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Determination of the pKa for Histidine-51 Residue in the Ternary Complex of Horse Liver Alcohol Dehydrogenase

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Abstract □ The pKa value of histidine-51 residue was determined by the pH dependency of contents of NADH bound to the active site in the horse liver alcohol dehydrogenase and % inactivation with diethyl pyrocarbonate treatment of the enzyme. The pKa for His-51 was ~7.15 in the ternary complex and ~6.7 in the enzyme itself.

Keywords □ Horse liver ADH, ternary complex, His-51, pKa.

A mechanism for acid/base catalysis in horse liver alcohol dehydrogenase, based on the X-ray crystallographic structure involves a proton-relay system in which the hydroxyl group of the alcohol ligated to the catalytic zinc is connected to the hydroxyl group of Ser-48, the 2'-hydroxyl group of the NAD^+-ribose and the imidazole group of His-51 through hydrogen bonding[1]. In this proposal, His-51 as a proton acceptor is supposed to be essential in the enzymatic reaction.

Several pKa values have been reported for the reactions that might reflect the role of His-51 : 4.3 in the rate of dissociation of CF_3CH_2OH[2], 6.4 in the oxidation of benzyl alcohol[3] and of ethanol[4], 5.9 and 8.2 in 1-propanol oxidation[5], 7.6 in E-NAD^+ dissociation[6] and association of CF_3CH_2OH[7], and 9.2 in binding of NAD^+ and NADH[6]. Chemical modification of free enzyme with diethyl pyrocarbonate inactivated enzyme rapidly at higher pH with pKa of 9.6[8]. Since pKa values can be assigned indirectly by kinetic studies, we employed a specific chemical modification of histidine in the ternary complex of alcohol dehydrogenase with diethyl pyrocarbonate to determine the microscopic pKa of His-51 in the interconversion of ternary complexes, in addition to the determination of NADH contents bound to active site of enzyme-NAD^+-ethanol : acetaldehyde ternary complex at various pHs.

EXPERIMENTAL METHODS

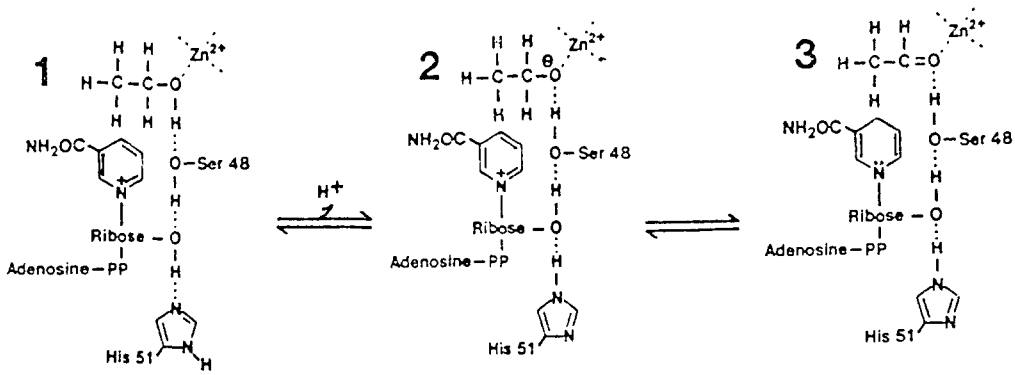
The crystalline horse liver alcohol dehydrogenase (Boerhinger Mannheim, Germany) was collected by centrifugation and resuspended in 2 m*l* of 1 mM sodium phosphate buffer (pH 7.0) containing 0.25 mM EDTA and 0.1 M Na_2SO_4 [buffer A]. The suspension was dialyzed against 400 m*l* of buffer A with five changes over 3 days at 4℃. After dialysis, the suspension was centrifuged at 4℃ for 10 min and the supernatant solution was used. The protein concentration was determined from the absorbance at 280 nm, by using a factor 0.455 A/cm per 1 mg/m*l*[9].

Enzyme assay

The activities of enzyme were assayed in 990 μ*l* of reaction buffer [$Na_2P_2O_7 \cdot H_2O$ 19.62g, semicarbazide·HCl 0.429g, glycine 0.746g, 95% ethanol 17.1 m*l*, H_2O 500 m*l*, pH 9.0 or 7.0] containing 1.35 mg of NAD^+ (Sigma, Grade III) per m*l* of the reaction buffer, with an addition of 10 μ*l* enzyme solution, at 25℃. The activity (U/m*l*) was calculated from the absorbance change per min at 340 nm [ΔA_{340}/min)×dilution factor/(6.22×0.01)].

Determination of pKa values

The pKa values were estimated by using a nonli-



Scheme 1.

near least square analysis program (NONLIN, by Metzler C.M., The Upjohn Pharm. Co., Kalamazoo, MI) to fit the equation $A=(A_{max}[H^+]+A_{min}K_a)/([H^+]+K_a)$ where A is the A_{328}, $[H^+]$ is the concentration of hydrogen ions, K_a is the proton dissociation equilibrium constant and A_{max} and A_{min} are the maximum and minimum value of A, respectively. The correlation coefficient for the fitting was more than 0.99. For inactivation data with diethyl pyrocarbonate, the values were taken from a midpoint of % inactivation changes.

