Leupeptin-binding site(s) in the mammalian multicatalytic proteinase complex

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The multicatalytic proteinase (MCP) complex is a major non-lysosomal proteinase which plays an important role in non-lysosomal pathways of protein degradation and which has recently been implicated in antigen processing. The mammalian MCP complex is composed of more than 20 different types of polypeptide, but it is not yet clear which of these components are responsible for its proteolytic activities. The complex has at least three distinct types of proteolytic activity. One of these, the so-called 'trypsin-like' activity, which involves cleavage on the carboxy side of basic amino acid residues, can be selectively

and completely inhibited by peptidyl arginine aldehydes (such as leupeptin and antipain), and is also the most sensitive to inhibition by thiol-reactive reagents. In the present study *N*-[ethyl-1-¹⁴C]ethylmaleimide has been used to specifically label thiol groups protected by leupeptin binding. The results suggest that one or two polypeptide components within the complex can be protected against modification by *N*-ethylmaleimide. These components may be responsible for the 'trypsin-like' activity of the complex or may be adjacent to the catalytic component(s) and play an important role in substrate binding.

INTRODUCTION

The multicatalytic proteinase complex (MCP) or proteasome is a high-molecular-mass proteinase (multicatalytic endopeptidase complex, EC 3.4.99.46) that is found in the nucleus and cytoplasm of eukaryotic cells (see [1,2] for reviews). MCP is a major non-lysosomal proteinase and appears to be involved in ubiquitin-dependent as well as ubiquitin-independent non-lysosomal pathways of protein degradation [3–5]. In addition, it is now believed to play a part in antigen processing [6,7]. The enzyme complex can degrade a variety of protein and peptide substrates [1]. It catalyses cleavage on the carboxy side of basic, hydrophobic or acidic amino acid residues [8]. These activities, which have been referred to, although possibly inappropriately, as 'trypsin-like', 'chymotrypsin-like' and peptidylglutamyl-peptide hydrolase activities respectively, appear to be catalysed at distinct sites [9–11].

Like other MCPs, the rat liver proteinase is composed of at least 24 subunits arranged in a hollow cylindrical structure. Highly purified proteinase preparations contain more than twenty different polypeptides, all of molecular mass 22–35 kDa and pI values ranging from 4 to 9 [12]. Comparisons of N-terminal amino acid sequences of several rat MCP polypeptides [13] and of complete amino acid sequences deduced from cDNA sequences [14], as well as similar observations made for subunits of the proteinase isolated from *Drosophila* [15], human [16], and yeast have shown that several of the subunits are encoded by members of the same gene family. Sequence comparisons have so far failed to demonstrate any close similarity between MCP polypeptides and other known proteinases, although it is assumed that several of the subunits have proteolytic functions. Despite many studies of the proteinase isolated from a variety of different sources, there is little information regarding the function of individual subunits, and it is not clear which of the many polypeptides are responsible for the distinct proteolytic activities.

As part of a broader study of possible relationships between the different polypeptides of rat liver MCP [12,13], the purpose

of the present experiments was to investigate which subunit(s) of the rat liver MCP complex are associated with the 'trypsin-like' activity. Here we have used this term to refer to the activity which is responsible for catalysing the hydrolysis of the synthetic peptide substrate *N*-Boc(t-butoxycarbonyl)-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (LSTR-AMC), although its specificity is not the same as that of trypsin (H. Djaballah and A. J. Rivett, unpublished work). Leupeptin, a peptide arginine aldehyde, which is a reversible inhibitor of serine and cysteine proteinases, selectively inhibits the 'trypsin-like' activity [8,10]. This activity is also the most sensitive to inhibition by thiol-reactive reagents, and in the present study *N*-[ethyl-1-¹⁴C]ethylmaleimide ([¹⁴C]-NEM) has been used successfully to label subunits which are protected by leupeptin binding. A preliminary account of these results has already been presented [17].

