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The Nature of the Amino Acid Residues Involved in the Inactivation of Gluconate 6-Phosphate Dehydrogenase by Iodoacetate*

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The gluconate 6-phosphate dehydrogenase, which catalyzes the reaction

$$\text{Gluconate 6-phosphate} + \text{TPN} \rightleftharpoons \text{ribulose 5-phosphate} + \text{TPNH} + \text{H}^+ + \text{CO}_2$$

has been crystallized from *Candida utilis* (1). The kinetics (2) and the stereochemistry of the reaction (3) have been studied, but very little is known of the nature of the functional groups or amino acids involved in the catalysis. This report deals with the role of one or two sulfhydryl groups in the activity of the enzyme. Rapid inactivation has been found to take place upon treatment with iodoacetate, and complete inactivation has been achieved when 1.8 moles of iodoacetate are bound per mole of dehydrogenase. The alkylated amino acid has been identified as cysteine.

EXPERIMENTAL PROCEDURE

Materials

The gluconate 6-phosphate dehydrogenase was prepared as previously described (1) with minor modifications. Labeled iodoacetic acid-1-^{14}C with a specific activity of 2.2 mc per mmole was obtained from New England Nuclear Corporation. Other substances were commercial preparations.

Methods

Reaction with Iodoacetate—The reaction mixture contained, in a final volume of 1 ml, gluconate 6-phosphate dehydrogenase (specific activity, 240 units per mg of protein) in a final concentration ranging from 1.5 to 2.6 mg per ml; Tris buffer, pH 8.6, 0.25 M; and iodoacetate-1-^{14}C, 1.2×10^{-2} M (2.58×10^6 c.p.m. per μmole).[1] The mixture was incubated at 20°, and samples were removed at intervals for determination of enzyme activity. Usually 30 minutes were sufficient for complete inactivation of the enzyme. To terminate carboxymethylation, the pH was lowered to 7 by the addition of 0.2 ml of 1 M acetate buffer, pH 4.2. The protein was precipitated by the addition of 670 mg of ammonium sulfate; the precipitate was washed three times with 1-ml portions of 80% saturated ammonium sulfate and then dissolved in 1 ml of water. The protein was dialyzed against 0.02 M acetate buffer, pH 5.8, and then against several changes of distilled water at 4° for 48 hours. The effectiveness of this procedure in removing the excess of iodoacetate was ascertained by a control in which iodoacetate-^{14}C was added to untreated protein immediately after acidification and before precipitation with ammonium sulfate. After application of the above procedure, only a very small amount of radioactivity was found associated with the protein.

Sucrose Gradient Centrifugation—Density gradient centrifugations, performed essentially as described by Martin and Ames (5), were carried out for 15 hours at 37,000 r.p.m. and 15°. Fructose diphosphate aldolase and glycerophosphate dehydrogenase of rabbit muscle were placed in each gradient as markers. Calculations of s_{20} values were based on several runs and were in good agreement, *i.e.* within ± 0.3 S. The s_{20} value obtained for the gluconate 6-phosphate dehydrogenase was 5.6. On the assumption of a spherical protein, this corresponds to an approximate molecular weight of 100,000.

Protein Determination—Protein was determined by the Folin method (6); lyophilized crystalline gluconate 6-phosphate dehydrogenase was used as the standard.

Enzyme Assay—The activity of the gluconate 6-phosphate dehydrogenase was measured in the test system reported by Horecker and Smyrniotis (7).

Protein Hydrolysis—The hydrolysis of the iodoacetate-treated protein was carried out in 6 N HCl for 20 hours at 110° by the procedure suggested by Moore and Stein for the highest recovery of *S*-carboxymethylcysteine (8). The hydrolysate was taken to dryness under reduced pressure in a rotary evaporator, dissolved and taken to dryness five times from water, and finally dissolved in water.

RESULTS

Inactivation of Gluconate 6-Phosphate Dehydrogenase by Iodoacetate—The activity of gluconate 6-phosphate dehydrogenase as function of time of reaction with iodoacetate at 20° is shown in Fig. 1. Virtually no inactivation was obtained in the controls, or with iodoacetate at pH 7.6 and 5.7. Inactivation was 80% complete in 20 minutes at pH 8.6. Thus there is a strong dependence upon pH. As shown in Fig. 2 (*left*), gluconate 6-phosphate strongly protects against inactivation by iodoacetate. With 2.5×10^{-3} M iodoacetate, the inactivation was about 80% in 40 minutes, but in the presence of 4×10^{-3} M gluconate 6-phosphate, inactivation was only slightly greater than the control (approximately 25% inactivation). TPN,

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[1] Owing to the volatility of free iodoacetic acid, the samples to be counted were first allowed to react with cysteine at pH 10 (4).

the cosubstrate, was ineffective in preventing inactivation by iodoacetate (Fig. 2, *right*).

