Effects of Photooxidation of Histidine-38 on the Various Catalytic Activities of Glyceraldehyde-3-phosphate Dehydrogenase†

Sharron H. Francis,‡ Blanche P. Meriwether, and Jane Harting Park*

ABSTRACT: Photooxidation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase in the presence of Rose Bengal specifically modified one histidine-38 residue per monomer and thereby inhibited the dehydrogenase, transferase, and esterase activities of this multifunctional enzyme. Glyceraldehyde-3-phosphate and NAD^+ both provided partial protection against inactivation suggesting that photooxidation may occur in the vicinity of the active center. The mechanism of the inhibition has been studied by examining the catalytic steps in the various reactions. Photooxidation did not effect (1) the initial rate of oxidation of glyceraldehyde-3-phosphate to form the *S*-acyl-enzyme intermediate, (2) the binding of the coenzyme NAD^+, or (3) the S → N transfer of an acetyl group from the active-site cysteine-149 to lysine-183. Photooxidation did impair (1) the formation of the catalytically active *S*-acetyl-enzyme intermediate with acetyl phosphate and (2) the deacetylation of the *S*-acyl-enzyme complex by phosphorolysis, arsenolysis, or hydrolysis. The data indicate that the photooxidation of histidine-38 does not effect the initial oxidation step of the dehydrogenase reaction but rather the second transferase step whereby the acyl group is removed from the enzyme. The effects of photooxidation are compared to the effects of NAD^+ and ATP on the active center.

Using acetaldehyde as a model substrate, we have shown that the dehydrogenase reaction proceeded by a two-step mechanism (Harting and Velick, 1954a). First, the aldehyde was oxidized in the presence of the enzyme (ESH) and NAD^+ to an *S*-acetyl-enzyme. In the second step the acetyl group was transferred to inorganic phosphate to yield acetyl phosphate. Arsenate could substitute for phosphate to give an unstable acetyl arsenate which was hydrolyzed to acetic acid (Scheme I).

In accordance with this mechanism we also demonstrated two transferase reactions which proceeded through the same acetyl-enzyme intermediate, namely, the ^{32}P exchange and arsenolysis reactions (Harting and Velick, 1954b) (Scheme II). The enzyme is designated as $ESH_{(NAD^+)}$ to indicate the requirements of the SH groups and NAD^+ for these reactions (Harting and Velick, 1954b). The same mechanism and reactions are encountered with the biological substrate, glyceraldehyde 3-phosphate.

As a further extension of these studies, the enzyme was also found to have an esterase activity in which *p*-nitrophenyl acetate was hydrolyzed by a two-step mechanism (Park *et al.*, 1961) involving the formation of the common *S*-acetyl-enzyme intermediate and the deacetylation to yield acetic acid (Scheme III). The simplicity of this reaction, which required neither NAD nor inorganic phosphate, made it a pivotal reaction for the study of the mechanism of enzyme action.

The participation of a specific cysteine residue in the catalysis is well documented and the sequence of the amino acids surrounding the active-site sulfhydryl group has been determined by Harris *et al.* (1963). However, the second step (deacetylation) has not been extensively studied. Since model *S*-acetyl compounds, such as *S*-acetylglutathione or *S*-acetyl-CoA, are too stable to account for the rate of deacetylation of the *S*-acetyl-enzyme complex, Olson and Park (1964) suggested that a histidine moiety might facilitate the hydrolytic step. This suggestion is substantiated by the similarity of the spectrum of the *N*-acetylimidazole to the catalytically active acetyl-enzyme complex (Harting and Chance, 1953), the pH dependence of the hydrolytic step (Olson and Park, 1964), solvent deuterium isotope effect (Lindquist and Cordes, 1968), fluorescence studies (Velick, 1958), kinetic experiments (Behme and Cordes, 1967), and the model reactions of histi-

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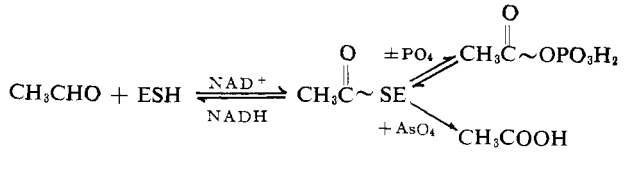
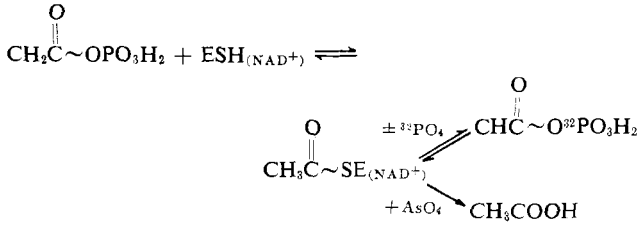
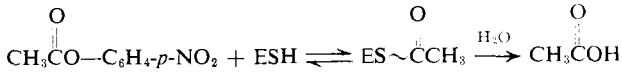


SCHEME II: Transferase Reaction.



SCHEME III: Esterase Reaction.



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dine with various thio esters (Jencks and Carriuolo, 1959; Bruice, 1959).

More recently, the involvement of the histidine moiety in the esterase reaction was experimentally demonstrated by the photooxidation of the enzyme in the presence of Rose Bengal (Bond *et al.*, 1970). Without any other modifications, one very sensitive histidine residue per monomer was specifically photooxidized in 2 min at 0°, and the tetradecapeptide containing the modified histidine had the following sequence: Val-Asp-Val-Val-Ala-Ile-Asn-Asp(Pro,Phe)Ile-Asp-Leu-*His*. Selective inhibition of the deacetylation of the *S*-acetyl-enzyme was achieved by this photooxidation procedure (Bond *et al.*, 1970). Neither the initial rate nor the extent of acetylation of the active-site sulfhydryl group was inhibited. Since this selective inhibition of the deacetylation step was to our knowledge unique among enzymes with esterolytic activity, the inhibitory effect of photooxidation was tested on the various other activities of the dehydrogenase. The enzymatic role of histidine-38 is discussed both from a conformational and mechanistic viewpoint. Since ATP and ADP can also selectively inhibit the deacetylation step (Francis *et al.*, 1970b), the effects of photooxidation and adenine nucleotides on the various activities of the enzyme are also compared.

Experimental Procedures

3-Phosphoglyceraldehyde dehydrogenase was crystallized from rabbit muscle by the method of Cori *et al.* (1948). The protein-bound NAD^+ was removed with charcoal (Taylor *et al.*, 1948) and the apoenzyme was dialyzed at least 2.5 hr against 5 mM Tris–1 mM Versene buffer, pH 6.8. This preparation was identical with that used in the photooxidation studies of the esterase activity of the enzyme (Bond *et al.*, 1970). The enzyme concentration was determined from the extinction coefficient at 280 mμ, and the molecular weight of the enzyme was taken as 140,000 (Fox and Dandliker, 1956).

