THE ROLE OF TRYPTOPHAN IN NEUTRAL FRUCTOSE DIPHOSPHATASE*

S. J. Benkovic**, W. A. Frey, C. B. Libby
and J. J. Villafranca

Department of Chemistry
The Pennsylvania State University
University Park, Pennsylvania 16802

Received January 10, 1974

SUMMARY. Neutral rabbit liver fructose-1,6-diphosphatase which does not contain tryptophan has been isolated and shown to possess catalytic properties identical to that for the neutral enzyme which contains tryptophan. Criteria include pH activity optimum, EDTA and oleate activation, AMP inhibition and specific activity. However, the enzyme lacking tryptophan responds differently to proteolysis by subtilisin exhibiting a more rapid loss of sensitivity to AMP inhibition.

Purified neutral rabbit liver fructose 1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.13.11) has been reported to contain four tryptophan residues per molecule of enzyme† (1,2). Since the previously studied alkaline form of the enzyme contained no tryptophan (3), the presence of this amino acid provided yet another criterion in the purification of pure neutral enzyme. Previous researchers have also shown that digestion of neutral FDPase by subtilisin converts neutral to alkaline enzyme and removes from each of the four subunits a peptide(s) containing the only tryptophan residue in the subunit (4). Because tryptophan is among the most highly fluorescent of biologically

* This work was supported by a grant from the National Institute of Health, GM-13306 (SJB), and an appropriation from the Pennsylvania State University.

** Career Development Awardee of the National Institutes of Health.

† Neutral enzyme exhibits higher activity at pH 7.5 in contrast to the alkaline form whose activity is maximal at pH 9.6.

occurring compounds, changes in the fluorescence of the single tryptophan residue were used to monitor conformational changes (4). The observed changes induced by denaturing agents were accompanied by alterations in the catalytic properties of the protein that mimicked those observed during treatment by subtilisin. These results were interpreted to show that removal of the NH_2-terminal region (tryptophan-containing peptide), or localized conformational changes involving the same region of the protein, produce a shift in the pH-activity profile of the enzyme to alkaline pH (4). Thus alterations in the tryptophyl-containing peptide region appeared to be associated with the functional properties of the neutral enzyme. Recent preparations of "neutral" FDPase in this laboratory have yielded a protein in which tryptophan is absent. Its characteristics, possible origin, and differences relative to the tryptophan containing enzyme are the subject of this communication.

Materials & Methods

D-Fructose-1,6-diphosphate, $NADP^+$, D-fructose, L-cysteine HCl, and auxiliary assay enzymes were purchased from Sigma Chemical Co. Buffer and inorganic salts were reagent grade.

Neutral FDPase was purified from young rabbit livers obtained after fasting according to the procedure of Pogell et al. (5) with the following modifications. Following pH adjustment from 7.0 to 6.0 and subsequent centrifugation, the supernatant was dialyzed overnight against 16 volumes of doubly-distilled deionized water. The dialysate was again centrifuged at 13,000 g for 40 minutes and then added dropwise to the slurry of CM-cellulose. Washing and elution were carried out as described. Eluted enzyme was concentrated by dialysis against saturated ammonium sulfate adjusted to pH 7.0. The centrifuged precipitate was redissolved in 50mM

Tris-HCl, pH 7.5, and dialyzed against the same buffer to remove any remaining salt. Enzyme homogeneity was estimated by dissociation of subunits in sodium dodecyl sulfate and electrophoresis as described by Weber and Osborn (6).

The enzyme was assayed spectrophotmetrically by following the rate of NADPH accumulation at 340nm in the presence of excess glucose-6-P dehydrogenase and glucose-6-P isomerase. The routine assay mixture at 25° for neutral activity (used during purification and for the pure enzyme) contained 50mM Tris-HCl, pH 7.5; 5mM $MgSO_4$; 0.1mM EDTA; 0.2mM $NADP^+$; 0.1mM fructose-1,6-diP; 3.5 units of glucose-6-P isomerase; 0.7 unit of glucose-6-P dehydrogenase; fructose 1,6-diphosphatase; and any other compounds as noted (final pH of 7.3). The assay for oleate activation and AMP inhibition contained 30μM oleate and 0.10mM AMP, respectively, with no EDTA in the oleate assay. Activity at alkaline pH was determined using the assay mixture described for neutral pH except that 0.1M glycine, adjusted to the pH 9.6 with NaOH, was substituted for the Tris-HCl (final pH of 9.3). Specific activity is defined as units per mg of protein. Protein concentration was calculated from the absorbance at 280nm. The absorbance of an enzyme solution containing 1.0mg of neutral FDPase (dry weight) per ml in a 1.0cm light path was 0.71 at 280nm and 0.37 at 260nm.

