Evidence of a Critical Histidine Residue in 6-Phosphogluconate Dehydrogenase from *Candida utilis**

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ABSTRACT: Photooxidation of 6-phosphogluconate dehydrogenase, in the presence of methylene blue, leads to a loss of enzymatic activity, which follows first-order kinetics. Amino acid analysis shows that histidine is the only amino acid significantly affected by photooxidation when the sulfhydryl groups are protected with *p*-hydroxymercuribenzoate. The loss of 90% of the enzymatic activity occurs with the oxidation of less than two histidine residues out of the 13 present in the enzyme molecule. The effects of pH and ionic strength on the rate of photoinduced inactivation of the enzyme correspond to that expected for the photooxidation of the imidazole group. The results provide direct chemical evidence for the involvement of a histidyl residue in the mechanism of action of 6-phosphogluconate dehydrogenase.

It has been previously reported that at least a cysteine and a lysine residue, together with a phosphate attracting group, are crucial to the catalytic activity of 6-phosphogluconate dehydrogenase from *Candida utilis*. For example, the reaction of iodoacetate (Grazi *et al.*, 1965) or chlorodinitrobenzene (Rippa *et al.*, 1966) with a single cysteine residue, or the binding of pyridoxal 5′-phosphate (Rippa *et al.*, 1967a) to a single lysine residue of the protein molecule, inactivate the enzyme.

In order to gain additional information regarding functional amino acid residues at the active center of the enzyme, we have studied the effect of photooxidation on the catalytic activity. The present report is concerned with the effect of various experimental conditions on the rate of dye-sensitized photooxidation of 6-phosphogluconate dehydrogenase and with the relationships between the inactivation of the enzyme and the destruction of amino acid residues.

The results provide direct chemical evidence that a histidine residue is involved either in the mechanism of action or in the maintenance of the proper conformation of 6-phosphogluconate dehydrogenase.

Materials and Methods

6-Phosphogluconate dehydrogenase (6-phospho-D-gluconate:NADP[1] oxidoreductase (decarboxylating) EC 1.1.1.44), type I, crystalline, was isolated from *C. utilis* as previously described (Rippa *et al.*, 1967b) with minor modifications, *i.e.*, the time of the heat treatment was increased from 6 to 12 min. The preparations used in the present work had a specific activity of 250, equivalent to 40 μmoles of 6-phosphogluconate oxidized/min per mg of protein at 22°, when assayed as described (Rippa *et al.*, 1967b). The difference in the specific activity between this preparation and the previous one is due to the minor modification reported above.

6-Phosphogluconate, TPN^+, TPNH, and *p*-hydroxymercuribenzoate were purchased from Sigma Chemical Co, St. Louis, Mo. Methylene blue was purchased from BDH. All other chemicals were of analytical reagent grade. Solutions were prepared in deionized, doubly quartz-distilled water. Prior to the experiments, the enzyme was freed from ammonium sulfate and other salts by dissolving the crystals in a minimum amount of 0.05 M phosphate buffer (pH 6.2) and passing the enzyme solution through a Sephadex G-25 column equilibrated with the same buffer. The enzymatically active fractions were pooled, analyzed for protein concentration and activity, and utilized for all the experiments reported in the present paper.

Apparatus for Photooxidation. For the photooxidation experiments, the protein sample was pipetted into a tube (1.2 cm wide, 2 cm high) which was enclosed in a glass water jacket through which water at 27° was circulated. The sample solution was continuously stirred with a magnetic bar. The light source was a 200-W lamp positioned 8 cm above the free surface of the sample.

Procedure for the Photooxidation. Methylene blue, at a final concentration of 0.001%, was added in the dark to the enzyme solution. At zero time a suitable aliquot was removed for activity measurement or for amino acid analysis. The light was then switched on and small aliquots were removed at the desired time intervals. The aliquots were diluted, if necessary, and immediately tested for enzymatic activity. It was not necessary to apply a volume correction (Ray and Koshland, 1962) for inactivation kinetics since the sum of the aliquot volumes removed did not exceed 5% of the initial volume (1 ml). In other experiments, where the loss

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[1] Abbreviations used in this paper are as defined in *Biochemistry 5*, 1445 (1966).

