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Mechanism of Alkylation of Rabbit Muscle Glyceraldehyde 3-Phosphate Dehydrogenase*

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ABSTRACT: The kinetics and stoichiometry of alkylation of the active-site thiols of glyceraldehyde 3-phosphate dehydrogenase (GPD) by iodoacetate (IAc) and iodoacetamide (IAM) were studied under a wide variety of conditions. The adherence to second-order kinetics (under most conditions), the maximum stoichiometry of 4 moles of inhibitor (IAc and IAM) per mole of enzyme tetramer, and the linear dependence of enzymic activity on the extent of alkylation, all strongly support a model for the enzyme in which all four thiols react equivalently and independently with the two inhibitors. Further supportive evidence for such a model is that enzyme species that are only partially alkylated undergo further alkylation at a rate similar to native, unmodified enzyme. These results are discussed with regard to the known functional heterogeneity of the active sites in the reaction with substrates and coenzyme (NAD^+). The kinetics deviate from second-order under some conditions: a hyperbolic dependence of the alkylation rate of NAD^+-bound GPD on the concentration of IAc indicates that IAc forms a reversible complex with GPD prior to the irreversible inactivation. On the other hand, the alkylation rates are linearly dependent on IAM concentration over the accessible concentration range. The alkylation rates are strongly dependent on the concentration of NAD^+. When the concentration of NAD^+ and GPD are comparable, the kinetics in the presence of a large excess of either IAc or IAM deviate from pseudo-first order. The rate of alkylation by IAc increases as the reaction proceeds whereas the rate of alkylation by IAM decreases as the reaction proceeds. These results are qualitatively consistent with the hypothesis that there is a redistribution of NAD^+ among alkylated and unalkylated subunits during the course of the inactivation process and that bound NAD^+ can be a positive or negative effector of reactivity dependent on the charge of the reactant. The pH–rate profile for the carboxymethylation of GPD suggests that the rate depends on the ionization of groups of pK_a = 5.5 and 5.2 in NAD^+-free and NAD^+-bound enzyme, respectively. The rate of inhibition by IAM depends on an acidic group of pK_a = 8.0–8.1 (although the rates are unexpectedly large at low pH). This dichotomy of pK_a's has also been found in studies of the pH dependence of enzyme acylation with neutral and anionic substrates. Evidence is presented that the active-site thiols do have an apparent pK_a = *ca.* 8.0, but that reaction with anionic inhibitor (IAc) and anionic substrate (FAP) is strongly influenced by enzymic equilibria other than the simple ionization of the essential thiols.

Recently, the properties of rabbit muscle GPD[1] have been studied extensively. The complex kinetics and the binding properties of NAD^+ have indicated that the active sites are functionally nonequivalent and perhaps not independent. NAD^+ binding studies on the rabbit muscle enzyme have shown the existence of two or more different NAD^+

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[1] Abbreviations used are: GPD, D-glyceraldehyde 3-phosphate dehydrogenase; IAc, iodoacetate; IAM, iodoacetamide; bicine, *N,N*-bis[2-hydroxyethyl]glycine; FAP, β-(2-furyl)acryloyl phosphate.

binding sites; the first molecule of NAD^+ bound reflecting the smallest dissociation constant (Fahien, 1966; De Vijlder and Slater, 1968; Conway and Koshland, 1968). Acyl-enzyme formation from substrates also demonstrates at least two types of sites (Malhotra and Bernhard, 1968; MacQuarrie and Bernhard, 1970), although the tetrameric enzyme is presumably composed of monomers of identical amino acid sequence (Harris and Perham, 1965; Harrington and Karr, 1965).

The reaction of substrates with the enzyme occurs *via* a thiol ester acyl-enzyme intermediate (Krimsky and Racker, 1955; Harris *et al.*, 1963). The sites on the enzyme which act as the acyl acceptors for substrates and which are alkylated by iodoacetate have been identified as belonging to a group of four reactive "essential thiols" (Harris *et al.*, 1963). Because of the reactive nature of the thiols, the enzyme is readily inactivated by SH reagents. Racker (1965) found that GPD was inactivated by *N*-ethylmaleimide, iodoacetamide, and that inactivation by iodoacetate was especially rapid in the presence of NAD^+. *p*-Chloromercuribenzoate is an effective inhibitor but it requires more than 4 moles of inhibitor to completely inactivate the enzyme (Szabolesi and Elodi, 1958; Wasserman *et al.*, 1969). Compounds which are known oxidants of SH groups are also often inhibitory. Among these reagents are tetrathionate (Pihl and Lange, 1962), *o*-iodosobenzoate (Rafter and Colowick, 1957; Parker and Allison, 1969), and iodine (Ehring and Colowick, 1964).

In addition to its role in the oxidation–reduction reaction with substrates, NAD^+ plays a part in regulating other kinetic and thermodynamic properties of the enzyme (see, *e.g.*, Park *et al.*, 1961, Listowsky *et al.*, 1965, and Malhotra and Bernhard, 1968). NAD^+ has also been implicated to interact with the sulfhydryl groups because of the effect of NAD^+ on the reactivity of the thiols and the effect of modifying the thiols on NAD^+ binding (Racker and Krimsky, 1952; Friedrich, 1965; Furfine and Velick, 1965; Trentham and Gutfreund, 1968).

In this communication, we report on the kinetic behavior of the essential thiols in their reaction with the irreversible inhibitors, iodoacetate and iodoacetamide. The effect of NAD^+ concentration and pH on the reaction rates was investigated. The rates of alkylation by iodoacetate and iodoacetamide, were found to respond in fundamentally different ways to changes in both the NAD^+ concentration and pH. These new results, when compared to previous findings on the effect of these same variables on substrate reactivity, are relevant to the catalytic mechanism.

Materials and Methods

Preparation of D-*Glyceraldehyde 3-Phosphate Dehydrogenase.* GPD was prepared from rabbit muscle by the method of Ferdinand (1964), with the modification that a blender and larger volumes of EDTA solution were used in the extraction (Bloch *et al.*, 1971). The native enzyme, as isolated, contains bound 3-phosphoglyceroyl groups and nucleotides (Bloch *et al.*, 1971). To remove the acyl groups when desired, the enzyme suspension was centrifuged and the precipitate was dissolved in 0.01 M ethylenediamine hydrochloride–0.1 M KCl–0.001 M EDTA (pH 7) at 0–5° containing 0.01 M sodium arsenate and 1×10^{-4} M NAD^+. The protein concentration was about 30 mg/ml. After standing for about 30 min, the enzyme was charcoal treated by either the method of Krimsky and Racker (1963) or De Vijlder and Slater (1968) to remove bound nucleotide, and then passed through a 1.2×25 cm Bio-Gel P-30 column equilibrated in 0.01 M ethylenediamine–0.1 M KCl–10^{-3} M EDTA (pH 7.0) at 0–5°. After charcoal treatment the A_{250}/A_{260} was generally about 1.85. All kinetic results were obtained within 6 hr of charcoal treatment and filtration through Bio-Gel.

Protein concentration was determined by using an extinction coefficient at 280 nm of 0.92 mg^{-1} ml cm^{-1} for native enzyme (Ferdinand, 1964) or the extinction coefficients of Fox and Dandliker (1956) for enzyme which had been charcoal treated. The molecular weight was taken as 144,000 (Jaenicke *et al.*, 1968; Hoagland and Teller, 1969).

Assays of Enzyme Activity. GPD activity was determined, using DL-glyceraldehyde 3-phosphate, NAD^+, and phosphate, by the method of Ferdinand (1964).