DL-Glyceraldehyde-3-phosphoric acid diethyl acetal, barium salt was purchased from Calbiochem. Acetyl phosphate was prepared as described by Stadtman and Lipmann (1950) and *p*-nitrophenyl acetate as outlined by Balls and Wood (1956). NAD^+, ATP, and ADP were obtained from Sigma Chemical Co. and Rose Bengal from Eastman.

The photooxidation of the enzyme was performed as previously described (Bond *et al.*, 1970). The procedures for the dehydrogenase, arsenolysis, and esterase assays were carried out as detailed in Figures 1 and 2 and Table II. The methods for the measurements of acetyl phosphate and NAD^+ binding are given in the legends of Tables III and V, and Figure 4.

Results

Inhibitory Effect of Photooxidation on Dehydrogenase Activity and the Protective Action of NAD^+. The effects of photooxidation on the oxidation of 3-phosphoglyceraldehyde to 1,3-diphosphoglyceric acid are shown in Figure 1. Photooxidation of the dehydrogenase produced a 50% inhibition of the rate of the formation of NADH. The per cent inhibition ranged from 40 to 60%. Treatment with Rose Bengal in the dark had no effect (not shown). The enzyme, which was photooxidized in the presence of NAD^+ at a molar ratio of enzyme to NAD^+ of 1:100, was 27% inhibited (Figure 1). NAD^+ only protected the enzyme when added prior to the light treatment. NAD^+ is a competitive inhibitor with Rose Bengal, suggesting interaction at a common site (Bond *et al.*, 1970).

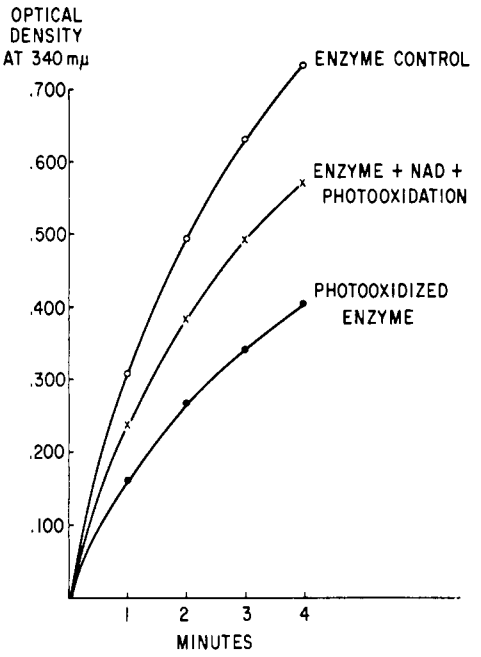


FIGURE 1: Protection by NAD^+ against the inhibitory effects of photooxidation on the dehydrogenase activity of the enzyme. One portion of dialyzed NAD^+-free enzyme, 0.025 μmol, was photooxidized for 2 min at 4° in the presence of Rose Bengal (RB:enzyme molar ratio = 0.5:1) as described under Experimental Procedure. A dark control was incubated with Rose Bengal in the dark (RB:enzyme molar ratio = 0.5:1). In another enzyme sample 2.5 μmol of NAD^+ was added prior to photooxidation. The reaction mixture for the dehydrogenase activity contained: 3-phosphoglyceraldehyde dehydrogenase, 2.5×10^{-5} μmol; sodium arsenate, 4 μmol; cysteine, 8 μmol; NAD^+, 1 μmol; 3-phosphoglyceraldehyde, 1.0 μmol; and pyrophosphate buffer, pH 8.6, 146 μmol; total volume, 3 ml; temperature, 25°. The reaction was initiated by the addition of substrate, and the rate of NADH formation was followed in a Beckman spectrophotometer at 340 mμ.

Since ADP competes with NAD^+ for a binding site (Francis *et al.*, 1970b; Constantinides and Deal, 1969; and Yang and Deal, 1969), its protective effect against photooxidation was also examined (Table I). The NAD^+-free enzyme was incubated with ADP at a molar ratio of enzyme to ADP to 1:300 and then photooxidized under the usual conditions. After dilution to an extent that ADP was no longer inhibitory in the dehydrogenase assay system, the enzymatic activity was tested. It can be seen that the adenine nucleotide did not protect the enzyme against photooxidation but NAD^+ afforded considerable protection. These data are compatible with previous findings which showed that NAD^+ and Rose Bengal were competitive inhibitors with high affinities for the enzyme (Bond *et al.*, 1970) whereas ADP, although competitive, was bound far more weakly than NAD^+ (Francis *et al.*, 1970b).

Effects of 3-Phosphoglyceraldehyde, p-Nitrophenyl Acetate, or Arsenate on the Photooxidation of the Dehydrogenase. As shown on lines 6 and 7 of Table I, preincubation with the natural substrate, 3-phosphoglyceraldehyde, provided partial protection against photooxidation. On the other hand, the model substrate, *p*-nitrophenyl acetate, did not protect the enzyme. This concentration of *p*-nitrophenyl acetate was sufficient to acetylate completely the active-site sulfhydryl groups. It should also be noted that *p*-nitrophenyl acetate did not protect the esterase activity against the inhibitory effect of photooxidation (Bond *et al.*, 1970).

Preincubation of the enzyme with arsenate at a molar ratio

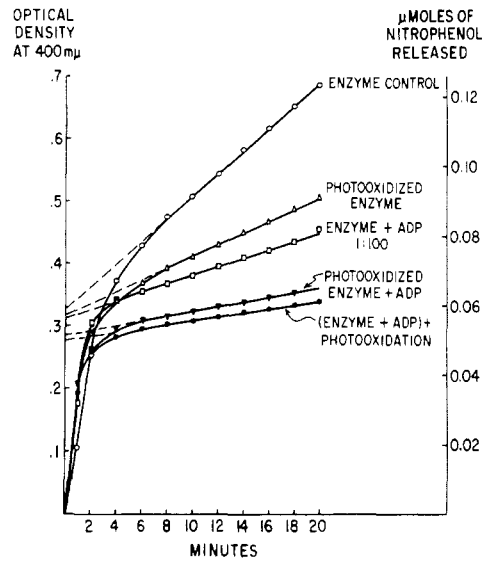


FIGURE 2: The effects of photooxidation and ADP on the esterase activity of 3-phosphoglyceraldehyde dehydrogenase. 3-Phosphoglyceraldehyde dehydrogenase was treated with charcoal and dialyzed as described in Figure 1. One portion of the NAD^+-free enzyme was photooxidized as described in Figure 1. In another sample, 0.02 μmol of this control enzyme was incubated with 2.0 μmol of ADP for 5 min at 0°. In the experiments shown in the two lower curves, ADP was incubated with the enzyme for 5 min prior to the photooxidation or added immediately thereafter. The reaction mixture for the esterase assay contained the following: 3-phosphoglyceraldehyde dehydrogenase, 0.02 μmol; *p*-nitrophenyl acetate, 1.2 μmol; sodium barbital buffer, pH 8.2, 96 μmol; and ADP adjusted to pH 7.0, 2.0 μmol as indicated in the figure. The molar ratio of enzyme to ADP was 1:100. The reaction was started by the addition of *p*-nitrophenyl acetate; total volume, 3 ml; temperature, 4°. The reaction was followed in a Beckman spectrophotometer measuring the increase in absorption at 400 mμ due to the liberated phenol. All curves were corrected for the nonspecific hydrolysis of *p*-nitrophenyl acetate as described in a previous publication (Park *et al.*, 1961).

