EVIDENCE FOR THE MODIFICATION OF FRUCTOSE 1,6-BISPHOSPHATASE BY TWO DISTINCT LYSOSOMAL PROTEASES

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SUMMARY: Two lysosomal proteases have been detected, each capable of catalyzing a different modification of the NH_2-terminal region of rabbit liver fructose bisphosphatase. Protease I has optimum activity near pH 5.0, in contrast to Protease II, which is most active only at lower pH. The peptide formed by the action of Protease I is acid-insoluble, with a molecular weight of approximately 7,000, whereas Protease II releases a small acid-soluble peptide, containing the tryptophan residue that is located near the NH_2-terminus. In fasted rabbits, Protease II appears to be selectively released from the lysosomes.

We have previously reported two distinct modifications of fructose 1,6-bisphosphatase (Fru-P_2ase)[1] attributable to lysosomal proteases. One is the conversion of the native enzyme, having a neutral pH optimum and a subunit molecular weight of 36,000, to a form with alkaline pH optimum and a subunit molecular weight of 29,000 (1). The second modification was observed in animals fasted for 36-96 hours, and is characterized by the loss of the single tryptophan residue located near the NH_2-terminus of the molecule, without significant change in the molecular weight or pH optimum (2). These results suggested that at least two proteolytic activities may be involved in the modification of the enzyme. We have now obtained evidence for the presence of these two activities in liver lysosomes, and for the selective release of one of these activities into the cytosol fraction prepared from the livers of fasted animals.

[1]Abbreviations: Fru-P_2ase, fructose 1,6-bisphosphatase.

METHODS AND MATERIALS

Preparation of lysosomes. The heavy particle fraction containing lysosomes was isolated from the livers of fed and fasted animals as previously described (3). The final pellets obtained from 10 g of fresh liver were suspended in 1 ml of 0.25 M sucrose, containing 1 mM EDTA, pH 7.0. (Particulate Fraction). The particles were disrupted by freezing and thawing 10 times, and the membranes removed by centrifugation for 45 min at 50,000 xg. To obtain the cytosol fraction the original 10,000 g supernatant solution from the preparation of the lysosome fraction (Supernatant II, reference 3) was centrifuged at 100,000 xg for 45 min.

Purification of Fru-P_2ase. Native neutral Fru-P_2ase was purified from the livers of fed rabbits as previously described (4), except that the extract was heated to 60° for 3 min (5) before chromatography on phosphocellulose. The specific activity, assayed at pH 7.5 and 23° as previously described (4), was 15 units per mg of protein. The enzyme contained 4 equivalents of tryptophan per mole, and the subunit molecular weight determined by disc gel electrophoresis in Na dodecyl sulfate was 36,000. This will be referred to as the native enzyme (H_4-form).

Analysis of Reaction Products after Proteolytic Modification. The content of heavy subunits (MW = 36,000) and light subunits (MW = 29,000) was determined by disc gel electrophoresis in the presence of Na dodecyl sulfate, as previously described (4) and the gels scanned with a Gilford Model 240 Spectrophotometer equipped with a Model 2410 linear transport unit. Other methods as specified in the Figures and Tables.

Materials. Phosphocellulose (Whatman P-11) was washed with alkali and acid as previously described for the purification of Fru-P_2ase (4) and stored as a wet paste at 2°.

RESULTS

Evidence for the presence in rabbit liver lysosomes of two distinct proteases capable of modifying Fru-P_2ase was obtained by a comparison of

