Control of Coenzyme Binding to Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: The rate of association of NAD^+ with wild-type horse liver alcohol dehydrogenase (ADH) is maximal at pH values between p*K* values of about 7 and 9, and the rate of NADH association is maximal at a pH below a p*K* of 9. The catalytic zinc-bound water, His-51 (which interacts with the 2′- and 3′-hydroxyl groups of the nicotinamide ribose of the coenzyme in the proton relay system), and Lys-228 (which interacts with the adenosine 3′-hydroxyl group and the pyrophosphate of the coenzyme) may be responsible for the observed p*K* values. In this study, the Lys228Arg, His51Gln, and Lys228Arg/His51Gln (to isolate the effect of the catalytic zinc-bound water) mutations were used to test the roles of the residues in coenzyme binding. The steady state kinetic constants at pH 8 for the His51Gln enzyme are similar to those for wild-type ADH. The Lys228Arg and Lys228Arg/His51Gln substitutions decrease the affinity for the coenzymes up to 16-fold, probably due to altered interactions with the arginine at position 228. As determined by transient kinetics, the rate constant for association of NAD^+ with the mutated enzymes no longer decreases at high pH. The pH profile for the Lys228Arg enzyme retains the p*K* value near 7. The His51Gln and Lys228Arg/His51Gln substitutions significantly decrease the rate constants for NAD^+ association, and the pH dependencies show that these enzymes bind NAD^+ most rapidly at a pH above p*K* values of 8.0 and 9.0, respectively. It appears that the p*K* of 7 in the wild-type enzyme is shifted up by the H51Q substitutions, and the resulting pH dependence is due to the deprotonation of the catalytic zinc-bound water. Kinetic simulations suggest that isomerization of the enzyme−NAD^+ complex is substantially altered by the mutations. In contrast, the pH dependencies for NADH association with His51Gln, Lys228Arg, and Lys228Arg/His51Gln enzymes were the same as for wild-type ADH, suggesting that the binding of NAD^+ and the binding of NADH are controlled differently.

The rate constant for the association of NAD^+ with *Eq*ADH[1] has a bell-shaped pH dependence described by p*K* values of about 7 and 9 (*1*, *2*), whereas the rate of NADH association decreases above a p*K* value of around 9 (*1*, *3*). The functional groups in the protein that control coenzyme binding have not been established. Groups in the coenzyme binding site that could ionize in the pH range of 6−10.5 are the water bound to the catalytic zinc, the imidazolium moiety of His-51, and the ϵ-amino group of Lys-228 (*4*).

The ionization of the zinc-bound water has been suggested to be responsible for the p*K* value of 9 (*5*). However, the water is linked to His-51 through a hydrogen bond system that includes the hydroxyl group of Ser-48 and a water molecule (in the free enzyme) or the 2′-hydroxyl group of the nicotinamide ribose (in the complex with the coenzyme), and this system should have two microscopic p*K* values, which have not been determined (*6*).

Lys-228, which stabilizes the binding of the coenzyme by forming a hydrogen bond with the adenosine 3′-hydroxyl group of the coenzyme and electrostatically attracting the negatively charged pyrophosphate of the coenzyme, could also contribute to the pH dependence (*4*). Acetimidylation of 24 of the 30 ϵ-amino groups per subunit, including Lys-228, shifts the p*K* value of 9 for association of NAD^+ to a value of $\geq$11 (*2*), which is consistent with a p*K* value for the acetimidyl group of 12.5 (*7*).

The controversy surrounding the origins of the pH dependencies for coenzyme binding and the contributions of the residues were addressed by using site-directed mutagenesis and transient kinetics. His-51 was changed to a glutamine, which is isosteric with histidine and is able to form a hydrogen bond with the coenzyme. Lys-228 was changed to an arginine residue, which maintains a positive charge in the coenzyme binding pocket and structurally resembles the acetimidylated lysine. His-51 and Lys-228 were both substituted in the Q51R228 enzyme, leaving the zinc-bound water as the most likely group in the active site that is ionizable in the observed pH range.

