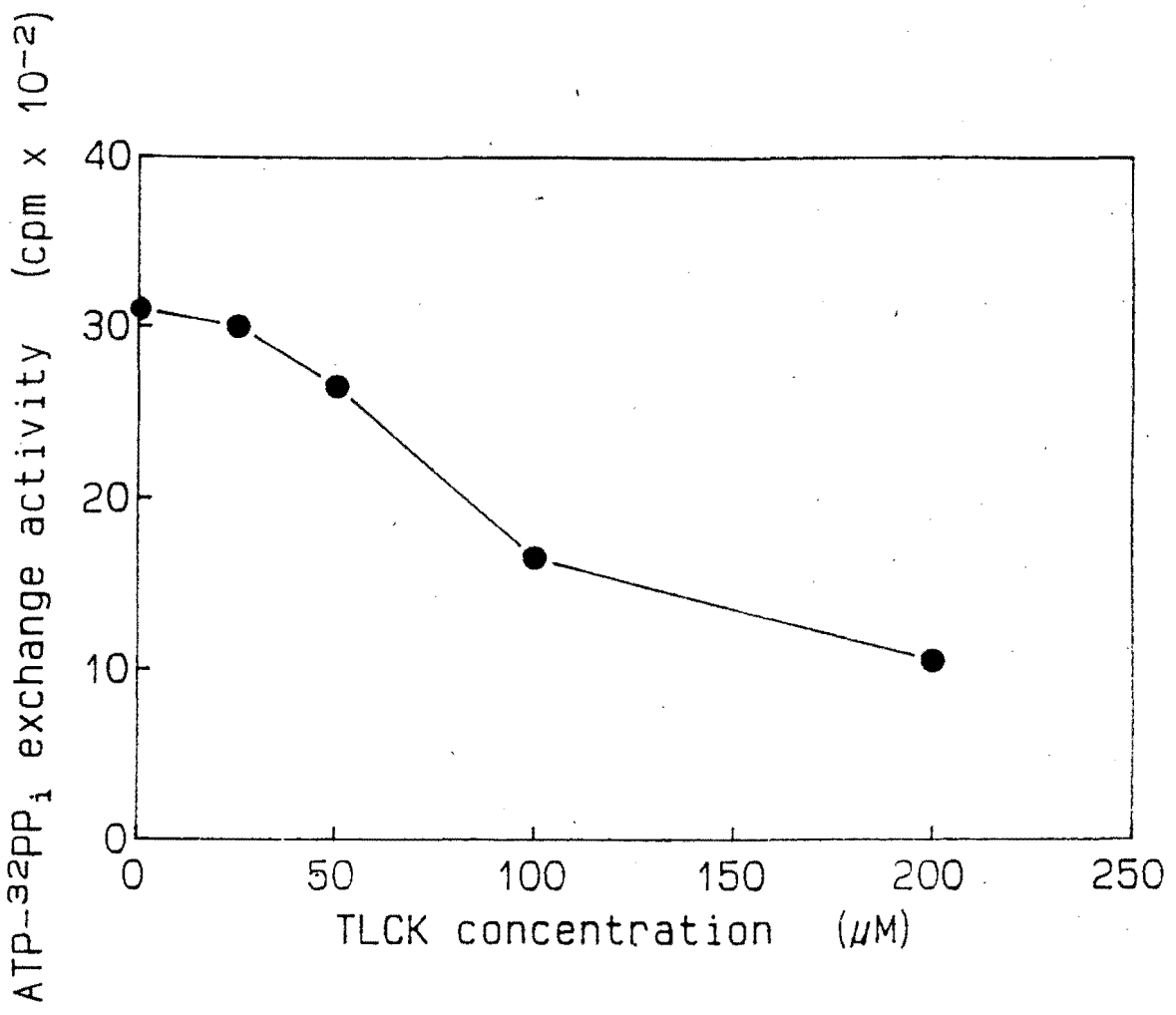


Fig. 3.12 Effect of TLCK on the ubiquitin-adenylate forming activity of E1

100 μg fraction II protein was incubated with 1 μg unlabelled ubiquitin and TLCK at the indicated concentrations for 2 hr at 37°C. Ubiquitin adenylate-forming activity was measured by ATP-$^{32}PP_i$ exchange, as described in section 3.2.6.(●)

3.4 DISCUSSION

The possibility of auto-ubiquitination of ubiquitin-charged enzymes arose from the observation that incubation at pH 7.4 of fraction II with ^{125}I-ubiquitin and ATP slowly led to the formation of ubiquitinated forms of E1 and several E2 species. The reaction was greatly enhanced when the incubated extracts were brought to a more alkaline pH; under these conditions ubiquitinated forms of E1 and three of the five E2's appeared in much larger amounts. These auto-conjugates were shown to be derived directly from ubiquitin thiolesters of E1 and E2 and to involve the formation of a peptide bond. Unfortunately, no primary structure or higher strucures for E1 or any of the E2's are known and it has not been possible to determine whether a particular residue on these proteins is regularly a ubiquitin acceptor for auto-conjugation. One may assume that a spatially adjacent lysine or N-terminal residue on E1 or E2 is involved in nucleophilic attack on a ubiquitin thiolester of E1 or E2, resulting in peptide bond formation.

Only one form of ubiquitin-E1 complex with an M_r value of 115 000 was consistently found in these incubations, which suggests that only one ubiquitin molecule (M_r = 8 650) was conjugated per E1 monomer (M_r = 105 000). Finley et al. (1984) have also reported that partially purified E1 fractions from the thermolabile mouse mammary carcinoma cell line, ts85, after a 5 min incubation at 37°C in the presence of ^{125}I-ubiquitin and ATP, contained ubiquitinated E1, which was stable to 2-mercaptoethanol and heat treatment. The amounts of ubiquitinated E1 formed in ts85 extracts were much higher than those found in extracts similarly prepared from the parental strain, FM3A and from rabbit reticulocytes. After heat treatment at the non-permissive temperature (40°C), the ts85 extracts formed multi-ubiquitinated E1's of up to four ubiquitins per E1

monomer. More recently, Mayer et al. (1989a) purified E1 from ts85 and FM3A to homogeneity and found E1 to be associated with a number of proteins of slightly higher molecular weight and suggested that these may be ubiquitinated forms of E1 which occurred in vivo or which had formed as a result of the purification procedure. Hatfield & Vierstra (1989), however, have purified several E1 species from wheat germ extracts and reticulocytes but have shown with antibodies to E1 and ubiquitin that these proteins are isoforms of E1 and do not contain any ubiquitin.

The auto-conjugated E2's had M_r values on SDS-PAGE of 37 000, 33 000 and 24 000. Difficulty was had in correlating these values with the known molecular weights of the E2 species in rabbit reticulocytes as the number of ubiquitin molecules bound per E2 species was not known. The fact, however, that the chicken skeletal muscle auto-conjugates were of molecular weights similar to those obtained in the reticulocyte extracts, suggests that these two systems have similar, if not identical E2 species. As yet there is no explanation of why only three of the five E2's were auto-conjugated. Both E1 and four of the five E2's of rabbit reticulocytes exist intracellulaly in a weak subunit interaction as dimers and tetramers (Haas & Bright, 1988). Auto-ubiquitination could thus have involved inter-subunit as opposed to intra-subunit reactions, which may imply that those E2 forms that do not transacylate are monomeric species of E2.

At present, the physiological significance of auto-ubiquitinated forms of the ubiquitin-charging enzymes in regulating the ubiquitin pathway in living cells is unknown. It has been shown in this study that their formation was not unique to chicken muscle, as rabbit reticulocyte extracts similarly formed alkali-induced auto-conjugates. The observation by Finley et al. (1984) that the thermolabile E1 of ts85 is readily ubiquitinated and forms multi-ubiquitinated species at the non-permissive

temperature, suggests that auto-ubiquitination could be a mechanism for the removal of abnormal E1's from the cell. Such auto-conjugates could be the result of a typical conjugation pathway catalyzed by E3 or could occur by direct intramolecular transacylation; in the latter case, the conformation of E1 from ts85 cells could be such that the transacylation reaction is enhanced. A preliminary indication was obtained in chicken skeletal muscle extracts that the thiolester-forming ability of E1 may be reduced after auto-conjugation at neutral and alkaline conditions; the affected E1 molecules might have been degraded by the ubiquitin conjugate-degrading protease, or they might have been de-ubiquitinated by isopeptidases. It has been shown in this study that isopeptidase(s) are capable of disassembling the E1 and E2 auto-conjugates, suggesting that the latter possibility exists. Multi-ubiquitinated E1 species of the ts85 cells would, however, have to be formed for the protein to be degraded by the ubiquitin conjugate-degrading enzyme, in keeping with the model that only multi-ubiquitinated proteins are recognized and degraded by such a protease (Chau et al., 1989).

Pre-incubation of the ubiquitin-depleted fraction with the protease inhibitor, TLCK, decreased the adenylate-forming and the thiolester-transferring activities of E1 and diminished the ubiquitin conjugation to the other endogenous proteins. At the same time, auto-ubiquitination of E1 at pH 7.4 was greatly increased. The alkylation of a histidine or cysteine residue of the adenylate-forming site of E1 could have resulted in a conformational change in the protein, causing a lysine residue in the thiolester site to become orientated in such a way as to enable transacylation of the ubiquitin thiolester to readily occur. This would result in an increased rate of auto-conjugation at pH 7.4. Not all of the ubiquitin thiolesters of E1, however, were auto-conjugated in the presence of TLCK, as was shown by the 28% increase in auto-ubiquitinated E1 after NaOH-induction of TLCK-treated fractions.

TLCK has been described by Schnebli (1975) as inhibiting the growth of virally transformed 3T3 fibroblasts (SV3T3) by blockage of their cell cycles possibly in the G_2 phase. The thermolabile mutants, ts85 and ts20, when grown at non-permissive temperatures are also arrested in this phase; an effect that is known to be caused by the thermal inactivation of E1 (Finley et al., 1984; Kulka et al., 1988) and suggests that ubiquitin conjugation is necessary for progression through G_2. The specific growth inhibition response to TLCK observed in the SV3T3 cells could thus have been due to decreased activity of E1 caused by intracellular alkylation of the protein. If this can be confirmed, the inhibition may turn out to be a useful probe in the role of E1 in cellular processes.

Although there is no direct evidence to indicate how the adenylate- and thiolester-forming sites are orientated to each other, the possible mechanism proposed here for the action of TLCK involving a conformational change in the adenylate-forming site affecting the thiolester-forming site, suggests that the two sites are spatially adjacent. This is supported by Mayer et al. (1989a) who have shown that incubation at a non-permissive temperature of the thermolabile mutant ts85, causes a rapid and irreversible inactivation of both the adenylate and thiolester sites. Such a mechanism is attractive when considering the role of E1 in transferring ubiquitin from the adenylate to the thiolester site, for subsequent transfer to an E2 molecule; a mechanism made easier if the two sites are in spatial proximity.

The occurrence <u>in vitro</u> and possibly <u>in vivo</u> of these auto-modifying reactions of proteins bearing thiolester-bound ubiquitin on specific active-site residues, is a reminder of the incompletness of our present understanding of the detailed mechanism(s) by which physiological ubiquitination of target proteins takes place. It is possible that such auto-modification could be involved in determining the rate at which cellular proteins are ubiquitinated; it is thus

clear that such mechanisms require further molecular clarification and categorization.

CHAPTER 4

THE QUANTITATION OF UBIQUITIN AND THE FURTHER CHARACTERIZATION OF THE CONJUGATING FACTORS IN CHICKEN SKELETAL MUSCLE

4.1 INTRODUCTION

Although chicken skeletal muscle extracts are capable of forming endogenous ubiquitin-protein conjugates (See chapter 2), little is known in muscle systems about the intracellular levels of free and bound ubiquitin. Two techniques have been used to quantitate ubiquitin pools in intact tissues or cultured cell lines; one of these involves the quantitative detection of ubiquitin by immunological methods, using antibodies specific for free ubiquitin or ubiquitin conjugates (Haas, 1988). The other method involves microinjection of ^{125}I-ubiquitin into cultured cells and measurement of the conjugates formed (Carlson & Rechsteiner, 1987). Both methods have yielded values in a similar range, although there appears to be a variation in the intracellular ubiquitin pools of different cell types. In only one case have the levels of free ubiquitin and that of conjugates been examined in muscle tissue: this was done by Riley et al. (1988) with an elaborate immunochemical method, although the thiolester forms could not be measured. A direct method for determining the level of such ubiquitin pools was accordingly sought in this present study.

Although ubiquitin activating enzymes (E1) and the ubiquitin carrier proteins (E2) have been well characterized in reticulocytes (for review, see Hershko, 1988) and E1 has been isolated from wheat germ extracts (Hatfield & Vierstra, 1989), the activities of these conjugating factors have not been well defined in muscle tissue. It was therefore decided to purify the activating enzyme(s) and carrier proteins from chicken skeletal muscle with a view to their further characterization.

4.2 METHODS

4.2.1 Quantitation of free, thiolester-bound and conjugated ubiquitin in chicken skeletal muscle

25 g of exsanguinated chicken skeletal muscle was homogenized and centrifuged as described in section 2.2.1; the resulting supernatant was the starting material for the measurement of the free, conjugated and thiolester-bound forms of ubiquitin.

4.2.1.1 Quantitation of free ubiquitin

Half of the supernatant from section 4.2.1 (40 ml), was applied to a DEAE-cellulose column (1.6 x 10 cm) previously equilibrated in 25 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM DTT, all at pH 7.4 (Buffer A). The column was washed with four column volumes of Buffer A and the unbound material collected (Fraction I). Subsequent heat treatment of fraction I to 90°C caused much protein to be denatured and this was removed by centrifugation at 20 000 rpm for 15 min. The resulting supernatant was concentrated by lyophilisation and subjected to another heating step to 90°C. The ubiquitin content of this material was analyzed, relative to a ubiquitin standard, by running the samples on a 5-20% gradient SDS polyacrylamide gel under reducing conditions as described in section 2.2.11. The Coomassie blue-stained ubiquitin protein was excised from the gel and the stain extracted in 25% pyridine; the absorbance was read at A_{605} on a Spectroplus spectrophotometer in the fume hood.

4.2.1.2 Quantitation of conjugated ubiquitin

The bound material of the DEAE-cellulose column from section 4.2.1.1 was batch-eluted with three column volumes of 0.5 M KCl in Buffer A. Salt was removed by dialysis against Buffer A, after which the residual ATP was removed and the ubiquitin conjugates disassembled by incubating the sample with 20 mM 2-deoxy-glucose and 2 U/ml hexokinase overnight at 37°C. The resulting free ubiquitin was heat-treated and quantitated as described in section 4.2.1.1.

4.2.1.3 Quantitation of ubiquitin thiolesters

In order to cleave the ubiquitin thiolesters, 5 M hydroxylamine at pH 7.0, was added to the remaining 40 ml of the fresh clarified muscle homogenate from section 4.2.1, to a final concentration of 0.5 M, and the mixture was then incubated in the presence of an ATP-depleting system for 20 min at 37°C. The sample was then dialyzed against Buffer A to remove hydroxylamine, and applied to a DEAE-cellulose column (1.6 x 10 cm) previously equilibrated in Buffer A. The unbound material was collected and the free ubiquitin heat-treated and quantitated as described in section 4.2.1.1.

4.2.2 Partial purification of E1 and E2

Ubiquitin-affinity chromatography was used to purify E1 and E2 from fraction II, basically according to the method of Hershko et al. (1983) and as described in section 3.2.2. Fraction II (20 mg protein), was pre-incubated for 10 min at 37°C in the presence of 2 mM ATP and 2 mM magnesium acetate, prior to applying to the ubiquitin-affinity column which had previously been equilibrated in 50 mM Tris-HCl, 2 mM ATP, 2 mM magnesium acetate, all at pH 7.2. The column

was treated sequentially with 0.25 M KCl to remove unadsorbed material, 50 mM Tris-HCl, 2 mM AMP, 0.04 mM sodium pyrophosphate (pH 7.0) for E1 elution, 50 mM Tris-HCl, 10 mM DTT (pH 7.0) for E2 elution and 50 mM Tris-HCl, 2 mM DTT (pH 9.0) for any bound E3 and for residual E1 and E2 elution. Between each elution, the column was washed with 25 mM Tris-HCl. All samples were collected on ice and ovalbumin was added to a final concentration of 0.2 mg/ml in order to stabilize the enzyme activities. The samples were dialyzed overnight against a buffer containing 25 mM Tris-HCl, 0.5 mM DTT, 0.1 mM EDTA, 1 mM magnesium actetate, 10% glycerol (pH 7.5).

4.2.3 Thiolester-forming activities of partially purified E1 and E2 fractions

Approximately 140 μg of protein from each eluted fraction of the ubiquitin-affinity column was incubated with 4 μg ^{125}I-ubiquitin (4 x 10^5 cpm), 5 mM ATP, 2.5 mM magnesium acetate, 1 mM DTT, 0.04 units of inorganic pyrophosphatase (final volume of 400 μl) for 30 min at 37°C. Samples were treated with NaOH and run on 5-20% gradient polyacrylamide gels, as described in section 3.2.4. The stable alkali-induced auto-conjugates were quantitated by cutting the relevant areas from the Coomassie blue-stained gel and the radioactivity in ^{125}I-ubiquitin counted in a gamma counter.

4.2.4 Gel filtration of E1

600 μg of hydroxylamine-treated fraction II protein was applied to a Sephacryl S-300 column (0.6 x 120 cm) equilibrated with 50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM DTT all at pH 7.4, at a flow rate of 3 ml/hr. Fractions of 1 ml were collected and assayed for ubiquitin-dependent ATP-$^{32}PP_i$ exchange activity as described in section 3.2.6.

4.3 RESULTS

4.3.2 Quantitation of cytosolic ubiquitin

Although ubiquitin has been purified to near-homogeneity from chicken skeletal muscle (see section 2.2.5), the unquantified losses associated with the procedure interfered with the assessment of the in vivo levels and also gave no information about the levels of thiolester-bound or conjugated ubiquitin pools within skeletal muscle. Haas & Bright (1985) have shown that in rabbit reticulocytes up to 83% of the ubiquitin pool is involved in conjugates, whilst Carlson & Rechsteiner (1987) have shown 15% of ubiquitin to be involved in thiolester linkages in HeLa cells. These results suggest that conjugated and thiolester-bound ubiquitin constitute a major part of intracellular ubiquitin pools. An attempt was accordingly made to measure the levels of free, conjugated and thiolester-bound ubiquitin in chicken skeletal muscle by a protocol shown in Fig. 4.1 and described in section 4.1.1. The rationale of this methodology involved the release of free ubiquitin from both the ubiquitin conjugates and thiolester-bound ubiquitin by certain enzymatic and chemical treatments. The released ubiquitin was partially purified and quantitated by measuring, after SDS-PAGE, the amount of Coomassie blue-stained ubiquitin protein relative to a ubiquitin standard of known protein concentration. In order to release ubiquitin from the protein conjugates, fraction II was incubated at 37°C for 16 hr with an ATP-depleting system; this allowed isopeptidases present in fraction II to cleave the isopeptide bonds between ubiquitin and the substrate protein, while lack of ATP stopped such conjugates from re-forming. Ubiquitin could also readily be released from thiolester linkages to E1 and E2 by treatment of these fresh extracts with high concentrations of neutral

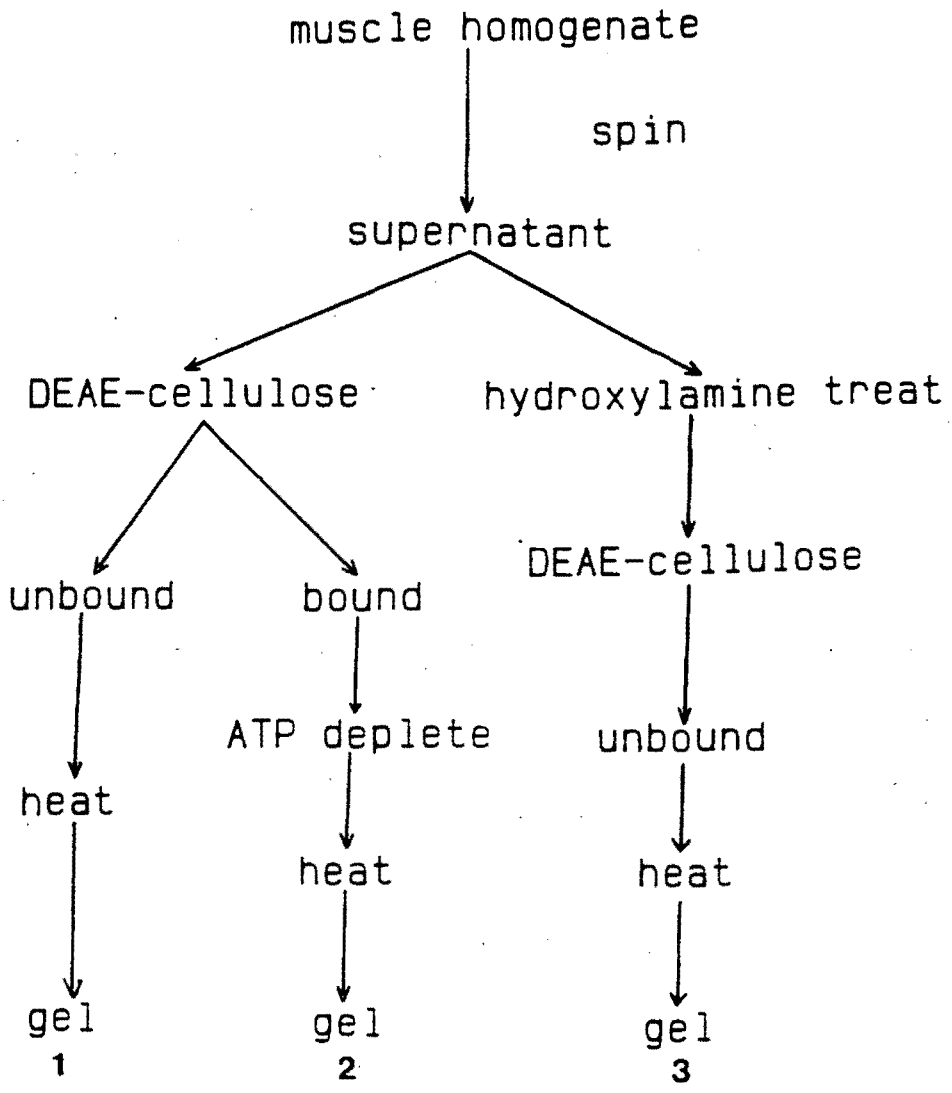


Fig. 4.1 Scheme for the detection of free, conjugated and thiolester-bound ubiquitin in skeletal muscle extracts

Free (1), conjugated (2) and thiolester-bound (3) ubiquitin fractions were prepared as described in section 4.2.1 using the above protocol.

hydroxylamine, a compound known to cleave thiolester bonds. As this treatment was done prior to DEAE-cellulose chromatography, the resulting unbound material contained both the free ubiquitin and thiolester-associated ubiquitin. The difference between extracts that had been hydroxylamine-treated and those that had not, reflected the amount of ubiquitin present in thiolester linkage.
Ubiquitin could clearly be identified in Coomassie blue-stained gels of the partially purified samples (Fig. 4.2) and could be quantitated by pyridine extraction of these proteins (Table 4.1). Two separate attempts of quantitation, using different muscle samples, although from the same animal, were performed. The average amount of free ubiquitin from the two separate runs was 24 μg/g muscle (eqivalent to 57.1 pmol/mg protein), which represented 71% of the total ubiquitin pool, whilst 18% of the total ubiquitin pool was conjugated and 11% was in thiolester linkage (Table 4.1).

