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Biochemical studies on the bovine pituitary multicatalytic proteinase complex (MPC): Evidence for a new distinct catalytic component and mechanism of dialysis activation

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Biochemical studies on the bovine pituitary multicatalytic proteinase complex (MPC): evidence for a new distinct catalytic component and mechanism of dialysis activation

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ABSTRACT

Biochemical studies on the bovine pituitary multicatalytic proteinase complex: evidence for a new distinct catalytic component and mechanism of dialysis activation

by

Bo Yu

Adviser: Professor Sherwin Wilk

The multicatalytic proteinase complex (MPC) is a 700 KDa proteinase containing multiple polypeptide subunits (Mr = 24,000 - 31,000) arranged as four rings in a cylinder-shaped particle. MPC contains at least three distinct catalytic activities, named as chymotrypsinlike, trypsinlike and peptidylglutamyl peptide bond (PGP) hydrolysing. In addition, MPC hydrolyzes selected proteins such as α and β crystallin, oxidized glutamine synthetase, oxidized hemoglobin, and casein. This latter activity is referred to in this thesis as caseinolytic activity. A characteristic feature of the caseinolytic activity is its latency which is thought to be an important mechanism for protection of the cell from uncontrolled proteolysis. Activation has been achieved by treatment with SDS, polycations, dialysis against distilled water and by heating.

The relationship of the activities of MPC toward synthetic substrates to the caseinolytic activity has not been clarified. The mechanism for the dialysis activation remains unclear. The studies described in this thesis provide strong evidence that the caseinolytic activity is distinct and elucidate the mechanism of the

dialysis activation.

After treatment with N-acetylimidazole (a mild acetylating reagent), the trypsinlike and PGP activities of MPC are markedly inhibited, the chymotrypsinlike activity is almost unaffected, but the caseinolytic activity is increased dramatically. This suggests that the active site responsible for degradation of β-casein is distinct from the other three. Furthermore, hydrolysis of β-casein by the acetylated enzyme generated a stable intermediate which could be further degraded by native MPC. Thus, the degradation of casein by MPC seems to be a sequential process with initial cleavage catalyzed by a distinct caseinolytic component. Removal of EDTA from MPC by dialysis against Tris-HCl buffers produced a time dependent activation of oxidized insulin B chain hydrolysis with predominant cleavage at the Glu^{13}-Ala^{14} bond. After dialysis, MPC loses its chymotrypsinlike, trypsinlike, and PGP activities almost completely, and at least one subunit is autolyzed. The resulting molecule is partially dissociated indicating that autolysis destabilizes the complex. By altering the profile of catalytic activities of the multicatalytic proteinase complex, autolysis may serve as a mechanism for regulation of this macromolecule. Other unpublished studies support the independence of the caseinolytic component and point to the existence of yet another distinct activity which can initiate the degradation of the oxidized B chain of insulin.

FORMAT OF THESIS

This thesis is prepared according to the guidelines of the City University of New York which permit the direct incorporation of published research articles as chapters. The thesis has a general introduction, two papers as chapters and a general discussion. Each chapter contains a specific introduction, materials, methods and results sections as well as discussion and references. The references for the introduction and discussion follow the discussion. Copyright permission for each chapter has been obtained from the publisher.

DEDICATION

This thesis is dedicated to
my wife, Shoulan Yang and my father and mother.

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LIST of ABBREVIATIONS:

ADP, adenosine 5'-pyrophosphate;
ATP, adenosine 5'-phosphate;
Boc, t-Butoxycarbonyl;
Boc-VEAL-NA, Boc-Val-Glu-Ala-Leu-NA;
Cbz (Z), benzyloxycarbonyl;
DCI, 3,4-dichloroisocoumarin;
DMSO, dimethyl sulfoxide;
EDTA, ethylenediaminetetra-acetate;
FPLC, Fast Protein Liquid Chromatography;
HPLC, High Performance Liquid Chromatography;
MHC, major histocompatibility complex;
MPC, Multicatalytic Proteinase Complex ;
NAI, N-acetylimidazole;
NEM, N-ethylmaleimide;
pNA, p-nitroanilide;
2NA, 2-naphthylamide;
PGP, peptidylglutamyl peptide bond hydrolyzing;
SDS, sodium dodecyl sulfate;
TLC, Thin Layer Chromatography;
Tris, 2-amino-2-hydroxymethyl-propane-1, 3-diol (tris(hydroxy-methyl) methylamine);

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Chapter 1

Overview

The multicatalytic proteinase complex (MPC) is the only proteinase known with distinct multiple proteolytic activities contained within a single molecule. This 700 kDa molecule is a multisubunit complex arranged in a cylindrical structure. Although MPC is found in the nucleus the predominant cellular localization is the cytosol. MPC is an abundant enzyme (up to 1% of soluble protein in some cells) and has optimal activity at physiological pH. Its unique catalytic character combined with its localization in the cytosol suggest an important role in intracellular protein degradation. Prior to a discussion on MPC, a brief review of the major aspects of eukaryotic intracellular protein degradation is presented.

A. Intracellular Protein Degradation

In recent years, an accumulation of data has demonstrated that intracellular protein degradation plays a role virtually equivalent in significance to protein biosynthesis in the regulation of cellular function.

At present, several intracellular protein degradation systems are generally recognized (Hershko & Ciechanover 1982, 1992; Jones 1991; Goldberg 1976; Pontremoli & Melloni 1986). A major portion of intracellular protein degradation is localized within the lysosomal compartment (Glaumann, H., and Ballard, F. J. (ed.) 1987). Those proteins resident in the extracellular spaces and bound within

the phospholipid bilayer of the cell membrane are endocytosed into the lysosome and digested by acid pH optima proteinases known as cathepsins. The primary physiological functions of this pathway are hydrolysis of membrane proteins during normal protein turnover and energy production by the breakdown of cytosolic proteins into amino acids during poor nutritional conditions (Dice, 1987, 1990). The lysosomal system also participates in antigen processing and presentation carried out by the Major Histocompatibility Complex (MHC) Class II expressing cells, such as macrophages. In this way, the extracellular antigens are endocytosed into the lysosome and hydrolyzed into peptides which then form a complex with a MHC II molecule (Allen 1987; Puri & Factorovich 1988).

Intracellular proteins are also degraded via the ubiquitin system (figure 1). Many cytosolic and nuclear proteins, either abnormal, short lived or long lived, are believed to be degraded by this system (Hershko & Ciechanover 1992 ; Rechsteiner 1988). Generally, the proteins targeted for degradation are initially modified by ubiquitin, and then the ubiquitinated proteins are hydrolyzed to peptides by a 26S proteinase complex.

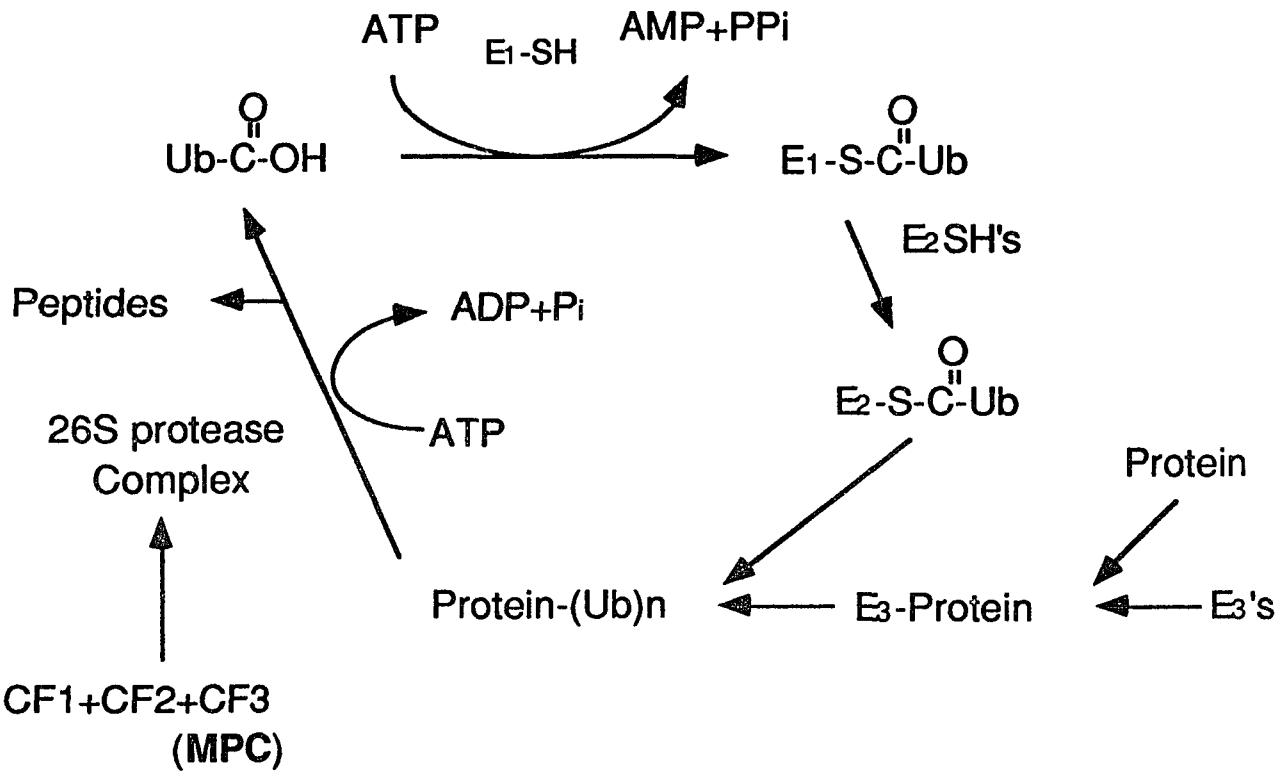


Figure 1 Current view of the major events in the ubiquitin pathway. Abbreviations: Ub, ubiquitin; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzymes; E3, ubiquitin-protein ligases; CF1, CF2 and CF3, conjugate-degrading factors; MPC, multicatalytic proteinase complex. Mordified from Hershiko (1992)

Ubiquitin, a 76 amino acid heat shock protein, is first activated by a specific ubiquitin-activating enzyme, E_1. The activated ubiquitin is then transferred to another enzyme, E_2 which can now react with a target protein-enzyme, E_3 complex which has formed separately. This reaction forms an ubiquitin-protein conjugate through an isopeptide bond between the C-terminal glycine of ubiquitin and ε-amino group of a lysine residue on the

target protein. The conjugates are usually forms of polyubiquitinated protein which are then proposed to be degraded by a 26S proteinase complex in an ATP-dependent manner. The factor CF3, has been shown to be identical to MPC (see section B.6.) and is therefore thought of as the catalytic core within the 26S proteinase complex. The formation of 26S from CF1, CF2 and CF3 (MPC) requires ATP (Eytan, et al 1989). After the degradation of the conjugate, the released ubiquitin can be used repeatedly.

Recently, abundant evidence suggests the existence of an additional protein degradation pathway parallel to the ubiquitin pathway (Orlowski 1990; Jones 1991). In such a pathway, many abnormal, damaged or endogenous cytosolic proteins are thought to be degraded directly by MPC. Many aspects of this pathway are poorly understood; the endogenous substrates and substrate recognition signals are not known; and proteinase regulation by endogenous inhibitors and activators are not well defined. Clearly, MPC must be tightly regulated in the cell, so as to avoid the breakdown of normal proteins which are thought to be exposed to MPC in the cytosol. In addition, increasing attention has been focused on the possible role of MPC in antigen processing (see section B.6.).

In addition to the intracellular protein degradation pathways mentioned above, there are some other pathways including the calpain system (Pontremoli and Melloni 1986; Croall and DeMatino 1991) and the many endoplasmic reticulum processing enzymes (Dalbey and von Heijne, 1992; Seidah and Chretien 1992; Muller,

1992). Calpains are calcium dependent neutral proteinases which are believed to be involved in cytoskeletal protein degradation, proteolytic modification, activation of protein kinase C, and limited proteolysis of some soluble proteins. The breakdown of calpain substrates is in response to intracellular or extracellular stimuli signaled by changes in calcium concentration. The signal peptidase and processing enzymes are among a group of proteinases required to remove targeting peptides from proprotein substrates and to generate a functional hormone from precursor molecules. These proteinases have a very clear and significant function in intracellular processing and transport.

B. Multicatalytic Proteinase Complex

B.1. Discovery and Nomenclature

The multicatalytic proteinase complex first discovered in bovine pituitary by Wilk and Orlowski (1979, 1980, 1981,1983) has been described under many different names including: macropain, high molecular weight proteinase, neutral protease, ingensin, 20S protease and proteasome (Dahlmann, et al. 1988, Orlowski and Wilk, 1988). The common identity among these proteinases was quickly recognized. In addition, a 19S - 20S particle called prosome was shown to be identical to MPC on basis of size, proteolytic activity, subunit pattern on SDS-PAGE, immunological cross-reactivity and the appearance in the electron microscope (Arrigo et al. 1988,

Falkenburg et al, 1988). The multicatalytic proteinase (complex) and proteasome are the most widely accepted names to date for this novel proteinase. Recently, MPC has been officially named as Multicatalytic endopeptidase complex (E.C. 3.4.99.46) by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB, 1992). Since the studies described have been conducted and reported prior to the introduction of the newest nomenclature, this thesis uses multicatalytic proteinase complex as recommended by Orlowski and Wilk (1988).

B.2. The existence of MPC in tissues or cells

MPC has been found in various organisms throughout the plant and animal kingdom: yeast (Achstetter et al. 1984), plants (Schliephacke et al. 1991), lobster (Mykles 1989), fish muscle (Folco et al. 1988), mouse liver (Rivett 1985), rat liver (Tanaka et al. 1986), rat skeletal muscle (Dahlmann et al. 1985a), bovine lens (Wagner et al. 1982; Ray and Harris 1985), bovine pituitary (Wilk et al. 1979), porcine skeletal muscle (Ishiura et al. 1985), human erythrocytes (McGuire and DeMartino, 1986), human lung (Zolfaghari et al. 1987a), human placenta (Nojima et al. 1986) and kidney (Zolfaghari et al, 1987b). A similar enzyme complex, not identical to the eukaryotic MPC, has been isolated from the archaebacterium *Thermoplasma acidophilum* (Dahlmann et al. 1989). Evidence points to the archaebacteria enzyme as an ancestor of the eukaryotic MPC (see latter). The ubiquitous nature of MPC from archaebacteria to

human indicates a role in some unknown but fundamental biological process in the cell.

It has also been reported that MPC constitutes about 1 % of the total cellular proteins (Tanaka et al. 1988a) with a half life of 5-10 days (Hendil 1988: Tanaka and Ichihara 1989). Recently, a model of two MPC pools has been proposed according to the results of protein measurement by immunochemical methods and mRNA levels from northern blots. The two pools differ in turnover rate with the larger pool possessing a slow rate and the reverse for the smaller pool (Shimbara et al. 1992).

A distribution study (Tanaka et al. 1986) in rat tissues found MPC to be widely distributed with the highest level in liver (360μg/g tissue), kidney, spleen, lung and small intestine. Much lower levels were found in extracts of heart, brain and muscle. Similar results were found using bovine tissue (Ray and Harris 1987). The physiological meaning of this pattern of distribution is not yet clear.

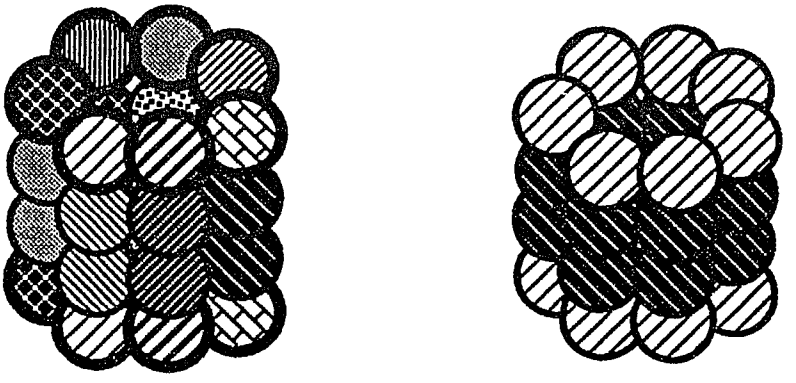
Immunohistochemical localization studies showed that MPC was present in both the nucleus and cytoplasm (Tanaka et al. 1986). Another localization study measured immunoreactive MPC in subcellular fractions (Tanaka et al. 1986) and showed that 83% is cytoplasmic with the remaining MPC associated with the microsomes. Therefore MPC is mainly a cytoplasmic proteinase. MPC has also been isolated from both cytoplasm and the nuclei (Strack, et al 1992).

B. 3. Structure

The unique structure of MPC is one of its characteristic features. First, this enzyme is a high molecular weight (700 kDa), multisubunit complex (Wilk and Orlowski 1983) with a sedimentation coefficient of 19S - 20S (Arrigo et al. 1988). 8-10 polypeptide bands ranging from 24 kDa to 32 kDa on one dimensional SDS-PAGE are easily observed (Orlowski and Michaud 1989). More than 13 kinds of polypeptides can be seen when MPC is separated by reverse-phase HPLC or by two dimensional electrophoresis (Tanaka et al. 1988b). However, the exact number of subunits within the complex is not yet clear.

Electron microscopy reveals a stacked arrangement of the subunits creating a hollow cylindrical structure of four layers with a diameter of 11-16 nm, a length of 16 nm and a tunnel of 1-4 nm diameter (Kopp et al. 1986). Most recent studies indicate that each layer consists of seven subunits, resulting in a complex of twenty-eight total subunits (Baumeister et al. 1988; Dahlmann et al. 1989; Puhler et al. 1992). The model in figure 2 represents the current view of the quaternary structure of MPC. It is very interesting that a homologous complex isolated from archaebacteria also has a similar cylindrical structure with four stacked rings, even though the archaebacterial enzyme contains only two different subunits (designated as α and β) (Danlmann et al. 1989, 1992), and expresses only chymotrypsin-like and caseinolytic activities (see section B4). Figure 2A suggests a complex dimeric arrangement (Kopp et al 1992)

for MPC. To date, all attempts to dissociate the complex in order to isolate catalytically active subunits have been unsuccessful. This indicates that the integrity of the complex is essential for expression of its proteolytic activities (Wilk and Orlowski, 1983; Rivett, 1989; Nothwang et al. 1992). An interesting question concerns the physiological purpose of such a complex structure. A similar structure has also been proposed for an unrelated molecule, GroEL (Zwickl et al 1990).



A B

Figure 2: The current models of the multicatalytic proteinase complex (A) and the archeabacterial multicatalytic proteinase (B) based on the electron microscopic data of Kopp et al. (1986, 1992), Baumeister et al. (1988), Dahlmann et al. (1989) and Puhler et al. (1992).

The complementary DNA sequences of 10 subunits of human MPC have now been cloned. Surprisingly, these gene products are homologous to one another but not to any other known proteinase or other gene families. Furthermore, the amino acid sequences from various sources are found to be highly conserved from yeast to man (Tanaka et al. 1992). A complete sequence has been determined for the α and β subunits of the archaebacterial enzyme. An interesting finding is that all cloned eukaryotic MPC subunits can be grouped into either α-type or β-type according to the sequence relationship between the archaebacterial enzyme and eukaryotic enzyme. This raises the possibility that the archaebacterial enzyme is an evolutionary ancestor of eukaryotic MPC (Zwickl et al. 1991, 1992).

It can be envisioned that throughout evolution two genes developed into probably fifteen genes, which then acquired control of a multitude of activities (see section B4). However, the quaternary structure and the number of subunits of the gene products remained conserved from archaebacteria. This fact indicates that the cylindrical structure is very important for proteolytic activity.

B. 4. Multiple Activities

The most unique property of MPC is its multiple proteolytic activities as indicated by its name. In the cornerstone paper, Wilk and Orlowski (1983), presented evidence that the pituitary enzyme was a multicatalytic proteinase complex which could cleave bonds

on the carboxyl side of hydrophobic, basic and acidic amino acid residues. The synthetic compounds, cbz-gly-gly-leu-p-nitroanilide, cbz-D-ala-leu-arg-2-naphthylamide and cbz-leu-leu-glu-2-naphthylamide were used as model substrates. According to the P1 position of the substrates, cleavage at -leu-pNA, -arg-2NA and -glu-2NA, were described as chymotrypsin-like (hydrophobic), trypsin-like(basic) and peptidylglutamyl-peptide (PGP) hydrolyzing (acidic) activity respectively. In addition, the multiple activities could be differentiated by different inhibitors and activators. The trypsin-like activity could be completely inhibited by leupeptin while other activities remained unaffected. On the other hand, the peptidylglutamyl-peptide hydrolyzing activity could be dramatically activated by 0.04 % sodium dodecyl sulfate whereas the chymotrypsin-like and trypsin-like activities are suppressed. With the inhibitor Cbz-Gly-Gly-leucinal, the chymotrypsin-like activity is selectively blocked.

Since the initial report, the concept of multicatalytic activities has been widely accepted (Dahlmann et al. 1985a, Ray and Harris et al. 1985, Wagner et al. 1985, Rivett 1985, Tanaka et al. 1986, McGuire et al. 1986). In these studies, many synthetic substrates (summarized in table 1) were used for the assay of enzyme activity and the study of substrate specificity.

Table 1 Activity toward synthetic peptide substrates by MPC and Archbacteria proteasome

Substrate	Enz Act		Substrate	Enz Act	
	Euk	Arc		Euk	Arc
P4 P3 P2 P1 P1'			P4 P3 P2 P1 P1'		
Z-Gly-Gly-Leu-pNA	+		Z-Gly-Gly-Arg-MCA	+	-
Hip-Phe-Ala-Ala-Phe-pAB	+		Z-Ala-Arg-Arg-MCA	+	-
Boc-Phe-Ala-Ala-Phe-pAB	+		Z-Arg-Arg-MCA	+	-
Bz-Gly-Ala-Ala-Phe-pAB	+		Boc-Leu-Gly-Arg-MCA	+	
Glt-Ala-Phe-Phe-pAB	+		Boc-Val-Leu-Lys-MCA	+	
Glt-Gly-Gly-Phe-MCA	+	+	Bz-Phe-Leu-Arg-pNA	+	
Suc-Ala-Ala-Phe-MCA	+	+	D-Ala-Leu-Arg-pNA	+	
Ala-Ala-Phe-MCA	+	+	Z-Arg-Arg-MNA	+	
Suc-Leu-Leu-Val-Tyr-MCA	+	+	Z-Gly-Gly-Arg-MNA	+	
Glt-Ala-Ala-Phe-MNA	+		Z-Arg-Arg-Arg-MNA	+	
Suc-Ala-Ala-pNA	+		Z-Ala-Arg-Arg-MNA	+	
Suc-Ala-Ala-Ala-pNA	+		Z-Val-Leu-Arg-MNA	+	
Glt-Gly-Gly-Phe-MNA	+		D-Val-Leu-Lys-pNA	+	
Glt-Ala-Ala-Phe-MNA	+		Z-Ala-Arg-Lys-MNA	+	
Glt-Phe-Leu-Phe-MNA	+		Arg-MCA	-	-
Suc-Ala-Ala-Ala-MNA	-		Bz-Arg-pNA	-	
Suc-Ala-pNA	-		Bz-Arg-MCA	-	-
Bz-Tyr-pNA	-		Z-Arg-MNA	-	
Leu-MNA	-		Z-Arg-Arg-AFC	-	
			Glt-Gly-Arg-MCA	-	-
			Z-Phe-Arg-MCA	-	-
Bz-Phe-Val-Arg-MCA	+		Arg-Arg-MNA	-	
Tos-Pro-Arg-MCA	+	-	D-Val-Leu-Lys-MNA	-	
Bz-Leu-Met-Arg-pNA	+				
Z-D-Ala-Leu-Arg-2NA	+		Z-Leu-Leu-Glu-2NA	+	-
Bz-Val-Gly-Arg-MCA	+	-	Suc-Ala-Glu-MCA	+	-

Three letter code for amino acid is used.

Enz Act, enzyme activity; Euk, eukaryotic MPC;

Arc, archaebacteria MPC; pAB, p-aminobenzoate;

AMC, 7-amido-4-methylcoumarin; Boc, t-butyloxycarbonyl;

Bz, benzoyl; Glt, glutaryl;

MNA, 4-methoxy-2-naphthylamide; p-NA, p-nitoanilide;

2NA, 2-naphthylamide; Tos, tosyl;

Suc, succinyl; Z, carbobenzoxy.

"+" indicates the compound can be hydrolyzed by MPC,

"-" indicates the compound cannot be hydrolyzed by the enzyme. The substrate subsites are denoted by a method introduced by Schecter and Berger (1967).

Table 1 shows not only the multicatalytic activities of MPC, but also some other points revealing substrate specificity. First, for the trypsin-like activity of MPC, a basic amino acid at the P1 position is not by itself sufficient for hydrolysis, unlike that of trypsin itself, and the P3 substituent can have a dramatic effect. Thus Arg-Arg-MNA and Glt-Gly-Arg-MCA cannot be hydrolyzed, but Z-Ala-Arg-Arg-MNA and Z-Gly-Gly-Arg-MCA can be. Arginine appears to be better than lysine at the P1 position. The chymotrypsin-like component does not have a very strict requirement for the P1 amino acid residue. However, a hydrophobic amino acid at the P1 position is not by itself sufficient. It should also be pointed out that several compounds such as, Suc-Leu-Leu-Val-Tyr-MCA and Bz-Gly-Ala-Ala-Phe-pAB are not only substrates for the chymotrypsinlike (hydrophobic) activity, but also appear to be good substrates for the PGP (acidic) catalytic component according to their SDS-

stimulatory behavior and identical pH optimum for the SDS-stimulated hydrolysis of chromogenic substrates (Wilk et al. 1991, Orlowski and Michaud 1989). Thus, the hydrolyzing activity towards these two substrates (Suc-Leu-Leu-Val-Tyr-MCA and Bz-Gly-Ala-Ala-Phe-pAB) can be dramatically increased in the presence of 0.04% SDS and the pH optimum for hydrolyzing Bz-Gly-Ala-Ala-Phe-pAB in the presence of SDS is the same as for Z-Leu-Leu-Glu-2NA. This phenomenon was interpreted as either due to cleavage only by the PGP component (which apparently can also cleave after hydrophobic amino acid provided that hydrophobic aminos acid are present in position P2-P4) or by cleavage by both PGP and chymotrypsinlike components (Wilk et al. 1991, Orlowski and Michaud 1989). The latter explanation is supported by evidence from studies on the archaebacterial multicatalytic protease. The archaebacterial enzyme apparently lacks a PGP activity yet it can cleave Suc-Leu-Leu-Val-Tyr-MCA. It cannot hydrolyze Z-Leu-Leu-Glu-2NA or Suc-Ala-Glu-MCA (Dahlmann et al., 1992). A third possibility is that the hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA is due to a distinct catalytic component (Djaballah et al. 1992). Orlowski and coworkers recently demonstrated that MPC has at least two distinct PGP components (1991). Low affinity and high affinity activity sites were observed for the PGP substrate, Z-Leu-Leu-Glu-2NA (Djaballah and Rivett 1992).

