The Effect of Iodination upon the Catalytic and Regulatory Activities of Fructose 1,6-Diphosphatase*

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ABSTRACT: Iodination of fructose 1,6-diphosphatase from *Candida utilis* in the presence of fructose 1,6-diphosphate leads to rapid desensitization to the enzyme's specific allosteric effector, adenosine 5′-monophosphate, with no loss of catalytic activity. Complete loss of enzymic activity is observed when iodination is performed in the absence of substrate. Hydrolysis of the protein treated with ^{131}I reveals that both desensitization and enzymic inactivation are associated with the iodination of tyrosine residues.

These data support those previously obtained using dinitrophenylation techniques (Rosen, O. M., and Rosen, S. M. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 1156) which demonstrated the importance of tyrosyl residues in the maintenance of catalytic and regulatory properties of the enzyme.

We have previously reported (Rosen and Rosen, 1966) that fructose 1,6-diphosphatase isolated from *Candida utilis* can be rendered insensitive (desensitized) to the inhibitory effect of 5′-AMP[1] by dinitrophenylation of the enzyme in the presence of FDP. Desensitization is specifically prevented by the presence of 5′-AMP during dinitrophenylation. In the absence of FDP, dinitrophenylation leads to loss of catalytic activity. Desensitization and enzymic inactivation are each associated with the formation of 2 ϵ-DNP-lysyl and 2 *O*-DNP-tyrosyl residues/mole of enzyme.

We now wish to report that iodination, like dinitrophenylation, can desensitize or inactivate FDPase. The formation of iodotyrosine during iodination supports the contention that tyrosine residues are critically involved in the maintenance of both catalytic activity and sensitivity to 5′-AMP.

Materials and Methods

FDPase was purified and crystallized from *C. utilis* (Rosen *et al.*, 1965). The purified enzyme has a molecular weight of 100,000 and catalyzes the conversion of 80 μmoles of fructose 1,6-di-P to fructose-6-P/min per mg of protein. Enzyme assays were performed at 25° with a Beckman DU spectrophotometer attached to a Gilford multiple-sample absorbance recorder. Unless otherwise stated they were performed at pH 7.5 in the presence of 0.5 mM EDTA (Rosen *et al.*, 1965). Protein was measured according to Lowry *et al.* (1951) using crystalline bovine serum albumin as the reference standard.

All chemicals were obtained reagent grade from commercial sources.

^{131}I was obtained as $Na^{131}I$ in dilute NaOH from Isoserve Corp., Boston, Mass. 3-Monoiodo-L-tyrosine was purchased from Aldrich Chemical Co. Inc., Milwaukee, and 3,5-diiodo-L-tyrosine from Nutritional Biochemicals Corp., Cleveland. Samples of mono- and diiodohistidines were gifts from Dr. Jan Wolff, Clinical Endocrinology Branch, National Institute of Arthritis and Metabolic Diseases.

Paper chromatography was performed on Whatman No. 1 paper. Chromatograms were developed for 16 hr with (a) 1-butanol–acetic acid–water (4:1:5) descending, (b) pyridine–2 N acetic acid (8:2) descending, and (c) butanol–pyridine–water (6:4:3) descending. Standard iodinated amino acid derivatives were identified with ninhydrin and Pauly's reagent.

Paper chromatograms were scanned for radioactivity with a 4-π Baird-Atomic strip scanner equipped with an integrator. ^{131}I radioactivity was measured in a well-type scintillation counter. The iodinating reagent was made by mixing equal volumes of 0.2 N KI and 0.2 N I_2. Appropriate aliquots of ^{131}I were added to the iodinating reagent to make the final specific activity approximately 5000 cpm/mμequiv of iodine. Iodine solutions were freshly prepared 1 hr prior to iodination.

