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Articles

Involvement of Histidine Residues in the Activity of Horse Liver Alcohol Dehydrogenase†

Manfred Hennecke and Bryce V. Plapp*

ABSTRACT: X-ray crystallographic studies indicate that His-51 in alcohol dehydrogenase may participate in a proton relay system during enzymatic catalysis [Eklund, H., Plapp, B. V., Samama, J.-P., & Brändén, C.-I. (1982) *J. Biol. Chem. 257*, 14349–14358], but there is no direct chemical evidence for this role. Diethyl pyrocarbonate (0.5–2 mM, pH 8, 25 °C) rapidly inactivated alcohol dehydrogenase, which was acetimidylated on all accessible lysine residues in order to prevent their modification. The reaction appeared to be specific for histidine residues, and the enzyme could be reactivated with 0.5 M hydroxylamine. The ethoxyformylated enzyme could still bind coenzymes, substrate analogues, and bipyridine, but with decreased affinity. The relationship between enzyme activity and the number of histidine residues modified showed that two histidine residues are modified during inactivation. NADH and isobutyramide significantly reduced the rate of inactivation, and the loss of activity then correlated with the modification of one to two histidine residues. The pH dependence of the inactivation showed the unusually high pK value of 9.6, which we attribute to the ionization of the water bound to zinc in the proton relay system. Although the histidine residue involved in the inactivation has not been identified, we conclude that one histidine residue (probably His-51) is essential for enzymatic activity.

X-ray crystallographic studies of the ternary complex of horse liver alcohol dehydrogenase (EC 1.1.1.1) with NAD^+ and *p*-bromobenzyl alcohol show that the alcohol oxygen is directly ligated to the catalytic zinc atom (Eklund et al., 1982). There is no obvious base (such as His-195 in lactate dehydrogenase) that could function as a proton acceptor in catalysis, and the substrate is buried deeply in the structure without contact with the aqueous phase. So that the transfer of the proton from the hydrophobic substrate binding pocket to the surface of the enzyme could be facilitated, a proton relay system including the zinc-bound hydroxyl group, the hydroxyl group of Ser-48, the 2′-hydroxyl group of the nicotinamide ribose, and the imidazole group of His-51 was proposed (Eklund et al., 1982). Various pH dependency studies have shown that one or more groups on the enzyme with pK values in the range of 6.4–7.6 must be unprotonated for maximum activity, and thus, the imidazole group of His-51 could participate in this proton relay [for a review, see Klinman (1981)]. To provide evidence for such a system, we have used diethyl pyrocarbonate since this reagent reacts with histidine residues [for a review, see Miles (1977)] and would disrupt the hydrogen-bond relay system. However, since this reagent also modifies lysine residues (Melchior & Fahrney, 1970) and horse liver alcohol dehydrogenase is activated by modification of Lys-228 with imido esters or isocyanates (Plapp, 1970; Zoltobrocki et al., 1974; Sogin & Plapp, 1975; Dworschack et al., 1975; Dworschack & Plapp, 1977a,b), we used alcohol dehydrogenase that was first acetimidylated on all accessible lysine residues. Previous studies on the reaction of horse liver alcohol dehydrogenase with diethyl pyrocarbonate used native enzyme, and the results were complicated due to counteracting effects of activation and inactivation (Morris & McKinley-McKee, 1972).

Experimental Procedures

Materials. Crystalline liver alcohol dehydrogenase, NAD^+, and NADH (grade I) were purchased from Boehringer Mannheim. Ethyl acetimidate was obtained from Eastman, diethyl pyrocarbonate was from Aldrich, and 2,2′-bipyridine and 5,5′-dithiobis(2-nitrobenzoic acid) were from Sigma. Bromoacetic acid was recrystallized from diethyl ether–petroleum ether. Other chemicals were of the highest grade available from commercial sources.

Acetimidylation. The enzyme was acetimidylated in 0.5 M triethanolamine hydrochloride buffer, pH 8.0, 25 °C, with four additions of $^1/_{20}$ volume of 2.1 M ethyl acetimidate hy-

† From the Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242. *Received April 1, 1983.* This work was supported by Grant AA00279 from the National Institute on Alcohol Abuse and Alcoholism, U.S. Public Health Service.

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drochloride (0.34 g freshly dissolved and neutralized with 1.0 mL of 1.9 M K_2CO_3) at 60-min intervals. After the fourth addition of ethyl acetimidate, the activity had increased 6-fold and remained constant. The modified enzyme was freed of ethanol and byproducts by gel filtration and dialysis. The acetimidylated enzyme was not activated by methyl picolinimidate and therefore seemed to be completely modified on Lys-228 (Sogin & Plapp, 1975). Amino acid analysis (Plapp & Kim, 1974) showed that 24 of the 30 lysine residues per subunit of enzyme were modified, which is essentially complete modification of all accessible lysine residues (Dworschack et al., 1975; Plapp et al., 1983).

Carboxymethylation. Acetimidylated enzyme was carboxymethylated on Cys-46 with bromoacetic acid in the presence of imidazole (Reynolds & McKinley-McKee, 1975). This enzyme showed a residual activity of 2.5% of the acetimidylated enzyme with ethanol as substrate and 11.7% with 1-butanol as substrate, as was found for carboxymethylated native enzyme (Reynolds & McKinley-McKee, 1975).

