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Effects of Photooxidation of Histidine-38 on the Acetylphosphatase Activity of Glyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: 3-Phosphoglyceraldehyde dehydrogenase requires a reduced cysteine residue at position 149 and a histidine residue at position 38 for maximal rates of three of its activities, namely, the dehydrogenase, arsenolysis, and esterase activities. However, the fourth activity, that of acetylphosphatase, is manifest only when the active-site sulfhydryl residue is oxidized. Therefore, it was of considerable interest to determine whether histidine-38 was required for the phosphatase activity. The essentiality of this amino acid was demonstrated by the quantitative photooxidation of histidine-38 in the presence of Rose Bengal. NAD^+ provided partial protection against the inactivation of the acetylphosphatase activity by photooxidation. The stoichiometry of NAD^+ binding to the oxidized enzyme was not significantly affected by photooxidation. Added imidazole or histidine did not substitute for the photooxidized histidine in the acetylphosphatase assay. The mechanism of the inhibition of the phosphatase reaction is discussed, and the effects of photooxidation on the various enzymatic activities are compared.

In addition to the transferase and esterase activities of phosphoglyceraldehyde dehydrogenase, we have also observed a phosphatase activity associated with this enzyme (Harting, 1954). In the absence of reducing agents, such as cysteine, 3-phosphoglyceraldehyde dehydrogenase was oxidized by air and acquired an acetylphosphatase activity (Harting, 1954; Park and Koshland, 1958). Rafter and Colowick (1957) found that this slow rate of hydrolysis of acetyl phosphate or 1,3-diphosphoglyceric acid could be markedly increased by oxidizing the enzyme with iodosobenzoate under strictly controlled conditions (Rafter, 1957; Rafter and Colowick, 1957). The phosphatase activity can be inhibited by sulfhydryl compounds, *i.e.*, cysteine or glutathione, and the dehydrogenase activity thereby restored (Harting, 1954; Park and Koshland, 1958). It has been demonstrated that cysteine-149 in the monomer of 332 amino acids was the active-site residue which was responsible for the reversible interconversion of the dehydrogenase and phosphatase activities (Harris

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et al., 1963; Harris and Perham, 1965). Although the dehydrogenase and phosphatase activities are mutually exclusive, Park and Koshland (1958) noted two properties which implicated an interrelationship between the reactions. First, both catalyses are NAD^+ dependent and, secondly, both activities involve an acyl cleavage of the C–O bond of the acyl phosphates (Park and Koshland, 1958; Cohn, 1956).

Since the phosphatase activity was the unique reaction in not requiring sulfhydryl groups, it was of interest to examine the enzymatic effects of the double modification, oxidation of cysteine-149 with iodosobenzoate and photooxidation of histidine-38 by Rose Bengal. The experimental results in this paper demonstrated a third common property of the dehydrogenase and phosphatase activities, namely, the requirement of histidine-38 for maximal activity.

Experimental Procedures

3-Phosphoglyceraldehyde dehydrogenase was crystallized from rabbit muscle as described by Cori *et al.* (1948). The NAD^+ was removed with charcoal (Taylor *et al.*, 1948) and the apoenzyme was photooxidized as previously outlined in the studies of the dehydrogenase, transferase, and esterase activities of the enzyme (Bond *et al.*, 1970; Francis *et al.*, 1973). Acetyl phosphate was prepared as described by Stadtman and Lipmann (1950). The procedure for the assay of the phosphatase activity is outlined in Tables I and II. The method for measurements of NAD^+ binding is given in the footnote to Table IV.

Results

Characterization of the Inhibitory Effects of Photooxidation on the Acetylphosphatase Activity of the Dehydrogenase. In testing the effect of photooxidation on the phosphatase activity, the enzyme was first photooxidized for 2 min and then treated with *o*-iodosobenzoate in order to oxidize the sulfhydryl groups. As shown in the upper panel of Table I, photooxidation produced an inhibition of 65%. The percentage inhibition varied from 50 to 75%. Increasing the time of photooxidation to 5 or 10 min caused only a small increase in the inhibition. As shown in the lower panel of Table I, oxidation of the sulfhydryl groups prior to photooxidation of the enzyme did not significantly alter the percentage inhibition. The activity of this control enzyme was somewhat lower than that of the preceding experiment due to the instability of the oxidized enzyme in the absence of NAD^+.

When the phosphatase reaction was followed over a 30-min period, the activity was inhibited about 50% throughout the assay. The photooxidized enzyme could be assayed 1 hr after the light treatment, and the results were not affected. This indicated that major structural changes were not occurring in the course of time.