Table 2 Cleavage of Peptide substrates by MPC

Peptide	Structure and sites of cleavage
Bradykinin:	R-P-P-G-F↓-S-P-F-R
Angiotensin II:	D-R-V-Y↓-I↓-H-P-F
LH-RH:	pE-H-W-S↓-Y↓-G-L-A-P-G-NH2
Neurotensin:	pE-L-Y-E↓-N↓-K-P-R-R-P-Y-I↓-L
Substance P:	R-P-K-P-Q-Q-F-F↓-G-L-M-NH2
Oxidized insulin B chain:	F-V-N-Q↓-H-L-C-G-S-H-L↓-V-E↓-A-L↓-Y-L↓-V-C↓-G-E-R-G-F-F-Y-T-P-K-A

Abbreviations: LH-RH, luteinizing hormone - releasing hormone. One letter code for amino acid is used. Data compiled from Wilk and Orlowski 1980, Rivett 1985, Dick et al. 1991.

In addition to the chromogenic substrates in Table 1, many peptides have been used to study substrate specificity. The use of peptides as substrate not only revealed that MPC had a very broad specificity, but also showed other complexities (Table2). The results from studies with natural peptide substrates demonstrated again that the nature of the P1 amino acid residue is by itself not sufficient to define a catalytic component (hydrophobic, basic, and acidic). For example, the enzyme does not cleave after all hydrophobic amino acids. Moreover, when studying the hydrolysis of oxidized insulin B chain, the trypsin-like component was found to have the ability to cleave a peptide bond at the carboxyl side of glutamine (Dick et al. 1991). This latter finding does not fit the

definition of trypsin-like activity i.e. cleavage of a peptide bond on the carboxyl side of a basic amino acid residue. Similar inconsistencies in definition occurred for PGP activity, i.e. the PGP component in addition to cleaving after an acidic amino acid, can cleave some substrates after a hydrophobic amino acid. These inconsistencies indicate that a precise definition of the substrate specificity of each component remains to be determined.

Another important aspect about substrate specificity is that MPC can hydrolyze some proteins, such as: α, β crystallin (Wagner et al. 1982), methyl globin, bovine serum albumin, ^{125}I-hemoglobin, oxidatively damaged hemoglobin, and glutamine synthetase (Rivett 1985), lyzozyme, β-casein, dephosphorylated-β-casein (Orlowski et al. 1989). It is interesting that the activity responsible for hydrolysis of proteins is latent. MPC has been isolated in two forms designed latent and activated in terms of its activity toward radiolabeled casein, although the biochemical basis of latency remained unclear (Tanaka et al 1986; McGuire et al. 1989a; Weitman and Etlinger, 1992). Protein degradation could be increased by polylysine, SDS, and N-ethylmaleimide (Dahlmann et al. 1985b; Tanaka et al. 1986). The property of latency is consistent with the view that this intracellular proteinase can be very dangerous if it is not under tight control.

Because there are so many potential cleavage sites in one polypeptide chain, it is difficult to decide which components are responsible for protein degradation. One possibility is that the

chymotrypsinlike, trypsinlike, PGP activities all participate equally. Another possiblity is that one activity is more critical than others. A third possibility is that proteolysis is catalyzed by a distinct fourth component. My studies on this question showed that the initial cleavage of proteins was carried out by a distinct component. (See, chapter 2).

B. 5. Inhibitors and activators

Many studies characterizing the effect of different compounds on the activities of MPC have been conducted. Some of them are summarized in table 3:

Table 3 Effect of various compounds and treatments on the catalytic activities of MPC

compound and treatment	Final Conc. (mM)	Activity (%) Chym.	Try.	PGP	Caseinolytic
None		100	100	100	100
DTT	1	105	100	115	107
	1				126
Iodoacetamide	5	108	112	120	118
	1	115	89		
DTNB	0.1	116	91		
	1	40	45		
	5	5	2		18
p-Hydroxymercuribenzene	1	0	0	0	0
sulphonic acid					
p-Hydroxymercuribenzoate	0.1				<1
	0.1	1	1		
p-chloromercuribenzoate	0.01	9			
Mersalyl acid	1	0	0	71	0
E-64	1	100	100	100	100
Ep-475	1	75	70		
	50ug/ml	100	100		100
Leupeptin	0.16	93	9	100	
	0.125	67	11	96	102
	0.005	100	30	100	
	50ug/ml	95	0		100
	1		2		63
	0.1		8		79
	0.01mg/ml	100	6		
Antipain	1		3		62
	0.01mg/ml	100	15		
PMSF	10	45	88		53
	1		100		92
Phe-Pro-Arg-CH2Cl	0.046	100	100		
Phe-Phe-Arg-CH2Cl	0.046	100	100		
Dansyl-Glu-Gly-Arg-CH2Cl	0.046	100	100		
N-ethylmaleimide	1	60	4	66	
	5	2	0		25
	5	107	4		
	1	109	36		

	0.1	107	81		
	5	100	4		
	0.5				252
Z-Gly-Gly-Leucinal	2	12	170	88	
Z-L-L-F-CHO		Ki=0.46 µm			
Ac-L-L-nL-CHO		Ki=5.7 µm			
Ac-L-L-M-CHO		Ki=33 µm			
DFP	1	80	117		
	10	5	75		20
	1		100		88
	0.11	100	100	100	
TLCK	1		100		96
TPCK	1		100		95
Chymostatin	0.125	49	19	128	322
	50ug/ml	20	71		100
	1		55		67
Pepstatin	50ug/ml	100	100		100
	0.2	100			
EDTA	1	100	100		100
o-Phenanthroline	1		100		100
Hemin	0.1		1		6
3,4 dichloroisocoumarin	0.004	15	260	54	
Elastatinal	50ug/ml	86	100		100
SDS	1.38	63	3	1490	
	0.69	399	150		
	1.725				352
Palmitic acid	4.32	133	1	369	
Linoleic acid	2.85	29	33	524	
	0.1	973	655		
Spermidine	0.2	94	99	293	
Benzamidine	1		100		99
Pentamidine	1		94		51
Propamidine	1		94		73
Stearic acid	0.1	127	156		
Oleic acid	0.1	1054	655		
Linolenic acid	0.1	118	211		
Arachidic acid	0.1	136	143		
Behenic acid	0.1	355	485		
polylysine	1mg/ml	54	83	21	
	1mg/ml				537
Magnesium ions	1	100	84	203	250
	10	91	50	220	440
Dialysis			184	95	2620
Heat	50°C				189

Chym, chymotrypsinlike activity; Try, trypsinlike activity; PGP, peptidylglutamylpeptide bond hydrolyzing activity; DTT, dithiothreitol; DTNB, 5,5'-dithiobis-(2-nitrobenzoate); E-64, L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane; E-475, L-trans-epoxysuccinyl-leucylamido(3-methyl)butane; PMSF, phenylmethanesulphonyl fluoride; DFP, diisopropyl fluorophosphate; Z-L-L-F-CHO, (benzyloxycarbonyl)-Leu-Leu-phenylalaninal; Ac-L-LnL-CHO, N-acetyl-Leu-Leu-norleucinal; Ac-L-L-M-CHO, N-acetyl-Leu-Leu-methioninal; TLCK, tosyl-lysine chloromethyl ketone; TPCK, tosylamido-2-phenylethyl chloromethyl ketone; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulphate.

Data from Wilk and Orlowski, 1980, 1983; Dahlmann et al 1985a, 1985b; Tanaka et al. 1986; Orlowski and Michaud, 1989; Rivett 1985, 1989; McGuire et al. 1986, 1988; Pereira, et al. 1992; Vinitsky et al. 1992.

As pointed out in section B.4, the effect of inhibitors and activators on MPC demonstrated that there were multiple catalytic sites on this molecular complex. In addition, studies on the effect of various inhibitors on the activities of MPC demonstrated another property of MPC - cooperative interactions between the catalytic components of the enzyme complex. Thus whereas cbz-Gly-Gly-Leucinal inhibits the chymotrypsinlike activity, it also activates the trypsinlike activity (Wilk and Orlowski, 1983). Low concentrations of 3,4-dichloroisocoumarin have a similar effect by inactivating the

chymotrypsinlike activity and activating the trypsinlike activity (Orlowski and Michaud, 1989).

Another purpose of studying the effect of inhibitors on MPC is to classify its proteolytic activities. Although no clear conclusion about classification can be made based on inhibitor studies, it has been generally assumed that the chymotrypsinlike, trypsinlike, and peptidylglutamylpeptide bond hydrolyzing activities belong to the class of serine proteinases. This conclusion is based on the 3,4-dichloroisocoumarin data (Orlowski, 1990). Recently, the archaebacterial MPC has been shown to behave like a serine proteinase by its sensitivity to DFP and DCI (Dahlmann et al. 1992). However there is no structural homology to any known serine proteinase. MPC may represent a new type of serine proteinase. Alternatively components of the active site may be contributed by more than one subunit. Obviously, the final classification requires additional data from other kinds of studies, as for example X-ray crystallography of an enzyme-substrate complex. Alternatively, site-directed mutagenesis may be of value.

As shown in table 3, most of the inhibitors used in the early studies are general proteinase inhibitors, Cbz-gly-gly-Leucinal was the first inhibitor specifically synthesized for MPC a decade ago (Wilk and Orlowski 1983). Recently, progress has been made in synthesizing potent specific inhibitors, such as Cbz-Leu-Leu-phenylalaninal (Vinitski et al. 1992). It is obvious that these kinds

of studies are very important, and will contribute much in clarifying the physiological functions of MPC.

In addition to the synthetic inhibitors, endogenous protein inhibitors have been reported (Li, et al. 1991, Ma, et al. 1992a). The endogenous inhibitors have been suggested as one component of the 26S proteinase complex (Driscoll et al. 1992). A 240-kDa endogenous inhibitor can be ubiquitinated. (Li and Etilinger 1992).

Table 3 also shows activators of PGP and caseinolytic activities (proteinase activity). Some of them may be physiological, such as fatty acids and magnesium ions. In addition, some treatments (dialysis and heat) were also reported to activate the caseinolytic activity of MPC. Recently, some exciting discoveries on endogenous protein activators have been reported. (Ma et al, 1992b; Dubiel et al 1992; Yukawa et al, 1991; Di Cola 1992). The endogenous activator, so called PA28 or 11S, can dramatically increase the activities of MPC toward synthetic substrates. Preliminary data showed that this activator is a novel protein. (Ma et al, 1992b; Dubiel et al 1992). A general question concerns the mechanisms of activation. I conducted studies on the mechanism of the dialysis effect (see chapter 3).

B. 6. The possible physiological function:

Multicatalytic proteinase complex is generally believed to play an important role in extralysosomal protein degradation. Although many investigators were attracted to study its possible

physiological function, its significance for cellular function is not clear yet. The following are some very attractive hypotheses.

1) The catalytic core of the 26 S proteinase complex

As briefly mentioned in page 5, Rechsteiner and coworkers discovered a 26S proteinase complex which could hydrolyze ubiquitinated proteins. The biochemical properties of this giant particle are still unclear (Hough et al. 1987). Investigators have attempted to demonstrate that the catalytic core of the 26S proteinase is MPC (Eytan et al 1989, Driscoll and Goldberg, 1990). Supporting the idea that MPC is the catalytic core of the 26S, the following evidence has been offered: 1) the 26S could hydrolyze the typical synthetic substrates used by MPC; 2) 26S showed the presence of the 20S subunit pattern on SDS-PAGE (multiple bands of 24-35 kDa); 3) the antibody raised against purified MPC could cross react with some bands (25-35 kD) of 26S complex; 4) when 20S was immunoprecipitated from a cellular extract, the extract could no longer hydrolyze ubiquitinated proteins (Tanaka and Ichihara 1988c, McGuire and DeMartino 1989b). Although overwhelming evidence supports the idea that MPC is the catalytic core of 26S, some reports still argue that 26S and MPC have no relationship at all (Kuehn et al. 1992; Seelig et al. 1991).

2) The possible proteolytic enzyme for endogenous antigen processing.

All vertebrates have a sophisticated immune system. In this system, there are two distinct pathways for antigen presentation

(Monaco, 1992). One is an endolysosomal pathway: foreign antigen is ingested by endocytosis, degraded in the endolysosomes and some fragment of this foreign antigen (bound on MHC [major histocompatibility complex] II molecule) are returned to the cell surface to stimulate the CD_4 T cell. The other pathway is cytosolic. The endogenous antigen (virus protein, tumor protein) is degraded in the cytosol, the fragment of antigen is then transported into the endoplasmic reticulum (ER) by putative transporters, some fragment (8-10mer) is presented to CD_8 T cell by MHC-I molecule. Unlike the MHC-II pathway, the proteolytic enzyme in the MHC-I pathway responsible for degradation of antigens in the cytosol has not been identified yet. Parham (1990) postulated that the proteolytic enzyme might be the MPC. Since then, much effort has been made to prove Parham's hypothesis. The discovery of Ring 10 and Ring 12 genes which locate in the class II region of the human MHC and resemble two subunits of MPC, made this hypothesis more attractive (Glynne et al. 1991; Kelly et al. 1991; Martinez and Monaco 1991). However recent studies cast some doubt on this hypothesis. Momburg et al. (1992) and Arnold et al. (1992) presented evidence that MPC subunits encoded by Ring 10 and Ring 12 were not essential for antigen presentation. So far, there is no direct evidence to prove the role of MPC in antigen processing, or the converse.

3) Other speculative functions

Besides the above, MPC has been proposed to participate in other cellular functions such as removal of damaged cellular proteins (Rivett 1985); modulation of translational activity (Kuehn et al. 1990); participation in tumorigenesis (Kumatori et al 1990) and embryogenesis (Klein et al. 1990) of animal cells; possible participation in the acrosome reaction (Matsumura and Aketa, 1991); and possible involvment in the pathology of certain neurodegenerating diseases (Kojima and Omori, 1992; Ishiura, 1991) All these postulates need much more data for confirmation. Furthermore, the physiological functions discussed above relate to the enzyme in the cytosol. The implication of the finding that the enzyme is also found in the nucleus and contains a consensus nuclear translocation motif (Tanaka et al. 1992) is unclear.

C. Remarks

The ultimate goal of MPC studies is to understand its physiological function. To this end, it is very important to obtain a very clear understanding of the biochemistry of MPC, including its structure, regulation and basic enzymological properties. As for basic enzymology, many questions remain to be answered. How many activities are present in this macromolecular complex? What is the relationship of the different catalytic components? What is the relationship of the catalytic activities to the quaternary structure of the complex? What is the molecular basis for latency? What are

the defining features of endogenous protein substrates. With a knowledge of its enzymology, inhibitors can be designed to aid in the exploration of its physiological function. This thesis attempts to address two of these questions.

Chapter 2

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Chemical Modification of The Bovine Pituitary Multicatalytic Proteinase Complex by N-acetylimidazole: Reversible Activation of Casein Hydrolysis

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SUMMARY

The effect of N-acetylimidazole, a mild acetylating reagent, on the catalytic activities and subunit structure of the bovine pituitary multicatalytic proteinase complex (MPC) was studied. The trypsinlike activity (cleavage of Cbz-D-Ala-Leu-Arg-2-naphthylamide) and the peptidylglutamyl-peptide bond hydrolyzing (PGP) activity (cleavage of Cbz-Leu-Leu-Glu-2-naphthylamide) of MPC were rapidly inactivated by N-acetylimidazole, whereas the chymotrypsinlike activity (cleavage of Cbz-Gly-Gly-Leu-p-nitroanilide) was inactivated slowly. However, the hydrolysis of β-dephosphoralated casein was markedly stimulated. Hydrolysis of β-dephosphoralated casein by the acetylated enzyme generated a stable intermediate (21kDa) which could be further degraded by native MPC. Treatment of acetylated MPC with hydroxylamine reversed the changes in trypsinlike and caseinolytic activities but did not restore the PGP activity. N-acetylimidazole did not dissociate MPC but altered its migration on non-dissociating gels presumably by acetylation of ε-amino groups of lysine residues. Hydroxylamine did not alter the gel electrophoretic appearance of the acetylated enzyme. These results indicate that acetylation of thiol or tyrosyl groups changes the trypsinlike and caseinolytic activities, and that amino group acetylation inhibits the PGP activity. Degradation of casein by MPC appears to be a sequential process with initial cleavage catalyzed by a component distinct

from the chymotrypsinlike, trypsinlike and PGP activities. The latter three components likely participate in the secondary proteolysis of the generated intermediates.

INTRODUCTION

The multicatalytic proteinase complex $(MPC)_2$ first characterized from bovine pituitaries (1-3) is a 700 kDa proteinase containing multiple polypeptide subunits (Mr = 22,000 - 34,000) (reviewed in 4-6) arranged as four rings in a cylinder-shaped particle (7). MPC contains at least three distinct catalytic activities defined on the basis of hydrolysis of synthetic chromogenic substrates (3) and is present in all eukaryotic cells in relatively high concentrations (4-6). This macromolecule has recently received considerable attention because of the recognition that MPC is a major extra-lysosomal proteinase and therefore plays an essential role in intracellular protein turnover, and the demonstration of its identity to the "prosome", a 20S ribonucleoprotein particle also present in all eukaryotic cells (8,9).

MPC contains three defined catalytic activities. The chymotrypsinlike activity cleaves the substrate Cbz-Gly-Gly-Leu-pNA. The trypsinlike activity cleaves Cbz-D-Ala-Leu-Arg-2NA, and the peptidylglutamyl peptide bond (PGP) hydrolysing activity cleaves Cbz-Leu-Leu-Glu-2NA (3). In addition to its action on synthetic chromogenic substrates, MPC hydrolyzes selected proteins such as α and β crystallins (10,11), oxidized glutamine synthetase (12), oxidized hemoglobin (13), and casein (14-16). A characteristic feature of the action of MPC on protein substrates (referred to here as caseinolytic activity) is its latency (16,17). The latency is thought to be an important mechanism for protection of the cell

from uncontrolled proteolysis since MPC is mainly present in the cytosol. Preparations with latent proteolytic activity have been obtained by purifying the enzyme with glycerol-containing buffers (16,17). Activation has been achieved by treatment with SDS (16,17), polycations (16,18), dialysis against distilled water (17) and by heating (19).

The relationship of the three catalytic activities of MPC defined by synthetic chromogenic substrates to the caseinolytic activity has not been clarified. It is not known to what degree the individual components contribute to the degradation of casein, whether the activities act in concert or whether casein degradation is initiated by a single component. It has been suggested that the PGP component is responsible for casein hydrolysis since both PGP and caseinolytic activities can be stimulated by SDS and since the PGP activity is the most sensitive to inhibition by proteins (3). The relationship of the subunit structure of MPC to its catalytic activities also remains unclear. All attempts thus far to dissociate the complex have led to the loss of all activities (3).

Chemical modification of MPC represents an approach which has the potential of clarifying structure-function relationships in this macromolecule. N-acetylimidazole first introduced by Riordan et al.,(20) is a mild and selective protein acetylating reagent. It preferentially acetylates exposed tyrosine residues but can also react with thiol groups and ε-amino groups of lysine. N-Acetylimidazole reversibly dissociates ovine brain glutamine

synthetase, an enzyme composed of eight identical subunits, to tetramers (21). We report here the results of our studies with this reagent on MPC. These experiments further confirm the multicatalytic nature of this macromolecule and provide evidence for the existence of a fourth catalytic component responsible for the initial hydrolysis of dephosphorylated β-casein.

EXPERIMENTAL PROCEDURES

Materials:

Frozen bovine pituitaries were obtained from Pel-Freez Inc. (Rogers AK). Cbz-Gly-Gly-Leu-pNA, Cbz-D-Ala-Leu-Arg-2NA, and Cbz-Leu-Leu-Glu-2NA were synthesized as previously described (1,22). N-Acetylimidazole and dephosphorylated β-casein were purchased from Sigma Chemical Corporation (St. Louis, MO). Molecular weight markers were from Pharmacia LKB Biotechnology (Piscataway, NJ). DEAE-cellulose was obtained from Whatman Inc. (Clifton NJ). Ultrogel AcA 22 was from IBF Biotechnics Inc. (Savage MD). All other reagents were of the highest purity available. MPC was purified to apparent homogeneity from 100g of bovine pituitaries as previously described (23, 24).

Protein determination:

Protein concentrations were determined by the Lowry method (25) with bovine serum albumin as standard.

Polyacrylamide gel electrophoresis:

Electrophoresis under non-denaturing conditions was carried out on 4.5% polyacrylamide gels. SDS-PAGE was conducted on 12.5% gels as described (26). Gels were stained for protein with either Coomassie blue or Silver stain.

Treatment of MPC with N-acetylimidazole:

N-acetylimidazole was stored under vacuum in a desiccator. Solutions were freshly prepared immediately before use. 100 µg of MPC were treated with 3 mg of N-acetylimidazole in 1 ml 0.01M Tris EDTA buffer, pH 7.5 for different periods of time at room temperature. In most cases the reaction was terminated by addition of a molar excess of tyrosine, and the reaction mixture dialyzed for 5 h at 4°C against a 0.01M Tris EDTA buffer, pH 7.5.

Deacetylation by Hydroxylamine:

When the effect of deacetylation was studied, 22 mg of Tris base and 9 mg of hydroxylamine hydrochloride were added to 100 µg of the N-acetylimidazole modified enzyme in 1 ml 0.01M Tris EDTA, pH 7.5. The solution was kept at 4°C for 6 h, and then dialyzed against a 0.01M Tris EDTA buffer, pH 7.5 at 4° C.

Enzymatic Assays:

Assays with synthetic chromogenic substrates were carried out as previously described (3). The reaction mixtures contained 10 µl substrate (10 mM in dimethyl sulfoxide), 50 µl enzyme (ca. 5 µg), and 190 µl buffer (0.05M Tris-HCl pH 7.5 for Cbz-Gly-Gly-Leu-pNA, or 0.05M Tris-HCl pH 8.0 for Cbz-D-Ala-Leu-Arg-2NA, and Cbz-Leu-Leu-Glu-2NA). Samples were incubated at 37°C and the reactions were terminated by addition of 250 µl of 10% trichloroacetic acid. The p-nitroaniline or 2-naphthylamine released were measured

spectrophotometrically by a diazotization procedure at 540nm and 580nm respectively.

The degradation of dephosphorylated β-casein was determined by a gel electrophoretic method. 70 μg of dephosphorylated β-casein were incubated at 37°C with approximately 2 μg of native or chemically modified MPC in a total volume of 175 μl 0.05 M Tris-HCl, pH 8.0. 75 μl aliquots of the reaction mixture were subjected to SDS-PAGE, and the gels stained with Coomassie blue. After destaining with a solution containing 40% methanol(v/v) and 10% acetic acid(v/v), the bands corresponding to the dephosphorylated β-casein were excised with a razor blade, transferred to small test tubes, and eluted by shaking overnight with 1.5 ml of 25% pyridine at room temperature. Blanks were obtained by excising a band with the same size from the background surface of each gel and subjecting it to the same treatment. As controls, 75 μl aliquots of each reaction mixture at time zero were run in every gel. The absorbance of the eluate was measured spectrophotometrically at 605 nm (27).

Quantitation of an intermediate protein generated from the degradation of dephosphorylated β-casein by N-acetylimidazole-modified MPC:

MPC was modified by treatment with N-acetylimidazole for 20 min as described above. Dephosphorylated β-casein was incubated for 2 hours at 37°C with the modified enzyme to obtain the intermediate protein. The reaction mixture (total volume 175 μl)

contained 70 μg of dephosphorylated β-casein, 3 μg of modified MPC and 0.05 M Tris-HCl pH 8.0. The reaction was terminated by addition of an equal volume of 10% trichloroacetic acid. The mixture was centrifuged for 10 min at 14,000x g, the supernatant fractions decanted, and the residue washed with acetone. After drying, the residue was dissolved in 145 μl of 0.05M Tris-HCl, pH 8.0. The mixture was either directly subjected to SDS-PAGE or incubated at 37°C with different MPC preparations. After incubation, the samples were subjected to SDS-PAGE for quantitation.

RESULTS

Effect of N-acetylimidazole on the catalytic activities of MPC:

MPC was treated with N- acetylimidazole for different periods of time as described above, and after termination of the reaction and dialysis the catalytic activities were measured with synthetic chromogenic substrates and with dephosphorylated β-casein.

The trypsinlike activity (measured with Cbz-D-Ala-Leu-Arg-2NA), and the PGP activity (measured with Cbz-Leu-Leu-Glu-2NA) rapidly declined after exposure to N-acetylimidazole (Fig. 1-A). By contrast the chymotrypsinlike activity (measured with Cbz-Gly-Gly-Leu-pNA) was much less sensitive to this treatment. Thus 15 min exposure to N-acetylimidazole resulted in a preparation containing essentially only chymotrypsinlike activity (Fig. 1-A). The caseinolytic activity was measured by monitoring the disappearance of dephosphorylated β-casein after SDS-PAGE of the incubation mixture. The results of a representative experiment are shown in Fig 1-B. Treatment with N-acetylimidazole markedly activated the breakdown of dephosphorylated β-casein. An approximate five-fold increase in the breakdown of dephosphorylated β-casein was seen after a 30 min treatment. In different preparations of treated MPC the time required to achieve a maximal stimulation of caseinolytic activity varied from 15 to 60 min, and the magnitude of stimulation varied from 5 to 10 fold. Nevertheless, in all preparations, N-

acetylimidazole treatment produced a marked stimulation of the caseinolytic activity, at times in which the trypsinlike and PGP activities were virtually totally inhibited, and the chymotrypsinlike activity either unchanged or moderately inhibited. Prolonged treatment with N-acetylimidazole (e.g. 3h) led to total inhibition of the caseinolytic activity (data not shown). Thus the stimulation of the caseinolytic activity of MPC is markedly dependent upon its degree of acetylation.

The pattern of cleavage of dephosphorylated β-casein by acetylated MPC differed from that of the native enzyme. Thus degradation of dephosphorylated β-casein by the acetylated enzyme led to the production of a stable intermediate protein which migrated on SDS-PAGE as 21 kDa (Fig. 2, lane 2) whereas accumulation of this intermediate peptide was not seen when casein was incubated with native MPC. It should be noted that the migration of dephosphorylated β-casein on SDS-PAGE is anomalous. Thus dephosphorylated β-casein migrates as a 29 kDa protein although its true molecular weight is close to 24 kDa (28). Therefore the true molecular weight of the intermediate protein may be close to 14 kDa. The intermediate protein could be precipitated by trichloroacetic acid, as described in the experimental procedures and used as substrate for subsequent proteolysis. The intermediate protein was fully degraded when incubated with native MPC, but almost resistant to degradation by the acetylated enzyme (Fig. 3).

Reversibility by hydroxylamine:

Hydroxylamine deacetylates acetyltyrosine and acetylcysteine but not acetyllysine. The effect of treatment of acetylated MPC with hydroxylamine on the catalytic activities of MPC was studied. MPC was treated with N-acetylimidazole for 20 min followed by hydroxylamine as described above. Hydroxylamine significantly restored most of the trypsinlike activity, but had no effect on the PGP activity (Fig.4). Of particular interest was the effect of hydroxylamine on the activated caseinolytic activity. The activation was reversed almost to basal levels by hydroxylamine (Fig.2, lane 3). Thus the caseinolytic specific activity of untreated MPC was 2.0 ± 0.3 mg of dephosphorylated β-casein degraded/mg MPC/h (N=2) and the caseinolytic specific activity of N-acetylimidazole-treated MPC was 19.2 ± 0.8 (N=3), whereas after deacetylation with hydroxylamine, the specific activity declined to 5.2 ± 0.5 (N=3). However when MPC was reacted with N-acetylimidazole for 3h all activities were virtually totally inhibited and hydroxylamine was unable to restore any of them (data not shown).

