Changes in Activity and Molecular Properties of Fructose 1,6-Bisphosphatase During Fasting and Refeeding

(glucogenesis/proteolysis/rabbit liver/rabbit kidney/lysosomes/phosphoenolpyruvate/phosphoenolpyruvate carboxykinase/phosphofructokinase)

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ABSTRACT During prolonged starvation, fructose 1,6-bisphosphatase (EC 3.1.3.11) activity in rabbit liver and kidney shows a transient decrease during the first 36 hr, before rising at 96 hr to levels severalfold higher than those found in the livers of fed animals. Proteolytic activity appears in the 105,000 × g supernatant fraction within several hours of starvation, and continues to increase during the entire 96-hr period. On refeeding, the activities return to nearly the control levels within 24 hr. The catalytic properties of fructose 1,6-bisphosphatase isolated from the livers of fasted rabbits are similar to those of the enzyme from fed animals, but its structure is modified, since it no longer contains the single tryptophan residue located near the NH₂-terminus in the native enzyme. Thus this tryptophan residue is not required for the neutral pH optimum. The structural changes and the transient decrease in activity may be related to the observed increase in "free" proteolytic activity.

We have previously reported that exposure of rabbits to brief cold and fasting caused a decrease in the activity of liver fructose 1,6-bisphosphatase (Fru-P₂ase EC 3.1.3.11) that appeared to be correlated with an increase in the number and proteolytic activity of liver lysosomes (1). The decrease in Fru-P₂ase activity was unexpected, since this enzyme is required for glucogenesis (2) and its activity would be expected to increase under the above conditions.

We have now analyzed the changes in Fru-P₂ase levels in both livers and kidneys of rabbits fasted for longer periods, up to 96 hr, and compared these changes with those of two marker enzymes, phosphoenolpyruvate (PEP)-carboxykinase (EC 4.1.1.32) and 6-phosphofructokinase (PFK, EC 2.7.1.11). Johnson et al. (3) have shown that the glucogenic enzyme, PEP-carboxykinase, increases dramatically in the livers of fasted rabbits, while Weber et al. (4) have reported profound decreases in the level of the key glycolytic enzyme, PFK, in livers of fasted rats. We have confirmed the reciprocal changes in activities of these two enzymes and found that after 36 hr the level of Fru-P₂ase begins to rise and thereafter increases in parallel with that of PEP-carboxykinase. The activities of all these enzymes returned rapidly to the control levels when the animals were refed. The increases in activity of the two glucogenic enzymes, Fru-P₂ase and PEP-carboxykinase, occurred concurrently with large increases in proteolytic activity in both liver and kidney, that persisted through the period of prolonged fasting. The levels of proteolytic activity also returned rapidly to normal on refeding.

Recently, several laboratories have reported the isolation of native forms of Fru-P₂ase from liver and kidney of several mammalian species (5-8). The native enzymes from rabbit liver and kidney are characterized by having optimum activity at neutral pH, and each is composed of four subunits, whose molecular weight is approximately 36,000 (6, 7). Each subunit contains a single tryptophan residue (7, 9, 10), located in the NH₂-terminal region of the molecule (11). Exposure of the purified native enzyme to proteolytic enzymes, or to lysosomal fractions, has been shown to cause marked changes in the catalytic properties of the enzyme (9, 12, 13), notably a shift from a neutral to an alkaline pH optimum. The changes in catalytic properties appear to be due to the conversion of the native subunits to a lighter form, having a molecular weight of approximately 29,000. In view of these effects of proteolytic enzymes on the native enzyme in vitro, we examined the catalytic and molecular properties of the enzyme isolated from the fasted animals. Despite the large increases in proteolytic activity, however, Fru-P₂ase in both liver and kidney retained its neutral pH optimum, and the enzyme purified from the livers of the fasted animals was still composed of the heavier subunit, with only traces of a lighter species. However, while there were no gross changes in molecular weight, we did find evidence for a more subtle proteolytic modification of the NH₂-terminus, since the enzyme isolated from the livers of fasted animals had lost the single tryptophan residue from this region of the molecule. In several respects, the enzyme isolated from the fasted animals was found to resemble the form previously isolated from the livers of fasted rabbits in the winter (12). The present results demonstrate (1) that the single tryptophan residue lies very close to the NH₂-terminus, since it can be removed without change in molecular weight, (2) that its presence is not required for the neutral pH optimum, and (3) that it is removed by a proteolytic modification in vivo, possibly related to the increase in activity of lysosomal proteases.