Ethoxyformylation. Diethyl pyrocarbonate was freshly diluted with anhydrous acetonitrile for each experiment. The concentration of reagent was determined by the reaction with imidazole (Dickenson & Dickinson, 1975). Acetimidylated or acetimidylated–carboxymethylated alcohol dehydrogenase (1 mg/mL, 25 μN) was incubated at 25 °C with 0.5–2.0 mM diethyl pyrocarbonate in 33 mM sodium phosphate buffer, pH 8.0, containing 0.25 mM ethylenediaminetetraacetic acid (EDTA). After inactivation, the enzyme was freed of byproducts by gel filtration. The time course of acylation and inactivation by diethyl pyrocarbonate was followed in a cuvette by recording continuously the absorbance change at 242 nm and withdrawing samples at various times for dilution (1:10) into 46 mM sodium phosphate buffer, pH 7.0, containing 0.25 mM EDTA and 1 mg/mL bovine serum albumin for determination of residual enzyme activity (Dickenson & Dickinson, 1975). The number of histidine residues modified was calculated from the absorption difference at 242 nm between ethoxyformylated and nonethoxyformylated enzyme at the same concentration by using $\epsilon = 3200\ M^{-1}\ cm^{-1}$ (Ovádi et al., 1967). The second-order rate constant for incorporation of diethyl pyrocarbonate could be determined in a conventional spectrophotometer up to a pH value of 8.6.

Enzyme Assays. Unmodified, acetimidylated, and ethoxyformylated–acetimidylated enzymes were assayed with ethanol as substrate (Plapp, 1970). Carboxymethylated enzyme, with its low residual activity, was assayed with 10 mM 1-butanol and 460 μM NAD^+ in 42 mM glycine–NaOH buffer, pH 10.0.

Binding Studies. NADH binding in the absence or presence of isobutyramide was determined by fluorescence measurements with a Hitachi MPF-2A spectrofluorometer with excitation at 330 nm and emission at 410 nm (Yonetani & Theorell, 1962). NADH binding in the absence or presence of isobutyramide was also determined by spectrophotometric titration at 355 nm using the difference extinction coefficient between NADH and the enzyme·NADH·isobutyramide complex of 2500 $M^{-1}\ cm^{-1}$ (Taniguchi et al., 1967). NAD^+ binding was determined by spectrophotometric titration at 300 nm in the presence of 10 mM pyrazole, where the difference extinction coefficient between NAD^+ and the enzyme·NAD^+·pyrazole complex is 7200 $M^{-1}\ cm^{-1}$ (Theorell & Yonetani, 1963). The modified enzyme was also titrated with 2,2′-bipyridine (Sigman, 1967).

Other Analytical Procedures. Normal concentrations of liver alcohol dehydrogenase were calculated by using a specific absorption coefficient of $A^{1\%}_{1cm} = 4.55$ at 280 nm (Bonnichsen, 1950) and an equivalent weight of 40 000 (Jörnvall, 1970). Total sulfhydryl content after inactivation was determined in 8 M urea by the method of Ellman (1959). All of the spectrophotometric analyses were carried out with a Cary 118 recording spectrophotometer at 25 °C.

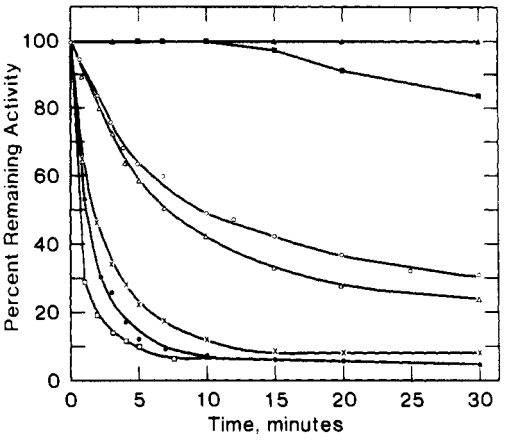


FIGURE 1: Kinetics of inactivation of horse liver alcohol dehydrogenase by diethyl pyrocarbonate in the absence or presence of ligands. Acetimidylated enzyme (25 μN) was incubated at 25 °C in 33 mM sodium phosphate buffer, pH 8.0, with 1 mM diethyl pyrocarbonate in the absence of any ligand (□) or in the presence of 300 μM NAD^+ (●), 140 μM NADH (×), 2 mM AMP (△), 10 mM bipyridine (○), 50 μM NADH plus 0.1 M isobutyramide (■), or 72 μM NAD^+ plus 50 mM trifluoroethanol (▲). The concentrations of NAD^+ or NADH were at least 10 times the K_d values (Zoltobrocki et al., 1974), and the ternary complexes were fully formed as determined by spectroscopic titration. The protecting ligands do not react significantly with diethyl pyrocarbonate under these conditions.

Data Analysis. Dissociation constants and the number of coenzyme binding sites were obtained by fitting the points in a Scatchard plot or in a Stinson–Holbrook plot (Stinson & Holbrook, 1973) to a straight line with a least-squares program (NONLIN, C. M. Metzler, The Upjohn Co., Kalamazoo, MI) or by using the FLKI program (L. Cocco, Department of Pharmacology, St. Jude Children's Research Hospital, Memphis, TN). K_d values and extinction coefficients for bipyridine binding were calculated by using the HYPER program or, if the double-reciprocal plot of the data was not linear, the TWOONE program (Cleland, 1979). The NONLIN program was also used to calculate pK values by fitting the rate constants to equations derived for various mechanisms giving a pH dependence (Dworschack & Plapp, 1977b) and to calculate rate constants for hydrolysis of diethyl pyrocarbonate, inactivation of enzyme, or modification of histidines.

Results

Inactivation by Diethyl Pyrocarbonate. Incubation of acetimidylated alcohol dehydrogenase with relatively small amounts of diethyl pyrocarbonate resulted in a rapid loss of enzymatic activity (Figure 1). The rate of inactivation was retarded when the enzyme was treated with diethyl pyrocarbonate in the presence of coenzymes, substrates, and other ligands. At saturating levels of NAD^+ and NADH, the rate of inactivation was slightly retarded. AMP and 2,2′-bipyridine at saturating levels partially protected, but only in ternary complexes with NADH and isobutyramide or NAD^+ and trifluoroethanol was the enzyme protected well against inactivation.