Electrophoretic analysis of N-acetylimidazole- modified MPC:

Non-dissociating and dissociating gel electrophoresis were used to determine if acetylation of MPC by N-acetylimidazole resulted in a structural modification of the enzyme. The proteinase was treated with N-acetylimidazole for 20 min. After removing a

portion of the acetylated enzyme, the remainder was treated with hydroxylamine as described. When the acetylated enzyme was subjected to non-dissociating gel electrophoresis (Fig. 5, lane 1), a single protein band with a faster mobility than the untreated enzyme(Fig. 5, lane3) was observed. The acetylated enzyme treated with hydroxylamine also migrated more rapidly on a non-dissociating gel (Fig. 5, lane2) than the native enzyme. To determine whether the change in mobility was due to an alteration of charge or to an alteration of size, the elution profile of the modified enzyme was examined on an AcA 22 column (5 cm X 45 cm). Modified and untreated enzymes co-eluted indicating that the faster electrophoretic migration was due to a modification of the charge of the enzyme, likely by acetylation of amino groups. The patterns on SDS-PAGE of the modified, and hydroxylamine treated preparations were not distinguishable, but differed from the subunit pattern of the native enzyme (Fig. 6). Changes in the migration of individual subunits are likely due to acetylation of amino groups, since acetylated amino groups are resistant to hydroxylamine. Thus although acetylated and deacetylated preparations differed markedly with respect to their caseinolytic and trypsinlike activities, their appearance on gels was similar. The similar pattern of acetylated, and hydroxylamine treated (deacetylated) preparations indicates that activation of the caseinolytic component by N-acetylimidazole is due to the acetylation of relatively few hydroxylamine-sensitive groups.

Reversibility of changes in enzymatic activities:

The activation-deactivation cycle was fully reversible. Thus the caseinolytic activity could be activated by N-acetylimidazole, reversed to basal levels by hydroxylamine, and reactivated by treating once more with N-acetylimidazole (Fig. 7). Similarly the trypsinlike activity which is inhibited by N-acetylimidazole and partially restored by hydroxylamine, could once more be inhibited by N-acetylimidazole (data not shown).

Analysis of substrate binding to the acetylated enzyme:

MPC was treated with N-acetylimidazole for 20 min and the kinetics of hydrolysis of the chymotrypsinlike substrate Cbz-Gly-Gly-Leu-pNA analyzed. No difference was seen in the hydrolysis of the substrate by native or acetylated MPC (results not shown).

DISCUSSIONS

MPC was originally described on the basis of its activity toward synthetic chromogenic substrates. Three distinct catalytic activities cleaving peptide bonds at the carboxyl end of hydrophobic, basic and acidic amino acids were differentiated (3). Subsequent studies demonstrated that MPC also degrades proteins (10-16). The relationship of the proteinase activity to the three defined catalytic components has not been clarified. Our studies on the chemical modification by N-acetylimidazole provide strong evidence that the degradation of proteins such as dephosphorylated β-casein is initiated by a distinct fourth catalytic component.

Prior to these experiments it had been assumed that the hydrolysis of proteins was catalyzed by the PGP component (3, 23). This conclusion was based on the observation that the PGP component is the most sensitive to inhibition by proteins (3) and that hydrolysis of Cbz-Leu-Leu-Glu-2NA, the substrate used to define the PGP component is markedly stimulated by SDS as is the hydrolysis of proteins under certain conditions. The experiments presented here cannot support this assumption. Thus treatment with N-acetylimidazole almost totally inhibits the PGP activity, while markedly stimulating the hydrolysis of dephosphorylated β-casein (Fig. 1 A and B). If the effect of N-acetylimidazole was simply to sterically hinder access of the substrate to the active site, the opposite effect would have been predicted, i.e. that the chromogenic

substrate could still gain access to the active site while binding of the bulky protein would be prevented. On the other hand, if secondary conformational changes resulted in an opening of the PGP site to facilitate the access of casein, it would be difficult to account for the inhibition of hydrolysis of the PGP substrate. Moreover loss of PGP activity could not be reversed by hydroxylamine, whereas activation of dephosphorylated β-casein degradation could be reversed to inactivation by hydroxylamine. It could be argued that acetylation of an amino group prevents binding of the acidic substrate Cbz-Leu-Leu-Glu-2NA but not the binding of dephosphorylated β-casein. However this explanation could not account for the resistance of the 21kDa intermediate protein to hydrolysis by acetylated MPC.

Several investigators reported that the caseinolytic activity could be stimulated by SDS (16,17,28). In our preparations we could never detect any stimulation of casein degradation by SDS, although we always obtained strong activation of the PGP component by SDS. Activation of casein degradation by SDS is apparently a function of the activation state of the isolated enzyme. Thus McGuire et al., (17) found that SDS only stimulated the caseinolytic activity of the latent form of MPC but not the activated form of MPC. The relatively high caseinolytic activity of our preparation indicates that we have isolated the activated form of this enzyme.

Although our results strongly suggest that the caseinolytic activity is due to a distinct fourth component of the enzyme

complex, we cannot rule out the possibility that the chymotrypsinlike activity is responsible for casein degradation. Thus a conformational change may facilitate the access of casein to the chymotrypsinlike catalytic site, whereas this change would not be expected to have any effect on the access of the small synthetic chromogenic substrate to the active site. However other studies in this laboratory render this identity of the chymotrypsinlike and caseinolytic components highly unlikely. Thus we have found that the caseinolytic activity can be markedly stimulated by magnesium ions, whereas this cation inhibits the chymotrypsinlike activity (Pereira, M.E., Yu, Bo and Wilk, S. submitted for publication).

Of particular interest is the observation that the hydrolysis of dephosphorylated β-casein by the N-acetylimidazole modified enzyme generates a stable intermediate protein migrating as 21 kDa on SDS-PAGE (Fig. 2). This intermediate protein does not accumulate when dephosphorylated β-casein is hydrolyzed by native MPC. The intermediate protein is degraded by native MPC (Fig. 3). These experiments support a role for the trypsinlike and PGP components in secondary proteolysis. In this model, protein degradation by MPC is seen as an ordered sequential process in which initial cleavage (limited proteolysis) is catalyzed by a caseinolytic component and further degradation of the generated intermediates is catalyzed by the other components. On the basis of analysis of the pattern of degradation of the oxidized B chain of insulin by MPC, Dick et al., (29) have also proposed a sequential hydrolysis of peptide bonds.

Many questions remain unanswered such as the catalytic mechanism responsible for proteolysis (the sequences of all cloned subunits bear no structural homology to known proteinases), and the specificity of the site catalyzing the initial degradation of dephosphorylated β-casein. The role of MPC in the degradation of ubiquitinated proteins still remains unclear.

Treatment of MPC with N-acetylimidazole does not result in dissociation. The faster migration of the enzyme on non-dissociating gels is likely due to charge modification by acetylation of amino groups. Both native and modified enzymes were similarly eluted on a gel filtration column. A significant change in the pattern of subunits on SDS-PAGE gels is seen even after brief exposure to N-acetylimidazole. Of interest is the observation that hydroxylamine is able to reverse both the activation of casein and the inhibition of the trypsinlike component while not reversing the subunit pattern to that seen with the native form. This indicates that the catalytic changes affected by N-acetylimidazole are due to the modification of relatively few tyrosine or cysteine residues. Further studies will be required to elucidate the molecular mechanisms underlying the catalytic changes produced when MPC is reacted with N-acetylimidazole.

REFERENCES

1. Wilk, S., and Orlowski, M.
   Cation-sensitive neutral endopeptidase : Isolation and specificity of bovine pituitary enzyme.
   J. Neurochem., 35: 1172-1182. (1980)

2. Orlowski, M. and Wilk, S
   A multicatalytical protease complex from pituitary that forms enkephalin and enkephalin containing peptides
   Biochem. Biophys. Res. Comm. 101: 814-822 .(1981)

3. Wilk, S., and Orlowski, M.
   Evidence that pituitary cation-sensitive neutral endopeptidase is a multicatalytic protease complex.
   J. Neurochem., 40: 842-849. (1983)

4. Orlowski, M.
   The multicatalytic proteinase complex, a major extralysosomal proteolytic system.
   Biochemistry, 29: 10289-10297. (1990)

5. Rivett, J. A.
   The multicatalytic proteinase of mammalian cells
   Arch. Biochem. Biophys., 268:1 - 8. (1989)

6. Tanaka, K.J. and Ichihara, A.
   Proteasomes (multicatalytic proteinase complexs) in eukaryotic cells.
   Cell Structure and function,15: 127-132. (1990)

7. Kopp, F., Steiner, R., Dahlmann, B., Kuehn, L., and Reinauer, H.

Size and shape of the multicatalytic proteinase from rat skeletal muscle.
Biochim. Biophy. Acta. 872: 253-260. (1986)

8. Fulkenburg, P-E., Haass, C., Kloetzel, P-M., Niedel B., Kopp, F., Kuehn, L., Dahlmann, B.
Drosophila small cytoplasmic 19S ribonucleoprotein is homologous to the rat multicatalytic proteinase.
Nature. 331: 190-192. (1988)

9. Arrigo, A.-P., Tanaka, K., Goldberg, A. L., & Welch, W. J.
Identity of the 19S 'prosome' particle with the large multifunctional protease complex of mammalian cells (the proteasome)
Nature. 331: 192-194). (1988)

10. Wagner, B. J., Margolis, J. W., Farnsworth, P. N. and Fu, S.-C.
Calf lens neutral proteinase activity using calf lens crystallin substrates
Exp. Eye Res., 35: 293 - 303. (1982)

11. Ray, K. and Harris, H.
Purification of neutral lens endopeptidase: close similarity to a neutral proteinase in pituitary.
Proc. Natl. Acad. Sci. U.S.A., 82: 7545-7549. (1985)

12. Rivett, J. A.
Purification of a liver alkaline protease which degrades oxidatively modified glutamine synthetase. Characterization as a high molecular weight cysteine proteinase.
J. Biol. Chem. 260:12000-12606. (1985)

13. Sacchetta, P., Battista, P., Santrarone, S., and Di Cola, D.

Isolation of two high-molecular-mass proteinases from huamn erythrocytes.
Biochim. Biophys. Acta, 1037: 337 -343. (1990)

14. Dahlmann B., Kuehn, L., Rutschmann M. and Reinauer H.
Purification and characterazation of a multicatalytic high-molecular-mass proteinase from rat skeletal muscle
Biochem. J., 228: 161-170. (1985)

15. Ray, K. and Harris, H.
Comparative studies on lens neutral endopeptidase and pituitary neutral proteinase: two closely similar enzymes
FEBS Lett., 194: 91 - 95. (1986)

16. Tanaka, K., Ii, K., Ichihara, A., Waxman, L., Goldberg, A. L.
A high molecular weight protease in the cytosol of rat liver. I Purification, enzymological properties and tissure distribution.
J. Biol. Chem. . 261:15197-15203 (1986)

17. McGuire, M. J., McCullough, M. L., Croall, D. E. and Dematino, G. N.
The high molecular weight multicatalytic proteinase, macropain, exists in a latent form in human erythrocytes.
Biochim. Biophys. Acta, 995: 181-186. (1989)

18. Mellgren, R.L.
Interaction of human erythrocyte multicatalytic proteinase with polycations.
Biochim.. Biophys. Acta, 1040: 28 - 34. (1990)

19. Mykles, D.L.

Purification and characterization of a multicatalytic proteinase from crustacean muscle: comparison of latent and heat-activated forms.
Arch. Biochem. Biophys., 274: 216 - 228. (1989)

20. Riordan, J.F., Wacker, W.E.C. and Vallee, B.L.
N-Acetylimidazole: areagent for determination of "free" tyrosyl residues of proteins.
Biochemistry, 4: 1785 - 1765. (1965)

21. Wilk, S., Meister, A., and Haschemeyer, R.H.
Dissociation of native octameric brain glutamine synthetase to a tertramer by treatment with N-acetylimidazole.
Biochemistry, 9: 2039 - 2043. (1970)

22. Wilk, S., Pearce, S. and Orlowski, M.
Identification and partial purification of a cation-sensitive neutral endopeptidase from bovine pituitary.
Life Sci., 24: 457 - 464. (1979)

23. Orlowski, M. and Michaud, C.
Pituitary multicatalytic proteinase complex. specificity of components and aspects of proteolytic activity
Biochemistry, 28: 9270-9278. (1989)

24.. Pereira, M. E. and Wilk, S.
Phosphorylation of the multicatalytic proteinase complex from bovine pituitaries by a copuriying cAMP-dependent protein kinase.
Arch. Biochem. Biophys., 283: 68 - 74. (1990)

25. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J.
Protein measurment with the folin phenol reagent.
J. Biol. Chem., 193: 265 - 275. (1951)

26. Laemmli, U.K.
Cleavage of structural proteins during the assembly of the head of bacteriophage.
Nature , 227: 680 - 685. (1970)

27. Fenner, C., Traut, R.R., Mason, D. T. and Wikman-Coffelt, J.
Quantification of coomassie blue stained proteins in polyacrylamide gels based on analyses of eluted dye.
Anal. Biochem. 63: 595 - 602. (1975)

28.. Dumas, B.R., Brignon, G., Grosclaude, F. and Mercier, J-C.
Structure primaire de la caseine b bovine.
Eur. J. Biochem. 25, 505-514. (1972)

29.Dick, L. R., Moomaw, C. R., DeMartino, G. N. and Slaughter, C. A.
Degradation of oxidized insulin B chain by the multiproteinase complex Macropain (proteasome).
Biochemistry. 30: 2725-2734. (1991)

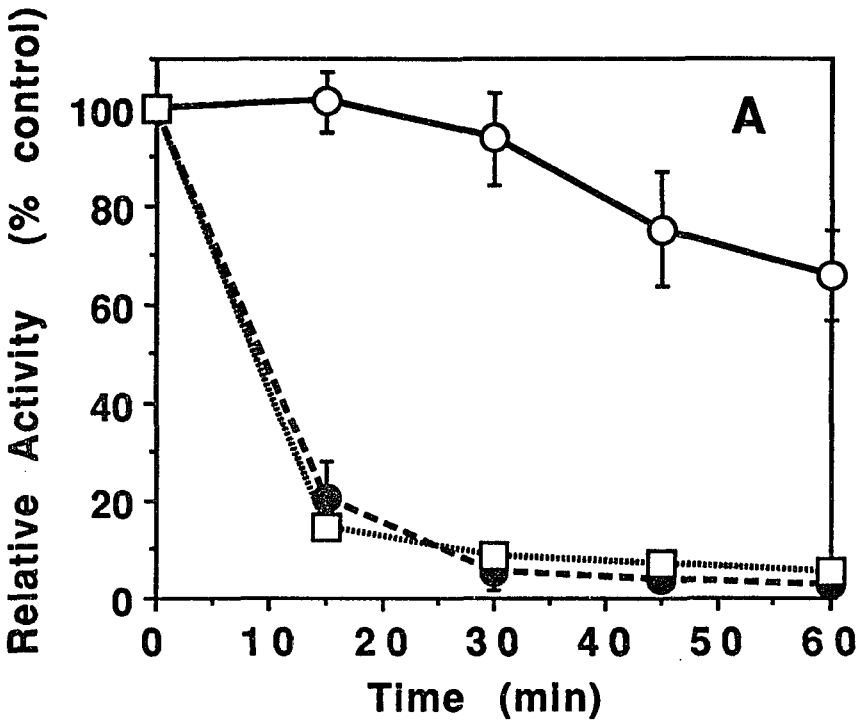
Figure 1. Effect of N-acetylimidazole on the catalytic activities of MPC measured with synthetic chromogenic substrates and with dephosphorylated β-casein.

MPC was reacted with N-acetylimidazole as described in experimental procedures. At different times of exposure to N-acetylimidazole, the reaction was terminated by addition of tyrosine and the reaction mixture dialyzed. Enzymatic activities were assayed with synthetic substrates **(A)** and dephosphorylated β-casein **(B)** as described in experimental procedures. In **A** all activities are expressed relative to zero time treatment (100%). Each point represents the mean ± SEM of 3-5 experiments,

–○– Cbz-Gly-Gly-Leu-pNA; –●– Cbz-Leu-Leu-Glu-2NA;

–□– Cbz-D-Ala-Leu-Arg-2NA.

In **B** the values are from a representative experiment.


A
Relative Activity (% control)
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80
60
40
20
0
0
10
20
30
40
50
60
Time (min)

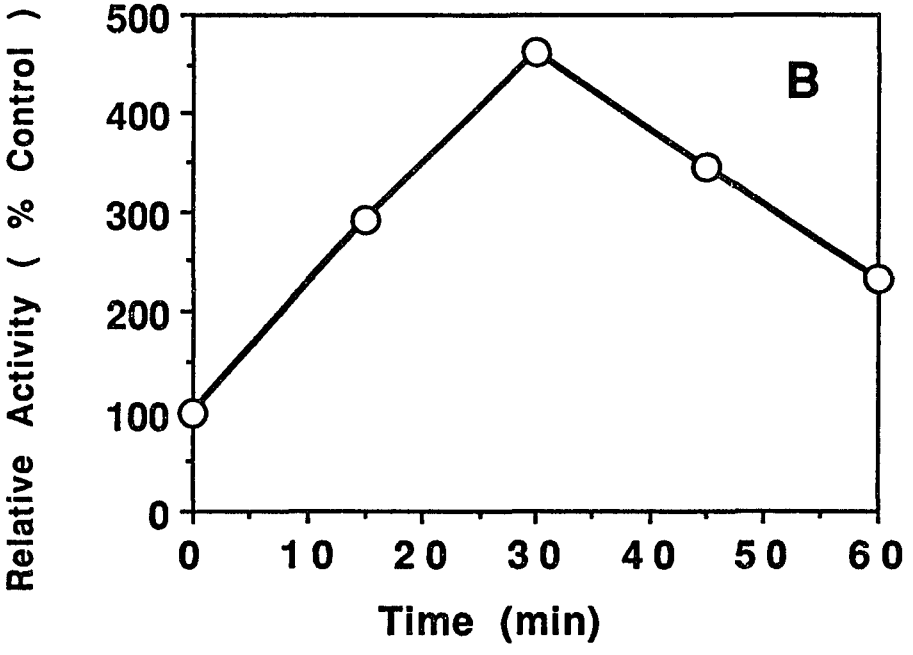

B
Relative Activity (% Control)
500
400
300
200
100
0
0
10
20
30
40
50
60
Time (min)

Figure 2. Polyacrylamide gel electrophoresis of dephosphorylated β-casein incubated with untreated and N-acetylimidazole modified MPC.

30 μg of dephosphorylated β-casein were incubated with 1.28 μg MPC for 3 hours 37° C. Lane 1: incubation with untreated MPC; lane 2: incubation with MPC treated for 20 minutes with N-acetylimidazole; lane 3: MPC treated for 20 minutes with N-acetylimidazole followed by a 6 hour treatment with hydroxylamine. Molecular weight markers are shown in lane 4.

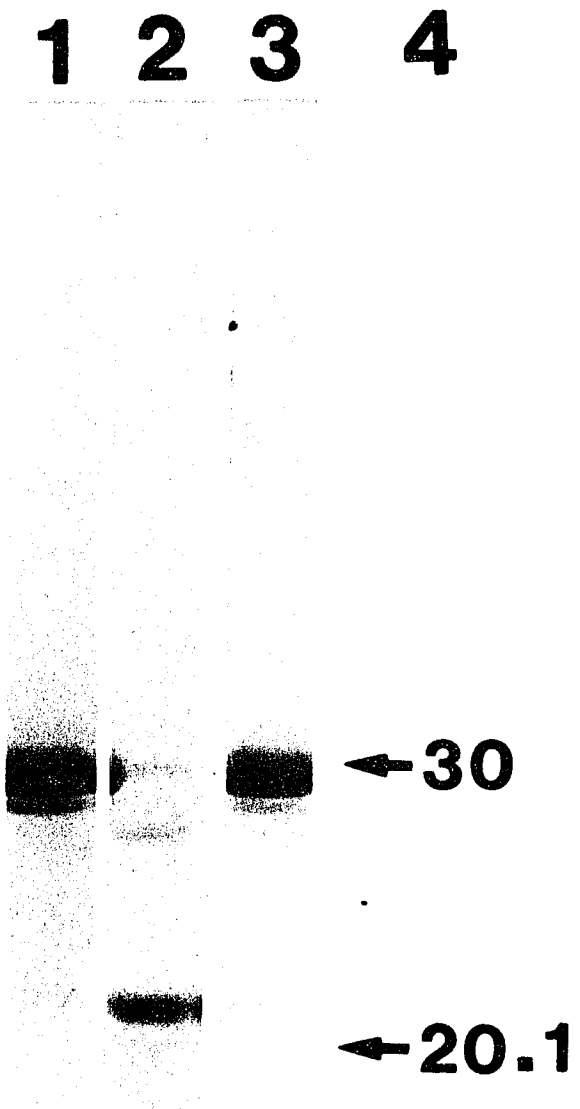

1 2 3 4
30
20.1

Figure 3 Effect of untreated and N-acetylimidazole-modified MPC on the degradation of the 21 kDa dephosphorylated β-casein intermediate

Dephosphorylated β-casein was incubated for 2 hours at 37° C with N-acetylimidazole-modified MPC. The reaction mixture (175 µl) contained 70 µg dephosphorylated β-casein, 3 µg modified MPC, and 0.05M Tris-HCl pH 8.0. The reaction was terminated by addition of an equal volume of 10% trichloroacetic acid. After centrifugation (10 min. at 14,000 X g), the residue was washed with acetone and then dissolved in 145 µl 0.05M Tris-HCl, pH 8.0. The mixture was incubated at 37° C with modified MPC and native MPC. After incubation, the samples were subjected to SDS PAGE. Lanes 1 and 2, intermediate + modified MPC incubated for 2h at 37°C (no degradation). Lanes 3 and 4, intermediate + untreated MPC incubated for 2h at 37° C (14 % degradation). Lanes 5 and 6, intermediate + modified MPC incubated 4h at 37° C (8 % degradation). Lanes 7 and 8, intermediate + untreated MPC incubated 4h 37° C (66 % degradation). Lane 9 indicates the position of the molecular weight marker.

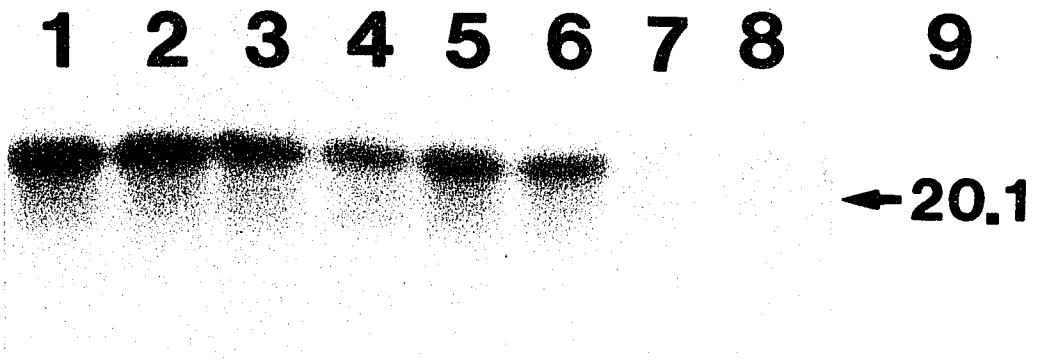

1 2 3 4 5 6 7 8 9
20.1

Figure 4 Effect of hydroxylamine on the cleavage of synthetic chromogenic substrates by N-acetylimidazole-treated MPC.

A) MPC treated with N-acetylimidazole for 20 min.

B) N-acetylimidazole-modified enzyme treated with hydroxylamine for 6h as described in experimental procedures. All activities are expressed relative to untreated MPC which is assigned a value of 100. Mean ± SEM of 3 experiments.

* Significantly different from N-acetylimidazole-treated MPC ($p < 0.05$)

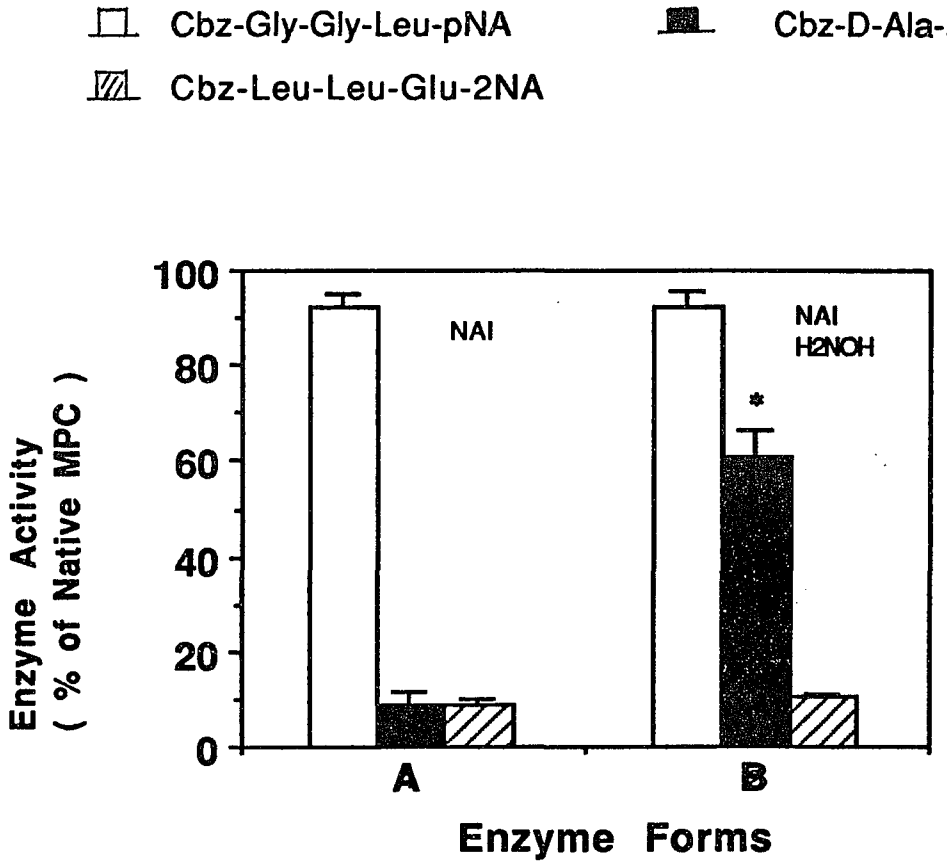


Figure 5 Polyacrylamide gel electrophoresis of the untreated and N-acetylimidazole treated MPC under non-dissociating conditions

lane 1, MPC treated for 20 minutes with N-acetylimidazole; lane 2 , MPC treated for 20 minutes with N-acetylimidazole and then for 6 hours with hydroxylamine; lane 3 , native MPC. Each lane contains 6 ug MPC. Protein visualized by Coomassie Blue staining.

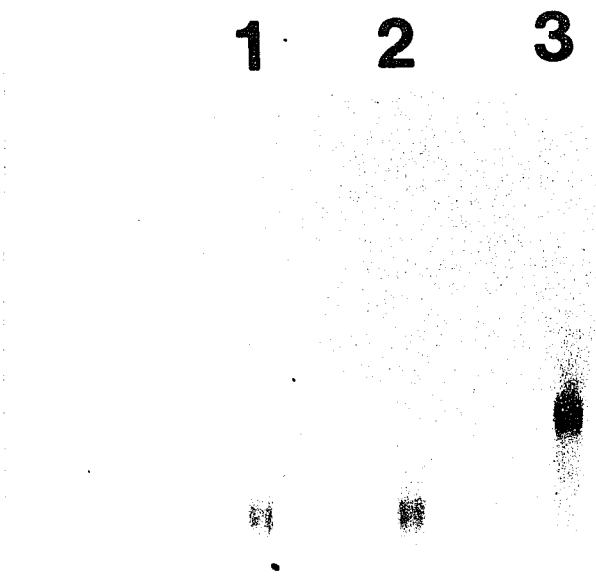


Figure 6 SDS-PAGE of the enzymes under dissociating and reducing conditions.