TABLE 1: Effects of the Presence of ADP, Substrates, or Arsenate during the Photooxidation Procedure.[a]

Preliminary Treatment of Enzyme Prior to Dehydrogenase Assay	Ratio of Enzyme to Compd in the Preliminary Incubation	NADH Produced/ 4 min (μmol)	Inhibition (%)
None		0.12	
Photooxidation		0.06	50
+ ADP	1:300	0.12	0
+ ADP + photooxidation	1:300	0.06	50
+ NAD^+ + photooxidation	1:100	0.09	25
+ 3-phosphoglyceraldehyde	1:100	0.12	0
+ 3-phosphoglyceraldehyde + photooxidation	1:100	0.09	25
+ *p*-nitrophenyl acetate	1:40	0.12	0
+ *p*-nitrophenyl acetate + photooxidation	1:40	0.05	58
+ arsenate	1:400	0.11	10
+ arsenate + photooxidation	1:400	0.06	50
+ arsenate	1:1000	0.08	33
+ arsenate + photooxidation	1:1000	0.06	50

[a] The NAD^+-free enzyme was photooxidized as described in Figure 1. Another sample of the NAD^+-free enzyme was preincubated with ADP at a molar ratio of 1:300 for 10 min at 0° and then photooxidized as indicated in the table. All other compounds were added 2 min before photooxidation. The dehydrogenase assay was then performed as described in Figure 1. When 3-phosphoglyceraldehyde was preincubated with the enzyme, the reaction was started by the addition of NAD^+.

of 1:400 or 1:1000 did not afford protection against photooxidation. However, it is difficult to assess the effects of arsenate on the photooxidation of the enzyme since the enzyme control was inhibited by both concentrations of arsenate and the per cent inhibition progressively increased with time.

Comparison of the Effects of Photooxidation on the Esterase and Dehydrogenase Activities. For comparative purposes, the inhibitory effects of photooxidation on the esterase and dehydrogenase activities were measured simultaneously on the same preparation. The esterase assay is the simplest system for independently determining both the formation and breakdown of the *S*-acetyl-enzyme intermediate. For example, in Figure 2 the top curve shows an initial rapid release of *p*-nitrophenol due to the formation of the *S*-acetyl-enzyme complex. This initial burst is followed by a slower and linear liberation of *p*-nitrophenol caused by the breakdown and re-formation of the *S*-acetyl-enzyme. Extrapolation of the linear portion of the curve to zero time gives an intercept which is a measure of the moles of acetyl groups bound to the enzyme. This corresponded to 2.8 mol/mol of enzyme which is slightly lower than the molar ratio of acetyl groups to enzyme of 3.0–3.5 found with dehydrogenase preparations under more optimal conditions (Park *et al.*, 1961). Photooxidation of the same enzyme used in Figure 1 had no inhibitory effect on the rate or extent of acetylation of the reactive sulfhydryl groups by *p*-nitrophenyl acetate. However, the rate of deacetylation of the *S*-acetyl-enzyme was inhibited by 56%. This is essentially identical with the 50% inhibition of the overall dehydrogenase activity shown in Figure 1.

Since deacetylation of the *S*-acetyl-enzyme intermediate was also selectively inhibited by adenine nucleotides (Francis *et al.*, 1970a), the combined effect of the inhibitors was investigated (Figure 2). ADP was selected for these studies because it was the most potent inhibitor at 4°. In this experiment, photooxidation of the enzyme for 2 min in the presence of Rose Bengal inhibited the deacylation 47%, and ADP alone reduced the deacylation by 62%. Neither photooxidation nor ADP significantly decreased the extent or rate of initial acetylation of the enzyme (Bond *et al.*, 1970; Francis *et al.*, 1970a). Photooxidation in the presence of ADP or the addition of ADP to photooxidized enzyme effected a 78% inhibition of the deacylation. This inhibition was of a cumulative type similar to that observed for glutamine synthetase by Woolfolk and Stadtman (1964, 1967). However, the combination of the two inhibitory processes produced an 18% reduction in the extent of acetylation as shown by the intercepts of the two lower curves on the ordinate, and this impairment could account for the increase in the inhibition of the deacetylation. Since 78% inhibition was observed when the ADP was added after photooxidation, it is clear that the nucleotide did not effect the light reaction *per se*.

The effect of NAD^+ on the photooxidation cannot be appraised in this assay system because NAD^+ itself inhibits

TABLE II: Effect of Photooxidation on Binding of NAD^+ to 3-Phosphoglyceraldehyde Dehydrogenase.[a]

Conditions for Incubation	Photo-oxidation	Mol of NAD^+ Bound per Mol of Enzyme
Veronal, pH 8.0	0	2.8
	+	2.4
Veronal, pH 8.0 + cysteine + arsenate	0	2.8
	+	2.4

[a] The control or photooxidized enzyme (0.05 μmol) was added to the Veronal buffer (0.02 M, pH 8.0) to simulate the esterase assay conditions or Veronal buffer with cysteine (0.001 M) and arsenate (0.005 M) to resemble the arsenolysis assay conditions. 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 free NAD^+ was forced through the filter with 60 psi of nitrogen pressure. The enzyme was washed with 3-ml portions of Veronal buffer until 20 ml had been collected. The enzyme was then collected from the chamber and suitable samples of the eluate and enzyme solution were counted in a Packard scintillation counter to determine the enzyme-bound NAD^+.