Relationship of Iodoacetate-^{14}C Labeling to Loss of Enzymatic Activity—In order to establish the number of equivalents of iodoacetate needed to inactivate the enzyme, iodoacetate-^{14}C was incubated with the dehydrogenase. At intervals, samples were taken, the reaction was stopped, and the excess iodoacetate was removed as described under "Methods." The amount of dehydrogenase inactivated by alkylation with iodoacetate was calculated from activity measurements in the presence of iodo-

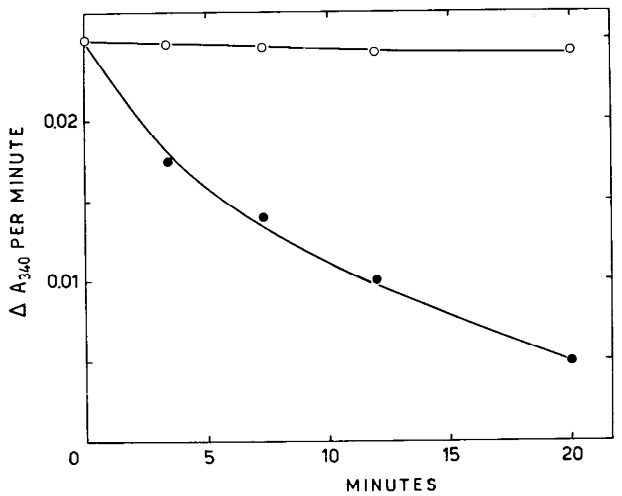

FIG. 1. Inactivation of gluconate 6-phosphate dehydrogenase by iodoacetate. The incubation mixture contained, in a final volume of 0.2 ml, gluconate 6-phosphate dehydrogenase (specific activity, 240 units per mg of protein), 0.4 mg; iodoacetate, 12×10^{-3} M; and either 0.25 M Tris buffer, pH 8.6 or 7.6, or 0.25 M acetate buffer, pH 5.7. Controls without iodoacetate were incubated at each pH. At intervals, samples were removed for the determination of enzymatic activity. ○, controls without iodoacetate, pH 8.6, 7.6, and 5.7, and tests with iodoacetate, pH 7.6 and 5.7; ●, tests with iodoacetate, pH 8.6.

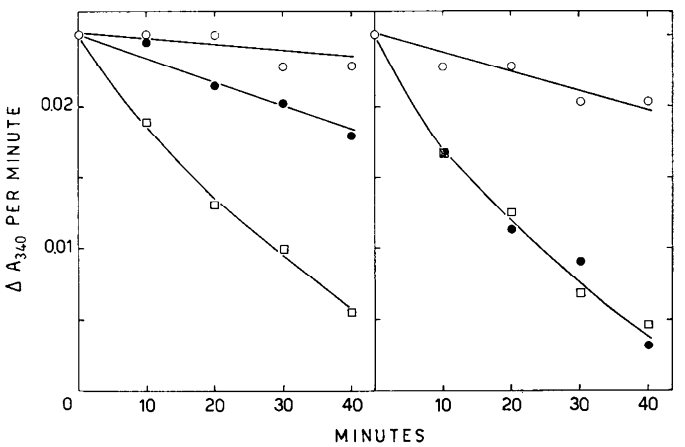

FIG. 2. Protection of gluconate 6-phosphate against inactivation by iodoacetate. The incubation mixture (final volume, 0.2 ml) contained gluconate 6-phosphate dehydrogenase (specific activity, 240 units per mg of protein), 0.4 mg, and Tris buffer, pH 8.6, 0.25 M. Other additions were 2.5×10^{-3} M iodoacetate and either 4×10^{-3} M gluconate 6-phosphate or 1.2×10^{-3} M TPN. At intervals, samples were removed for the determination of enzymatic activity. ○, controls without iodoacetate or substrate; □, iodoacetate added; ●, iodoacetate and gluconate 6-phosphate added (*left*) or iodoacetate and TPN added (*right*).

TABLE I

Relationship of iodoacetate-^{14}C labeling to loss of enzymatic activity

The composition of the reaction mixtures is given under "Methods."