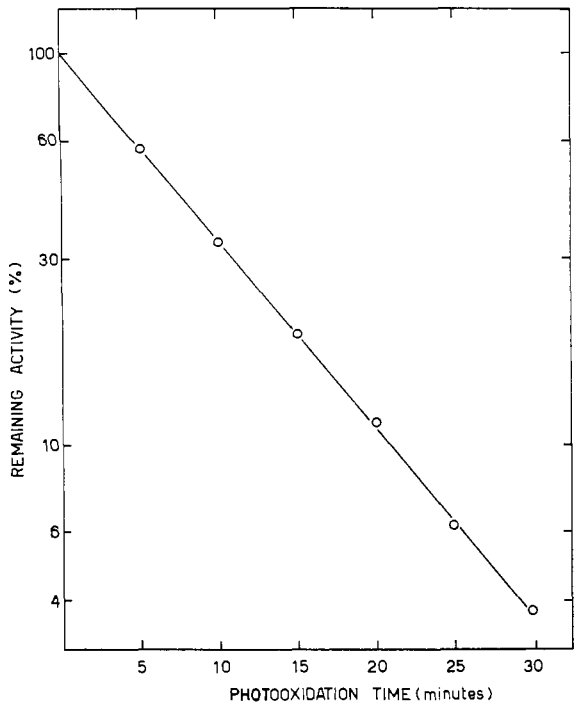

FIGURE 1: Kinetics of the inactivation of 6-phosphogluconate dehydrogenase by photooxidation. Reaction conditions: 1 ml of the reaction mixture contained 4 mg of enzyme, 50 mM phosphate buffer (pH 6.2), and 0.001% methylene blue. The ionic strength was 0.1 and the temperature was 27°. The light was then turned on. Aliquots (5 μl) were removed at the indicated time intervals and after 100- to 300-fold dilution with 50 mM phosphate buffer (pH 6.2) were assayed for enzymatic activity. A control experiment was run in the dark in identical conditions.

of enzymatic activity was correlated to the destruction of amino acids, the entire sample was utilized after it had reached the desired per cent of inactivation. The protein was freed from methylene blue by Sephadex G-25 filtration and was used for the acid and/or basic hydrolysis and for the spectrophotometric analyses of sulfhydryl and tryptophanyl residues. The same procedure was used for the control samples containing protein and methylene blue but kept at 27° in the dark.

Amino Acid Analyses. Amino acid analyses were performed in duplicate, according to the method of Spackman *et al.* (1958), on a Spinco Model 120B automatic amino acid analyzer, equipped with a high-sensitivity cuvet (6-mm light path) and a recorder for the 4–5 mV range. Acid hydrolysis was carried out under vacuum in sealed, Pyrex tubes at 110° for 24 hr. Alkaline hydrolyses for the determination of methionine and tryptophan were carried out as described (Noltmann *et al.*, 1962). Sulfhydryl groups were determined spectrophotometrically (Boyer, 1954). In addition, tryptophan and tyrosine contents were determined also spectrophotometrically (Goodwin and Morton, 1946). The amino acid residues susceptible to photooxidation were compared with two other amino acid residues which were stable to photooxidation and acid or basic hydrolysis. The results obtained were then compared with the amino acid analyses of the control sample kept in the dark.