Iodination was performed with mechanical stirring at 4° in 0.2 M sodium carbonate buffer (pH 9.2). In a typical experiment 100 μg of FDPase was dissolved in 0.2 ml of buffer with or without FDP or AMP. Iodine (1 μl of the iodinating reagent) was added and aliquots (1 μl) were removed from the incubation mixture for assay. After the desensitization or inactivation was completed an excess of sodium metabisulfite (10 μl of a 20-

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[1] Abbreviations used: FDPase, fructose 1,6-diphosphate phosphatase; 5′-AMP, adenosine 5′-monophosphate; FDP, fructose 1,6-di-P; MIT, 3-monoiodo-L-tyrosine; DIT, 3,5-diiodo-L-tyrosine; MIH, 2- or 5-monoiodo-L-histidine; TEA, triethanolamine.

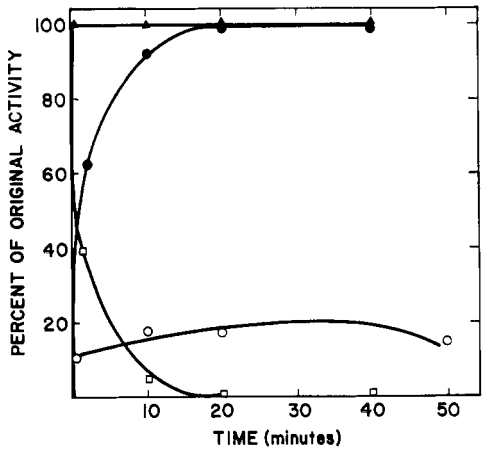

FIGURE 1: Effect of iodination on catalytic activity and sensitivity to AMP. Iodination of 100 μg of FDPase was carried out as described in Methods. The iodinated enzyme was assayed for catalytic activity and sensitivity to 5′-AMP. Activity and sensitivity to AMP were determined as a function of the time of iodination. (▲) Iodination carried out in the presence of 10 mM FDP; assay performed in the absence of AMP. (●) Iodination carried out in the presence of 10 mM FDP; assay carried out in the presence of 1.0 mM AMP. (○) Iodination carried out in the presence of 1.0 mM AMP and 10 mM FDP; assay performed in the presence of 1.0 mM AMP. (□) Iodination performed in the absence of both FDP and AMP; assayed in the absence of AMP.

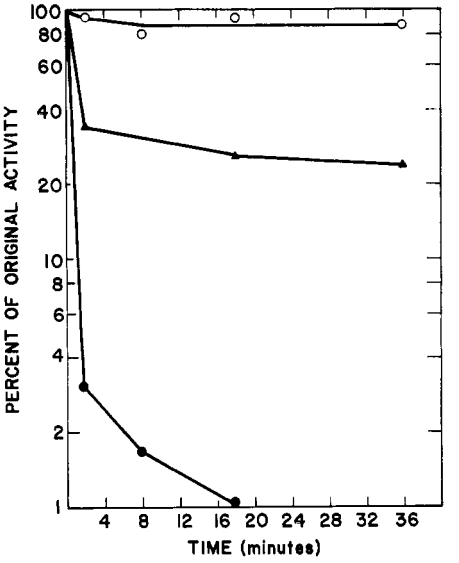

FIGURE 2: The protective effect of AMP on enzymic inactivation by iodine. Iodination of FDPase was carried out as described in Methods and catalytic activity was measured at various intervals. (○) Iodination in the presence of 5.0 mM FDP. (▲) Iodination in the presence of 5.0 mM AMP. (●) Iodination carried out in the absence of both AMP and FDP.

mg/ml solution) was added. (This concentration of metabisulfite had no effect on enzymic activity or sensitivity to AMP.) The reaction mixture was then dialyzed against multiple changes of distilled water for 24 hr. At the end of this time the contents were removed for protein determination and measurement of radioactivity. Protein recovery was essentially complete.