Lane 1: untreated MPC. Lane 2: MPC treated for 20 minutes with N-acetylimidazole. Lane 3: MPC treated for 20 minutes with N-acetylimidazole followed by 6 hour treatment with hydroxylamine. Each lane contains 2 µg enzyme protein. Lane 4 shows the position of a 30 kDa molecular weight marker. Proteins visualized by silver staining.

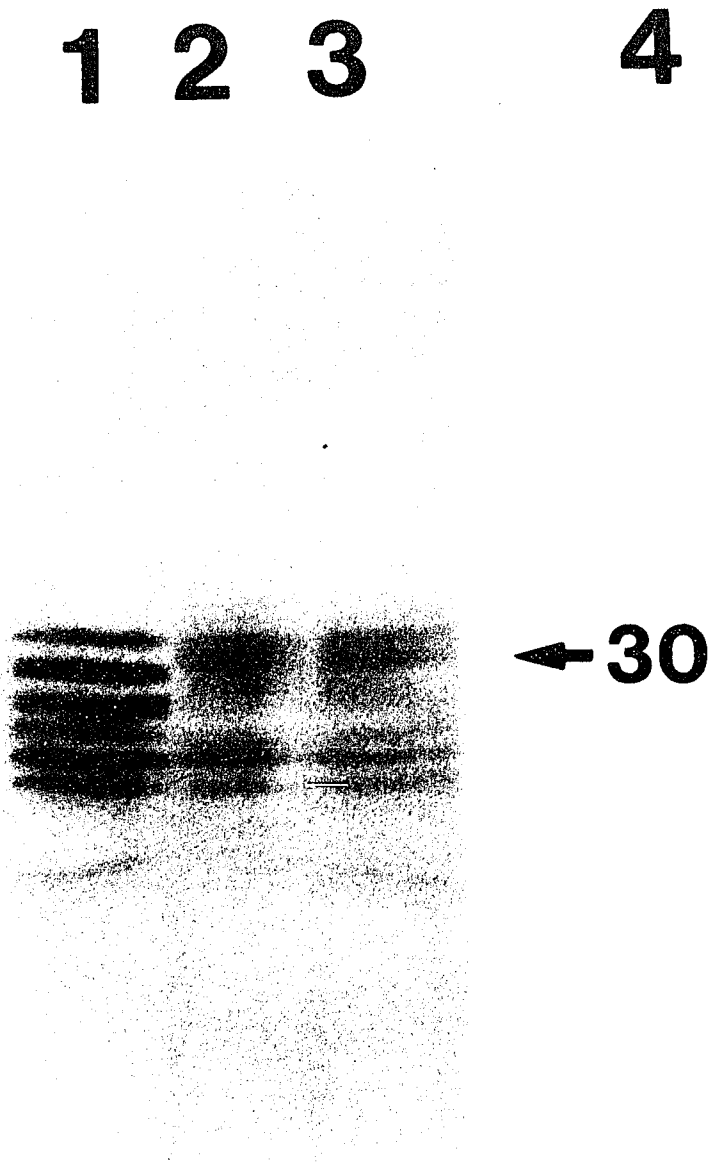
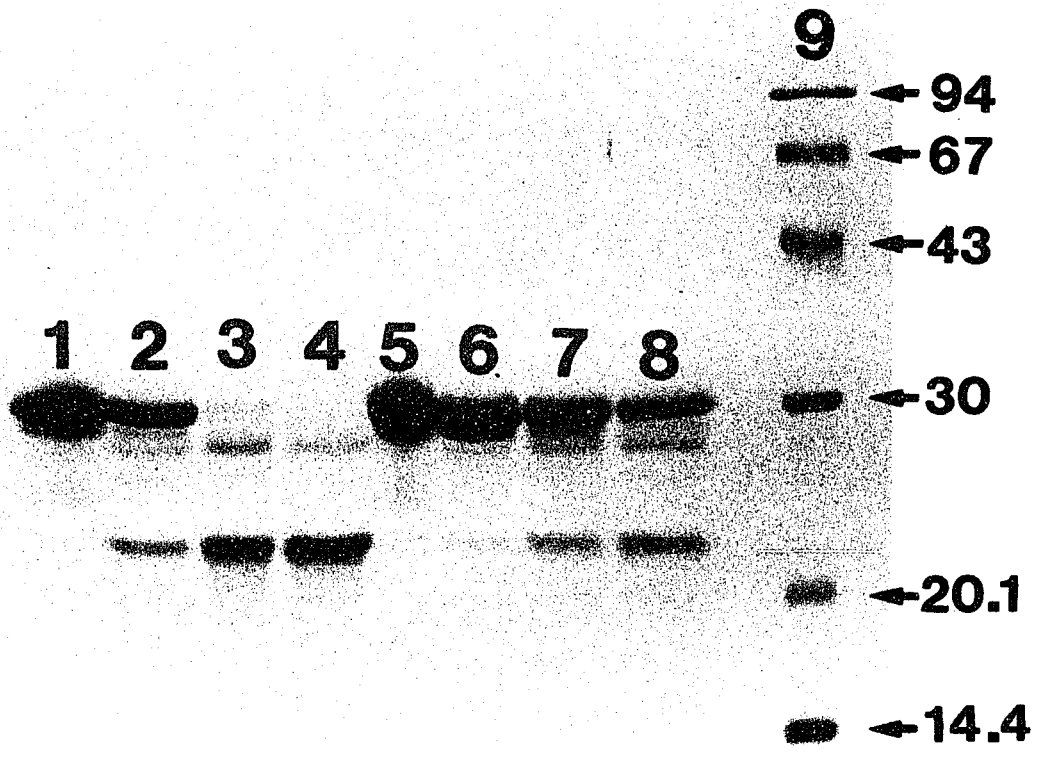


Figure 7 Repetitive activation of MPC by N-acetylimidazole.

Lanes 1-4, dephosphorylated β-casein incubated for 0, 90, 180 and 240 min. with MPC treated consecutively with N-acetylimidazole, hydroxylamine an N-acetylimidazole again. Lanes 5-8, dephosphorylated β-casein incubated for 0, 90, 180 and 240 min. with MPC (treated consecutively with N-acetylimidazole and hydroxylamine). Each lane contains 30 μg dephosphorylated β-casein and 1.28 μg enzyme protein. Lane 9: Molecular weight markers. Proteins visualized with Coomassie blue staining.



Chapter 3

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Changes in the Structure and Catalytic Activities of the Bovine Pituitary Multicatalytic Proteinase Complex Following Dialysis

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SUMMARY

The multicatalytic proteinase complex (proteasome) contains at least four distinct active sites catalyzing the degradation of selected chromogenic substrates (trypsinlike, chymotrypsinlike and peptidylglutamyl peptide hydrolyzing activities) and proteins such as β-casein. Oxidized insulin B chain was recently proposed as a model substrate for protein degradation by the multicatalytic proteinase complex [Dick, L.B., et al., (1991) Biochemistry 30, 2725-2734]. We studied the dialysis-induced activation of the hydrolysis of oxidized insulin B chain by this enzyme. Removal of EDTA from purified preparations of bovine pituitary multicatalytic proteinase complex by dialysis against Tris-HCl buffers led to marked changes in the catalytic properties and structure of the enzyme. Dialysis produced a time dependent activation of oxidized insulin B chain hydrolysis with predominant cleavage at the Glu^{13}-Ala^{14} bond. A new chromogenic assay was developed for measurement of this activity. Activation was accompanied by a virtually total inactivation of the chymotrypsinlike, trypsinlike and peptidylglutamyl peptide hydrolyzing activities. SDS-PAGE revealed a loss of the 24 kDa subunit and appearance of a new band at 21 kDa. N-terminal amino acid analysis established that the 21 kDa band was autolytically derived from the 24 kDa subunit. Evidence for partial dissociation and/or aggregation indicated that autolysis destabilizes the complex. By altering the profile of catalytic

activities of the multicatalytic proteinase complex, autolysis may serve as a mechanism for regulation of this macromolecule.

INTRODUCTION

The multicatalytic proteinase complex $(MPC)_2$ (also referred to as the proteasome) was first characterized on the basis of the effect of inhibitors and activators on the hydrolysis of chromogenic peptide substrates by the bovine pituitary enzyme (1-3). This unique high molecular mass macromolecule (approx. 700 kDa) has been found in all eukaryotic cells thus far examined. It is composed of subunits of molecular mass ranging from about 24-34 kDa, although its exact subunit composition has still not been clearly defined (see 4,5 for reviews). Electron microscopic examination reveals a cylindrical particle composed of four rings each containing 6 or 7 subunits (6,7). Molecular cloning of several of the subunits demonstrates conservation of primary structure from archaebacteria to mammals (8). Electron microscopy also shows conservation of quaternary structure despite the fact that the archaebacteria enzyme only contains two non-identical subunits (designated α and β) (8). The primary structure of cloned subunits bears no homology to other known proteinases, although inter-subunit homology is found. MPC is therefore a product of a unique closely related gene family. The amino terminal sequences of cloned subunits are structurally related to either the α- or β-subunits of the archaebacteria enzyme. The β-subunits have been proposed to catalyze proteolysis whereas the α-subunits have been proposed to be involved in regulation or nuclear targeting (8). Although MPC is likely to be of considerable importance in cellular

function, the exact nature of its function remains unclear. Its abundance, pH optima and predominantly cytosolic localization suggest that MPC plays a major role in non-lysosomal proteolysis. A still higher molecular mass 26S ubiquitinated-protein degrading complex was purified from rabbit reticulocyte lysates (9). A role for MPC as the catalytic core of the 26S complex has been proposed (10,11) and disputed (12). MPC has also been proposed as catalyzing proteolysis in the antigen presentation pathway for major histocompatibility complex class I molecules (13-15).

The number of distinct catalytic activities associated with MPC is not known. On the basis of hydrolysis of chromogenic substrates and their differential inhibition and activation, three catalytic activities were originally defined (3). These were termed chymotrypsinlike (cleavage after a leucyl residue in the substrate Cbz-Gly-Gly-Leu-pNA), trypsinlike (cleavage after an arginyl residue in the substrate Cbz-D-Ala-Leu-Arg-NA), and peptidylglutamyl peptide bond hydrolyzing (PGP) (cleavage after a glutamyl residue in the substrate Cbz-Leu-Leu-Glu-NA). We have recently presented evidence that the hydrolysis of β-casein is initiated by a distinct fourth active site (termed caseinolytic). This activity is stimulated by 3,4-dichloroisocoumarin, a reagent that inhibits the other three activities (16). Degradation of β-casein is also stimulated by N-acetylimidazole, a reagent that inhibits the trypsinlike and PGP activities (17). We proposed that the three activities defined with chromogenic substrates catalyze secondary degradation of

intermediate peptides formed by the primary action of the caseinolytic component on protein substrates (18). Evidence supporting a channeling of peptide intermediates between different catalytic centers has been presented (19).

Recently Dick et al., (19) studied the degradation of oxidized insulin B chain by human erythrocyte MPC. They found that hydrolysis of this substrate required activation of MPC by dialysis. This was attributed to removal of glycerol and NaCl. The phenomenon of activation of a latent activity was similar to that found when casein was used as substrate (20). They therefore suggested that the oxidized insulin B chain would be an appropriate model substrate for the degradation of proteins. Since we were interested in further characterizing the caseinolytic component, we explored the degradation of oxidized insulin B chain by MPC and the nature of the changes in the properties of the enzyme produced by dialysis. We present evidence that removal of EDTA by dialysis produces autolytic changes in the structure of MPC and profoundly alters its catalytic properties.

EXPERIMENTAL PROCEDURES

Materials - Frozen bovine pituitaries were obtained from Pel Freez, Rogers AK. Hydroxylapatite was from Calbiochem, LaJolla, CA. Dialysis membrane (10 mm flat width) was obtained from Spectrum Medical Industries, Houston, TX. The membranes were routinely boiled in an EDTA solution and then extensively washed with deionized water. Ultrafiltration membranes (PM-10) were from Amicon, Inc., Beverly MA. Oxidized insulin B chain, dephosphorylated β-casein, 3,4-dichloroisocoumarin, and Leu-pNA were from Sigma, St. Louis, MO. Intermediates for peptide synthesis were obtained from Bachem, Inc. Philadelphia, PA. Cbz-Gly-Gly-Leu-pNA, Cbz-D-Ala-Leu-Arg-NA and Cbz-Leu-Leu-Glu-NA were synthesized as previously described (1, 21). Polygram Sil G/UV plastic sheets for TLC (40X80 mm) were obtained from Brinkmann Instruments, Westbury, NY. Immobilon membranes were a product of Millipore Inc. Bedford, MA. All other reagents were of the highest purity available.

Synthesis of Boc-Val-Glu-Ala-Leu-NA - This compound was prepared by solution phase synthesis. Amino terminal stepwise elongation was achieved by reaction with Boc-amino acid-N-hydroxysuccinimide esters. The γ-carboxyl group of the glutamate intermediate was protected as the t-butyl ester. Deprotection of intermediates was achieved by treatment with trifluoroacetic acid at room temperature for 30 min. The final product was recrystallized from ethanol-H_2O. The purity of the product was

confirmed by TLC (visualization by UV 254 nm) on silica gel G/UV plates (chloroform:ethanol 9:1); Rf= 0.29; and by HPLC (gradient B, see below) (Rt =30.4 min). Amino acid analysis: Val: 1.0; Glu: 0.9; Ala: 1.0; Leu; 0.9.

Synthesis of Ala-Leu-NA - This compound prepared by solution phase synthesis was an intermediate isolated in the course of the synthesis of Boc-Val-Glu-Ala-Leu-NA. The purity of the product was confirmed by TLC and by HPLC as described above. TLC (butanol:acetic acid:H_2O, 4:1:1) Rf = 0.8, HPLC (gradient B, see below) Rt = 21.0 min.

Purification of enzymes - a) MPC was purified by a modification of the procedure described by Orlowski and Michaud (22). Aliquots of enzyme obtained after the final step of purification (approx. 1 mg) were chromatographed on a 0.7cm X 2 cm hydroxylapatite column and eluted with a 100 ml linear pH 7.0 potassium phosphate buffer gradient (0.05 M-0.4 M). Fractions containing enzymatic activity (determined with Cbz-Gly-Gly-Leu-pNA) were pooled and dialyzed against a 10 mM Tris-EDTA buffer, pH 7.5. The enzyme was stored in this buffer at 4°C.

b) Membrane alanyl aminopeptidase (EC 3.4.11.2) was purified to apparent homogeneity from rabbit kidney membranes as described (23).

A unit of enzymatic activity is defined as that amount of enzyme liberating 1 μmole aromatic amine/h.

Measurement of enzymatic activities - The chymotrypsinlike, trypsinlike, and PGP activities of MPC were determined by measuring the release of chromogen from the substrates Cbz-Gly-Gly-Leu-pNA, Cbz-D-Ala-Leu-Arg-NA and Cbz-Leu-Leu-Glu-NA respectively as described (3).

Hydrolysis of Boc-Val-Glu-Ala-Leu-NA was measured by quantitating the release of free NA by incubation at 37°C in the presence of MPC and excess purified membrane alanyl aminopeptidase. Incubation mixtures contained 10 μl substrate (10mM in dimethyl sulfoxide), 200 μl MPC (80μg/ml), 25 μl (3.9 U) membrane alanyl aminopeptidase, and 15 μl 0.05M Tris-HCl, pH 8.0.

Hydrolysis of oxidized insulin B chain by MPC was determined by measuring the disappearance of the substrate by HPLC. A 1mg/ml stock solution of oxidized insulin B chain was prepared by dissolving the peptide in 0.05M Tris-HCl buffer, pH 8.0. The incubation mixture contained 180 μl MPC (16 μg), 31.5 μl substrate, and 58.5 μl 0.05M Tris-HCl, pH 8.0. The reaction was stopped by addition of an equal volume of 2% trifluoroacetic acid and 25 μl taken for HPLC analysis as described below. Oxidized insulin B chain was quantitated by measurement of peak height.

Hydrolysis of dephosphorylated β-casein was determined by a gel electrophoretic method as previously described (17).

The activity of membrane alanyl aminopeptidase was measured with the substrate Leu-pNA as described (23).

HPLC analysis of peptides - HPLC was performed on a Waters 600E liquid chromatograph. Peptides were separated on a C8 reverse phase Aquapore RP-300 7μ column (4.6x250 mm), (Applied Biosystems, Foster City, CA). For analysis of oxidized insulin B chain degradation, a linear gradient of 0 - 50% acetonitrile with 0.05% trifluoroacetic acid was generated over a period of 30 min (gradient A). The flow rate was 1 ml/min. Peptides were monitored by UV detection at 210nm. For analysis of the hydrolysis of Boc-Val-Glu-Ala-Leu-NA a linear gradient of 15-70% acetonitrile/0.05% trifluoroacetic acid generated over a period of 34 min was used (gradient B). The eluate was monitored by UV detection at 254nm. For collection of products of oxidized insulin B chain hydrolysis for amino acid analysis, a gradient was generated by increasing the acetonitrile concentration linearly from 0 to 17 % in 10 min and from 17 to 35% in 35 min (gradient C). The flow rate was 1ml/min. The peptides were monitored by UV detection at 210 nm.

FPLC Analysis of Proteins - FPLC was conducted on a Pharmacia system. A Superose 12 HR 10/30 column was equilibrated with a Tris-HCl buffer (0.05M, pH 8.0) containing NaCl (0.1M) and sodium azide (0.01%). The flow rate was 0.8 ml/min.

A Mono Q column was equilibrated with a Tris-HCl buffer (0.01M, pH 8.0) containing NaCl (0.1M) and sodium azide (0.01%) (buffer A). An eluting buffer (buffer B) was prepared by increasing the concentration of NaCl in buffer A to 1.0 M. The flow rate was 2 ml/min. The chromatographic conditions were: 2 min following injection, a linear gradient of buffer B from 0-100% was generated over 10 min, maintained at 100% for an additional 2 min and then returned to initial conditions.

Amino acid analysis and N-terminal sequence determination - Peptides separated by HPLC were manually collected, lyophilized and submitted to the Protein Chemistry core facility of the Mount Sinai School of Medicine for amino acid analysis and N-terminal sequence determination.

N-terminal sequence analysis of the 24 kDa subunit of MPC and a derived 21 kDa protein was performed by the Yale Protein and Nucleic Acid Facility. MPC after the last step of purification was dialyzed against a Tris-HCl buffer as described below and subjected to SDS-PAGE. Proteins were transferred to Immobilon membranes, stained with Coomassie blue, and the bands of interest excised for sequence analysis.

Gel electrophoresis - Non-dissociating PAGE was run on 5% acrylamide gels in a 0.25M Tris-glycine buffer, pH 8.3. SDS-PAGE

was conducted on 12.5% gels (24). Gels were stained for protein with Coomassie blue or Silver staining.

Quantitation of MPC Subunits - MPC following SDS-PAGE was transferred to Immobilon membranes and the membranes stained with Coomassie blue. Intensities of individual bands were quantitated by image analysis (Data Translation System, model DT 2255) as described (25).

Protein determination - Protein concentrations were determined by the method of Lowry et al., (26).

RESULTS

Changes in the catalytic properties of MPC following dialysis - The oxidized insulin B chain was proposed as a model substrate for protein hydrolysis by MPC (19). In our initial attempts to use this substrate we observed negligible hydrolysis (Fig. 1A). However when the enzyme obtained after the final purification step in a 10 mM Tris-EDTA buffer, pH 7.5, was dialyzed for 15 h against a Tris-HCl buffer (0.05M, pH 8.0), hydrolysis was markedly activated (Fig. 1B). Dialysis increased the specific activity toward oxidized insulin B chain at least 6 fold. The two prominent degradation products were collected after HPLC and characterized by amino acid analysis. Amino acid analysis was consistent with a cleavage at Glu^{13}-Ala^{14}, as previously found by others (19, 27) (data not shown). To confirm the cleavage site, the first three N-terminal amino acids of peak 2, (Fig. 1B) were determined. The amino acids found were Ala, Leu and Tyr respectively, verifying the cleavage site as Glu^{13}-Ala^{14}.

Although the dialysis-induced activation initially appeared to be the same phenomenon reported by Dick et al., (19) we observed other changes in the properties of dialyzed MPC. The activities toward the three chromogenic substrates (Cbz-Gly-Gly-Leu-pNA, Cbz-Leu-Leu-Glu-NA, Cbz-D-Ala-Leu-Arg-NA) were markedly decreased (Table 1). Moreover the ability of SDS to activate the remaining PGP activity was also markedly reduced after dialysis. The activity toward β-casein was essentially unchanged (before

dialysis: 1.97 ± 0.28 mg β-casein degraded/h/mg MPC; after dialysis: 2.30 ± 0.56 mg /h/mg, N = 4). Changes in the properties of MPC following dialysis were irreversible. Attempts to restore basal activities by back dialysis against Tris-EDTA buffers were not successful.

Effect of modification of dialysis conditions on the catalytic activities of MPC - To investigate the mechanism of the dialysis-induced changes, we systematically varied the dialysis conditions. Results of these studies are summarized in Table 2. The enzyme prior to dialysis was present in a Tris-EDTA buffer (0.01M, pH 7.5). An increase in the molarity of the final Tris-HCl buffer accelerated the change. Maximal activation occurred at 0.1 M Tris-HCl (Fig. 2). The optimal pH for this effect was 8-8.5 (data not shown). The possibility that the chloride ion was responsible for the change was ruled out by dialysis against Tris-acetate, Tris-citrate and Tris-phosphate buffers. Activation was still observed when dialysis was conducted against these buffers (Table 2). Removal of EDTA was intimately associated with the changes. No alterations occurred when the enzyme was dialyzed against Tris-EDTA buffers of pH 7.5 or 8.0 at molarities of 0.01 or 0.05. The dialysis membrane itself however was also found to play a role in the alteration of the properties of MPC. Thus, exchanging the Tris-EDTA buffer for a Tris-HCl buffer by ultrafiltration did not alter the properties of MPC. Thus the described alterations in the properties of MPC are

associated both with the dialysis membrane and with the removal of EDTA. The enzyme was also dialyzed against Tris-EDTA buffers containing a molar excess of metal ion above that of EDTA (metal ion concentration 5mM, EDTA concentration 2.34 mM). Metal ions examined were Ca^{++}, Mg^{++} and Zn^{++}. No changes in the properties of the enzyme were observed under these conditions.

Gel electrophoretic examination of the dialyzed enzyme - To determine whether dialysis altered the structure of MPC, we compared the subunit pattern of the enzyme by SDS-PAGE before and after dialysis. Alterations in the subunit pattern following dialysis were apparent. Most prominent was the disappearance of the lowest molecular mass band (24 kDa) , and the appearance of a new band at 21 kDa (Fig. 3). The time course of the changes in the catalytic activities of MPC following dialysis was compared to the time course of disappearance of the 24 kDa band and appearance of the 21 kDa band. Densitometry was used to semi-quantitate the staining intensities of these bands. Changes in the subunit pattern were found to highly correlate with changes in the activities toward the three chromogenic substrates (Fig. 4 A, B). Activation of oxidized insulin B chain hydrolyzing activity appeared to precede the other effects (Fig. 4C). We were unable however to obtain preparations in which the oxidized insulin B chain hydrolyzing activity was stimulated without some loss of the three chromogenic activities.

When the enzyme was examined by non-dissociating gel electrophoresis before and after dialysis against Tris-HCl buffer, no change in the migration pattern was seen. However the intensity of staining of the dialysis activated enzyme by either Coomassie blue or amido black was markedly decreased (Fig. 5). This result was unexpected since the staining intensities of the subunits following dialysis did not change (Fig. 3). When the gel was cut into 2 mm slices and assayed for oxidized insulin B chain hydrolyzing activity, all the activity was associated with the undissociated protein band (data not shown). No evidence for free subunits could be found on non-dissociating gels.

FPLC analysis of the dialyzed enzyme - Enzyme preparations before and after dialysis against a 0.1M Tris-HCl buffer (pH 8.0) were subjected to FPLC on Superose or Mono Q columns. The peak corresponding to intact enzyme as measured by absorbance at 280 nm was reduced as a function of time of dialysis (Fig. 6). The loss of enzymatic activity toward the chromogenic substrates preceded the loss of intact protein. Thus at a time in which enzymatic activities toward chromogenic substrates declined by about 75%, at least 70% of the enzyme remained undissociated. After 8 h dialysis against the 0.1 M buffer, only about 29% of the enzyme remained undissociated. Fractions collected after Mono Q chromatography were analyzed by SDS-PAGE. In the non-dialyzed enzyme preparation, MPC subunits were seen only in the fraction containing

undissociated enzyme, whereas in the dialyzed preparation, other fractions contained subunits (Fig. 7).

Use of a chromogenic substrate to determine the activity responsible for oxidized insulin B chain hydrolysis - The primary cleavage of oxidized insulin B chain by dialysis activated MPC occurs at the Glu^{13}-Ala^{14} bond. This suggested the possibility that this activity could be more conveniently measured with a chromogenic substrate containing Glu in the P1 position rather than by the HPLC method. Although hydrolysis of a Glu-Ala bond would be expected to be catalyzed by the PGP activity, hydrolysis of Cbz-Leu-Leu-Glu-NA is markedly inhibited following dialysis (Fig. 4B). The possibility that the oxidized insulin B chain hydrolyzing activity requires amino acid substituents in the P1' and P2' positions for expression was considered. A chromogenic substrate Boc-VEAL-NA was designed based on the amino acid sequence around the scissile bond of oxidized insulin B chain. It was predicted that cleavage of this substrate would yield Boc-Val-Glu and Ala-Leu-NA as products. Addition of excess membrane alanyl aminopeptidase (EC 3.4.11.2) to the incubation mixture would be expected to generate free NA from the latter peptide product. Hydrolysis of this substrate indeed reflected the activity responsible for oxidized insulin B chain degradation for the following reasons:

a. Incubation of dialysis activated MPC with Boc-VEAL-NA alone did not release free NA. Incubation of Boc-VEAL-NA with membrane alanyl aminopeptidase alone also did not release free NA.

Addition of membrane alanyl aminopeptidase to the incubation mixture containing substrate and dialysis activated MPC released NA, indicating internal cleavage of the chromogenic substrate (Table 3).

b. HPLC analysis of an incubation mixture of dialyzed MPC and Boc-VEAL-NA revealed a major UV_{254} absorbing product (Fig. 8). The retention time of the product was identical to that of authentic Ala-Leu-NA. Amino acid analysis of the product identified Ala and Leu in a 1:1 molar ratio as the only amino acids. Cleavage at the Glu-Ala bond was therefore confirmed.

c. The dialysis activated enzyme poorly hydrolyzed Cbz-Leu-Leu-Glu-NA but cleaved Boc-VEAL-NA with a specific activity of 1230 nmol/h/mg, an activity similar to that of oxidized insulin B chain as substrate (1020 nmol/h/mg), (Table 3).

d. Although SDS at a concentration of 0.04% markedly stimulates the hydrolysis of Cbz-Leu-Leu-Glu-NA by native MPC (2, 3) it inhibited the cleavage of oxidized insulin B chain by the dialysis activated enzyme (Table 4) (see below). SDS also inhibited the cleavage of Boc-VEAL-NA by the dialysis activated enzyme (data not shown).