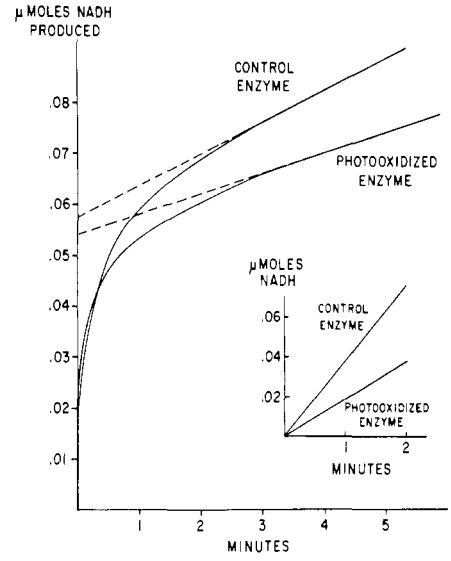


FIGURE 3: The effect of photooxidation on the isolated oxidation step of the dehydrogenase reaction. The control and photoxidized enzymes were prepared as outlined in Figure 1. The reaction mixture for the oxidation contained: glyceraldehyde-3-phosphate dehydrogenase, 0.02 μmole (2.8 mg); cysteine, 8 μmol; NAD^+, 8 μmol; sodium barbital buffer, pH 8.2, 96 μmol; temperature, 4°. The reaction was started by the addition of glyceraldehyde-3-phosphate, 0.6 μmol. Back extrapolation of the linear portion of the plot to zero time intersects the ordinate at the point where the number of micromoles of NADH equals the number of bound acyl groups. The insert in the lower right-hand corner is a measure of the overall dehydrogenase reaction in the presence of 4.0 μmol of arsenate. The reaction conditions were the same as above except that 3×10^{-5} μmol of enzyme was employed.

the acetylation of the enzyme with *p*-nitrophenyl acetate (Park *et al.*, 1961).

Studies on the Catalytic Step of the Dehydrogenase Reaction Inhibited by Photooxidation. In order to determine the mechanism of the inhibition of the dehydrogenase reaction, the effect of photooxidation was tested on (1) the oxidation of glyceraldehyde-3-phosphate to form the *S*-acetyl-enzyme intermediate, (2) the NAD^+ binding, and (3) the transferase step involving the arsenolysis of the acyl-enzyme intermediate. First, the rate of oxidation of the substrate (Figure 3) was determined in the absence of acceptors such as arsenate or phosphate and in the presence of a large amount of enzyme (2.8 mg). The maximum amount of glyceraldehyde 3-phosphate giving a suitable rate for measurement in the Gilford spectrophotometer at 4° was 0.6 μmol. During the first minute, the initial rapid rate of NADH formation represents the oxidation of substrate to form the *S*-acyl-enzyme intermediate and the slower linear phase shows the breakdown and re-formation of the active complex. At two substrate concentrations, 0.6 and 0.3 μmol, the rate of oxidation catalyzed by the photooxidized enzyme was equal to or slightly greater than that of the control enzyme for at least 20 sec. This time period represents the acylation of about 2.2 active sites. The back extrapolation of the linear portion of the curve to zero time gives an intercept which is a measure of the micromoles of acyl groups bound to the enzyme. The number of acyl groups bound per molecule of enzyme was 2.9. Photooxidation reduced the number of binding sites by about 5% to 10%. The small insert in the lower right-hand corner of the figure shows the effect of photooxidation on the overall dehydrogenase reaction using arsenate as an acceptor. Since this reaction under similar conditions of buffer and temperature was 50% inhibited by photooxidation, it can be inferred that photooxidation does not effect the initial oxidation step but does impair the deacylation of the enzyme.

The effect of photooxidation on NAD^+ binding was tested under conditions similar to those employed for assays of the esterase and arsenolysis activities. The data of Table II show that photooxidation reduced NAD^+ binding by only 10–15% under conditions used in these assays.

Experimentally, arsenolysis is the most convenient test of transferase activity of this enzyme (Harting and Velick, 1954b). As shown in Table III the extent of cleavage of the model substrate, acetyl phosphate, by the photooxidized enzyme was 40% inhibited. The percentage of inhibition of the arsenolysis activity varied from 35 to 45%. Over a tenfold range of arsenate concentration, the inhibition remained constant. The per cent inhibition also remained constant throughout the course of the assay. In order to determine the mechanism of the inhibition of this NAD^+-dependent transferase reaction, the effect of photooxidation on (1) the acetylation of the enzyme with [^{14}C]acetyl phosphate, and (2) the arsenolytic deacetylation of the *S*-acetyl-enzyme was next examined.

Inhibitory Effect of Photooxidation on the Acetylation of the Dehydrogenase with [^{14}C]Acetyl Phosphate. The effect of photooxidation on the acetylation of the dehydrogenase with [^{14}C]acetyl phosphate was measured under the conditions used for the arsenolysis assay, namely, pH 8.0 and room temperature. These are optimal conditions for acetylation since 4.0 mol of [^{14}C]acetyl groups are bound per mole of tetramer (Park *et al.*, 1970). Photooxidation produced a 40% reduction in the formation of the [^{14}C]acetyl-enzyme complex with only 2.5 mol of bound [^{14}C]acetyl groups. This effect could account for the inhibition (45%) of the arsenolysis reaction described in Table III. The distribution of the acetyl groups

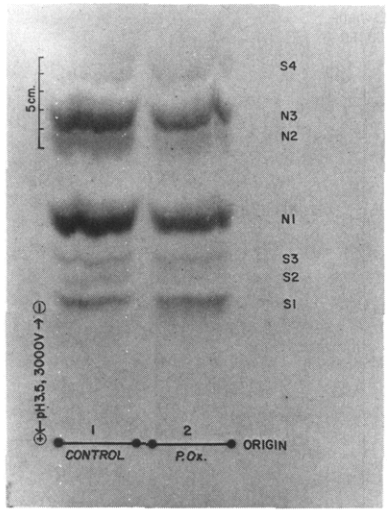


FIGURE 4: Radioautograph of [^{14}C]acetyl peptides from the control and photooxidized dehydrogenases acetylated with acetyl phosphate. The [^{14}C]acetyl-enzymes which were used for this radioautograph were selected from the experiments discussed under Results. The control and photoxidized enzymes (with 4.0 and 2.5 equiv of bound [^{14}C]acetyl groups, respectively) were digested with pepsin and lyophilized, and 2.0 mg of the sample was ionophoresed on Whatman No. 1 paper in pyridine–acetate buffer, pH 3.5 (Park *et al.*, 1970). On the radioautograph, the *S*-acetyl peptides and the *N*-acetyl peptides are labeled S1 to S4 and N1 and N3, respectively. Panels 1 and 2 represent the peptides from the control and photooxidized (P.Ox) enzymes.

between cysteine-149 and lysine-183 will be subsequently discussed in connection with Figure 4.

