Activation of the multicatalytic proteinase from rat skeletal muscle by fatty acids or sodium dodecyl sulphate

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A multicatalytic proteinase from rat skeletal muscle contains active site(s) catalysing the degradation of benzoyl-Val-Gly-Arg 4-methyl-7-coumarylamide, succinyl-Ala-Ala-Phe 4-methylcoumarylamide and [^{14}C]methylcasein as well as benzyloxycarbonyl-Leu-Leu-Glu 2-naphthylamide. These activities are 7–14-fold activated by 1 mM-sodium dodecyl sulphate. The activation leads to a higher susceptibility to the proteinase inhibitor chymostatin and to a lower ability to be inhibited and precipitated by antibodies raised against the non-activated enzyme. Since no changes in M_r or subunit composition were observed in the SDS-activated form, some conformational changes seem to occur during the activation step. More pronounced activation was observed in the presence of physiological concentrations of fatty acids; oleic acid at 100 μM concentrations stimulated the proteinase about 50-fold. In contrast with the non-activated proteinase, the activated enzyme considerably degrades muscle cytoplasmic proteins *in vitro*. Thus it is not unlikely that, *in vivo*, potential activators such as fatty acids can induce the multicatalytic proteinase to participate in muscle protein breakdown.

Intracellular protein degradation is catalysed by proteolytic enzymes that are localized within the lysosomal compartment as well as in the extralysosomal, cytoplasmic compartment. To prevent uncontrolled action of these proteinases, a situation that would lead to wastage and cell death, these enzymes are generally associated with proteinase inhibitors (Lenney, 1980). This proteinase–proteinase inhibitor interaction is probably one mechanism to regulate intracellular protein catabolism. In addition, a number of other mechanisms have been discussed that may be involved in the control of the highly co-ordinated breakdown of intracellular proteins (for reviews, see Goldberg & Dice, 1974; Goldberg & St. John, 1976; Holzer & Heinrich, 1980). Regulation may occur at both levels, by post-translational modification of the substrate proteins (Beynon, 1980) as well as by modulation of the proteolytic activities, e.g. by

Abbreviations used: DTT, dithiothreitol; NMec, 4-methyl-7-coumarylamide; NNap, 2-naphthylamide; SDS, sodium dodecyl sulphate, Bz, benzoyl; Suc, succinyl (3-carboxypropionyl).

activators like Ca^{2+} (Murachi *et al.*, 1981) or ATP (Hershko & Ciechanover, 1982; Tanaka *et al.*, 1983).

We have recently isolated a high-molecular-mass proteinase from rat skeletal muscle tissue. This enzyme has been designated multicatalytic proteinase, on the basis of observations that the catalytic sites for the substrates Bz-Val-Gly-Arg-NMec, Suc-Ala-Ala-Phe-NMec, Z-Leu-Leu-Glu-2NNap and [^{14}C]methyl casein were antagonistically affected by several compounds [Dahlmann *et al.*, 1983a; the preceding paper (Dahlmann *et al.*, 1985)].

Wilk & Orlowski (1980) have identified a similar enzyme in bovine pituitary. More recently, those authors reported that the Z-Leu-Leu-Glu-2-NNap-hydrolysing activity can be activated by low concentrations of SDS (Wilk & Orlowski, 1983).

In the present study we have investigated the effect of SDS and structurally related compounds on the hydrolytic activities of the multicatalytic proteinase from rat skeletal muscle with the aim of gaining insight into the mechanism of activation.

Experimental

Materials

The substrates Bz-Val-Gly-Arg-NMec and Suc-Ala-Ala-Phe-NMec were products from Bachem Feinchemikalien AG, Bubendorf, Switzerland. [^{14}C]Methylcasein ($0.82\mu\text{Ci}/\text{mg}$) was obtained from New England Nuclear, Boston, MA, U.S.A. The substrate Z-Leu-Leu-Glu-2-NNap was generously given by Dr. D. Wilk, Mount Sinai School of Medicine, New York, NY, U.S.A. SDS was purchased from Pierce Chemical Co., Rockford, IL, U.S.A., and AG 11 A8 anion-exchange resin was from Bio-Rad, Richmond, CA, U.S.A. The saturated and unsaturated fatty acids, as well as sodium oleate and oleoyl ethyl ester were from Sigma Chemie G.m.b.H., Taufkirchen Germany.