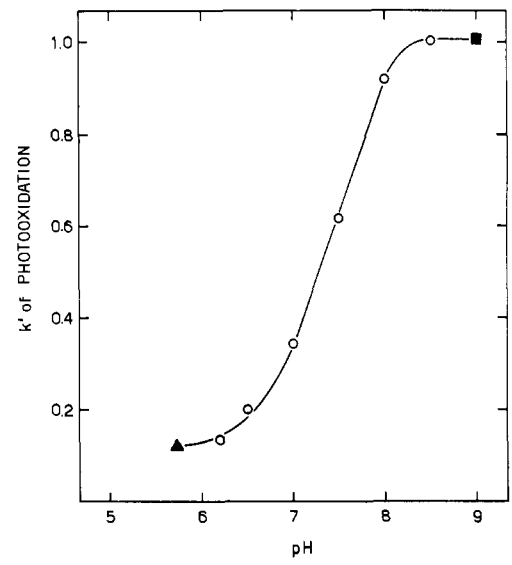

FIGURE 2: Effect of the pH on the rate of photoinactivation of 6-phosphogluconate dehydrogenase. Apparent first-order velocity constants for the loss of enzymatic activity were obtained graphically from semilogarithmic plots analogous to that reported in Figure 1. All buffers used were 5× mM with the ionic strength adjusted to 0.15 with NaCl. The enzyme concentration was 0.2 mg/ml; all other conditions as in the legend of Figure 1. (▲) Acetate, (○) phosphate, and (■) ethanolamine.

Results

Effect of Photooxidation on the Enzymatic Activity. 6-Phosphogluconate dehydrogenase is inactivated by photooxidation in the presence of methylene blue. As shown in Figure 1 the loss of enzymatic activity follows first-order kinetics down to 90% inactivation. The enzyme undergoes no loss of activity when exposed to methylene blue in the dark or to a light source in the absence of methylene blue.

Effect of Hydrogen Ion Concentration and Ionic Strength on the Rate of Inactivation. The rate of inactivation is dependent on the pH of the reaction mixture. As shown in Figure 2, on increasing pH from 6 to 8.5 there is an almost eightfold increase in the inactivation rate. The ionic strength was also found to affect the rate of inactivation. Figure 3 shows that a tenfold increase of the ionic strength causes a twofold decrease in the inactivation rate.

Photooxidation in the Presence of Substrates. In order to see if the presence of substrates in the photooxidation mixture altered the rate of enzyme inactivation, 6-phosphogluconate, TPN^+, TPNH, ribulose 5-phosphate, and CO_2 have been tested. None of the substrates, even at a concentration 30-fold higher than its K_m value, changed the rate of photoinactivation.

Effect of the Photooxidation on the V_{max} and K_m of the Enzyme. Partially photooxidized enzyme was subjected to catalytic rate studies as a function of the 6-phosphogluconate concentration. The results obtained (Figure 4) show that only the maximal velocity is affected by the photooxidation. Thus the photooxidation produces an inactive enzyme species not participating in the substrate binding or one in which there are sep-

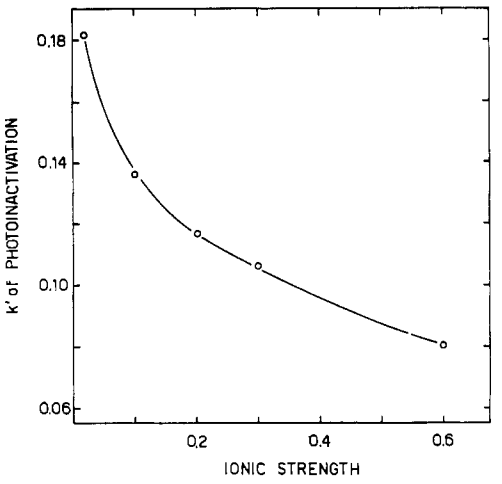

FIGURE 3: Effect of the ionic strength on the rate of photoinactivation of 6-phosphogluconate dehydrogenase. Apparent first-order velocity constants for the rate of photoinactivation were obtained graphically as described in the legend of Figure 2. Increasing amounts of NaCl were added to the reaction mixture to obtain the desired ionic strength; enzyme concentration 0.2 mg/ml. Other conditions as described in the legend of Figure 1.

arate sites for substrate binding and for catalysis, and in which the site for catalysis has been rendered completely nonfunctional.

