Conversion of Neutral to Alkaline Liver Fructose 1,6-Bisphosphatase: Changes in Molecular Properties of the Enzyme
(rabbit/subtilisin/allosteric/tryptophan fluorescence)

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ABSTRACT Removal of the NH₂-terminal region of fructose 1,6-bisphosphatase from rabbit liver by digestion with subtilisin, or changes in conformation in this region of the protein produced by exposure to low concentrations of urea, result in similar changes in catalytic and allosteric properties of the enzyme. These changes include shift of the pH optimum to more alkaline pH, and loss of sensitivity to inhibition by AMP. The conformation changes are monitored by changes in the fluorescence of the single tryptophan residue, which is located near the NH₂-terminus. Thus, the tryptophan-containing peptide appears to determine the functional properties of the native enzyme.

We have shown that fructose 1,6-bisphosphatase can be isolated from rabbit liver in two homogeneous forms that differ from each other with respect to functional and molecular parameters (1-3). One form is the classical enzyme described by Gomori (4), with optimum activity at pH 9.2 and a molecular weight of 130,000 (5). More recently, we have described the isolation of the enzyme in what we believe to be its native form (2, 3), characterized by a pH optimum in the neutral range and by an increased sensitivity to inhibition by AMP. The native enzyme is also significantly larger, with a molecular weight of about 143,000. We have also found that digestion with subtilisin converts the native enzyme to an enzyme species whose properties closely resemble those of the alkaline form (3). This conversion is paralleled by removal from each of the four subunits of a peptide or peptides, of molecular weight totalling about 6000, containing the only tryptophan residue in the molecule.

In this paper we present evidence for the location of the tryptophan-containing peptide(s) at the amino-terminal end of the peptide chains. It may, therefore, be concluded that this region of the molecule is essential to maintain the protein in the conformation that determines the neutral pH optimum.

Taking advantage of the presence of a tryptophan residue as a natural fluorophore in this part of the protein, we have investigated the effects of exposure to urea on the structural and functional properties of the enzyme. We have observed that urea, besides enhancing the fluorescence intensity of the native form of the enzyme, causes a red shift, indicating a decreased quenching in the tryptophanyl fluorescence. These alterations in the fluorescence emission spectrum are accompanied by changes in the catalytic properties of the protein that mimic those observed during treatment with subtilisin. Thus, either removal of the tryptophan-containing peptide or localized conformational changes, such as that induced by urea (probably involving the same region of the protein), produce similar changes in the pH-activity profiles of the enzyme.

MATERIALS AND METHODS

Materials. Δ-Fructose 1,6-bisphosphate, TPN, AMP, and sodium dodecyl sulfate were purchased from the Sigma Chemical Co., St. Louis, Mo. N-Bromosuccinimide was obtained from Eastman Organic Chemicals, Rochester, N.Y., and was recrystallized twice from acetone. Ultra-pure urea was purchased from Mann Research Laboratories, New York. Hydrazine (anhydrous grade) was obtained from ICN, City of Industry, Calif. Hexosephosphate isomerase and glucose 6-phosphate dehydrogenase were purchased from the Boehringer Mannheim Corp., Mannheim, Germany. Subtilisin Carlsberg was obtained from Novo Industri, Copenhagen, carboxypeptidase A (diisopropylfluorophosphate-treated) was from the Worthington Biochemical Corp., Freehold, N.J., and aminopeptidase was from Röhm GmBH, Darmstadt, Germany.

Methods. Neutral enzyme was purified from rabbit livers according to Traniello et al. (3). Its activity was assayed spectrophotometrically at 23° by following TPN reduction in the presence of excess hexosephosphate isomerase and glucose 6-phosphate dehydrogenase. The reaction mixture (1 ml) contained 20 mM triethanolamine-20 mM diethanolamine (pH 7.5 or 9.5, as indicated), 0.1 mM TPN, 0.1 mM fructose bisphosphate, 1 μg each of glucose 6-phosphate dehydrogenase and hexosephosphate isomerase, 2 mM MgCl₂ and 0.1 mM EDTA. The final pH values in the cuvettes were 7.5 and 9.2, respectively.

Enzyme activity was also estimated from the rate of appearance of inorganic phosphate, as measured by the method of Fiske and Subbarow (6). The incubation mixture (0.5 ml) contained 20 mM triethanolamine-20 mM diethanolamine (pH 7.5 or 9.5, as indicated), 2 mM MgCl₂, 0.2 mM EDTA, 0.4 mM FDP, and amounts of the enzyme required to release about 0.1 μmol of Pi in 10 min at 22°. The reaction was stopped by the addition of 5 N H₂SO₄. One unit of enzyme activity was defined as the amount that caused the hydrolysis of 1 μmol of FDP per min under these assay conditions.