Methods

The preparation of the multicatalytic proteinase from rat skeletal-muscle tissue and activity measurement were performed as described previously (Dahlmann *et al.*, 1985).

The isolation of the immunoglobulin G fraction from rabbit anti-(multicatalytic proteinase) serum and the immunoinhibition assays were essentially performed as described previously (Dahlmann *et al.*, 1982). Crossed immunoelectrophoresis was carried out as described by Weeke (1973), with 5% (v/v) anti-(multicatalytic proteinase) serum in 1% (w/v) agarose gels for the second dimension. Electrophoresis buffer was 24mM-barbital, pH 8.6. Electrophoresis in the first dimension was at a constant voltage gradient of 20V/cm for 45 min and at 10°C, followed by electrophoresis in the second dimension, run at 1.2V/cm for 16h and at 10°C. Washing, drying and staining with Coomassie Blue R250 of the agarose gel was performed as described by Johansson (1972).

Removal of SDS from the SDS-activated enzyme was carried out by using a column (1.5cm $\times$ 13cm) of AG 11 A8 resin equilibrated in distilled water (Kapp & Vinogradov, 1978). The flow rate was 60ml/h and 2ml fractions were collected. Routinely, a 2ml portion of proteinase solution, containing about 3–4mg of enzyme protein, was applied to the column after the enzyme had been concentrated and desalted by ultrafiltration in an Amicon cell (YM-30 membrane) or in Centricon 30 microconcentrators (Amicon Corp., Danvers, MA, U.S.A.). After chromatography, the fractions with proteolytic activity were pooled and dialysed against 20mM-Tris/HCl/1mM-EDTA/1mM-Na N_3 /0.1% (v/v) mercaptoethanol, pH 7.5.

The isolation and separation of cytoplasmic proteins from skeletal muscle as well as measurement of their proteolytic degradation by using

Coomassie Blue G250 was performed as described previously (Dahlmann *et al.*, 1983b).

Protein concentration was determined with Coomassie Blue G250 by the method of Sedmak & Grossberg (1977), with control serum (LAB TROL; Dade, Miami, FL, U.S.A.) as a standard.

Results

When the activities of purified rat skeletal-muscle multicatalytic proteinase were tested in the presence of increasing concentrations of SDS, all four activities were found to be enhanced, and each activity was maximally activated at 0.03% SDS. At higher SDS concentrations the activities gradually decreased to basal values or below (Table 1).

In order to investigate whether activation of the enzyme was accompanied by a dissociation into subunits of the high- M_r proteinase or whether it was due to the separation of an inhibitory peptide, the activated enzyme was subjected to gel filtration on TSK HW 55 (S) after removal of SDS by chromatography on AG 11 A8 resin (Dahlmann *et al.*, 1983a). However, it was found that the activated enzyme, when tested with Bz-Val-Gly-Arg-NMec, Suc-Ala-Ala-Phe-NMec or [^{14}C]methylcasein as substrate, was eluted from the column at the same position as the non-activated enzyme (result not shown). Further, no difference in mobility or in subunit composition was detected with the activated proteinase as compared with the non-activated enzyme when analysed by electrophoresis in polyacrylamide gels under non-denaturing conditions and by SDS/polyacrylamide-gel electrophoresis respectively (results not shown). Since, also, an activation of the enzyme did not occur when the non-activated enzyme was chromatographed on AG 11 A8 resin (results not shown), all these findings argue against the presence of an inhibitory peptide that may be removed in the activation step.

A difference between the non-activated and SDS-activated enzyme becomes, however, apparent by analysis by rocket immunoelectrophoresis (Fig. 1). Whereas the rocket obtained with the untreated enzyme formed a double peak, this phenomenon was not observed with the SDS-activated enzyme (Fig. 1).