Inhibitory Effect of Photooxidation on the Arsenolytic Deacetylation of the S-Acetyl-Enzyme Intermediate. Photooxidation also inhibited directly the arsenolysis of the *S*-acetyl-enzyme complex as shown in Table IV. The control and photooxidized enzymes were acetylated briefly with [^{14}C]-*p*-nitrophenyl acetate at pH 7.0 and 0° to give *S*-acetyl-enzyme complexes. When arsenate and NAD^+ were subsequently added to decompose the enzyme–substrate complex, the arsenolysis of the photooxidized enzyme was significantly inhibited (50%). Only the arsenolysis of the preformed *S*-acetyl-enzyme complex was measured since the added NAD^+

TABLE III: Effect of Photooxidation on the Enzyme-Catalyzed Arsenolysis of Acetyl Phosphate.[a]

Arsenate Added (μmol)	Acetyl Phosphate Hydrolyzed: Control Enzyme (μmol)	Dark Control Enzyme (μmol)	Photo-oxidized Enzyme (μmol)	Inhibition of the Photo-oxidized Enzyme (%)
4.0	2.2	2.2	1.3	41
2.0	2.3		1.4	39
0.4	2.0		1.2	40

[a] The photooxidized and dark control enzymes were prepared as described in Figure 1 and Experimental Procedures. The reaction mixture for the arsenolysis assay contained the following: 3-phosphoglyceraldehyde dehydrogenase, 0.028 μmol; NAD^+, 1 μmol; cysteine, 1.5 μmol; sodium arsenate, 0.4, 2, or 4 μmol; and sodium barbital buffer, pH 8.0, 40 μmol. The reaction was initiated by the addition of 4 μmol of acetyl phosphate: total volume, 2.36 ml; temp, 25°. After 30 min the disappearance of acetyl phosphate was assayed by the method of Lipmann and Tuttle (1945).

TABLE IV: Inhibition of the Arsenolytic Deacetylation of the *S*-Acetyl-Enzyme by Photooxidation.[a]

Type of Enzyme Acetylated	Subsequent Additions	Mol of [^{14}C]Acetyl Groups Bound per Mol of Enzyme
Control	None	2.6
	Arsenate + NAD^+	0.6
Photooxidized	None	2.3
	Arsenate + NAD^+	1.5

[a] The NAD^+-free dehydrogenase was photooxidized as outlined earlier (Bond *et al.*, 1970). The control and photooxidized enzymes (0.07 μmol) were acetylated with *p*-nitrophenyl [^{14}C]acetate (2.4 μmol) for 5 min in Tris buffer, pH 7.0, at 0°. Arsenate (1.6 μmol) and NAD^+ (0.8 μmol) were then added as indicated above. The reaction was terminated after 7 min by the addition of a mixture of acetone–ether–1 N HCl (20:5:1). The moles of [^{14}C]acetyl groups bound per mole of enzyme were determined by the previously described method (Park *et al.*, 1961).

TABLE V: Effect of Photooxidation on *S*-Acetylation and *N*-Acetylation Reactions of Enzyme with [^{14}C]Acetyl Phosphate at pH 7.0 and 8.5.[a]

Experimental Conditions	Photo-oxidation	Mol of [^{14}C]-Acetyl Groups Bound per Mol of Enzyme
Tris buffer, pH 7.0	0	3.9
	+	3.0
Tris buffer, pH 8.5	0	4.4
	+	2.9
Tris buffer, pH 7.0 with subsequent adjustment to pH 8.5	0	3.0
	+	2.5

[a] The NAD^+-free control or photooxidized enzyme (0.07 μmol) was incubated with [^{14}C]acetyl phosphate (10 μmol) for 45 min at room temperature in a total volume of 1.7 ml. The enzymes in the first four experiments were incubated in 0.1 M Tris buffer adjusted to pH 7.0 or 8.5 as indicated in the table. For the experiments in the last two lines, the enzymes were incubated 45 min at pH 7.0. The pH was then adjusted to 8.5 with 0.1 N KOH, and the incubation continued for 10 min in order to facilitate the S → N transfer reaction. The procedure of determining the moles of bound [^{14}C]acetyl groups was described previously (Park *et al.*, 1961).

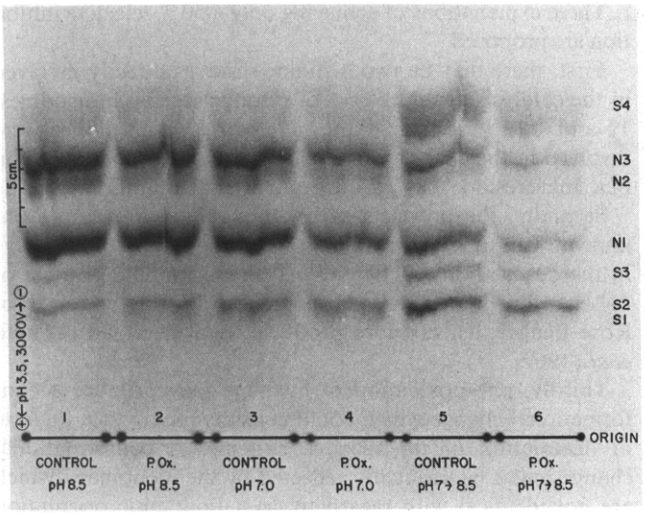


FIGURE 5: Radioautograph of the [^{14}C]acetyl peptides of the dehydrogenase illustrating the S → N transfer reaction with the control and photooxidized enzymes. The [^{14}C]acetyl-enzymes shown in this radioautograph were taken directly from the experiment of Table V. The peptides from the control and photooxidized (P.Ox) enzymes were incubated with acetyl phosphate at pH 7.0 or 8.5 as indicated on the figure. Panels 5 and 6 show the peptides from the enzymes which were acetylated at pH 7.0 for 45 min and then incubated at pH 8.5 for 10 min. This pH adjustment promotes the acetylation of the lysine moiety as noted in comparing panels 3 and 4 with panels 5 and 6.

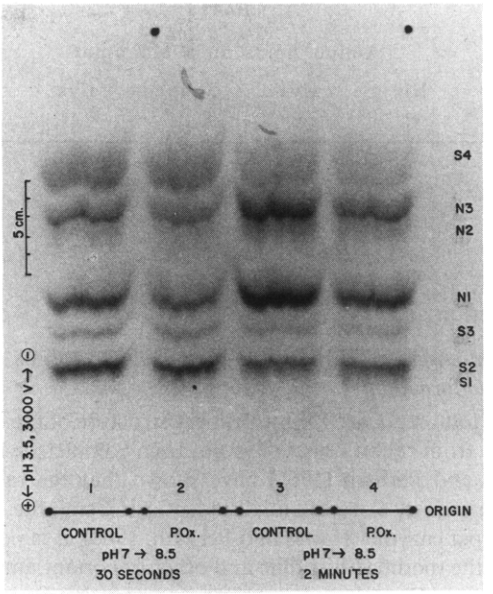


FIGURE 6: Radioautograph of the [^{14}C]acetyl peptides of the control and photooxidized dehydrogenases showing the time course of the S → N transfer reaction. The control and photooxidized enzymes were incubated with [^{14}C]acetyl phosphate for 20 min at pH 7.0 and 0°. The pH was then adjusted to 8.5 and the acetylation allowed to continue for 30 sec (panels 1 and 2) or 2 min (panels 3 and 4). The molar ratios of the acetyl groups to enzyme were 2.0, 1.5, 1.6, and 1.5 in panels 1–4, respectively.

prevents the reacetylation of the enzyme by *p*-nitrophenyl acetate (Park *et al.*, 1961).