Experiment	Observed inactivation		Protein			Iodoacetate-^{14}C fixed		Iodoacetate fixed[d]
	Iodoacetate present (A)	Controls (B)	Total[a]	Active[b]	Iodoacetate-inactivated[c]			
	%	%	mμmoles			c.p.m.	mμmoles	equivalents
1	44.8	10.5	3.8	2.1	1.3	5,700	2.27	1.74
2	58	11	3.8	1.6	1.78	7,700	3.1	1.7
3	60	10	8	3.2	4	18,470	7.19	1.79
4	76	11	2.16	0.52	1.4	6,580	2.56	1.83
5	92.4		11.9	0.9	11	52,000	20.4	1.85

[a] Based on a molecular weight of 100,000.
[b] Calculation: (100% − A) × total protein.
[c] (A − B) × total protein.
[d] Millimicromoles of iodoacetate fixed per mμmole of protein inactivated by iodoacetate.

TABLE II

Effect of substrate on binding of iodoacetate-^{14}C to enzyme

The reaction mixture contained gluconate 6-phosphate dehydrogenase (specific activity, 240 units per mg of protein), 2 mg per ml; Tris buffer, pH 8.6, 0.25 M; iodoacetate-1-^{14}C, 2.5×10^{-3} M; and gluconate-6-phosphate, 4×10^{-3} M, as indicated. After 20 and 40 minutes of incubation with iodoacetate, the residual enzymatic activity was determined. At these times, aliquots of the reaction mixture were removed and freed from excess iodoacetate, and both the protein content and radioactivity were determined.

Compound tested	Iodoacetate-^{14}C bound after 20 minutes calculated on basis of			Iodoacetate-^{14}C bound after 40 minutes, calculated on basis of		
	Total enzyme	Total enzyme inactivated	Enzyme inactivated by iodoacetate[a]	Total enzyme	Total enzyme inactivated	Enzyme inactivated by iodoacetate[a]
	moles/mole enzyme			moles/mole enzyme		
None	0.77	1.5	1.8	1.06	1.5	1.75
Gluconate 6-P, 4×10^{-3} M	0.4	1.53	2.2	0.65	1.7	2.3

[a] See Table I.

acetate and in a control without iodoacetate (see Table I). The number of moles of iodoacetate fixed was obtained from the number of counts per minute fixed and the specific activity of the iodoacetate-^{14}C. In five experiments, 1.7 to 1.85 (average, 1.8) equivalents of iodoacetate were consumed. Thus an average of 1.8 moles of amino acid were carboxymethylated.

Effect of Substrates on Binding of Iodoacetate to Enzyme—The results of these experiments are reported in Table II and are expressed as moles of iodoacetate-^{14}C bound per mole of enzyme after 20 and 40 minutes of incubation. In the presence of 4×10^{-3} M gluconate 6-phosphate, there was 48% protection of the dehydrogenase from binding of iodoacetate after 20 minutes and 38% protection after 40 minutes. A further increase in substrate concentration did not appreciably improve the protection.

TABLE III

Radioisotope recoveries during isolation of carboxymethylcysteine

Step	Recovery
	c.p.m.
Radioactive protein before hydrolysis	121,000
Hydrolysate	97,000
Total recovered after elution from electrophoretogram	69,000
Recovered from main radioactive band	55,200
Remaining radioactivity recovered	13,800

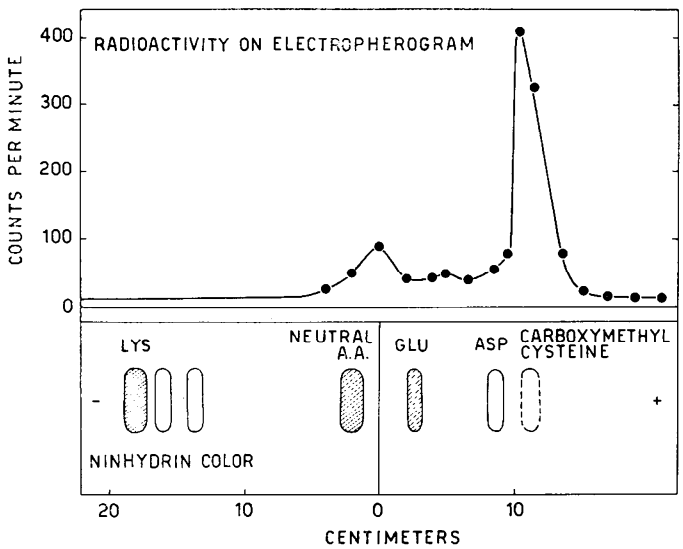

FIG. 3. Electrophoretic separation of labeled amino acid derivatives. The hydrolysate derived from 3 mg of labeled gluconate 6-phosphate dehydrogenase (97,000 c.p.m.) was divided in two parts, each of which was streaked in an 8-cm band on Whatman No. 3MM paper. Marker spots of lysine, glutamic acid, aspartic acid, and carboxymethylcysteine were placed in adjacent lanes. Electrophoresis was conducted for 80 minutes at 1,500 volts in 2% pyridine-1% acetic acid, pH 5.2. The areas of radioactivity were located by cutting the paper into pieces 1 cm wide and assaying them in a gas flow Geiger-Müller counter. The nonradioactive lanes were sprayed with ninhydrin to reveal the location of amino acids.