MATERIALS AND METHODS

Materials

[¹⁴C]NEM (sp. radioactivity 40 Ci/mol) was purchased from New England Nuclear. LSTR-AMC was purchased from The Peptide Institute, and Ala-Ala-Phe-7-amido-4-methylcoumarin (AAF-AMC), *N*-Cbz(benzyloxycarbonyl)-Leu-Leu-Glu- β -naphthylamide (LLE-NA), leupeptin and NEM were from Sigma. MCP was purified from fresh rat livers as described previously [12].

Amino acid analysis and the determination of protein concentration

Protein concentrations were routinely determined by the method of Bradford [18], with BSA as standard, because MCP was found to give a colour reaction in the dye-binding assay [18] that is almost identical with that of BSA ([MCP] = 0.96 $\times$ concentration determined using BSA). This value was determined by using results based on amino acid analysis. MCP (dialysed against

Abbreviations used: AAF-AMC, Ala-Ala-Phe-7-amido-4-methylcoumarin; Boc, t-butoxycarbonyl; Cbz, benzyloxycarbonyl; LLE-NA, Cbz-Leu-Leu-Glu- β -naphthylamide; LSTR-AMC, *N*-Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin; NEM, *N*-ethylmaleimide; MCP, multicatalytic proteinase complex; 2D, two-dimensional.

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50 mM ammonium acetate buffer, pH 7.0) was prepared for amino acid analysis following the addition of a known amount of norleucine standard. Hydrolysis of MCP samples, untreated or oxidized by performic acid, was performed by treatment with 6 M HCl for 24, 48 or 72 h at 110 °C. The results were calculated using a value of 700 kDa for the molecular mass of the proteinase complex. An absorption coefficient ($A_{280}^{0.1\%}$) of 1.3 was determined by measuring the absorbance of the above standard MCP solution.

Proteinase assays

Assays of proteinase activity were carried out for 30 min at 37 °C in 50 mM Hepes/KOH buffer, pH 7.5 or 8.0, with either 20 or 50 μ M LSTR-AMC, 20 or 50 μ M AAF-AMC or with 0.4 mM LLE-NA, as described previously [10].

Labelling of the site(s) catalysing 'trypsin-like' activity

A differential labelling protocol was designed to label selectively subunit(s) which bind leupeptin. MCP (0.5 mg/ml in 50 mM Hepes buffer, pH 7.5) was preincubated with leupeptin (47 μ M) for 30 min at 20 °C prior to addition of unlabelled NEM (10 mM) and further incubation for 2–3 h at 20 °C. At the end of this period the 'trypsin-like' activity was recovered either by dialysis overnight at 4 °C against 50 mM Hepes buffer, pH 7.5, or by dialysis for 1 h and treatment with 0.1 % NaBH₄ for 1 h, followed by dialysis. Finally, the MCP (0.1 mg/ml) was incubated for 1 h at 20 °C, usually with 0.1 mM [¹⁴C]NEM, then dialysed against 50 mM Hepes buffer, pH 7.5, to remove the excess NEM. Samples prepared by this differential-labelling protocol were compared with samples treated with [¹⁴C]NEM only. Radiolabelled protein samples, usually approx. 2400 c.p.m./ μ g for MCP treated with [¹⁴C]NEM only and approx. 200 c.p.m./ μ g for differentially labelled samples, were analysed by SDS/PAGE and two-dimensional PAGE (2D) [19,20] and fluorography [21]. The amount of MCP loaded on to gels was usually 10 μ g for SDS/PAGE gels and 30–40 μ g for 2D-PAGE gels. Dried gels were exposed to Fuji RX film at –70 °C for up to 1 week (SDS/PAGE gels) or 2–4 weeks (2D-PAGE gels). The longer time required to reveal spots on the 2D-PAGE gels probably reflects a loss of protein during transfer to the second dimension.