Effect of Photooxidation on the S → N Transfer of the Acetyl Group from Cysteine to Lysine. Under the conditions of Tables I–III, namely, pH 8.0 and room temperature, acetyl phosphate acetylates the reactive cysteine-149 and then the acetyl group migrates to lysine-183 by an S → N transfer reaction (Mathew *et al.*, 1965; Mathew *et al.*, 1967). The distribution of the acetyl groups between the cysteine and lysine moieties can be ascertained by radioautography as shown in Figure 4. The control and photooxidized [^{14}C]acetyl-enzymes, which are described in the preceding paragraphs, were digested with pepsin and the resulting *S*-acetyl peptides (S1 to S4) or *N*-acetyl peptides (N1 to N3) were identified by subsequent electrophoresis and radioautography (Mathew *et al.*, 1965). In confirmation of the quantitative ^{14}C analyses of the acetyl-enzymes, the radioautograph of Figure 4 showed more radioactivity associated with the control (panel 1), which binds 4.0 equiv of [^{14}C]acetyl groups, than with the photooxidized enzyme (panel 2) binding only 2.5 [^{14}C]acetyl groups. However, the relative intensities of the various peptides from the control and photooxidized enzymes were about equivalent. The *N*-acetyl bands are sufficiently dark to suggest that photooxidation did not inhibit the S → N transfer reaction. In order to verify this suggestion, the acetylation reactions were studied in more detail under a variety of conditions (Table V, Figures 5 and 6).

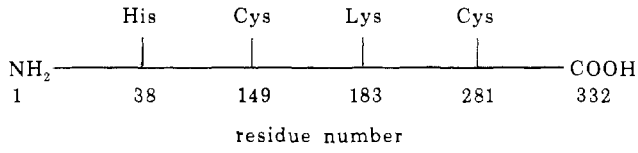
Control and photooxidized enzymes were acetylated in Tris buffer, pH 7.0 or 8.5 (Table V). Photooxidation reduced the amount of acetylation by about 25% at pH 7.0 and 8.5. This inhibition is less than the 40–45% in the acetylation of the enzymes for Figure 4 due to variations in buffers and pH. At pH 8.5 the control enzyme bound 4.4 mol of acetyl groups per mol of enzyme. This number is slightly greater than the theoretical value of 4.0 for a tetrameric enzyme. However, as previously shown, there is a small amount of dual labeling of both the reactive cysteine and lysine moieties (Park *et al.*, 1966; Park *et al.*, 1970).

When the acetyl-enzyme complexes were examined by electrophoresis and radioautography (Figure 5), the peptides obtained from the control enzyme labeled at pH 8.5 were predominately N-acetylated fragments (panel 1, bands N1, N2, N3). The photooxidized enzyme (panel 2) had the same peptide pattern indicating significant S → N transfer. The intensities of the bands were somewhat lighter thereby confirming the data on the impaired acetylation (Table V). In the case of the peptides acetylated at pH 7.0, there was an increase in the amount of the *S*-acetyl peptides (panel 3, band S1 to S4) and a decrease in the intensity of the *N*-acetyl peptides because the rate of the S → N transfer reaction is greatly reduced at neutral pH (Mathew *et al.*, 1965; Park *et al.*, 1970). Again, the photooxidized enzyme at pH 7.0 (panel 4) has essentially the same pattern as the control. When the enzyme was acetylated with acetyl phosphate at pH 7.0 with subsequent adjustment to 8.5 for 10 min, primarily *N*-acetyl peptides were obtained with both the control and photooxidized enzymes (panels 5 and 6).

In a separate experiment the time course of the N-acetylation reaction was followed at 30 sec and 2 min (Figure 6). The control and photooxidized enzymes were acetylated at 0° and pH 7.0 in order to prepare an *S*-acetyl-enzyme with minimal amounts of *N*-acetyl residues (Park *et al.*, 1970). The pH was then adjusted to 8.5 for 30 sec (panels 1 and 2) or 2 min (panels 3 and 4). During the longer incubation period the amount of N-acetylation increased as evidenced by the comparative darkening of bands N1, N2, and N3 of the control enzymes (panels 1 and 3). Photooxidation does not impede the S → N transfer of the acetyl group as shown by comparing panel 1 with 2 or panel 3 with 4.

CHART I

Amino Acids on a Monomer



Discussion

I. Relationship of Histidine-38 to Enzyme Catalysis and Conformation

The total sequence for the primary structure of the dehydrogenase from rabbit muscle has not been completed; however, Harris and Perham (1965) have shown that the rabbit and the pig sequence are essentially identical. Using the sequence of the pig enzyme (Harris and Perham, 1968), it is possible to locate the modified histidine and other important amino acids as shown in Chart I.

The functions of four important residues can be designated as follows: *histidine-38*, essential for maximum rate of deacetylation of the *S*-acetyl-enzyme, in the dehydrogenase, arsenolysis, and esterase activities (Bond *et al.*, 1970; Bond and Park, 1967; Halcomb *et al.*, 1968); *cysteine-149*, active-site sulfhydryl participating in the *S*-acetyl-enzyme formation for dehydrogenase, arsenolysis, and esterase activities (Harris *et al.*, 1963; Mathew *et al.*, 1967); *lysine-183*, acetylated in the S → N transfer reaction and involved in NAD^+ binding (Mathew *et al.*, 1965; Mathew *et al.*, 1967); *cysteine-281*, essential for maintaining the proper conformation of the enzyme (Park, 1966; Park *et al.*, 1967). Two of the critical amino acids, histidine-38 and cysteine-149, are separated by 111 residues but could readily be approximated in the folded chain. With the use of cross-linking reagents (Givol, 1969; Shaltiel and Tauber-Finkelstein, 1971), this has been shown to be true for the cysteine-149 and lysine-183 which are involved in the S → N transfer reaction (Mathew *et al.*, 1967; Park *et al.*, 1970). As yet no cross-linking reagent has been found for histidine-38 and cysteine-149; however, the partial protection of the photosensitive residue by both substrate and NAD^+ suggests that histidine-38 may be in the vicinity of the active center.

Explanation of the Partial Inhibition of the Photooxidized Enzyme. All four enzymatic activities, namely, the dehydrogenase, transferase, esterase, and phosphatase reactions have a common histidine requirement for maximal activity and are about 50% inhibited by photooxidation (Bond and Park, 1967). In this study photooxidation also produced approximately 50% reduction of the deacetylation step in the esterase and arsenolysis reactions (Figure 2 and Table IV). It should be noted that a deacetylation rate of 50% is still considerably above that expected for a typical thio ester such as *S*-acetylglutathione. The cysteine residue at the active site of the photooxidized enzyme is about 600 times more reactive than cysteine alone as measured by the acetylation with *p*-nitrophenyl acetate (Olson and Park, 1964). It is difficult to predict in a quantitative manner how much the rate of deacylation of this highly reactive cysteine would be inhibited by photooxidation of a nearby histidine. The reactivity of the *S*-acetyl-enzyme may not be decreased to that of a typical thio ester until the conformation of the native protein is completely disrupted as seen in the acetone precipitation of the *S*-acetyl-enzyme (Park *et al.*, 1961).