4.3.2 Partial purification of E1 and E2

In order to characterize E1 and E2 from chicken skeletal muscle, an attempt to purify E1 and E2 from fraction II proteins by a ubiquitin-affinity column was made. The thiolester-forming activities of E1 and E2 were measured by their ability to form alkali-induced auto-conjugates, as described in section 3.3.1. The distribution and extent of purification of E1 and E2 from both chicken skeletal muscle and rabbit reticulocytes after affinity column steps is shown in Table 4.2. In both systems, E1 co-eluted with E2 with the high DTT and pH 9.0 buffers, instead of with the AMP-PP_i buffers designed specifically for E1 elution. This resulted in E1 and E2 not being separated from each other but they were, however, removed from most of the fraction II proteins as shown by their approximately 10-fold and 6-fold purification in the case of the reticulocytes and

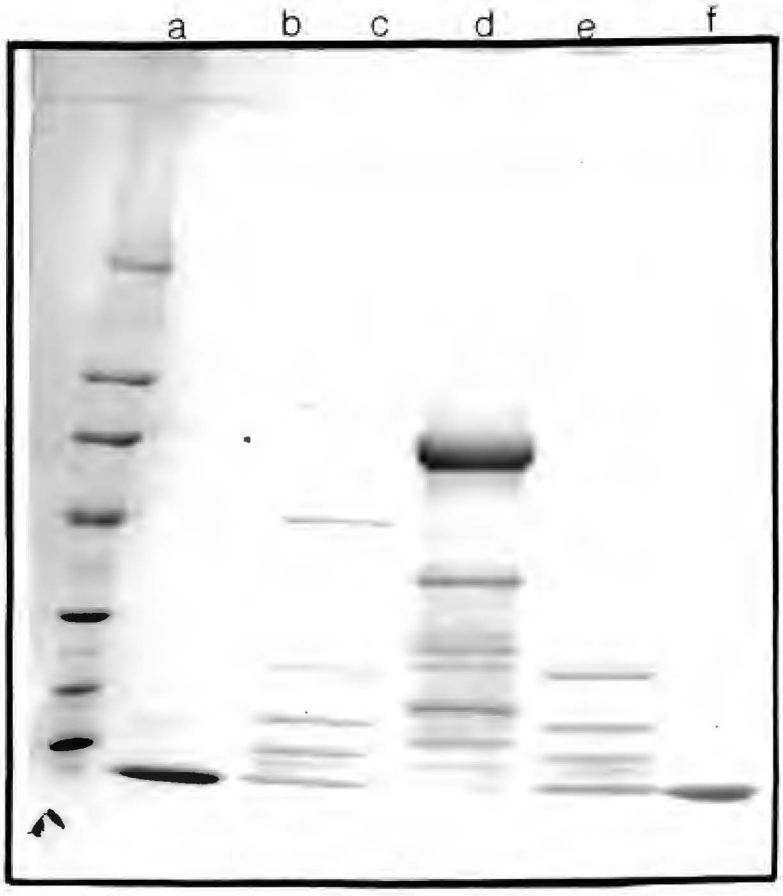


Fig. 4.2 SDS-PAGE of ubiquitin samples prior to pyridine extraction

Samples were treated with 1% SDS and 5% 2-mercaptoethanol, heated to 90°C for 2 min and run on a 5-20% gradient SDS gel as described in section 4.2.1. **Lane a:** molecular weight markers; myosin heavy chain (M_r = 200 000), phosphorlase b (M_r = 94 000), bovine serum albumin (M_r = 68 000), ovalbumin (M_r = 43 000), carbonic anhydrase (M_r = 30 000), soybean trypsin inhibitor (M_r = 20 100), α-lactalbumin (M_r = 14 400). **Lane b:** 20 μg ubiquitin standard. **Lane c:** 19 μg free ubiquitin sample. **Lane d:** 53 μg conjugated ubiquitin sample. **Lane e:** 33 μg thiolester-containing ubiquitin sample. **Lane f:** 20 μg ubiquitin standard.

Table 4.1 Quantitation of ubiquitin pools by pyridine extraction

Ubiquitin pools, from two separate experiments were quantitated by pyridine extraction of Coomassie-stained gels, as described in section 4.2.1.

Sample	µg protein/ g muscle	pmol ubiquitin/ mg protein	% total cytosolic ubiquitin
Free	28.8	63.0	72.0
	20.6	51.0	70.0
Conjugated	6.8	14.8	17.0
	5.4	13.4	18.3
Thiolester	3.9	9.1	10.0
	3.4	8.5	11.6
Total cytosolic ubiquitin	39.5	87.0	
	29.4	73.1	

TABLE 4.2 QUANTITATION OF E1 AND E2 FROM THE UBIQUITIN-AFFINITY COLUMN

20 mg fraction II protein from either chicken skeletal muscle extracts or rabbit reticulocyte extracts was subjected to affinity chromatography, as described in section 4.2.2. The activities of E1 and E2 were determined by measuring the NaOH-induced auto-ubiquitination, as described in section 4.2.3.

Fraction	Total protein	Total activity		Recovery		Specific activity		Purification	
		E_1	E_2	E_1	E_2	E_1	E_2	E_1	E_2
	mg	cpm x 10^{-4}		%		cpm x 10^{-4}/mg		(-fold)	
Reticulocytes									
Fraction II	25.0	316.4	733.2	100	100	12.7	29.4	1.0	1.0
Unbound	22.5	104.2	255.9	33	35	4.6	11.4	0.4	0.4
AMP-PPi eluate	0.5	0.6	5.3	0.2	0.7	1.1	11.9	0.1	0.4
DTT eluate	0.5	11.4	66.8	3.6	9.1	25.3	148.4	2.0	5.1
pH 9.0 eluate	0.4	41.1	152.6	13	21	97.8	363.3	7.7	12.4
Muscle									
Fraction II	25.0	255.0	639.3	100	100	10.2	25.6	1.0	1.0
Unbound	21.7	126.9	329.6	50	52	5.8	15.2	0.6	0.6
AMP-PPi eluate	0.3	2.1	5.6	0.8	0.9	6.4	17.0	0.6	0.7
DTT eluate	0.5	7.2	16.7	2.8	2.7	14.7	34.1	1.4	1.3
pH 9.0 eluate	0.5	23.9	114.6	9.4	18.0	46.9	224.7	4.6	8.8

muscle extracts, respectively. The specific activities of the muscle enzymes were lower than those of the reticulocyte enzymes; this was partially due to the fact that smaller amounts of the muscle fraction II bound to the affinity column (although, in both cases substantial activity losses were caused by E1 and E2 not binding to the affinity column). Comparing the reticulocyte findings with those of Hershko et al. (1983) it appeared that the total activities of E2 were higher, and those of E1, lower than those of these authors. This could have resulted from the use of the auto-conjugation assay as opposed to measuring ubiquitin conjugates by ion-exchange resins.

4.3.3 Gel filtration of E1

Although E1 was not purified further from the ubiquitin-affinity column, an estimate of the native molecular weight of E1 was made by analytical gel filtration of fraction II samples, coupled with assays for E1 activity by the ubiquitin-dependent ATP-$^{32}PP_i$ exchange reaction. Prior to column analysis, it was found that fraction II transferred $^{32}P_i$ to ATP under the assay conditions, but the reaction was ubiquitin-independent. As fraction II had previously been shown to contain ubiquitin in conjugated and thiolester forms (section 4.3.1) it was treated with hydroxylamine and the free ubiquitin removed by DEAE-cellulose chromatography. Subsequent ATP-$^{32}PP_i$ exchange assays containing treated fraction II showed a marked ubiquitin dependence (Fig. 4.3) and 5 μg of added ubiquitin was now required to attain the same level of activity prior to the hydroxylamine treatment. (Note: this fact, however, did not mean that 5 μg ubiquitin/60 μg fraction II protein was removed from the treated samples but rather suggested that of the exogenous ubiquitin added to the assay system, only a portion contained the diglycine C-terminus and was thus able to form the ubiquitin adenylate).

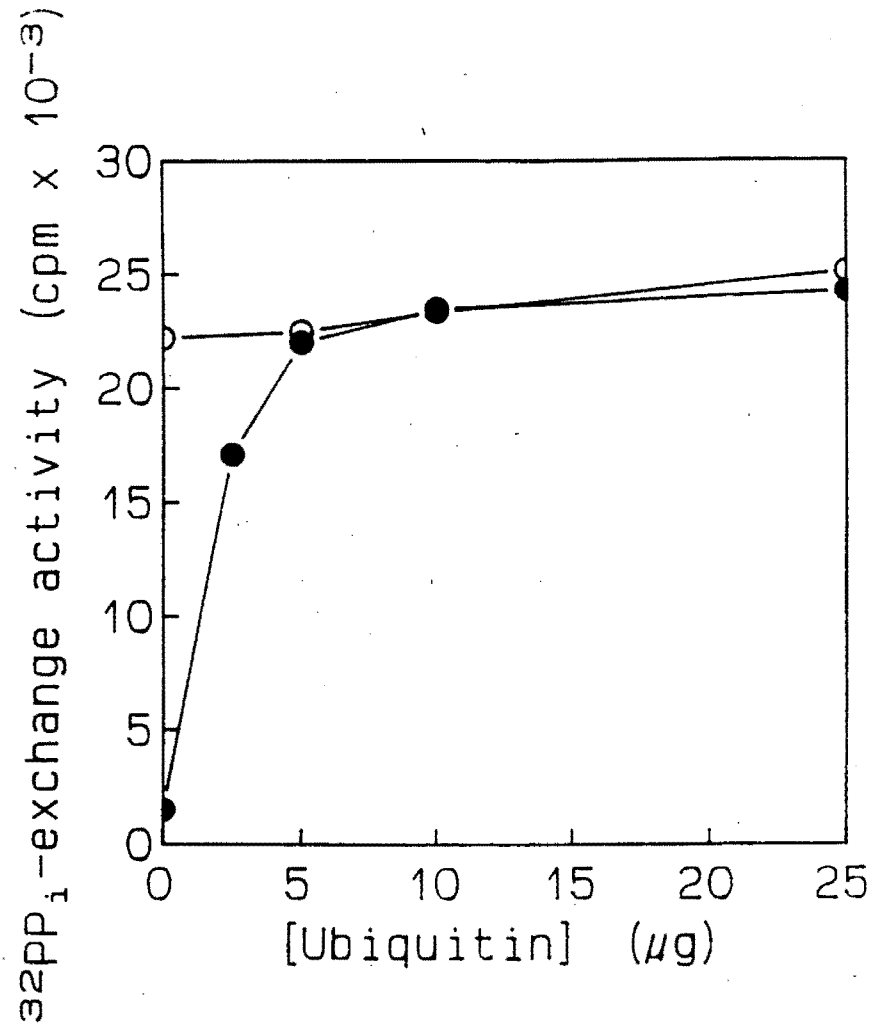


Fig. 4.3 Ubiquitin-dependence of the ATP-$^{32}PP_i$ exchange activity

100 μg fraction II protein (o) or hydroxylamine-treated fraction II (●) was incubated at the incubated ubiquitin concentrations with 5 mM ATP, 1 x 10^5 cpm $^{32}PP_i$, 50 mM NaF, in a final volume of 140 μl for 20 min at 37°C. The transfer of $^{32}PP_i$ to ATP was measured as described in section 3.2.6.

Gel filtration of hydroxylamine-treated fraction II showed a major form of E1 eluting with an M_r of 98 000, and a less prominant form eluting in a broad shoulder of activity with a larger M_r value of 180 000 (Fig. 4.4). These presumably corresponded to the monomeric and dimeric forms of E1, respectively and the finding suggested that the E1 monomer is the dominant species in muscle extracts as prepared.

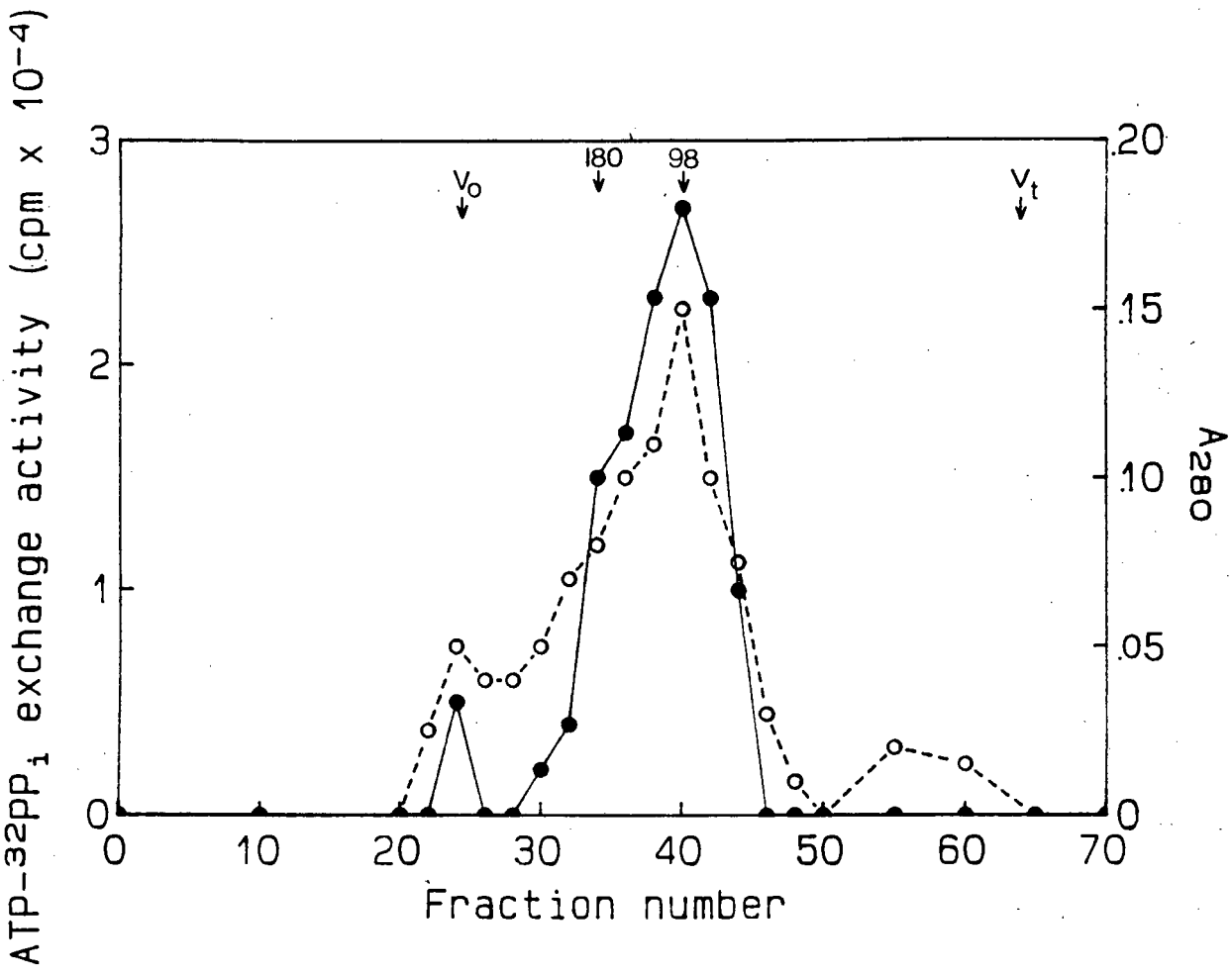


Fig. 4.4 Gel filtration of E1

600 μg of hydroxylamine-treated fraction II protein was applied to a 0.6 x 120 cm Sephacryl S-300 column as described in section 4.2.4. Ubiquitin-dependent ATP-$^{32}PP_i$ exchange activity (●) was measured as described in section 3.2.6. A_{280} (O---O). The indicated M_r values ($x10^{-3}$) were obtained by calibrating the column with blue dextran (V_o), catalase (M_r = 232 000), aldolase (M_r = 158 000), BSA (M_r = 68 000), ovalbumin (M_r = 42 000) and ATP (V_t).

4.4 DISCUSSION

Estimation of the free ubiquitin level in chicken muscle tissue gave an average value of 57.1 pmol ubiquitin/mg protein (equivalent to 25 μg ubiquitin/g tissue). Riley et al. (1988) have quantitated ubiquitin levels from various rat skeletal muscles, using an immunochemical technique, and have shown free ubiquitin levels in the different muscles ranged from 61.2 pmol/mg protein in soleus muscle to 32.7 pmol/mg protein in plantaris muscle. Rat EDL muscle is notably the closest in fibre type to chicken breast muscle and had a total ubiquitin content of 36.6 pmol/mg protein; approximately two-thirds of the value that was obtained in this study from chicken skeletal muscle. These differences, however, are not surprising considering the very different assay conditions used.

It is difficult to compare these values with those obtained either by purification of ubiquitin from tissue samples, (which do not reflect the *in vivo* ubiquitin levels due to losses in the purification procedures), or in cultured cells (where ubiquitin concentrations have usually been measured in terms of pmol/10^6 cells). As a general indication, free ubiquitin values from purified tissues have ranged from 2-4 μg ubiquitin/g in the case of etiolated oat shoots (Vierstra et al., 1985), to 150 μg ubiquitin/g hemoglobin in red blood cells (Jabusch & Deutsch 1983), and in cultured cells from 7.1 pmol/10^6 cells in chicken embryo fibroblasts (Bond et al., 1988) to 300 pmol/10^6 cells in African green monkey cells (Haas & Bright, 1985). The values for free ubiquitin in chicken breast muscle and those obtained by Riley et al. (1988) fall into this general range.

In comparing the ubiquitin pools from different tissues, it is especially interesting to examine the relative amounts of ubiquitin in different forms. About 18% of muscle ubiquitin was present as conjugates, 11% was in thiolester

form and the rest (71%) was apparently free. Assuming that the ubiquitin antibody used by Riley et al. (1988) recognized thiolester-bound ubiquitin as well as conjugated ubiquitin, similar levels were obtained by these authors in rat skeletal muscle (approximately 25% of the ubiquitin conjugated, and 75% free, irrespective of the absolute ubiquitin levels).

Comparing muscles with other tissues reveals that the levels of conjugates were lower in muscles than in cultured cells or in reticulocytes; an upper limit was seen in reticulocytes where 83% of the ubiquitin pool was conjugated (Haas & Bright, 1985) whilst a lower limit was found in mouse fibroblasts, where 31% of the ubiquitin was conjugated (Haas et al., 1987). The possibility that tissue extracts contain more free ubiquitin than do cultured cells has been suggested by Jahngen et al. (1986) who have shown that 60% of ubiquitin was free in cultured lens epithelial cells but this increased to 95% when lens epithelium was homogenized and quantitated. Whether this was due to homogenizing conditions allowing isopeptidases to act on ubiquitin conjugates, or was a reflection of the more heterogeneous cell population in the organized tissue, is not known, but the possibility remains that levels of ubiquitin conjugates in whole tissues may be lower than in cell cultures.

The attempted purification of E1 and E2 from chicken breast muscle by ubiquitin-affinity chromatography was not as successful as anticipated, due firstly, to E1 co-purifying with E2, and secondly, to apparently lower purification values and to losses (about 50%) arising from the fact that E1 and E2 did not bind substantially to the affinity column. Ciechanover et al. (1982) have demonstrated a form of E1 that does not bind to ubiquitin-affinity columns, arising from a defect in its thiolester-forming ability, despite an active adenylate-forming site. As the assay used was dependent on the formation of a ubiquitin-thiolester, such an E1 species would have been undetected in the assay

system and this factor could accordingly not have been responsible for the lack of E1 binding to the column. Alternatively, it is possible that the ubiquitin on the affinity column had been partially removed or degraded by isopeptidases or proteases from fraction II in previous column runs; this is especially pertinant as the reticulocyte system which was run prior to the muscle system, showed lower activity losses.