MATERIALS AND METHODS

Materials. n-Fructose 1,6-bisphosphate (Na₄ salt), NADP, NADH, AMP, ADP, sodium dodecyl sulfate, inosine 5'-

† During the preparation of this manuscript, we learned of a similar observation by S. J. Benkovic, W. A. Frey, C. B. Libby, and J. J. Villafranca (1974) (Biochem. Biophys. Res. Commun. 57, 196-203), who also found that tryptophan was not essential for neutral activity, and that the enzyme lacking tryptophan was more sensitive to proteolytic modification. We are grateful to Dr. Benkovic for providing this information in advance of its publication.

Abbreviations: Fru-P₂ase, fructose 1,6-bisphosphatase; PFK, phosphofructokinase; PEP, phosphoenolpyruvate.
triphasphate, glycelyglycine, Cbz-Glu-Tyr, and Cbz-Glu-Phe were purchased from the Sigma Chemical Co., St. Louis, Mo.

Carboxypeptidase A (diisopropyl fluorophosphate-treated) was obtained from the Worthington Biochemical Corp., Freehold, N.J.

Reduced glutathione, oxaloacetic acid, lactate dehydrogenase, pyruvate kinase, glycerol 1-phosphate dehydrogenase-triosephosphate isomerase, and aldolase were all obtained from Boehringer, Mannheim, Germany; fructose 1,6-bisphosphatase was purified according to Tranilii et al. (9).

Animals. Adult female brown rabbits, weighing 2.5-2.8 kg, were obtained from the local Italian market. Control animals were fed ad libitum on a stock diet (Rieper Mangime C-L, purchased from A. Rieper, Bolzano, Italy) and kept at 25° ± 2°. Fasted rabbits were maintained at the same temperature, with water ad libitum. Refeeding was with the same diet. The animals were killed by cervical dislocation and the livers and kidneys removed immediately and cooled in crushed ice.

Subcellular Fractionation. Subcellular fractions were prepared as described by Pontremoli, et al. (1), except that the Supernatant II was clarified by centrifugation at 105,000 × g for 30 min. This fraction was employed for assay of Fru-Pase, PEP-carboxykinase and PFK, and also for soluble proteolytic activity.

Enzyme Assays. Fru-Pase activity was assayed spectrophotometrically or by the liberation of P1, as previously described (9), in the presence of 2 mM MgCl2. One unit was defined as the amount required to catalyze the hydrolysis of 1 μmole of Fru-P1 per min. Protein concentrations were calculated from the absorbance at 280 nm, assuming an absorbance of 0.73 for a solution containing 1 mg/ml. Specific activity is expressed as units/mg of protein.

PFK activity was assayed spectrophotometrically at pH 8.2 following the oxidation of NADH in the presence of excess aldolase and glyceral 1-phosphate dehydrogenase-triosephosphate isomerase, as described by Mansour (14). PEP-carboxykinase activity was assayed at 30° by the method of Nordlie and Lardy (15), as modified by Foster et al. (16).

Proteolytic Activity. Proteolytic activities were assayed with Cbz-Glu-Tyr and Cbz-Glu-Phe as substrates (17, 18), as previously described (1).

RESULTS

Effects of Fasting and Refeeding on the Activities of Liver and Kidney Fru-Pase. The activity of liver Fru-Pase declined during the first 36 hr to approximately one-half the original value (Fig. 1A). Thereafter, it rose rapidly and after 96 hr of fasting it exceeded the control level by more than 2-fold. Refeeding was followed by a rapid return to the control range. Similar responses to fasting and refeeding were observed for Fru-Pase in rabbit kidney (Fig. 1B).

Effects of Fasting and Refeeding on the Activities of PEP-Carboxykinase and PFK. PEP-carboxykinase and PFK are key enzymes of gluconeogenesis and glycolysis that show reciprocal changes in activity levels in fasted animals (3, 4). We therefore assayed these enzymes in the livers and kidneys of the fasted and refed rabbits (Fig. 1A and B). Within the first 50 hr of fasting PFK activity declined to about 40% and 15% of the initial values, respectively, in liver and kidney, and remained at these low levels as long as the animals were fasted. On the other hand, the level of PEP-carboxykinase increased during fasting until it reached levels 15- to 50-fold higher than those observed in the livers and kidneys of fed animals. Both enzymes returned to the control range within 24 hr after refeeding, as reported by previous workers (3, 4). Unlike Fru-Pase, however, PEP-carboxykinase activity did not show the initial decline, perhaps because this activity was already very low in the fed animals.