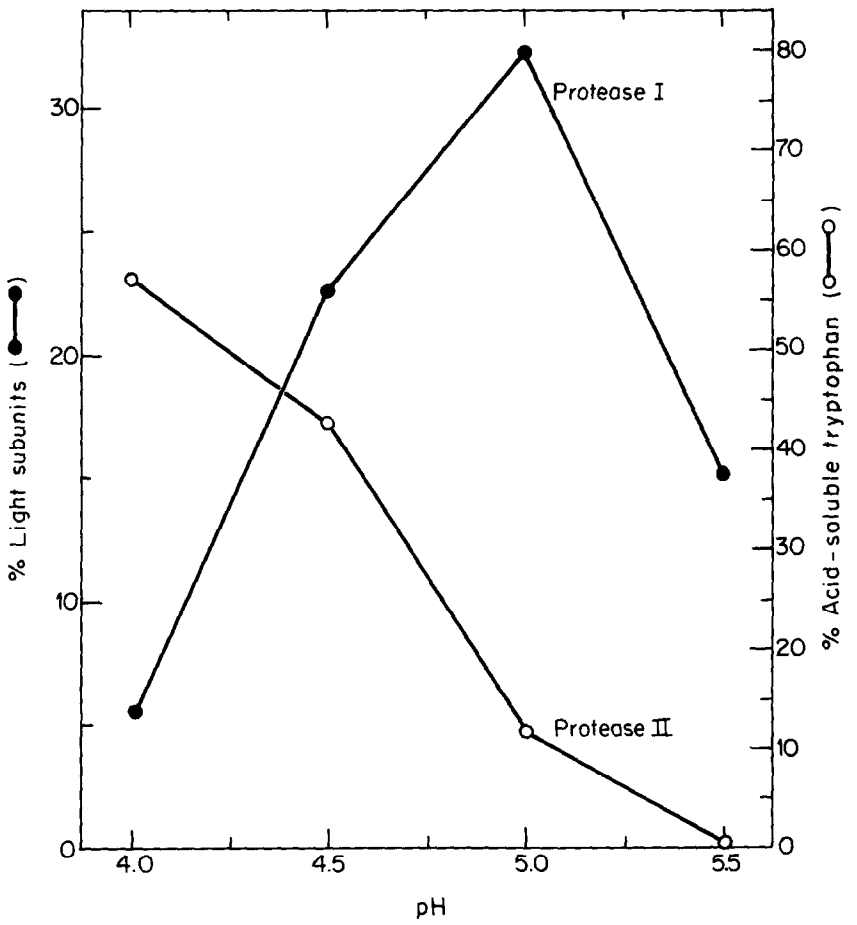

Fig. 1. Digestion of Fru-P_2ase (H_4 form) with proteolytic enzymes from rabbit liver. The particulate fraction was prepared from livers of fed rabbits and the enzymes released by freezing and thawing as described under Methods. The incubation mixtures, 1.0 ml, contained 2 mg of purified native Fru-P_2ase, 0.1 M acetate buffer at the indicated pH, 5 mM cysteine and particle contents equivalent to 0.1 mg of protein. After incubation for 120 min at 37°C, 0.1 ml of 50% CCl_3COOH was added and the suspension centrifuged. Aliquots of the supernatant solution (50 µl) were diluted to 2.0 ml with 6 M urea, containing 0.02 M triethanolamine - 0.02 M diethanolamine buffer, pH 9.2, and analyzed for tryptophan by fluorescence emission at 350 nm, with excitation at 273 nm, using the Aminco-Bowman Spectrofluorometer. Free tryptophan was used as the standard. The precipitate was suspended in 0.2 ml of 0.01 M phosphate buffer, pH 7.0, containing 2% Na dodecyl sulfate and 2% mercaptoethanol and analyzed in disc gel electrophoresis (4) for the content of heavy and light subunits.

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the products formed at pH 4.0 and pH 5.5 (Fig. 1). At the lower pH 58% of the total tryptophan was released as a small acid-soluble peptide without

change in the apparent molecular weight. At pH 5.5, on the other hand, no acid-soluble peptides containing tryptophan were formed, although 15% of the subunits were converted to the lighter species. At intermediate pH's a mixture of products was formed. The results of disc gel electrophoresis of the products obtained at pH 5.0-5.5 confirmed the presence of a peptide of molecular weight of about 7,000, which was not seen when the reaction was carried out at pH 4.0. Thus the site of cleavage was different at the two pH's.