Characterization of the oxidized insulin B chain degrading activity - The profile of inhibitors of the oxidized insulin B chain degrading activity was determined after activation of MPC by dialysis. This activity was sensitive to inhibition by cysteine blocking reagents,

but unaffected by the serine proteinase inhibitor 3,4-dichloroisocoumarin, the metalloproteinase inhibitors EDTA and 1,10 phenanthroline and the aspartyl proteinase inhibitor pepstatin. Leupeptin was without effect whereas the peptide aldehyde Cbz-Gly-Gly-Leucinal, designed as an inhibitor of the chymotrypsinlike activity (3) moderately inhibited hydrolysis of oxidized insulin B chain (Table 4).

The dialysis activated enzyme did not hydrolyze α-casein, α-crystallin or lysozyme as determined by gel electrophoretic analysis of incubation mixtures following prolonged incubation (data not shown).

N-terminal sequence analysis of the 24 kDa and 21 kDa bands - The N-terminus of the 24 kDa band was not blocked and therefore could be directly sequenced. The sequence was found to be identical in 13/13 positions to the bovine lens MPC L2 subunit (28), and identical in 12/12 positions of the lowest molecular mass band of the human erythrocyte enzyme (subunit ε) (29). A close relationship was also found to the recently cloned rat liver RC1 subunit (30), mouse BALB/c B cell lymphoma subunit (31) and human Ring 10 gene (14) (13/15 positions for all three) and to the rat liver subunit 6 (32) (11/15 positions) (Table 5). N-terminal sequence analysis of the 21 kDa band demonstrated that it was autolytically derived from the 24 kDa subunit as suggested by gel electrophoretic analysis of the dialyzed enzyme (Fig. 3).

DISCUSSION

Recent studies conducted in this laboratory provided evidence that the initial degradation of β-casein by bovine pituitary MPC is catalyzed by an active site distinct from the previously defined chymotrypsinlike, trypsinlike and PGP activities (16,17). The degradation of proteins such as β-casein by MPC is said to be a latent activity requiring in some preparations various treatments for activation (20,33). The trypsinlike and chymotrypsinlike activities are fully expressed even in so-called latent preparations, whereas the PGP activity in preparations that are latent or activated with respect to casein degradation can be further stimulated by SDS (3, 20). Dick et al., (19) reported that the hydrolysis of the oxidized insulin B chain by MPC was also a latent activity. To be expressed it required dialysis against distilled H_2O or against a pH 7.2, 10 mM Tris-HCl buffer containing 0.5mM DTT. Our studies extend these findings. Removal of EDTA by dialysis produced marked changes in both the structure and profile of catalytic activities of purified bovine pituitary MPC. Although dialysis markedly stimulated the hydrolysis of oxidized insulin B chain as reported, the properties of bovine pituitary MPC following dialysis were significantly different from the preparation described by Dick et al. A striking difference was that our preparation after 15 h dialysis against a 0.05M Tris-HCl buffer, pH 8.0 lost essentially all of its trypsinlike, chymotrypsinlike and PGP activities. This is reflected in the HPLC profile of the incubation mixture of MPC with

oxidized insulin B chain where a single cleavage predominates (Fig. 1B) in contrast to the more complex HPLC profile obtained by Dick et al. The temporal changes in the loss of catalytic activities following removal of EDTA by dialysis parallel changes in subunit structure, i.e. loss of a 24 kDa subunit and appearance of a 21 kDa band (Fig 4 A-C; Fig 3). Activation of oxidized insulin B chain hydrolysis appears to somewhat precede the subunit changes and the loss of other catalytic activities. N-terminal sequence analysis confirms that the structural modification leading to the disappearance of the 24 kDa subunit, and the appearance of a new 21 kDa component is due to autolysis.

The effect of proteinase inhibitors on oxidized insulin B chain hydrolysis distinguishes this activity from the chymotrypsinlike and peptidylglutamyl peptide bond hydrolyzing activities. The latter two are inhibited by 3,4-dichloroisocoumarin at the concentration used (16, 22) whereas the oxidized insulin B chain degrading activity is slightly stimulated by this reagent. Leupeptin, a strong inhibitor of the trypsinlike activity (2,3), also does not affect the oxidized insulin B chain degrading activity. Of the inhibitors tested, the most effective were the cysteine blocking reagents. The lack of stimulation by dithiothreitol and the rather weak inhibition by iodoacetamide make it unlikely that this activity is a classical cysteine proteinase.

The mechanisms underlying the dialysis induced alterations in the properties of MPC remain to be determined. Removal of EDTA is

required, and the dialysis membrane itself is also involved since the enzyme is unchanged when EDTA is removed by ultrafiltration. Dialysis-induced changes are also optimal at an alkaline pH and favored by an increase in the buffer molarity. One explanation consistent with these observations is that autolysis is metal ion catalyzed. The dialysis membrane may serve as the source of the metal ion or alternatively the metal ion may be derived from the buffer solution and the dialysis membrane may provide a surface to facilitate catalysis. However we could not reproduce these changes by dialyzing the enzyme against Tris EDTA buffers containing excess Mg^{++}, Ca^{++} or Zn^{++}. Studies on the effect of other metal ions are ongoing.

Analysis of the dialyzed enzyme either by gel electrophoresis under non-dissociating conditions or by FPLC on Superose or Mono Q columns revealed that only a fraction of the total protein in solution remained as the undissociated macromolecule. Since all of the oxidized insulin B chain degrading activity was associated with the undissociated macromolecule, the specific activity of the dialysis activated preparations expressed in terms of total protein in solution is considerably underestimated. When the dialyzed enzyme was examined by SDS-PAGE, no loss in the staining intensities of the subunits was seen. The loss of undissociated complex is therefore not due to autolysis to small peptides. The autolytic changes following dialysis are associated with a destabilization of the quaternary structure of the complex resulting in dissociation

and/or aggregation of protein (Fig. 7). However loss of the chymotrypsinlike, trypsinlike and PGP activities appears to occur prior to gross structural changes. Thus at early times after dialysis the molecule was largely undissociated whereas the chromogenic activities were markedly depressed. Therefore structural modifications within the undissociated macromolecule are sufficient to cause the loss of these activities.

The N-terminal sequence of the autolyzed subunit displays strong homology to several other N-terminal MPC sequences reported in the literature and to a sequence contained in the cloned human ring 10 gene (14), rat liver RC1 subunit (30), and mouse BALB/c B cell (31) (Table 5). Although the primary sequences of cloned MPC subunits bear no homology to known proteinases, the ring 10 sequence has structural motifs found in the subtilisin family of serine proteinases (14). According to its N-terminal sequence, 24 kDa subunit is of the β-type, proposed as a catalytic subunit (8). Two studies employing radiolabeled DFP have correlated the chymotrypsinlike activity with the smallest MPC subunit (34, 35). Although the 24 kDa subunit is the smallest one in our preparation, sequence data is necessary to determine its identity to the radiolabelled subunits.

The relationship of the active site of the oxidized insulin B chain degrading activity to the other defined active sites remains to be determined. Since the enzyme undergoes structural changes during dialysis, one cannot rule out the possibility that the oxidized

insulin B chain degrading activity results from a modification of one of the other previously defined active sites leading to a change in specificity. The PGP activity would appear to be a likely candidate since oxidized insulin B chain is primarily cleaved after a glutamyl residue. This possibility however is rendered unlikely by observations that clearly distinguish the oxidized insulin B chain degrading activity from the PGP activity. Thus the PGP activity is inhibited by the mechanism based serine proteinase inhibitor 3,4-dichloroisocoumarin (16, 22), whereas the cleavage of oxidized insulin B chain by the dialyzed enzyme is mildly stimulated by this reagent (Table 4). SDS in contrast to its marked stimulation of the PGP activity, inhibits oxidized insulin B chain hydrolysis.

Although oxidized insulin B chain may be an appropriate model substrate for the caseinolytic activity as proposed (19), it should be pointed out that under our conditions of dialysis, the hydrolysis of β-casein is unaffected, whereas the hydrolysis of oxidized insulin B chain is markedly stimulated. The relationship of these two activities remains to be determined. Native MPC has little activity toward oxidized insulin B chain. Therefore the trypsinlike, chymotrypsinlike and PGP activities behave as oligopeptidases directed toward peptides smaller than 30 amino acids. We have proposed that these activities are involved in the final stages of protein degradation by MPC (18).

These studies have demonstrated a series of changes in the structure and catalytic properties of MPC following dialysis. With

respect to the temporal ordering of these changes, the results presented in fig. 6 demonstrate that disruption of the quaternary structure of the complex is the final event. It is however not possible to determine with certainty from the data presented whether the activated oxidized insulin B chain degrading activity is responsible for autolysis of the 24 kDa subunit or whether activation is a consequence of the autolytic changes. The data presented in fig. 4 suggest that activation precedes autolysis and that autolysis is associated with the loss of the other activities. It is of interest that we (16) and others (20, 36, 37) have also observed occasional loss of the heaviest subunit of MPC, and this loss has been associated with an increase in caseinolytic activity (37). Autolytic modification of MPC may represent a mechanism for regulation of the catalytic activities of MPC as is the case for calpain (38). Further studies will be necessary to determine whether autolysis is more than an *in vitro* phenomenon. A proposal that autolysis is important for the regulation of MPC levels in eukaryotic cells was made by Tanaka and Ichihara on the basis of their experiments on urea-induced autolysis (39).

REFERENCES

1. Wilk, S., and Orlowski, M.
   Cation-sensitive neutral endopeptidase : Isolation and specificity of bovine pituitary enzyme.
   J. Neurochem., 35: 1172-1182. (1980)

2. Orlowski, M. and Wilk, S
   A multicatalytical protease complex from pituitary that forms enkephalin and enkephalin containing peptides
   Biochem. Biophys. Res. Comm. 101: 814-822 .(1981)

3. Wilk, S., and Orlowski, M.
   Evidence that pituitary cation-sensitive neutral endopeptidase is a multicatalytic protease complex.
   J. Neurochem., 40: 842-849. (1983)

4. Orlowski, M.
   The multicatalytic proteinase complex, a major extralysosomal proteolytic system.
   Biochemistry, 29: 10289-10297. (1990)

5. Rivett, J. A.
   The multicatalytic proteinase of mammalian cells
   Arch. Biochem. Biophys., 268:1 - 8. (1989)

6. Baumeister, W., Dahlmann, B., Hergerl, R., Kopp, F., Kuehn, L. & Pfeifer, G.
   Electron microscopy and image analysis of the multicatalytic proteinase
   FEBS letter, 241: 239-245. (1988)

7. Puhler, G. et al.

Subunit stoichiometry and three-dimensional arragement in proteasomes from *Thermoplasma acidophilum*
The EMBO Journal , 11:1607-1616. (1992)

8. Zwickl, P., Grziwa, A., Puhler, G., Dahlman, B., Lottspeich, F.and Baumeister, W.
Primary structure of the *Thermoplasma* proteasome and its implications for the structure, function, and evolution of the multicatalytic proteinase
Biochemistry, 31: 964-972. (1992)

9. Hough, R., Pratt, G. and Rechsteiner, M.
Purification of two high molecular weight proteases form rabbit reticulocyte lysate.
J. Biol. Chem., 262: 8303-8313. (1987)

10. Eytan, E., Ganoth, D., Armon, T., Hershko, A.
ATP-dependent incorporation of 20S protease into the 26S complex that degrades proteins conjugated to ubiquitin.
Proc. Natl. Acad. Sci. USA, 86: 7751-7755. (1989)

11. Driscoll, J. and Goldberg, A. L.
The proteasome (multicatalytic proteinase) is a component of the 1500-kDa proteolytic complex which degrades ubiquitin-conjugated proteins.
J. Biol. Chem., 265, 4789-4792. (1990)

12. Kuehn, L., Dahlmann, B. and Reinauer, H.
Evidence indicating that the multicatalytic proteinase of rabbit reticulocytes is not incorporated as a core enzyme into a 26S proteinase complex.
Arch. Biochem. Biophys., 295: 55-60. (1992)

13. Brown, M. G., Driscoll, J. and Monaco, J.J.

Structural and serological similarity of MHC-linked LMP and proteasome (multicatalytic proteinase) complex.
Nature, 353: 355-357. (1991)

14. Glynne, R., Powis, S. H., Beck, S., Kelly, A., Kerr, L-A. and Trowsdale, J.
A proteasome-related gene between the two ABC transporter loci in the class II region of the humen MHC.
Nature, 352: 357-360. (1991)

15. Ortiz-Navarette, V., Seelig, A., Gernold, M., Frentzel, S., Kloetzel, P.M. and Hammerling, G.J.
Subunit of the '20' proteasome (multicatalytic proteinase) encoded by the major histocompatibility complex.
Nature, 353: 662-664. (1991)

16. Pereia, M.E., Nguyen, T., Wagner, B. J., Margolis, J. W., Yu, B. and Wilk, S.
3,4-Dichloroisocoumarin-induced activation of the degradation of β-casein by the bovine pituitary multicatalytic proteinase complex.
J. Biol. Chem., 267: 7949-7955. (1992)

17. Yu, B., Pereira, M.E., and Wilk, S.
Chemical Modification of The Bovine Pituitary Multicatalytic Proteinase Complex by N-acetylimidazole: Reversible Activation of Casein Hydrolysis
J. Biol. Chem., 266: 17396-17400. (1991)

18. Wilk, S., Pereira, M.E. and Yu, B.
Probing the specificity of the bovine pituitary multicatalytic proteinase complex by inhibitors, activators, and by chemical modification.
Biomed. Biochim. Acta, 50: 471-478. (1991)

19. Dick, L. R., Moomaw, C. R., DeMartino, G. N. and Slaughter, C. A.
Degradation of oxidized insulin B chain by the multiproteinase complex Macropain (proteasome).
Biochemistry, 30: 2725-2734. (1991)

20. McGuire, M. J., McCullough, M. L., Croall, D. E. and Dematino, G. N.
The high molecular weight multicatalytic proteinase, macropain, exists in a latent form in human erythrocytes.
Biochim. Biophys. Acta, 995: 181-186. (1989)

21. Wilk, S., Pearce, S. and Orlowski, M.
Identification and partial purification of a cation-sensitive neutral endopeptidase from bovine pituitary.
Life Sci., 24: 457 - 464. (1979)

22. Orlowski, M. and Michaud, C.
Pituitary multicatalytic proteinase complex. specificity of components and aspects of proteolytic activity
Biochemistry, 28: 9270-9278. (1989)

23. Wilk, S. and Thurston, L.S.
Inhibition of angiotensin III formation by thiol derivatives of acidic amino acids.
Neuropeptides, 16: 163-1623. (1990)

24. Laemmli, U.K.
Cleavage of structural proteins during the assembly of the head of bacteriophage.
Nature, 227: 680-685. (1970)

25. Pereira, M.E., Yu, B. and Wilk, S.

Enzymatic changes of the bovine pituitary multicatalytic proteinase complex, induced by magnesium ions.
Arch. Biochem. Biophys., 294: 1-8. (1992)

26. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.
Protein measurment with the folin phenol reagent.
J. Biol. Chem., 193: 265-275. (1951)

27. Rivett, J. A.
Purification of a liver alkaline protease which degrades oxidatively modified glutamine synthetase. Characterization as a high molecular weight cysteine proteinase.
J. Biol. Chem. 260:12000-12606. (1985)

28. Shivanandappa, T., Margolis, J.W. and Wagner, B.J.
Conserved N-terminal sequences in homologous subunits of the multicatalytic proteinase complex (proteasome)
Curr. Eye Res. 10: 871-876. (1991)

29. Lee, L.W., Moomaw, C.R., Orth, K., McGuire, M.J., DeMartino, G. and Slaughter, C.
Relationships among the subunits of the high molecular weight proteinase, macropain (proteasome).
Biochim. Biophys. Acta, 1037: 178-185. (1990)

30. Aki, M., Tamura,T., Tokunaga, F., Iwanaga, S., Kawamura, Y., Shimbara, N., Kagawa, S., Tanaka, K. and Ichihara, A.
cDNA cloning of rat proteasome subunit RC1, a homologue of Ring 10 located in the human MHC class II region.
FEBS, 301: 65-68. (1992)

31. Frentzel, S., Graf, U., Hammerling, G.J. and Kloetzel, P-M.
Isolation and characterization of the MHC linked b-type proteasome subunit MC13 cDNA

FEBS. 302: 121-125. (1992)

32. Lilley, K.S., Davison, M.D. and Rivett, A.J.
N-terminal sequence similarities between components of the multicatalytic proteinase complex.
FEBS Lett. 262, 327-329. (1990)

33. Tanaka, K., Ii, K., Ichihara, A., Waxman, L., Goldberg, A. L.
A high molecular weight protease in the cytosol of rat liver. I Purification, enzymological properties and tissure distribution.
J. Biol. Chem. . 261:15197-15203 (1986)

34. Sato, S. and Shiratsuchi, A.
'Chymotrypsin-like' activity of chicken liver multicatalytic proteinase resides in the smallest subunit.
Biochim. Biophys. Acta. 1041: 269-272. (1990)

35. Wagner, B.J., Margolis, J.W. and Yin, J.
Covalent labelling of bovine lens multicatalytic proteinase complex with ^{3}H diisopropyl fluorophosphate.
Curr. Eye Res. 10: 485-489. (1991)

36. Rivett, A.J. and Sweeney, S.T.
Properties of subunits of the multicatalytic proteinase complex revealed by the use of subunit-specific antibodies.
Biochem. J. 278: 171-177. (1991)

37. Weitman, D. and Etlinger, J. D.
A monoclonal antibody that distinguishes latent and active forms of the proteasome (multicatalytic proteinase complex)
J. Biol. Chem., 267: 6977-6982. (1992)

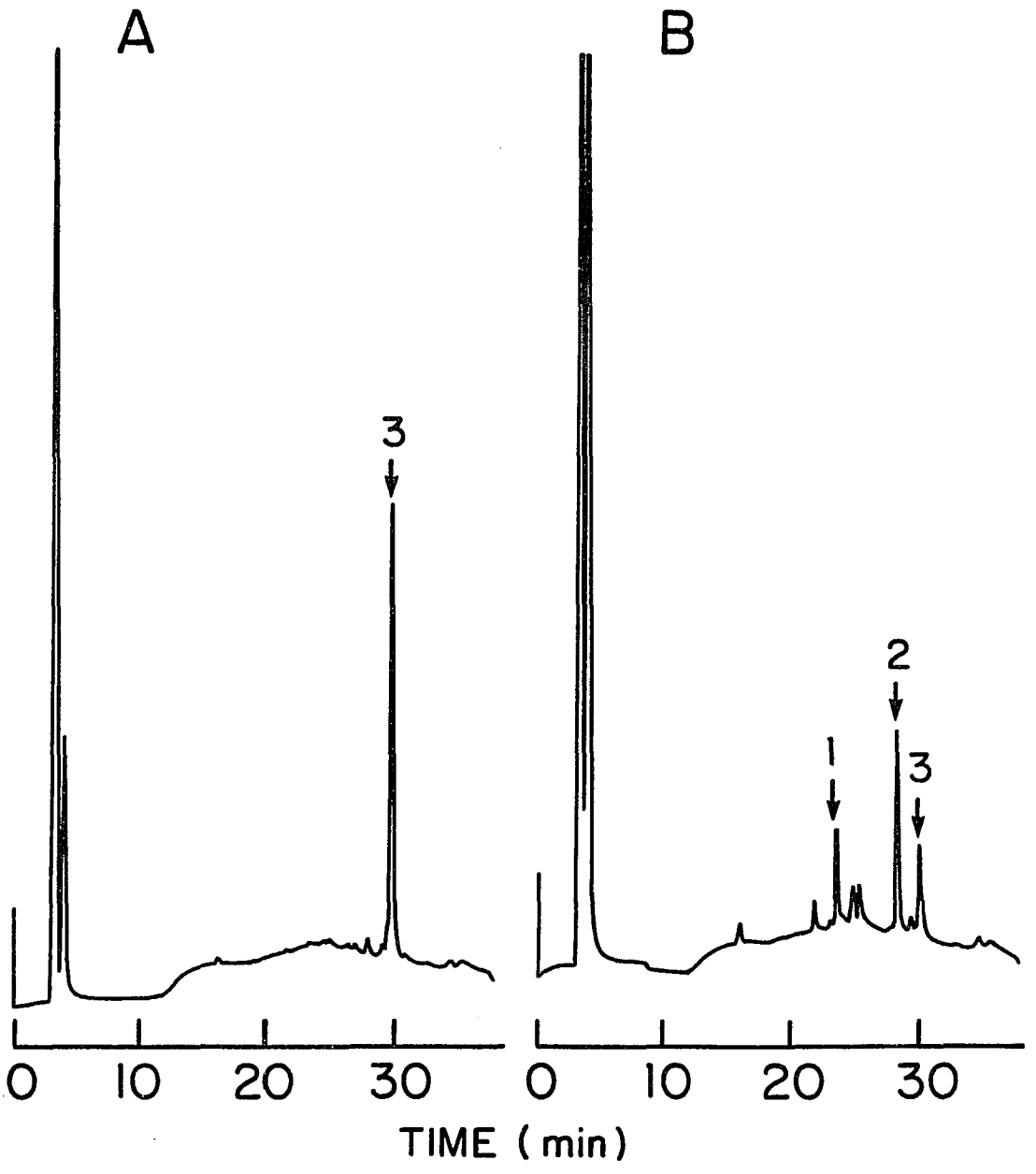
38. Suzuki, K., Tsuji, S., Kubota, S., Kimura, Y. and Imahori, K.

Limited autolysis of Ca^{2+}-activated neutal protease (CANP) changes its sensitivity to Ca^{2+}-ions.
J. Biochem., 90: 275-278. (1981)

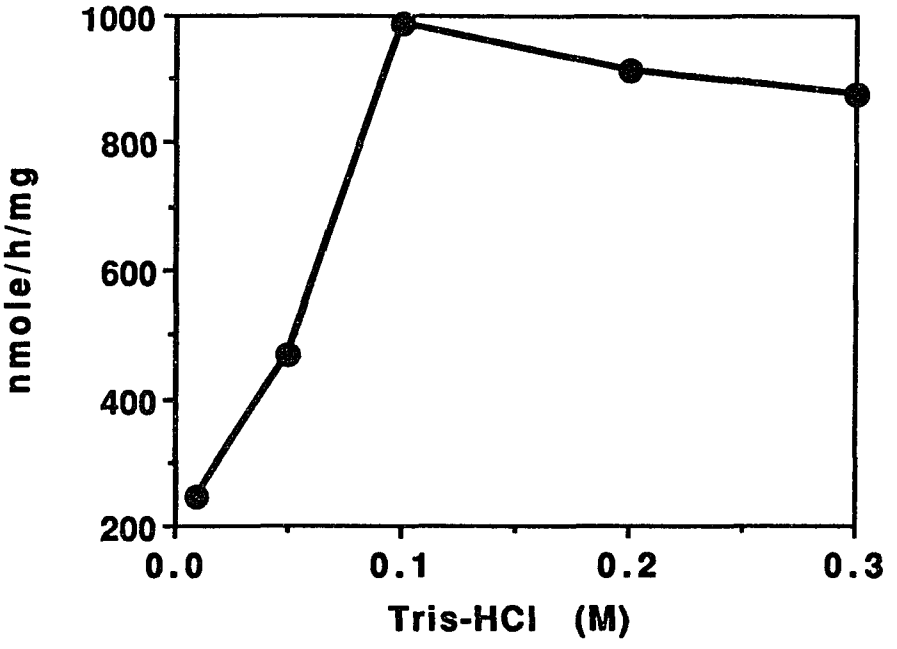
39. Tanaka, K. and Ichihara, A.
Autodegradation of rat liver proteasomes (large multicatalytic proteinase complex).
Biochem. Biophys. Res. Comm., 158: 548-554. (1989)

1A. HPLC chromatogram of a mixture of 16 µg MPC in 0.01M Tris-EDTA buffer, pH 7.5 and 32 µg oxidized insulin B chain after 30 min incubation at 37°C. Chromatographic conditions and composition of incubation mixture as described in Methods. Detector sensitivity: 0.05 AUFS. Peak 3: oxidized insulin B chain 1-30.

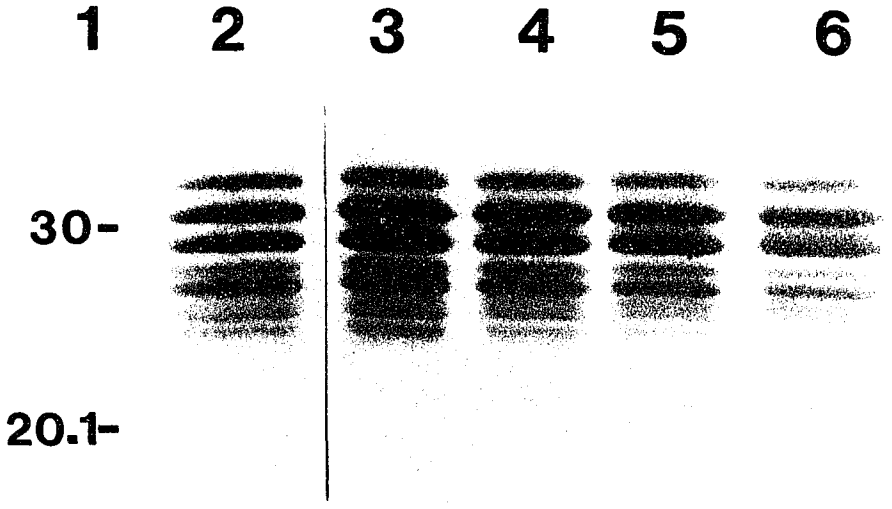
1B. HPLC chromatogram of a mixture of 16 µg MPC (from above experiment) dialyzed against a 0.05M Tris-HCl buffer, pH 8.0 and 32 µg oxidized insulin B chain after 30 min incubation at 37°C. Chromatographic conditions as above. Peak 1: oxidized insulin B chain 1-13. Peak 2: oxidized insulin B chain 14-30. Peak 3: oxidized insulin B chain 1-30.


A
B
3
1
2
3
0
10
20
30
0
10
20
30
TIME (min)

2. Oxidized insulin B chain hydrolyzing activity as a function of the molarity of Tris-HCl buffers. MPC purified as described in Methods was dialyzed against Tris-HCl buffers of pH 8.0 for 5 h, and then assayed for oxidized insulin B chain degrading activity as described in Methods.



3. SDS-PAGE of MPC as a function of time of dialysis. MPC purified as described in Methods was dialyzed against a 0.05M Tris-HCl buffer, pH 8.0. At the time periods indicated, 9.6 μg were subjected to SDS-PAGE as described. Lane 1 indicates position of molecular mass markers; Lane 2, no dialysis; Lane 3, 3h dialysis; Lane 4, 6h dialysis; Lane 5, 9h dialysis; Lane 6, 20 h dialysis.



4. Changes in the catalytic activities of MPC and its SDS-PAGE profile as a function of time of dialysis. MPC prepared as described in Methods was dialyzed against a 0.05M Tris-HCl buffer, pH 8.0. Aliquots of enzyme were withdrawn at the time periods shown, assayed for enzymatic activity and subjected to SDS-PAGE.