TABLE IV

Paper chromatography of main radioactive component isolated by paper electrophoresis

Two aliquots of the main radioactive component isolated by paper electrophoresis (5000 c.p.m.) were placed on separate sheets of Whatman No. 1 paper. These were developed by descending chromatography in 95% ethanol-3 N ammonia-butanol, 1:2:2, or in 80% phenol-water. The radioactivity was located as described in Fig. 3, and the authentic compound was located by spraying with 0.25% ninhydrin in acetone.

Solvent systems	Authentic carboxymethylcysteine	Unknown compound
	R_F	R_F
Butanol-ethanol-ammonia	0.39	0.39
80% Phenol-water	0.29	0.29

Identification of Amino Acid Residues Carboxymethylated—Lysyl, histidinyl, and cysteinyl residues may be carboxymethylated readily by iodoacetate at pH 8.6. Owing to this lack of specificity, identification of the carboxymethylated amino acids was necessary. A sample containing 3 mg of gluconate 6-phosphate dehydrogenase, 90% inactivated by iodoacetate (40,400 c.p.m. per mg of protein), was lyophilized, dissolved in 1 ml of 6 N HCl, and hydrolyzed as described under "Methods." The hydrolyzed material was finally dissolved in 0.4 ml of water. The recovery of radioactivity after the hydrolysis was 80% of the initial value (Table III). The resulting amino acid mixture was then separated by paper electrophoresis at pH 5.2. As shown in Fig. 3, three ninhydrin-positive bands moved toward the anode, whereas another group of four migrated toward the cathode. However, the main radioactive component corresponded to one ninhydrin band approximately 11 cm from the origin toward the positive side (Fig. 3). This radioactive band moved to the same location as authentic *S*-carboxymethylcysteine and was well separated from aspartic and glutamic acids, which, at this pH, move toward the anode. The remaining radioactivity was located near the origin. After elution, the radioactive band corresponding to the carboxymethylcysteine accounted for 80% of the radioactivity which could be eluted from the entire electrophoretogram. The eluted material was then concentrated and analyzed chromatographically. The results of the chromatographic analysis in 80% phenol-water (4) and in ethanol-ammonia-butanol systems (9) (Table IV) show that the R_F values of the unknown were identical with those of authentic carboxymethylcysteine.

DISCUSSION

The results presented above furnish direct evidence for the importance of sulfhydryl groups in the activity of the gluconate 6-phosphate dehydrogenase. The data are in agreement with the conclusions drawn from *p*-hydroxymercuribenzoate titrations reported by Rippa and Pontremoli (10). The gluconate 6-phosphate dehydrogenase is rapidly inactivated by iodoacetate at pH 8.6, the reaction being strongly influenced by the hydrogen ion concentration. Full inactivation is achieved by carboxymethylation of 1.8 amino acid residues per enzyme molecule. This stoichiometry between carboxymethylation and inactivation is not influenced by the extent of inactivation. This very likely means that the only two enzymatic species which exist during the reaction are fully active and fully inactive dehydrogenase. The studies on the nature of the amino acid residues involved in inactivation show that about 80% of the radioactivity can be accounted for as carboxymethylcysteine. The observation that a fraction of a mole of other amino acid residues reacts with iodoacetate was not unexpected, but these side reactions are probably not quantitatively sufficient to influence the enzymatic activity appreciably.

Of the two substrates (TPN and gluconate 6-phosphate), only gluconate 6-phosphate protects the enzyme against iodoacetate. This means that the high reactivity of the cysteinyl residues involved in the inactivation by iodoacetate is in some way decreased in the enzyme-gluconate 6-phosphate complex. This may reflect either changes in the conformation of the enzyme after reaction with substrate or proximity of the cysteinyl residues to the active site, so that gluconate 6-phosphate can sterically impede carboxymethylation.

SUMMARY

Gluconate 6-phosphate dehydrogenase was inactivated by treatment with iodoacetate at pH 8.6. The inactivation was complete after 1.8 amino acid residues per molecule of enzyme had been carboxymethylated. Carboxymethylcysteine accounted for 80% of the iodoacetate fixed. The substrate, gluconate 6-phosphate, afforded protection against inactivation and carboxymethylation of the enzyme. It is therefore concluded that one or more cysteinyl residues are sufficiently near the active site so that their carboxymethylation leads to complete loss of activity.

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