Levels and Subcellular Distribution of Proteolytic Activity in Fasted and Refed Rabbits. Proteolytic activity increased in both the livers and kidneys of the fasted animals, and continued to increase during starvation up to 96 hr (Fig. 2). Most of this increase was due to the appearance of activity in the 105,000 × g supernatant fraction. In contrast to the control animals, where all of the proteolytic activity was associated with the lysosomal pellet, in the fasted animals an increasing fraction of the proteolytic activity was present in the cytoplasmic fraction. This may be related to the changes in lysosomal morphology reported earlier (1). It is remarkable that both the proteolytic activity (Fig. 2) and morphological properties (unpublished observations by Prof.

Fig. 1. Changes in the activities of liver (A) or kidney (B) enzymes. The experimental conditions were as described under Materials and Methods. Fasting was begun at zero time, and refeeding at 96 hr. The numbers in parentheses represent the number of animals used in each experiment, and the vertical lines the experimental variation. In some experiments, organs from two to three animals were combined for extraction and analysis. The results are expressed as units/g of fresh tissue. After refeeding for 48 hr the activities were as follows: Fru-Pase: liver, 3.6 units/g; kidney, 2.8 units/g; PFK: liver, 0.6 unit/g; kidney, 0.7 unit/g; PEP-carboxykinase: liver, 0.1 unit/g; kidney, 0.2 unit/g.
Fig. 2. Levels and distribution of liver and kidney proteolytic activities during starvation and refeeding. Total and free proteolytic activities were measured in the 800 × g and 100,000 × g supernatant solutions of both liver and kidney homogenates (see Methods), with a mixture of Cbz-Glu-Tyr and Cbz-Glu-Phe, as previously described (1). "Total" and "free" proteolytic activities represent those assayed in the 800 × g and 100,000 × g supernatant solutions, respectively, and are expressed as units/g fresh tissue. After refeeding for 48 hr the activities were as follows: total proteolytic activity: liver, 0.6 units/g; kidney, 1.7 units/g; "free" proteolytic activity: not detectable.

A. T. Franzi) are restored to normal within 24 hr after refeeding.

Molecular Properties of Fru-Pase Isolated from Livers of Control and Fasted Rabbits. Fru-Pases purified from the livers of fed or starved animals were identical with respect to specific activity (15.0 ± 0.2 units/g) or in the ratio of activity at pH 9.2 to that at pH 7.5 (0.40-0.42). When analyzed by disc gel electrophoresis in the presence of Na dodecyl sulfate, the preparations from 36- and 96-hr fasted animals showed small amounts of a lighter subunit, but the bulk of the subunits (85-90%) were indistinguishable in molecular weight from those of the control enzyme (Fig. 3 and Table 1). Analysis with carboxypeptidase A revealed no changes in the carboxyterminal sequence, which was -Gly-His-Ala-COOH for all three preparations. However, the preparations from fasted animals contained less than one tryptophan per subunit, suggesting that the enzyme had suffered some loss of amino acids from the NH₂-terminus. In order to exclude the possibility that this loss of tryptophan was an artifact of isolation, caused by the presence of free proteolytic activity in the isonicotic sucrose extracts from which the enzyme is purified (see Fig. 2), we incubated the enzyme from fed animals for 3 hr at 25°C with the liver cytosol fraction from a 96-hr fasted rabbit. The 3-hr period was selected because it corresponded to the time required for purification of the enzyme, and the incubation was carried out at pH 5.0 as well as at pH 6.7. The enzyme recovered from these reaction mixtures by the usual purification procedures showed (1) no change in the pH 7.5/pH 9.2 activity ratio, (2) no change in the Na dodecyl sulfate disc gel electrophoresis pattern, and (3) no loss of tryptophan. The content of the latter was 4.18 moles/mole of enzyme for