To identify the iodinated amino acids, a solution of the iodinated protein was taken to dryness in a Buchler Rotary Evapomix and resuspended in 0.2 ml of potassium phosphate buffer (0.05 M, pH 7.5). The contents were then boiled for 5 min and cooled to room temperature. Pronase (20 μg) was added and the tube was placed in a 37° incubator for 36 hr under toluene. The digested protein was concentrated by lyophilization and appropriate aliquots were applied to paper for chromatography. After development, the paper chromatograms were scanned for radioactivity. Radioactive areas were cut out, eluted with water, and either retreated with pronase or incubated in the presence of 2.0 mM $MnCl_2$ with leucine aminopeptidase (50 μg/ml) in potassium phosphate buffer (0.05 M, pH 8.0) for 2 hr at 40°. The iodinated derivatives so treated were then rechromatographed with appropriate standards and scanned. In all cases, their mobility in the chromatographic systems remained unaltered.

Results

Iodination of FDPase in the presence of substrate (FDP) and inhibitor (5′-AMP) does not alter enzyme activity or sensitivity to 5′-AMP (Figure 1). When AMP is not present, however, iodination renders the enzyme insensitive to the inhibitory effects of 5′-AMP although catalytic activity remains unchanged. Iodination in the absence of FDP inactivates the enzyme (Figure 1). Inorganic phosphate does not protect against desensitization or inactivation. The presence of 5′-AMP, however, diminishes the rate of inactivation which occurs when FDP is omitted (Figure 2). At equimolar concentrations it is less effective then FDP in maintaining catalytic activity during iodination. Protection by AMP against inactivation of the rabbit liver FDPase with acetylimidazole has been reported by Pontremoli *et al.* (1966b).

Enzyme which has been inactivated by iodination in the presence or absence of AMP alone showed relative desensitization to inhibition by AMP. At comparable levels of inactivation enzyme iodinated in the presence of AMP showed a slightly greater retention of sensitivity than did the enzyme treated in its absence (Table I). We have also observed (O. M. Rosen and S. M. Rosen, unpublished observation) that the presence of AMP minimally retards the process of desensitization during dinitrophenylation in the absence of substrate. These observations are in agreement with those of Pontremoli *et al.* (1966b) who, however, demonstrated a more profound protection of the regulatory function of liver FDPase during acetylation with acetylimidazole.

TABLE I: The Effect of Iodination in the Presence of AMP upon Sensitivity of FDPase to AMP.[a]

Iodination Performed in Absence of AMP		Iodination Performed in Presence of AMP	
Residual Catalytic Act. (μmoles/min mg)	Inhibn of Residual Act. by AMP (%)	Residual Catalytic Act. (μmoles/min mg)	Inhibn of Residual Act. by AMP (%)
73	95	72	95
56	85	57	90
47	75	46	85
34	55	32	65
24	30	25	40
15	10	16	25

[a] FDPase (100 μg) was iodinated in the presence of 5.0 mM AMP as well as in its absence and the reaction was stopped after arbitrary intervals ranging from 15 sec to 10 min by the addition of metabisulfite. Catalytic activity and sensitivity to 1.0 mM AMP were measured. Enzyme fractions that had been inactivated to a similar extent are compared on the same line.

Iodination at pH 7.5 (TEA buffer, 0.05 M) has no effect on either enzymic activity or sensitivity to 5′-AMP. Under these conditions less than 1 equiv of iodine is bound to the protein. Iodination at pH 9.2 (see Methods), however, yields an iodinated protein (see below). The number of equivalents of iodine incorporated for each mole of enzyme was variable since the reaction of iodine with protein occurred rapidly and was difficult to control.

The time required for desensitization or inactivation varied considerably from experiment to experiment. Analyses of both the number and types of iodinated derivatives obtained after short (1–2 min) periods of iodination and after longer (10–20 min) incubations revealed that none of the variation detected could be correlated with length of time of iodination provided that desensitation or inactivation was complete.