An altered reactivity of the SDS-activated enzyme with antibodies raised against the non-activated enzyme could also be observed in an immunoinhibition test. Thus a considerably larger amount of IgG was necessary to inhibit as well as to precipitate the enzyme in the activated form as compared with the non-activated enzyme (Fig. 2). This suggests that some conformational change is occurring in the proteinase molecule during the

Table 1. Effect of SDS on the activities of the multicatalytic proteinase

SDS was dissolved in 20 mM-Tris/HCl/1 mM-EDTA/1 mM-Na ₃ PO ₄ /0.1% (v/v) mercaptoethanol, pH 7.5, and mixed with purified enzyme to obtain final concentrations of SDS as indicated. The enzyme concentration was 5 µg/assay, except for Z-Leu-Leu-Glu-2NNap, where 11 µg/assay was used. Enzyme and SDS were incubated for 10 min at 37°C before the hydrolysis of the various substrates was measured. Activities are expressed in pmol/min per ml, except that towards [¹⁴ C]methylcasein, which is expressed in c.p.m. per 80 min incubation. The data are means for triplicate experiments, with a variation of less than 5% between these replications.									
Enzyme activity									
Final SDS concn. (%)	Substrate	Bz-Val-Gly-Arg-NMec	Suc-Ala-Ala-Phe-NMec	Z-Leu-Leu-Glu-2NNap	[¹⁴ C]Methylcasein				
0	...	91	14	79	58				
0.01		423	48	264	85				
0.02		577	105	802	453				
0.03		639	164	1159	603				
0.04		477	88	710	168				
0.05		93	6	75	162

Fig. 1. Crossed immunoelectrophoresis of multicatalytic proteinase
A 2.0 µg portion of multicatalytic proteinase (a), as well as of SDS-activated multicatalytic proteinase (b), was subjected to crossed immunoelectrophoresis with 5% (v/v) rabbit anti-(rat multicatalytic proteinase) serum. To remove SDS the activated enzyme was chromatographed on AG 11 A8 anion-exchange resin before immunoelectrophoresis.

Fig. 2. Immunotitration of multicatalytic proteinase
Multicatalytic proteinase (open symbols) and SDS-activated multicatalytic proteinase (closed symbols) after removal of SDS by chromatography on AG 11 A8 resin was incubated with rabbit anti-(rat multicatalytic proteinase) IgG for 60 min at 37°C. The mixture was then centrifuged for 10 min at 45000g. Residual hydrolytic activity was determined in the supernatants with Bz-Val-Gly-Arg-NMec (○, ●) and Suc-Ala-Ala-Phe-NMec (△, ▲) as substrates. The activities are given as percentage of controls containing the proteinase solution and the IgG dilution buffer (0.15M-NaCl/0.1M-sodium phosphate, pH 7.4). Enzyme was dissolved in 20 mM-Tris/HCl/1 mM-EDTA/1 mM-Na₃PO₄/0.1% (v/v) mercaptoethanol, pH 7.5

activation step. This idea is also supported by the finding that, in the SDS-activated state, not only are the Bz-Val-Gly-Arg-NMec- and Suc-Ala-Ala-Phe-NMec-hydrolysing activities inhibited by chymostatin but the casinolytic activity, previously activated by chymostatin in the non-activated

enzyme, is now equally inhibited (Table 2). The same effect was observed when leupeptin was used instead of chymostatin (results not shown).

In an attempt to find activating molecules more likely to play a physiological role than does SDS, we tested saturated fatty acids with various even-numbered-carbon-chain lengths. As shown in Fig. 3, all fatty acids tested stimulated the caseinolytic activity of the multicatalytic proteinase irrespective of their chain length and concentration, but the stimulatory effect on the proteolytic activity was the most prominent with stearic, arachidic and behenic acids. The optimum chain length was $C_{20:0}$, and the presence of this fatty acid at a concentration of $200\text{ }\mu\text{M}$ provoked a 40-fold increase in caseinolytic activity.

To study the effects of unsaturated fatty acids on the proteolytic activity, we tested the influence on the caseinolytic activity of the multicatalytic proteinase of oleic ($C_{18:1}$), linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids and compared it with that of stearic acid ($C_{18:0}$). Oleic acid, the most abundant among unsaturated fatty acids in muscle, stimulates the proteolytic activity to a significant extent, at concentration as low as $0.02\text{ }\mu\text{M}$. At $100\text{ }\mu\text{M}$, the highest concentration of $C_{18:1}$ acid tested, the enzyme activity was about 50-fold increased over control values (Fig. 4). By contrast, to achieve a stimulation of the proteinase activity by stearic, linoleic and linolenic acids, $100\text{ }\mu\text{M}$ concentrations of these fatty acids were required. SDS does not stimulate the proteinase at micromolar concentrations (Fig. 4) and, as mentioned above, 0.03% SDS, equivalent to 1 mM , is needed to maximally activate the enzyme. Sodium oleate activated the enzyme to the same low extent as did linolenic acid, and no activation was observed with oleoyl ethyl ester (results not shown), indicating that the carboxy group of the fatty acid is important in the activation process.