<table>
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<tr>
<th>Conditions</th>
<th>% heavy</th>
<th>% light</th>
<th>COOH-terminal sequence</th>
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<td></td>
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<td>87</td>
<td>13</td>
<td>3.38</td>
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* The enzyme was purified from livers of rabbits as described in Materials and Methods.
† Estimated by integration of the areas under the peaks in Fig. 3.
‡ Released during incubation with carboxypeptidase A for 30 min, as described by Traniello et al. (9). Amino-acid analyses were carried out with a Beckman model 120B amino acid analyzer as described by Spackman et al. (19).
§ Determined by the method of Edelhoch (20).
the original preparation, and 3.89 and 3.72 moles/mole of enzyme for those incubated with cytosol at pH 6.7 or pH 5.0, respectively. It may be concluded that the absence of tryptophan in the preparation from animals fasted for 36 and 96 hr is indeed a property of the Fru-P2ase present in the liver cells under these conditions.

**DISCUSSION**

The previously reported (1) decrease in Fru-P2ase activity in the livers of rabbits during the first 36 hr of exposure to cold and fasting raised doubts as to whether our animals were indeed responding to these gluconeogenic conditions. We therefore exposed them to longer periods of fasting and also measured the changes in activities of two marker enzymes of glycolysis and gluconeogenesis, namely PFK and PEP-carboxykinase. The expected changes in the activities of the two marker enzymes were observed within 18 hr after food was withdrawn, indicating that the animals were indeed responding to the gluconeogenic stimulus. In the animals fasted for 96 hr, we observed an increase in the activity of Fru-P2ase, particularly in liver, that paralleled the increase in PEP-carboxykinase during the later period. The decrease in Fru-P2ase during the first 36 hr remains unexplained, but it should be pointed out that the activity of this enzyme, even at its minimum, was severalfold greater than that of PEP-carboxykinase, and it does not become rate-limiting for the overall process even when its activity is low. The changes in activity of Fru-P2ase may be related to changes in its regulatory properties.

Of particular interest is the large increase in "free" proteolytic activity, as measured with the Cbz-Glu-Tyr, Cbz-Glu-Phe mixture, since this may be related to the changes in levels and molecular properties of Fru-P2ase. It remains to be established whether other lysosomal proteases are also increased, and also whether this activity is present in the cytoplasm in vivo, or released during the isolation of lysosomes because of their increased fragility in the starved animals (1). If proteolytic enzymes are indeed released into the cytoplasm in vivo, then the rapid disappearance of this activity within 24 to 48 hr of refeeding suggests their rapid turnover. It is also of interest that despite the evidence suggesting a large increase in proteolytic activity in both liver and kidney, the gluconeogenic enzymes, Fru-P2ase and PEP-carboxykinase, reach levels manifolds higher than those found in fed animals.

Two properties of Fru-P2ase in the fasted livers and kidney are particularly noteworthy. The first is the loss of tryptophan, which occurs without significant change in molecular weight. We have previously shown (11) that the single tryptophan residue is located in the NH2-terminal region of the subunit; the present results suggest that it may lie very close to the NH2-terminus. The second is the appearance of traces of a lighter subunit, which suggests that a modification similar to that observed when the enzyme is exposed to lysosomes in vitro (12) has occurred in vivo. However, in vivo, the accumulation of lighter subunits is far more extensive, reaching at least 50% of the total, and is accompanied by a shift in the pH optimum of the enzyme from neutral to alkaline (12). In the intact starved animals, on the other hand, the lighter subunit does not accumulate, suggesting that modified enzyme molecules containing the lighter subunit may be rapidly degraded. We have previously shown that the neutral enzyme isolated from winter rabbits, also containing a trace amount of lighter subunits, is more sensitive to proteolytic enzymes such as subtilisin (12). We have also found that the shift in pH optimum as a function of the content of lighter subunits is a highly cooperative process (13). Thus it is barely detectable when the lighter subunits account for only 10% of the total, and nearly complete when the proportion reaches 35%, when it is estimated that 80% of the tetrameric molecules contain at least one light subunit.

We have interpreted the available evidence in terms of proteolytic modification of Fru-P2ase in the fasted animals, resulting from the increased endogenous proteolytic activity. However, the presence of two genes for Fru-P2ase, one expressed in the fed animals and the other in the fasted animals, has not been excluded.

Although the enzymes purified from the livers and kidneys of starved animals resemble the enzymes from fed animals in catalytic properties, particularly in having a neutral pH optimum, their regulatory properties may be altered.

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