A. Changes in 21kDa and 24 kDa bands. Proteins quantitated by image analysis as described in Methods.

--◍-- 21 kDa band

--o-- 24 kDa band

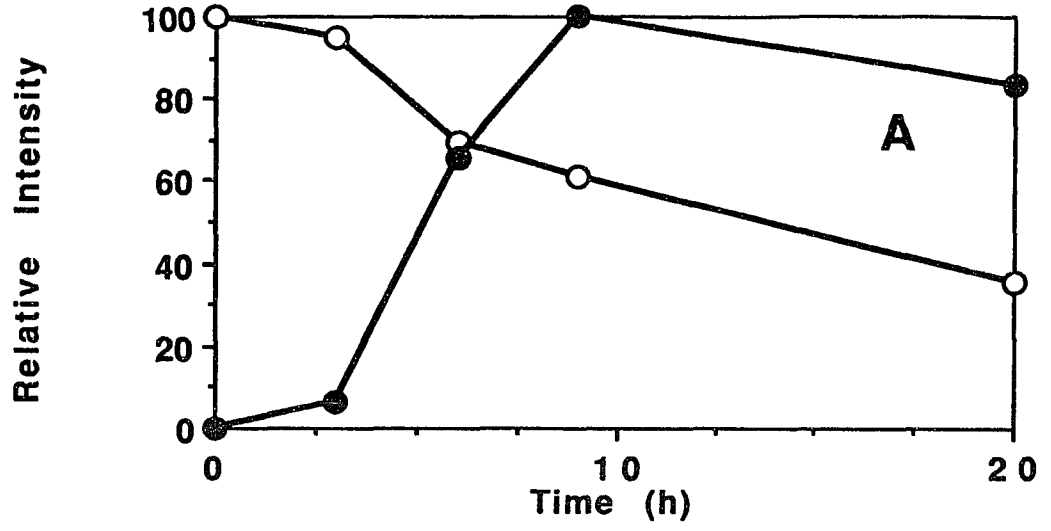
B. Changes in the chymotrypsinlike, trypsinlike and PGP activities of MPC as a function of time of dialysis. Activities measured with chromogenic substrates as described in Methods.

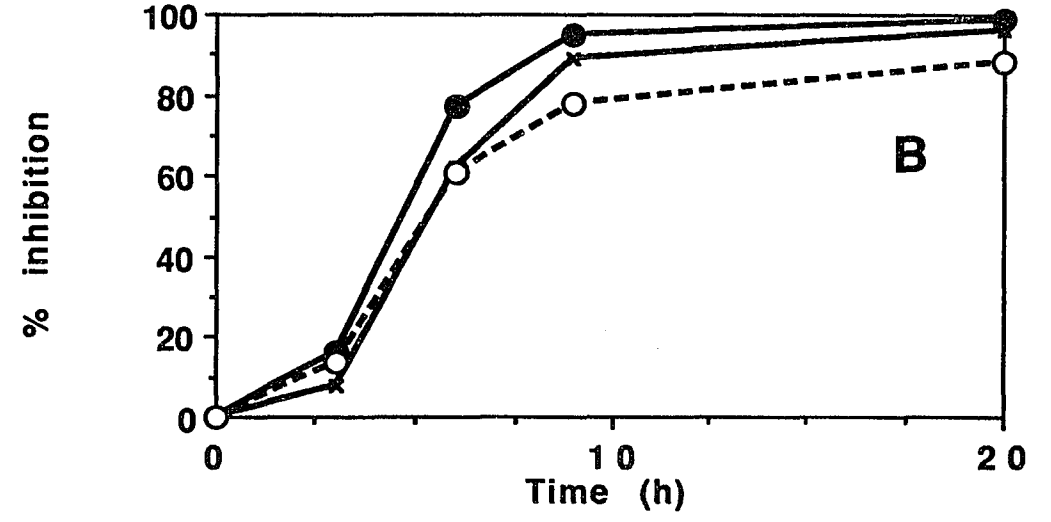
--x-- chymotrypsinlike activity

--●-- trypsinlike activity

--O-- PGP activity

C. Changes in the oxidized insulin B chain hydrolyzing activity as a function of time of dialysis. The hydrolysis of oxidized insulin B chain was determined by HPLC as described in Methods.


Relative Intensity
100
80
60
40
20
0
A
0
10
20
Time (h)


% inhibition
100
80
60
40
20
0
B
0
10
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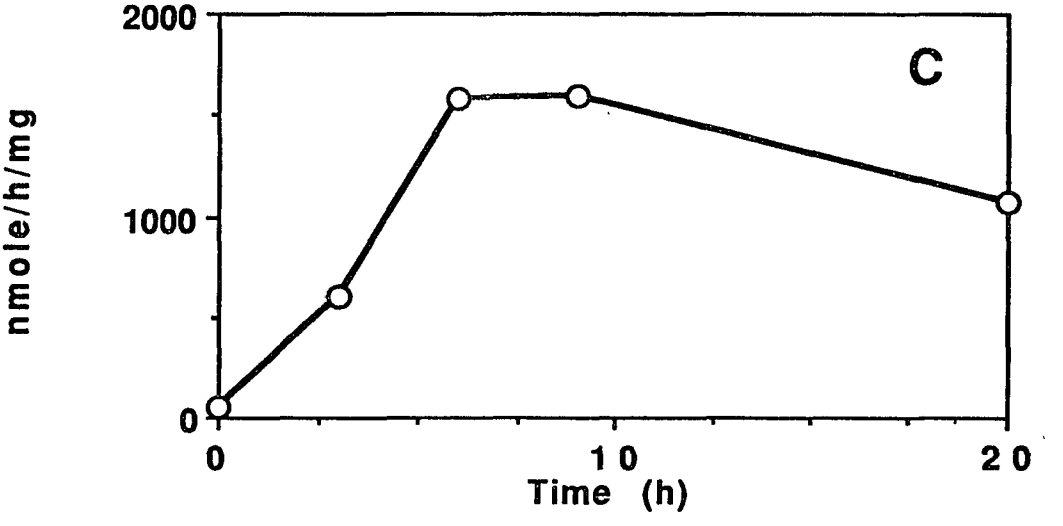
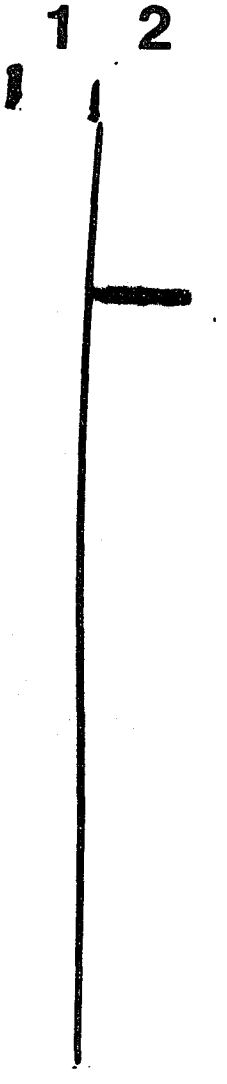
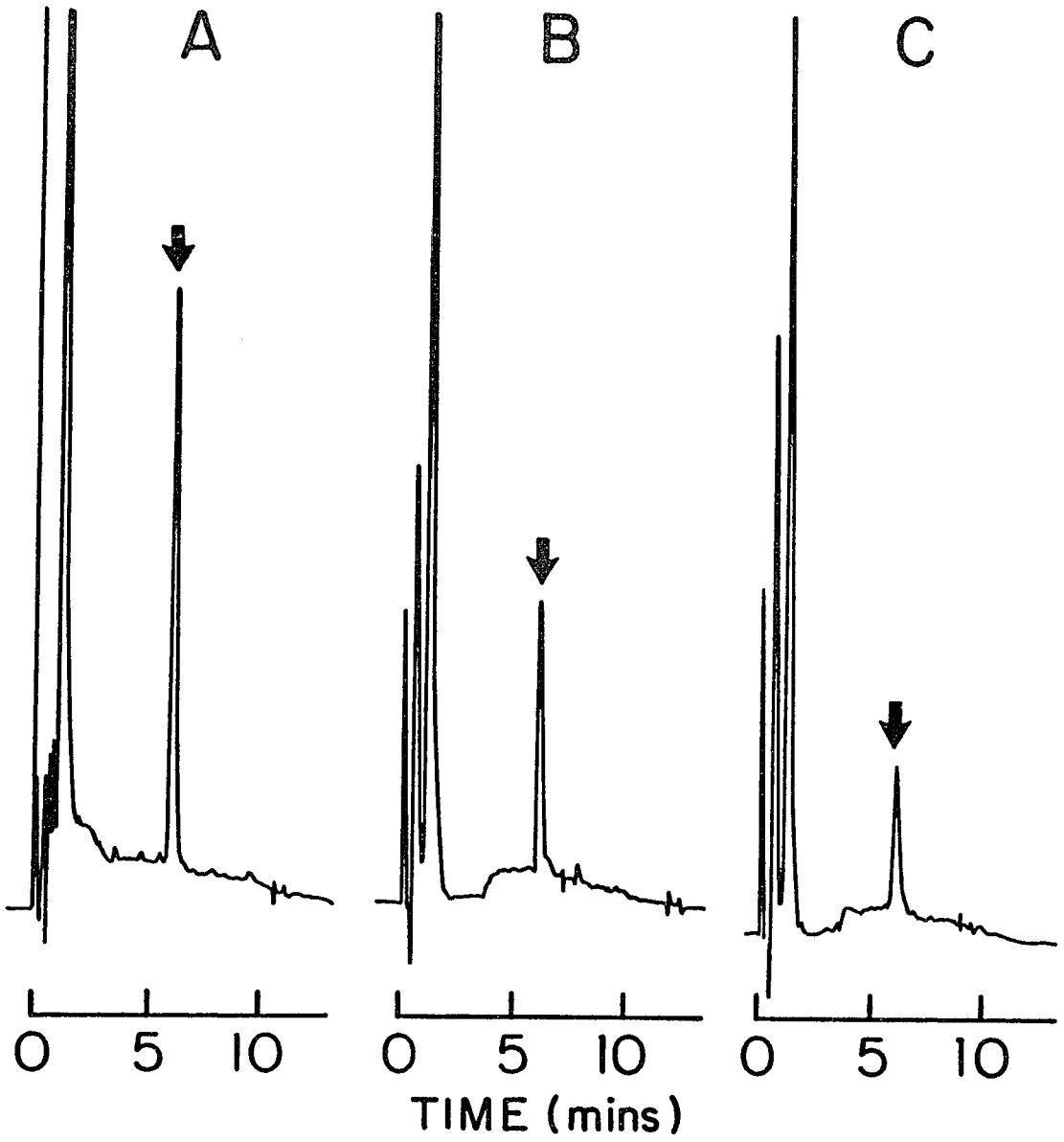
Time (h)


nmole/h/mg
2000
1000
0
C
0
10
20
Time (h)

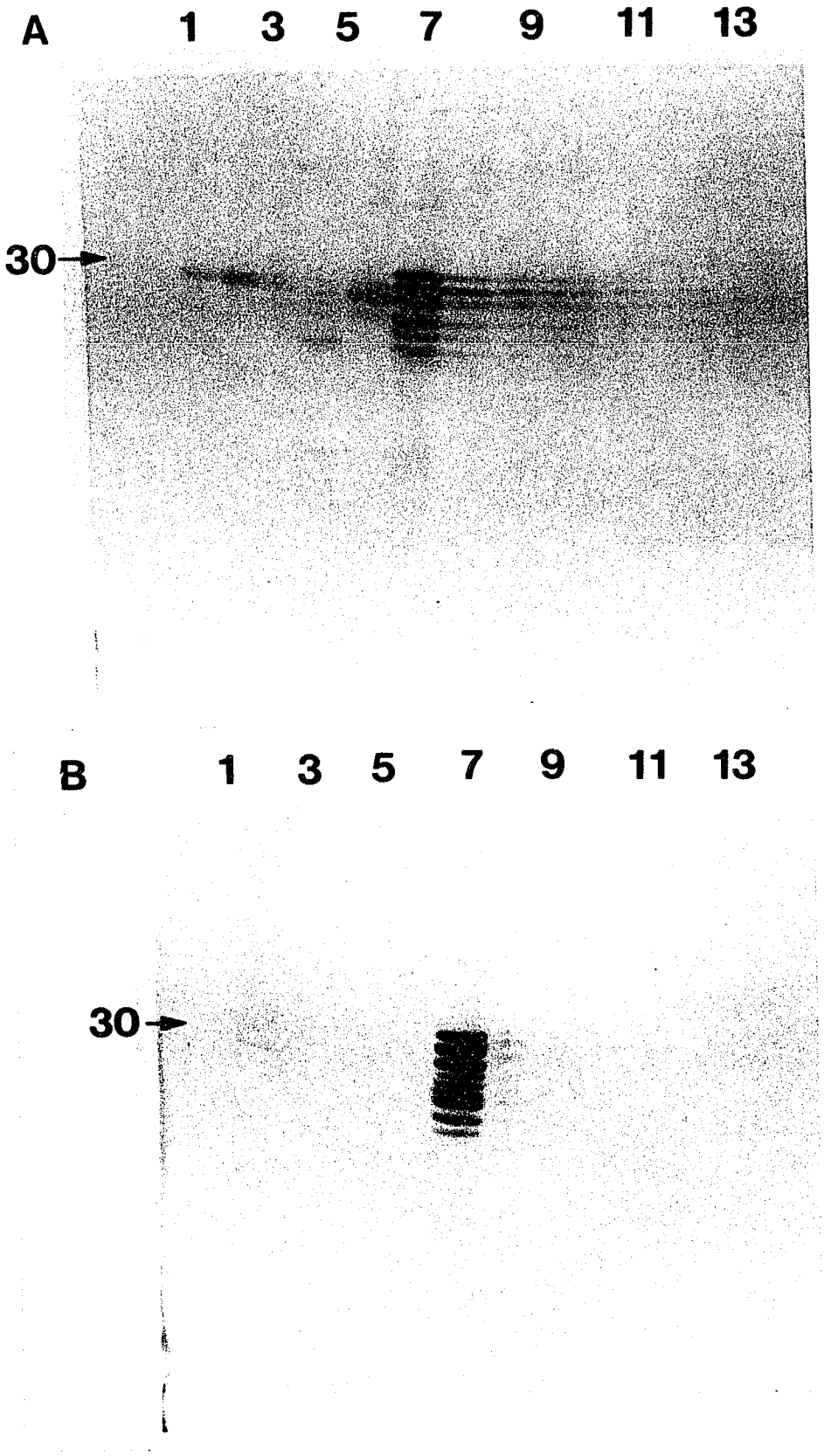
5. Non-dissociating gel electrophoresis of MPC before and after 15 h dialysis against a 0.05 M Tris-HCl buffer, pH 8.0. Lane 1, 16 μg dialyzed MPC. Lane 2, 16 μg MPC prior to dialysis. Gels were stained with Coomassie blue.



6. Mono Q chromatography of MPC before and after dialysis against 0.1M Tris-HCl buffer, pH 8.0. A: MPC (17 µg) prior to dialysis. B: MPC (17 µg after 4 h dialysis. C: MPC (17 µg) after 8 h dialysis. FPLC conditions as described in Methods. Ordinate: absorbance at 280 nm.



7. SDS-PAGE of fractions eluted from a Mono Q column. MPC (170 μg) was dialyzed for 15 h against a 0.05 M Tris-HCl buffer, pH 8.0, and injected onto a Mono Q column. 2 ml fractions were collected and subjected to SDS-PAGE. Proteins were visualized by silver staining. Panel A: enzyme prior to dialysis. Panel B: enzyme after dialysis. Lane 1 corresponds to the first 2 ml of eluate. Lane 7 corresponds to the peak of undissociated enzyme. The numbers on the left are the position of molecular mass marker.


A
1 3 5 7 9 11 13
30
B
1 3 5 7 9 11 13
30

8. HPLC chromatograms of incubation mixtures of native MPC (16 μg) (A) and MPC dialyzed for 15 h against a 0.05M Tris-HCl buffer, pH 8.0 (B), and Boc-Val-Glu-Leu-Ala-NA (65μg). Samples incubated for 30 min. at 37°C. Chromatographic conditions as described in Methods. Detector sensitivity 0.05 AUFS. Peak 1: Ala-Leu-NA; Peak 2: Boc-Val-Glu-Ala-Leu-NA.

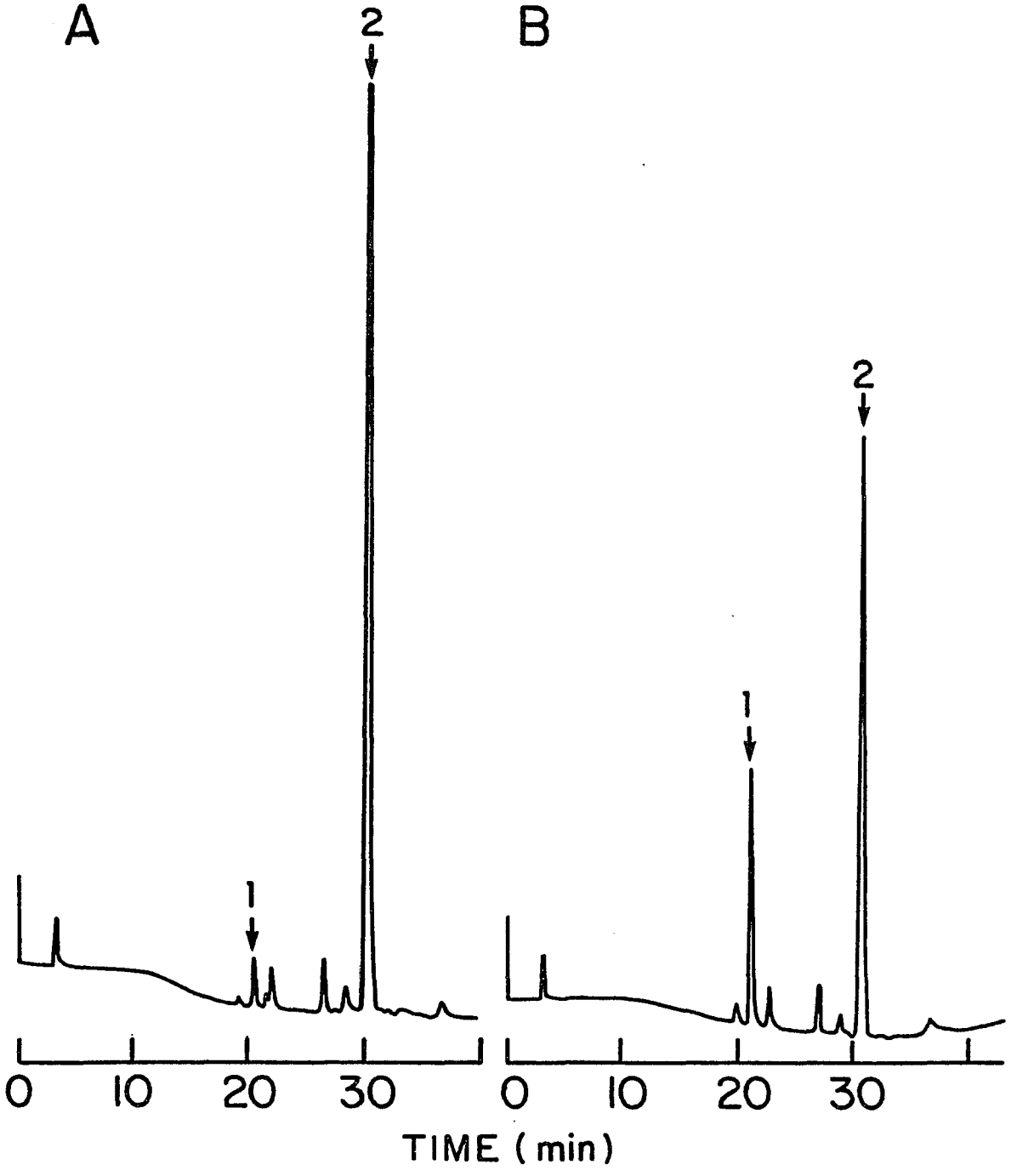

A
2
B
2
1
1
0
10
20
30
0
10
20
30
TIME (min)

Table 1

Effect of dialysis on the catalytic activities of MPC defined by hydrolysis of chromogenic substrates

	Specific Activity (μmole/h/mg)	
	MPC[b]	Dialyzed MPC[c]
Chymotrypsinlike[a]	3.8 ± 0.7	0.14 ± 0.02
Trypsinlike	2.2 ± 0.2	0.14 ± 0.02
Peptidylglutamyl peptide bond hydrolyzing	2.7 ± 0.5	0.39 ± 0.06
Peptidylglutamyl peptide bond hydrolyzing + SDS (0.04%)	49.0 ± 3.9	1.10 ± 0.28

a. The chymotrypsinlike activity was determined with Cbz-Gly-Gly-Leu-pNA, the trypsinlike activity with Cbz-D-Ala-Leu-Arg-NA and the peptidylglutamyl peptide bond hydrolyzing activity with Cbz-Leu-Leu-Glu-NA as described in Methods. b. MPC was purified as described in Methods. The enzyme was present in a 10mM Tris-EDTA buffer (pH 7.5). c. MPC was dialyzed for 15 h against a Tris-HCl buffer (0.05M, pH 8.0). Each value is expressed as the mean ± S.E.M., N = 6 to 10.

Table 2
Effect of dialysis buffers on the catalytic activities of MPC

Dialysis Buffer	RELATIVE ACTIVITY			
	Ins. $B_{[ox]}$[a]	Chy	Try	PGP
0.01M Tris-EDTA				
pH 7.5	100	100	100	100
pH 8.0	87	88	89	90
0.05M Tris-EDTA				
pH 7.5	135	99	101	102
pH 8.0	56	95	112	105
0.01M Tris-HCl				
pH 7.5	965	52	65	68
pH 8.0	982	20	29	43
0.05M Tris-HCl				
pH 7.5	1083	11	18	38
pH 8.0	1252	3	6	13
0.05M Tris-Acetate				
pH 8.0	1407	19	17	32
0.05M Tris-H_3PO_4				
pH 8.0	928	11	18	33
0.05M Tris-Citrate				
pH 8.0	1184	16	34	20

MPC purified as described in Methods and present in a 10 mM Tris-EDTA buffer, pH 7.5, was dialyzed for 15 h against the buffers shown.

a) Abbreviations: Ins. $B_{[ox]}$, oxidized insulin B chain degrading activity; Chy, chymotrypsinlike activity; Try, trypsinlike activity;

PGP, peptidylglutamyl peptide bond hydrolyzing activity. Activities determined as described in Methods. Activities expressed relative to the activities of MPC in 0.01 M Tris-EDTA buffer, pH 7.5, which have been assigned values of 100. The data represent single experiments.

Table 3

Comparison of the hydrolysis of oxidized Insulin B chain, Boc-Val-Glu-Ala-Leu-NA and Cbz-Leu-Leu-Glu-NA by MPC preparations

	Activity (nmol/h/mg)		
Enzyme Preparation	Insulin Box	Boc-VEAL-NA	Cbz-LLE-NA
MPC in 0.01M Tris-EDTA pH 7.5	156 ± 37	204 ± 10	2700 ± 500
MPC dialyzed against 0.05M Tris-HCl, pH 8.0	1020 ±160	1230 ± 44	390 ± 60

Values expressed as mean ± S.E.M. of the following number of determinations: for Insulin Box N=6; for Boc-VEAL-NA, N=2; for Cbz-LLE-NA, N=10. Activities determined as described in methods.

Table 4

Effect of inhibitors on the degradation of oxidized insulin B chain by dialysis activated multicatalytic proteinase complex

Inhibitor	Conc. (mM)	Relative Activity
None		100
N-ethylmaleimide	0.2	45
PCMB	0.4	7
iodoacetamide	5	80
3,4-dichloroisocoumarin	0.004	121
EDTA	2.7	100
leupeptin	0.1	96
Cbz-Gly-Gly-Leucinal	0.23	60
SDS	1.38	23
dithiothreitol	1	100
1,10-phenanthroline	0.5	96
pepstatin	0.04	106
α1-antichymotrypsin	0.001	94

MPC was purified as described in Methods and dialyzed for 15 h against a 0.05 M Tris-HCl buffer, pH 8.0. Hydrolysis of oxidized insulin B chain was determined by HPLC as described in Methods. Inhibitors were tested without preincubation.

Table 5

Comparison of the N-terminal sequence of the 24 kDa subunit to the N-terminal sequences of the 21 kDa fragment and other MPC subunits

Sources	Amino Acid Sequence	Identities
Bovine Pituitary 24 kDa subunit	T-T-T-L-A-F-K-F-R-H-G-V-I-V-A-A	
Bovine Pituitary 21 kDa fragment	X-X-X-L-A-F-K-F-R-H-G-V-I-V-A-A	13/13
Bovine lens L2 (28)	X-X-X-L-A-F-K-F-R-H-G-V-I-V-A-A	13/13
Human Ring 10 T-cell (15)	T-T-T-L-A-F-K-F-**Q**-H-G-V-I-**A**-A-**V**	13/15
Rat Liver RC1 (30)	T-T-T-L-A-F-K-F-**Q**-H-G-V-I-V-A-**V**	13/15
Mouse BALB/c B cell lymphoma (31)	T-T-T-L-A-F-K-F-**Q**-H-G-V-I-V-A-**V**	13/15
Human erythrocyte ε (29)	X-X-X-L-A-F-K-F-R-X-G-V-I-V-A-A	12/12
Rat Liver Subunit 6 (32)	T-T-**I**-L-A-F-K-F-**Q**-**E**-G-V-I-**L**-A-X	11/15

Reference numbers given in parentheses. X designates an amino acid whose identity is uncertain. Non-equivalent amino acids indicated in bold type.

Chapter 4

Characterization of the caseinolytic activity and the DCI resistant component (unpublished results)

In chapter 2, I presented evidence showing that the caseinolytic activity is distinct from the other components (chymotrypsinlike, trypsinlike and PGP activities) of MPC. However, some properties of the caseinolytic component are unclear, such as its substrate specificity, and its mechanistic classification. Pereira et al. (1992) have provided strong evidence to show that the caseinolytic activity is not a serine proteinase. They showed that a serine proteinase inhibitor, 3,4-dichloroisocoumarin, not only did not inhibit the degradation of casein, but even activated casein degradation. I report here that the caseinolytic activity is also not a cysteine proteinase.

DCI can stimulate the caseinolytic activity of MPC (Pereira et al. 1992), as well as an activity cleaving peptide bonds after branched chain amino acids (Cardozo et al. 1992). Oxidized insulin B chain has been proposed to be a model substrate for protein degradation by Dick et al. (1991). It was interesting to see whether DCI treatment could change the hydrolysis of oxidized insulin B chain. The primary cleavage of this peptide is at the Glu^{13}-Ala^{14}bond. The data shown here indicate that hydrolysis of insulin B chain may be catalyzed by an activity distinct from the chymotrypsinlike, trypsinlike, PGP, and caseinolytic activities.

EXPERIMENTAL PROCEDUDURE:

Treatment of MPC with thiol blocking reagents and assay of enzyme activity:

N-ethylmaleimide (NEM) was dissolved in DMSO, and stored at -20°C. MPC (100μg/ml) in 0.01M Tris EDTA, pH 7.5, was treated with NEM at a final concentration of 1mM, for different periods of time at room temperature. The final concentration of DMSO was 4%. The treated enzyme then was dialyzed for 15h. at 4°C against a 0.01M Tris EDTA buffer, pH 7.5. A similar treatment was carried with iodoacetamide except that this reagent was freshly prepared in DMSO at a final concentration of 5 mM.

The peptidase and caseinolytic activities were measured under the conditions described in chapter 2.