Fatty acids also showed a stimulatory effect on both the Bz-Val-Gly-Arg-NMec- and Suc-Ala-Ala-Phe-NMec-hydrolysing activities, but to a much lower extent than on the caseinolytic activity (Table 3).

Fig. 3. Effect of saturated fatty acids on the activity of the multicatalytic proteinase

A 0.05 ml portion of purified enzyme ($11\text{ }\mu\text{g}$), 0.05 ml of fatty acid dissolved in $20\text{ mM-Tris/HCl}/1\text{ mM-EDTA}/1\text{ mM-NaN}_3/0.1\%$ (v/v) mercaptoethanol, $\text{pH } 7.5$, containing 6% (v/v) dimethyl sulphoxide and 0.05 ml of $0.1\text{ M-potassium phosphate}/30\text{ mM-cysteine}$, $\text{pH } 8.0$, were incubated for 10 min at 37°C . A 0.05 ml portion of $[^{14}\text{C}]$ methylcasein was then added and the proteolytic activity was measured after a further incubation for 60 min at 37°C . The final concentrations of fatty acids were $0.02\text{ }\mu\text{M}$ ($\blacksquare$), $1.56\text{ }\mu\text{M}$ ($\square$), $6.25\text{ }\mu\text{M}$ ($\blacktriangle$), $25\text{ }\mu\text{M}$ ($\triangle$), $100\text{ }\mu\text{M}$ ($\bullet$) and $200\text{ }\mu\text{M}$ ($\circ$). The proteolytic activities are given as percentage of the control value obtained without fatty acid.

Table 2. Effect of chymostatin on the activities of the multicatalytic proteinase

A 0.05 ml portion of non-activated ($80\text{ }\mu\text{g/ml}$) or SDS-activated multicatalytic proteinase ($80\text{ }\mu\text{g/ml}$) after chromatography on AG 11 A8 anion-exchange resin and dialysis against $20\text{ mM-Tris/HCl}/1\text{ mM-EDTA}/1\text{ mM-NaN}_3/0.1\%$ (v/v) mercaptoethanol, $\text{pH } 7.5$, was mixed with 0.05 ml of a solution containing chymostatin, dissolved in the same buffer, but containing 10% (v/v) dimethyl sulphoxide, at the concentration indicated. The mixture was left for 10 min at 21°C and proteolytic activities were measured by addition of the substrate solutions. The activities are given as the percentage of the control value obtained without chymostatin. The data are mean values for three (Bz-Val-Gly-Arg-NMec, Suc-Ala-Ala-Phe-NMec) and two ($[^{14}\text{C}]$ methylcasein) experiments, with variations of less than 5 and 10% respectively.

Substrate	Activity state of proteinase	Chymostatin final concn. (mM) ...	Activity (%)					
			0	1	7.8	62.5	125	500
Bz-Val-Gly-Arg-NMec	Non-activated		100	81	62.9	26.1	19.4	9.1
	Activated		100	82	51.3	13.2	6.7	2.5
Suc-Ala-Ala-Phe-NMec	Non-activated		100	109	109	58.2	49.1	23.6
	Activated		100	90.9	61.8	27.3	18.2	18.2
$[^{14}\text{C}]$ Methylcasein	Non-activated		100	115	152	343	322	132
	Activated		100	88	89	84	83	70

Table 3. Effect of fatty acids on the Bz-Val-Gly-Arg-NMec- and Suc-Ala-Ala-Phe-NMec-hydrolysing activities of the multicatalytic proteinase

A 0.05ml sample of fatty acids (final concn. 100 μM) dissolved in 6% (v/v) dimethyl sulphoxide/20mM-Tris-HCl/1 mM-EDTA/1 mM-Na₃N/0.1 (v/v) mercaptoethanol, pH 7.5, was mixed with 0.05 ml of multicatalytic proteinase (100 μg/ml) and preincubated for 10min at 37°C, before 0.1 ml of substrate solution was added to measure proteolytic activity. Assays were carried out in triplicate and there was less than 5% variation between these replicates.