Specific activity is expressed as units/mg of protein. Protein concentration was measured by the method of Büchler (7) or of Lowry et al. (8), or was calculated from the absorbance at 280 nm. The absorbance of a dialyzed enzyme solution containing 1.0 mg of neutral enzyme (dry weight) per ml in a 1.0-cm light path was 0.73 at 280 nm and 0.370 at 260 nm.

Dissociation of protein in dodecyl sulfate–10% polyacrylamide gels at pH 7.0 was performed as described by Weber and Osborn (9). Electrophoresis was for 5 hr, with a current of 5
mA per tube, until the tracking dye reached the bottom of the gel. Hemoglobin, trypsin, lactate dehydrogenase, aldolase, and 6-phosphogluconic dehydrogenase were used as standards for estimation of the molecular weight of the enzyme subunits.

Digestion with carboxypeptidase A was performed as described by Lacko et al. (10). For each experiment, 50 nmol of enzyme was dialyzed against 50 mM Tris·HCl-0.1% Na dodecyl sulfate (pH 7.5). Carboxypeptidase A was added to a final ratio of 1 mol/10 mol of subunit. Incubation was at 23°C. Aliquots were removed at zero time (before addition of carboxypeptidase) and at 5, 20, 40, and 120 min, acidified with 2 N HCl, diluted with 0.1 M citrate buffer (pH 2.4), and analyzed with a Beckman model 120 B automatic amino-acid analyzer.

Digestion with aminopeptidase M was at 23°C with 50 nmol of native or subtilisin-digested enzyme, previously dialyzed against 50 mM Tris·HCl buffer (pH 7.5). Aminopeptidase M was added to a final ratio of 1 mol/20 mol of subunit. Aliquots were removed at various time intervals and processed as for

the carboxypeptidase digestion. Tryptophan content was estimated with N-bromosuccinimide (11).

Fluorescence spectra were recorded at room temperature with an Amino–Bowman spectrophotofluorometer fitted with a xenon arc light source. Measurements were made in 1-cm path length cells in a volume of 2.0–4.0 ml. The enzyme (0.50–1.4 mg) was equilibrated in 20 mM diethanolamine–20 mM triethanolamine buffer, at various pH values. The excitation wavelength was 288 nm and the emission was recorded from 300 to 400 nm. All emission intensities are expressed in arbitrary fluorescence units. Measurements of relative quantum yields were performed by the procedure of Kirby and Steiner (12), with l-tryptophan as the standard.

RESULTS

Changes in primary structure and catalytic activity during digestion by subtilisin

The changes in catalytic, regulatory, and molecular properties during digestion of enzyme by subtilisin are shown in Fig. 1. We observe a significant increase in the catalytic activity at pH 9.2, with little change in the activity at pH 7.5. The sensitivity to AMP inhibition decreases, and the modified enzyme obtained after digestion for 180 min is inhibited only 25% by 0.1 mM AMP. As reported (3), treatment with subtilisin reduces the molecular weight of the subunits from about 36,000 to about 30,000 (Fig. 1).

Analysis of the carboxy-terminal and amino-terminal residues during digestion by subtilisin

There was no change in the pattern of amino acids released by carboxypeptidase after digestion of the enzyme with subtilisin for 45 and 180 min (Fig. 2). In each case, nearly four equivalents of alanine were released, followed by histidine and glycine, suggesting the following sequence: Gly-His-Ala-COOH. The presence of four COOH-terminal alanine residues per mol of enzyme throughout the digestion was confirmed by hydrazinolysis (13). Thus, the proteolytic attack by subtilisin does not appear to affect the carboxy-terminal region of the protein.

The native protein was resistant to digestion with aminopeptidase M, indicating either the presence of NH₂-terminal proline or masking of the NH₂-terminal residue (Table 1). After exposure to subtilisin for 45 min, when about 50% of the heavier subunit was replaced by the new lighter species (see Fig. 1), digestion of the dialyzed modified enzyme with aminopeptidase (see Methods) resulted in the release of nearly two leucine residues per mol of enzyme, quantitatively con

Table 1. Release of amino acids by aminopeptidase M after digestion with subtilisin

<table>
<thead>
<tr>
<th>Min of digestion with subtilisin</th>
<th>Released by aminopeptidase M† (mol/mol of enzyme)</th>
<th>Tryptophan content‡ (mol/mol of enzyme)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Gly</td>
<td>Ile</td>
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<tr>
<td>0</td>
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</tr>
<tr>
<td>45</td>
<td>3.72</td>
<td>1.48</td>
</tr>
<tr>
<td>180</td>
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* The conditions are described in the legend to Fig. 1.
† See text for experimental details. The values given were obtained after digestion with aminopeptidase M for 2 hr.
‡ By the procedure of Patchornik et al. (11).
firming the pattern of Na dodecyl sulfate-gel electrophoresis (Table 1). Smaller quantities of alanine, isoleucine, and glycine were also detected, suggesting the following NH$_2$-terminal sequence: NH$_2$-Leu-Ala-Ile-Gly. At 180 min, about four equivalents of leucine were released, and the quantities of the other residues that followed suggested that the new molecular form of the enzyme is composed of four identical polypeptide chains. It is interesting to note that the loss of the four tryptophan equivalents that are present in the native enzyme parallels the appearance of the new amino-terminal residues (Table 1). Two tryptophan residues are lost at about 45 min, and all four are lost after 180 min.