Gel filtration analysis of E1 after hydroxylamine treatment showed that E1 in the muscle extract was present both as a dimer and a monomer, which is similar to the findings of Haas & Bright (1988) who have shown E1 to elute as a broad activity peak and have suggested that E1 exists as a dimer in weak subunit associations. The ts85 cell line (Mayer et al., 1989) and reticulocytes (Ciechanover et al., 1981) have been reported to have E1's with native molecular weights of 200 000, however, indicating that E1 was present as a dimeric structure. Again, Hatfield & Vierstra (1989) have shown that wheat germ E1 elutes with an M_r value of 115 000, implying that in this tissue E1 is present in a monomeric form. The hydroxylamine treatment to which fraction II was subjected prior to gel filtration may have caused some subunit dissociation and thus although in chicken skeletal muscle the monomeric E1 species was dominant, this may not reflect the situation in vivo.

Unfortunately, unless purified samples are used, the native M_r values of the E2 species are difficult to determine. As shown in section 3.3.1, only the ubiquitinated E2 species occurring in the low molecular weight region were detected. The arsenite-sensitive E2 species with an M_r value of 230 000 recently identified by Klemperer et al. (1989) was not detected in the muscle system nor in the reticulocyte system under the assay conditions used.

Although the intracellular distribution of ubiquitin in chicken skeletal muscle has been examined, the dynamics of such a system are unknown. Whether the 30% of ubiquitin involved in conjugates and in thiolester linkages is

rapidly turned over by disassembly or by the degradation of substrate proteins, or whether they represent many stable ubiquitin conjugates, is not known. Information of this kind will enhance our understanding of the role of the ubiquitin pathway, not only in muscle, but in all eukaryote cells.

CHAPTER 5

THE BREAKDOWN AND DEGRADATION OF UBIQUITIN-PROTEIN CONJUGATES

5.1 INTRODUCTION

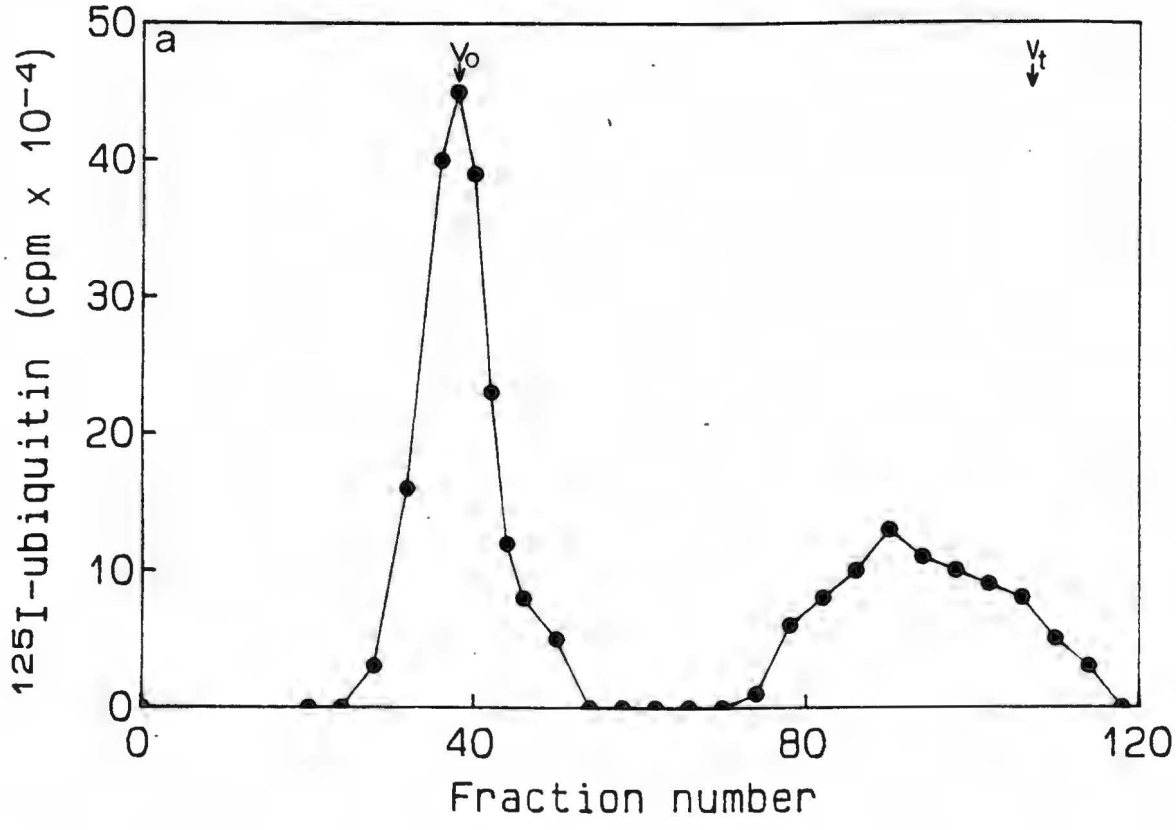
In order to assess the importance of the ubiquitin-dependent pathway in chicken skeletal muscle, it is necessary to investigate whether the ubiquitin conjugates, which have been shown to be formed in extracts prepared from this tissue (see chapter 2) are subsequently degraded to TCA-soluble fragments, or whether they represent stably modified intracellular proteins. Two separate pathways are known to exist in rabbit reticulocytes for conjugate breakdown; the one involves the ATP-independent disassembly of ubiquitin conjugates to free ubiquitin and intact protein by isopeptidases (for review see Rose, 1988) and the other involves ATP-dependent degradation of ubiquitinated proteins to peptide fragments by a specific protease that recognises ubiquitin conjugates (Hough et al., 1987); this latter entity has been termed the ubiquitin conjugate-degrading enzyme (UCDEN) by some authors (Waxman et al., 1987). Although isopeptidases have been shown to disassemble ^{125}I-lysozyme-ubiquitin conjugates in reticulocytes (Hough & Rechsteiner, 1986; Hough et al., 1986; Hershko et al., 1984b) no such activity has been described in skeletal muscle. Conflicting reports have appeared concerning the presence of the ubiquitin conjugate-degrading enzyme in muscle; whilst Fagan et al. (1987) and Matthews et al. (1989) have demonstrated such an activity in rabbit skeletal muscle, Gehrke & Jennissen (1987) were unable to detect it in rabbit cardiac muscle despite the presence of an active ubiquitin-conjugating system acting on endogenous proteins.

An attempt was accordingly made in the present study to identify both isopeptidase(s) and ubiquitin conjugate-degrading protease(s) in chicken skeletal muscle.

5.2 METHODS

5.2.1 ^{125}I-ubiquitin-conjugate formation and purification

^{125}I-ubiquitin conjugates were formed by incubating 9 mg fraction II protein, in a final volume of 10 ml, containing 100 μg ^{125}I-ubiquitin (1 x 10^7 cpm), 5 mM ATP, 2 mM magnesium acetate, 1 mM DTT, 25 mM Tris-HCl, all at pH 7.5 for 2 hr at 37°C. The reaction was terminated by the addition of NEM to a final concentration of 5 mM and the mixture was then incubated for a further 10 min period. Marked breakdown of ^{125}I-ubiquitin conjugates had previously been observed in such systems, presumably arising from the action of endogenous hydrolases present in fraction II. These activities were accordingly inactivated in the experimental samples by alkali and heat treatment (incubation for 2 min at 90°C, at pH 9.0), after which all proteins were solubilized by the addition of formic acid to a final concentration of 5%. The samples were then applied to a 2 x 100 cm Sephadex G-100 column, previously equilibrated with 5% formic acid. Fractions of 2.5 ml were collected and counted in a gamma counter (Fig 5.1a). Fractions containing ^{125}I-ubiquitin activity were dialyzed against 25 mM Tris-HCl (pH 7.5) to remove the formic acid, and the purity of the conjugates was assessed by SDS-PAGE followed by autoradiography. Fig. 5.1b shows that the ^{125}I-ubiquitin conjugates eluted in the V_o of the G-100 column and were separated from most of the free ^{125}I-ubiquitin which eluted later in the column.



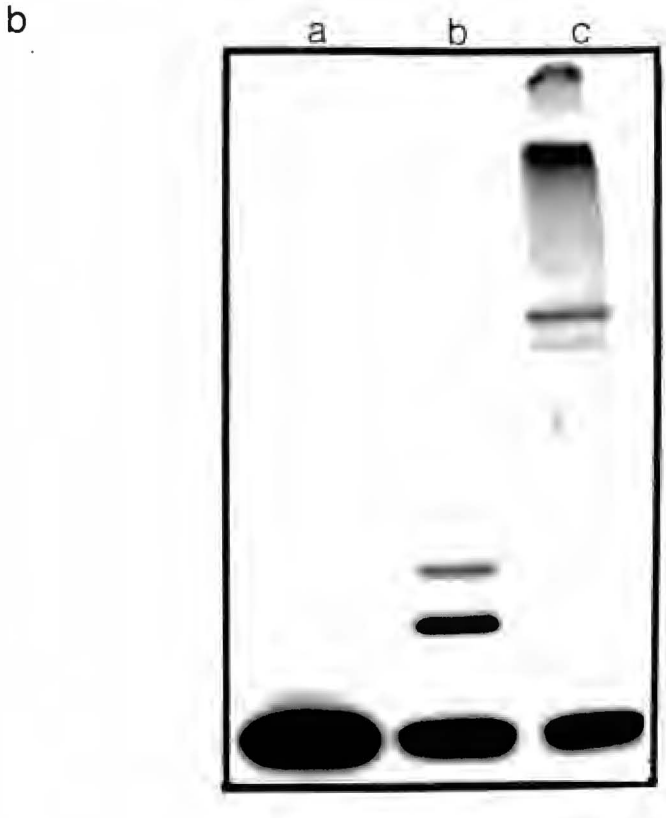


Fig. 5.1 (legend overleaf)

Fig. 5.1 Separation of ^{125}I-ubiquitin conjugates from ^{125}I-ubiquitin

Fig. 5.1a Gel filtration of ^{125}I-ubiquitin conjugates

^{125}I-ubiquitin conjugates were formed as described in section 5.2.1 and applied to a 2 x 100 cm Sephadex G-100 column equilibrated in 5% formic acid. Fractions were collected and counted in a gamma counter ( ). The V_o and V_t values were determined by calibrating the column with blue dextran (V_o) and ATP (V_t).

Fig. 5.1b Autoradiography of column fractions

Appropriate fractions were pooled and dialyzed against 25 mM Tris-HCl (pH 7.5) followed by heat treatment at 90°C in the presence of 1% SDS and 5% 2-mercaptoethanol. Samples were applied to a 5-20% gradient SDS gel and the ^{125}I-ubiquitin conjugates visualized by autoradiography. **Lane a:** V_t fraction. **Lane b:** mid-column fraction (fractions 55-75). **Lane c:** V_o fraction.

5.2.2 Gel filtration of muscle and reticulocyte fractions on Sepharose CL-6B

Clarified extracts of muscle and reticulocytes (300 μl) were run on a 0.9 x 60 cm Sepharose CL-6B column, previously equilibrated with 25 mM Tris-HCl, 10% glycerol, 0.1 mM DTT, 0.1 mM EDTA, all at pH 7.5. Fractions of 1 ml were collected and their proteolytic activities measured by the breakdown of ^{125}I-lysozyme to TCA-soluble fragments, as described in section 5.2.5.

5.2.3 Assay of isopeptidase activity

Purified ^{125}I-ubiquitin conjugates (5 x 10^4 cpm) were incubated with 40 μg of either the V_o, mid-column or V_t regions of fraction II run on Sepharose CL-6B as described in section 5.2.2. Various activators and inhibitors were added as described in the text and all the samples were incubated in a final volume of 200 μl for up to 8 hr. TCA-soluble material was measured as described in section 5.2.5 and the ^{125}I-ubiquitin-conjugate distribution analyzed by 5-20% SDS-PAGE and autoradiography as described in section 2.2.11. The gels were quantitated by cutting up the relevant areas of the dried Coomassie blue-stained gel and counted in a gamma counter.

5.2.4 Degradation of endogenous proteins

4 mg of either dialyzed crude extract (to remove free tyrosine) or fraction II protein were incubated in the presence of 2 mM magnesium acetate, 1 mM DTT and 25 mM Tris-HCl at pH 7.5. Where indicated, 5 mM ATP and 10 μg ubiquitin were added and the samples incubated for 6 hr at 37°C. Assays were terminated by the addition of TCA to a final concentration of 10% and the tyrosine release

measured according to the method of Waalkes & Udenfriend (1957).

5.2.5 Degradation of exogenous proteins

100 µg fraction II protein was incubated at 37°C for the indicated time periods with 5 µg (5 x 10^5 cpm) of iodinated substrate protein in the presence of 2 mM magnesium acetate, 1 mM DTT, 25 mM Tris-HCl, pH 7.5. When ATP- and ubiquitin-dependent activities were measured, 5 mM ATP and 10 µg ubiquitin were added in a final volume of 200 µl. Incubations were terminated by the addition of 100 µl 10 mg/ml BSA, 100 µl 20% TCA, stored at 0°C for 20 min, centrifuged at 2 000 rpm for 15 min and the supernatant counted in a gamma counter. Activators and inhibitors were included as described in the text.

5.3 RESULTS

5.3.1 Presence of isopeptidases in chicken skeletal muscle

In an attempt to show isopeptidase activity in chicken skeletal muscle, the release of ^{125}I-ubiquitin from ^{125}I-ubiquitin conjugates was measured in the absence of ATP. Incubations, with purified ^{125}I-ubiquitin conjugates of either the V_o, mid-column or V_t regions of fraction II, previously run on Sepharose CL-6B (see section 5.2.2), showed that the mid-column region was most active in disassembling the ^{125}I-ubiquitin conjugates, as judged by their disappearance on autoradiographs (Fig. 5.2b); this reaction was ATP-independent, required Mg^{2+} and was inhibited by NEM. Very little TCA-soluble material formed, indicating that the process did not involve the degradation of ubiquitin but rather its removal from the conjugates in a manner identical to that described for isopeptidases in rabbit reticulocytes (Hershko et al., (1984b); Hough & Rechsteiner, 1986; Hough et al., 1986). A less active ^{125}I-ubiquitin conjugate-disassembling activity was also present in the V_t region of the Sepharose CL-6B column (Fig. 5.2c) whilst the V_o region was inactive (Fig. 5.2a). As the major isopeptidase activity of fraction II eluted in the mid-column region of the Sepharose CL-6B column, it was estimated to have an M_r value between 1 x 10^5 and 1 x 10^6, which is in agreement with some of the molecular weights obtained for reticulocyte isopeptidases (Hough et al., 1986; cited in Hershko & Rose, 1987).

Further evidence that chicken skeletal muscle contained isopeptidase activity came from the studies with the NaOH- and TLCK-induced auto-conjugates of E1 described in sections 3.3.3 and 3.3.5.1, respectively; breakdown of these auto-conjugates, as well as other ^{125}I-ubiquitin conjugates, occurred in an ATP-independent manner and in

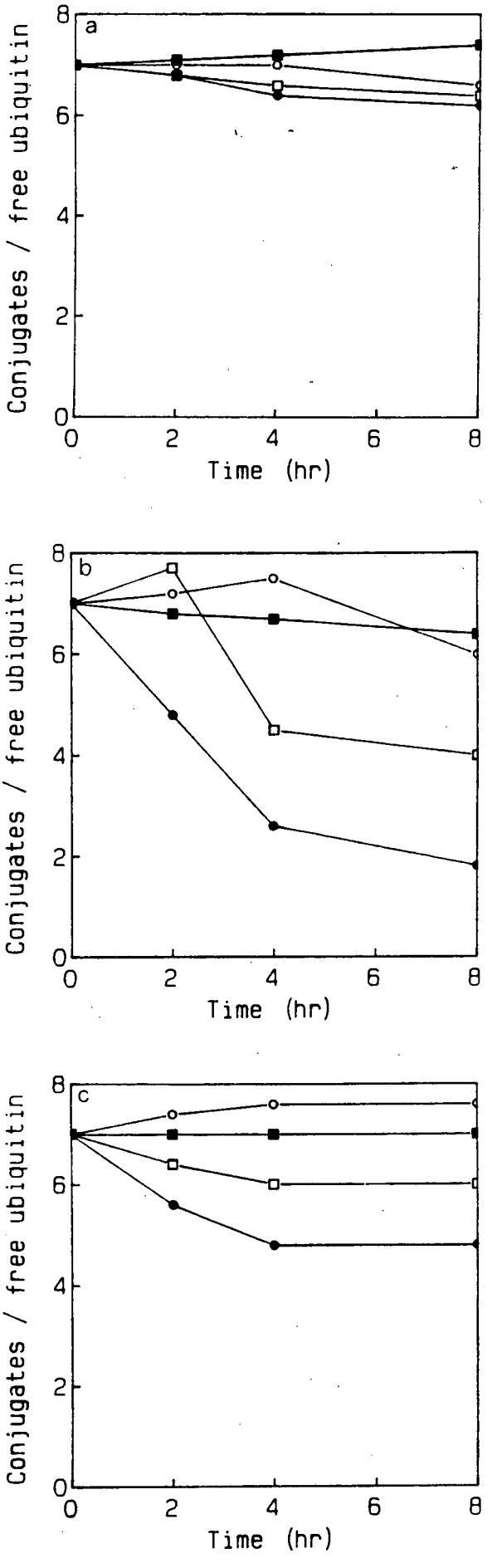


Fig. 5.2 (legend overleaf)

Fig. 5.2 Isopeptidase activity of fraction II

Fig. 5.2a Previously prepared ^{125}I-ubiquitin conjugates (1 x 10^4 cpm) as in section 5.2.1 were incubated with 40 μg of the V_o region of a fraction II sample chromatographed on Sepharose CL-6B in mixtures containing 2.5 mM DTT, 25 mM Tris-HCl (pH 7.5) (■); 2.5 mM DTT, 25 mM Tris-HCl (pH 7.5) 2.5 mM magnesium acetate (●); 2.5 mM DTT, 25 mM Tris-HCl (pH 7.5), 2.5 mM magnesium acetate, 5 mM ATP (□) or with 2.5 mM DTT, 25 mM Tris-HCl (pH 7.5), 2.5 mM magnesium acetate, 5 mM NEM (○). Samples were incubated for the times indicated at 37°C and the isopeptidase activity quantitated by measuring the disappearance of ^{125}I-ubiquitin from the conjugates as described in section 5.2.3. The ratio of ^{125}I-ubiquitin remaining in conjugates to free ^{125}I-ubiquitin was calculated.

Fig. 5.2b As in Fig. 5.2a but using 40 μg of the mid-column activity of the fraction II sample on Sepharose CL-6B instead of the V_o region.

Fig. 5.2c As in Fig. 5.2a but using 40 μg of the V_t region of the Sepharose CL-6B column.

the case of the alkali-induced auto-conjugates was inhibited by NEM, hemin and PMSF.
These data suggest that chicken skeletal muscle contains at least one isopeptidase with an activity similar to that described in rabbit reticulocytes.

5.3.2 Ubiquitin-dependent degradation of proteins

Although muscle has been found to contain a variety of endoproteases (for review, see Pennington, 1977), it is not known if the ubiquitin conjugate-degrading activity characteristic of reticulocytes (Waxman et al., 1987; Hough et al., 1987) and rabbit skeletal muscle (Fagan et al., 1987; Matthews et al., 1989) is present in chicken skeletal muscle. Accordingly, attempts were made to measure the proteolytic activities present in chicken skeletal muscle extracts that acted on endogenous and exogenous proteins in a ubiquitin-dependent manner, and to compare them with activities present in reticulocytes.

5.3.2.1 Degradation of endogenous proteins

Proteolytic activities present in crude muscle and reticulocyte extracts (prior to DEAE-cellulose chromatography), as well as those remaining in fraction II preparations, were assayed by their ability to release tyrosine from endogenous proteins in an ATP-dependent and, in the case of fraction II, ubiquitin-dependent manner. Table 5.1 shows that the proteolytic activities of crude extracts of muscles and reticulocytes were stimulated 2-fold and 3-fold, respectively, by the addition of ATP; both systems, however, also contained substantial ATP-independent proteolytic activity (Table 5.1). Fraction II preparations, although they were able to degrade endogenous proteins in the absence of ubiquitin, showed a 4-fold

Table 5.1 Degradation of endogenous proteins by muscle and reticulocyte fractions

4 mg of crude extract or fraction II protein were incubated as described in section 5.2.4 for 6 hr at 37°C. Assays were terminated by the addition of TCA and the tyrosine release measured as described in section 5.2.4.

Sample	Alone	+ ATP	+ ATP + ubiquitin
	(nmol tyrosine/6 hr)		
Muscle crude extract	4.2	6.6	ND
Muscle fraction II	2.1	3.7	5.2
Reticulocyte crude extract	2.6	6.9	ND
Reticulocyte fraction II	1.0	2.4	8.7

(reticulocytes) and a 1.75-fold (muscles) increase in activity when ubiquitin was added. These results suggest that muscle not only contains a less ubiquitin-dependent degrading ability (on a specific activity basis) than reticulocytes but also that other proteolytic systems not involving ubiquitin are present in both tissues.

5.3.2.2 Degradation of exogenous proteins

In order to see whether muscle and reticulocyte fraction II preparations degraded ^{125}I-labelled exogenous proteins, lysozyme, bovine serum albumin (BSA), and phosphorylase kinase (PK) were iodinated and the ATP- and ubiquitin-dependent formation of TCA-soluble fragments measured. Reticulocyte fraction II was capable of degrading ^{125}I-lysozyme, ^{125}I-BSA and ^{125}I-PK, in an ATP-dependent manner, which was increased 3-fold, 2.5-fold and 1.5-fold, respectively, when ubiquitin was added (Fig. 5.3). Muscle fraction II, although capable of degrading all three substrates in a ubiquitin-independent manner gave an increase in the degradation of ^{125}I-lysozyme of only 1.5-fold when ubiquitin was added (Fig. 5.3). This was not due to ubiquitin being limiting in the system, as increasing the amounts of ubiquitin did not alter the extent to which ^{125}I-lysozyme was degraded (Fig. 5.4). Nor were these activities due to endogenous ubiquitin masking the ubiquitin-dependence of fraction II, as hydroxylamine-treated and untreated fraction II degraded ^{125}I-lysozyme to a similar extent in the presence of ubiquitin (results not shown). Rabbit cardiac muscle fraction II, however, was not able to degrade ^{125}I-lysozyme in a ubiquitin-dependent manner (Fig. 5.3) despite ATP-dependent and ATP-independent activities existing in accordance with the findings by Gehrke & Jennissen (1987) and DeMartino (1983).