The difference spectrum between ethoxyformylated and acetimidylated enzyme showed the formation of a product with an absorption maximum at 242 nm, characteristic of the

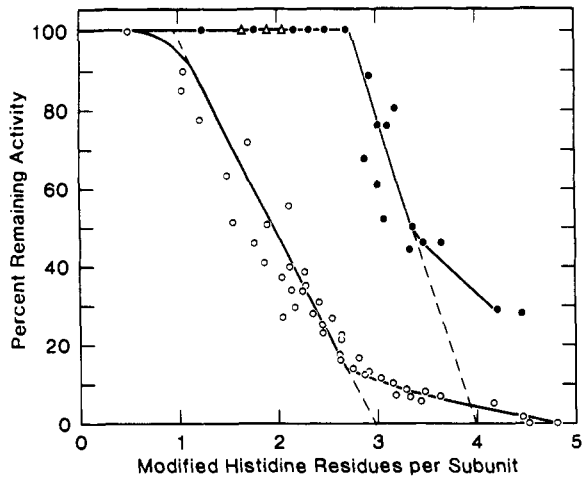


FIGURE 2: Relationship between enzyme activity and the number of histidine residues ethoxyformylated. In the chambers of tandem cells, 25 μN enzyme was incubated at 25 °C in 33 mM sodium phosphate buffer, pH 8.0, with different concentrations of diethyl pyrocarbonate (0.5, 1, and 2 mM) in the absence of any ligand (○) or in the presence of 50 μM NADH plus 0.1 M isobutyramide (●) or 72 μM NAD^+ plus 50 mM trifluoroethanol (△).

N-(carbethoxy)histidine residue. A maximum of 4.8 histidine residues could be ethoxyformylated per subunit of the acetimidylated enzyme before the enzyme denatured. When the enzyme was modified with 1 mM diethyl pyrocarbonate at pH 8.0, NADH and isobutyramide protected about one histidine residue against modification, and NAD^+ and trifluoroethanol protected about two histidine residues against modification in 30 min of reaction.

A plot of the percentage of remaining activity against the number of modified histidine residues indicates how many histidine residues could be involved in activity (Figure 2). With no protecting ligands, one histidine residue could be modified without loss of enzyme activity, and activity was lost when two more histidine residues were modified. When protected by NADH and isobutyramide, almost three histidine residues could be modified without loss of activity, and the slope shows that modification of one to two histidine residues is correlated with the loss of enzymatic activity. When protected by NAD^+ and trifluoroethanol, only two histidine residues could be modified at pH 8.0, and no inactivation was observed until the enzyme began to denature. We conclude that at least one histidine residue, or perhaps two, is essential for activity. The activity remaining after three histidine residues were modified could indicate that ethoxyformylated enzyme has partial activity.

Specificity of Reaction for Histidine Residues. Horse liver alcohol dehydrogenase contains 14 cysteine residues per subunit (Jörnvall, 1970), of which two, Cys-46 and Cys-174, are ligated to the catalytic zinc ion (Eklund et al., 1976). Modification of Cys-46 with iodoacetate (Harris, 1964; Li & Vallee, 1964) or Cys-174 with 5-diazo-1*H*-tetrazole (Sogin & Plapp, 1976) inactivates the enzyme. To determine if cysteine residues were modified by diethyl pyrocarbonate, we titrated modified enzyme with 5,5′-dithiobis(2-nitrobenzoic acid). Acetimidylated and ethoxyformylated–acetimidylated enzymes gave values of 13.6 and 13.2 cysteine residues per subunit of enzyme, respectively. Titration of carboxymethylated–acetimidylated enzyme gave a value of 12.7 cysteine residues per subunit of enzyme, that is, a difference of 0.9 cysteine residue, because of the selective modification of Cys-46. Therefore, the difference of 0.4 cysteine residue between acetimidylated and ethoxyformylated enzyme is not sufficient to account for inactivation by modification of cysteine residues.

To obtain some information about which histidine residue is important for activity, we examined acetimidylated enzyme, which was also carboxymethylated on Cys-46. Although the carboxymethylated sulfur of Cys-46 still coordinates the zinc ion, the environment around the zinc ion is disturbed (Zeppezauer et al., 1975), and the ligation of His-67 to the zinc could be changed. When the carboxymethylated enzyme was modified by diethyl pyrocarbonate, the rate of reaction and the correlation between the number of modified histidine residues and the loss of enzymatic activity were the same as those in noncarboxymethylated enzyme. Thus, Cys-46 and probably His-67 are not involved in the inactivation.

The inactivation of alcohol dehydrogenase was apparently not due to the modification of any residue other than a histidine residue. Tyrosine residues did not react with diethyl pyrocarbonate, since no spectral change was observed at 278 nm and O-ethoxyformylation of a tyrosine residue shows a negative difference spectrum at 278 nm (Mühlrad et al., 1967). Acetimidylation of lysine residues rules out modification of these residues.

Hydroxylamine cleaves the ethoxyformyl groups from modified histidine and tyrosine residues (Melchior & Fahrney, 1970), and several dehydrogenases inactivated by diethyl pyrocarbonates are known to be reactivated by treatment with hydroxylamine (see literature notes in Table II). Treatment of the totally inactivated enzyme with 0.5 M hydroxylamine for 24 h at pH 8.0 and 25 °C restored 70% of the original enzymatic activity, concomitant with the total disappearance of the peak at 242 nm. Removal of some denatured enzyme by gel filtration produced an enzyme with the same specific activity as the original acetimidylated enzyme. The modified histidine residues also hydrolyze in the absence of hydroxylamine, especially at high pH values. At pH 9.3, 25 °C, the inactivated enzyme regained 70% of the enzymatic activity in 45 min.