EXPERIMENTAL PROCEDURES

Mutagenesis. pBPP/ADH-E/K228R and pBPP/ADH-E/H51Q were created by site-directed mutagenesis with the Amersham Mutagenesis kit, version 2, starting with the plasmid pBPP containing the cDNA for the horse E isoenzyme (*8*). The oligodeoxyribonucleotide 5′-CA GAT GAC CAG GTG GTT AG-3′ (the mutation site is underlined) was used as the primer for mutagenesis of pBPP/ADH-E/H51Q.

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[1] Abbreviations: *Eq*ADH, horse liver alcohol dehydrogenase, E isoenzyme; H51Q, His-51 substituted with Gln; K228R, Lys-228 substituted with Arg; Q51R228, double-mutant with H51Q and K228R substitutions.

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The oligodeoxyribonucleotide 5′-AC AAA GAC AGG TTT GCA AAG-3′ was used as the primer for the mutagenesis of pBPP/ADH-E/K228R. The double Q51R228 mutant was constructed from the two single mutants by digestion with *Pfl*MI and *Bst*EII and religation of the two fragments containing the single-point mutations. Both *Pfl*MI and *Bst*EII enzymes cut only once in the two mutant plasmids. The mutations were confirmed by restriction mapping and DNA sequencing.

Purification. The mutant enzymes were purified by a published procedure (*8*). The enzymes were homogeneous as determined by SDS−PAGE. The concentration of active enzyme sites for the purified enzymes was determined by titration with NAD^+ in the presence of pyrazole (*9*), which indicated that about 60% of the protein subunits could bind NAD^+.

Steady State Kinetics. Initial velocities of the enzyme activity were determined by measuring the change in absorbance at 340 nm with a Cary 118C spectrophotometer interfaced with a computer with a Data Translation 2805 A/D board. An SLM fluorimeter (model 4800 C) was also used with excitation at 340 nm and emission at 460 nm. Data were collected by using a computer interfaced with the fluorimeter. Initial velocities were estimated by a linear or parabolic fit of the data. The kinetic constants were calculated with programs from Cleland (*10*). $LiNAD^+$ (grade I) and Na_2NADH (grade I) were obtained from Boehringer Mannheim.

Transient Kinetics. Transient kinetics were studied with the BioLogic SFM 3 stopped-flow instrument (dead time of 2.3 ms), which allows concentrations of substrates to be varied systematically with three syringes. The reaction for coenzyme binding was followed by the quenching of the protein fluorescence ($\lambda_{ex} = 294$ nm, $\lambda_{em} = 310-384$ nm) or by the increase in absorbance at 294 nm due to formation of the enzyme−NAD^+−pyrazole complex. Transient data were collected for varied concentrations of NAD^+ or NADH and analyzed with the BioKine software. The progress curves fit a first-order process. The kinetic simulation program KINSIM and the automatic fitting routine FITSIM (*11*) were used to estimate the microscopic rate constants from the transient progress curves collected from the BioKine stopped-flow instrument by fitting several progress curves simultaneously.

pH Dependence Studies. Buffers consisting of 10 mM $Na_4P_2O_7$, 0.25 mM EDTA, and sufficient H_3PO_4, NaH_2PO_4, and Na_2HPO_4 to produce the desired pH and a final ionic strength of 0.1 were used in the pH range of 5.5−9.5 (*12*). Buffers at pH ≥9.5 had a final composition of 10 mM $Na_4P_2O_7$, 0.25 mM EDTA, and 5 mM sodium carbonate for buffering capacity. The buffers were made double-strength and diluted 2-fold in the final reaction mixture with enzyme in weakly buffered solutions.

RESULTS

Steady State Results. Kinetic constants were determined from steady state analysis of H51Q, K228R, and Q51R228 enzymes (Table 1). The data for both the oxidation of ethanol and the reduction of acetaldehyde for the three mutant enzymes fit to a sequential bi mechanism. The equilibrium constants calculated from the Haldane relationship for the three mutated enzymes agree well with the experimentally determined value of 10 pM (*13*), indicating that cumulative errors in the kinetic constants are small. The kinetic constants for the H51Q enzyme were almost identical to those for wild-type ADH (*14*).