Compound	Substrate ...	Hydrolysis			
		Bz-Val-Gly-Arg-NMec		Suc-Ala-Ala-Phe-NMec	
		(pmol/min per ml)	(%)	(pmol/min per ml)	(%)
None		105	100	11	100
Stearic acid		164	156	14	127
Oleic acid		688	655	116	1054
Linoleic acid		688	655	107	973
Linolenic acid		222	211	13	118
Arachidic acid		150	143	15	136
Behenic acid		509	485	39	355

Fig. 4. Effect of saturated and unsaturated fatty acids as well as SDS on the activity of the multicatalytic proteinase Purified proteinase (11 μg) was incubated with stearic acid (■), oleic acid (△), linoleic acid (●), linolenic acid (○) and SDS (□) respectively at the indicated concentrations. Conditions, buffer and assay conditions of proteolytic activity were as described in the legend to Fig. 3.

To investigate whether the activation of the multicatalytic proteinase may be of relevance for the degradation of substrates that more closely resemble the physiological ones, cytoplasmic proteins from rat skeletal muscle were subjected to the

Table 4. Degradation of muscle cytoplasmic proteins by multicatalytic proteinase

Cytoplasmic proteins dissolved in 20mM-Tris/HCl, pH 7.5 and 9.5 respectively and multicatalytic proteinase were incubated in a shaking water bath for 22h at 37°C. Proteinase was dissolved in 20mM-Tris/HCl/1 mM-EDTA/1 mM-Na₃N/0.1% (v/v) mercaptoethanol, pH 7.5; from the activated proteinase SDS was removed by chromatography on AG 11 A8 resin. Controls contained Tris buffer without the enzyme. Degradation of the cytoplasmic protein was measured by using the Coomassie Blue G250-binding test described by Dahlmann *et al.* (1983b), and results are given as the percentage of the control value. Assay mixtures with activated and non-activated enzyme contained the same amount of enzyme protein at a ratio of substrate to enzyme of 20:1. Assays were carried out in duplicate, and there was less than 5% variation between these replicates.

Cytoplasmic protein fraction	pH	Degradation by multicatalytic proteinase (%)	
		Non-activated	Activated
A	7.5	6.3	13.0
	9.5	2.2	18.8
B	7.5	0	0
	9.5	0	55.1
C	7.5	0	0
	9.5	0.84	32.4

action of non-activated and SDS-activated proteinase. The cytoplasmic proteins had been fractionated into four groups (A,B,C,D) by anion-exchange chromatography (Dahlmann *et al.*, 1983b). Fraction-D proteins, having pI values between 5.3 and 3.5 (Dahlmann *et al.*, 1983b), were not used as substrates in these experiments, since they contained Bz-Val-Gly-Arg-NMec- and Suc-

Ala-Ala-Phe-NMec-hydrolysing activity. As shown in Table 4, the non-activated enzyme degraded only group-A and group-C proteins to a small extent. When the same amount of SDS-activated enzyme was incubated with the substrate proteins, the degradation of group-A proteins was increased 2–3-fold and group-B and -C proteins were now degraded to a considerable extent at pH 9.5.

Discussion

The present data show that the activities of the multicatalytic proteinases from rat skeletal muscle can be stimulated by SDS. Maximum activation of the purified enzyme was obtained at concentration of 0.03% SDS in the activation mixture. Although a similar concentration of SDS was required to activate the multicatalytic proteinase from bovine pituitary (Wilk & Orlowski, 1983), an important difference between the enzymes from the two sources resides in the fact that, in the enzyme from pituitary, only the Z-Leu-Leu-Glu-2NNap-hydrolysing activity is activated, whereas the other two activities are inhibited by SDS. In contrast, in the enzyme from rat skeletal muscle, all four activities tested are activated by SDS. The action of SDS appears to lead to the formation of a persistently activated state of the enzyme, in that, after the removal of SDS by chromatography on AG 11 A8 anion-exchange resin, about 80% of the enzyme activity loaded on to the column was recovered (results not shown). In the activation experiments the detergent was in a 3000-fold molar excess over the enzyme. Since Kapp & Vinogradov (1978) determined the ratio of SDS to protein to be 0.1–1.4 mol/mol after chromatography on AG 11 A8 and as no differences in electrophoretic mobility were found in our experiments between non-activated and SDS-activated enzyme after removal of SDS, we conclude that the major part of SDS was removed by this anion-exchange-chromatographic step without loss of activation of the enzyme.