The apparently low levels of ubiquitin-dependent proteolytic activity in muscle fraction II could either

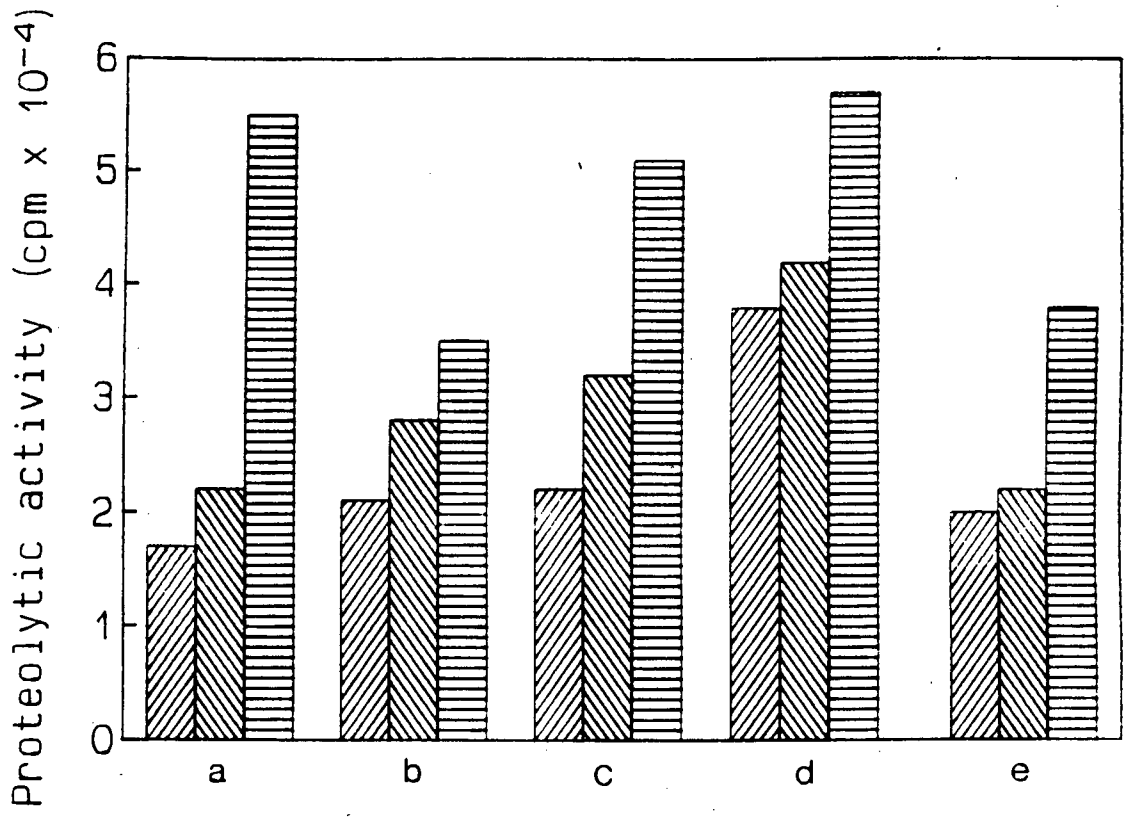


Fig 5.3 Proteolytic activities of reticulocyte and muscle extracts

5 μg ^{125}I-protein (as indicated) was incubated for 4 hr with 100 μg fraction II protein at 37°C in the presence of 2 mM magnesium acetate, 1 mM DTT, 25 mM Tris-HCl, pH 7.5 (▨), with 5 mM ATP (▧) and with 5 mM ATP and 10 μg ubiquitin (▤). Reactions were terminated by the addition of TCA and the TCA-soluble material analyzed as described in section 5.2.5.

(a): reticulocyte fraction II with ^{125}I-lysozyme. **(b):** muscle fraction II with ^{125}I-lysozyme. **(c):** reticulocyte fraction II with ^{125}I-BSA. **(d):** reticulocyte fraction II with ^{125}I-PK. **(e):** reticulcte fraction II with ^{125}I-fraction II.

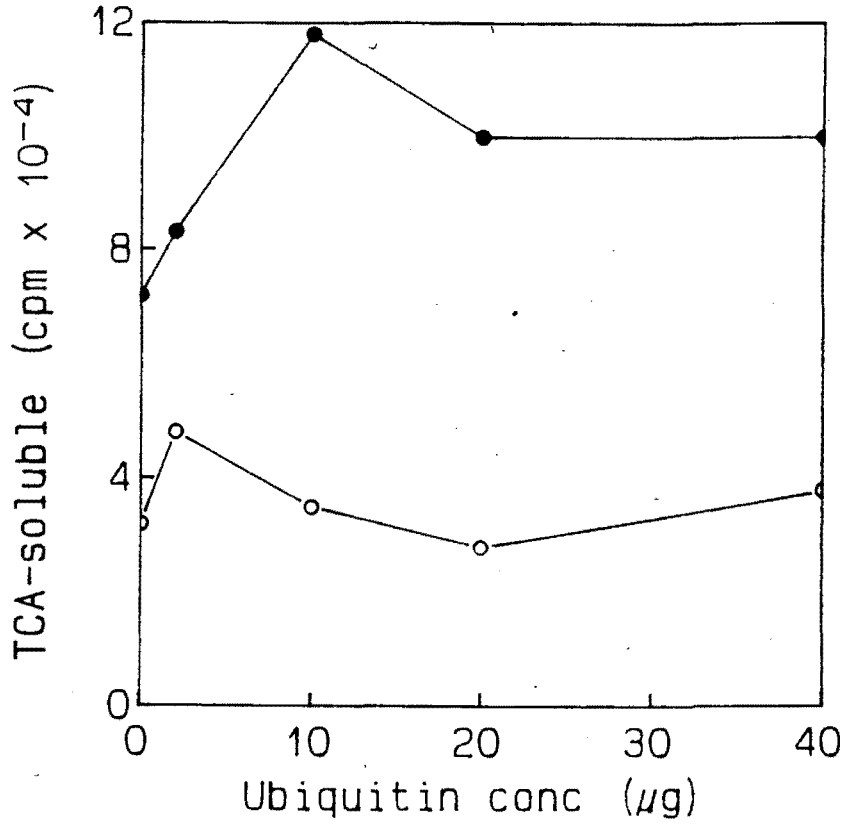


Fig. 5.4 Ubiquitin-dependence of ^{125}I-lysozyme degradation by muscle fraction II

100 μg fraction II protein was incubated with 1 μg ^{125}I-lysozyme (1 x 10^5 cpm), 2.5 mM DTT, 2.5 mM magnesium acetate at varying ubiquitin concentrations in the presence (●) or absence (○) of 5 mM ATP for 4 hr at 37°C. TCA-soluble material was analyzed as described in section 5.2.5.

have been the result of poor formation rates of specific ubiquitin-conjugates destined for degradation or, they could have reflected a low activity of the proteolytic system. This was examined by assessing whether addition of the reticulocyte material to muscle material would increase the degradation of exogenous proteins in the muscle system. Fraction IIA and fraction IIB were accordingly prepared from muscle and reticulocytes by ammonium sulphate fractionation and their ability to degrade ^{125}I-lysozyme in an ATP- and ubiquitin-dependent manner was assayed, both singly, or in homologous or heterologous mixed incubations. Whilst fraction IIA had a detectable but low proteolytic activity in both muscle and reticulocytes, fraction IIB had substantial ATP-dependent and ubiquitin-independent activities (Fig. 5.5). A mixture of homologous fraction IIA and fraction IIB was associated with ubiquitin-dependent degradation of ^{125}I-lysozyme; the fractions from reticulocytes being markedly more active than those from muscle. Incubations of reticulocyte fraction IIA with muscle fraction IIB gave a ubiquitin-dependence similar to that found in reticulocyte fraction II, however, whilst incubations of muscle fraction IIA with reticulocyte fraction IIB gave activities similar to those found in muscle fraction II (Fig. 5.5). This suggested that reticulocyte fraction IIA conferred on the muscle system the ability to degrade ^{125}I-lysozyme in a ubiquitin-dependent manner, whilst muscle fraction IIA contained lower levels of this factor. Whether this was due to a low level of ubiquitin conjugate-degrading activity in muscle preparations or to a lower conjugate forming activity in muscle fraction IIA preparations, as shown previously in section 2.3.5, could not be deduced from these experiments. The observation, however, that endogenous proteins which did form ubiquitin conjugates (see section 2.3.1) were degraded in an ATP- and ubiquitin-dependent manner to a lesser extent by muscle fraction II than reticulocyte fraction II (Fig. 5.3), indicated it was more likely that

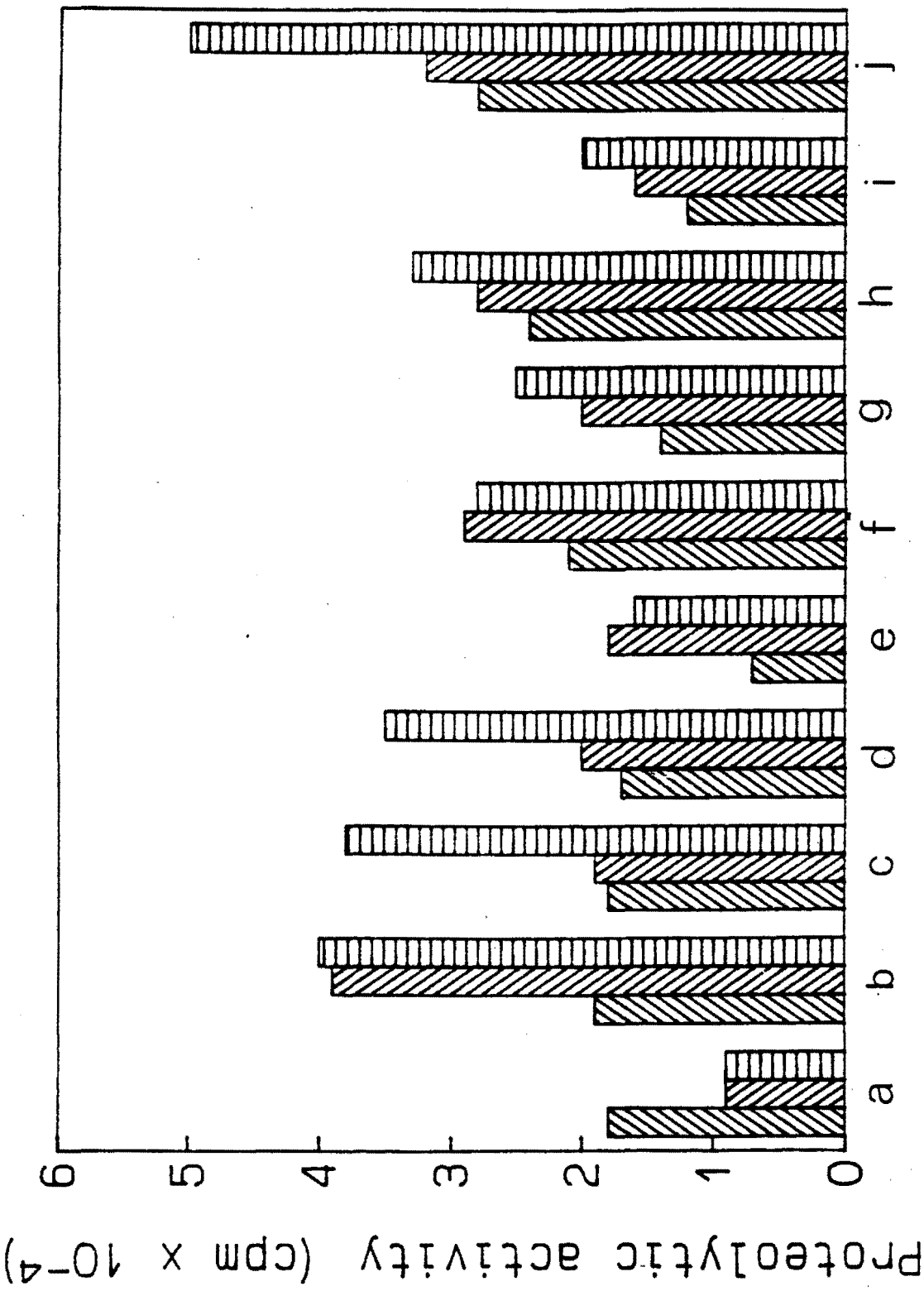


Fig. 5.5 (legend overleaf)

Fig. 5.5 ^{125}I-lysozyme degradation of fraction IIA and fraction IIB from muscle and reticulocytes

Fraction II extracts, as indicated, were incubated with 1 μg ^{125}I-lysozyme, 1 mM DTT, 2 mM magnesium acetate, 25 mM Tris-HCl, pH 7.5, (▨), in the presence of 5 mM ATP (▧) and in the presence of 5 mM ATP and 10 μg ubiquitin (▤) for 4 hr at 37°C. The amount of ^{125}I-lysozyme degraded to TCA-soluble material was measured as described in section 5.2.5.

(a): 50 μg reticulocyte fraction IIA. **(b):** 50 μg reticulocyte fraction IIB. **(c):** 50 μg reticulocyte fraction IIA and 50 μg reticulocyte fraction IIB. **(d):** 100 μg reticulocyte fraction II. **(e):** 50 μg muscle fraction IIA. **(f):** 50 μg muscle fraction IIB. **(g):** 50 μg muscle fraction IIA and 50 μg muscle fraction IIB. **(h):** 100 μg muscle fraction II. **(i):** 50 μg muscle fraction IIA and 50 μg reticulocyte fraction IIB. **(j):** 50 μg reticulocyte fraction IIA and 50 μg muscle fraction IIB.

the conjugate-degrading activity was in fact present but at lesser levels in chicken skeletal muscle.

5.3.3 Gel filtration of fraction II

The possibility of a ubiquitin conjugate-degrading activity being present in fraction IIA was considered and attempts were made to separate this activity from other proteases as well as to estimate its native M_r by gel filtration. Although the ubiquitin conjugate-degrading enzyme has been reported to degrade only ^{125}I-lysozyme-ubiquitin conjugates (Hough et al., 1987), Waxman et al. (1987) have shown a fraction containing this protease to degrade ^{125}I-lysozyme to TCA-soluble material in an ATP- and ubiquitin-independent manner, albeit at a low rate. Thus ^{125}I-lysozyme was used as a substrate to monitor ubiquitin conjugate-degrading protease activity as well as other proteolytic activities present in fraction II. The resulting profile of eluted subfractions obtained by gel chromatography of muscle and reticulocyte fraction II preparations on Sepharose CL-6B recorded that reticulocyte fraction II contained two separable proteolytic activities, one of which eluted in the V_o and thus had a native M_r value greater than 1 x 10^6, and another which eluted in the mid-column region corresponding to an M_r of approximately 400 000 (Fig. 5.6a); muscle fraction II preparations, however, contained little of the V_o activity but similar levels of the lower molecular weight activity (Fig. 5.6b). Gel filtration analysis of fractions IIA and IIB showed that the V_o activity was present only in fraction IIA, that of reticulocytes being substantially higher than the muscle activities (Fig. 5.6c and d), whilst the mid-column activity was present in fraction IIB at similar levels in both muscle and reticulocytes (Fig. 5.6e and f).

Of interest was the finding that fraction II contained an ATP-dependent but ubiquitin-independent activity which

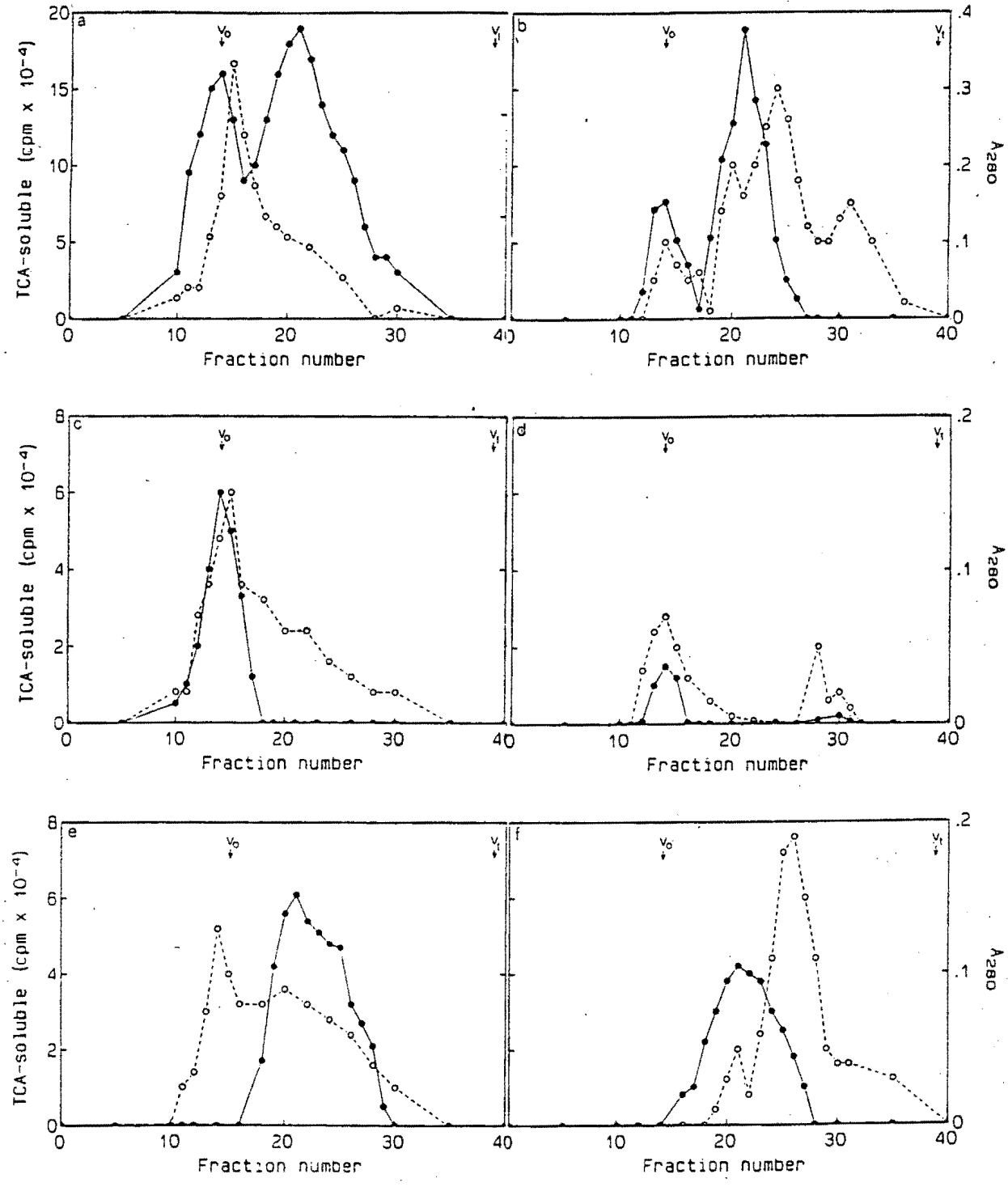


Fig. 5.6 (legend overleaf)

Fig. 5.6 Gel filtration of fraction II extracts

Fraction II extracts, as indicated, were applied to a 0.9 x 60 cm Sepharose CL-6B column equilibrated in 25 mM Tris-HCl (pH 7.5), 10% glycerol, 0.1 mM DTT. 0.1 mM EDTA at a flow rate of 3 ml/hr. Fractions (1 ml) were collected and the proteolytic activities of the fractions measured (●) by the breakdown of ^{125}I-lysozyme to TCA-soluble fragments as described in section 5.2.5. A_{280} (o---o). The V_o and V_t values were determined by calibrating the column with chylomicrons (V_o) and ATP (V_t).
(a): 600 μg reticulocyte fraction II. **(b):** 600 μg muscle fraction II. **(c):** 300 μg reticulocyte fraction IIA. **(d):** 300 μg muscle fraction IIA. **(e):** 300 μg reticulocyte fraction IIB. **(f):** 300 μg muscle fraction IIB.

could be partially supported also by GTP but not by UTP or CTP (results not shown). The ATP-dependence was lost on gel filtration, however, suggesting that it involved a number of factors which could be separated by gel filtration.
Taking into account the large M_r value of the V_o material, and the fact that this activity was more prominant in reticulocyte than in muscle extracts, it appears likely that the V_o activity was the ubiquitin conjugate-degrading enzyme described by some authors (Hough et al, 1987; Fagan et al., 1987; Waxman et al., 1987). The mid-column activity could correspond either to an ATP-stabilized protease found in rat skeletal muscle (Ismail & Gevers, 1983; Dahlmann et al., 1983) and in rat cardiac muscle (Gehrke & Jennissen, 1987; DeMartino, 1983) or to the widespread ATP-independent, multi-catalytic protease known as the proteasome and initially described in lens tissue by Wilk & Orlowski (1983). The activities were accordingly further characterized.

5.3.4 Stability of the muscle and reticulocyte proteolytic activities

The ubiquitin conjugate-degrading activity has been shown in reticulocytes to be labile and is readily inactivated by incubations at 37°C in the absence of ATP (Hershko et al., 1979; Hough et al., 1987; Waxman et al., 1987) whilst the proteasome was stable at high temperatures (Hase et al., 1980; Ray & Harris, 1986; Makinodan et al., 1988), thus allowing discrimination between these two activities to be made. The V_o material of the Sepharose CL-6B column was in fact somewhat unstable; half the activity was lost in 2 days upon storage at 4°C, whilst the mid-column activity was stable for two weeks at this temperature. This latter activity was also stable to short incubations of 42°C in the absence of ATP, but the activity of the V_o material was too low to measure with this treatment (results not shown).