Methods of Measuring Alkylation Rates. The rates of reaction of GPD with iodoacetate and iodoacetamide were measured by three procedures. (a) The inactivation was followed by withdrawing aliquots of the reacting solution, diluting the mixture in 50 volumes of ice-cold ethylenediamine buffer (see above) to stop the reaction, and assaying the activity of the diluted enzyme within 3 min. The reactions were performed in test tubes maintained at 25 ± 0.1° in a water bath. (b) The alkylation was followed optically by measuring the decrease in absorption at 360 or 370 nm in the presence of NAD^+ or at 255 nm in the absence of NAD^+. The reactions were carried out in 0.8-ml quartz cuvets of 1-cm path length using a Cary Model 14 spectrophotometer thermostated at 25 ± 0.5°, or alternatively using a stopped-flow spectrophotometer (see below). (c) The incorporation of [^{14}C]alkyl groups was followed in the manner described in point a except 20- or 50-μl aliquots were removed and quenched in 3 ml of cold 5% trichloroacetic acid, and, after 30-min incubation in the cold, collecting the ^{14}C-labeled enzyme on 0.45 μ Millipore filters (HAWP 024). The filters were washed three times with 3 ml of 5% trichloroacetic acid and once with 3 ml of ethanol, dried, and counted in a Packard Tri-Carb scintillation spectrometer using 10 ml of scintillator/filter.

The quenching procedures were found to completely stop the alkylation reactions. The reliability of the reported rate constants is estimated to be ±10%.

The extent of reaction of the alkylating reagents with *N*-acetyl-L-cysteine and with dimethylcysteamine were followed, under conditions of excess thiol, by measuring the decrease in ultraviolet absorption at 270 nm due to the disappearance iodoacyl linkage (Finkle and Smith, 1958). The reactions were carried out at 25° using a Cary Model 14 spectrophotometer.

Where indicated, measurements of the rates of alkylation of NAD^+-bound enzyme were followed in a stopped-flow rapid-mixing spectrophotometer (Bernhard *et al.*, 1967) by the disappearance of the broad absorption band due to the E–NAD^+ complex (Krimsky and Racker, 1955). Enzyme in the presence of NAD^+ was mixed with iodoacetate or iodoacetamide at 25° and the increase in transparency at 370 nm was followed as a function of time. The reactions at high inhibitor concentration, especially at the high concentrations used with iodoacetamide, were less reproducible due to back-diffusion of reagents from the observation chamber into the reactant syringes. This was found to be a limiting factor in determining the concentration range that could be studied.

Preparation of Alkylated Enzyme from Acyl-Enzyme. Partially alkylated enzyme was prepared by adding excess iodoacetate to acyl-enzyme, isolating the alkylated acyl-enzyme, and removing the acyl groups. Native enzyme

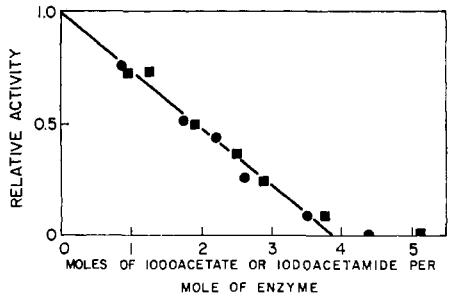

FIGURE 1: Inhibition of native enzyme (mol wt 1.4×10^5) with iodoacetate and iodoacetamide. Enzyme was incubated with various ratios of either iodoacetate (●) or iodoacetamide (■) to enzyme; enzyme = $1.1–1.7 \times 10^{-4}$ M at 25° in 0.01 M ethylenediamine–0.1 M KCl–0.001 M EDTA (pH 7). Activity is measured after completion of the reaction by the method of Ferdinand (1964).

TABLE I: The Apparent Second-Order Rate Constants (in M^{-1} sec^{-1}) for the Reaction of IAc and IAM with GPD at pH 7 and 25°.

	Apo-GPD		NAD^+-Bound GPD	
Method	IAc[a]	IAM[b]	IAc[c]	IAM[d]
1. ^{14}C incorporation	11.0	16.2	133	6.1
2. Enzyme activity	9.5	14.7	127	5.4
3. Optical absorption	11.3[e]	16.2[e]	136[f]	5.2[f]

[a] GPD, 15.2 μM; IAc, 0.41 mM. [b] GPD, 10.3 μM; IAM, 0.64 mM. [c] GPD, 15.2 μM; IAc ,0.109 mM; NAD^+, 0.17 mM. [d] GPD, 10.3 μM; IAM, 2.8 mM; NAD^+, 0.3 mM. [e] Rates monitored at 255 nm. [f] Rates monitored at 360 nm.

containing 1 mole of (3-phosphoglyceroyl) acyl group/mole of enzyme was charcoal treated as described above. The enzyme (9.3×10^{-5} M) was then incubated with 6.6×10^{-4} M [^{14}C]-IAc and after standing 30 min at room temperature, the solution was passed through a 1.2 × 25 cm Bio-Gel P30 column equilibrated in 0.01 M ethylenediamine–0.1 M KCl–10^{-3} M EDTA (pH 7.0) at 0–5°. The solution was then adjusted to pH 8.4 by the addition of 0.2 M $Na_4P_2O_7$ and after standing 15 min in the cold, the solution was dialyzed at 5° for 4 hr to remove the hydrolyzed acyl groups and the pyrophosphate. The resultant alkylated enzyme was free of covalently bound acyl groups and contained 3 moles of ^{14}C/mole of enzyme.

The kinetics of alkylation of acyl-enzyme were performed in the absence of NAD^+ in a solution containing enzyme, 20 μM, and IAc, 544 μM. In the presence of 110 μM NAD^+, the enzyme was 16 μM and [^{14}C]IAc was 121 μM. Partially alkylated enzyme was prepared as described above and the alkylation kinetics were performed as follows: in the absence of NAD^+, solutions contained enzyme, 4.4 μM, and [^{14}C]IAc, 663 μM. In the presence of NAD^+, solutions contained enzyme, 13.3 μM; IAc, 132 μM, and NAD^+, 120 μM. In the absence of NAD^+, all alkylation rates were measured by ^{14}C incorporation, and in the presence of excess NAD^+, rates were measured using absorption changes in the "Racker" band (see above).

Measurement of Acylation Rates. The rates of acylation of GPD by a nexcess of the pseudosubstrate FAP (Malhotra and Bernhard, 1968) was measured at 25° in solutions of nezyme (8 μM) and NAD^+ (310 μM) at various concentrations of FAP. At higher FAP concentrations (>2–3 mM) the pseudo-first-order rate became progressively less dependent on [FAP]. A maximal (saturating) acylation rate constant was determinable from the adherance of the data to a linear double-reciprocal plot of FAP concentration *vs.* pseudo-first-order rate constant. The buffers used were sodium acetate (0.1 ionic strength) and 0.05 M bicine–0.1 M HCl.

Reagents. Iodoacetic acid (Matheson Coleman & Bell) was recrystallized twice from carbon tetrachloride and dried before use. Solutions of iodoacetic acid were neutralized with KOH before use. Iodoacetamide (Calbiochem) was recrystallized twice from water and dried. FAP was prepared as described previously (Malhotra and Bernhard, 1968). Other reagents, as follows, were commercial products used without further purification. NAD^+ and the barium salt of DL-glyceraldehyde 3-phosphate diethyl acetal were purchased from Boehringer und Soehne; EDTA from Matheson Coleman & Bell; 2-dimethylaminoethanethiol (dimethylcysteamine) hydrochloride from Aldrich Chem. Co.; *N*-acetyl-L-cysteine and bicine from Sigma Chemical Co. [^{14}C]Iodoacetate and [^{14}C]iodoacetamide were obtained from Amersham-Searle Corp., diluted with 5 equiv of nonradioactive reagent in water and stored at −17°.