Table 1: Steady State Kinetic Constants for Wild-Type, H51Q, K228R, and Q51R228 Alcohol Dehydrogenases[a]

kinetic constant	native[b]	H51Q[c]	K228R	Q51R228
K_a (μM)	3.9	6.2	27	96
K_b (mM)	0.35	0.50	1.1	1.4
K_p (mM)	0.40	1.1	0.73	5.8
K_q (μM)	5.8	4.8	21	37
K_{ia} (μM)	27	20	190	270
K_{iq} (μM)	0.50	0.57	1.7	8.0
V_1 (s^{-1})	3.5	1.1	7.3	1.6
V_2 (s^{-1})	47	54	18	15
K_{eq} (pM)[d]	16	13	23	13
turnover number (s^{-1})[e]	1.8	1.1	6.4	0.90

[a] Kinetic constants were determined at 25 °C in 33 mM sodium phosphate and 0.25 mM EDTA buffer (pH 8.0) by varying the substrate and coenzyme concentrations in initial velocity studies. K_a, K_b, K_p, and K_q are the Michaelis constants for NAD^+, ethanol, acetaldehyde, and NADH, respectively. K_{ia} and K_{iq} are the dissociation constants for NAD^+ and NADH, respectively, and were determined by product inhibition experiments. V_1 is the turnover number for ethanol oxidation, and V_2 is the turnover number for acetaldehyde reduction. Errors for the fitted parameters were in the range of 10−20%. [b] From ref *36*. [c] From ref *14*. [d] K_{eq} is the Haldane relationship calculated from $V_1K_pK_{iq}/V_2K_bK_{ia}$; the established value is 10 pM. [e] Turnover number determined in a standard enzyme assay at 25 °C (*37*), based on titration of active sites with NAD^+ in the presence of pyrazole.

The K228R substitution has modest effects on the kinetic constants, increasing the dissociation constants for NAD^+ and NADH, K_{ia} and K_{iq}, by 7- and 3-fold, respectively. This is probably due to the different interactions of the arginine residue in the coenzyme binding pocket. Binding of coenzyme fragments, adenosine, AMP, and ADP ribose, compared to that of the wild-type enzyme (*15*), was only altered about 2-fold by the K228R substitution, indicating that local interactions with the adenosine moiety were not affected. The larger effects on the binding of NAD^+ and NADH may indicate that the substitution affects the coupling of binding to the conformational change that occurs for wild-type enzyme, as demonstrated by X-ray crystallography (*16*).

The Michaelis and dissociation constants for the Q51R228 enzyme increase to values larger than those of the wild-type enzyme and either of the singly mutated enzymes. The dissociation constants for NAD^+ and NADH, K_{ia} and K_{iq}, are 10- and 16-fold larger than the respective values for wild-type ADH (Table 1).

pH Dependence of NAD^+ Association. The rate constants for NAD^+ association were determined from stopped-flow kinetics for the three mutant enzymes over a pH range of about 6−10.5, where the protein is stable. The pH dependencies and the association rate constants for the three mutant enzymes differ significantly from those for wild-type ADH (Figure 1 and Table 2).

The rate of NAD^+ association with the K228R enzyme exhibited very little pH dependence (Figure 1), and the data were best fit to a mechanism (eq 2 in Table 2) involving a single ionizable group with a pK value of 6.4. The maximum association rate constant from the pH dependence of the K228R enzyme is reduced by 13-fold compared to that of wild-type ADH. The pH dependencies for NAD^+ association