Thus in order to specifically measure the stabilties of the ubiquitin-dependent as well as the ubiquitin-independent activities, fraction II was incubated at 42°C in the presence and absence of ATP, after which proteolytic activities were measured at 37°C. Both muscle and reticulocyte preparations contained a heat-labile ubiquitin-dependent degrading activity which was stabilized by ATP (Fig. 5.7). Although both muscle and reticulocyte activities were inactivated by heat to similar extents, the absolute values in muscle were lower in accordance with the results obtained in section 5.3.1. The ubiquitin-independent activity in muscle was partially heat-labile and could be stabilized by ATP, whilst in reticulocytes the ubiquitin-independent activity was largely heat-stable; this suggested that muscle contained both a heat-stable and a heat-labile ubiquitin-independent activity which could have corresponded to the proteasome and ATP-stimulated activities, respectively.

The proteasome has been shown to be activated at high temperatures and in some cases is only detected during incubations at 60°C (Makinodan et al., 1987). To see if this was the case in chicken skeletal muscle extracts, fraction II and the Sepharose CL-6B mid-column activity were incubated at 60°C for 1 hr in the presence of ^{125}I-lysozyme. A two- and four-fold increase in ATP-independent activity was caused by the fraction in the two cases, respectively (results not shown), indicating that a proteasome-like activity was present in muscle and could be identified on a Sepharose CL-6B column.

5.3.5 Inhibitors and activators of the muscle and reticulocyte proteases

The V_o and mid-column activities of the Sepharose CL-6B column were exposed to a variety of inhibitors and activators in an attempt to further characterize these

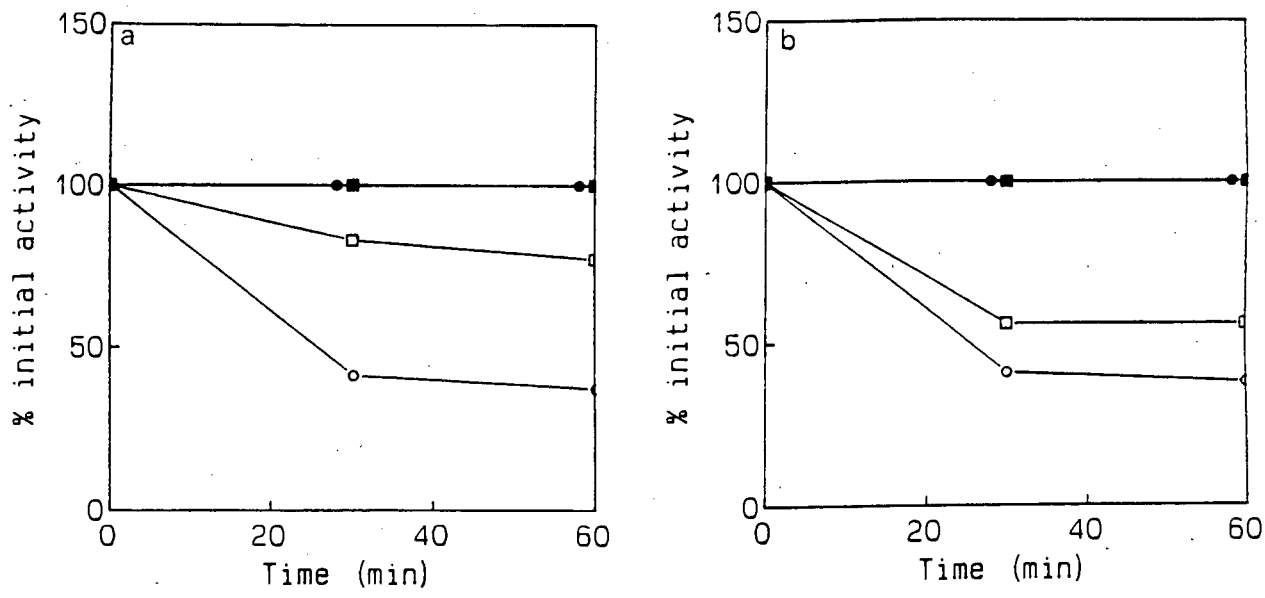


Fig. 5.7 Heat stabilities of muscle and reticulocyte fraction II

Fig. 5.7a 100 μg reticulocyte fraction II was pre-incubated at 42°C in the presence (● , ■) and absence (○ , □) of 5 mM ATP, for 30 and 60 min. Samples were then assayed for ubiquitin-dependent proteolytic activity (● , ○) as described in section 5.2.5, ATP-dependent but ubiquitin-independent activity (■) as described in section 5.2.5 and ATP-independent activity (□).

Fig. 5.7b As above but with 100 μg muscle fraction II.

activities. As shown in Table 5.2, NEM, chymostatin, hemin and $ZnCl_2$ inhibited both the V_o and mid-column activities. Some differences, however, were apparent between these two activities; in particular, DTT was essential for the mid-column activity, whilst EDTA, leupeptin and TLCK were less inhibitory for the V_o material. The ubiquitin conjugate-degrading enzyme has been shown to be similarly inhibited by NEM, hemin and chymostatin (Hough et al., 1987; Waxman et al., 1987). Comparison of the mid-column activity with the proteasome was more difficult due to the multi-catalytic properties of this protease and the controversy as to whether it is a thiol protease (Dahlmann et al., 1985; Rivett, 1988) or a serine protease (Tanaka et al., 1986; Yamamota et al., 1986). The activities described here agree with some of those described for the proteasome from fish muscle (Folco et al., 1988) which similarly showed a DTT requirement, ATP-independence, and marked inhibition by NEM and chymostatin. Unlike other reports (Dahlmann et al., 1985; Saitoh et al., 1989), SDS was not an activator of this mid-column activity, suggesting that this protease, if equivalent to the proteasome, could already have been in an activated form.

Although lysozyme has not been shown to be a good substrate for the proteasome (Hough et al., 1987), incubations of the Sepharose mid-column activity with casein, an established proteasome substrate (Ray & Harris, 1986) reflected similar properties, suggesting that this proteasome-like activity could also degrade lysozyme.

Thus chicken skeletal muscle appears to contain three proteolytic activities; firstly, a labile, ubiquitin-dependent protease that can be stabilized by ATP; secondly, a heat-stable ATP-independent activity that displays increased activity at high temperatures, (similar to the proteasome) and, thirdly an ATP-stabilized, heat-labile activity, independent of ubiquitin and similar to the protease described by Ismail & Gevers (1983). For definite

Table 5.2 Inhibitors of muscle and reticulocyte proteolytic activities

20 μg of pooled V_o or mid-column activity was incubated with 2 μg ^{125}I-lysozyme (2 x 10^5 cpm), 1 mM DTT, 2 mM magnesium acetate for 4 hr at 37°C with the indicated effectors. The TCA-soluble material released was measured as described in section 5.2.5 and the % activity remaining calculated.

	Muscle	Reticulocytes	
	mid-column	V_o	mid-column
Inhibitor	% activity remaining		
None	100	100	100
5 mM ATP	72	100	98
5 mM NEM	0	0	0
12.5 mM EDTA	35	85	46
250 μM chymostatin	10	6	21
500 μM TLCK	81	ND	ND
100 μM $ZnCl_2$	10	15	8
10 μM hemin	0	0	0
100 μg/ml leupeptin	54	78	60
0.01% SDS	6	8	4
No DTT	5	35	12

identification of these activities, further purification and characterization will be necessary.

5.4 DISCUSSION

The presence in chicken skeletal muscle of an isopeptidase activity that disassembles ubiquitin conjugates to yield free ubiquitin and (presumably) intact proteins has been established in this study; although this component of the ubiquitin pathway has been characterized in reticulocytes, calf thymus and other eukaryote cells (for review see Rose, 1988) it had not previously been identified in skeletal muscle. The enzyme appeared to have a native M_r value between 1×10^5 and 1×10^6. Other isopeptidases have been described in reticulocytes with native M_r values of 400 000, 300 000, 100 000 (cited in Hershko & Rose, 1987) and 200 000 (Hough et al., 1986). A smaller species (M_r = 38 000) appears to be involved in histone de-ubiquitination (Matsui et al., 1982) and other hydrolases specific for non-protein adducts have also been described with M_r values of 30 000 (Pickart & Rose, 1986). These lower M_r species could have been responsible for the small amounts of isopeptidase activity found in the V_t of the Sepharose CL-6B column. Inhibition by NEM is typical of isopeptidases: recently, Mayer & Wilkinson (1989) identified four hydrolases in bovine thymus all of which were inhibited by NEM. Although some heavy metal cations have been reported to inhibit isopeptidase activity, Mg^{2+} is not amongst these (Matsui et al., 1982); in fact Mg^{2+} increased isopeptidase activity in chicken skeletal muscle, indicating that it may be essential for activity in this tissue.

The protease responsible for the degradation of ubiquitin conjugates has been characterized mainly in rabbit reticulocytes (Hough et al., 1987; Waxman et al., 1987; Ganoth et al., 1988), although similar activities and partial characterizations have also been described in rabbit skeletal muscle (Fagan et al., 1987; Matthews et al., 1989) and BHK fibroblasts (McGuire et al., 1988). In rabbit cardiac muscle (DeMartino, 1983; Gehrke & Jennissen,

1987), rat skeletal muscle (Dahlmann et al., 1983; Ismail & Gevers, 1983) and erythroleukemia cells (Waxman et al., 1985), however, a ubiquitin-independent but ATP-dependent protease has been identified.

An ATP-dependent activity was present in crude extracts of both reticulocytes and chicken skeletal muscle, capable of degrading both endogenous and exogenous proteins. Whether this activity was ubiquitin-dependent, however, could not be established because endogenous ubiquitin was present in the extract. Degradation of both endogenous and exogenous proteins was ubiquitin-dependent in fraction II, that of the reticulocyte system being more active than muscle (4-fold increase as compared to a 1.5-fold increase, respectively). A comparison with other systems in which ^{125}I-lysozyme has been used as a substrate showed that rabbit skeletal muscle had a 2-fold increase in ubiquitin-dependent activity (Matthew et al., 1989; Fagan et al., 1987) whilst 12-fold and 10-fold increases in ubiquitin-dependent activity were shown in BHK cells (McGuire et al., 1988) and reticulocytes (Waxman et al., 1987), respectively. Muscle thus appears to contain lower levels of ubiquitin-dependent activity than other tissues. This does not arise from low rates of ubiquitin conjugate formation, as the muscle extracts degraded endogenous proteins at a lower rate than did the reticulocyte system. The entity reponsible was characterized in muscle extracts as having an M_r value, stability and inhibitor sensitivity similar to that of the ubiquitin conjugate-degrading enzyme of rabbit reticulocytes (Waxman et al., 1987; Hough et al., 1987).

Muscle extracts also contained a stable ATP- and ubiquitin-independent proteolytic activity that was similar to the proteasome extensively described in a rat skeletal muscle (Dahlmann, 1983). Recently it has been shown in reticulocytes that the proteasome is identical to the CF-3 component of the ubiquitin conjugate-degrading enzyme (Eytan et al., 1989) and this is supported by immunological

cross-reactivity between the two proteins (Matthews et al., 1989) as well as similar inhibitor profiles (Hough et al., 1987). Thus it is possible that the proteasome in muscle tissue is a component of the disassembled ubiquitin conjugate-degrading enzyme.

Other workers have reported the proteasome as being involved in an ATP-dependent, and ubiquitin-independent degradation of proteins in rabbit muscle (Driscoll et al., 1989), BHK cells (McGuire et al., 1989,), reticulocytes (Tanaka et al., 1988) and in erythroleukemia cells (Tsukahara et al., 1988b). Further ATP-stimulating activities, although not specifically associated with the proteasome, have also been detected (Gehrke & Jennissen, 1987; Ismail & Gevers, 1983; DeMartino, 1983). A heat-labile but ATP-stabilized, non-ubiquitin-dependent component was present in fraction II from chicken muscle, although this activity was lost on gel filtration. Recently, Fagan & Waxman (1989) have partially purified a similar ATP-requiring protease from chicken skeletal muscle which degrades non-ubiquitinated proteins. This protease had an M_r value = 600 000, contained an essential serine residue and was highly labile. It is thus possible that the ATP-dependent activity described in these studies is similar or identical to that described by Fagan & Waxman (1989) and the activity is lost on gel filtration due to its instability. On the other hand, Dahlmann et al. (1989b) have shown that an ATP-dependent but ubiquitin-independent proteolytic activity is actually due to an α_2-macroglobulin-cathepsin complex present in the blood or extracellular fluid of certain tissues, which contaminates such tissues upon homogenization or cell lysis. The possibility that the ATP-dependent activity found in chicken skeletal muscle fraction II is such a complex cannot be ignored.

The fact that chicken skeletal muscle apparently contains less ubiquitin dependent-degrading enzyme than does rabbit reticulocytes, again raises the question of the importance

of the ubiquitin pathway in muscle protein breakdown. The known instability of the ubiquitin conjugate-degrading enzyme suggests, however, that higher levels may exist in muscle cells *in vivo*. Alternatively, some other proteolytic pathway may be more important in protein breakdown in this tissue. The relationships between the different proteolytic pathways not only of muscle but also of other tissues, appear to be exceedingly complex; whether the separate proteolytic activities represent individual degradation pathways, or whether they are inter-dependent remains unsolved. Elucidation of such operations and the controlling mechanisms involved remain a major challenge in the field of protein degradation.

CHAPTER 6

THE CALPAIN PROTEOLYTIC PATHWAY IN CHICKEN SKELETAL MUSCLE

6.1 INTRODUCTION

Apart from the ubiquitin-dependent pathway, another major proteolytic system of the cytoplasm is that of the calpains. Although a similar pool of potential substrates is available for both the calpains and the ubiquitin-dependent pathways, to date, there is no indication of these two pathways being related. This may reflect their very different substrate specificities; while the ubiquitin-dependent pathway is involved in the degradation of short-lived and abnormal proteins, the calpains are apparently implicated in the selective and limited proteolysis of certain proteins. Although the two pathways may not be associated, the potential importance of the calpain system in muscle deserves further attention.

One of the roles of the calpains in skeletal muscle appears to be limited proteolysis at the Z-disc (Reddy et al., 1983; Ishiura et al., 1980) and, in particular, it participates in the degradation of α-actinin, resulting in the disassembly of the myofibrillar structure. The fact that an increase in calpain activity is associated with dystrophic muscle (Neerunjun & Dubowitz, 1979; Johnson & Hammer, 1988), with vitamin E deficiency (Otsuka et al., 1985; Dayton et al., 1979) and with denervation of muscle (Hussain et al., 1987), suggests that calpain may have an important role to play in the pathological turnover of myofibrillar proteins.

Calpain activities as well as an endogenous calpain inhibitor (calpastatin) have been identified in most tissues (for review, see Pontremolli & Melloni, 1986). Usually two forms of the calpain species are present; one is active at μM, and the other at mM Ca^{2+} concentrations. Although it was initially suggested that the lower Ca^{2+}-requiring form might be derived from the higher Ca^{2+}-requiring form by autolysis (Suzuki et al., 1981a), the finding that calpain I is usually composed of a heterodimer

with M_r values of 83 000 and 30 000, whilst calpain II is a heterodimer of 80 000 and 30 000 (Ando et al., 1988a; Kitahara et al., 1984) is clearly inconsistant with this notion. How calpain II, however, operates at physiological Ca^{2+} concentrations is not known, although an association of this species with the cell membrane to form an autolyzed form requiring less Ca^{2+} has been proposed (Mellgren et al., 1987).

In the case of chicken skeletal muscle, there is disagreement about the number of calpain species: thus Ishiura et al. (1979) identified only one form of calpain activity which had a Ca^{2+} sensitivity much lower than that normally found in some mammalian forms (Kawashima et al., 1984), while Wolfe et al. (1985) found three calpain species active at low, intermediate and high Ca^{2+} concentrations, respectively.

An attempt has accordingly been made in this study to characterize the number of calpain species present in chicken breast muscle with the hope of clarifying the discrepancies that exist in this tissue.

6.2 METHODS

6.2.1 Separation of calpain(s) from calpastatin in chicken skeletal muscle extracts

All procedures, unless stated, were performed at 4°C. Exsanguinated chicken breast muscle (10 g) was homogenized in an Ultra-turrax homogeniser at 0.75 of maximum setting, in a buffer containing 10 mM Tris-HCl, 50 mM NaCl, 4 mM EDTA, 0.1 mM DTT, 10% (v/v) glycerol, all at pH 7.4. The homogenate was centrifuged at 50 000 g for 40 min and the resulting supernatant diluted five times with ice-cold distilled-deionized water. The diluted supernatant was applied to a DEAE-cellulose column (1.6 x 20 cm), previously equilibrated with 10 mM Tris-HCl, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 10% (v/v) glycerol, pH 7.4. The column was washed with 10 column volumes of the same buffer and the bound protein was then eluted at a flow rate of 15 ml/hr, using a 300 ml linear salt gradient from 10 mM NaCl to 500 mM NaCl. Fractions of 3.75 ml were collected and the calpain and calpastatin activities measured (see 6.2.4 and 6.2.5, respectively).

6.2.2 Phenyl-sepharose chromatography

The pooled calpain I or calpain II peaks from the DEAE-cellulose column were adjusted to a final salt concentration of 0.3 M NaCl and applied, at a flow rate of 10 ml/hr, to a 1.6 x 10 cm phenyl-sepharose column, which had previously been equilibrated in 10 mM Tris-HCl, 0.1 mM DTT, 0.1 mM EDTA, 0.3 M NaCl, all at pH 7.4. The column was washed with five column volumes of the above buffer and 200 ml of a 0.3 M to 0.01 M NaCl gradient applied to elute bound calpain activity. Fractions of 2.5 ml were collected and assayed for calpain activity (see section 6.2.4).

6.2.3 Gel filtration of calpain and calpastatin samples

The relevant calpain and calpastatin fractions from either the DEAE-cellulose or phenyl-sepharose columns were applied to a Sephacryl S-300 column (0.6 x 150 cm) previously equilibrated with 50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM DTT, 50 mM NaCl, pH 7.4, at a flow rate of 4 ml/hr. Fractions of 1 ml were collected and the calpain or calpastatin activities measured (see sections 6.2.4 and 6.2.5, respectively).

6.2.4 Assays of calpain activity

Calpain activity was generally measured by the formation of TCA-soluble material from $^{14}CH_3$-casein (methylated by the method of Rice & Means, 1971) in an assay mixture containing 2 mM $CaCl_2$, 12 μg $^{14}CH_3$-casein (2 x 10^4 cpm), 0.1 mM DTT, 10 mM Tris-HCl, all at pH 7.4, together with 0-100 μg of enzyme fraction in a final volume of 200 μl. The mixture was incubated at 37°C for 1 hr and the reaction terminated by the addition of 100 μl BSA (10 mg/ml) and 100 μl 20% TCA. The samples were kept at 4°C for 20 min followed by centrifugation at 2 000 rpm for 15 min and the counting of a sample of the supernatant in a β-scintillation counter. Control samples contained 2 mM EDTA instead of $CaCl_2$.

In the assessment of calpastatin activity or Ca^{2+} sensitivity of calpain, the more accurate fluorometric method dependent on the release of tyrosine from denatured casein was used. The assay mixtures contained 4 mM $CaCl_2$, 5 mg/ml denatured casein, 0.1 mM DTT, 50 mM Tris-HCl, all at pH 7.4, together with 0-300 μg of enzyme fraction in a final volume of 500 μl. The mixture was incubated at 37°C for 60 min and the reaction terminated by the addition of 13.3% (w/v) TCA to a final concentration of 10%. The precipitated proteins were removed by centrifugation at

2 000 rpm and the tyrosine content of the supernatant determined fluorimetrically by the method of Waalkes & Udenfriend (1957) in a Perkin-Elmer fluorimeter.

6.2.5 Assay of calpastatin activity

Calpastatin activity was measured by mixing an aliquot of each selected column fraction with a known amount of pooled calpain II activity. The mixture was incubated at 0°C for 15 min prior to the addition of the remaining components of the standard calpain assay incubation mixture. The calpastatin activity was expressed as a percentage of control activity.

6.2.6 Quantitation of membrane-bound calpains

6.2.6.1 Preparation of samples

Chicken skeletal muscle (4 g) was homogenized in 8 volumes of buffer A (50 mM Tris-HCl, 0.1 mM DTT, 1 mM PMSF, 150 nM pepstatin A, 20 μM leupeptin, 40 μM chymostatin, 50 μM $CaCl_2$), centrifuged at 105 000 g for 60 min in a SW-40 Ti rotor and the supernatant collected ("Ca^{2+} cytosolic fraction"). The cell pellet was suspended in Buffer A and re-centrifuged at 105 000 g for 60 min; the resulting "Ca^{2+}- cytosolic fraction" was pooled with that from the previous step and EGTA added to a final concentration of 1 mM. In order to elute bound calpain from the particulate fraction, Buffer A containing 1 mM EGTA was added to the "Ca^{2+}- particulate fraction" and centrifuged as before; the resulting supernatant containing EGTA-extracted calpains was collected. Control samples were treated identically but always in the presence of 1 mM EGTA to give rise to the "EGTA-cytosolic fraction" and the "EGTA-particulate fraction".

6.2.6.2 Quantitation of calpains and calpastatin

The levels of calpain and calpastatins present in the above fractions were quantitated by phenyl-sepharose chromatography. NaCl was added to an aliquot of sample, corresponding to 0.5 g of starting material, at a final concentration of 0.5 M. The sample was applied to a 0.8 x 2 cm phenyl-sepharose column previously equilibrated with Buffer B (20 mM Tris-HCl, 0.1 mM DTT, 1 mm EDTA, 0.5 M NaCl, all at pH 7.4). The unbound protein containing calpastatin was collected in five column volumes of Buffer B, whilst the bound calpains were eluted with 8 ml of Buffer C (20 mM Tris-HCl, 0.1 mM DTT, 1 mM EDTA). Calpain and calpastatin activities were assayed as previously described.