RESULTS AND DISCUSSION

The peptide arginine aldehyde inhibitors leupeptin and antipain are specific inhibitors of the 'trypsin-like' activity of the MCP complex. At a concentration of around 50 μ M leupeptin or antipain, residual activity with LSTR-AMC after a 30 min preincubation was less than 20 %, whereas activities with the other types of substrate are not significantly affected (Table 1). Leupeptin was chosen for further experiments. The inhibition by leupeptin is apparently of the slow-binding nature. For reactions started by the addition of enzyme, the progress curves are non-linear, and preincubation of enzyme and inhibitor was required to obtain the maximum inhibitory effect (Figure 1). For some proteinases, apparent slow binding occurs because of the rate of interconversion of inactive forms of leupeptin into the active inhibitor, which only constitutes 2 % of the total [22]. However, in the case of MCP, the on-rate is substantially lower than the rate of conversion from inactive into active inhibitor. Leupeptin forms a hemiacetal derivative of the catalytic serine residue of serine proteinases [23].

Inhibition of MCP by leupeptin can be reversed slowly, either by dialysis to remove the leupeptin or by reaction with NaBH₄,

to produce the peptidyl alcohol, which is non-inhibitory at concentrations up to at least 100 μ M. Complete re-activation of the leupeptin-inhibited 'trypsin-like' activity can be achieved by dilution into an assay mix containing 20 mM NaBH₄, but no re-activation was observed when the enzyme was diluted for assay in the absence of NaBH₄ (Figure 1). The result of addition of NaBH₄ to leupeptin-treated MCP depended on the precise concentrations of leupeptin, and without dilution sometimes resulted in only a 30–50 % recovery of activity in 1 h, even with repeated additions of NaBH₄ (Figure 1). These observations reflect the low K_i value, determined to be less than 1 μ M (results not shown) and the slow off-rate of leupeptin, which is also evidenced by the long dialysis period required to recover full activity of leupeptin-treated MCP in the absence of NaBH₄. Conditions for complete recovery of LSTR-AMC-hydrolysing activity were found for the labelling experiments (see below).

Of the thiol-reactive reagents tested previously [10], NEM (1 mM) was found to inhibit the 'trypsin-like' activity of rat liver MCP, while having rather little effect on other activities of the complex. Almost complete inhibition of proteolytic activity

Table 1 Effect of leupeptin, antipain and NEM on rat liver MCP activities

Assays of proteinase activity were carried out for 30 min at 37 °C in 50 mM Hepes buffer, pH 8.0, after preincubation of proteinase with inhibitor for 30 min at 20 °C in the same buffer followed by a 10-fold dilution into the assay mix.

Inhibitor	Concn.	Activity (% of control) with:		
		LSTR-AMC	AAF-AMC	LLE-NA
None	—	100	100	100
Leupeptin	47 μ M	8	88	94
Antipain	47 μ M	28	99	103
NEM	5 mM	11	102	47

Figure 1 Inhibition of 'trypsin-like' activity of the multicatalytic proteinase complex by leupeptin and NEM

Preincubations were carried out at 20 °C with 0.1 mg/ml MCP in 50 mM Hepes, pH 7.5, and then assays were carried out for 10 min at 37 °C following addition of LSTR-AMC. ■, Control enzyme with NaBH₄ added at the time indicated by the arrow; ●, enzyme treated with 5 mM NEM. □, Enzyme protected with 47 μ M leupeptin and treated with NEM, followed either by addition of NaBH₄ or by 24 h dialysis. Enzyme treated with leupeptin but not NEM followed a similar activity profile. The dotted vertical line indicates the start of the 24 h dialysis. The bar indicates the range of values for the recovery of activity following treatment with NaBH₄ (see the text). Similar levels of LSTR-AMC hydrolase activity were recovered in samples treated with leupeptin but no NEM.

assayed with LSTR-AMC as substrate can be achieved within 1 h with 5 mM NEM (Table 1). At this higher concentration, NEM does inhibit the peptidylglutamyl-peptide hydrolase activity, but this observation does not affect the labelling experiments, because leupeptin is specific for the 'trypsin-like' activity except when used at concentrations above 100 μ M. Inhibition of 'trypsin-like' activity by NEM, unlike that by leupeptin, is irreversible and unaffected by NaBH_4 (Figure 1). If MCP is preincubated with leupeptin prior to the addition of NEM, the LSTR-AMC-hydrolysing activity can be recovered following removal of excess NEM and leupeptin (Figure 1). However, the resulting modified active enzyme appears to be much less sensitive to inhibition by subsequent treatment with NEM than the untreated enzyme, and often a maximum of only 30–70% inhibition could be obtained. If, as we now believe to be the case, the thiol groups are not essential for catalytic activity, these observations could be explained by a minor change in conformation in the modified enzyme. Alternatively they might be due to incomplete removal of bound leupeptin if residual trypsin-like activity were activated to give an apparent 100% recovery.