We have previously reported (2) that the proteolytic activity toward Cbz-Gly-Tyr in rabbit liver extracts increases nearly 3-fold during fasting, and that this increase is largely due to the appearance of proteolytic activity in the supernatant fraction. We have now observed a parallel increase in the total Fru-P_2ase converting enzyme activity, as measured by the conversion of heavy to light subunits (Table I, Protease I), and also in the activity responsible for the release of the small acid-soluble peptide containing tryptophan (Table I, Protease II). Both activities increase nearly 3-fold, but the activity of Protease I remains confined to the lysosomal fraction, while nearly two-thirds of the activity of Protease II is found in the supernatant fraction.

In order to verify that the lysosomes were still intact, and to exclude the possibility that the release of Protease II activity was the result of mechanical disruption of fragile lysosomes during their isolation from the fasted livers, we measured the proteolytic activity without freezing and thawing under both isotonic and hypotonic conditions (Table II). Exposure of lysosomes to hypotonic solution containing Triton X-100 caused a 5-6 fold increase in the proteolytic activity, assayed with a mixture of Cbz-Glu-Phe and Cbz-Glu-Tyr as the substrate. The ratio of activity in hypotonic versus isotonic solution was the same for the particles prepared from livers of fed and fasted animals, indicating that there was no significant difference in lysosomal fragility. These results confirm that

TABLE I

Activity of Proteases in the Particulate and Cytosol Fractions of Livers from Fed and Fasted Rabbits[a]

Conditions	Disrupted Particles[b]		Cytosol fraction[c]		Total	
	Protease I	Protease II	Protease I	Protease II		
	nmoles/min/gm liver		nmoles/min/gm/liver		I	II
Fed	70	269	0	0	70	269
Fasted 36 hr	143	364	16	235	159	599
Fasted 60 hr	169	274	22	392	191	666
Fasted 96 hr	196	258	22	437	218	695

[a]The particulate and supernatant fractions were prepared as described under Methods, and the particles disrupted by freezing and thawing and the membrane fragments removed. The conditions of incubation with Fru-P_2ase were as described in the legend to Fig. 1, except that the quantity of supernatant fraction employed was 0.2 ml, containing 6 mg of protein. Protease I activity was measured at pH 5.0, and Protease II activity at pH 4.0.

[b]The incubation mixtures were analyzed as described in the legend to Fig. 1. The activity of Protease I is deduced from the fraction of light subunits, that of Protease II from the appearance of tryptophan in the CCl_3COOH-soluble peptides.

[c]For analysis of the products formed with the supernatant fraction the Fru-P_2ase was recovered by chromatography on phosphocellulose. The incubation mixture was diluted to 8 ml with H_2O and the pH adjusted to 7.5. Phosphocellulose was added until the pH reached 5.6, and the suspension was transferred to a small column (0.6 cm diam.), and the resin washed with 0.22 M acetate buffer, pH 5.75, containing 0.1 mM EDTA. Fru-P_2ase was eluted with the same buffer containing 2 mM Fru-P_2. The fractions containing Fru-P_2ase activity were collected and analyzed for heavy and light subunits as described in the legend to Fig. 1. Protease I activity represents the % of light subunits formed per min. Protease II activity was evaluated by the loss of tryptophan, measured spectrofluorometrically after digestion with pronase and chymotrypsin (Abrams, Sasaki and Horecker, unpublished procedure). In each case the units of proteolytic activity are expressed as nmoles of Fru-P_2ase subunits modified per min per gm of fresh liver.

the lysosomes were still intact, and that the activity in the lysosomal fraction was not due to proteases bound to lysosomal particles or membranes.