Kinetics and pH Dependence of Inactivation. Because of the similar reactivities of two histidine residues (Figure 2), it is difficult to determine how many residues are required for activity. If the histidine residues had different p*K* values, it might be possible to selectively modify the essential histidine residues. By changing the pH of the reaction buffer in a range from 6.5 to 8.6, we were able to change the reactivity of some histidine residues (Figure 3). At pH 6.5, about two histidine residues could be modified without loss of activity, in accordance with Dickenson & Dickinson (1975), whereas at pH 8.6, modification of the first two histidine residues inactivated the enzyme. At every pH value, however, about two histidine residues reacted during inactivation. Nevertheless, these results suggested that the various histidine residues had different p*K* values, and we therefore determined the pH dependencies for inactivation and modification.

Diethyl pyrocarbonate hydrolyzes in aqueous solutions. In 33 mM sodium phosphate buffer, pH 8.0, and at 25 °C, we determined the half-time of diethyl pyrocarbonate to be 15 min, in accordance with Dickenson & Dickinson (1975). When the rate of decrease in concentration of reagent due to reaction with enzyme is negligible, the rate of disappearance of enzyme activity (A) may be expressed as

$$A = A_0 \exp((-k/k')I_0[1 - \exp(-k't)]) \qquad (1)$$

where I_0 is the initial concentration of diethyl pyrocarbonate, k is the bimolecular rate constant for reaction of enzyme with the reagent, and k' is the pseudo-first-order rate constant for hydrolysis of reagent (Gomi & Fujioka, 1983). With a value

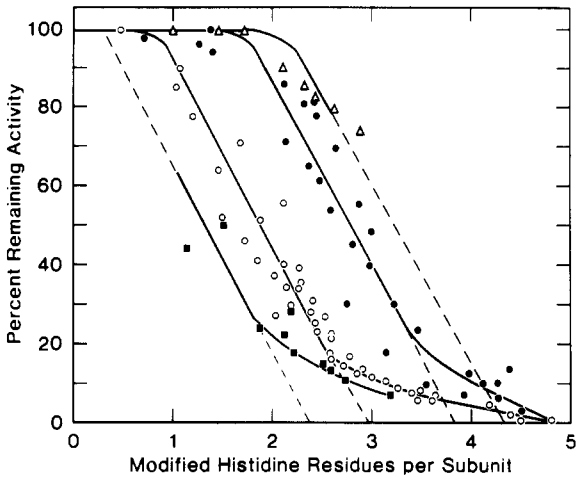


FIGURE 3: Relationship between enzyme activity and the number of histidine residues ethoxyformylated at different pH values. 25 μN enzyme was incubated at 25 °C in 0.1 ionic strength sodium phosphate buffers, pH 6.5 (△), 7.0 (●), 8.0 (○), or 8.6 (■), with different concentrations of diethyl pyrocarbonate (0.5, 1, and 2 mM) in the chambers of tandem cells. At pH 6.5, the enzyme denatured when more than three residues per subunit of enzyme were modified. At pH 8.6, the reaction was too fast to be followed at low values of incorporation.

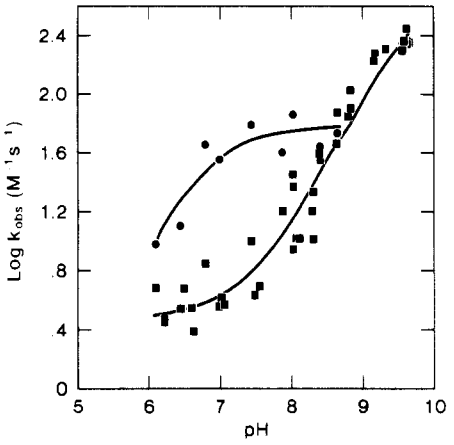


FIGURE 4: Effect of pH on inactivation and incorporation of carbethoxy groups. The modified enzyme (25 μN) was incubated with 0.5 mM diethyl pyrocarbonate in 0.1 M sodium phosphate buffer at the pH values indicated at 25 °C. At each pH, the second-order rate constants for inactivation and for incorporation of reagent were determined. The points in the figure represent the experimental values for incorporation of diethyl pyrocarbonate (●) and inactivation (■), and the curves are calculated from the logarithmic forms of the equations $k_{obsd} = k/(1 + [H^+]/K_a)$ with k = 56 M^{-1} s^{-1} and pK_a = 6.8 for incorporation of reagent and $k_{obsd} = (k_{min} + (k_{max}K_a/[H^+]))/(1 + K_a/[H^+])$ with k_{min} = 2.8 M^{-1} s^{-1}, k_{max} = 510 M^{-1} s^{-1}, and pK_a = 9.6 for inactivation of alcohol dehydrogenase.

of k' = 7.7 × 10^{-4} s^{-1}, the data obtained from experiments at several concentrations fitted this equation well, and a bimolecular rate constant (k) of 26 M^{-1} s^{-1} was calculated for the inactivation at pH 8.

This bimolecular rate constant increased with increasing pH up to 10 (Figure 4). The inactivation rate at higher pH values was not determined, because the enzyme denatures at high pH values, and the rate of hydrolysis of diethyl pyrocarbonate and *N*-(carbethoxy)histidine becomes as fast as the reaction with diethyl pyrocarbonate. The data fitted best to a pH dependency curve with a pK value of 9.6 ± 0.2, unusually high for histidine residues. A maximal second-order rate constant at high pH of 510 ± 30 M^{-1} s^{-1} and a minimal second-order rate constant at low pH of 2.8 ± 0.3 M^{-1} s^{-1} were calculated.