Direct treatment with [^{14}C]NEM of MCP unprotected by leupeptin caused labelling of many of the polypeptide components of the complex (Figures 2a and 2b). The number of groups modified by NEM was measured by the incorporation of label from [^{14}C]NEM, and a value of up to approx. 30 residues per MCP molecule was obtained, depending upon the concentration of NEM used (0.1–5.0 mM) and the time of reaction (30 or 60 min). It is possible that the reaction was not entirely specific for cysteine residues [24], but labelling of the enzyme could be blocked by prior treatment with unlabelled reagent (10 mM). A total number of 113 cysteine residues per molecule was determined from the amino acid analysis. The cysteine content is somewhat higher than that reported for rat liver MCP by Tanaka et al. [25], which may be explained by our improved analysis of this amino acid (as cysteic acid) following performic acid oxidation. There appear to be some differences in amino acid composition for MCPs isolated from different sources [26,27].

In enzyme samples which had been protected by leupeptin during treatment with unlabelled NEM and subsequently labelled with [^{14}C]NEM (approx. 2 mol/mol of MCP) after removal of the leupeptin, predominantly one labelled band (molecular mass 29 kDa) was observed on autoradiograms of SDS/PAGE gels (results not shown). Analysis by two-dimensional PAGE showed either one (pI 5.7; Figure 2d) or two (pI 5.7 and 6.0; Figure 2c) major labelled spots. There are a number of possible explanations for the apparent variation in these results. Figure 2(c) was obtained following incubation at a higher concentration of [^{14}C]NEM. Leupeptin may protect more than one site, or the results may be explained by differences in the relative amounts of the polypeptides concerned, as observed on gels stained for protein (Figures 2a and 2e), and it is also possible that the two major labelled polypeptides are related. Background labelling of other polypeptides was observed in the experiment in which the higher concentration of [^{14}C]NEM was used (Figure 2c).

Figure 2 2D-PAGE of labelled MCP

(a) A 2D-PAGE gel of MCP stained for protein with Coomassie Blue. (b) MCP control sample unprotected by leupeptin and treated only with [^{14}C]NEM (0.1 mM), detected by fluorography. (c) and (d) Two separate MCP preparations preincubated with leupeptin (47 μ M) and unlabelled NEM (10 mM) prior to treatment with [^{14}C]NEM (0.1 mM, c; 1.25 mM, d), showing detection of labelled polypeptides by fluorography. The sample in (c) was inhibited by 29% in 60 min and contained 220 c.p.m./ μ g of MCP, whereas the sample in (d) was inhibited by 42% in 30 min and contained 232 c.p.m./ μ g of MCP. (e) Silver-stained gel used to obtain the fluorogram shown in (c). Arrows indicate the spots identified in (c) and (d).

On the basis of the reactivity of MCP with some inhibitors of serine proteinases, we now believe the enzyme to be a novel type of serine protease [28], and the 'trypsin-like' activity can be completely inhibited by the serine-proteinase inhibitor 4-(2-aminoethyl)benzenesulphonyl fluoride. The results presented here suggest that the 'trypsin-like' activity of MCP may be associated with only one or two polypeptide components of the complex. It seems unlikely that the labelled cysteines are catalytic residues. Therefore it is possible that polypeptides identified in the present study are adjacent to catalytic subunits and involved only in substrate binding rather than being catalytic subunits themselves. However, the conclusion that an activity is catalysed by only one or two subunits within the MCP complex is consistent with results of recent studies showing loss of the 'chymotrypsin-like' activity in a yeast mutant lacking the MCP PRE1 gene [5].