DISCUSSION

Two modified forms of Fru-P_2ase subunits have been identified in the livers of fasted rabbits (2). One form, accounting for approximately 90% of

TABLE II

Proteolytic Activity of the Particulate Fraction

under Isotonic and Hypotonic Conditions

Conditions	Proteolytic activity in		
	A. Isotonic solution[a] μmoles/hr/gm liver	B. Hypotonic solution[b] μmoles/hr/gm liver	B/A
Fed	2.9	15.1	5.2
Fasted 96 hr	3.5	20.4	5.8

[a]The particulate fraction was isolated as described under Methods and the proteolytic activity measured with Cbz-Glu-Phe and Cbz-Glu-Tyr as previously described (1). The reaction mixtures (1.0 ml) contained 2 mM each of Cbz-Glu-Phe and Cbz-Glu-Tyr in 0.05 M acetate buffer, pH 6.0 containing 0.25% sucrose, and 50 μl (2 mg protein) of lysosomal suspension. Incubation was at 37°C, and aliquots were removed at 15, 30, 60, 90, and 120 min for ninhydrin analysis, referred to glycine as the standard. The results are expressed as μmoles of free amino acid formed per hr per gm of fresh liver.

[b]The conditions were as described under footnote a, except that the reaction mixture contained no sucrose, and 0.1% Triton X-100 was added to the buffer.

the total subunits, has the same apparent molecular weight as the native subunits found in the enzyme from fed animals, but has lost the tryptophan residue located at, or close to, the NH_2-terminus (6). The second form, also devoid of tryptophan but having a much smaller molecular weight, accounts for only 10-15% of the total subunits. We have now identified two distinct proteolytic activities, designated Proteases I and II, respectively, that appear to be responsible for these modifications. Both are present in the lysosomal fraction, and released by freezing and thawing. Protease I shows optimum activity at pH 5.0, and cleaves a peptide bond approximately 60-70 amino acids removed from the NH_2-terminus. The peptide remains associated with enzyme, but is released when the protein is denatured and can be detected when the products are analyzed by disc gel electrophoresis in Na dodecyl sulfate. Protease II is active at more acid pH, and releases a small acid-soluble peptide containing the tryptophan residue.

Of particular interest is the observation that Protease II is selectively and progressively released during fasting. In animals fasted for 96 hrs nearly two-thirds of this activity is recovered in the supernatant fraction prepared in isotonic sucrose, in contrast to Protease I, which remains associated with the lysosomal fraction. That these lysosomes are still intact is apparent from the fact that their activity toward Cbz-Glu-Phe(Tyr) remains largely cryptic until the lysosomes are broken by Triton X-100 in isotonic solution. We have previously reported that the enzyme responsible for the hydrolysis of Cbz-Glu-Phe(Tyr), probably cathepsin A, is also present in the cytoplasmic fraction of livers from fasted animals (2). The present results establish that this enzyme is distinct from Protease I, since the latter is not released under these conditions. Protease II may also differ from cathepsin A, since it appears to catalyze a very different type of cleavage.

Although Protease II shows little or no activity at pH 5.5 or above, it apparently does act *in vivo*, because the same modification is observed in the enzyme isolated from the livers of fasted rabbits. The conditions required for this activity are now under investigation.

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REFERENCES

1. Pontremoli, S., Melloni, E., Balestrero, F., Franzi, A. T., De Flora, A., and Horecker, B. L. (1973) Proc. Nat. Acad. Sci. 70, 303-305.
2. Pontremoli, S., Melloni, E., Salamino, F., De Flora, A., and Horecker, B. L. (1974) Proc. Nat. Acad. Sci., in press.
3. Pontremoli, S., Melloni, E., Salamino, F., Franzi, A. T., De Flora, A., and Horecker, B. L. (1973) Proc. Nat. Acad. Sci. 70, 3674-3678.
4. Traniello, S., Melloni, E., Pontremoli, S., Sia, C. L., and Horecker, B. L. (1972) Arch. Biochem. Biophys. 149, 222-231.
5. Traniello, S., Pontremoli, S., Tashima, Y., and Horecker, B. L. (1971) Arch. Biochem. Biophys. 146, 161-166.
6. Pontremoli, S., Melloni, E., De Flora, A., and Horecker, B. L. (1973) Proc. Nat. Acad. Sci. 70, 661-664.