Hydrolysis of oxidized insulin B chain by DCI-treated MPC and NAI treated MPC:

MPC was preincubated with DCI under the conditions described by Pereira (1992). Briefly, 4μM DCI was incubated with the enzyme (100μg/ml) in 0.01 M Tris EDTA buffer, pH 7.5, at room temperature for 30 min. N-acetylimidazole treated MPC was prepared as described in chapter 2. 8 μg of either the DCI treated MPC or NAI treated MPC (100 μg/ml) was then incubated with 12.5 μg oxidized insulin B chain (1mg/ml) in 0.01 M Tris EDTA, pH 7.5 and 1mM DTT buffer, for 60 min. The incubation mixture was assayed by HPLC as described in chapter 3. The retention times were used as a parameter for comparison.

RESULTS AND DISSCUSSION:

Effect of NEM on the catalytic activities of MPC:

MPC was preincubated with NEM for different periods of time as described in the experimental procedures. After removal of free NEM by dialysis, the catalytic activities were measured with synthetic chromogenic substrates (Z-Gly-Gly-Leu-pNA, Z-D-Ala-Leu-Arg-2NA and Z-Leu-Leu-Glu-2NA) and with dephosphorylated β-casein.

The trypsinlike activity was the most sensitive to NEM treatment. The chymotrypsinlike and PGP activities declined at slower rates (Fig. 1A). By contrast, the caseinolytic activity was stimulated by the same NEM treatment (Fig. 1B). A similar result was also observed with another thiol blocking reagent, iodoacetamide. A detailed study on the effect of NEM on the trypsinlike activity has been reported by Dick et al. (1992). They also observed that the trypsinlike component is the most sensitive to NEM.

These results provide further evidence in support of my proposal that the casenolytic activity is a distinct component. In addition, the results also demonstrate that the caseinolytic component is not a cysteine proteinase. The caseinolytic component cannot at present be classified within the known categories of proteinases.

The degradation of oxidized insulin B chain by DCI-treated MPC and N-acetylimidazole (NAI) treated MPC:

As 3,4-dichloroisocoumarin and N-acetylimidazole can activate the hydrolysis of β casein by MPC, it was of interest to see whether these treatments also increase the degradation of oxidized insulin B chain. I compared the HPLC profile of the degradation products of oxidized insulin B chain catalyzed by the DCI treated enzyme, the dialysis activated enzyme, the NAI treated enzyme and the native enzyme (fig 2). First, the results show that N-acetylimidazole treated enzyme does not hydrolyze oxidized insulin B chain at an increased rate (fig 2C), however, the DCI treated enzyme degrades oxidized insulin B chain at a rate at least as high as the dialysis activated enzyme (fig 2A and 2B). Table 1 summarizes the results of those treatments on the caseinolytic activity and hydrolysis of oxidized insulin B chain.

As shown in chapter 2, the hydrolysis of β-casein by the N-acetylimidazole treated enzyme was markedly activated. This treatment apparently only produced stimulation of an initial cleavage leading to the accumulation of an intermediate protein product. The results in this chapter show that the N-acetylimidazole treated enzyme does not have any increased activity toward oxidized insulin B chain (fig 2C and table 1).

Table 1 Comparison of the hydrolysis of casein and hydrolysis of oxidized insulin B chain by various MPC preparations

	Substrate	
MPC preparation	β casein	oxidized Insulin B chain
NAI	++++	+
DCI	++++	++++
Dialysis	+	++++
Native	+	+

DCI, 3,4-dichloroisocoumarin-treated enzyme; NAI, N-acetylimidazole-treated enzyme; dialysis, dialyzed MPC; native, native MPC; ++++, high activity; +, low activity.

In chapter 3, I showed that the dialyzed enzyme preparation could hydrolyze oxidized insulin B chain very rapidly (also see fig 2B, 2D and table 1). However, its ability to hydrolyze β casein was essentially unchanged (page 78).

These results taken together support the idea that the active site catalyzing the hydrolysis of oxidized insulin B chain may be distinct from the active site catalyzing the hydrolysis of β casein.

The results with DCI treated enzyme are more complicated. As shown in fig 2 A, the DCI treated enzyme hydrolyzes oxidized insulin

B chain at a very high rate. Pereira et al. showed that DCI-treated enzyme could activate the degradation of β casein, but without any intermediate accumulation (1992). Pereira at al. speculated that there might be some other unidentified components in the complex which were also activated by DCI and responsible for secondary degradation. The retention times of the products of the DCI treated and dialysis activated enzyme are identical, fig 2A and 2B demonstrating that both enzyme preparations cleave at the same sites. However the peak heights of the products differ markedly in both preparations. The dialysis activated enzyme predominantly cleaves the oxidized B chain of insulin at a single site i.e. Glu^{13}-Ala^{14}. The DCI treated enzyme appears to have other stimulated activities. Although the identities of the products have not been determined, it is reasonable to assume that the other cleavages are at branched chain amino acids (Cardozo et al. 1992). This would explain why the DCI-treated enzyme can degrade β casein without any accumulation of intermediates. The intermediates are degraded by the branched chain amino acid hydrolyzing activity.

One may suggest on the basis of the above studies that MPC contains multiple catalytic activities. There are:

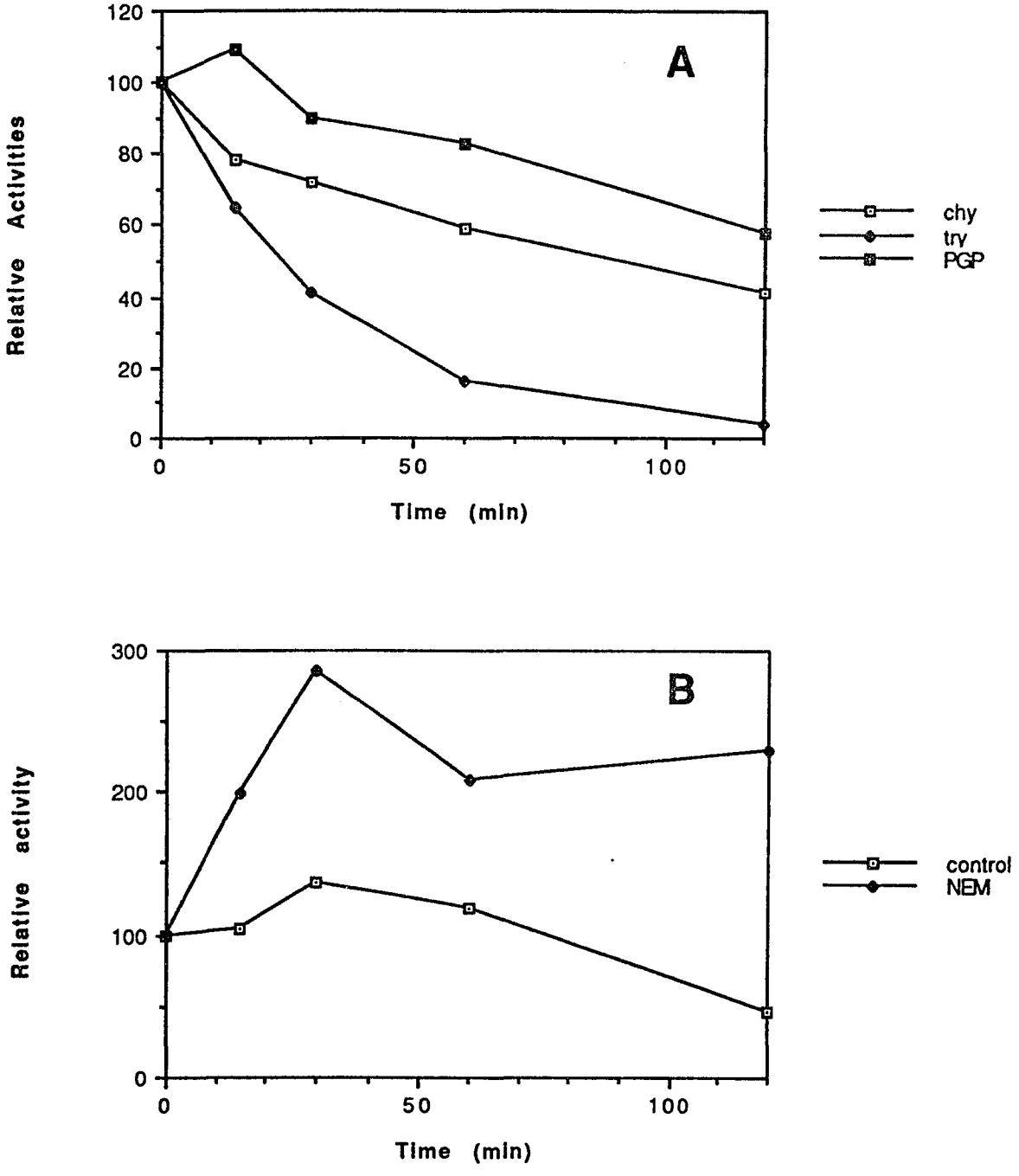
1) chymotrypsinlike,
2) trypsinlike,
3) peptidylglutamylpeptide bond hydrolyzing activity,
4) caseinolytic (an activity catalyzing limited proteolysis of proteins-specificity undefined),

5) Insulin B chain degrading (cleaving Glu^{13}-Ala^{14} bond),

6) DCI resistant branched chain amino acid degrading. Obviously, further studies will be required to clarify the nature of the catalytic components of MPC.

Fig. 1 Effect of NEM on the catalytic activities of MPC

MPC was pretreated with N-ethylmaleimide as described under experimental procedures. At different times of preincubation, the enzyme was dialyzed to remove free NEM. Enzymatic activities were assayed with chromogenic substrates (A) and dephosphorylated-β-casein (B) as described under "experimental procedures in chapter 2". All activities are expressed relative to zero time treatment (100%). Each point represents the mean of three experiments.


A
Relative Activities
120
100
80
60
40
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50
100
Time (min)
chy
try
PGP
B
Relative activity
300
200
100
0
0
50
100
Time (min)
control
NEM

Fig 2 Panel A, HPLC chromatogram of a mixture of 16 μg of DCI treated MPC and 32 μg of oxidized insulin B chain in 0.01 M Tris-EDTA and 1mM DTT buffer pH 7.5 after 60-min incubation at 37°C. MPC was preincubated with 4μM DCI 30 min at room temperature. Chromatographic conditions as described in Methods. Detector sensitivity: 0.05 AUFS.

The retention time of peaks:

1, 30.0 min; 2, 28.4 min; 3, 25.6 min

4, 25.0 min; 5, 23.6 min; 6, 22.0 min

Panel B, HPLC chromatogram of oxidized insulin B chain by dialysis activated MPC. HPLC chromatogram of a mixture of 16 μg MPC dialyzed against a 0.05M Tris-HCl buffer, pH 8.0 and 32 μg oxidized insulin B chain after 60 min incubation at 37°C. Chromatographic conditions as described in Methods. Detector sensitivity: 0.05 AUFS.

The retention time of peaks:

1, 30.0 min (oxidized insulin B chain 1-30);

2, 28.4 min (oxidized insulin B chain 14-30);

3, 25.6 min; 4, 25.0 min;

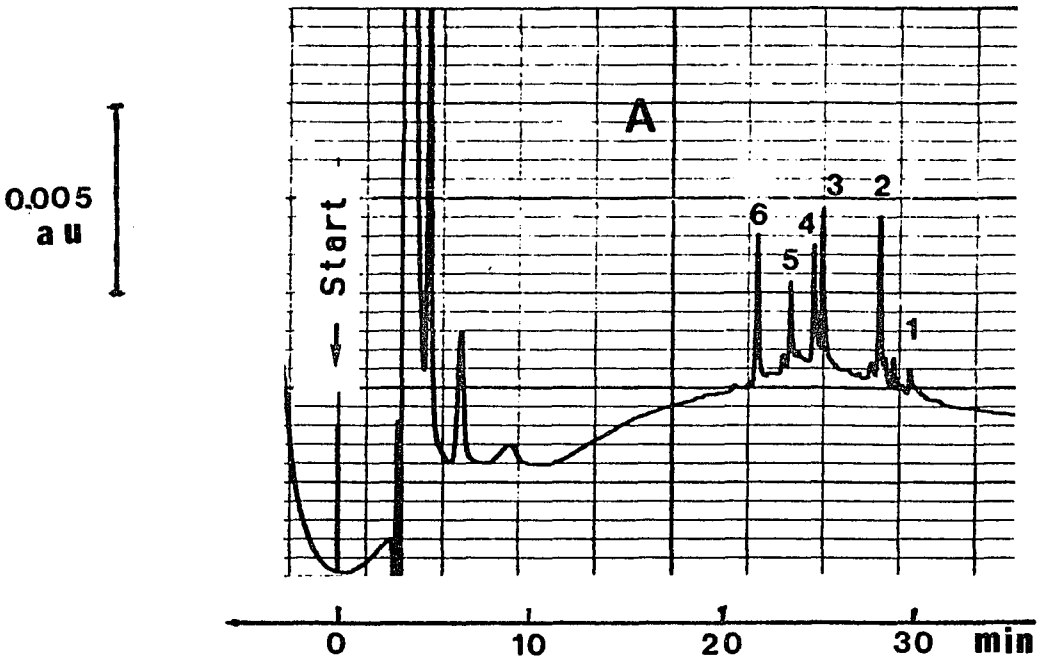
5, 23.6 min (oxidized insulin B chain 1-13);

6, 22.0 min.

Panel C, HPLC chromatogram of oxidized insulin B chain by NAI treated MPC. All conditions are same as panel A, except that

different enzyme preparation was been used. Peak 1 is the oxidized insulin B chain 1-30.

Panel D, HPLC profile of oxidized insulin B chain incubated with native enzyme. Incubation and assay conditions are as same as panel A. Peak 1 is the oxidized insulin B chain 1-30.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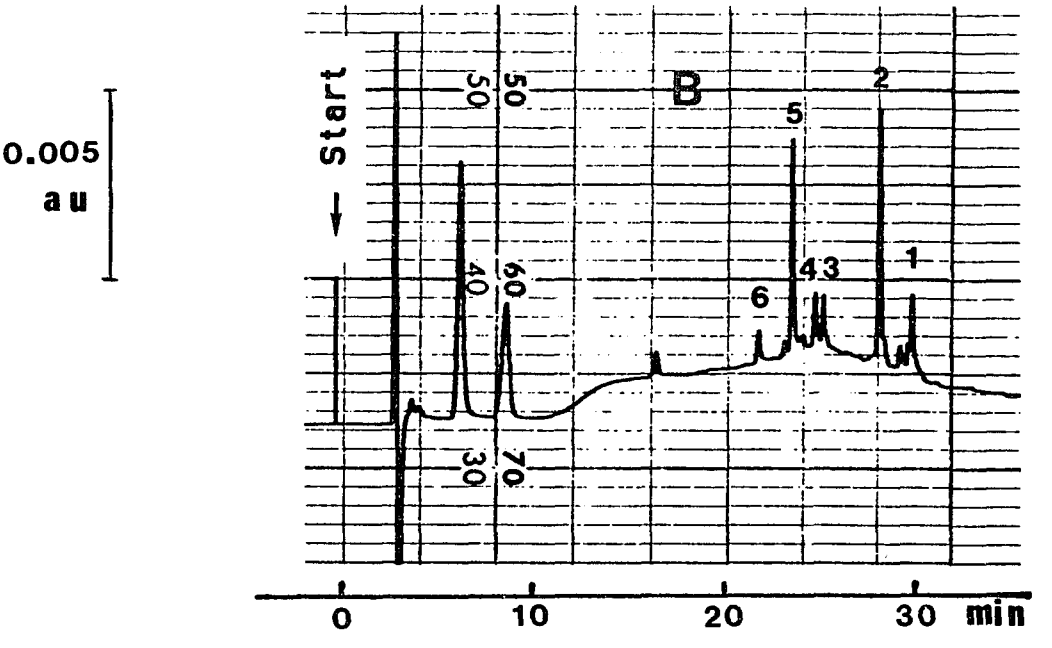
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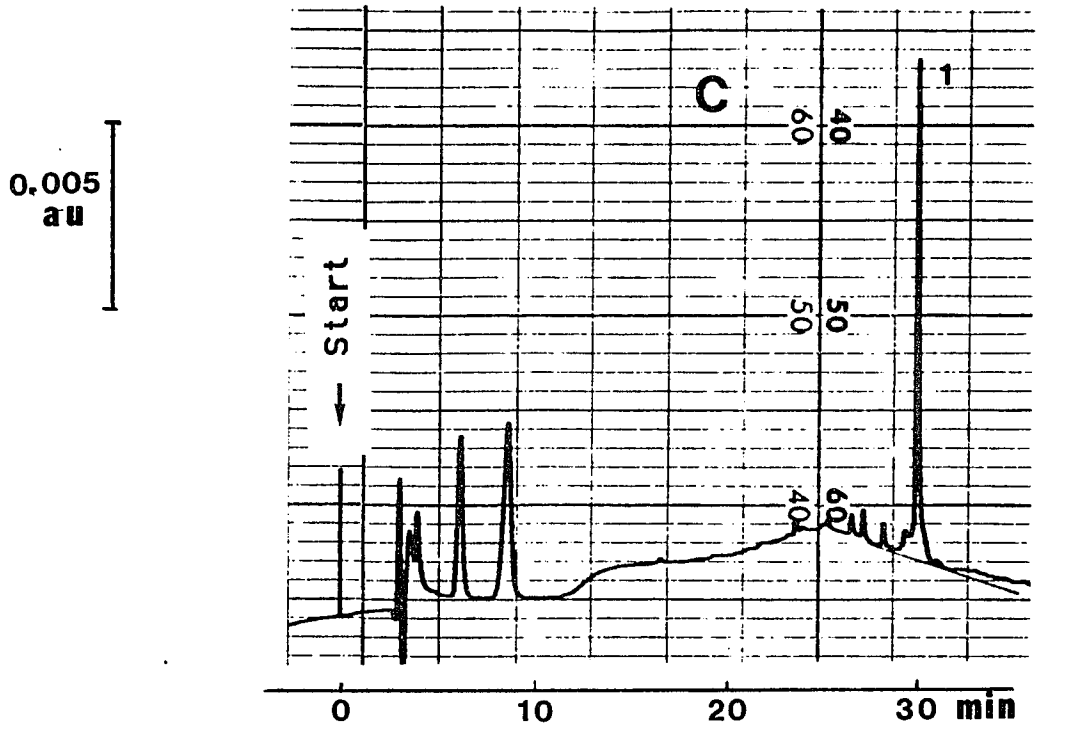
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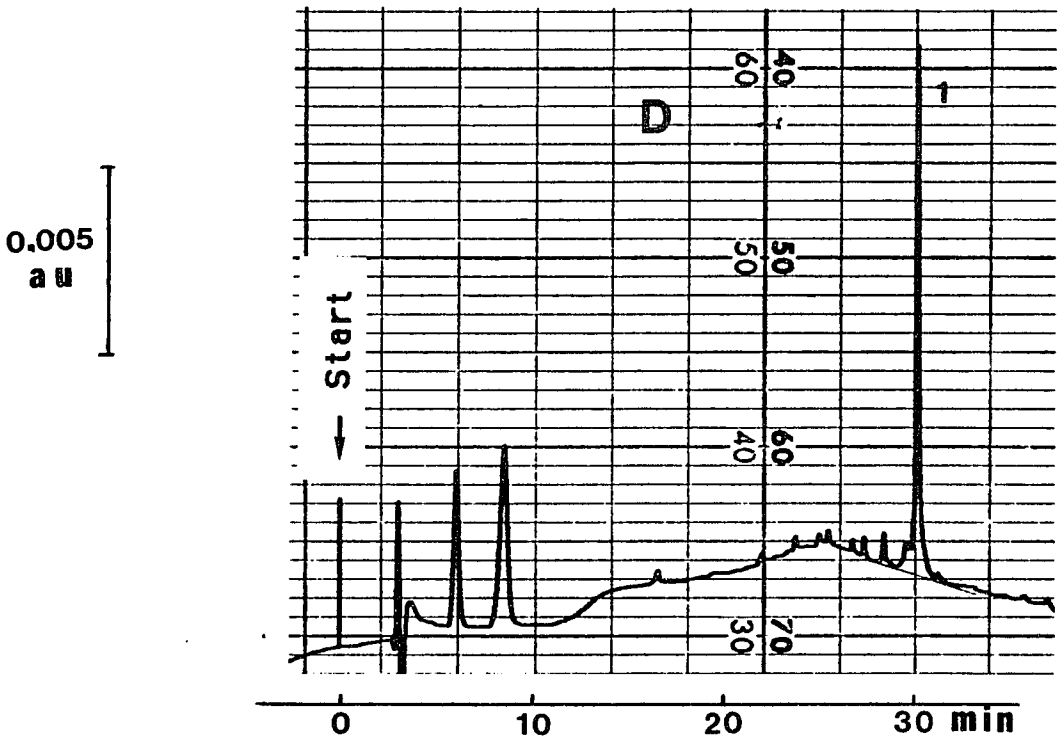
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Chapter 5

DISCUSSION

Since the discovery of MPC by Wilk and Orlowski (1980) a decade ago, many scientists from many different fields have been attracted to the study of this enzyme. Among these studies, characterization of the basic enzymological nature of the molecule is of fundamental significance. MPC was originally described on the basis of its activity toward synthetic chromogenic substrates. Three activities - chymotrypsinlike (cleavage of Cbz-Gly-Gly-Leu-p-nitroanilide), trypsinlike (cleavage of Cbz-D-Ala-Leu-Arg-2-naphthylamide) and peptidylglutamyl peptide bond hydrolyzing activity (cleavage of Cbz-Leu-Leu-Glu-2-naphthylamide) - were originally defined (Wilk and Orlowski, 1980, 1983; Orlowski and Wilk, 1981). Subsequently, MPC was demonstrated to be capable of hydrolyzing proteins (Ray an Harris, 1985; Rivett, 1985; Dahlmann et al. 1985a; Tanaka et al. 1986a). The multicatalytic nature of this enzyme is no longer questioned, however the exact number of catalytic sites and the relationship between them remains unclear. In this thesis project, chemical modification and physico-biochemical methods have been employed to further explore the multiple activities of the bovine pituitary multicatalytic proteinase complex.

The relationship of the caseinolytic activity (degradation of protein) to the three defined catalytic activities has been the subject of other studies (Tanaka et al. 1986; Dahlmann et al. 1985; Rivett, 1989; McGuire et al. 1989; Orlowski and Michaud 1989;

Mykles and Haire 1991), but it was still unclear if the caseinolytic activity was an activity distinct from the other defined activities. Some evidence suggested that the PGP hydrolyzing activity was responsible for the hydrolysis of protein substrates (Orlowski and Michaud 1989). My studies on the chemical modification of MPC by N-acetylimidazole provided strong evidence that the caseinolytic activity was in fact a distinct fourth catalytic component. After treatment with N-acetylimidazole, the trypsinlike and PGP activities of MPC were markedly inhibited, and the chymotrypsinlike activity almost unaffected, whereas the caseinolytic activity was increased many-fold. Furthermore, the pattern of degradation of casein by MPC could be best explained by a sequential process with initial cleavage catalyzed by a component distinct from the chymotrypsinlike, trypsinlike and PGP hydrolyzing activities.

Several other types of experiments have led to the same conclusion. When treated with the serine proteinase inhibitor 3,4-dichloroisocoumarin, the caseinolytic activity of bovine pituitary and lens MPC could be stimulated while the other three catalytic activities were inactivated (Pereira et al. 1992). A newly identified protein activator (PA28) of MPC from bovine red blood cells and bovine heart could activate the chymotrypsinlike, trypsinlike and PGP hydrolyzing activities as high as 200 fold in a positive allosteric manner, however, the activator failed to stimulate the hydrolysis of large protein substrates such as casein and lysozyme (Ma et al. 1992 b). A similar observation has been reported by Dubiel

et al. (1992). Thus an 11S regulator from human red blood cells could activate the hydrolysis of synthetic substrates but not the activity toward protein substrates, such as, ubiquitin-lysozyme conjugates, bovine serum albumin and lysozyme. A study on the lobster muscle MPC revealed that MPC had three stable and functionally distinct states in vitro: defined as basal, heat-activated and SDS-activated forms (Mykles and Haire, 1991). Heating the basal form stimulated the caseinolytic activity with little effect on the activities measured with synthetic substrates. SDS stimulated the PGP hydrolyzing activity and inhibited the chymotrypsinlike, trypsinlike and caseinolytic activities. These observations indicated that heating induced a proteinase activity distinct from the three defined activities. My studies on the effect of dialysis on MPC provided additional evidence that the caseinolytic activity is distinct from the others (see page 137).

There are many ways reported to effect a stimulation of the caseinolytic activity, e. g. N-acetylimidazole (chapter 2), 3,4-dichloroisocoumarin (Pereira 1992a), thiol blocking reagents (chapter 4) , autolysis, magnesium ions and heating at 52°C (Pereira 1992b). It is reasonable to propose that the chemical modification of the enzyme (N-acetylimidazole, DCI, thiol blocking reagents) causes conformational changes in the complex resulting in a relaxed structure of the enzyme capable of accomodating bulky protein substrates. Recently, Djaballah et al (1992) reported that manganese ions could induce a transition of MPC from a compact to an extended

conformation as revealed by electron microscopy. It is very likely that magnesium ions have a similar effect on the complex. The loss of the heaviest subunit by autolysis also increases caseinolytic activity. It can be imagined that the loss of one subunit causes structural changes resulting in an increased accessibility of protein substrates to a previosly inaccessible active site. Heating probably induces the complex to attain a relaxed conformation through mild disruption of its structure.

According to the above discussion, the multiple activities of MPC can be probably grouped into proteinase (for protein substrate) and peptidase (for short peptide substrate) components. This concept (Ma et al. 1992b and Dubiel et al. 1992)) is very logical for a functioning macromolecular complex. It is reasonable to inquire as to the advantages offered by an enzyme complex. A partial answer can be obtained by inquiring as to the interrelationship of the multiple activities of the complex. It is interesting that at the time I addressed this question, it was also considered by two other groups who proposed a channelling theory to describe the relationship of the multiple activities. One group studied yeast MPC (proteinase yscE) (Heinemeyer, et al. 1991). They found a *pre1-1* mutation which was reflected as a lack of chymotrypsinlike activity due to a subunit mutation. Strains carrying the *pre1-1* mutation showed enhanced sensitivity to stresses such as incorporation of the amino acid analogue canavanine into proteins or a combination of poor growth medium and elevated temperature. Under these stress

conditions *pre1-1* mutant cells exhibited decreased protein degradation and accumulation of ubiquitin - protein conjugates. They proposed that the site responsible for the 'chymotrypsinlike' activity is the first in a 'channelling' mechanism, which binds to and cleaves ubiquitinated proteins after neutral amino acids and thereafter delivers the peptide fragments in a tightly bound form to the other proteolytically active domains on the molecule for complete degradation. Another group studied the degradation of oxidized insulin B chain by purified MPC (Dick, et al. 1991). The kinetics of insulin B chain degradation indicated that MPC can catalyze sequential hydrolysis of peptide bonds in a single substrate molecule via a reaction pathway that involves channeling of peptide intermediates between different catalytic centers within the multienzyme complex. All these hypotheses are compatible with my finding that β-casein can be sequentially degraded by the multiple components of MPC, however, the initial cleavage seems to be conducted by a previously unknown catalytic component. The rationale of the hypotheses on organized activities can be seen from studies of other enzyme complexes. Although there are not too many well studied multienzyme complexes, all of them share one principal feature i.e. that the multiple activities in the complex are well organized; e.g. pyruvate dehydrogenase multienzyme complex, tryptophan synthase multienzyme complex, carbamoyl phosphate synthase, aspartate carbamoyltransferase (Price and Stevens, 1989). In addition to this common reason for existing as a complex, i.e. high

efficiency by shortening transit time and substrate channeling, the organized activities of MPC have been considered as a way of avoiding diffusion of possible deleterious peptide intermediates into the environment (Heinemeyer et al., 1991). A related concept is that organization of the activities cannot be separated from the cylindrical structure of MPC and that this structure must have an important function. An interesting idea is that protein substrates are degraded in the tunnel of MPC (Parham, 1990; Goldberg 1992), however there is no any data to support this concept at present.