Three explanations of achieving only a 50% selective inhibition are proposed.

First, there may be two histidine residues directly involved in the catalysis as in the case of ribonuclease with histidines-12 and -119 (Crestfield *et al.*, 1963). If two histidines were involved in the deacylation reaction, then the photooxidation of a single residue may produce only partial inhibition.

Secondly, the products of photooxidation are as yet unknown. The modified histidine may itself be partially active in the deacylation reaction. When the α-amine derivative of *N*-benzoylhistidine was photooxidized in the presence of Rose Bengal, ten or more products were identified (Tomita *et al.*, 1969).

Thirdly, photooxidation of histidine may produce a conformational change which could considerably reduce the rate of deacylation of the reactive cysteine. A conformational change might be unequally reflected by the monomers, which are not identical with regard to crystallographic orientation (Watson and Banaszak, 1964), NAD^+ binding (Kirschner *et al.*, 1966; Conway and Koshland, 1968), or acetylation with model compounds such as β-(2-furyl)acryloyl (Malhotra and Bernhardt, 1968).

Lactic dehydrogenase from dog fish muscle has essential cysteine and histidine residues which have been identified by both chemical (Fondy *et al.*, 1965; Woenckhaus *et al.*, 1969) and crystallographic techniques (Rossman *et al.*, 1971). As with triose phosphate dehydrogenase, it was possible to obtain complete inhibition by stoichiometrically reacting the enzyme with *p*-hydroxymercuribenzoate but only partial inhibition by modification of the specific histidine residue. Nonetheless the histidine residue was deemed essential because not only the activity but also the NAD^+ sulfite binding capacity was reduced and ADP ribose or NAD^+ protected lactic dehydrogenase against alkylation of the histidine by 3-(2-bromo-1-[^{14}C]acetyl)pyridine. The crystallographic map at 2.5 Å confirms the presence of an active-center histidine residue which has a surrounding amino acid sequence similar to that of the alkylated histidine moiety (Rossman *et al.*, 1971). Thus there may be two categories of "essential" amino acid residues—one which is obligatory for catalysis and a second that merely facilitates activity by the enhancement of binding or proper conformations.

Role of Histidine-38 in the Enzymatic Reactions. Extrapolating from a study on model compounds and the esterase activity, Olson and Park (1964) have suggested an enzymatic mechanism facilitated by histidine residues. As one possibility they discussed an interaction between the sulfhydryl group and a histidine residue which would enhance the reactivity of the sulfhydryl group and promote the subsequent deacylation of the acetylated cysteine by a nucleophilic attack of the histidine moiety. Alternatively, the histidine could function in a general base catalysis. On the basis of deuterium exchange studies and kinetic data, Cordes and his coworkers (Lindquist and Cordes, 1968; Behme and Cordes, 1967) have presented strong evidence that histidine residues are involved in the esterase activity. We concur with these authors who cautiously state: "At the moment, there exists no definite basis on which to decide whether deacylation occurs with nucleophilic or general base catalysis, or by an alternate route, or a combination of these."

In examining the effect of pH on lactic dehydrogenase, Schwert *et al.* (1967) suggested that ionizable groups are participating in the catalytic reaction with pH values appropriate for a thiol, an imidazolium, and an ϵ-amino group. The authors proposed that the imidazolium is the "source and

sink" for the protein in the reaction. A cooperative catalysis promoted by a cysteine and histidine may be a rather general phenomenon as the proteolytic enzymes, papain and streptococcal proteinase, have also been found to have these two essential residues in the active center (Husain and Lowe, 1968; Liu, 1967).

It is also possible that the inhibition of the various activities of the photooxidized enzyme is a manifestation of a conformational change. If this were the case, it would nonetheless be interesting to have identified a specific histidine critically involved in the structural maintenance of that part of the active center required for four different types of reactions. Any conformational change must occur at the time of photooxidation since the inhibition did not progressively increase during the 20-min assay of the esterase activity. Moreover, the photooxidized enzymes could be assayed an hour after the light treatment, and the results were not effected. Progressive inhibition might be expected if major structural changes were continuing to occur in the photooxidized enzyme molecule. Further experimentation is in progress to determine the detailed mechanism of the inhibition by photooxidation.

Harris and Perham (1968) have compared the complete primary sequences of the dehydrogenase from pig and lobster. In connection with the present work, histidine-38, which appears to be an important residue in the mammalian enzyme, is replaced by a glutamic acid in the lobster protein. Superficially, this might tend to minimize the role of histidine-38. Indeed, the authors state ". . . only five histidines and two cysteines are common to both chains, indicating that at least six of the eleven histidines and two of the four cysteines in the pig sequence are not essential for maintaining the tertiary or quaternary structure of the enzyme molecule" (Harris and Perham, 1968). However, this prediction is not fully supported by the experimental data. The two cysteines, at positions 244 and 281, in the pig dehydrogenase are not found in the lobster enzyme which has cysteine residues in positions 22, 130, and 150. However, cysteine-281 is critical for the structural maintenance of the muscle enzyme since the acetylation of cysteine-281 causes rapid and complete precipitation of the protein. As a control, the yeast enzyme, which does not contain a cysteine at position 281, cannot be precipitated under these conditions of acetylation (Park, 1966; Park *et al.*, 1967). Therefore, it is clear that the data from the primary sequence cannot be extrapolated with certainty to the interpretation of the nature of the tertiary or quaternary structure. Analogous reasoning may also apply to the histidine residues. The great differences in the enzymological (Allison and Kaplan 1964), immunological (Allison and Kaplan, 1964), and crystallographic (Brand *et al.*, 1967) properties of the mammalian and arthropod enzymes must be reflected somewhere in the primary structure. This conclusion is in general agreement with the ideas which Nolan and Margoliash (1968) derived from the comparative studies on the evolutionary aspects of cytochrome *c*.

A similarity between the spectrum of the acetyl-enzyme complex of the dehydrogenase and *N*-acetylimidazole has been observed by Harting and Chance (1963) using the substrate analog, acetyl phosphate, and the native enzyme with bound NAD^+. The spectrum was observed between 220 and 260 mμ with a sensitive scanning spectrophotometer. Malhorta and Bernhardt (1968) have examined spectrophotometrically the complex formed with the enzyme and the model compound β-(2-furyl)acryloyl phosphate. The formation of a thio ester would produce a spectrum with a maximum at 336 mμ and a furylacryloyl imidazole at 340 mμ (Conway and Koshland, 1968). However, the authors were unable to determine the mode of binding since, as they noted, the spectrum of the acyl chromophore in the native FA-enzyme was evidently perturbed and the maximum shifted to 344 mμ. A stable complex precipitated by perchloric acid had the spectrum of a thio ester at 337 mμ. Thus it is difficult to draw a conclusion about the forms of the catalytically active enzyme–substrate intermediate on the basis of spectrophotometric data alone.