Attempt to Substitute Imidazole or Histidine for the Photooxidized Residue as Assayed in the Phosphatase Reaction. Imidazole and histidine were added to the reaction mixture to see if they would substitute for the photooxidized histidine residues of the enzyme (Table II). Imidazole failed to alleviate the inhibition of the photooxidized enzyme. Histidine reduced the activity of the control enzyme by 32% and did not allay the inhibition of the photooxidized dehydrogenase, Thus, 10 μmol of imidazole or histidine could not replace functionally 0.04 μmol of the modified histidine residues in the enzyme. As seen in the last line of Table II, imidazole or histidine alone had no effect on the stability of acetyl phosphate under these conditions.

TABLE I: Inhibition of Acetylphosphatase Activity of 3-Phosphoglyceraldehyde Dehydrogenase by Photooxidation of the Enzyme in the Reduced or Oxidized States.[a]

Conditions during Photooxidation	Photooxidation Time (min)	Acetyl Phosphate Hydrolyzed (μmol)	Inhibition (%)
Reduced SH			
Enzyme control	0	2.3	
Enzyme + Rose Bengal	0	2.1	8
Enzyme + Rose Bengal	2	0.8	65
Enzyme + Rose Bengal	5	0.6	74
Enzyme + Rose Bengal	10	0.6	74
Oxidized SH			
Enzyme control	0	1.8	
Enzyme + Rose Bengal	0	1.7	6
Enzyme + Rose Bengal	2	0.6	67
Enzyme + Rose Bengal	5	0.4	78
Enzyme + Rose Bengal	10	0.2	89

[a] The enzyme was treated with charcoal and dialyzed as noted in Experimental Procedures. The NAD^+-free enzyme was divided into two portions. For the first set of experiments, the enzyme was photooxidized as outlined in Experimental Procedures for varying lengths of time. Then 0.015 μmol of the enzyme was added to 40 μmol of sodium barbital buffer, pH 8.2, which contained 1 μmol of NAD^+ and 1 μmol of *o*-iodosobenzoate in a volume of 1.5 ml. The reagents were incubated for 10 min at 0° in order to oxidize the reactive sulfhydryl groups and promote the phosphatase activity. The phosphatase reaction was started by the addition of 4 μmol of acetyl phosphate and proceeded for 5 min, total volume, 2 ml; temperature, 25°. The disappearance of acetyl phosphate was measured by the hydroxamic acid procedure of Lipmann and Tuttle (1945). In the second set of experiments, the 0.025 μmol of NAD^+-free enzyme was first treated with 0.14 μmol of *o*-iodosobenzoate in 0.5 ml of 2.5 mM Tris–0.5 mM EDTA buffer, pH 7.0, at 0°. The oxidized enzyme was then photooxidized and assayed for phosphatase activity as described above.

NAD^+ Protection against the Inhibitory Effects of Photooxidation on the Phosphatase Activity. As shown in the first three lines of Table III, the presence of NAD^+ during the photooxidation partially prevented the loss of phosphatase activity and reduced the inhibition from 62 to 25%. NAD^+ added after the photooxidation procedure had no protective effect. Since ADP is a competitive inhibitor for NAD^+ and displaces bound [^{14}C]NAD^+ from the enzyme (Francis *et al.*, 1971b), protection by adenine nucleotides against photooxidation was also tested (Table III, lines 5–7). Under the conditions of the assay, ADP itself is slightly inhibitory (17%). It was clear, nevertheless, that the adenine nucleotide did not protect against photooxidation. The addition of ADP to a previously photooxidized enzyme produced the same additive inhibition of 83%. Thus ADP *per se* had no effect on the photooxidation.

TABLE II: Substitution of Imidazole or Histidine for the Modified Histidine Residue in the Acetylphosphatase Activity of Photooxidized 3-Phosphoglyceraldehyde Dehydrogenase.[a]

Experimental Conditions	Acetyl Phosphate Hydrolyzed (μmol)	Inhibition (%)
Enzyme control	2.5	
Photooxidized enzyme	1.0	60
Enzyme + imidazole	2.4	5
Photooxidized enzyme + imidazole	0.9	64
Enzyme + histidine	1.7	32
Photooxidized enzyme + histidine	0.6	76
No enzyme + imidazole or histidine	0	

[a] The control and photooxidized enzymes were oxidized with *o*-iodosobenzoate for 5 min at 0°. Then 10 μmol of imidazole or histidine was added as indicated, and the incubation continued for another 5 min. The hydrolysis reaction was started by the addition of 4 μmol of acetyl phosphate. After 5 min the reaction was terminated and assayed as described in Table I.