The mechanism of activation, however, remains unclear. Wilk & Orlowski (1983) suggested that the detergent removes an inhibitor from the enzyme. On the basis of the results of gel-filtration experiments and analysis by SDS/polyacrylamide-gel electrophoresis we have, however, reason to believe that this activation step is not due to the removal of a potential inhibitory protein, since no change in M_r or subunit composition was detected with the activated proteinase. On the other hand, alterations became apparent when the activated enzyme was allowed to react with antibodies raised against the non-activated proteinase. The double peak that formed between non-activated enzyme

and the antibody was resolved to a single peak for the pair SDS-activated enzyme/antibody. Such an immunological 'camel' precipitate formation has been reported for partly degraded membrane proteins (Bjerrum & Bøg-Hansen, 1976). Alternatively, partial dissociation into subunits could also explain such a precipitate formation, as has been observed with the fatty acid synthetase complex (Paskin & Mayer, 1976). If the latter is correct, the activated enzyme would be in a more stable conformation that does not dissociate into subunits under the conditions of immunoelectrophoresis.

Indeed, from the results obtained with the immunoinhibition experiments, we conclude that the activation of the enzyme may evoke some conformational alterations in the molecular structure, since the amount of antibodies needed for inhibition and precipitation of the activated enzyme is more than twice that amount of antibodies necessary to achieve the same with the non-activated form of enzyme.

Assuming that such an activation of the multicatalytic proteinase is of significance *in vivo*, fatty acids would be potential candidates as modulators of the proteinase activity. Saturated fatty acids of all chain lengths tested slightly activated the catalytic activity of the enzyme and the largest stimulatory effect was found with a carbon chain length of 20. Among the unsaturated fatty acids, oleic acid was found to be the most efficient fatty acid, stimulating the proteinase activity at concentrations as low as $0.02 \mu\text{M}$. A higher degree of unsaturation of the fatty acid reduced its ability to activate the proteinase, as shown for linoleic and linolenic acid.

To our knowledge the concentration of free fatty acids within the muscle cell is presently unknown and the significance of the stimulatory effect of fatty acids on the multicatalytic proteinase is therefore difficult to assess. The increased fatty acid concentration as reported for skeletal-muscle tissue of diabetic rats (Garland & Randle, 1963) could result in an increased activity of the multicatalytic proteinase, or, *in vivo* the proteinase is *per se* in the activated form if the basal intracellular concentration of free fatty acids is within the micromolar range. Opinions about the intracellular level of fatty acids appear to be contradictory and, as recently discussed by Van der Vusse & Reneman (1984), there are several reasons to believe that the concentration within the cells is lower than 100 nmol/g wet wt. of tissue. In dog myocardial biopsies, concentrations as low as $10 \mu\text{mol/litre}$ were estimated (Van der Vusse *et al.*, 1982). If in skeletal muscle the concentration of fatty acids is of similar magnitude, the multicatalytic proteinase would be in a moderately activated

state within the cell. Thus, during diabetes and starvation, when the concentration of fatty acids is known to increase within the muscle tissue (Garland & Randle, 1964), fatty acids could dramatically increase the activity of the multicatalytic proteinase, which may then participate in the increased protein breakdown observed under these catabolic conditions. Modulation of proteolytic activity by fatty acids was reported previously for human granulocyte elastase (Ashe & Zimmerman, 1977) and for rat mast-cell chymase (Kido *et al.*, 1984). The activities of both enzymes are inhibited by fatty acids, whereas fatty acid alcohol esters increase the activity of the granulocyte elastase. Thus further investigations, to see whether fatty acids are regulatory compounds for the activity of proteolytic enzymes, may be remunerative.

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