6.2.7 SDS-PAGE

5-20% gradient SDS gels were run as described in section 2.2.11. Proteins were visualized by silver staining according to the method of Merril et al. (1981) using a Bio-Rad Silver Stain kit.

6.3 RESULTS

6.3.1 Separation of calpains from calpastatin

Calpain activities were separated from endogenous inhibitors (calpastatins) by applying the clarified homogenate to a DEAE-cellulose column and eluting the bound proteins with a linear salt gradient. Two peaks of calcium-dependent proteolytic activity eluted from the column, the first at 0.1 M NaCl and the second at 0.25 M NaCl. Both of these were inhibited by leupeptin, EDTA, and E 64 but not by pepstatin and chymostatin, suggesting that they were calpains (Fig. 6.1). In accordance with similar profiles obtained by other workers (Dayton et al., 1981; Croall & DeMartino, 1983; Penny et al., 1985), these entities were provisionally called calpain I and II in their order of elution off DEAE-cellulose. A major calpastatin peak eluted at 0.06 M NaCl with a smaller peak at 0.13 M NaCl; the former has previously been found to be present in a number of tissues (Murachi et al., 1981b; DeMartino & Croall, 1984; Ishiura et al., 1982).

Wolfe et al. (1985) have also shown more than one calpain species in chicken skeletal muscle preparations but other workers have consistently identified only one "calpain II" with an unusual Ca^{2+} sensitivity lower than that normally obtained for this type of enzyme (Ishiura et al., 1978; Kawashima et al., 1984). It was thus necessary to investigate whether the calpain activity eluting off DEAE-cellulose at 0.1 M NaCl was that of a typical calpain I or of an autolytic product of a calpain II species.

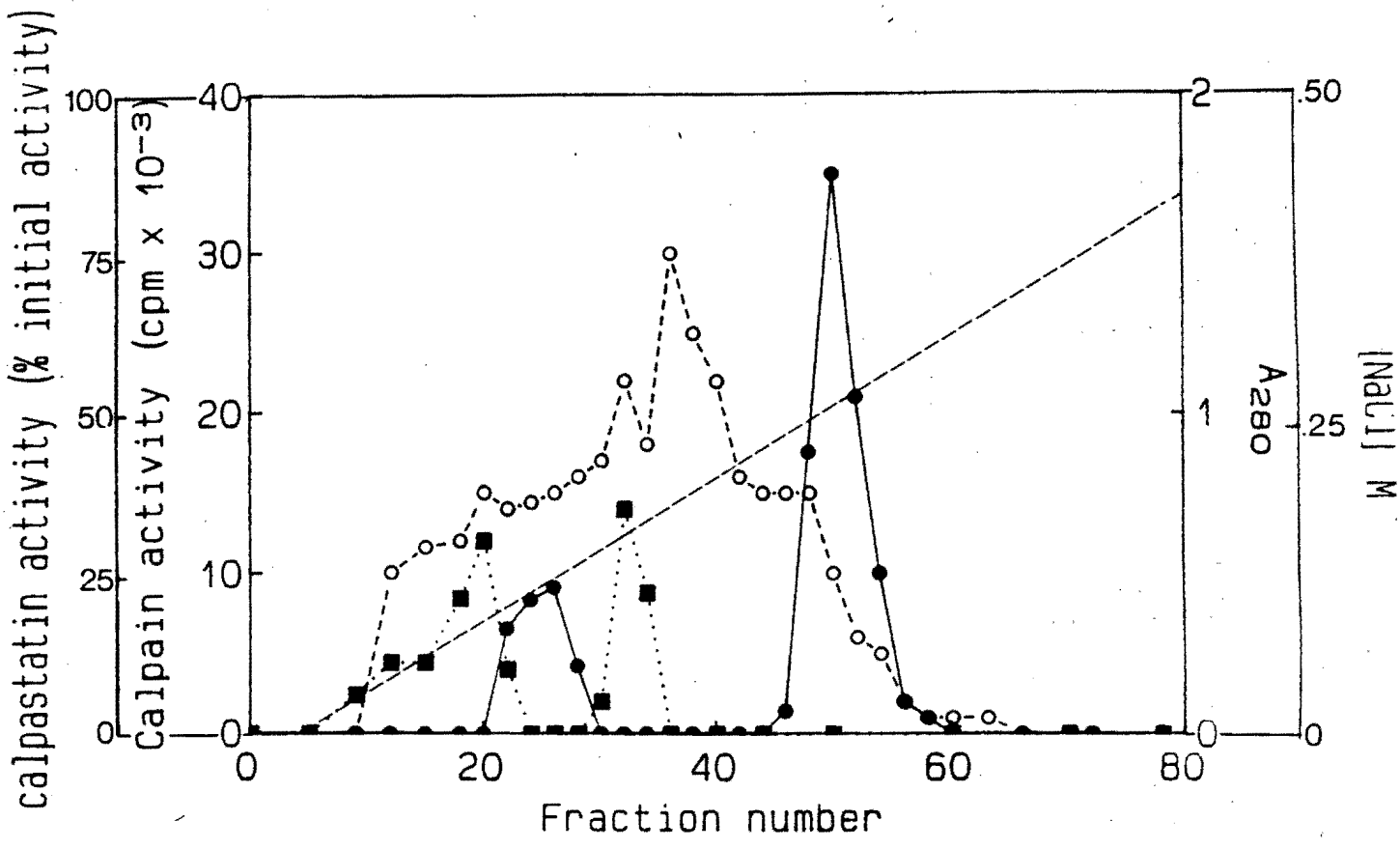


Fig. 6.1 DEAE-cellulose chromatography of calpain and calpastatin from adult chicken breast muscle

Homogenized chicken breast muscle (10 g) was centrifuged at 50 000 g for 40 min as described in section 6.2.1 and applied to a 1.6 x 20 cm DEAE-cellulose column equilibrated in 10 mM Tris-HCl, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol, all at pH 7.5. Bound protein was eluted in a linear 10 - 500 mM NaCl gradient and fractions of 3.75 ml collected. Calpain (●) and calpastatin (■) activities were measured as described in sections 6.2.4 and 6.2.5, respectively. A_{280} (O--O); NaCl (------).

6.3.2 Properties of the two calpains prepared from chicken muscle

A major feature of calpain I is its ability to be active at μM Ca^{2+} concentrations, far below those at which calpain II can be detected. This usually allows one to discriminate between the two species. Measurement of the Ca^{2+} sensitivities of the two calpains demonstrated that the first-eluting material was approximately ten times more sensitive to Ca^{2+} than the second (Fig. 6.2); thus it was half maximally activated at 20 μM Ca^{2+} whilst the other needed 180 μM Ca^{2+} to be stimulated to the same extent. Wolfe et al. (1985) have reported that a "calpain I" from chicken skeletal muscle (breast) was activated between 2-10 μM Ca^{2+}, whilst "calpain II" was active in the range of 250-350 μM Ca^{2+}. In chicken leg muscle, however, calpain II activity was half maximally activated at the much higher Ca^{2+} concentration of 570 μM (Ballard et al., 1988).

In order to determine if the particular assay conditions were responsible for the relatively high Ca^{2+} sensitivity, these calpain species were directly compared with the well-characterized calpains from rat liver. The calpain I activity from rat liver was half-maximally activated at 20 μM Ca^{2+} whilst the value for calpain II was 220 μM Ca^{2+} (Table 6.1); this should be compared with the literature value of 7 μM and 150 μM, respectively (DeMartino, 1981). Thus the two calpains of chicken breast muscle showed a Ca^{2+} sensitivity similar to those from a well-characterized mammalian source. Autolyzed calpain forms are, however, also known to be active at low Ca^{2+} concentrations (Suzuki et al., 1981a) and thus it was necessary to study further distinguishing properties of the two calpain species.

Differential heat stability is a useful test to discriminate between calpain I and calpain II activities, with that of calpain I being appreciably more heat-stable than calpain II (Inomata et al., 1984) while autolyzed forms are generally much more unstable (Suzuki et al.,

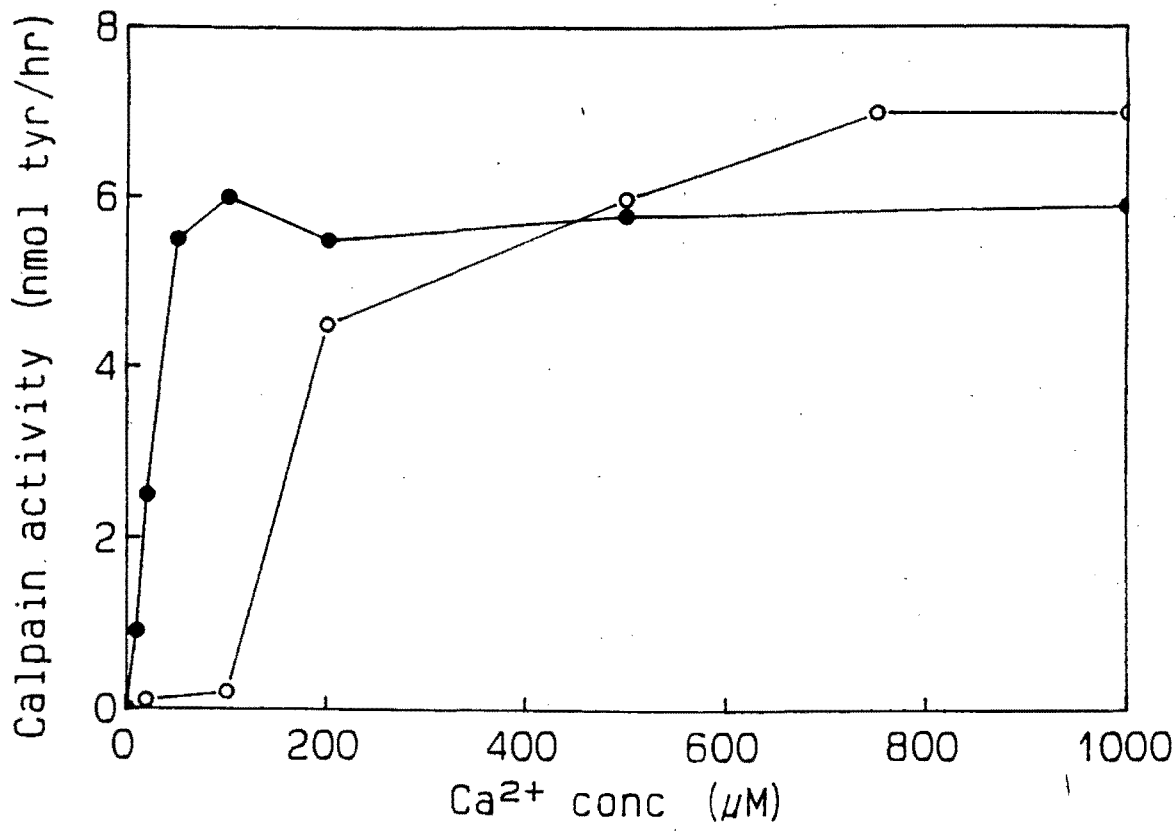


Fig. 6.2 Ca^{2+} sensitivities of calpain I and calpain II

50 μg pooled calpain I (●) and calpain II (○) fractions from the DEAE-cellulose column were incubated at varying Ca^{2+} concentrations in a mixture containing 5 mg/ml denatured casein, 0.1 mM DTT, 50 mM Tris-HCl, pH 7.5, in a final volume of 500 μl. The samples were assayed at 37°C for 60 min and the reaction terminated by the addition of 1.5 ml 13.3% TCA, and the amount of tyrosine released measured as described in section 6.2.4.

Table 6.1 Heat stabilities and Ca^{2+} sensitivities of rat liver and chicken skeletal muscle calpains I and II

50 μg of pooled chicken skeletal muscle and rat liver calpains I and II fractions from the DEAE-cellulose column were assayed for their temperature stabilities and Ca^{2+} sensitivities as described in Fig. 6.3 and Fig. 6.2, respectively.

Sample	Temperature for 50% inactivation (°C)	$[Ca^{2+}]$ for 50% activation (μM)
Rat calpain I	61	20
Rat calpain II	48	220
Chicken calpain I	45	20
Chicken calpain II	57	180

1981a). The "calpain I" from chicken skeletal muscle was more heat labile than calpain II, however, being 50% inactivated at 45°C while this point was reached in the case of calpain II only at 57°C (Fig. 6.3). In the case of calpains from rat liver, calpain I was more heat stable than calpain II (Table 6.1); rat liver calpain II had a stability profile that was more similar to chicken "calpain I" rather than to chicken "calpain II". Thus whilst the Ca^{2+} sensitivity of the activity eluting at 0.1 M NaCl off DEAE-cellulose was similar to that associated with a calpain I activity, its heat-lability was more suggestive of a partially autolyzed calpain.

6.3.3 Alterations in homogenization procedures

In order to determine if the homogenization procedure was associated with artifactual autolysis of a calpain species, variations in this procedure were designed and the resulting calpain I: calpain II ratios assessed after subsequent DEAE-cellulose chromatography. Table 6.2 shows that the continuous presence of the specific Ca^{2+} chelator, EGTA did not cause any diminution in the "calpain I" peak, suggesting that this entity was not the result of insufficient calcium chelation which could have been associated under these conditions with the calcium-dependent autolysis of a calpain species. Keeping the fresh muscle tissue at 4°C for 15 min before homogenization or storing it in liquid nitrogen prior to homogenization, similarly did not alter the calpain I : calpain II ratio. When EDTA was omitted from the homogenizing buffer, there was a marked decrease in "calpain I" activity, whilst calpain II activity was unaffected. The fact that changes in the "calpain I" levels occurred independently of calpain II suggested that "calpain I" was not derived from calpain II; taking into account the high Ca^{2+} sensitivity of calpain I species, the decrease in activity of this species

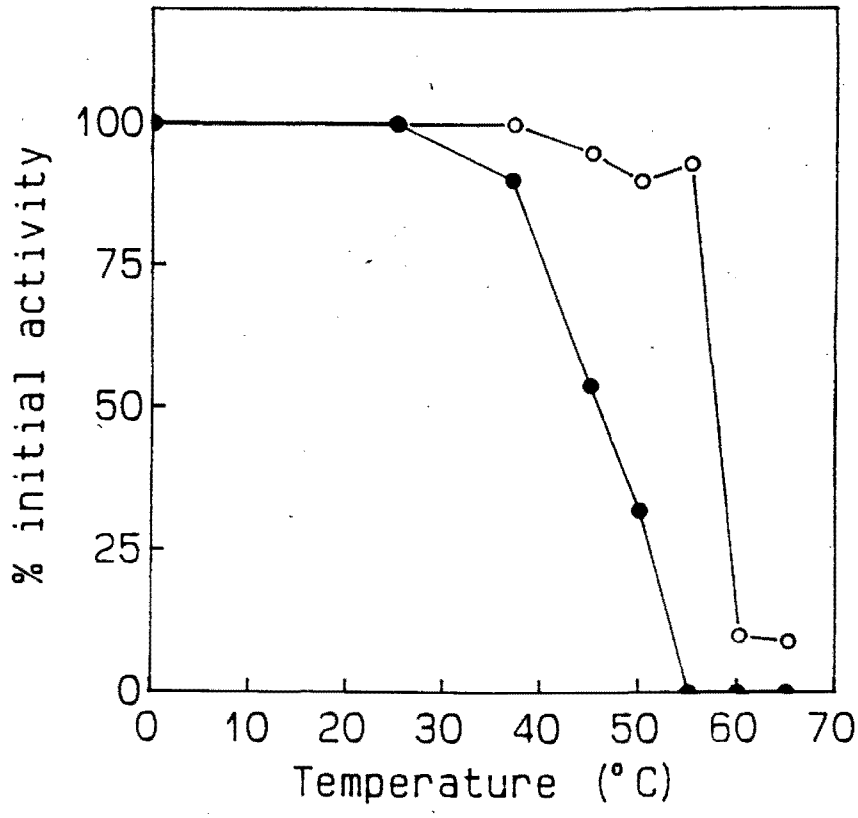


Fig. 6.3 Heat stabilities of calpain I and calpain II

50 μg pooled calpain I (●) and calpain II (○) fractions from the DEAE-cellulose column were pre-incubated at the varying temperatures for 10 min and the activity remaining measured by incubating the sample at 37°C for 1 hr in a mixture containing 2 mM $CaCl_2$, 12 μg $^{14}CH_3$-casein (2 x 10^4 cpm), 0.1 mM DTT, 10 mM Tris-HCl, all at pH 7.5 in a final volume of 200 μl. TCA-soluble material was measured as described (section 6.2.4).

Table 6.2 Effect of homogenization procedures on calpain I and calpain II activities

10 g of fresh exsanguinated chicken skeletal muscle was treated as indicated prior to centrifugation and DEAE-cellulose chromatography as described in section 6.2.1. The pooled calpain activities of the DEAE-cellulose column were assayed as in section 6.2.4 and the ratios of calpain I to calpain II calculated.

Homogenization procedure	Calpain I activity	Calpain II activity	Calpain I: Calpain II
	(cpm x 10^4)	(cpm x 10^4)	
Normal	2.8	4.3	0.060
4 mM EGTA	2.6	4.2	0.062
15 min on ice in 4mM EDTA	2.3	3.9	0.059
Stored in liqid nitrogen	2.2	3.5	0.064
No EDTA	0.8	3.7	0 021

was more likely to have been a result of its autolysis to a form more unstable during chromatography on DEAE-cellulose.

6.3.4 Autolysis of calpain II

In order to determine if an autolytic product of calpain II would elute at the same position as "calpain I" on DEAE-cellulose, calpain II was partially autolyzed during a 10 min incubation at 20°C in the presence of 1 mM $CaCl_2$; the reaction was stopped with 2 mM EDTA. The partially autolyzed calpain II sample was applied to a DEAE-cellulose column, the bound protein eluted with a linear salt gradient and the calpain activity(ies) assayed. A 50% decrease in calpain II activity was detected but no calpain activity appeared in any new fractions (results not shown), suggesting that the autolysis products of calpain II were very unstable. Thus the Ca^{2+}-dependent activity that eluted at 0.1 M NaCl on DEAE-cellulose was considered not to be an autolytic product of calpain II. Suzuki et al. (1981b,) have similarly shown the calpain II autolytic products to be unstable in chicken skeletal muscle; they did not re-chromatograph on DEAE-cellulose in any active form, although an inactive species was detected, using calpain antibodies which eluted at 0.08 M NaCl, which is close to the position of "calpain I". Other reports, however, suggest that some autolytic products of calpain II generally elute in an active form in the same position as the calpain II species (Nagainis et al., 1988; Gopalakrishna & Barsky, 1986). Possibly the degree of autolysis achieved is related to these differing results.

6.3.5. Partial purification of calpains I and II

In order to investigate the subunit structure of the two chicken muscle calpains, further purification was required.

Phenyl-sepharose chromatography is a useful step in calpain purification (Karlsson et al., 1985; Gopalakrishna & Barsky, 1986); pooled "calpain I" and calpain II fractions were separately subjected to hydrophobic interaction chromatography. Both species eluted from the phenyl-sepharose column only after the salt had been removed, "calpain I" reproducibly eluting slightly later than calpain II (Fig. 6.4). Although Karlsson et al. (1985) reported that calpain II eluted from phenyl-sepharose at 0.1 M NaCl, this was not the case with chicken skeletal muscle calpain II, suggesting that the chicken species was more hydrophobic than that derived from rabbit brain. SDS-PAGE analysis of these samples showed that calpain II was composed of two subunits with approximate M_r values of 80 000 and 30 000, while "calpain I" contained two major polypeptides with M_r values of 88 000 and 70 000 but no 30 000 species was found (Fig. 6.5a). Whether the M_r = 88 000 species is the "parent" calpain I or a protein contaminant, is not known. The "calpain I" species was thus apparently different in subunit structure from calpain II (Ohsumi et al., 1984; Ando et al., 1988a; Kitahara et al., 1984); further purification of "calpain I" proved difficult due to the instability of this entity.