We thank Dr. L. Packman (Department of Biochemistry, University of Cambridge) for advice on the preparation of samples for amino acid analysis and for carrying out the analyses, and Dr. K. Lilley for preparing the samples and calculating the results. A.J.R. is a Lister Institute-Jenner Research Fellow. This work was supported by the Medical Research Council and by the Lister Institute of Preventive Medicine.

REFERENCES

- Rivett, A. J. (1989) *Arch. Biochem. Biophys.* **268**, 1–8
- Orlowski, M. (1990) *Biochemistry* **29**, 10289–10297
- Eytan, E., Ganoth, D., Armon, T. and Hershtko, A. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7751–7755
- Rivett, A. J. (1990) *Curr. Opin. Cell Biol.* **2**, 1143–1149
- Heinemeyer, W., Kleinschmidt, J. A., Saidowsky, J., Escher, C. and Wolf, D. H. (1991) *EMBO J.* **10**, 555–562
- Brown, M. G., Driscoll, J. and Monaco, J. J. (1991) *Nature (London)* **353**, 355–357
- Glynne, R., Powis, S. H., Beck, S., Kelly, A., Kerr, L. A. and Trowsdale, J. (1991) *Nature (London)* **353**, 357–360
- Wilk, S. and Orlowski, M. (1983) *J. Neurochem.* **40**, 842–849
- Orlowski, M. and Michaud, C. (1989) *Biochemistry* **28**, 9270–9278
- Rivett, A. J. (1989) *J. Biol. Chem.* **264**, 12215–12219
- Djaballah, H. and Rivett, A. J. (1992) *Biochemistry* **31**, 4133–4141
- Rivett, A. J. and Sweeney, S. T. (1991) *Biochem. J.* **278**, 271–277
- Lilley, K. S., Davison, M. D. and Rivett, A. J. (1990) *FEBS Lett.* **262**, 327–329
- Tanaka, K., Fujiwara, T., Kumatori, A., Shin, S., Yoshimura, T., Ichihara, A., Tokunaga, F., Aruga, R., Iwanaga, S., Kakizuka, A. and Nakanishi, S. (1990) *Biochemistry* **29**, 3777–3785
- Haass, C., Pesold-Hurt, B., Multhaup, B., Beyreuther, K. and Klotzel, P.-M. (1990) *Gene* **90**, 235–241
- DeMartino, G. N., Orth, K., McCullough, M. L., Lee, L. W., Munn, T. Z., Moomaw, C. R., Dawson, P. A. and Slaughter, C. A. (1991) *Biochim. Biophys. Acta* **1079**, 29–38
- Savory, P. J. and Rivett, A. J. (1991) *Biochem. Soc. Trans.* **19**, 292s
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Bonner, W. M. and Laskey, A. R. (1974) *Eur. J. Biochem.* **46**, 83–88
- Schultz, R. M., Varma-Nelson, P., Ortiz, R., Kozlowski, K. A., Orawski, A. T., Pagast, P. and Frankfater, A. (1989) *J. Biol. Chem.* **264**, 1497–1507
- Ortiz, C., Tellier, C., Williams, H., Stolorow, N. J. and Scott, A. I. (1991) *Biochemistry* **30**, 10026–10034
- Smyth, D. G., Blumenfeld, O. O. and Konigsberg, W. (1964) *Biochem. J.* **91**, 589–595
- Tanaka, K., Yoshimura, T., Ichihara, A., Kameyama, K. and Takagi, T. (1986) *J. Biol. Chem.* **261**, 15204–15207
- Tanaka, K., Yoshimura, T., Kumatori, A., Ichihara, A., Ikai, A., Nishigai, M., Kameyama, K. and Takagi, T. (1988) *J. Biol. Chem.* **263**, 16209–16217
- Zolfaghari, R., Baker, C. R. F., Amirgholami, A., Canizaro, P. C. and Behal, F. J. (1987) *Arch. Biochem. Biophys.* **258**, 42–50
- Djaballah, H., Harness, J. A., Savory, P. J. and Rivett, A. J. (1992) *Eur. J. Biochem.*, in the press