Correlation between Activity Loss and Amino Acid Destruction. The comparison of the amino acid composition of the control and of the photooxidized enzyme

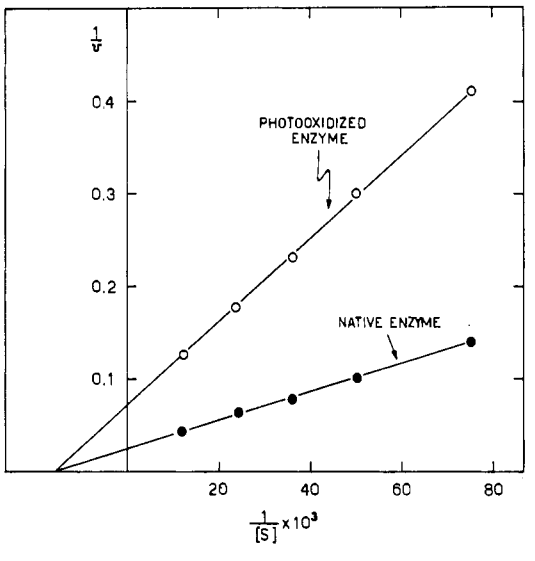

FIGURE 4: Lineweaver–Burk plot of data obtained with equal amounts of native and partially photoinactivated 6-phosphogluconate dehydrogenase. Enzyme (4 mg) was photooxidized under the standard conditions until approximately 70% of the enzymatic activity was lost. Methylene blue was removed on Sephadex G-25. The eluate contained 2.57 mg/ml of 6-phosphogluconate dehydrogenase, with a specific activity of 75 units/ml. The specific activity of the control sample, treated with methylene blue, kept in the dark, and passed through Sephadex, was 250. The activity was measured, with identical enzyme aliquots taken from the photooxidized enzyme and the control, over the range of 6-phosphogluconate concentrations indicated on the abscissa.

TABLE I: Recovery of Amino Acids after Photooxidation.[d]

Amino Acid	Control	Photooxidized	
		Untreated	PMB Treated
Histidine[a]	13	9.8[c]	11.3[c]
Cysteine[b]	8	4.9	8
Tyrosine[a,b]	36	36	36
Tryptophan[a,b]	12	12	12
Methionine[a]	16	16	16

[a] Determined on the amino acid analyzer after hydrolysis. [b] Determined spectrophotometrically. [c] Average of four determinations. [d] A sample of 6-phosphogluconate dehydrogenase (8.2 mg in 2 ml of 50 mM phosphate buffer (pH 6.2) containing 0.001% of methylene blue) was divided in two parts. One part was kept in the dark, and the other was subjected to the photooxidation until 92% of the original activity was lost. The two enzyme solutions were then passed through Sephadex G-25 columns and the protein samples were subjected to the amino acid analysis as indicated in the methods (untreated photooxidized). An identical sample was treated with *p*-hydroxymercuribenzoate (final concentration 0.2 mM) divided into two parts; one was kept in the dark and the other photooxidized. When the photooxidation caused 92% inactivation (measured as described in the legend of Figure 5), the two samples were treated with an excess of cysteine, passed through Sephadex columns, and submitted to amino acid analysis. In moles per mole of enzyme.

showed no differences in the content of any amino acid residue, other than histidine and cysteine. All 18 amino acids were examined and in Table I (second column) the values of those amino acids known to be affected by photooxidation are reported. Tyrosine, methionine, and tryptophan residues were not affected. The destruction of around 3.1 residues of cysteine and 3.2 residues of histidine resulted in the 92% loss of enzymatic activity.

Photooxidation in the Presence of Mercuribenzoate. It has been previously reported (Grazi *et al.*, 1965; Rippa *et al.*, 1966) that free SH groups are essential for the catalytic activity of the 6-phosphogluconate dehydrogenase, and that they can reversibly react with mercuribenzoate. In order to establish if the oxidation of the SH groups was responsible for the loss of the catalytic activity, observed in the experiments reported above, we have subjected an enzyme sample, in which all SH groups had previously reacted with mercuribenzoate, to photooxidation. At intervals aliquots were removed, treated with an excess of cysteine to remove the bound mercuribenzoate, and analyzed for enzymatic activity. As appears from Figure 5, the rate of inactivation under these conditions is identical with the rate obtained when free SH groups are present. When 92% inactivation was