Results

Stoichiometry of Alkylation. The stoichiometry of the alkylation reactions of enzyme with iodoacetate and iodoacetamide at pH 7 is detailed in Figure 1. For these inhibitors there is a linear relationship between the moles of added inhibitor and the extent of enzyme inactivation. A total of 4 moles of inhibitor is required to completely inactivate the tetrameric enzyme.

Time Course of Alkylation at pH 7. Three principal procedures were used to follow the reactions of the inhibitors with the enzyme: changes in enzyme activity, changes of optical absorption, and the incorporation of radioactivity into the enzyme (see Materials and Methods section). The absorption change at 255 nm should reflect the disappearance of the iodoacetate, which at this wavelength shows an extinction change upon reactions with thiols (Finkle and Smith, 1958) as well as any optical change due to a difference spectrum between unmodified and carboxymethylated protein. The optical change at 360 nm is due to a loss of the Racker band, a broad, low intensity absorption band characteristic of the native E–NAD^+ complex (Racker and Krimsky, 1952) and presumably dependent on the availability of free (unmodified) essential thiol.

The time course for enzymic inactivation is consistent with a second-order kinetic process (eq 1) (first order in enzyme

$$E + I \xrightarrow{k} \text{products}$$

$$v = k[E][I] \qquad (1)$$

and first order in inhibitor). Table I shows the calculated second-order kinetic constants for alkylation with iodoacetate and iodoacetamide in the absence of NAD^+, and in the presence of an excess of NAD^+. The adherence to second-order kinetics is consistent with an equivalent reactivity for all four thiol sites. No other sites of alkylation are detectable under these conditions although the molecule contains 12 other thiols as well as other potential alkylation sites (histidine,

TABLE II: Carboxymethylation Rates of Acylated and Partially Alkylated Enzymes.

Enzyme	Carboxymethylation Rate Constant,[a] sec^{-1} M^{-1}	
	Absence of NAD^+	Presence of NAD^+
E	11	136
Acyl-E	8	115
Alkyl-E	10	160

[a] Alkylation rates were determined in solutions of 0.01 M ethylenediamine–0.1 M KCl–0.001 EDTA (pH 7.0) at 25° as described in the Materials and Method section. The apparent second-order rate constants were calculated from the pseudo-first-order rate constants and the iodoacetate concentration.

TABLE III: The Apparent Second-Order Rate Constants for the Reaction of GPD with IAc and IAM at pH 7 and 25° in 0.01 M Ethylenediamine Hydrochloride–0.1 M KCl–mM EDTA.

GPD Concn (μM)	Apo-GPD, k_2, sec^{-1} M^{-1}		NAD^+-Bound GPD, k_2, sec^{-1} M^{-1}	
	IAc[a]	IAM[b]	IAc[c]	IAM[d]
28.4			128	
25.7	11.4			
21.3				5.4
20.4		15.5		
10.3	13.0	16.2	136	5.2
6.2			147	
4.1		16.6		
3.6				7.2
2.1	11.0			

[a] IAc, 0.55 mM. [b] IAM, 0.64 mM. [c] IAc, 0.25 mM: NAD^+, 0.3 mM. [d] IAM, 2.8 mM: NAD^+, 0.3 mM.

tyrosine, methionine, lysine, etc.). This specificity for four thiols per enzyme tetramer appears to hold for iodoacetamide as well as iodoacetate: the carboxamidomethylation and carboxymethylation rate constants obtained by any one of the three methods of following the reaction agrees with any other to within experimental error (Table I).

The radioactivity incorporated into the enzyme was used to calculate the number of moles of alkyl group reacted per mole of enzyme. It was found that under the conditions stated in the legend of Table I, 3.9–4.0 moles of carboxymethyl groups is incorporated per mole of enzyme after approximately 10 half-lives of reaction, either in the total absence of or in the presence of excess NAD^+. Similar results were obtained for the incorporation of carboxamidomethyl groups.

Alkylation Rates of Acylated and Partially Alkylated Enzymes. An alternative explanation for an "apparent" equal reactivity among all four thiols is that the alkylation reaction is so highly cooperative that the rate-limiting reaction at the first thiol group within a tetramer is followed by extremely rapid subsequent alkylation at the other sites. Such a mechanism would also predict second-order kinetics over the complete time course. Assuming this model for a highly cooperative alkylation reaction, a solution of partially alkylated enzyme would consist predominantly of unalkylated and tetraalkylated enzyme species (eq 2). On the basis of

$$E + RX \xrightarrow{\text{slow}} RE \xrightarrow{\text{fast}} R_2E \xrightarrow{\text{fast}} R_3E \xrightarrow{\text{fast}} R_4E \qquad (2)$$

such a cooperative mechanism, the alkylation rate for a partially alkylated enzyme (*e.g.*, RE, R_2E) would be expected to be much greater than the rates with native enzyme. The following experiment was designed to test this prediction.

Partially acylated and partially alkylated enzymes were prepared (see Materials and Methods section) as schematized in eq 3 where Ac is an acyl group and R is an alkyl group.

$$E + \text{excess Ac-P} \longrightarrow Ac_2E \xrightarrow{\text{excess RX}} Ac_2ER_2 \xrightarrow{\text{arsenate}} ER_2 \qquad (3)$$

Previous work has been shown that only two of the four thiols are readily acylated (Malhotra and Bernhard, 1968; MacQuarrie and Bernhard, 1970, 1971) and hence, that "diacyl-enzyme" molecules all contain two acyl groups per tetramer. Thus, in the absence of intermolecular subunit rearrangements, the alkylated enzyme (RE) prepared from acyl-enzyme (AcE) is a mixture of enzyme species all containing at least two alkyl groups per molecule.

The carboxymethylation rates of the free thiols within the acyl- (AcE) and alkyl- (RE) modified tetramer were measured for both apo- and NAD^+-bound GPD. The alkylation rates are summarized in Table II. The rate constants found for both acyl- and alkyl-E are similar to those found for unmodified enzyme under similar conditions of NAD^+ concentration. The similarity of the alkylation rates indicates that there is no substantial alteration in the nucleophilic character of the active thiols when other active sites are modified by either acylation or alkylation, and adds further support to the idea that the four specific thiols undergo alkylation independently of one another. In the reaction of IAc with acyl-enzyme, the alkylation rates indicate that the origin of the stoichiometric limitation (two acyl groups per tetramer) does not reside solely in a decreased nucleophilicity of the active-site thiols.

The alkylation stoichiometry was calculated from the ^{14}C incorporation into the modified enzymes. Acyl-enzyme, containing about 1 mole of 3-phosphoglyceroyl group/mole of enzyme, was found to incorporate 3 moles of ^{14}C into the enzyme upon alkylation. Partially alkylated enzyme, prepared from acyl-enzyme (eq 3), contained about three [^{14}C]carboxymethyl groups per enzyme and possessed 22% of native enzyme activity. Further alkylation resulted in complete inactivation and a total incorporation of nearly four [^{14}C]carboxymethyl groups per enzyme. The rates of alkylation of the modified enzymes (Table II) were unchanged (within experimental error) when the original acyl-enzyme contained two acyl groups per mole of enzyme.

Concentration Dependencies of Alkylation Rates. The apparent second-order rate constants for alkylation of GPD by IAc and IAM at various concentrations of reagents are shown in Tables III and IV. The rate constants are independent of enzyme concentration under the stated conditions. The observed rate constants for alkylation of NAD^+-bound

TABLE IV: The Apparent Second-Order Rate Constants for the Reaction of GPD[a] with Iodoacetate and Iodoacetamide in the Absence of NAD^+ at pH 7 and 25° in 0.01 M Ethylenediamine Hydrochloride–0.1 M KCl–0.001 M EDTA.