Effect of Photooxidation on NAD⁺ Binding to the Reduced and Oxidized Enzymes. The first and third lines of Table IV show that the reduced enzyme bound about 10% more NAD^+ than the enzyme oxidized with iodosobenzoate. Photooxidation of these enzymes decreased the amount of bound NAD^+ by only 15%. Therefore, photooxidation of histidine-38 does not have a significant effect on coenzyme binding as measured under these conditions.

TABLE III: Combined Effects of Photooxidation and NAD^+ or ATP on the Phosphatase Activity.[a]

Treatment of Enzyme	Acetyl Phosphate Hydrolyzed (μmol)	Inhibition (%)
Enzyme control	2.4	
Enzyme + photooxidation	0.9	62
(Enzyme + NAD^+) + photooxidation	1.8	25
Photooxidized enzyme + NAD^+	2.4	62
Enzyme + ADP	2.0	17
(Enzyme + ADP) + photooxidation	0.4	83
Photooxidized enzyme + ADP	0.4	83

[a] In order to test the effect of photooxidation in the presence of NAD^+, 0.025 μmol of the enzyme was first incubated in the presence or absence of 2.5 μmol of NAD^+ for 20 min at 0° in a volume of 0.5 ml. Enzyme samples were then photooxidized. As shown on the last three lines of the table, another sample of the enzyme was incubated with 7.5 μmol of ADP for 10 min at 0° and then photooxidized. As shown on the fourth and seventh lines, the enzyme was photooxidized first and then incubated with NAD^+ or ADP. The phosphatase assays were performed as outlined in Table I.

TABLE IV: Effect of Photooxidation on Binding of NAD^+ to Reduced and Oxidized 3-Phosphoglyceraldehyde Dehydrogenase.[a]

Conditions for Incubation	Photooxidation	Mol of NAD^+ Bound/Mol of Enzyme
Veronal, pH 8.0	0	2.8
	+	2.4
Veronal, pH 8.0	0	2.6
+ iodosobenzoate	+	2.2

[a] The experimental conditions were similar to those employed for the dehydrogenase or phosphatase reactions. In the experiments shown on the first two lines, the control or photooxidized enzyme (0.05 μmol) was added to the Veronal buffer (0.02 M, pH 8.0). The enzyme and the [^{14}C]NAD (0.025 μmol) were incubated for 10 min at 0° in a total volume of 2 ml and then placed in the Amicon Ultrafiltration Cell, Model 52. The moles of NAD^+ bound per mole of enzyme were determined by the procedure outlined in the preceding paper (Francis *et al.*, 1973). In the case of the last two experiments the control or photooxidized enzyme (0.05 μmol) was incubated for 10 min with iodosobenzoate (1.0 μmol) and then [^{14}C]NAD (0.25 μmol) for another 10 min. The NAD^+ binding was determined as mentioned above.

Discussion

Histidine-38 of the oxidized enzyme retained its remarkable sensitivity to photooxidation and remained in an exposed conformation similar to that of the reduced enzyme. This fact was demonstrated in Table I where treatment with iodosobenzoate prior to the brief 2-min photooxidation did not alter the per cent inhibition of the phosphatase reaction. Moreover, these data showed that the interaction of a reduced cysteine-149 was not required for the inhibitory effects of photooxidation during the catalysis (Table I).

The increased inhibition of the acylphosphatase activity observed with prolonged photooxidation of the iodosobenzoate treated enzyme (Table I) may represent the slower destruction of a second histidine residue which could be responsible for the activity remaining after the rapid destruction of histidine-38. Apitz-Castro and Suarez (1970) found that photooxidation of α-glycerophosphate dehydrogenase with Rose Bengal destroyed one histidine residue per enzyme molecule and produced a concomitant 50% inhibition of the enzymatic activity. Chemical modification of two histidine residues by diazonium-1-*H*-tetrazole produced complete inhibition of the enzyme (Apitz-Castro and Suarez, 1970). The reasons for achieving approximately 50% inhibition of the various activities of the photooxidized 3-phosphoglyceraldehyde dehydrogenase were given detailed consideration in the preceding publication (Francis *et al.*, 1973). The possible explanations for the inhibition of the various activities of this enzyme were also discussed in the preceding paper from both conformational and mechanistic viewpoints (Francis *et al.*, 1973).