Although photooxidation has some obvious drawbacks as a procedure for modifying a specific histidine residue, it has the advantage of not introducing a bulky moiety in the sequence. The chemical changes incurred by photooxidation are probably less than by alkylation with a reagent such as the previously mentioned 3-(2-bromo-1-[^{14}C]acetyl)pyridine. However, it would be advantageous to supplement the photooxidation results by chemical identification of a functional histidine residue and such studies are in progress.

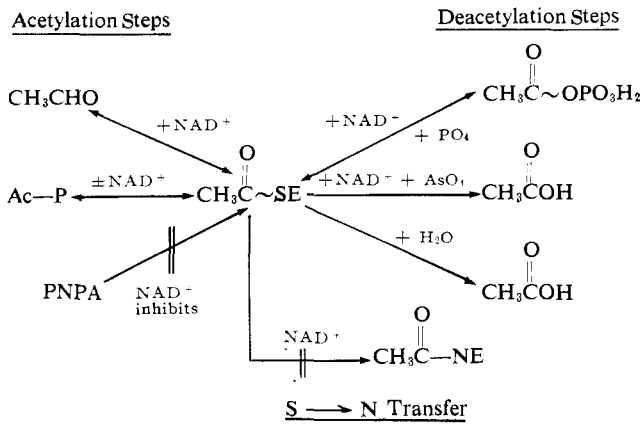
Substrate and NAD^+ Binding to the Enzyme. *p*-Nitrophenyl acetate did not protect the enzyme against photooxidation (Table I), and the acetylation of the enzyme with this substrate was not inhibited by photooxidation (Figure 2). On the other hand, 3-phosphoglyceraldehyde protected the enzymes against photooxidation (Table I) and the acetylation of the enzyme by acetyl phosphate was partially inhibited by photooxidation (Table IV). These findings suggest that the negatively charged, phosphorylated substrates may block the access of Rose Bengal or interact with the histidine moieties. Velick also deduced from his experiments on the fluorescence of the enzyme–NADH complex that there might be interactions between the charged phosphate moieties and histidine residues (Velick, 1958).

Since photooxidation did not significantly decrease the stoichiometry of the NAD^+ binding to the enzyme (Table III), histidine-38 does not appear to provide any direct site of attraction for the coenzyme nor is it critical in maintaining the conformation of the NAD^+ binding site. However, these experiments were not designed to detect subtle changes in the positioning of NAD^+. For example, the native and carboxymethylated dehydrogenase bind the same amount of NAD^+. However, by more sensitive techniques the carboxymethylated enzyme was shown to have lost the $E \cdot NAD^+$ spectrum between 320 and 400 mμ (Racker and Krimsky, 1952), and the bound NAD^+ was readily susceptible to deamination by takadiastase deaminase (Astrachan *et al.*, 1957). Further investigation would be required to relate the effects of photooxidation to the detailed properties of binding.

II. Interrelationships between NAD^+, Adenine Nucleotides, and Histidine-38

Structural Basis for the Differences in the Effects of NAD^+, Adenine Nucleotides, and Photooxidation of Histidine-38. The effects of NAD^+ are schematically presented in Scheme IV. For convenience, the reactions are shown taking place with the model compounds, acetaldehyde and acetyl phosphate. NAD^+ participates in almost every phase of catalysis as indicated in the diagram which shows NAD^+ as either absolutely required (+) or stimulatory (±). NAD^+ is known to facilitate substrate binding of aldehydes and acyl phosphates, oxidation of aldehydes to acyl-enzyme intermediates, formation of high-energy phosphates, and arsenolysis. As indicated by the double vertical lines, NAD^+ protects the enzyme by inhibiting the acetylation reaction with the nonphysiological substrates, such as *p*-nitrophenyl acetate (PNPA), and by inhibiting the S $\rightharpoonup$ N transfer. It is biologically advantageous, therefore, that this key enzyme of the glycolytic cycle has such a high

SCHEME IV

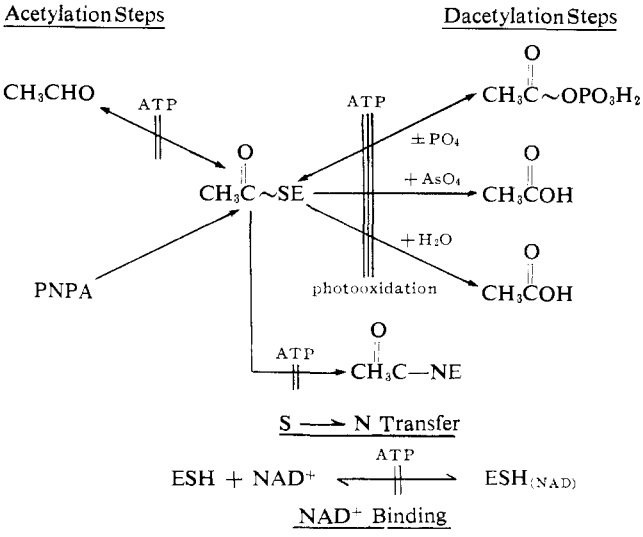


affinity for NAD^+ and actually exists as the enzyme–NAD^+ complex in the cell (Chance, 1954).

The second diagram (Scheme V) shows that the effects of ADP and ATP are generally opposite to those of NAD^+. As indicated by the vertical lines, the adenine nucleotides inhibit formation of the *S*-acyl-enzyme intermediate with glyceraldehyde 3-phosphate or acetyl phosphate, coenzyme binding, and three catalytic activities of the protein. These effects of ATP and NAD^+ can be correlated with their modes of bindings. The loosely bound ATP has several negative charges and could interact with the histidine moiety which is required for maximal catalytic activity. On the other hand, the firmly bound NAD^+, which has a rigid conformation and one negative charge, must be so positioned that it does not prevent substrate interactions with the functional residues. The common property of ATP and NAD^+, namely, the inhibition of the S ⟶ N transfer to lysine-183, may relate to a common binding site at this residue (Mathew *et al.*, 1967; Park *et al.*, 1970).

As indicated by the triple vertical lines in Scheme V, photooxidation of histidine-38 can inhibit all the catalytic activities by blocking the common deacetylation step. However, histidine-38 is not required in the S ⟶ N transfer to lysine-183 and is not implicated in NAD^+ binding. Thus the effects of photooxidation are confined to a more limited portion of the active center.

SCHEME V



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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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