Iodoacetate Concn (mM)	Iodoacetate, k_{obsd}, sec^{-1} M^{-1}	Iodoacetamide Concn (mM)	Iodoacetamide, k_{obsd}, sec^{-1} M^{-1}
1.10	11.7	1.28	16.4
0.55	13.0	0.642	16.2
0.24	12.6	0.29	18.5
0.10	12.4		

[a] GPD, 0.0103 mM.

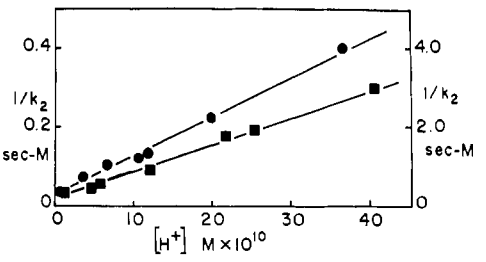

FIGURE 3: pH dependence of the rate of alkylation of *N*-acetyl-L-cysteine by iodoacetate and iodoacetamide. k_2 is the apparent second-order rate constant for the reaction of IAc (■) and IAM (●) with *N*-acetyl-L-cysteine in 0.05 M bicine–0.1 M KCl at 25°. Iodoacetate concentration is 0.23 mM; iodoacetamide, 0.22 mM; *N*-acetyl-L-cysteine varied from 2 to 4 mM. Left ordinate refers to reaction with IAM; right ordinate refers to reaction with IAc.

enzyme were unaffected by increasing the NAD^+ concentration above 0.3 mM. Alkylation of apo-GPD by either IAc or IAM is a second-order rate process (eq 1) within the limited range of concentrations that could be studied.

The reaction of the alkylating reagents with NAD^+-bound enzyme could be studied over a wider range of inhibitor concentration by observing changes in the Racker band using stopped-flow techniques. Figure 2 shows a plot of the pseudo-first-order rate constants *vs.* the inhibitor concentration. With iodoacetamide there is a linear dependence of rate on concentration. On the other hand, with iodoacetate the rate appears to saturate, characteristic of a reversible binding process prior to alkylation (eq 4). In such a mech-

$$E + ICH_2CO_2^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E(ICH_2CO_2^-) \xrightarrow{k_2} E\text{-}CH_2CO_2^- + HI \quad (4)$$

anism, the dependence of rate on iodoacetate concentration is formally analogous to the simple Michaelis–Menten expression (Meloche, 1967)

$$\frac{V_{inact}}{v_{inact}} = 1 + \frac{K_{inact}}{[I]} \quad (5)$$

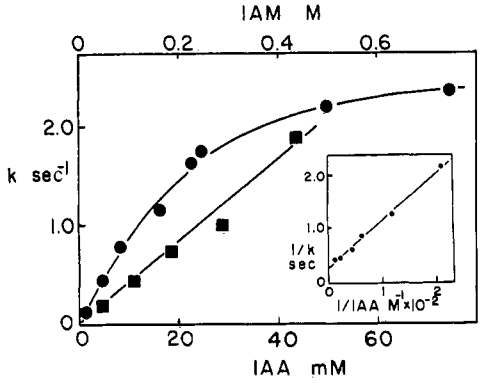

FIGURE 2: Alkylation rate as a function of iodoacetate or iodoacetamide concentration. The pseudo-first-order rate constant is plotted as a function of iodoacetate (●) or iodoacetamide (■) concentration. The rates were measured in a stopped-flow spectrophotometer by mixing enzyme (31 μM) and NAD^+ (350 μM) with alkyl halide. Other conditions are as described in Figure 1. The reaction is followed by the change in absorption at 360 nm. The insert is a double-reciprocal plot of rate constant *vs.* IAc concentration.

where [I] is inhibitor concentration, V_{inact} is inactivation velocity when [I] is infinite, v_{inact} is inactivation velocity at finite [I] and K_{inact} is $(k_{-1} + k_2)/k_1$ (eq 4) and represents the concentration of [I] giving half-maximum inactivation rate. Substituting rate constants for velocities (Meloche, 1967), eq 5 becomes $k_2/k_0 = 1 + K_{inact}/[I]$, where k_0 is the observed first-order rate constant. A plot of $1/k_0$ *vs.* 1/[I] is shown in the insert of Figure 2. The straight line provides evidence that iodoacetate and enzyme do form a dissociable complex prior to irreversible inactivation. From the slope and intercept of the plot, the kinetic constants K_{inact} = 25 mM and k_2 = 3.6 sec^{-1} were calculated.

Alkylation of Model Thiols. Alkylation of native enzyme by both iodoacetate and iodoacetamide was compared to the rate of alkylation of the model compounds *N*-Ace-L-Cys and Me_2CysNH_2. Under the conditions specified (pH 7 and 25°), NAD^+-free or NAD^+-bound enzyme was 20–5000 times more reactive than either of the model compounds. The rates of reaction of *N*-Ac-L-Cys with IAc and IAM were about 0.02 and 0.13 M^{-1} sec^{-1}, respectively. With Me_2CysNH_2 the corresponding rates were 0.57 and 1.2 M^{-1} sec^{-1}. The largest difference in reactivity is observed in the carboxymethylation of the model compounds *vs.* the E-NAD^+ complex. This difference could reflect a rate enhancement due to the binding of iodoacetate to the enzyme (eq 4), a lower pK_a for the thiols in the enzyme than in the model compounds, or a greater nucleophilicity of the enzyme thiols. These possibilities were investigated by studying the pH dependence of the alkylation rates of the enzyme and the model compounds.

Haloacetates and haloacetamides react with thiols by the mechanism of eq 6 in which the predominantly reactive

$$RSH \overset{K_A}{\rightleftharpoons} RS^- + H^+$$
$$RS^- + R'X \xrightarrow{k} RSR' + X^- \quad (6)$$

thiol species is the anion (Lindley, 1960). When the ionization of the thiol is rapid compared to k, the observed alkylation rate is given by eq 7, which predicts a linear dependence

$$k_{obsd} = \frac{K_A k}{K_A + [H^+]} \quad (7)$$

of $1/k_{obsd}$ on (H^+). From such a plot (Figure 3) the values of k and pK_a were calculated for the reactions of *N*-Ac-L-Cys with iodoacetate and iodoacetamide as summarized in

TABLE V: Reaction of *N*-Acetyl-L-cysteine with Iodoacetate and Iodoacetamide at 25° in 0.05 M Bicine and 0.1 M KCl.

	Iodoacetate	Iodoacetamide
k^a, sec^{-1} M^{-1}	4.35	33.0
pK_a	9.46	9.52

[a] The rate constants and pK_a's are calculated from the data shown in Figure 3.

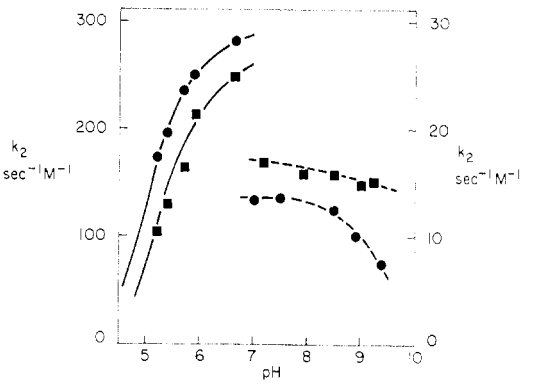

FIGURE 4: Carboxymethylation rate of GPD as a function of pH. In the absence of NAD^+, enzyme was 20 μM and IAc was 380–560 μM. The solid line is calculated assuming a p*K* of 5.5. In the presence of 300 μM NAD^+, enzyme was 10–22 μM and IAc was 280–380 μM. The solid line is calculated assuming a p*K* of 5.2. The reactions were followed by the change in optical absorption (see Materials and Methods section) at 25° in either sodium acetate buffer (ionic strength 0.1) or 0.05 M imidazole hydrochloride in the pH range 5–7 or in 0.05 M bicine–0.1 M KCl in the pH range 7–10. The left ordinate refers to the reaction in the presence of NAD^+ (●); the right ordinate refers to reaction in the absence of NAD^+ (■).