The external addition of histidine as a substitute for the photooxidized histidine was not an entirely satisfactory experiment because the added histidine decreased the phosphatase activity of the control enzyme (Table II). The inhibition may have been due to a reduced activity of the enzyme or to the

acetylation of histidine yielding *N*-acetylhistidine. This product would give a positive hydroxamic reaction and falsely indicate that acetyl phosphate had not been decomposed. This point was not further investigated since the activity of the photooxidized enzyme was not altered by added histidine or imidazole. Nonetheless the phosphatase assay is technically the most suitable for appraising the results of added histidine or imidazole. In the esterase assay the effects of histidine and imidazole are difficult to evaluate because these compounds elicit a rapid hydrolysis of *p*-nitrophenyl acetate at pH 8.2 (Jencks and Carriuolo, 1959a, 1959b). The arsenolysis assay also presents complications arising from nonenzymatic side reactions such as the imidazole-dependent transfer of the acyl group of acetyl phosphate to sulfhydryl compounds to form acyl thio esters (Jencks and Carriuolo, 1959a).

A competitive inhibition between NAD^+ and Rose Bengal has been demonstrated kinetically and by equilibrium dialysis (Bond *et al.*, 1970); therefore, NAD^+ partially protected the phosphatase activity against the inhibition by photooxidation (Table III). These data suggested that histidine-38 might be a functional residue for coenzyme binding. However, since photooxidation did not significantly alter the stoichiometry of NAD^+ binding, histidine-38 was not an essential ligand for coenzyme interactions.

Reducing agents, such as cysteine and glutathione, are known to stabilize the protein conformation and the NAD^+–enzyme complex (Astrachan *et al.*, 1957). This fact is in accord with the finding that the reduced enzyme bound 2.8 mol of NAD^+/mol of protein and the oxidized enzyme bound only 2.6 mol of NAD^+ (Table IV). Photooxidation alone decreased the NAD^+ binding of the control about 15%. A comparison of the control and the oxidized-photooxidized enzyme showed an overall decrease of 25% from 2.8 to 2.2 mol of bound NAD^+.

The range of the per cent inhibition was slightly greater for the phosphatase activity (50–73%) than for the dehydrogenase (40–60%) or arsenolysis activity (35–45%). This difference might be attributed to the 10–15% difference in NAD^+ binding between the oxidized enzyme required for the phosphatase reaction and the reduced enzyme employed for the dehydrogenase and arsenolysis activities (Table IV). The same general trends were noticed in our earlier work which showed that the phosphatase activity was the reaction most sensitive to inhibition by ATP and ADP (Francis *et al.*, 1971a, 1971b).

Since a covalent acetyl-enzyme intermediate of the phosphatase activity has not as yet been isolated, [^{14}C]acetyl phosphate binding studies were not feasible. Thus it is not possible to compare more specifically the effects of photooxidation on the substrate binding or investigate the mechanism of the inhibition in greater detail.

The production of the most active phosphatase activity for this enzyme was achieved by Ehring and Colowick (1969) using stoichiometric amounts of iodosobenzoate or iodine as the oxidizing agents. They concluded from their data that the active center sulfhydryl group (–SH) was not converted to a disulfide (S–S) but rather to a sulfenic acid (–SOH). On the basis of quite different experiments, Parker and Allison (1969) and Allison and Connors (1970) also reached the same conclusion and postulated further that the active enzyme–substrate complex might be an enzyme–sulfenylcarboxylate intermediate (ESOC(=O)CH_3).

Ehring and Colowick (1969) discovered a second phenomenon of importance. The first mole of added iodine elicited the acetylphosphatase activity, but the second mole of iodine resulted in the irreversible inactivation of both the phosphatase and the dehydrogenase activities (Ehring and Colowick, 1969). This finding was particularly noteworthy because it demonstrated that a second residue was required for both activities. The second residue was extremely reactive as the inactivation was accomplished with stoichiometric amounts of iodine in a few seconds at pH 7.0 and 0°. Three reasonable candidates for this reactive residue were tryosine, tryptophane, and histidine. On the basis of chemical studies, histidine was the most likely residue. The authors speculated as to whether an iodine-sensitive histidine moiety might be identical with the photosensitive histidine-38 which was involved in the deacylation of the dehydrogenase and the esterase reaction (Bond *et al.*, 1970). One difference between the experiments of the laboratories of Colowick and Park is that iodine treatment produces a 100% inactivation and photooxidation only about 50% inactivation. This could be due to variations in the oxidation products of the attacked histidine moiety. For example, chemical studies of the photooxidation of histidine in the presence of Rose Bengal revealed at least ten different products (Tomita *et al.*, 1969). This multiple product formation could account for the incomplete and somewhat variable inactivation of the enzyme activities.