6.3.6 Gel filtration of "calpain I" and calpain II

In order to estimate the native M_r values of "calpain I" and calpain II, pooled DEAE-cellulose samples were chromatographed on Sephacryl S-300. Calpain II reproducibly eluted at a position consistent with an M_r value of 120 000 (Fig. 6.5b), while "calpain I" eluted with a slightly smaller M_r value of 90 000 (Fig. 6.5c), in agreement with the SDS-PAGE subunit data described above. Hydrophobic interactions of "calpain I" with the column matrix were unlikely to have been responsible for a lower apparent M_r

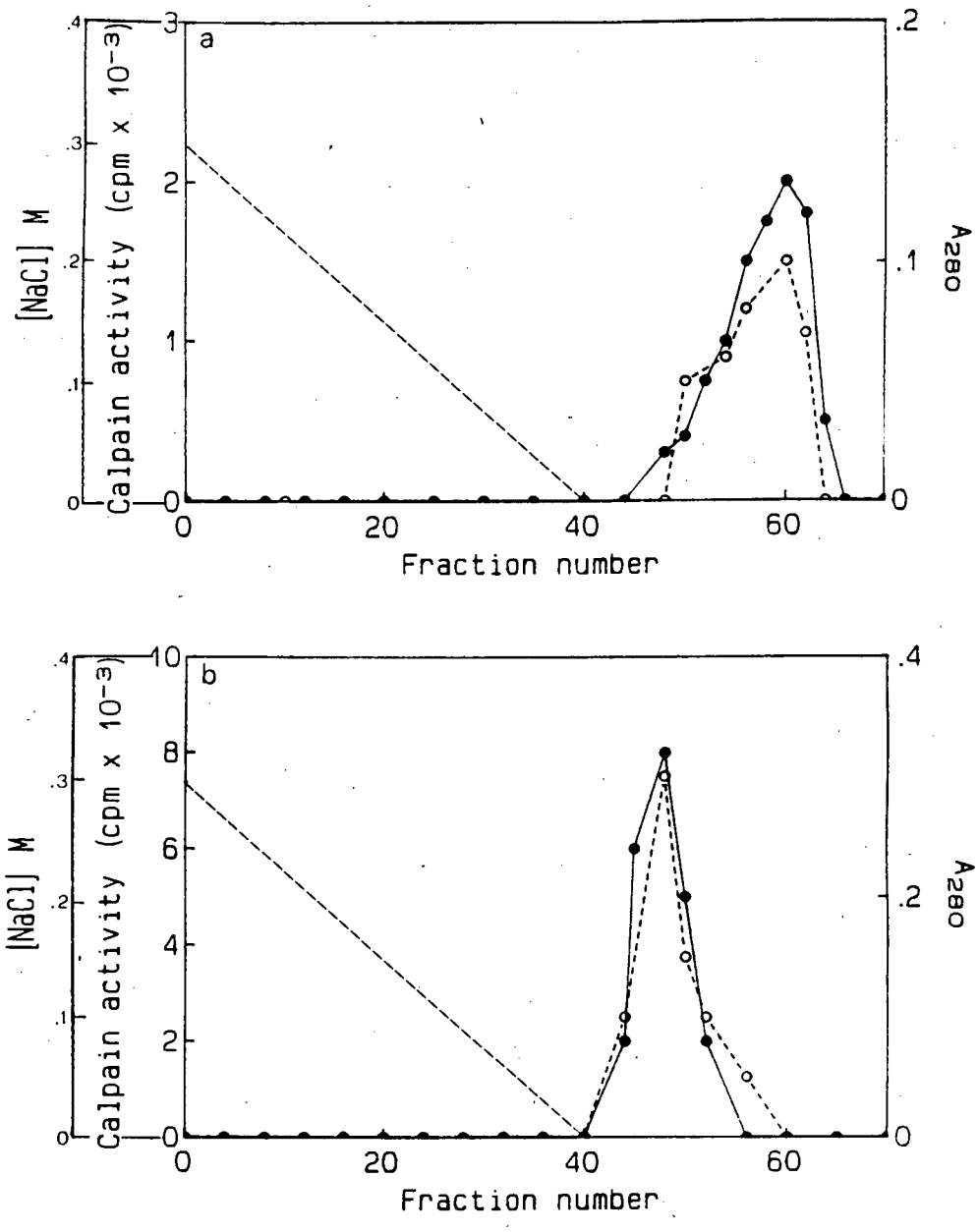
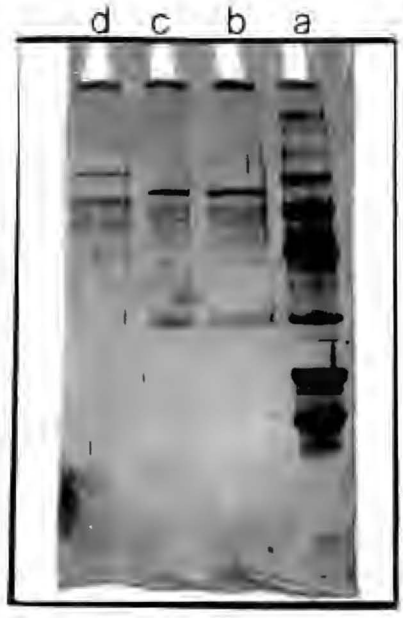


Fig. 6.4 Phenyl-sepharose chromatography of "calpain I" and calpain II

20 mg of "calpain I" (Fig. 6.4a) and 30 mg calpain II (Fig. 6.4b) from the DEAE-cellulose column were adjusted to 0.3 M NaCl and applied to a 1.6 x 10 cm phenyl-sepharose column equilibrated in 10 mM Tris-HCl, 0.1 mM DTT, 0.1 mM EDTA, 0.3 M NaCl, all at pH 7.5. Unbound protein was washed from the column and the bound protein eluted in a linear 0.3 M - 0.01 M NaCl gradient and the calpain activity (●) measured as described in section 6.2.4. A_{280} (O---O); NaCl (-----).

a



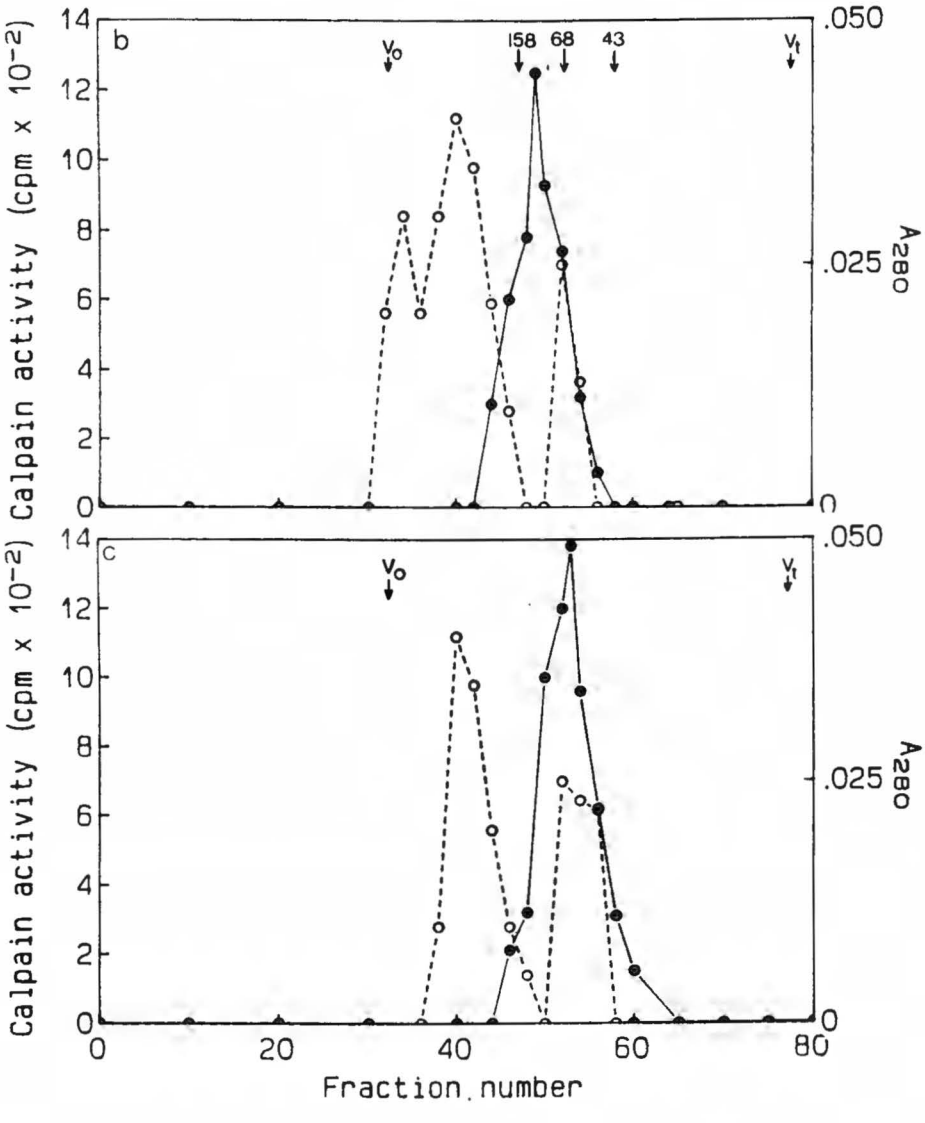


Fig. 6.5 (legend overleaf)

Fig. 6.5 Subunit structure and M_r values of the calpains

Fig. 6.5a SDS-PAGE of calpains

Pooled calpain samples from the phenyl-sepharose column were heated to 90°C for 2 min in the presence of 1% SDS and 5% 2-mercaptoethanol and electrophoresed as described in 6.2.7. **Lane a:** molecular weight markers: myosin heavy chain (M_r = 200 000), phosphorlase b (M_r = 94 000), bovine serum albumin (M_r = 68 000), ovalbumin (M_r = 43 000), carbonic anhydrase (M_r = 30 000), soybean trypsin inhibitor (M_r = 20 100), α-lactalbumin (M_r = 14 400). **Lane b:** 8 g calpain II. **Lane c:** 10 μg calpain II. Lane d: 8 μg calpain I.

Fig. 6.5b and c Gel filtration of "calpain I" and calpain II

1 mg calpain II (Fig. 6.5.b) and 1 mg "calpain I" (Fig. 6.5c) from the DEAE-cellulose column (Fig 6.1) was applied to a 0.9 x 60 cm Sephacryl S-300 column equilibrated in 50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM DTT, 50 mM NaCl, all at pH 7.4, at a flow rate of 4 ml/hr. Fractions were collected and the calpain activity measured (●) as previously described (6.2.5). A_{280} (O----O). The indicated M_r values ($x10^{-3}$) were obtained by calibrating the column with blue dextran (V_o), catalase (M_r = 232 000), aldolase (M_r = 158 000), BSA (M_r = 68 000), ovalbumin (M_r = 42 000) and ATP (V_t).

of this species, as all samples were run at an ionic strength of 0.1 M.
The lower than-expected M_r values of "calpain I", the subunit size, and the enzyme's instability, suggested that this species was not a typical calpain I. Since it seemed not to be derived from calpain II by autolytic degradation, one is left with the possibility that it was derived from a precursor calpain I. Reaction of these calpain species with specific antibodies to calpain I and calpain II should enable one to determine their origin; an attempt to use anti-bovine calpain I and calpain II antibodies as well as anti-mouse calpain I antibodies (kindly donated by Dr D.Goll) was uninformative due to the lack of cross-reactivity between these species (data not shown).

6.3.7 Calpastatin activities

Although the major calpastatin activity in the muscle extracts eluted from ion-exchange columns at 0.06 M NaCl, a smaller activity was more retarded, eluting at 0.13 M NaCl. In embryonic tissue a third calpastatin activity was detected eluting at 0.2 M NaCl. Whilst the calpain specific activity remained at a constant level during chicken development, all of the calpastatin activities decreased during the growth to 7-week chickens. The most striking change was in the content that of the 0.2 M NaCl species: this underwent a 2-fold decrease in activity from the 15-day embryo to the 19-day embryo stage and was totally absent in the 8-day and 7-week chickens (results not shown; A.A. Smith. 1981).
The major calpastatin activity emerging from the DEAE-cellulose column was more inhibitory to both "calpain I" and calpain II as its concentration was increased; "calpain I" was slightly more sensitive than calpain II (Fig. 6.6). Unfortunately, as no completely purified enzymes were prepared, the molar amounts of calpastatin bound per

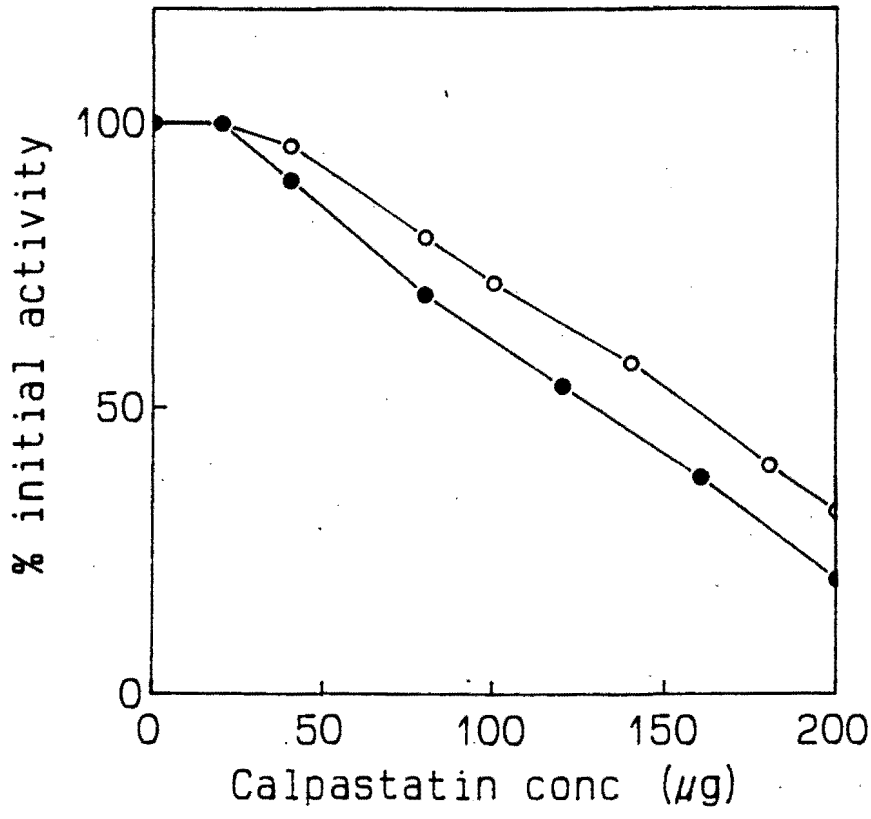


Fig. 6.6 Inhibition of calpain activity by extracts containing calpastatin

An amount of calpain I (●) and calpain II (○) with an activity equivalent to 10 nmol tyrosine/hr was incubated with the indicated concentrations of partially purified calpastatin (obtained from the DEAE-cellulose column) at 0°C for 15 min, after which 4 mM $CaCl_2$, 5 mg/ml denatured casein, 0.1 mM DTT, 50 mM Tris-HCl (pH 7.4) was added in a final volume of 500 μl. The mixture was incubated at 37°C for 60 min and the calpastatin activity measured as described in section 6.2.3.

calpain molecule could not be determined. The calpastatin was heat-stable and showed an unchanged degree of inhibition after it had been heated to 90°C for 5 min (results not shown). On gel filtration, it eluted in a broad peak of activity with a major form at an M_r of 270 000 (Fig. 6.7).

6.3.8 Membrane binding of the calpains

A major problem in the assessment of the cellular function of calpain II is the high Ca^{2+} concentration required for its activity. Recent studies have demonstrated that binding of calpains to cell membranes can occur at μM calcium concentrations; and once bound, the enzymes can be autolysed, in the presence of Ca^{2+} and phospholipids to a form requiring less Ca^{2+} (for review, see Suzuki et al, 1987; Pontremoli & Melloni, 1986; Mellgren, 1987). It was thus of interest to see if the calpains of chicken skeletal muscle bound to cell membranes in the presence of Ca^{2+} with and without molecular weight changes, indicative of autolytic products. Chicken muscle was accordingly homogenized in the presence and absence of calcium, the resulting cytosolic and particulate fractions isolated and the amounts of calpain activity, both cytosolic and membrane-bound, quantitated. Phenyl-sepharose chromatography was chosen for calpain quantitation due to the total removal of calpastatin from such fractions. In the case of both the Ca^{2+}- and the EGTA-treated cytosolic fractions, calpastatin did not bind to phenyl-sepharose and was separated from the calpain activities which eluted when the salt had been removed (Fig. 6.8a and b). Calpain activity was present in the "Ca^{2+}-particulate fraction" rather than in the "EGTA-particulate fraction" (Fig. 6.8c and d), suggesting firstly, that calpain bound to the membrane fraction only in the presence of Ca^{2+}, and secondly, that calpastatin did not bind to the membranes.

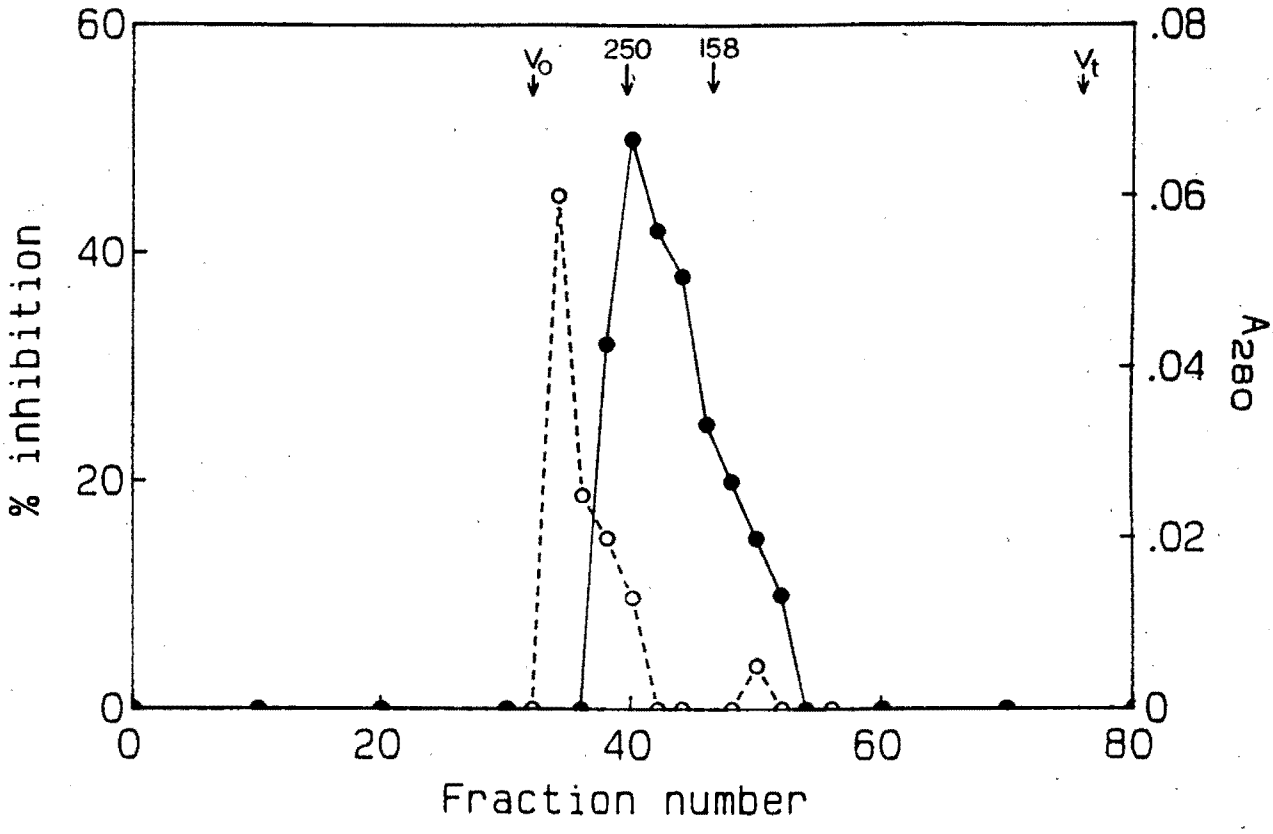


Fig. 6.7 Gel filtration of calpastatin

1 mg of pooled calpastatin, obtained from the DEAE-cellulose column was applied to a 0.6 x 150 cm Sephacryl S-300 column and run as previously described (section 6.2.3). Calpastatin activity (●) was measured by the decrease in the release of tyrosine of a known activity of calpain II, as described in section 6.2.5. A_{280} (O---O). The indicated M_r values ($x10^{-3}$) were obtained by calibrating the column with blue dextran (V_o), catalase (M_r = 232 000), aldolase (M_r = 158 000), BSA (M_r = 68 000), ovalbumin (M_r = 42 000) and ATP (V_t).

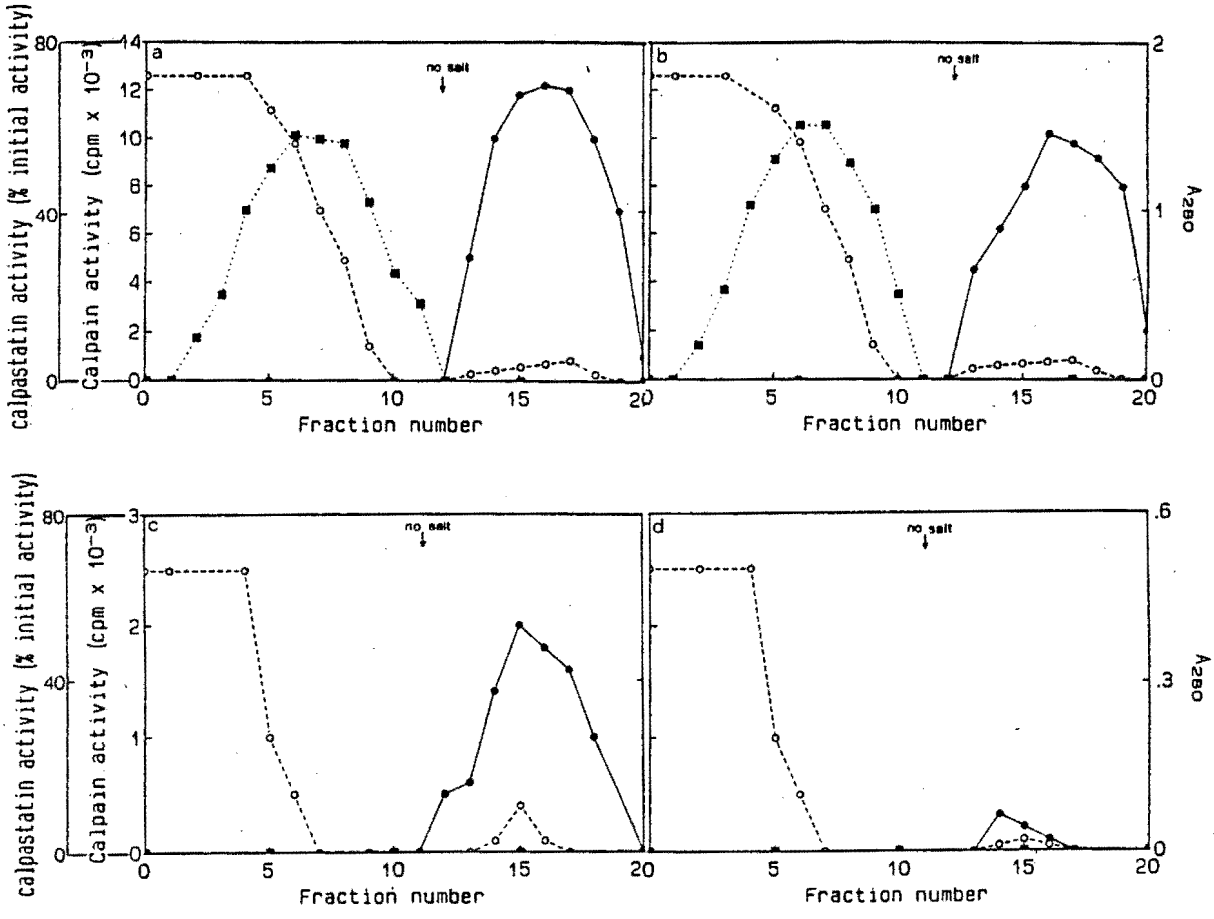


Fig. 6.8 Phenyl-sepharose chromatography of membrane- and non-membrane-associated calpains

Calpain samples were prepared as described in section 6.2.6.1 and quantitated by phenyl-sepharose chromatography. NaCl was added to a final concentration of 0.5 M to an aliquot of sample corresponding to 0.5 g of starting material. The sample was applied to a 0.8 x 2 cm phenyl-sepharose column and run as described in section 6.2.6.2. Calpastatin (■·····■) and calpain activities (●——●) were measured as previously described in sections 6.2.5 and 6.2.4, respectively. A_{280} (O--O).