***Acetimidylation*[8]**

The enzyme was acetimidylated in 0.5 M triethanolamine hydrochloride buffer (pH 8.0), at 25℃, with four additions of 1/20 volume of 2.1 M ethyl acetimidate hydrochloride (Sigma Chemical Co., 0.34g freshly dissolved and neutralized with 1.0 m*l* of 1.9 M K_2CO_3) at 60 min intervals. The modified enzyme was free of ethanol and of by-products by dialysis in buffer A.

Diethyl pyrocarbonate modification

Diethyl pyrocarbonate (Sigma Chemical Co.) was freshly diluted to be 0.1 M concentration in anhydrous acetonitrile for each experiment. The final concentration of the reagent in modification reactions was 1 mM. The enzyme activities were assayed at 0, 1, 2, 3, 4 and 5 min during modification reactions.

Ternary complex formation and internal equilibrium

The ternary complex contained 0.025 mN horse liver ADH (acetimidylated), 0.025 mM NAD^+ (Li

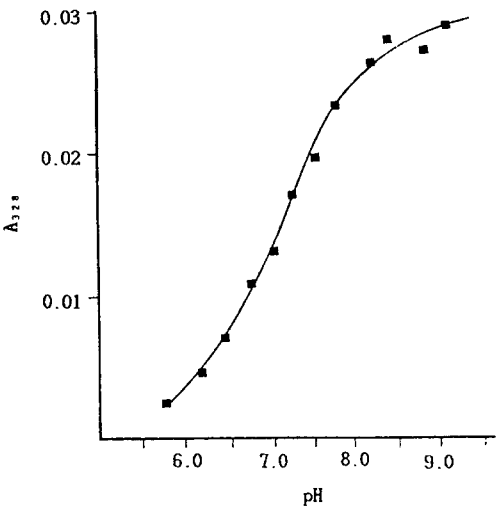


Fig. 1. The pH dependency of NADH concentration bound to the enzyme active site in the ternary complex of horse liver alcohol dehydrogenase-NAD^+-ethanol and acetaldehyde.

salt, Sigma), 100 mM ethanol and 10 mM acetaldehyde. The contents of NADH bound to enzyme active site were determined by measuring the absorbance at 328 nm which was due to the blueshift of NADH absorbance at 340 nm upon binding to the enzyme.

RESULTS AND DISCUSSION

In the excess of substrates, the enzyme should be forced into equilibrium in ternary complex, because the reaction mechanism of horse liver alcohol dehydrogenase has been known as ordered Bi

Table I. pH dependency of the contents of NADH in the active site of horse liver alcohol dehydrogenase ternary complex and inactivation of acetimidylated horse liver alcohol dehydrogenase and the ternary complex by diethyl pyrocarbonate

Enzyme complex	pKa of the formation of NADH bound to enzyme	pKa of inactivation of horse liver alcohol dehydrogenase with diethyl pyrocarbonate treatment	
ADH		6.70± 0.15 (6.55−6.85)	7.80± 0.10 (7.70−7.90)
ADH·NAD·CH_3CH_2OH·CH_3CHO	7.15± 0.05	7.13± 0.02 (7.10−7.15)	8.08± 0.18 (7.90−8.25)

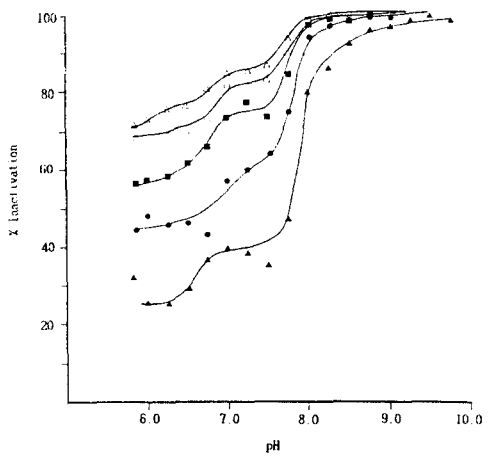


Fig. 2. The effect of pH on the inactivation of acetimidylated horse liver alcohol dehydrogenase with diethyl pyrocarbonate treatment (1 mM). 0.025 mN enzyme solution was used in 33 mM sodium phosphate or sodium pyrophosphate buffer at 25°C. ▲—▲ (1 min), ●—● (2 min), ■—■ (3 min), ○—○ (4 min) and △—△ (5 min treatment).

Bi[10]. Changes in the absorbance at 328 nm which reflect NADH bound to the active site of the enzyme could be employed to determine the contents of NADH in enzyme active site at various pHs, experimentally. Fig. 1 shows the pH dependency of the NADH contents in enzyme active site. With NONLIN fitting, the pKa resulted in 7.15± 0.05. In the assumption of proton relay system, the protonated state of His-51 might be one of factors to determine the interconversion of the ternary complex: E-NAD^+-alcohol$\rightleftarrows$E-NADH-aldehyde. The other factor would be the chemical property of a substrate.