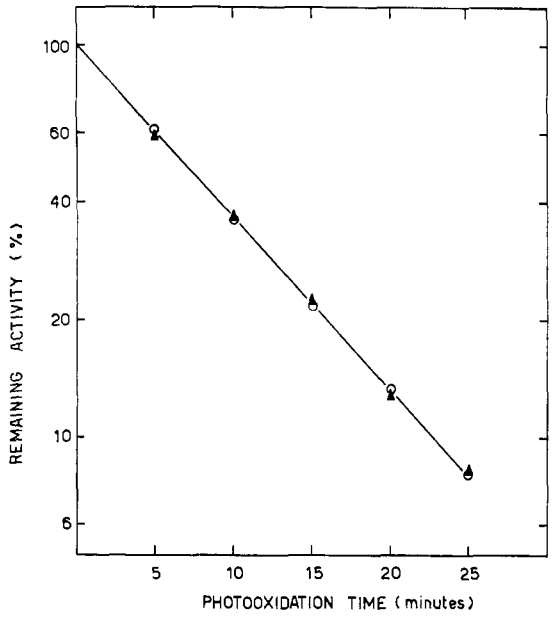

FIGURE 5: Kinetics of the photoinactivation of native and *p*-hydroxymercuribenzoate-treated 6-phosphogluconate dehydrogenase. Reaction conditions were the same as described for Figure 1. Mercuribenzoate was added to the enzyme solution at a final concentration of 0.2 mM. Complete inactivation of the enzyme and the blocking of all the SH groups occurred in less than 5 min. After the addition of methylene blue, an aliquot was removed as control and kept in the dark. The remaining solution was subjected to photooxidation. At intervals aliquots were removed and diluted 100-fold with phosphate buffer (50 mM, pH 6.2) containing 1 mM cysteine, and after 1 min at room temperature, the residual activity was tested under standard conditions. The control sample, which was kept in the dark and then treated with cysteine, yielded a complete recovery of activity throughout the experimental period. (○) Enzyme photooxidized in the standard conditions. (△) Enzyme photooxidized in the presence of *p*-hydroxymercuribenzoate.

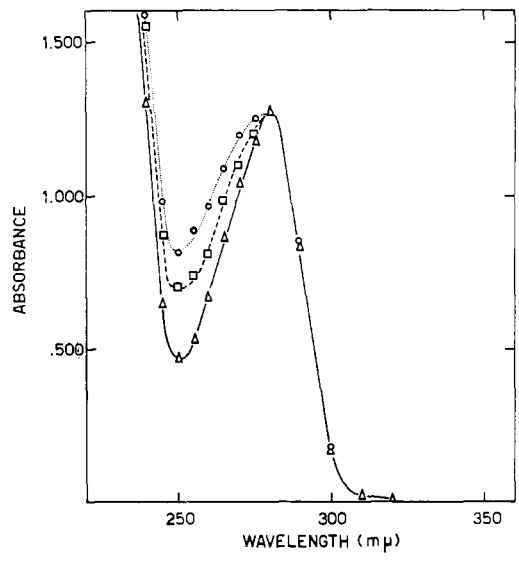

FIGURE 6: Absorption spectra of the native and of the photooxidized 6-phosphogluconate dehydrogenase. Native enzyme and the enzyme photooxidized with and without prior treatment with mercuribenzoate were utilized. All three samples were obtained according to the legend of Table I. The solutions contained 1 mg of protein/ml dissolved in 0.02 M phosphate buffer (pH 6.2). The values of the amino acid lost by photooxidation were the same reported in Table I. Solid line: native enzyme. Broken line: untreated enzyme photooxidized. Dotted line: enzyme treated with mercuribenzoate and then photooxidized.

reached, the remaining protein was treated with cysteine and passed through a Sephadex G-25 column. The determination of the amino acid composition was carried out on the photooxidized enzyme and on the control (enzyme subjected to all treatments, except exposure to light). The photooxidized inactive enzyme has the same SH content as the active control (Table I, last column). In this case the inactivation was correlated to the loss of approximately 1.7 residues of histidine.

Absorption Spectra of the Photooxidized Enzymes. The absorption spectra of the enzyme photooxidized in the absence or in the presence of mercuribenzoate differ from the spectrum of the native enzyme in the region of 250 mμ (Figure 6). The increase in absorbancy at this wavelength seems to be related to the number of histidine residues photooxidized. Our results indicate that an increase of 0.200 and 0.340 optical density units correspond to the photooxidation of 1.7 and 3.2 residues of histidine, respectively.