Results

The results of our tryptophan analyses are shown in Table I. There appears to be a small amount of tryptophan in the pre-concentration samples, although the amount is much less than had been reported in previous enzyme purifications (1,2). The tryptophan content of the post-concentration enzyme was also determined using the method of Spande & Witkop (7) which indicated

Table I

Tryptophan Content of Purified Enzyme

Test	Pre-Concentration* Moles Tryptophan/Subunit†	Post-Concentration** Moles Tryptophan/Subunit
Chemical††	0.104 ±0.004	0.047 ±0.004
Fluorescence¶	0.119 ±0.005	0.006 ±0.005

*Before precipitation with $(NH_4)_2SO_4$.

**Redissolved enzyme.

†Determined using a M.W. of 143,000 (subunit M.W. of 35,750).

††Method of Messineo & Mussarra, Int. J. Biochem. 3, 700 (1972).

¶From data obtained on enzyme samples (0.01mg/ml) excited at 280nm (emission at 348nm) in 6M guanidine-HCl recorded on an Aminco-Bowman spectrophotofluorometer standardized against measured quantities of L-tryptophan.

the absence of tryptophan in the concentrated neutral enzyme.

Although fluorescence intensity is enhanced in 6M guanidine-HCl (or 5M urea) this enhancement may be the result of decreased collisional quenching in the more viscous solution rather than an increased fluorescence yield due to unfolding and exposure of the chromophore as has been suggested (1). The intensity of FDPase tryptophan fluorescence was found to decrease with increasing concentrations of iodide ion. These data were plotted according to a modified Stern-Volmer equation for quenching of tryptophan in protein (8) and exhibited a linear plot with an intercept of one. This indicates that the small amount of tryptophan present in the pre-concentration enzyme [Table I] is entirely exposed to solution without the addition of guanidine-HCl. The finding that

Table II

Properties of Purified Enzyme

Property	Pre-Concentration (before pptn. with $(NH_4)_2SO_4$)	Post-Concentration (redissolved enzyme)	Literature Values	
Activity Ratio pH7.5/pH9.3	3.1	3.3	4	(5)
EDTA Neutral Activation	6.0	6.9	2	(2)
Oleate Activation	6.4	6.1	8.0	(5)
%AMP Inhibition	94.0	96.5	~95.0	(4)
Specific Activity	11.1	11.0	14.6 35*	(2) (5)

*Differences between our data and literature values arise from differences in temperature (25° vs. 30°) and additions (0.5M KCl) to assay mixtures.

the λ_{max} for emission appears at >340nm in the absence of guanidine-HCl also is consistent with exposed tryptophan residues (8).

Of particular interest is the observation that not all of the protein precipitated by $(NH_4)_2SO_4$ redissolves. A small amount of solid remains, apparently unaffected by increasing dilution. A suspension of this material gave a positive tryptophan test by both the chemical and fluorescence techniques indicating the tryptophan lost during concentration is contained in a protein(s) that will not redissolve in the buffer system. Previously reported methods of concentrating the enzyme by lyopholization, use of an Amicon filter, or ammonium sulfate precipitation (where the enzyme precipitate is resuspended in $(NH_4)_2SO_4$) would not result in removal of this tryptophan containing protein. Our insoluble

precipitate shows no activity in the assay system.

To test the possible involvement of the tryptophan containing residue on enzyme catalytic properties, the comparison shown in Table II was made before and after concentration. These results indicate that the presence or absence of tryptophan has no affect on the enzyme assay system. This is in agreement with an earlier observation (1) that chemical modification of the tryptophyl residues by reaction with 2-hydroxy-5-nitrobenzyl bromide has no observable effect on enzyme properties. Addition of the tryptophan-containing insoluble precipitate to the various assay systems also failed to show any observable change in enzyme properties. All of these results, therefore, indicate the non-involvement of the tryptophyl residue on the catalytic function of the enzyme.

The changes in activity of neutral FDPase upon digestion by subtilisin are shown in Figure 1. There is a sharp increase in the pH 9.3 activity, with only a slight decrease in activity at pH 7.5. Enzyme sensitivity to AMP inhibition decreases dramatically to 25% of the inhibition found with neutral FDPase. The observed changes parallel closely the previous results (4) for subtilisin digestion of neutral enzyme containing 4 moles of tryptophan per mole of enzyme except in one important aspect. The decrease in the sensitivity of the present protein to AMP inhibition precedes an increase in alkaline activity; for the FDPase which contains tryptophan the converse was observed -- alkaline activity precedes the decrease in AMP sensitivity.