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A Calorimetric Investigation of the Copper–Bovine Plasma Albumin Interaction†

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ABSTRACT: The thermodynamics of the copper–bovine plasma albumin interaction was investigated by a combination of microcalorimetry and equilibrium dialysis. Computer regression procedures were used to calculate the thermodynamic parameters for the first 10 steps of the reaction. At 20° in sodium acetate buffer, $I = 0.1$, pH 4.80, the enthalpy changes ranged from 4.0 to 2.2 kcal mol^{-1} for the first to the tenth bound ion, respectively. Similarly, the apparent standard free-energy changes ranged from -5.0 to -2.7 kcal mol^{-1} and the entropy changes from 31 to 17 cal mol^{-1} deg^{-1}. Two to three protons were released per mol of copper bound during the binding of the first 4 mol of copper. Hydrogen–deuterium exchange studies suggested that no conformational change occurred when 1 mol of copper was bound but did occur when 10 mol of copper was bound.

The thermodynamic parameters of the copper–bovine plasma albumin interaction were determined more than two decades ago from equilibrium dialysis data taken at two different temperatures (Klotz and Curme, 1948). The development of sensitive microcalorimeters, however, now permits direct measurement of the enthalpy changes associated with this interaction. Accordingly, a combination of calorimetric and equilibrium dialysis techniques should provide the desired thermodynamic parameters from data taken at one temperature. This paper reports the results of such a study and compares them to those reported previously. To aid interpretation of the data, studies were also performed regarding the effect of acetate ion, proton evolution, and conformational changes. The results from these are also reported.

Materials and Methods

Bovine Plasma Albumin Samples. Crystalline bovine plasma albumin (Pentex Corp., now Research Division, Miles Laboratories, Inc., lot nos. 15, 16 and 18) was used without further purification for most of the studies. Some albumin was purified, however, by defatting (Sogami and Foster, 1968) and deionizing (Dintzis, 1952).

Reaction Conditions. All reactions were performed at 20°. Generally, solutions were prepared in a sodium acetate buffer, $I = 0.1$, pH 4.80. In one set of experiments the molarity of the acetate ion in the buffer was varied to determine what effect this might have on the copper–albumin interaction.

Equilibrium Dialysis. Visking dialysis tubing (Union Carbide) was washed in boiling deionized water and soaked in buffer overnight before use. Dialysis bags (2 ml) were suspended in 8-ml volumes of buffer solution containing reagent grade cupric acetate and shaken for 18 hr. The albumin concentration used for these studies was 2.5%. Copper was assayed spectrophotometrically as the 2,9-dimethyl-1,10-phenanthroline chelate (Smith and McCurdy, 1952).

Microcalorimetry. A heatburst microcalorimeter similar to that described by Kitzinger and Benzinger (1960) was used to measure heat changes. The heat associated with the interaction of copper with albumin was obtained by mixing, in one of the bicompartmented vessels, a solution of 5% albumin with one of cupric acetate, while in the reference vessel a similar solution of albumin was mixed with acetate buffer. The heat of dilution of the cupric acetate was measured separately by mixing cupric acetate solutions with equal volumes of acetate buffer in the reaction vessel, while two equal volumes of acetate buffer were mixed in the reference vessel.

Analysis of Binding Data. Binding data were analyzed by use of eq 1 (Scatchard, 1949) which is applicable to processes involving sites that can be grouped into classes having similar intrinsic binding constants

$$\bar{\nu} = \sum_{j=1}^{m} \frac{N_j k_j[\mathrm{A}]}{1 + k_j[\mathrm{A}]} \qquad (1)$$

where $\bar{\nu}$ is the average number of mol of ligand bound per mol of protein, [A] is the equilibrium molarity of the unbound ligand, m is the number of classes of sites, N_j is the number of sites in class j, and k_j is the intrinsic constant for class j. This analysis was done by use of a computer program developed at the Division of Computer Research and Technology of the National Institutes of Health (Fletcher and Spector, 1968; Fletcher *et al.*, 1970). Subsequently, N_j and k_j values were used to find K_{ij} for each step of the reaction from eq 2 (Klotz, 1946), where K_{ij} is the association constant

$$K_{ij} = \frac{N_j - (i-1)}{i} k_j \qquad (2)$$

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