(a): EGTA cytosolic fraction. **(b):** Ca^{2+} cytosolic fraction. **(c):** Ca^{2+} particulate fraction. **(d):** EGTA particulate fraction.

Table 6.3 Quantitation of membrane-associated and non-membrane-associated calpain activities

Membrane-associated and non-membrane-associated calpain fractions were prepared and quantitated from three separate experiments as described in section 6.2.6. The % of calpain bound to the membrane fraction was calculated in each of the three separate experiments.

	Exp. 1	Exp. 2	Exp. 3
Sample	calpain activity (cpm x 10^{-3})		
Ca^{2+} cytosolic	40	14	7.6
EGTA cytosolic	46	16	9.3
Ca^{2+} particulate	6.7 (14.5%)	23 (14.1%)	14 (14.8%)
EGTA particulate	0.2	1.0	0.3

Fig. 6.9 (legend overleaf)

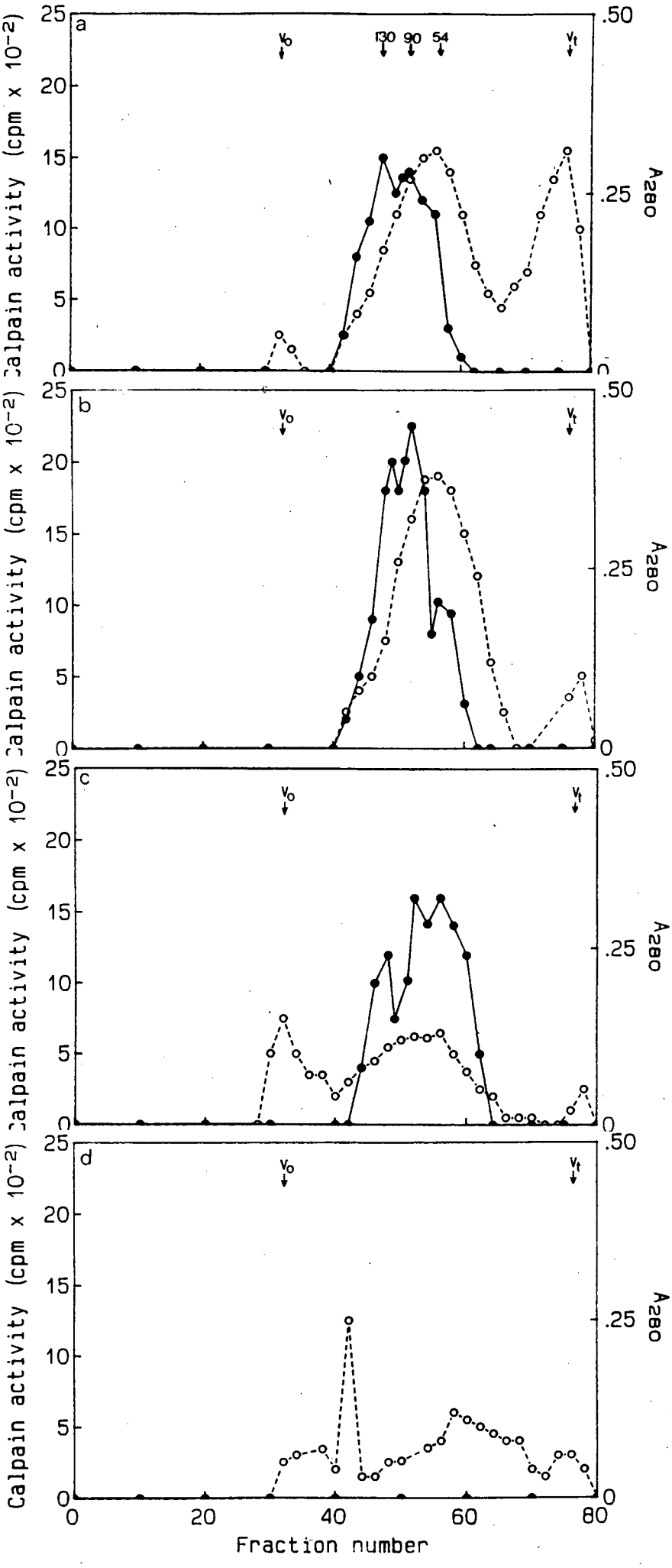


Fig. 6.9 Gel filtration of membrane and non-membrane associated calpain

1 mg of calpain sample, prepared as described in section 6.2.6.1 was applied to a 0.9 x 60 cm Sephacryl S-300 column equilibrated in 25 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM DTT, 50 mM NaCl, all at pH 7.5, at a flow rate of 4 ml/hr. Samples were collected and the calpain activity (●) assayed as previously described (section 6.2.4). A_{280} (O----O). The indicated M_r values ($x10^{-3}$) were obtained by calibrating the column with blue dextran (V_o), catalase (M_r = 232 000), aldolase (M_r = 158 000), BSA (M_r = 68 000), ovalbumin (M_r = 42 000) and ATP (V_t).
(a): EGTA cytosolic. **(b):** Ca^{2+} cytosolic. **(c):** Ca^{2+} particulate. **(d):** EGTA particulate.

6.4 DISCUSSION

The presence in the case of chicken muscle extracts of two calpain peaks eluting from DEAE-cellulose together with one major calpastatin peak, is similar to the profiles obtained by other workers in a variety of different tissues (Dayton et al., 1981; Kubota et al., 1986; Suzuki et al., 1982; Ando et al., 1988a); the data suggest that chicken skeletal muscle also contains two calpain activities. The less anionic entity had a higher Ca^{2+} sensitivity than the more anionic species and this fact also supports the notion that the two activities were those of the chicken versions of calpain I and calpain II, respectively. The heat stability, subunit structure and native M_r values of the "calpain I" species were not typical of calpain I from other sources, and were more similar to those of an autolyzed form of a calpain. Although Wolfe et al. (1985) in chicken skeletal breast muscle have also detected calpains sensitive to low and high calcium concentrations, as well as a third form only active at mM Ca^{2+} concentrations, the heat stabilities of these fractions were not described and no direct comparison between the low Ca^{2+}-requiring forms can be made. Nagainis et al. (1988) have, however, reported an autolytic product of calpain II in chicken breast muscle which was a dimer with M_r values of 77 000 and 18 000; the 77 000 species was further degraded to subunits with M_r's of 56 000 and 18 000. None of these entities corresponded to the 70 000 M_r species detected in this work, suggesting that the detected "calpain I" species was not an autolysis product of calpain II.

Recently, Fukui et al. (1988) have described, in the case of polymorphonuclear leukocytes, a form of calpain I that is less heat-stable than normal calpain I and is composed of only one subunit with an M_r of 70 000 co-existing with a "normal" calpain containing M_r subunits of 83 000 and 29 000, respectively. The unusual calpain I was formed after

disruption of the cells under nitrogen cavitation and was apparently derived from calpain I by non-autolytic proteolysis possibly brought about by other cytosolic proteases. Thus the less anionic entity may have been a calpain I which had become partially degraded during the homogenization procedure; whether lysosomal or cytosolic proteases might have been involved is not known. Similar low Ca^{2+}-requiring activities, with smaller M_r values than those expected for calpain I species, have also been described in rat cardiac muscle (Croall & DeMartino, 1983), hamster skeletal muscle (Johnson & Guindon-Hammer, 1987) and human skeletal muscle (McDermott et al., 1985). It is only the report of Fukui et al. (1988), however, which has conclusively shown the lower M_r- and more Ca^{2+}-sensitive species to be derived from calpain I and not from calpain II.

The calpain II activity described in this work from chicken breast muscle extracts was similar to that described in other reports in the same tissue, in terms of its elution position on DEAE-cellulose columns (Ishiura et al., 1978; Nagainis et al., 1988), its heat stability (Kubota & Suzuki, 1982,) and its calcium sensitivity (Kawashima et al., 1984). Although the Ca^{2+} sensitivity of this form was in fact slightly higher than in the case of some mammalian forms (Kawashima et al., 1984), other reports have shown both calpain I and calpain II to have Ca^{2+} sensitivity similar to that of chicken muscle (Yoshimura et al., 1983; DeMartino, 1981; Kitahara et al., 1984). Thus the calcium sensitivity of chicken skeletal muscle calpain II is not as unique as initially thought and the suggestion that chicken contains only one calpain species of intermediate Ca^{2+} sensitivity (Kawashima et al., 1984) seems unfounded. In any case, the notion that chickens contain a single calpain species in all tissues has recently been disputed by Murakami et al. (1988) who have identified both calpain I and calpain II in nucleated chicken erythrocytes.

A heat-stable calpastatin with a major M_r activity of 270 000 is present in chicken skeletal muscle. Species of similar size have been reported in human erythrocytes (Murachi et al., 1982), rat liver (DeMartino & Croall, 1984) and rabbit liver (Melloni et al., 1984). A calpastatin activity in chicken skeletal muscle has in fact been identified but this had an M_r of 68 000 (Ishiura et al., 1982). Although it is true that a variety of M_r values have been reported for the calpastatins, ranging from 300 000 (Nishiura et al., 1978)to 34 000 (Takahashi-Nakamura et al., 1981), recent work on the deduced amino acid sequence of calpastatin cDNA has shown this protein to have a true M_r of 77 000; it nevertheless migrates anomalously on SDS-PAGE, with an M_r = 107 000 (Takano et al., 1988). Thus the calpastatin form in chicken skeletal muscle is likely to be an assembly of four M_r = 77 000 subunits. The wide range of M_r values obtained in other studies is likely to reflect degradation of calpastatin (Otsuka & Goll, 1987) to forms which retain their inhibitory activity due to the repetitive functional domains characteristic of this protein (Emori et al., 1988; Takano et al., 1988).

Developmental changes in calpastatin specific activities in chicken muscle were shown in the case of the species eluting from DEAE-cellulose at 0.2 M NaCl. Recently, Takano et al. (1989) have similarly found a calpastatin species to elute from DEAE-cellulose at 0.2 M NaCl, but whether this form also undergoes developmental changes is not known. Whilst a similar loss of inhibitory activity occurred during the differentiation of rat skeletal myoblasts into myotubes (Kaur & Sanwal, 1981) and in the first 9 weeks of rat liver development (Tanaka et al., 1985), other workers studying chicken leg muscle have not shown any statistical changes in the calpastatin levels with respect to the growth rate (Ballard et al., 1988). Similarly, no changes in calpastatin levels were seen in rats between the ages of 6 to 12 months (Nakamura et al., 1988). Thus it appears that the fall in calpastatin levels occurs only during

early post-embryonic stages and not in all muscles or muscle fibre types, suggesting a selective role for calpain in developing muscle.

The ability of chicken skeletal muscle calpains to bind to a membrane fraction in vitro in the presence of μM Ca^{2+}, and to generate an active lower M_r species is in agreement with an existing model for the activation of calpain II to a form requiring less Ca^{2+} (Pontremoli & Melloni, 1986; Suzuki et al., 1987; Mellgren 1987). Although the origin of the M_r = 54 000 species is not known, the observation that similar active molecular weight species can be derived from calpain II by autolysis (Nagainis et al., 1988; Suzuki et al., 1981b), suggests that it is this calpain species which is involved.

Although calpastatin has been identified to be associated with the sarcolemma (Mellgren et al., 1987), no such membrane-associated activity was detected in this work. Instead, the calpastatin activity was present only in the cytosolic fractions, in agreement with the findings of other workers who have shown this species to be essentially cytosolic (Murachi et al., 1981b).

Gopalakrishna & Barsky (1986) have shown, in rat tissues, that calpain becomes membrane-associated in the presence of Ca^{2+} and is autolyzed to forms with higher Ca^{2+} sensitivity. Quantitation of these phenomena showed 31% and 47% of total calpain to be membrane-associated in the case of kidney and skeletal muscles, respectively; although a similar value was obtained in the present work for rat kidneys, the skeletal muscle values were much lower. This may reflect the low total specific activity present in this tissue.

A problem arising from the association of calpain to membrane fractions in the presence of Ca^{2+}, is that complex formation between calpain and excess calpastatin should also occur under these conditions yielding an inactive calpain form. Gopalakrishna & Barsky (1986) have shown that calpain is loosely associated with cell membranes in a

Ca^{2+}-independent manner, and that the association becomes stronger in the presence of Ca^{2+}, allowing some calpain species to avoid being complexed to calpastatin. It is possible that the situation in chicken homogenates is similar.

Chicken skeletal muscle thus contains an active Ca^{2+}-dependent proteolytic system containing two calpains and calpastatin. The exact functions of these entities in muscle tissue is still not clear, but the present evidence supports the notion that they are important constituents inter alia of signal transduction systems and remodelling processes with inappropriate destructive activity in certain diseased states.

CHAPTER 7
PERSPECTIVES

The findings reported in this thesis on the calpain and the ubiquitin-dependent pathways of protein degradation, whilst providing insights into the properties of these systems in chicken skeletal muscle, at the same time have raised some further fascinating, and as yet unanswered questions; some of these are addressed below.

1 The importance of the ubiquitin-dependent system in muscle protein degradation

Although ATP-dependent ubiquitin conjugation to endogenous proteins readily occurs in chicken skeletal muscle, the assayed specific activities of the ubiquitin conjugate-degrading enzyme and ubiquitin-protein ligase(s) are much lower than those of the same entities in reticulocytes. Should this in fact reflect the in vivo capacities of these enzymes, it is possible that the ubiquitin-dependent pathway may be expected to play a minor role in the breakdown of muscle proteins. Alternatively, it is possible that inactivation of these proteins occurs during the isolation procedures and thus higher capacities of these proteins may exist in vivo. It is always important to relate the capacity of the ubiquitin-dependent system to the in vivo rates of proteolysis of a particular tissue or cell type. Thus reticulocytes, which are self-destructing cells, would be likely to have a higher capacity of the ubiquitin-dependent pathway compared to the comparatively less proteolytic active system of muscle.

As the ubiquitin-dependent pathway in other cell types is concerned with the removal of denatured and short-lived proteins, a special role of this system in muscle could be the removal of damaged muscle proteins. It is well

established that endurance exercise, particularly marathon running, causes major trauma to the muscle fibres, including Z-line disruption (Hikida et al, 1983). Although much of the muscle damage is caused by the generation of mechanical forces (Schwane & Armstrong, 1983), other factors, including the generation of a more acidic intracellualr environment by lactic acid accumulation, as well as the muscle temperature rising to 41°C and above, may also be involved. One might expect that under such conditions a plethora of damaged or denatured proteins could be generated, and this may entail the activation of the heat-shock response. Although the role of ubiquitin in the heat-shock response has been well reported in chicken embryo fibroblasts (Bond & Schlesinger, 1985) little is known about this response in exercise-damaged muscle. The ubiquitin-dependent pathway may thus be very important under such stressed conditions.

2 TLCK-induced auto-ubiquitination of E1 and the cell cycle

The novel findings described here and reported elsewhere (Arnold & Gevers, 1990), of the NaOH- and TLCK-induced auto-ubiquitination of the E1 and E2 proteins, may have important consequences related to the cell cycle. A similar (if not identical) phenomenon of E1 ubiquitination associated with E1 inactivation has been described in the thermolabile mouse mammary carcinoma cell line, ts85, at non-permissive temperatures (Finley et al, 1984). Such E1 inactivation is responsible for abortive chromosome condensation occurring in the "pre-prophase" of the M-phase (Mita et al., 1980) and for the growth arrest of such cells, which mainly occurs in the G_2 phase, although some cells are blocked in S-phase (Ciechanover et al., 1984; Finley et al., 1984). The cell cycle block is thus rather staggered, suggesting that a proportion of the E1 molecules

remain active. Ubiquitination of individual subsets of substrate proteins under these conditions may thus be determined by the "affinity" of the individual E2 species for charged E1 and this may determine, for example whether histones, as opposed to abnormal proteins, might be the preferred substrates at lowered E1 levels. That E1 inactivation in the mutant cells at the non-permissive temperatures causes a nearly total inhibition of the ubiquitin-dependent degradation of the short-lived proteins as well as preventing uH2A formation, suggests a similar affinity of the various E2 species for charged E1. A lack of uH2A formation may definately be responsible for the abnormal chromosomal condensation seen in these cells at the non-permissive temperature (Mita et al, 1980).

It is possible that TLCK, because it promotes inactivating auto-ubiquitination of E1 (see section 3.3.5) may, in a manner analogous to the lesion in ts85 cell, cause a block in the cell cycle during the G_2 or very early M phase. Although Schnebli & Haemmerli (1974) showed TLCK to inhibit cell division whilst allowing DNA synthesis to occur in SV3T3 cells, it was difficult categorically to define at what stage in S, G_2 or early M-phase, TLCK acted.

Of great interest has been the recent finding of Luca & Ruderman (1989) that certain cyclins, proteins necessary for the entry of cells into the M-phase of their cycles, are rapidly degraded in an ATP- and Mg^{2+}-dependent manner which is inhibited by a high concentration of TLCK. Although it would be attractive to suggest that the inhibition of cyclin destruction results from the TLCK-induced auto-ubiquitination of E1, the requirement of cyclin destruction occurring specifically at metaphase (Luca & Ruderman, 1989) as opposed to the likelihood of TLCK-induced auto-ubiquitination of E1 causing a late G_2 block, argues against this possibility. Nevertheless, cyclin destruction in metaphase may still occur by the ubiquitin-dependent pathway, a possibility supported by the ATP- and Mg^{2+}-dependence, the sensitivity of cyclin

degradation to thiol reagents as well as the observation that TLCK is a partial inhibitor of the ubiquitin conjugate-degrading enzyme (Hough et al, 1987).

It would thus be of great interest to further investigate the effects of TLCK in cultured cells, order to determine firstly, whether TLCK inhibits cell cycles under well-defined cell culture conditions, secondly, at what stage of the cell cycle the arrest occurs, and thirdly, whether auto-ubiquitination of E1 is responsible. Should TLCK treatment produce states in cells of E1 depletion analogous to ts85 cells at the non-permissive temperature, then TLCK induction of auto-ubiquitinated E1 may turn out to be a powerful tool for the study of ubiquitin-dependent modification mechanisms. Such information would give further insights into the roles of this remarkable protein within the cell.

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APPENDIX

Chemicals and enzymes

These were obtained from the following suppliers:

BDH Chemicals Ltd. (Poole, England)	Hemin
Bio-Rad Laboratories (Richmond, CA, USA)	Bisacrylamide (NN'-methylene-bisacrylamide), TEMED (NNN'H-tetramethylene diamine), Silver Stain Kit, Bio-gel P-10.
Boehringer Mannheim (Mannheim, West Germany)	Bovine serum albumin, dithio-threitol, E 64 (N-[N-(L-3-trans-carboxyoxir-ane-2-carbo-xyl)-L-leucyl]-agmatine)
Kodak (Rochester, NY, USA)	Kodak XAR 5 X-ray film
Peptide Institute, Inc. (Osaka, Japan)	Leupeptin
Pharmacia Fine Chemicals (Uppsala, Sweden)	Sephacryl S-300, Sepharose CL-6B, Sephadex G-100, CNBr-activated Sepharose 4B, low molecular weight marker standards
Sigma Chemical Co (St. Louis, MO, USA)	ATP (adenosine 5'-triphosphate), 2,3, BPG (2,3 bi-phosphogly cerate), CTP (cytidine 5' tri-phosphate), creatine phospho-kinase, chymostatin, glycer-aldehde 3-phosphate, GTP (guan osine 5'-triphosphate, lyso-zyme, myoglobin, N-ethylmalei mide, PMSF (phenylmethylsulph-onyl flouride), pepstatin, phosphorylase kinase, phospho-creatine, ribonuclease, TLCK (N-α-p-tosyl-l-lysine chloro-methyl ketone), TPCK (N-tosyl

	-L-phenylalanine chloromethyl ketone, ubiquitin
Whatman (Kent, England)	DEAE-cellulose, CM-cellulose Glass fibre filters
E. Merck A.G. (Darmstadt, West Germany)	All other reagents (Analytical Grade)

Radioactive chemicals

Amersham (Bucks. England)	[^{14}C]-formaldehyde [^{125}I]sodium iodide
New England Nuclear (Boston, Mass., USA)	[^{32}P]orthophosphate

Instrumentation

Beckman Instruments, Inc. (Fulleston, CA, USA)	TJ 6 centrifuge, L8-70 ultra-centrifuge, Beta scintillation counter, Ready-Solv EP Scint-illation fluid, DU-50 Spectro-photometer
Hoefer Scientific Inst. (San Francisco, CA, USA)	Duel Temperature Slab Gel Dryer Model 1150
Packard Instruments, Inc. (Downers Grove, IL, USA)	Crystal II multidetector RIA system
Perkin Elmer (Norwalk, CT, USA)	LS-5 Luminescence Spectrometer
Pharmacia Fine Chemicals (Uppsala, Sweden)	Electrophoresis power supply 500/400