Table V. Dimethylcysteamine was found to have a similar reactivity (but a lower apparent pK_a).

pH Effects on the Alkylation Rates of GPD. Boross and Cseke (1967) showed that the reaction of iodoacetate with the NAD^+-bound enzyme depends on a dissociable group of $pK_a = 5.4$ which they assigned to the active thiols. This reaction was reinvestigated in both the presence and the absence of NAD^+ (Figure 4). In the presence of NAD^+, the rate data indicate an approximate pK_a of 5.2 while the apoenzyme exhibits an apparent pK_a of 5.5. Enzyme instability at lower pH prevented extending the data to below pH 5 and hence prevented a more accurate determination of the pK_a values. Since iodoacetate reacts with NAD^+-bound GPD *via* a dissociable enzyme–inhibitor complex, the apparent pK_a for the reaction may relate to the dissociation of a proton from the enzyme–iodoacetate complex or to dissociation of the complex itself rather than dissociation of a proton from the free enzyme. Proton dissociation from the free enzyme should be reflected in the pH–rate profile for iodoacetamide inhibition since this inhibitor apparently does not form a reversible complex with the enzyme. The results with iodoacetamide are shown in Figure 5A,B. In the absence of NAD^+, the pH–rate data can be satisfactorily described by dependence on a single ionizing group of $pK = 8.0$. In the presence of NAD^+, although there is still evidence that alkylation rate is dependent on a pK_a of about 8, the rate at low pH is much greater than that expected on the basis of a *required* protonic dissociation with $pK_a \sim 8$. Note that at low pH where other potential alkylation sites might compete, the rates of carboxamidomethylation are still pseudo-first order in the presence of excess IAM and that the stoichiometry, as measured by reaction with [^{14}C]IAM, is still approximately 4 moles/mole of tetramer.

Specificity of Alkylation with Iodoacetamide at pH 9.75. The fact that the enzymic reaction with iodoacetamide exhibits an apparent pK_a value different from that observed in the reaction with IAc could result from the reaction of IAM at high pH with a functional group other than the active thiol. To check the specificity under these conditions, the enzyme was incubated with various amounts of [^{14}C]IAM at pH 9.75; after standing, the reaction was stopped and the enzyme activity and the amount of enzyme-bound ^{14}C was determined. In addition, the enzyme was incubated with various amounts of nonradioactive IAM and after stopping the reaction and determining the enzyme activity, the partially inhibited enzyme was reacted with [^{14}C]IAc at pH 7 to determine the number of remaining essential thiols. Both of these results are illustrated in Figure 6. Just as in the case of iodoacetate or iodoacetamide at neutral pH (Figure 1), 4 moles of inhibitor is required to inactivate the enzyme tetramer. Furthermore, the loss of activity is associated with a loss of reactivity toward IAc and since IAc is specific for reaction with the active thiols (Harris *et al.*, 1963), the IAM is reacting predominantly with the four essential thiols. However, it was observed that more than four moles of reagent could be incorporated into the protein by incubation with excess [^{14}C]IAM for long periods of time at pH 9.75.

Effect of pH on the Rate of Acylation of GPD. The pH dependence of the reaction of enzyme with substrate was determined by measuring the rate of acylation of NAD^+-bound GPD by the pseudosubstrate β-(2-furyl)acryloyl phosphate (FAP). Figure 7 shows a plot of the (extrapolated) maximum first-order rate constant for acylation as a function of pH. The results show that for this dianionic substrate ($pK_a \sim 4.8$) there is only a small dependence of rate on pH in the pH range 6–9, just as is observed for reaction of the enzyme with the negatively charged iodoacetate (Figure 4).

Effect of NAD^+ on the Alkylation Kinetics. Previous work has shown that NAD^+ accelerates the reaction of enzyme with iodoacetate and inhibits alkylation with iodoacetamide (Racker, 1965; Trentham and Gutfreund, 1968; MacQuarrie

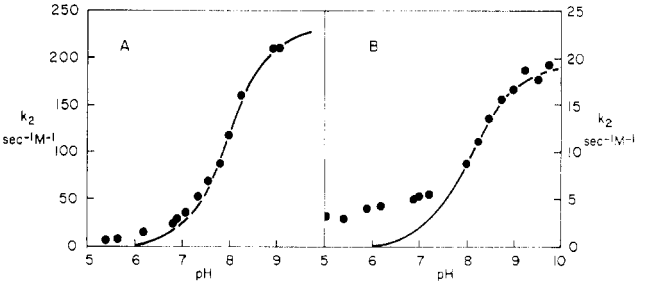

FIGURE 5: Carboxamidomethylation rate of GPD as a function of pH. (A) The reaction mixtures contained apo enzyme (20 μM) and IAM (0.28–2.4 mM). The reactions were followed at 255 nm. The curve is calculated assuming a pK_a of 8.0. (B) The reaction mixtures contained enzyme (15 μM), NAD^+ (0.5 mM), and IAM (1–2.3 mM). The reactions were followed at 370 nm. The buffers used are listed in the legend of Figure 4. The curve is calculated assuming a pK_a of 8.1.

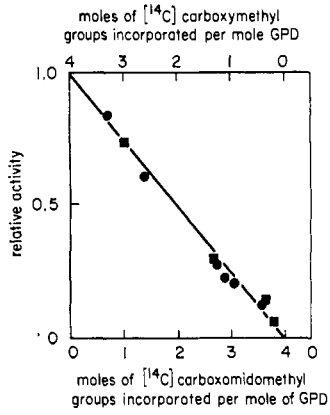

FIGURE 6: Stoichiometry of iodoacetamide inhibition at high pH. The experiment was performed in two parts: (a) enzyme (1.3×10^{-4} M) was incubated with various amounts of [^{14}C]IAM (ranging from 0 to 20 moles per mole of enzyme) in 0.05 M bicine–0.1 M KCl (pH 9.75). After standing 5 min at 25°, aliquots are removed, diluted, and assayed for enzymic activity and enzyme-bound radioactivity as described in the Materials and Methods section. The results are plotted as activity *vs.* the moles of [^{14}C]carboxamidomethyl groups per mole of enzyme (●). (b) Enzyme (9.5×10^{-5} M) was incubated with various amounts of IAM (ranging from 0 to 7 moles per mole of enzyme) as in part a. After standing 30 min at 25°, aliquots are diluted and assayed for enzyme activity as above and other aliquots are diluted 25 times into 0.01 M ethylenediamine–0.1 M KCl–0.001 M EDTA (pH 7) containing 8.3×10^{-5} M NAD^+ and 1.2×10^{-4} M [^{14}C]IAc. After reaction for 15 min at 25°, the solutions are assayed for enzyme-bound radioactivity. The results are plotted as activity *vs.* moles of [^{14}C]carboxymethyl groups per mole of enzyme (■).