In my model of sequential degradation (chapter 2), the three peptidase activities (chymotrypsinlike, trypsinlike and PGP) were originally proposed to participate in the secondary proteolysis of the generated 21 kDa intermediate. This proposal appears inconsistent with the detailed studies on the effect of DCI on MPC (Periera, 1992). First, DCI treated MPC has low chymotrypsinlike, trypsinlike and PGP activities, yet it hydrolyzes β-casein rapidly without any accumulation of intermediates. The intermediate generated by the N-acetylimidazole modified MPC can be degraded very easily by DCI treated MPC. Apparently, if the "caseinolytic component" is responsible only for catalyzing the initial cleavage, there must be other DCI-resistant components that are stimulated and are responsible for secondary degradation. A strong possibility is an activity identified by Cardozo et al. (1992). They reported that inactivation of the three peptidase activities by 3,4-dichloroisocoumarin (DCI) revealed the presence of an additional

DCI-resistant component. This component cleaved natural peptides including neurotensin, dynorphin, angiotensin II, the oxidized B-chain of insulin, and also proinsulin at a rate greater than that of the native uninhibited complex. Their studies on cleavage sites of this DCI-resistant catalytic activity suggested preferential cleavage of bonds on the carboxyl side of branched chain amino acids. Fig 2A in chapter 4 also shows that DCI stimulates cleavage of oxidized insulin B chain by MPC at multiple sites. Considering the difference between peptide substrates and protein substrates, it is difficult to say if this DCI resistant component, or components is responsible for the secondary degradation. The caseinolytic component itself cannot be responsible since N-acetylimidazole results are not consistent with this possibility. Further studies will be required to answer this question.

An activating effect of dialysis on MPC has been reported (McGuire et al. 1989; Dick et al 1991). The hydrolysis of [methyl-^{14}C] casein and oxidized insulin B chain could be increased by dialysis of MPC against water. I studied this phenomenon further and showed that dialysis not only caused an activation of oxidized insulin B chain hydrolysis, but also changed the other activities and the structure of MPC. After dialysis, MPC lost its chymotrypsinlike, trypsinlike, and PGP activities almost completely, the enzyme complex partially disintegrated and at least one subunit was autolyzed.

Dick et al. claim that oxidized insulin B chain is a model substrate for studying protein degradation, because both radiolabelled casein and oxidized insulin B chain could be rapidly hydrolyzed by dialyzed MPC. My data are not consistent with this conclusion. When MPC is activated by dialysis, only the hydrolysis of oxidized insulin B chain is increased dramatically, the degradation of β-casein remains the same as the native enzyme (see page 78). This result not only provides additional evidence that the caseinolytic activity is distinct, but also suggests that the hydrolysis of oxidized insulin B chain is carried out by a new activity. As summarized in Table 1, the dialysis of MPC did not activate the caseinolytic activity; on the other hand, N-acetylimidazole treated MPC having an activated caseinolytic activity did not have an increased activity toward oxidized insulin B chain. Although the activity toward oxidized insulin B chain appears to differ from the known activities, it cannot be said at this time with certainty that the oxidized insulin B chain activity reflects a new catalytic component. Further studies with inhibitors are required to validate this conclusion.

Table1. Comparison of catalytic activities of various MPC preparations

	Activity (%)				
	Chy.	Try.	PGP	Casein.	Insulin.
Native	100	100	100	100	100
NAI	95	5	10	750	100
DCI	1	7	9	1000	1400
Dialysis	3	6	13	115	1200

Chy. chymotrypsinlike activity; Try., trypsinlike activity; PGP, peptidylglutamyl peptide bond hydrolyzing activity; Casein, caseinolytic activity; Insulin, hydrolyzing activity toward oxidized insulin B chain; Native, native MPC; NAI, N-acetylimidazole treated MPC; DCI, 3,4-dichloroisocumarin treated MPC; Dialysis, dialysis treated MPC; * data from Periera et al. 1992 and Cardozo et al. 1992.

In summary, the multicatalytic proteinase complex contains more than three kinds of activities. A fourth activity (casenolytic activity) seems to be responsible for the initial cleavage of protein substrates. Clearly, this activity needs to be characterized further with respect to its substrate specificity, its catalytic mechanism, its regulation etc. Some evidence is presented in this thesis that the initial hydrolysis of oxidized insulin B chain may be catalyzed by a fifth component, and that there exist other DCI resistant component(s). Further studies will be required to characterize these activities and firmly establish their separate identities.

REFERENCES:

Allen, P. M., Babbitt, B. P. and Unanue, E. R.
T-Cell Recognition of lysozyme: the biochemical basis of presentation.
Immunol Rev. 1987, 98: 171-187

Achstetter, T., Ehmann, C., Osaki, A., & Wolf, D. H.
Proteolysis in eukaryotic cells: proteinase yscE, a new yeast peptidase.
J. Biol. Chem., 259:13344-13348. (1984)

Arrigo, A.-P., Tanaka, K., Goldberg, A. L., & Welch, W. J.
Identity of the 19S 'prosome' particle with the large multifunctional protease complex of mammalin cells (the proteasome)
Nature, 331: 192-194). (1988)

Baumeister, W., Dahlmann, B., Hergerl, R., Kopp, F., Kuehn, L., & Pfeifer, G.
Electron microscopy and image analysis of the multicatalytic proteinase
FEBS letter, 241: 239-245. (1988)

Cardozo, C., Vinitsky, A., Hidalgo, M. C., Michaud, C. and Orlowski, M
A 3,4-dichloroisocoumarin-resistant component of the multicatalytic proteinase complex.
Biochemistry, 31: 7373-7380. (1992)

Cenciarelli, C., Hou, D., Hsu, K-C., Rellahan, B. L., Wiest, D. L., Smith, H. T., Fried, V. A. and Weissman, A. M.
Activation-induced ubiquitination of the T cell antigen receptor.
Science, 257: 795-797. (1992)

Croall, D. E. and Demartino, G. N.
Calcium-activated neutral protease (calpain) system structure, function and regulation.
Physiological Review, 71: 813-847. (1991)

Dahlmann B., Kuehn, L., Rutschmann M. and Reinauer H.
Purification and characterazation of a multicatalytic high-molecular-mass proteinase from rat skeletal muscle
Biochem. J., 228: 161-170. (1985a)

Dahlmann B., Rutschmann M., Kuehn, L.and Reinauer H.
Activation of the multicatalytic proteinase from rat skeletal muscle by fatty acids or sodium dodecyl sulphate.
Biochem. J., 228: 171-177. (1985b)

Dahlmann, B., Kuehn, L., Ishiura, S., Tsukahara, T., Sugita, H., Tanaka, K., Revett, J., Hough,R. F., Rechsteiner, M., Mykles, D. L., Fagan, J. M., Waxman, L., Ishii, S., Sasaki, M., Kloetzel, P., Harris, H., Ray, K., Behal, F. J., Demartino, G. N., and McGuire, M. J.
The multicatalytic proteinase: a high-Mr endopeptidase
Biochem. J. 255, 750-751. (1988)

Dahlmann, B., Kopp, F., Kuehn, L., Niedel, B., Pfeifer, G., Hegerl and Baumeister, W.
The multicatalytic proteinase (prosome) is ubiqutous from eukaryotes to archaebacteria
FEBS letters. 251. 125-131. (1989)

Dahlmann, B. Kuehn, L., Grziwa, A., Zwickl, P., and Baumeister, W.
Biochemical properties of the proteasome from *Thermoplasma acidphilum*
FEBS, 208: 789-797. (1992)

Dalbey, R. E.and von Heijne, G.
Signal peptidases in prokaryotes and eukaryotes - a new protease family.
TIBS. 17,474-478. 1992

Dice, J. D.
Molecular determinants of protein half-lives in eukaryotic cells.
FASEB J., 1: 329-357. (1987)

Dice, J. D.
Peptide sequences that target cytosolic proteins for lysosomal proteolysis

TIBS. 15: 305-309. (1990)

Dick, L. R., Moomaw, C. R., DeMartino, G. N. and Slaughter, C. A.
Degradation of oxidized insulin B chain by the multiproteinase complex Macropain (proteasome).
Biochemistry. 30: 2725-2734. (1991)

Dick, L. R., Moomaw, C. R., Pramanik, B. C., DeMartino, G. N. and Slaughter, C. A.
Identification and localization of a cysteinyl residue critical for the trypsinlike catalytic activity of the proteasome
Biochemistry. 31: 7347-7355. (1992)

Di Cola , D.
Human erythrocyte contains a factor that stimulates the peptidase activites of multicatalytic proteinase complex
Italian J. Biochem., 41: 213-224. (1992)

Driscoll, J. and Goldberg, A. L.
The proteasome (multicatalytic proteinase) is a component of the 1500-kDa proteolytic complex which degrades ubiquitin-conjugated proteins.
J. Biol. Chem., 265, 4789-4792. (1990)

Driscoll, J., Frydman, J. and Goldberg, A. L.
An ATP-stabilized inhibitor of the proteasome is a component of the1500-kDa ubiquitin conjugate-degrading complex
Proc. Natl. Acad. Sci. USA, 89: 4986-4990. (1992)

Djaballah, H., and Rivett, J. A.
Peptidylglutamyl-peptide hydrolase activity of the multicatalytic proteinase complex: evidence for a new high-affinity site, analysis of cooperative kinetics, and the effect of manganese ions.

Biochemistry. 31: 4133-4141. (1992)

Djaballah, H., Harness, J. A., Savory, P. J. and Rivett, J. A.
Use of serine-proteinase inhibitors as probes for the different proteolytic activities of the rat liver multicatalytic proteinase complex.
Eur. J. Biochem., 209, 629-634. (1992)

Djaballah, H., Rowe, A. J., Harding, S.E., and Rivett, A. J.
Conformational changes in the multicatalutic proteinase complex assessed by sedimentation velocity, dynamic light scattering, and electron microscopy.
9th international ICOP conference on proteolysis and protein turnover. 1992

Doherty, F. J., and Mayer, R. J.
Intracellular protein degradation
IRL press at Oxford University Press. 1992

Dubiel, W., Pratt, G., Ferrell, K. and Rechsteiner, M.
Purification of an 11S regulator of the multicatalytic protease.
J. Biol. Chem., 267: 22369-22377. (1992)

Eytan, E., Ganoth, D., Armon, T., Hershko, A.
ATP-dependent incorporation of 20S protease into the 26S complex that degrades proteins conjugated to ubiquitin.
Proc. Natl. Acad. Sci. USA. 86: 7751-7755. (1989)

Falkenburg, P-E., Haass, C., Kloetzel, P-M., Niedel B., Kopp, F., Kuehn, L., Dahlmann, B.
Drosophila small cytoplasmic 19S ribonucleoprotein is homologous to the rat multicatalytic proteinase.
Nature. 331: 190-192. (1988)

Folco, E. J., Busconi, L., Martone, C. B. and Sanchez, J. J.
Multicatalytic proteinase in fish muscle
Arch. Biochem. Biophys., 267: 599-605. (1988)

Glaumann, H., and Ballard, F. J.
Lysosomes: their role in protein breakdown.

1987 Acdemic Press, London and New York

Glynne, R., Powis, S. H., Beck, S., Kelly, A., Kerr, L-A. and Trowsdale, J.
A proteasome-related gene between the two ABC transporter loci in the class II region of the human MHC.
Nature, 352: 357-360. (1991)

Goldberg, A. and John, A.C.St.
Intracellular protein degradation in mammalian and bacterial cells: part 2
Ann. Rev. Biochem., 45: 747-803. (1976)

Hase, J., Kobashi, K., Nakai, K., Mitsui, K., Iwata, K., & Takadera, T.
The quaternary structure of carp muscle alkaline protease.
Biochim. Biophys. Acta., 611: 205-213. (1980)

Heinemeyer, W., Kleinschmidt, J. A., Saidowsky, J., Escher, C and Wolf, D. H.
Proteinase yscE, the yeast proteasome/multicatalytic-multifunctional proteinase: mutants unravel its function in stress induced proteolysis and uncover its necessity for cell survival.
EMBO J. 10: 555-562. (1991)

Hendil, K, B.
The 19 S multicatalytic "prosome" proteinase is a constitutive enzyme in HeLa cells.
Biochem. Int. 17: 471-476. (1988)

Hershko, A., and Ciechanover, A.
The ubiquitin system for protein degradation
Ann. Rev. Biochem., 61: 761-807. (1992)

Hershko, A., and Ciechanover, A.
Mechanisms of intracellular protein breakdown
Ann. Rev. Biochem., 51: 335-364. (1982)

Hough, R., Pratt, G. and Rechsteiner, M.

Purification of two high molecular weight proteases form rabbit reticulocyte lysate.
J. Biol. Chem., 262: 8303-8313. (1987)

Ishiura, S., Sano, M., Kamakura, K., and Sugita, H.
Isolation of two forms of the high-molecular-mass serine proteinase, ingensin, from porcine skeletal muscle.
FEBS lett., 189:119-123. (1985)

Ishiura, S.
Proteolytic cleavage of the Alzheimer's disease amyloid A4 precursor protein.
J. Neurochem., 56:363-369. (1991)

Jones, E. W.
Three proteolytic systems in the Yeast Saccharomyces cerevisiae.
J. Biol. Chem., 266: 7963-7966. (1991)

Kelly, A., Powis, S. H., Glynne, R., Radley, E., Beck, S., and Trowsdale, J.
Second proteasome-related gene in human MHC class II region.
Nature, 353: 667-668. (1991)

Klein, U., Gernold, M.and Kloetzel, P-M.
Cell-specific accumulation of Drosophila proteasomes (MPC) during early development.
J. Cell. Biol. 111: 2275-2282. (1990)

Kojima, S. and Omori, M.
Two-way cleavage of β-amyloid protein precursor by multicatalytic proteinase.
FEBS letter, 304: 57-60. (1992)

Kopp, F., Steiner, R., Dahlmann, B., Kuehn, L., and Reinauer, H.
Size and shape of the multicatalytic proteinase from rat skeletal muscle.
Biochim. Biophy. Acta 872, 253-260. (1986)

Kopp, F., Dahlmann, B., and Hendil, K. B.

Evidence indicating that the human proteasome is a complex dimer.
J. Mol. Biol., In press. (1992)

Kuehn, L., Dahlmann, B. and Kopp, F.
Effect of the multicatalytic proteinase (prosome) on translational activity in rabbit reticulocyte lysates
FEBS Lett., 261: 274-278. (1990)

Kuehn, L., Dahlmann, B. and Reinauer, H.
Evidence indicating that the multicatalytic proteinase of rabbit reticulocytes is not incorporated as a core enzyme into a 26S proteinase complex.
Arch. Biochem. Biophys., 295: 55-60. (1992)

Kumatori, A., Tanaka, K., Inamura, N., Sone, A., Ogura, T., Matsumoto, T., Tachikawa, T., Shin, S.,and Ichihara, A.
Abnormally high expression of proteasomes in human leukemic cells.
Proc. Natl. Acad. Sci. U.S.A., 87: 7071-7075. (1990)

Li, X., Gu, M. and Etlinger, J. D.
Isolation and characterization of a novel endogenous inhibitor of the proteasome.
Biochemistry, 30: 9709-9715. (1991)

Li, X. S. and Etlinger, J. D.
Ubiquitinated proteasome inhibitor is a component of the 26 S proteasome.
Biochemistry, 31, 11963-11967. (1992)

Ma, C-H., Slaughter, C. A. and DeMartino, G. N.
Purification and characterization of a protein inhibitor of the 20S proteasome (macropain)
Biochim. Biophys. Acta, 1119: 303-311. (1992 a)

Ma, C-H., Slaughter, C. A. and DeMartino, G. N.
Identification, purification, and characterization of a protein activator (PA28) of the 20S proteasome (Macropain).
J. Biol. Chem., 267: 10515-10523. (1992 b)

Martinez C. K. and Monaco, J. J.
Homology of proteasome subunits to a major histocompatibility complex-linked LMP gene.
Nature. 353: 664-667. (1991)

Matsumura, K. and Aketa, K.
Proteasome (multicatalytic proteinase) of sea urchin sperm and its possible participation in the acrosome reaction.
Mol. Reprod. Develop., 29: 189-199 (1991)

McGuire, M. J., and Demartino, G. N.
Purification and characterization of a high molecular wight proteinase (macropain) from human erythrocytes
Biochim. Biophys Acta. 873: 279-289. (1986)

McGuire, M. J., McCullough, M. L., Croall, D. E. and Demartino, G. N.
The high molecular weight multicatalytic proteinase, macropain, exists in a latent form in human erythrocytes.
Biochim. Biophys. Acta. 995: 181-186. (1989a)

McGuire, M. J., and Dematino, G. N.
The latent form of macropain (high molecular weight multicatalytic protease) restores ATP-dependent proteolysis to soluble extracts of BHK fibroblasts pretreated with anti-macropain antibody.
Biochem. Biophys Res. Commun., 160: 911-916. (1989b)

Monaco, J. J.
A molecular model of MHC class - I - restricted antigen processing.
Immun. Today. 13: 173-179. (1992)

Mykles, D. L.,
High-molecular-weight serine proteinase from lobster muscle that degrades myofibrillar proteins.
J. Exp. Zool. 250: 244-252. (1989)

Mykles, D. L. and Haire, M. F.

Sodium dodecyl sulfate and heat induce distinct forms of lobster muscle multicatalytic proteinase: the heat-activated form degrades myofibrillar protein.
Arch. Biochem. Biophys., 288: 543-550. 1991

Muller, M.
Proteolysis in protein import and export: Signal peptide processing in eu- and prokaryotes.
Experientia, 48: 118-129. (1992)

NC-IUBMB
Enzyme nomeclature 1992: recommendations of the nomenclature committee of the international union of biochemistry and molecular biology on the nomenclature and classification of enzymes.
Academic Press, Inc., California. (1992)

Nojima, M. Ishiura. S., Yamamoto, T., Okuyama, T., Furuya, H., and Sugita, H.
Purification and characterization of a high-molecular-weight protease, Ingensin, from human placenta.
J. Biochem., 99:1605-1611. (1986)

Nothwang, H. G., Coux, O., Bey, F. and Scherrer, K.
Disruption of prosomes by some bivalent metal ions results in the loss of their multicatalytic proteinase activity and cancels the nuclease resistance of prosomal RNA.
Biochem. J., 287: 733-739. (1992)

Orlowski, M. and Wilk, S.
A multicatalytic protease complex from pituitary that forms enkephalin and enkephlin-containing peptides.
Biochem. Biophys. Res. Commun., 101: 814-822. (1981)

Orlowski, M., and Wilk, S.
'Multicatalytic proteinase complex' or 'multicatalytic proteinase: a high-Mr endopeptidase'.
Biochem. J., 255 :751. (1988)

Orlowski, M. and Michaud, C.

Pituitary multicatalytic proteinase complex. specificity of components and aspects of proteolytic activity
Biochemistry, 28: 9270-9278. (1989)

Orlowski, M.
The multicatalytic proteinase complex, a major extralysosomal proteolytic system.
Biochemistry, 29: 10289-10297. (1990)

Parham, P.
Transporters of delight.
Nature, 348: 674-675. (1990)

Pereira, M.E., Nguyen, T., Wagner, B. J., Margolis, J. W., Yu, B. and Wilk, S.
3,4-Dichloroisocoumarin-induced activation of the degradation of β- casein by the bovine pituitary multicatalytic proteinase complex.
J. Biol. Chem., 267: 7949-7955. (1992a)

Pereira, M.E., Yu, B. and Wilk, S.
Enzymatic changes of the bovine pituitary multicatalytic proteinase complex, induced by magnesium ions.
Arch. Biochem. Biophys., 294: 1-8. (1992b)

Pontremoli. S. and Melloni, E.
Extralysosomal protein degradation
Ann. Rev. Biochem. ,. 55: 455-481 (1986)

Price, N, C. and Stevens, L.
Fundamentals of Enzymology
Oxford University Press, Oxford New York, (1989)

Puri, J. and Factorovich, Y.
Selective inhibition of antigen presentation to cloned T cells by protease inhibitors.
J. Immunol. ,141, 3313-3317. (1988)

Puhler, G., Weinkauf, S., Bauchmann, L., Muller, S., Engel, A., Hegerl, R., and Baumeister, W.

Subunit stoichiometry and three-dimensional arragement in proteasomes from *Thermoplasma acidophilum*
The EMBO Journal , 11:1607-1616. (1992)

Ray, K. and Harris, H.
Purification of neutral lens endopeptidase: close similarity to a neutral proteinase in pituitary.
Proc. Natl. Acad. Sci. U.S.A., 82: 7545-7549. (1985)

Ray K. and Harris, H.
Lens neutral endopeptidase occurs in other bovine and human tissues.
Biochem. J., 248: 643-648. (1987)

Rivett, J. A.
Purification of a liver alkaline protease which degrades oxidatively modified glutamine synthetase. Characterization as a high molecular weight cysteine proteinase.
J. Biol. Chem. 260:12000-12606. (1985)

Rivett, J. A.
The multicatalytic proteinase. Multiple proteolytic activities.
J. Biol. Chem., 264: 12215-12219. (1989)

Schecter and Berger
On the size of the active site in protease. I. Papain.
Biochem. Biophys. Res. Comm., 27: 157-162 (1967)

Schliephacke, M., Kremp, A., Schmid H. Kohler, K., and Kull, U.,
Prosome (proteasomes) of higher plants
Europ J. of Cell Biol., 55: 114-121. (1991)

Seidah, N. G. and Chretien, M.
Proprotein and prohormone convertase of the subtilisin family, recent developments and future perspectives.
TEM 3, 133-140. (1992)

Seelig, A., Kloetzel, P-M., Kuehn, L. and Dahlmann, B.
Molecular interaction of the proteasome (multicatalytic proteinase). Evidence that the proteasome is not a constituent of the '26S' multienzyme complex.

Biochem. J., 280: 225-232. (1991)

Shimbara, N., Orino, E., Sone, S., Takashina, M., Shono, M., Tamura, T., Yasuda, H., Tanaka, K., Ichihara, A.
Regulation of gene expression of proteasomes (multi- protease complexes) during growth and differentiation of human hematopoietic cells
J. Biol. Chem., 267: 18100-18109. (1992)

Strack, P. R., Wajnberg, E. W., Waxman, L., and Fagan, J.
Comparison of the multicatalytic proteinases isolated from the nucleus and cytoplasm of chicken red blood cells
Int. J. Biochem., 24: 887-895. (1992)

Tanaka, K., Ii, K., Ichihara, A., Waxman, L., Goldberg, A. L.
A high molecular weight protease in the cytosol of rat liver. I Purification, enzymological properties and tissue distribution.
J. Biol. Chem., 261:15197-15203 (1986)

Tanaka, K., Yoshimura, T., Kumatori, A., Ichihara, A., Ikai, A., Nishigai, M., Kameyama, K., and Takagi, T.
Proteasomes (multiprotease complex) as 20S ring-shaped particles in a variety of eukaryotic cells.
J. Biol. Chem., 263: 16209-16217. (1988a)

Tanaka, K., Yoshimura, T.,Ichihara, A., Ikai, A., Nishigai, M., Morimoto, Y., Sato, M., Tanaka, N., Katsube, Y., Kameyama, K., and Takagi, T.
Molecular organization of a high molecular weight multi-protease complex from rat liver
J. Mol. Biol., 203: 985-996. (1988b)

Tanaka, K. and Ichihara, A.
Involvement of proteasomes (multicatalytic proteinase) in ATP-dependent proteolysis in rat reticulocyte extracts.
FEBS Lett. 236: 159-162. (1988c)

Tanaka, K. and Ichihara, A.
Half-life of proteasomes (multiprotease complex) in rat liver.
Biochem. Biophys. Res. Commun., 159: 1309-1315. (1989)

Tanaka, K., Tamura, T., Yoshimura, T.,Ichihara, A.
Proteasomes: protein and gene structures
New Biol., 4 :1-11. (1992)

Vinitsky, A., Michaud, C., Powers, J., and Orlowski, M.
Inhibition of the chymotrypsin-like activity of the pituitary multicatalytic proteinase complex.
Biochemistry, 31: 9421-9428. (1992)

Wagner, B.J., Margolis, J. M., Farnsworth, P. N. and Fu, S-C. J.
Calf lens neutral proteinase activity using calf lens crystallin substrates.
Exp. Eye Res., 35: 293-303. (1982)

Wagner, B.J., Margolis, J. M., Abramovitz, A. S., and Fu, S-C. J.
Differential inhibition of two proteolytic activities in bovine lens neutral proteinase preparations.
Biochem. J., 228: 517-519. (1985)

Weitman, D. and Etlinger, J. D.
A monoclonal antibody that distinguishes latent and active forms of the proteasome (multicatalytic proteinase complex)
J. Biol. Chem., 267: 6977-6982. (1992)

Wilk, S., Pearce, S., and Orlowski, M.
Identification and partial purification of a cation-sensitive neutral endopeptidase from bovine pituitaries.
Life Sci., 24, 457-464. (1979)

Wilk, S., and Orlowski, M.
Cation-sensitive neutral endopeptidase : Isolation and specificity of bovine pituitary enzyme.
J. Neurochem., 35: 1172-1182. (1980)

Wilk, S., and Orlowski, M.
Evidence that pituitary cation-sensitive neutral endopeptidase is a multicatalytic protease complex.
J. Neurochem., 40: 842-849. (1983)

Yukawa, M., Sakon, M., Kambayashi, J., Shiba, E., Kawasaki, T., Ariyoshi, H. and Mori, T.
Proteasome and its novel endogenous activator in human platelets.
Biochem. Biophys. Res. Commun. 178: 256-262. (1991)

Zolfaghari, R., Baker, C. R. F., Jr., Canizaro, P. C Amirgholami, A.,. & Behal, F. J.
A high-molecular mass neutral endopeptidase-24.5 from human lung.
Biochem. J., 241:129-135. (1987a)

Zolfaghari, R., Baker, C. R. F., Jr., Amirgholami, A., Canizaro, P. C. & Behal, F. J.
A multicatalytic high-molecular-weight neutral endopeptidase from human kidney.
Arch. Biochem. Biophys., 258:42-50. (1987b)

Zwickl, P., Pfeifer, G., Lottspeich, F., Kopp, F., Dahlmann, B.,and Baumeister, W.
Electron microscopy and image analysis reveal common principles of organization in two large protein complexes: groEL-type proteins and proteasome.
J. Struct. Biol., 103: 197-203. (1990)

Zwickl, P., Lottspeich, F., Dahlmann, B., and Baumeister, W.
Cloning and sequencing of the gene encoding the large (α-) subunit of the proteasome from *Thermoplasma acidophilum*
FEBS letters, 278:217-221. (1991)

Zwickl, P., Grziwa, A., Puhler, G., Dahlmann, B., Lottspeich, F.and Baumeister, W.
Primary structure of the *Thermoplasma* proteasome and its implications for the structure, function, and evolution of the multicatalytic proteinase
Biochemistry, 31: 964-972. (1992)