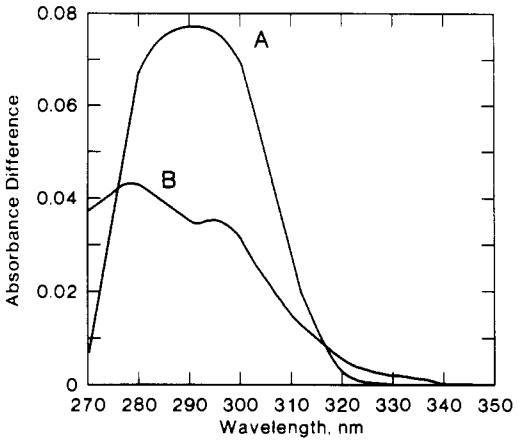


FIGURE 5: Difference absorption spectra of NAD^+·pyrazole complexes. Measurements were carried out with 25 μN enzyme, 80 μM NAD^+, and 10 mM pyrazole in 46 mM sodium phosphate buffer, pH 7.0. In the sample cuvette, one compartment (0.435-mm path length) contained the enzyme·NAD^+·pyrazole complex and the other only buffer. In the reference cuvette, one compartment contained only enzyme and pyrazole and the other NAD^+ in buffer. (A) Acetimidylated enzyme; (B) ethoxyformylated–acetimidylated enzyme.

The modification of histidine residues was biphasic, and the data from the absorbance change at 242 nm fitted best to eq 2 where the number of modified histidine residues (H) is a

$$H = H_1 \exp((-k_1/k')I_0[1 - \exp(-k't)]) + H_2 \exp((-k_2/k')I_0[1 - \exp(-k't)]) \quad (2)$$

function of H_1 (the number of histidine residues that react fast), H_2 (the number of histidine residues that react slowly), I_0 (the initial concentration of diethyl pyrocarbonate), k_1 and k_2 (the bimolecular rate constants for the fast and slow reactions, respectively), and k' (the first-order rate constant for hydrolysis of the reagent). The assumption of only two species of histidine residues (total of 4.2 residues) with different reaction velocities toward diethyl pyrocarbonate gave satisfactory fits with R^2 values of 0.999. In the pH range from 6.8 to 8.6, one to two histidines reacted rapidly (H_1) and two to three reacted slowly (H_2). At pH 6.5 and below, about three histidines were in the rapid class.

As shown in Figure 4, the bimolecular rate constant for reaction of histidine residues that reacted rapidly increased with increasing pH, and the data fitted a simple pH dependence, showing a typical pK value of 6.8 ± 0.2 and a maximal second-order rate constant of 56 ± 2 M^{-1} s^{-1} (Figure 4). At pH 8.6, the essential histidine residues became as reactive as the rapidly reacting, nonessential residues.

Coenzyme Binding. The binding of NADH and isobutyramide to the ethoxyformylated enzyme produced a spectral change at the same wavelength as was produced with the acetimidylated or unmodified enzyme, but the extinction coefficient decreased to about half of the value with the native enzyme (Table I). K_d values of NADH binding were determined by fluorescence measurements. Binding of NADH in the presence of 0.1 M isobutyramide to ethoxyformylated enzyme enhanced the fluorescence of NADH only 0.4 times as much as did binding to acetimidylated or unmodified enzyme.

The interaction of modified enzyme (ethoxyformylated and acetimidylated) with NAD^+ and pyrazole was different than that found for the acetimidylated or unmodified holoenzyme in that the specific peak at 290 nm was replaced by two peaks appearing at 280 and 295 nm (Figure 5). The K_d values for NAD^+ binding differed by 40-fold (Table I). After treatment with hydroxylamine and cleavage of all modified histidine

Table I: Binding Studies with Coenzymes and 2,2'-Bipyridine with Modified Horse Liver Alcohol Dehydrogenases[a]

ligand	enzyme	n	K_d (μM)	ϵ (mM^{-1} cm^{-1})
NADH	ADH[b]	1	0.38	2.5
	AI-ADH[c]	1 ± 0.05	0.5 ± 0.05	1.65 ± 0.1
	EF-AI-ADH[d]	*i*	*i*	*i*
NADH in the presence of 0.1 M isobutyramide[e]	ADH[b]	1	0.02	2.5
	AI-ADH[c]	1 ± 0.05	0.08 ± 0.01	2.6 ± 0.1
	EF-AI-ADH[d]	0.85 ± 0.05	0.6 ± 0.06	1.4 ± 0.2
NAD^+ in the presence of 10 mM pyrazole	ADH[e]	1	0.1	7.2
	AI-ADH[c]	1 ± 0.1	0.2 ± 0.1	7.2 ± 0.3
	EF-AI-ADH[d]	0.5 ± 0.1	8 ± 3	7 ± 1, at 295 nm
		0.5 ± 0.1	8 ± 3	9 ± 1, at 280 nm
	NH_2OH·EF-AI-ADH[f]	1 ± 0.1	0.2 ± 0.1	7.2 ± 0.5
2,2'-bipyridine	ADH[g]	1	400	11.2
	AI-ADH[c]	1	360 ± 20	11.5 ± 0.4
	EF-AI-ADH[d]	0.5	40 ± 20	11 ± 1
		0.5	2300 ± 300	11 ± 1
	CM-AI-ADH[h]	0.25	30 ± 20	5.7 ± 0.7
		0.75	3600 ± 500	5.7 ± 0.7
	zinc acetate	1	60 ± 6	42.5 ± 2

[a] All binding studies were done in 46 mM sodium phosphate buffer, pH 7.0, at 25 °C; 2.5 μN enzyme was used in fluorescence measurements, and 25 μN enzyme was used in spectrophotometric measurements. The values and their calculated or estimated standard deviations are given. [b] K_d values from Winer & Theorell (1960); ϵ values from Taniguchi et al. (1967). [c] AI-ADH is acetimidylated alcohol dehydrogenase. [d] EF-AI-ADH is ethoxyformylated and acetimidylated alcohol dehydrogenase. [e] Values from Theorell & Yonetani (1963). [f] NH_2OH·EF-AI-ADH is ethoxyformylated and acetimidylated alcohol dehydrogenated treated 24 h with 0.5 M hydroxylamine. [g] Values from Sigman (1967). [h] CM-AI-ADH is carboxymethylated and acetimidylated alcohol dehydrogenase. [i] No detectable binding.

residues, the enzyme showed the same NAD^+ and pyrazole binding behavior as did the original acetimidylated enzyme.