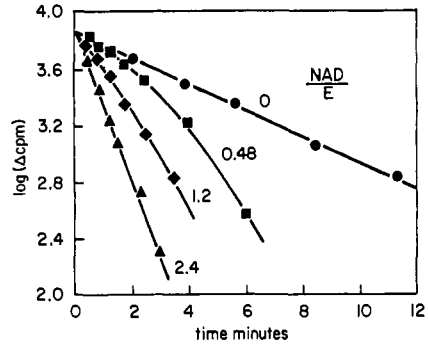

FIGURE 8: Carboxymethylation time course as a function of NAD^+ concentration. Enzyme (7.48 μM) was incubated with [^{14}C]IAc (2.31×10^{-4} M) and various ratios of NAD^+ (moles per mole of enzyme) as indicated. Enzyme-bound radioactivity was used to monitor the extent of the reaction. Other conditions are as described in Figure 1.

and Bernhard, 1970). The effect of varying the concentration of NAD^+ on the carboxymethylation rate was examined. In the presence of less than stoichiometric amounts of NAD^+, *i.e.*, when NAD^+ was less than four times the tetramer concentration, the kinetics were found to deviate from pseudo-first order under conditions of excess IAc. The apparent rate increased as the extent of the reaction increased. The results, using incorporation of [^{14}C]carboxymethyl groups to measure the extent of reaction, are shown in Figure 8. Changes in the Racker band were also found to follow complex kinetics (Figure 9) under conditions of low NAD^+ concentration. It should be noted that as the total optical density change increases, that is, as the Racker band extinction increases and the enzyme becomes saturated with NAD^+, the kinetics show less deviation from pseudo-first order until finally at high NAD^+ concentrations the kinetic processes are accurately described as pseudo-first order. Under conditions of less than stoichiometric NAD^+, the Racker band extinction is not necessarily an accurate reflection of the extent of the reaction.

Complex time courses were also observed for reaction of IAM with GPD when NAD^+ was approximately stoichiometric with the enzyme. The results are shown in Figure 10. In this case the apparent rate decreased with increasing extent of reaction.

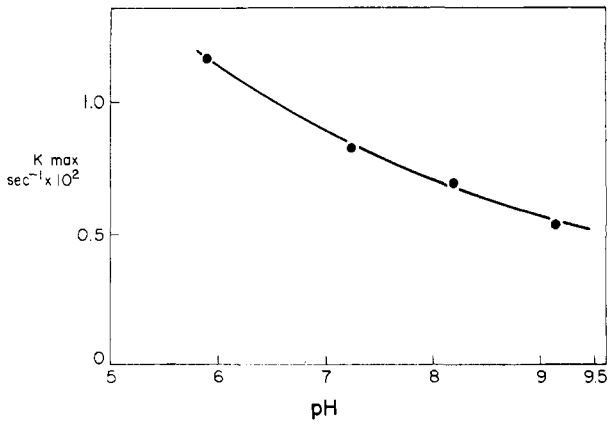

FIGURE 7: pH dependence of the acylation rate of GPD. The maximum first-order rate constant for acylation of GPD by FAP is plotted *vs.* pH as described in the Materials and Methods section.

Discussion

The reaction of either excess iodoacetate or iodoacetamide with GPD is pseudo-first order over more than 85% of the reaction in either the absence, or in the presence of a high concentration, of NAD^+. At completion of reaction, 4 moles of inhibitor is incorporated per enzyme tetramer. There is a linear correlation between the extent of carboxymethylation, the specific enzyme activity, and the intensity of the absorption band of the E–NAD^+ complex. These results are in agreement with previous findings (Racker, 1965; Trentham and Gutfreund, 1968; MacQuarrie and Bernhard, 1970; Fenselau and Weigel, 1970) and taken together with the extent of the reaction, demonstrate the specificity of alkylation. These results strongly support a model in which all *four* sites of the enzyme react independently and with equal rates toward the irreversible inhibitors, iodoacetate and iodoacetamide. On the other hand, reaction of substrates with the enzyme has shown that the four enzyme sites are unequal in reactivity and affinity. Heterogeneity of the active sites of the rabbit muscle enzyme has been observed in the binding of NAD^+ (De Vijlder and Slater, 1968; Conway and Koshland, 1968; Bloch, 1970), in acylation with acyl phosphates (Malhotra and Bernhard, 1968; MacQuarrie and Bernhard, 1970, 1971), and in acylation with glyceraldehyde 3-phosphate plus NAD^+ (Krimsky and Racker, 1963; Smith, 1966).

An alternative explanation to the equal reactivity of all four thiols is that there is a high degree of cooperativity in the alkylation reaction. In a cooperative mechanism, the rate-limiting step would be reaction with the first thiol in the tetramer. A solution of partially alkylated enzyme would

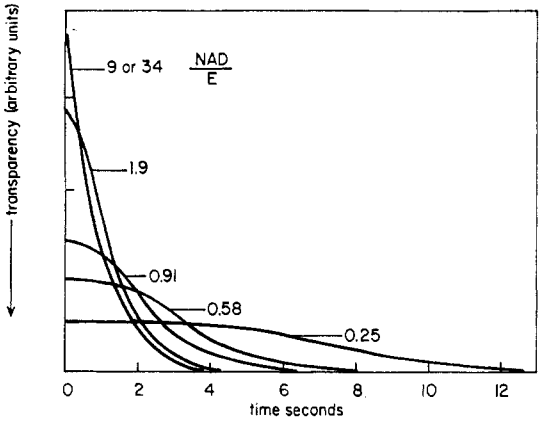

FIGURE 9: Carboxymethylation time course as a function of NAD^+ concentration as measured by changes in the Racker band. Enzyme (14.8 μM) was incubated with various ratios of NAD^+ as indicated and mixed with IAc (9.9 mM) in a stopped-flow spectrophotometer. The reaction is followed at 370 nm. Under these conditions, transparency is essentially proportioned to absorbance. Other conditions are as described in Figure 1.

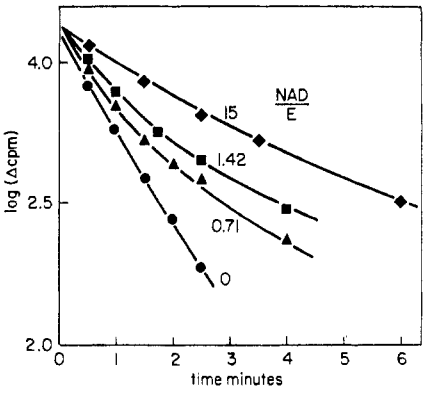

FIGURE 10: Carboxamidomethylation time course as a function of NAD^+ concentration. Enzyme (9.70 μM) was incubated with various ratios of NAD^+ (moles per mole of enzyme) as indicated and allowed to react with [^{14}C]IAM (9.2×10^{-4} M). Enzyme-bound radioactivity is used to monitor the extent of the reaction. Other conditions are as described in Figure 1.

then contain predominantly a mixture of tetraalkylated and unalkylated enzyme species. Such cooperativity is very unlikely since acylated enzyme and partially alkylated enzyme derived from acyl-enzyme undergo carboxymethylation at rates similar to those of unmodified enzyme.

It is not surprising that iodoacetate, and not iodoacetamide, reversibly complexes with the enzyme in view of the strong affinity of the enzyme for anions (Velick *et al.*, 1953) and the fact that the specific substrates are highly negatively charged. Similar reversible complex formation has been observed between iodoacetate and human carbonic anhydrase (Bradbury, 1969) which also strongly binds anions (Kernohan, 1965).

The formation of a GPD–IAc complex could account for the rate acceleration caused by NAD^+. The positive charge on the nicotinamide portion of the coenzyme might provide additional positive charge in the active site which could more strongly attract iodoacetate anion. This view is strongly supported by the finding that NADH, in which the reduced nicotinamide ring no longer has a positive charge, provides little rate acceleration (Trentham and Gutfreund, 1968). By facilitating the approach to the thiol, the noncovalent binding of IAc could also provide an explanation for the large apparent second-order rate constants for reaction with NAD^+-bound enzyme. Iodoacetamide, which shows no evidence of extensive binding to GPD, shows a smaller difference in rate of alkylation of GPD as compared with the model thiols.