In the presence of FDP and AMP a minimum of 3 equiv of iodine is incorporated into the protein. When AMP is absent (during the process of desensitization) an additional 3 equiv of iodine become protein bound. Three equivalents of iodine in excess of that required for desensitization is incorporated during enzymic inactivation in the absence of both AMP and FDP. The desensitized, iodinated enzyme exhibited properties reported previously for the native enzyme with respect to the pH/activity relationship, capacity for stimulation by EDTA, and molecular weight (as estimated by gel filtration on Sephadex G100).

The iodinated amino acid derivatives formed during desensitization or inactivation were identified as a mixture of monoiodotyrosine, diiodotyrosine, and monoiodohistidine by paper chromatography in three systems (Table II) and cochromatography with standard derivatives. Monoiodohistidine (1–3 moles/mole of enzyme) was detected when FDPase was iodinated in the presence

TABLE II: ^{131}I Uptake and Iodinated Amino Acids Formed during Desensitization and Inactivation of FDPase.[a]

Expt	Addn during Iodination: FDP	Addn during Iodination: AMP	Time of Iodination (sec)	Act. (μmoles/min mg of protein): Without AMP	Act. (μmoles/min mg of protein): With AMP	Equiv of I^{131} Incorp/Mole of Enzyme	MIH (moles)	MIT (moles)	DIT (moles)
1	+	+	160	80	3	3.8	1.0	0.9	0.9
	+	−	90	79	76	6.8	1.1	1.7	1.9
	−	−	80	<1	<1	9.7	1.1	2.8	2.7
2	+	+	80	79	2	7.8	2.0	1.8	1.8
	+	−	90	80	78	11.1	2.0	3.1	3.0
	−	−	120	<1	<1	14.2	1.7	4.1	4.0

[a] In two separate experiments, 100 μg of FDPase were iodinated in the presence and absence of FDP and AMP as described in Methods. After the addition of metabisulfite, aliquots were assayed for enzymic activity in the presence and absence of 1.0 mM AMP. Following dialysis, the contents of the dialysis sac were counted in a Nuclear-Chicago well-type scintillation counter and the equivalents of ^{131}I incorporated per mole of enzyme were determined. The FDPase was then digested with pronase and leucine aminopeptidase and the resultant hydrolysate was applied to paper for chromatography in solvent a. The molar ratio of amino acid derivatives was calculated using the integrator of the strip scanner and further checked by either counting segments of the paper in a liquid scintillation system using Bray's (1960) solution or by eluting from the paper with 1 N acetic acid and counting in a well-type scintillation counter.

of FDP and AMP. The concentration of iodohistidine did not increase during the process of desensitization or inactivation. Desensitization by iodination in the presence of FDP is accompanied by the appearance of equal amounts of monoiodotyrosine and diiodotyrosine. Enzymic inactivation occurs with the formation of an additional 1 mole of both mono- and diiodotyrosines (expt 1, Table II). No other radioactive peaks were present with the exception of less than 5% of the radioactivity which remained at the origin.

Discussion

The data presented above as well as those previously reported by this laboratory (Rosen and Rosen, 1966) demonstrate the essential role of tyrosyl residues in the maintenance of the catalytic and allosteric properties of the enzyme. Pontremoli *et al.* (1966a, b) have shown that tyrosyl residues are similarly important in the catalytic allosteric properties of rabbit liver FDPase.

Interpretation of data obtained from iodination is confused by the difficulty encountered in controlling the number of iodine atoms incorporated during the reaction and the possibility of side reactions such as oxidation of sulfhydryl groups on the protein (Hughes and Straessle, 1950). It is clear, however, that iodination of two tyrosyl residues occurs concomittantly with loss of sensitivity to 5′-AMP and that another two tyrosyl residues are iodinated during loss of enzymic activity (Table II). The results of these experiments, therefore, are in agreement with those obtained by dinitrophenylation.

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