Bipyridine Binding. 2,2'-Bipyridine chelates the catalytic zinc ions of horse liver alcohol dehydrogenase with a dissociation constant of 0.4 mM, and the complex shows an absorption maximum at 308 nm with a difference extinction coefficient of 1.1×10^4 M^{-1} cm^{-1} per zinc ion (Sigman, 1967). For examination of changes in bipyridine binding due to modification near the catalytic zinc ion, acetimidylated, ethoxyformylated–acetimidylated, and carboxymethylated–acetimidylated enzymes were titrated with bipyridine. With acetimidylated enzyme, no change of K_d or ϵ was found. With ethoxyformylated or carboxymethylated enzyme, the bipyridine binding changed significantly. In both cases, the Scatchard plot of bipyridine binding of modified enzyme yielded two K_d values, one lower and one higher than the K_d value for acetimidylated or unmodified enzyme (Figure 6, Table I). Bipyridine titration of 25 μM zinc acetate in 50 mM sodium *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonate (Hepes) buffer, pH 7.0, gave a K_d value of 0.06 mM, which is as low as the lower K_d value found for the inactivated enzymes.

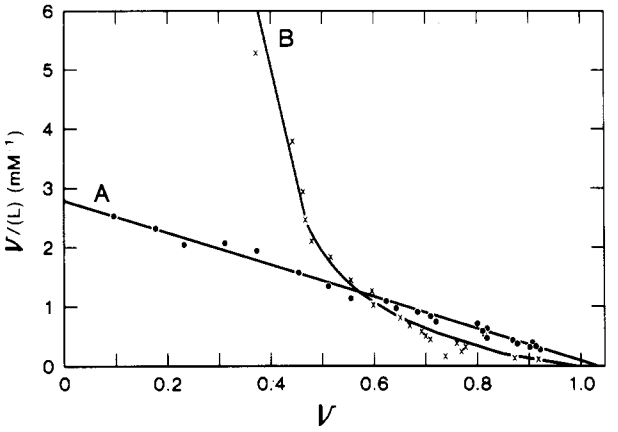


FIGURE 6: Binding of bipyridine to acetimidylated and ethoxyformylated alcohol dehydrogenase. (A) 25 μN acetimidylated alcohol dehydrogenase was titrated at 25 °C in 46 mM sodium phosphate buffer, pH 7.0, with 20 mM bipyridine solution. (B) Same as in (A) but using acetimidylated–ethoxyformylated enzyme with a residual enzymatic activity of 4%. The data were analyzed with the TWOONE program (Cleland, 1979) with the assumption that the two species have different K_d values but the same extinction coefficients. ν is the number of molecules of ligand bound per subunit of enzyme.

Discussion

Our results show that histidine residues are important for the activity of horse liver alcohol dehydrogenase. This is in contrast to the conclusions of Morris & McKinley-McKee (1972). They also used diethyl pyrocarbonate to modify histidine residues in horse liver alcohol dehydrogenase and reported a rapid activation followed by a slower, partial inactivation. We could reproduce this result with native enzyme at pH 6.1. We think that the activation is due to modification of Lys-228 and the inactivation to modification of histidine residues. In our studies, lysine residues were acetimidylated to prevent their modification by diethyl pyrocarbonate. Acetimidylated alcohol dehydrogenase is activated (Zoltobrocki et al., 1974; Dworschack & Plapp, 1977a), but crystallographic studies show that the tertiary structure of the protein is not altered even when large isonicotinimidyl groups are incorporated (Plapp et al., 1983). The acetimidylated enzyme exhibits the same p*K* for fluorescence quenching as does the unmodified enzyme (9.8), although the pH dependencies for coenzyme and substrate binding are not shifted as they are in native enzyme (Parker et al., 1978).

Diethyl pyrocarbonate inactivates acetimidylated enzyme, suggesting the involvement of histidine residues in activity. The difference spectrum between the inactivated and the acetimidylated enzymes shows a single peak at 242 nm, characteristic of *N*-(ethoxyformyl)histidine, and the lack of absorbance change at 278 nm excludes modification of a tyrosine residue. Acetimidylation of alcohol dehydrogenase rules out modification of lysine residues, and cysteine residues appear not to be modified, because the sulfhydryl content is nearly unchanged after modification. The reversibility of the inactivation of hydroxylamine is also consistent with the modification of only histidine residues.

Since (ethoxyformyl)histidine residues are unstable, it is not feasible to isolate a peptide containing the modified histidine residues. It is also clear that diethyl pyrocarbonate, which can

react with all accessible histidines in proteins, is not selective enough to distinguish among the essential one (or two) of the seven histidine residues per subunit of alcohol dehydrogenase.

In the three-dimensional structure of the apoenzyme determined by X-ray crystallography (Eklund et al., 1976), the imidazole groups of histidine residues 34, 105, and 138 are completely exposed to solvent.[1] His-348 has one of its imidazole nitrogens exposed. His-67, ligated to the catalytic zinc ion, and His-139, buried in a hydrophobic pocket, are not accessible to solvent. The imidazole nitrogens of His-51 are partly exposed in the apoenzyme and in the ternary complexes, although His-51 participates in binding coenzyme and changes position during the conformational change (Eklund et al., 1981). Histidine residues 34, 51, 105, 138, and 348 probably are those that react during inactivation (Figures 2 and 3). Of these residues, His-51 is most likely to be important for activity since its modification should inactivate by disrupting the proton relay system. Histidine residues 34, 105, 138, and 348 are so far from the active site that they probably are not involved in activity.