Boross and Cseke (1967) found that the rate of alkylation of NAD^+-bound GPD depended on the state of dissociation of an acidic group of $pK_a = 5.4$. This finding is in agreement with the pK_a of 5.2 $\pm$ 0.2 obtained in this study, in which the results were extended to include the pH dependence in the absence of NAD^+. The pH profile under these latter conditions can be described by an ionizing group with a $pK_a = 5.5 \pm 0.2$. The similarity of the pK's in the presence and absence of NAD^+ indicates that NAD^+ acts principally to facilitate IAc binding or to increase the intrinsic reactivity of the active thiols and not to significantly decrease the pK_a as suggested (Trentham and Gutfreund, 1968).

The rate of alkylation of NAD^+-free enzyme by iodoacetamide depends predominantly on an ionizing group of $pK_a = 8.0$, although the rate is slightly larger than expected at low pH. To test whether the rate of reaction of the NAD^+-free enzyme with IAM was reflecting the reactivity of the active thiols, the specificity of the reaction was examined at high pH. Just as at pH 7, 4 moles of inhibitor is required to inactivate the enzyme at pH 9.75, and the decrease in activity is proportional to the loss of active thiols as measured by reactivity toward IAc. For the NAD^+-bound enzyme, the pH dependence of the rate of alkylation by IAM at high pH can be accounted for by an ionizing group with a pK_a of 8.1. However, in the pH range 5–8, the deviation from the expected rate behavior is very marked with the observed rate constants being larger than anticipated for a single group ionizing independently of other groups on the enzyme. Clearly, in this case, more than one ionizing group is affecting the enzyme reactivity. Such a result could arise from electrostatic effects on the pK of the ionizing group (Tanford, 1962) or by a change in the protein structure. A similar unexpectedly high reactivity was found for the reaction of deoxyhemoglobin with iodoacetamide at low pH with the results being interpreted as due to a dissociation of the original tetramer to dimers in which the reactivity of the thiol groups is much increased (Guidotti, 1967). Because of the known dissociative properties of GPD (Hoagland and Teller, 1969; Fahien, 1966; Constantinides and Deal, 1968), such an explanation was considered but is ruled out by the absence of any significant effect of protein concentration on the alkylation rates.

An alternative hypothesis for the high rate of reaction at low pH is that IAM reacts with other groups in addition to the essential thiolate anions of the holo enzyme. Reaction with the protonated thiol, or interaction with a different group or groups, could account for the results. However, the large rate constant for the reaction between pH 5 and 6 (about 5 M^{-1} sec^{-1}) as well as the pseudo-first-order kinetics and the stoichiometry of four reactive groups per mole of enzyme, leads us to believe that this unexpectedly high reactivity at low pH is due to a structural and/or electrostatic change which affects the thiol reactivity of the enzyme molecule. Indeed, previous work (Bolotina *et al.*, 1967; Elodi *et al.*, 1960) suggests that below pH 7 the holo enzyme undergoes a structural transition.

The question then arises as to what proton-dissociable

groups (if any) can the observed pK_a values be assigned. Boross and Cseke (1967) assigned the pK of 5.4 to the ionization of the essential thiols. However, since inactivation by iodoacetate proceeds *via* formation of an enzyme–IAc complex, the pH dependence of the rates of alkylation may depend on proton dissociation from the enzyme–IAc complex (eq 8), dissociation of the complex itself (eq 9), or other equilibria preceding the rate-limiting step. The pK's of 5.2–5.5 are much too high to be accounted for by the ionization of iodoacetic acid alone.

$$E\cdot(IAc) + H^+ \rightleftharpoons H^+E\cdot(IAc) \quad (8)$$

$$E\cdot(IAc) + H^+ \rightleftharpoons H^+E + IAc \quad (9)$$

It was anticipated that the pH dependence of the rate of inactivation by iodoacetamide would not be influenced by the binding equilibria associated with iodoacetate reactivity since there is a linear dependence of alkylation rate on iodoacetamide concentration.

Therefore, it seems likely that the rate of reaction of IAM with GPD provides an accurate reflection of the state of ionization of the active thiols in the native enzyme. It appears that in apo-GPD as well as in NAD^+-bound GPD, the essential thiols have an apparent pK_a of 8.0–8.1, but that their reactivity, especially at low pH, depends on ionization of another group or groups. This assignment of pK_a values is supported by the work of Behme and Cordes (1967) who also assigned a pK_a of 8.1 to the active thiols on the basis of the rate of enzyme acylation with *p*-nitrophenyl acetate.

From the dependencies of the alkylation reactions alone it is clear that iodoacetate and iodoacetamide react with GPD by different mechanisms; a conclusion arrived at independently of considerations of the effect of inhibitor concentration on alkylation rate. That the difference in mechanism is due exclusively to the substantial noncovalent binding which occurs only with iodoacetate, is insufficient to explain the difference in pH–rate profiles for the two reactions. The same pH dependencies are observable in reaction with substrates. The rate of acetylation with *p*-nitrophenyl acetate shows dependence on an ionizable group of pK_a = 8.1 whereas furylacryloylation with FAP lacks this p$K_a \sim 8$ dependence. Several explanations are possible for these findings. First, and most obvious, is that the anionic reagents, IAc and FAP, react with different nucleophilic groups than do the neutral reagents, IAM and *p*-nitrophenyl acetate. This possibility seems remote in view of the structural studies (Harris *et al.*, 1963) on Ac-GPD (preparable from both nitrophenyl acetate and acetyl phosphate) and CM-GPD and the specificity discussed above and elsewhere (MacQuarrie and Bernhard, 1970). A second possibility is that the reaction of the anionic reagents does not depend on ionization of the active thiol of GPD, *e.g.*, iodoacetate and FAP may react equally well with both the SH and S^- forms of the enzyme and the apparent pK of about 5 may be due to dissociation of some other group or groups. Alternatively the apparent pK of the thiol group may be influenced by other equilibria associated with the reaction of the anionic substrates with the enzyme (Bruice and Schmir, 1959). For example, the equilibrium constant for an isomerization (eq 10) of the enzyme–anion complex would be included

$$\begin{array}{ccc} EAH & \overset{K_A}{\rightleftharpoons} & EA^- + H^+ \\ \Updownarrow & & \Updownarrow \\ E^*AH & \underset{K_A^*}{\rightleftharpoons} & E^*A^- + H^+ \end{array} \quad (10)$$

in the apparent pK_a for the reaction provided the isomerization occurred prior to the rate-limiting step. The effect of such an isomerization could be (if $K_A^* > K_A$) to lower the apparent pK_a for the overall reaction. Evidence for similar enzyme isomerizations (eq 10) have been found in studies of NAD^+ binding to yeast GPD (Kirschner *et al.*, 1966) and in both stopped-flow (Bloch, 1970) and temperature-jump studies (P. Lilliford and G. G. Hammes, 1970, personal communication) of NAD^+ binding to rabbit muscle GPD. The isomerization could provide a means for the negatively charged substrate (FAP) and inhibitor (IAc) to react at a rapid rate at pH values below the intrinsic pK_a of the thiol.

Thus the existence of an enzyme isomerization although an *ad hoc* assumption, nevertheless seems a reasonable possibility. In view of the above results, the need for caution in the interpretation of pH–rate data and especially in the assignments of pK values to specific enzyme groups, should be strongly emphasized.

A detailed analysis of the pH dependence of the inhibition of papain, an enzyme containing a single, uniquely reactive thiol, by chloroacetate and chloroacetamide led to several findings (Chaiken and Smith, 1969a,b) which are strikingly analogous to those found in this study. In the study of papain and for similar findings with streptococcal proteinase (Gerwin, 1967), the results have been interpreted (Chaiken and Smith, 1969a,b; Wallenfels and Eisele, 1968) by assuming that at low pH other groups on the enzyme affect the reactivity of the sulfhydryl group, possibly by electrostatic effects, and that there is a rate enhancement with haloacetates because another group or groups facilitate approach of the acetate to the thiol. Such an explanation for alkylation of GPD is entirely consistent with the present results but is, of course, insufficient to explain the ligand and charge-induced differences in pH dependence.