Discussion

Photooxidations in the presence of methylene blue, at pH 6.2 and 27°, cause the inactivation of the 6-phosphogluconate dehydrogenase purified from *C. utilis*. The inactivation follows a first-order kinetics until all the enzymatic activity is lost.

The dependence of the inactivation rate on the pH of the reaction mixture closely resembles that found in the cases of the photooxidation of the free histidine (Weil, 1965) and of the photoinactivation of several enzymes in which histidine residues appear to be involved in the mechanism of enzyme action (Westhead, 1965; Martinez-Carrion *et al.*, 1967; Chatterjee and Noltmann, 1967).

The considerable effect of the ionic strength on the photoinactivation rate of 6-phosphogluconate dehydrogenase points to the involvement of one ionizable group, whose ionization is suppressed at high ionic strength. A similar effect was found for the photoinactivation of phosphoglucose isomerase (Chatterjee and Noltmann, 1967). This evidence, considered in conjunction with the pH dependency, would thus appear to exclude methionine residues as direct participants in the inactivation process.

Chemical analysis has shown that, in our experimental conditions, no tyrosine, tryptophan, or methionine residues are destroyed by photooxidation. The photooxidation resulting in a 92% loss of enzymatic activity caused the destruction of approximately 3 histidine and 3 cysteine residues of the 13 and 8 residues, respectively, present in the native enzyme.

When photooxidation was performed on the enzyme in which all the sulfhydryl groups had reacted with mercuribenzoate, the kinetics of enzyme inactivation (evaluated in each sample after the removal of mercuri-

benzoate) corresponded to that observed with the enzyme not treated with mercuribenzoate. Chemical analyses revealed that, in this case, no cysteine residue was affected by photooxidation, and that only approximately 1.7 histidine residues were destroyed. Thus the inactivation of the enzyme, in our experimental condition, seems to be dependent only on the destruction of less than two histidyl residues.

Several hypotheses can be formulated to explain our results. Histidine residue(s) could be essential for the maintenance of the protein conformation necessary for the catalytic activity or could directly participate in the enzymatic reaction. This last hypothesis seems to receive support from the following two facts: (a) the reaction rate of the native enzyme shows half-maximal activity at pH 6.3 (Rippa *et al.*, 1967b), and (b) the high reactivity of one SH group (Grazi *et al.*, 1965; Rippa *et al.*, 1966). This high reactivity could be attributed to the presence of a neighboring imidazole group as postulated for the streptococcal proteinase (Liu, 1967). Furthermore the enzyme catalyzes the proton exchange between one substrate (the ribulose 5-phosphate) and the medium (Lienhard and Rose, 1964), a process for which the presence of a basic group in the active center of the enzyme seems to be required.

The lack of protection by substrates from photoinactivation cannot exclude the presence of a photosensitive histidine residue at the active center of the enzyme. In the case of ribonuclease, for instance, while the substrates protect two critical histidine residues from the alkylation by iodoacetate, they do not protect the same residues from the methylene blue induced photoinactivation (Kenkare and Richards, 1966). A similar lack of protection by substrate has also been observed in the case of the photoinactivation of aspartate aminotransferase (Martinez-Carrion *et al.*, 1967).

The difference between the absorption spectra of the native and photoinactivated enzyme is similar to that reported for lactic dehydrogenase (Millar and Schwert, 1963). In that case it was suggested that the spectral differences observed could be due to the presence of ultraviolet-absorbing materials extracted from the laboratory charcoal used to remove the methylene blue. In our case no charcoal was used, and the changes in absorbancy at 250 mμ are roughly related to the number of histidyl residues photooxidized. A derivative of the imidazole, namely the acetylimidazole, has been reported to have a maximum at 244 mμ (Stadtman, 1954). Work is in progress to establish the role played by the histidine, lysine, and cysteine residues in the mechanism of action of 6-phosphogluconate dehydrogenase.

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