Another evidence for the pKa of His-51 residue in the ternary complex was provided from the pH dependency of inactivation of enzyme activity with diethyl pyrocarbonate treatment. This reagent reacts with histidine residues in a protein[11]. However, since this reagent also modifies lysine residues[12] and horse liver alcohol dehydrogenase is activated by modification of Lys-228 with imido esters or isocyanates[13,14], the enzyme was acetimidylated prior to diethyl pyrocarbonate treatment. In Figs. 2 and 3, pH dependencies of % inactivation at various time points during diethyl pyrocarbonate treatment were shown for acetimidylated-enzyme and its ternary complex, respectively. The approximate pKa values were taken from a mid-point of % inactivation changes. The ranges of values were listed in Table I. As shown in the results, the enzyme has at least two histidine residues affecting on the enzymatic activity upon DEP treatment; one residue is reactive at high pH during early treatment time and another reactive at low pH. Henneke and Plapp[8] reported that about two histidine residues reacted during the inactivation of horse liver ADH by diethyl pyrocarbonate. Of the seven histidine residues in horse liver ADH subunit, His-51 in the proton relay system and His-67 ligated to the active site zinc are apparently crucial for enzyme activity[15,16]. Therefore, the two possible pKa values might be assigned to these two histidine residues. According to the results in Table I, the pKa value of NADH concentration at active site and the lower pKa vlaue of inactivation by diethyl pyrocarbonate are similar values, which may reflects the pKa of His-51 residue in the interconversion of the ternary complex ($1\rightleftarrows3$) of horse liver ADH as depicted in the

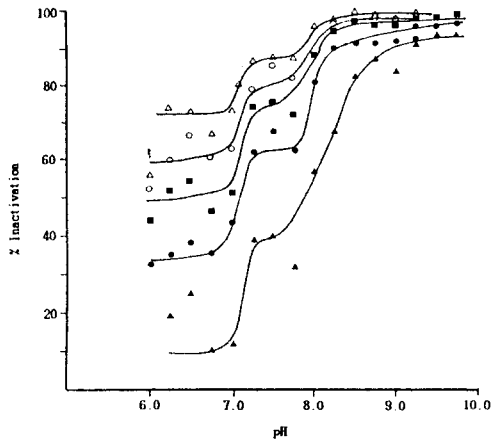


Fig. 3. The effect of pH on the inactivation of acetimidylated horse liver alcohol dehydrogenase-NAD^+-ethanol : acetaldehyde ternary complex with diethyl pyrocarbonate treatment (1 mM). 0.025 mN enzyme solution was equilibriated with 0.025 mM NAD^+ and substrate : 100 mM ethanol and 10 mM acetaldehyde in 33 mM sodium phosphate or sodium pyrophosphate buffer at 25°C. ▲—▲ (1 min), ●—● (2 min), ■—■ (3 min), ○—○ (4 min) and △—△ (5 min treatment).

scheme 1.

Sartorius *et al.*[17] reported the apparent pKa values for the ionization of alcohol within the ternary complex of Co(II)-horse liver alcohol dehydrogenase in the range of 5.0-7.5. Hennecke and Plapp[8] reported pKa 9.6 for fast inactivation rate for acetimidylated enzyme alone but there was another value around 7.2 for slow inactivation rate which they did not mentioned. This lower pKa value is close to our pKa 6.7 or 7.13 for enzyme itself and ternary complex, respectively. For the higher pKa value ($\geq$7.80) in the DEP modification, we tentatively assigned this pKa to that of His-67 which might be less tightly ligated to active site zinc at high pH, modified and led to loss of activity rapidly. The protection phenomena in the ternary complex with NADH and isobutyramide or NAD^+ and trifluoroethanol from inactivation with diethyl pyrocarbonate treatment suggest that His-67 is protected in the tightly closed form of the enzyme complex for chemical modification[8,18]. But His-67 in a normal ternary complex or enzyme alone might be modified at higher pH than 7.80 rapidly, which is resulting in a loss of activity. The reported pKa values 9.2 in binding of NAD^+ and NADH[6] and 9.6 for fast inactivation rate of free enzyme[8] might be related to the unligated state of His-67 around active site zinc.

CONCLUSION

In the interconversion of ternary complex of horse liver alcohol dehydrogenase, the pKa value of His-51 was determined by the pH dependency of the contents of NADH bound to enzyme active site and % inactivation upon diethyl pyrocarbonate treatment. The pH dependency of NADH in the ternary complex showed pKa 7.15$\pm$0.05. In the diethyl pyrocarbonate modification, % inactivation of enzyme activity gave rise to two pKa values: 7.13 and 8.08 in the ternary complex and 6.70 and 7.80 in enzyme itself. From results of two independent experiments, we could assigned pKa 7.15 to that of His-51. The pKa 7.8 or 8.1 might be assigned to His-67 residue ligated to active site zinc in the enzyme.

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