Since NAD^+ has such a large effect on the rate of reaction with iodoacetate, it was hoped that a study of the NAD^+ concentration dependence of the rate of carboxymethylation and carboxamidomethylation would provide an excellent system for determining the number and type of NAD^+ binding sites responsible for the changes in reactivity. This is of particular importance in view of the stoichiometric limitations observed for reaction with substrates (Malhotra and Bernhard, 1968; MacQuarrie and Bernhard, 1970) and the varied number and types of dissociation constants derived from studies of the binding of oxidized coenzyme (Furfine and Velick, 1965; Conway and Koshland, 1968; De Vijlder and Slater, 1968; Bloch, 1970). However, the complex time course of GPD alkylation observed in the presence of small amounts of NAD^+ precluded a quantitative analysis of the binding of NAD^+ to the native enzyme on the basis of the alkylation rate data alone. We qualitatively interpret the complex time course, with the rate increasing with time for carboxymethylation and decreasing with time for carboxamidomethylation, as due to a lowering in affinity of GPD for tightly bound NAD^+ upon alkylation (Trentham and Gutfreund, 1968; Friedrich, 1965). Under the conditions [E] $\gtrsim$ [NAD^+] nearly all the NAD^+ is bound to the native enzyme. Since alkylation displaces some of the coenzyme from sites which have reacted, the fraction of unreacted sites containing bound NAD^+ will increase with increasing extent of alkylation and hence will be time dependent. Since the rate of alkylation depends on the presence or absence of bound NAD^+, it follows that the time course is likely to be complex. Similar kinetic complexities may be

expected, and in fact have been observed (Malhotra and Bernhard, 1968; R. A. MacQuarrie and S. A. Bernhard, unpublished results) for other NAD^+-dependent reactions (for example, the transient acylation and deacylation of the enzyme) when NAD^+ and enzyme are present at comparable concentrations. Conway and Koshland (1968) found a complex dependence of the initial transient velocity of NAD^+ reduction by glyceraldehyde on NAD^+ concentration. They interpret the results as indicative of at least three different NAD^+ binding sites on native enzyme. However, in the absence of phosphate or arsenate, a significant build-up of acyl-enzyme would be expected. Since acylation, much like alkylation, alters the affinity for NAD^+ (Furfine and Velick, 1965; Malhotra and Bernhard, 1968), such complex results are expected, and cannot be used exclusively to calculate NAD^+ stoichiometry or affinities for native enzyme. Conway and Koshland (1968) also interpreted the NAD^+ effect on the rate of reaction of iodoacetate with enzyme in terms of a model in which the first (or four) molecules of NAD^+ bound causes a conformational change of *all* of the subunits of the enzyme. Their conclusions are dependent on the assumption that the reaction is true second order (eq 1) over the complete time course, since they measured the extent of the reaction as a function of NAD^+ after an arbitrary time period. Such a procedure could tend to make the apparent NAD^+ stoichiometry smaller than it actually is. Indeed, our results indicate clearly that more than 1 mole of NAD^+/mole of enzyme is required to produce the maximum effect on the rate and that possibly all 4 moles of bound coenzyme may be required.

No subunit–subunit interactions are evident in this alkylation study other than the above-mentioned redistribution of NAD^+ during the course of alkylation. It is apparent that reactions of the enzyme which involve substrate binding or other highly specific catalyzed reactions exhibit heterogeneity among sites, while those strictly "chemical" reactions, which occur with comparable facility in model compounds, may have essentially identical behavior with all four sites. This is not surprising since the enzyme-catalyzed reactions must involve a high degree of cooperation among the functioning units of the active site and thus are extremely sensitive to the precise alignment of these units. Such subtle changes may not be detected in the two-center reactions of a single amino acid side chain with a modifying reagent.

Acknowledgment

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Role of Metals in the Class II Aldolases. Spectral Studies of Cobalt Yeast Aldolase*

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ABSTRACT: The binding of 2 moles of cobalt(II) to each mole of yeast apoaldolase produces an active enzyme, exhibiting an absorption spectrum with maxima at 535 (155), 485 (125), 1290 (18), and 1640 mμ (18 M^{-1} cm^{-1}). The visible absorption bands are optically active, with circular dichroic bands of molecular ellipticities −2800 and −2500 (deg cm^2) per dmole, respectively. The addition of saturating concentrations of the substrate, fructose 1,6-diphosphate, results both in hypso- and hypochromic shifts of the main absorption band in the visible region. In contrast, neither K^+, an activator, nor phosphate ion, an inhibitor, affect these spectral properties of the cobalt enzyme. The optical asymmetry of the active-site cobalt atoms seems closely related to enzymatic activity, as is demonstrated by its correlation to the changes of the circular dichroic spectrum on titration to pH values of less than 8.

The present studies are discussed in the context of the proposal that the metal atom at the active site of yeast aldolase serves as an electrophile, aiding in bond polarization to facilitate the aldolytic cleavage of the substrate.

Cobalt(II) can substitute for zinc at the active site of fructose 1,6-diphosphate aldolase of *Saccharomyces cerevisiae* to form an active metalloenzyme (Kobes *et al.*, 1969b). Cobalt complexes, including cobalt enzymes, exhibit spectral properties which may reflect the environment of the metal atom. The state of cobalt at the active site of a metalloenzyme can be examined both in the presence and absence of inhibitors, activators, coenzymes and, under appropriate circumstances, in the enzyme–substrate complex. The information thus gained can contribute significantly to the elucidation of the role of the metal in the catalytic mechanism and/or substrate binding of the enzyme. Such mechanistic studies of yeast aldolase are of particular interest, since it has served as the prototype for the class II aldolases isolated from microbial sources. These aldolases are apparently metalloenzymes, usually containing zinc. They accomplish the same interconversion of substrates as the class I aldolases isolated from mammals and plants. Aldolases in the latter group, however, do not contain a metal but employ a reactive lysyl residue in catalysis (Grazi *et al.*, 1962; Rutter, 1964). Yet at least part of the mechanisms of the two classes of enzyme seem similar, as suggested by the formation in both of a catalytic intermediate with the characteristics of carbanions (Riordan and Christen, 1969). Definition of the basis for the catalytic role of the metal on the one hand, and of the lysyl residue on the other might augment significantly the understanding of the mechanism of enzymatic catalysis of aldol condensation reactions. Based on such considerations, the present study concerns the spectral characteristics of cobalt at the active site of yeast aldolase.

Two cobalt ions can bind to each mole of apoaldolase, generating an active enzyme exhibiting optically active visible absorption bands and a near-infrared absorption band. Interaction with substrate alters the spectral properties only slightly, as would be expected if the metal participates in catalysis as an electrophile, as postulated previously (Rutter, 1964; Kobes *et al.*, 1969b).

Experimental Section

Yeast aldolase was isolated from *Saccharomyces cerevisiae* by a modification of a procedure previously described (Rutter *et al.*, 1966). Enzyme prepared in this manner was physicochemically homogeneous by both ultracentrifugal and electrophoretic criteria. The sources of the reagents employed in enzyme assays have been detailed (Kobes *et al.*, 1969b). All other chemicals were of reagent grade, and all solutions were freed of trace metal contaminants either by extraction with dithizone in carbon tetrachloride (Thiers, 1957) or by passage over a Chelex-100 (Bio-Rad Corp.) column (Him-

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