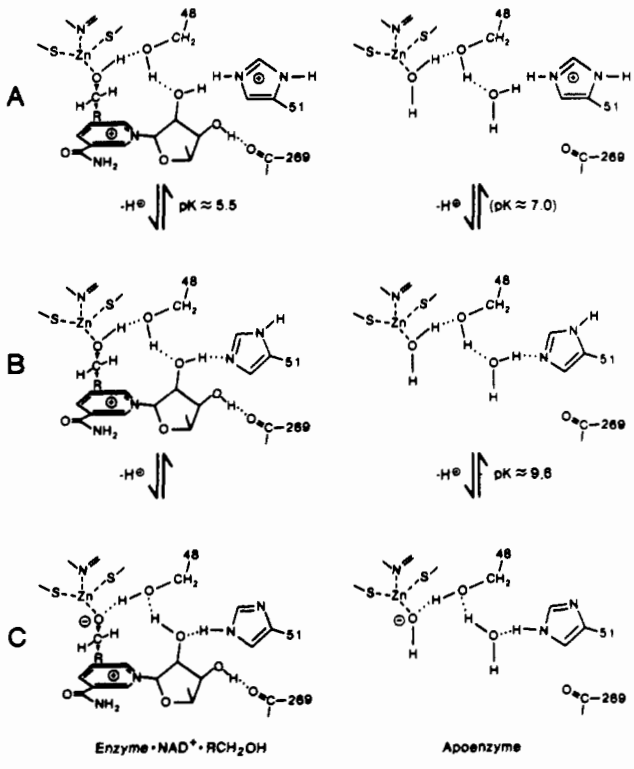
Modification of His-51 also should hinder the binding of coenzymes, and K_d values were indeed increased (Table I). The coenzymes apparently cannot bind in the same manner as in unmodified coenzyme, and the extinction coefficients for the holoenzyme are altered also. The appearance of two peaks at 280 and 295 nm in the enzyme·NAD·pyrazole spectrum might reflect the fact that the imidazole ring could be modified at either of the two nitrogens, producing two species. Modification at either nitrogen could disturb the environment in two different ways and produce two new peaks in the enzyme·NAD^+·pyrazole spectrum.

Although most of the evidence is consistent with the proposal that His-51 is essential for activity, we cannot exclude the possibility that modification of another residue also affects activity, either directly or by altering the conformation of the protein. For instance, modification of His-67, which is ligated to the zinc in the active-site pocket, could inactivate, change the coenzyme and bipyridine binding, and show a high pK value for the pH dependence of the reaction with diethyl pyrocarbonate. In enzyme carboxymethylated on Cys-46, the changed environment around the catalytic zinc hinders bipyridine binding as compared to unmodified enzyme, and the K_d value increases as a result (Table I). The smaller K_d value may be due to some free zinc, as it is reported that carboxymethylated alcohol dehydrogenase loses zinc upon storage (Li & Vallee, 1965). Ethoxyformylated enzyme shows the same behavior, suggesting that a residue in the neighborhood of the catalytic zinc ion is modified and that ethoxyformylated enzyme can also lose zinc.

On the other hand, there is some evidence that His-67 does not react with diethyl pyrocarbonate. If the enzyme is carboxymethylated on Cys-46, the binding between His-67 and the catalytic zinc ion should change, which should cause a change in the reaction with diethyl pyrocarbonate. However, the carboxymethylated and noncarboxymethylated enzymes reacted at similar rates and to similar extents, indicating that His-67 probably is not involved in the inactivation. Furthermore, even if His-67 is accessible in the free enzyme and in the binary complexes, it should be protected completely in a ternary complex. Protection with NADH and isobutyramide, however, slows down but does not prohibit inactivation. His-51 should still be exposed for reaction in the ternary complex.

[1] Hans Eklund, personal communication.

Scheme I



A significant result in these studies is that the inactivation shows a pK value of 9.6. The involvement of His-51 in the proton relay system could be the reason for the high pK value. In liver alcohol dehydrogenase, binding of many ligands is controlled by a pK value of about 9.6, and it has been suggested that the deprotonation of the zinc–water system is responsible (Kvassman & Pettersson, 1980). Recent solvent deuterium isotope results suggest that several protons may be in the system (Taylor, 1983). The protonation of the imidazole ring could be controlled by this system and therefore not show the pK value of about 7.0 observed for other dehydrogenases (Table II).

The pH dependency results can be explained in terms of the structures shown in Scheme I, which is based on the crystallographic results (Eklund et al., 1982). In the absence of coenzyme, the proton relay system is not installed, or if there is a water molecule as shown in structure B for the apoenzyme, the hydrogen-bonding system may not be as tight as in the holoenzyme. In that case, His-51, with an unprotonated nitrogen, is accessible to the reagent and should show the reactivity expected of model compounds. The bimolecular rate constant was about 4 M^{-1} s^{-1} in the pH range of 6–7. The slightly decreased reactivity compared with the model compounds could be due to the decreased accessibility of His-51.[1] With increasing pH, the system becomes deprotonated, and a negative charge is created which tightens the proton relay system and enhances the reactivity of the ND1 nitrogen, which is unprotonated and exposed for reaction. This leads to a bimolecular rate constant of 510 M^{-1} s^{-1}, which is about 20 times higher than that for the model compound and about 130 times higher than the reactivity of the same histidine residue at pH 6–7.