The results of COOH- and NH$_2$-terminal analyses support the view that subtilisin modifies the primary structure of enzyme by removing a tryptophan-containing peptide from the NH$_2$-terminal region of each subunit.

**Changes in the fluorescence emission spectrum of enzyme after exposure to urea**

The presence of the fluorophore residue in the NH$_2$-terminal region of the enzyme prompted us to check whether other conformational changes affecting this region might induce modifications in the catalytic properties of the enzyme similar to those produced by proteolysis. The possible effects of urea were of particular interest, since it has been reported that low concentrations can modify the catalytic properties of the enzyme (14) and decrease its sensitivity to inhibition by AMP (15).

The fluorescence emission spectrum of the native enzyme, when it was excited at 286 nm, was practically unchanged over the pH range from 5.4 to 9.2, with a peak at 341 nm that is characteristic of tryptophan (Fig. 3, left). Thus, the polarity of the environment of the tryptophan residue is unaffected by these changes in pH. A pronounced quenching of tryptophan fluorescence is apparent from the low relative quantum yield at pH 9.2, with Q = 0.06. Addition of 2 M urea did not modify the fluorescence emission spectrum qualitatively, although the fluorescence intensity was enhanced to some extent. Exposure to higher concentrations of urea resulted in a progressive increase of the fluorescence intensity, as well as in a shift of the emission maximum to higher wavelengths, both effects being optimally observed at pH 9.2. Thus, the peak of the tryptophan emission spectrum was centered at 351 nm in 5 M urea and at 355 nm in 10 M urea. Furthermore, the relative quantum yield of tryptophan fluorescence increased progressively as the urea concentration was increased, reaching a value of Q = 0.25 for enzyme in 5 M urea. The urea-dependent red shift in the fluorescence spectrum of the enzyme is fully reversible, as indicated by a progressive shift of the emission maximum to lower wavelengths upon serial dilution of a parent solution of enzyme in concentrated urea, until the maximum reaches 341 nm, as with the native protein.

These spectrofluorometric changes indicate that the tryptophan fluorescence can serve as an index of conformational transitions induced by urea, and that such transitions involve the progressive shift of this fluorophore residue into a more polar environment.

Parallel to these changes in the secondary and tertiary structure of the enzyme, urea evoked marked changes in its catalytic properties, at concentrations that did not inactivate. Thus, while the activity at pH 7.5 declined rapidly on increases in the urea concentration, the activity at pH 9.2 showed a significant increase up to 5 M urea (Fig. 3, right). The resulting ratio of the activity measured at pH 9.2 to that measured at pH 7.5 was very similar to that observed with the subtilin-digested enzyme, and was correlated with the conformational transitions. An additional urea-dependent change in the functional properties of enzyme was observed. This is desensitization to inhibition by AMP, produced by the same urea concentrations that resulted in the clear red-shift in the fluorescence of the protein (Fig. 4). These urea-dependent functional effects were also fully reversible on dilution.

Thus, the changes in both the catalytic and regulatory properties of enzyme induced by urea resemble closely those observed during digestion with subtilisin. Therefore, it may be concluded that the conformational transitions on exposure of the protein to urea affect the same region that is susceptible to proteolysis, namely the NH$_2$-terminal end.

**DISCUSSION**

The molecular basis for the changes in catalytic properties of enzyme during digestion with subtilisin has now been established. Data reported in this paper indicate that the conversion of the native "neutral" enzyme to an "alkaline" form is due to the loss of a tryptophan-containing peptide from the amino-terminal region of the enzyme. This peptide appears to determine the functional properties of the native enzyme, probably by imposing constraints on its secondary and tertiary...
structure. This hypothesis is supported by the fact that both a shift of the optimum activity to pH 9.2 and a substantially decreased susceptibility to AMP inhibition result from the urea-dependent unmasking of tryptophan. The same effects are induced by release of the tryptophan-containing peptide.

Whether tryptophan has a direct function in determining the properties of the native enzyme, including the neutral pH optimum and the susceptibility to the AMP effect, or whether it is simply a reporter for the native conformation of the enzyme, remains to be established.

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