Discussion

Recently it has been demonstrated that the tryptophan content of FDPase which exhibits a pH 7.5/pH9.3 activity ratio associated with the neutral enzyme is variable and depends on the environmen-

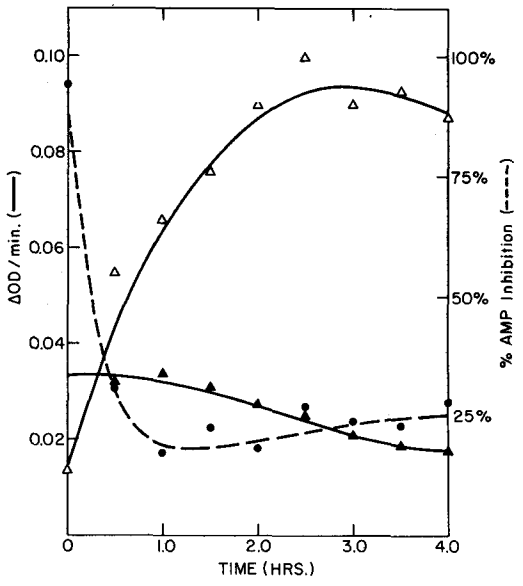

Figure 1. Changes in kinetic properties of neutral FDPase treated with subtilisin according to the procedure of Pontremoli et al. (4). Aliquots were removed at the indicated periods and assayed. Observed rates are indicated at pH 7.5 (filled triangles) and at pH 9.3 (open triangles). Susceptibility to AMP inhibition was determined in assay mixtures containing 0.1mM AMP (filled circles).

tal conditions to which the rabbits were subject (9) FDPase obtained from the livers of fasted rabbits showed a lower tryptophan content (1.3moles/mole of enzyme) than the enzyme isolated from livers of fed rabbits where the tryptophan level was 4.0moles/mole of enzyme. Likewise, lower amounts of tryptophan were found in the FDPase isolated from the livers of animals subject to cold. In view of our results, ratios for tryptophan content less than four but greater than zero may also result from incomplete removal of the tryptophan containing fragment from the protein. The sodium dodecyl sulfate gel electrophoresis patterns for all isolated neutral FDPases -- with or without tryptophan -- feature one major band corresponding in mobility

to that assigned the "heavy" subunit species suggesting that the tryptophan is within a small peptide.

Although the presence or absence of tryptophan in neutral FDPase does not appear to alter the catalytic properties of the enzyme, the absence of tryptophan affects the susceptibility of the protein to proteolysis in one important aspect. Treatment of our neutral FDPase with subtilisin resulted in rapid loss of sensitivity to AMP inhibition without a complementary increase in alkaline activity until later times. If the neutral FDPase which does not contain tryptophan represents the first species produced upon lysosomal modification of the enzyme, then further proteolysis can yield an FDPase still capable of functioning in gluconeogenesis but no longer subject to the same extent of AMP control (provided proteolysis is a means of enzymatic regulation). Whether fasting is the primary reason for our isolation of FDPase which does not contain tryptophan also remains a question for further study.

References

1. Sarngadharan, M. G. and Pogell, B. M. (1972) Biochem. Biophys. Res. Commun. 46, 1247.

2. Traniello, S., Melloni, E., Pontremoli, S., Sia, C. L. and Horecker, B. L. (1972) Arch. Biochem. Biophys. 149, 222.

3. Fernando, J., Pontremoli, S., and Horecker, B. L. (1969) Arch. Biochem. Biophys. 129, 370.

4. Pontremoli, S., Melloni, E., De Flora, A. and Horecker, B. L. (1973) Proc. Nat. Acad. Sci. 70, 661.

5. Carlson, C. W., Baxter, R. C., Ulm, E. H. and Pogell, B. M. (1973) J. Biol. Chem. 248, 5555.

6. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406.

7. Spande, T. F. and Witkop, B. (1967) Methods Enzymol. 11, 498.

8. Lehrer, S. S. (1971) Biochem. 10, 3254.

9. Pontremoli, S., Melloni, E., Salamino, F., Franzi, A. T., DeFlora, A. and Horecker, B. L. (1973) Proc. Nat. Acad. Sci. 70, 3674.