The involvement of the zinc-bound water in the system would explain why bipyridine partially protects against inactivation (Figure 1). Bipyridine binds to the zinc, displacing the water, and disrupts the proton relay system. Then His-51 would react without the facilitation of the system. NAD^+ or

Table II: Ethoxyformylation of Different Dehydrogenases[a]

dehydrogenase	source	inactivation by diethyl pyrocarbonate					reactivation by hydroxylamine			
		pH of reaction	% residual activity[b]	max no. of His modified[b]	no. of His essential[c]	pK	% activity	[NH_2OH] (M)	time	ref
malate	pig heart cytosol	6.5	0	0.6			100	0.7	10 min	*e*
malic enzyme	pig liver	7.0	10	25			100	0.5	10 min	*f*
octopine	*Pecten maximus*	6.0	0	2			96	0.75	24 h	*g*
lactate	rabbit muscle	6.0	0	3	1		90	0.25	15 min	*g*
lactate	pig heart	6.0	3	1	1	6.8				*h*
saccharopine	yeast	6.9	10	3	1	6.9	80	0.45	1 h	*i*
alcohol	yeast	7.0	0	2.5	1	7.1	78	none	7 days	*k*
alcohol	pea	6.0	0	1.22	1	7.0	88	1.0	40 h	*l*
alcohol	horse liver	6.1	56	4.0			50	0.1		*m*
AI-alcohol[d]	horse liver	6.5–10	0	4.8	1	9.6	70	0.5	24 h	*j*

[a] Values from the literature, except for acetimidylated alcohol dehydrogenase. [b] Percentage of residual activity as measured when the maximal number of histidine residues were modified. [c] Number of essential histidine residues as established or suggested by authors. [d] AI-alcohol is acetimidylated alcohol dehydrogenase. [e] Holbrook et al. (1974). [f] Chang & Hsu (1977). [g] Huc et al. (1971). [h] Holbrook & Ingram (1973). [i] Fujioka et al. (1980). [j] This study. [k] Dickenson & Dickinson (1975). [l] Cerovská et al. (1982). [m] Morris & McKinley-McKee (1972).

NADH should not protect very much, even though the hydroxyl group of the coenzyme is inserted into the system, since the zinc-bound water could still be present.

The nonessential histidine residues, which react faster than the essential one (or ones) at low pH values, had the expected pK value of 6.8 and a maximal second-order rate constant of 56 M^{-1} s^{-1}, which is about as fast as that reported for free histidine, imidazole, or N^{α}-acetylhistidine (about 24 M^{-1} s^{-1}; Holbrook & Ingram, 1973). The maximal second-order rate constant of 510 M^{-1} s^{-1} for reaction of histidine residues involved in inactivation is about 9 times larger than the constant for the nonessential ones. The essential histidine residue in lactate dehydrogenase (EC 1.1.1.27) had 10-fold higher reactivity than the model compounds (Holbrook & Ingram, 1973), which may be due to a histidine–aspartic acid pair at the active site (Birktoft & Banaszak, 1983). Likewise, the reactivity of alcohol dehydrogenase is consistent with the proposal that ionization of the zinc–water system affects the reactivity of His-51.

If the ionizations shown for the apoenzyme in Scheme I are correct, we should expect that the rate of inactivation would approach zero at low pH when form A is obtained. (A normal pK of 7 has been assumed.) Unfortunately, the data in Figure 4 did not fit well to the equation for such a system [eq 4 in Dworschack & Plapp (1977b)], and the enzyme is not stable enough to use lower pH values. Nevertheless, it is significant that the data fitted better to the equation derived for a system that had finite reactivity at low pH than they fitted to the equation for a system that had a single pK value and no reactivity at low pH (R^2 of 0.99 compared to 0.93).

An intriguing observation is that the enzyme·NAD^+·trifluoroethanol complex is very resistant to inactivation by diethyl pyrocarbonate. This is apparently not due to steric hindrance around His-51 in the holoenzyme complex since the ND1 nitrogen in crystalline complexes is accessible to solvent.[1] It does not appear to be due to a shift in the pK value of the essential histidine [as in the yeast alcohol dehydrogenase·NADH complex (Dickenson & Dickinson, 1977)], since the rate of inactivation was depressed over the whole pH range. Determination of the pH dependence for the competitive inhibition by trifluoroethanol of activity on ethanol shows that the pK of the acetimidylated enzyme·NAD^+ complex is 9.3, and the enzyme·NAD^+·trifluoroethanol complex has a pK of 5.5, with the K_I at high pH being 2.5 μM [data not shown; see also Parker et al. (1978)]. The resistance of the complex could be explained if His-51 has a hydrogen on the exposed ND1 nitrogen, as in structure B (or a tautomer of B) for the holoenzyme shown in Scheme I. The hydroxyl group of coenzyme binds the NE2 nitrogen of His-51 firmly in the proton relay system, and therefore, chemical reaction or protonation is hindered at that nitrogen. Thus, protonation of B could have the pK of 5.5. It should be noted that the current consensus in the literature is that the ionization of trifluoroethanol complexed with holoenzyme (B $\rightleftharpoons$ C) should have the pK value of 5.5. With native liver alcohol dehydrogenase, the pK value controlling hydride transfer in the complexes with NAD^+ and ethanol or benzyl alcohol is 6.4 (Brooks et al., 1972; Kvassman & Pettersson, 1978). Thus, it is believed that the zinc–alkoxide complex (C) undergoes the hydride transfer reaction (Cook & Cleland, 1981). Nevertheless, the enzyme kinetic data only require that the complex be unprotonated for maximal catalytic activity; the data do not allow one to specify which group has lost the proton. Since the kinetic data and our results with diethyl pyrocarbonate cannot be interpreted unambiguously, it is clear that other methods, such as NMR or neutron dif-

fraction, will be required to determine the state of protonation of His-51 and the proton relay system. For the moment, we conclude that at least one histidine residue is important for enzymatic activity.

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Registry No. NADH, 58-68-4; NAD, 53-84-9; isobutyramide, 563-83-7; bipyridine, 366-18-7; pyrazole, 288-13-1; alcohol dehydrogenase, 9031-72-5; L-histidine, 71-00-1; diethyl pyrocarbonate, 1609-47-8.

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