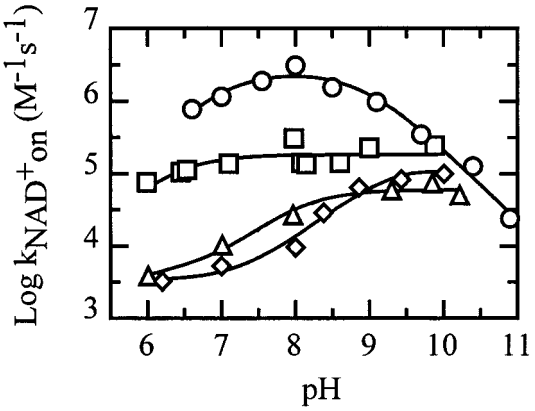
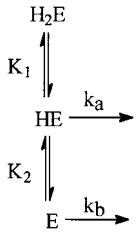


FIGURE 1: pH dependence of NAD^+ association with wild-type, H51Q, K228R, and Q51R228 enzymes. The rate constants were determined from transient kinetic analysis: wild-type (○), K228R (□), H51Q (△), and Q51R228 (◇). The data for the pH dependence of NAD^+ association with the wild-type enzyme are from ref *2*.

Scheme 1



with the K228R enzyme determined from steady state kinetics (V_1/K_a, data not shown) and transient data are comparable.

Data for both the H51Q and Q51R228 enzymes were best fit to a function describing a mechanism with one ionizable group (eq 3 in Table 2), where the unprotonated form of the enzyme binds coenzyme more rapidly than the protonated form. The Q51R228 enzyme has a pK value of 9.0, and the H51Q mutant has a pK value of 8.0. Compared to that of wild-type ADH, the maximal, pH-independent rate constants for binding decrease 40- and 20-fold for the H51Q and Q51R228 enzymes, respectively.

With the Q51R228 enzyme, the data for the pH dependence of the rate of NAD^+ association were collected by varying the concentrations of both NAD^+ and pyrazole. The data were fit to the equation for a sequential bi reaction (Figure 2, for example). The bimolecular rate constants, k/K_{NAD^+} and k/K_{Pyz}, and the limiting rate constant, k_{lim}, exhibited the same pH dependence (Figure 3). The data were best fit to an equation (eq 3 in Table 2) which describes a mechanism with one ionizable group where both forms of the enzyme are reactive. The pK values for k/K_{NAD^+}, k/K_{Pyz}, and k_{lim} were all about 9. These results suggest that the same ionizable group may be responsible for the observed pK values. The results for the Q51R228 and wild-type enzymes are distinctly different in the effect of pH on k/K_{Pyz} and k_{lim}, as the rate constants for the wild-type enzyme decrease with an increase in pH (*2*).

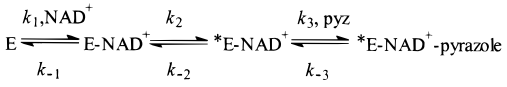
Simulations. The rate constants for association of NAD^+ with K228R, H51Q, and Q51R228 enzymes decrease by 20-, 110-, and 260-fold, respectively, compared to that of wild-type ADH at pH 8. An explanation for this must consider that binding of NAD^+ to the wild-type enzyme is a two-step process, involving a bimolecular association followed by the isomerization of the E−NAD^+ complex as shown in Scheme 2 (*2*).

Table 2: pH Dependence of Coenzyme Association with Wild-Type, K228R, H51Q, and Q51R228 Alcohol Dehydrogenases Determined from Transient Kinetics[a]

enzyme	equation	pK value	limiting rate
NAD^+ association			
wild-type[b]	1	6.9 and 9.0	$k_a = 2400\ mM^{-1}\ s^{-1}$
K228R	2	6.3 ± 0.2	$k_b = 180 \pm 30\ mM^{-1}\ s^{-1}$
H51Q	3	8.0 ± 0.2	$k_a = 3.4 \pm 0.9\ mM^{-1}\ s^{-1}$
			$k_b = 60 \pm 7\ mM^{-1}\ s^{-1}$
Q51R228	3	9.0 ± 0.2	$k_a = 3.3 \pm 0.7\ mM^{-1}\ s^{-1}$
			$k_b = 120 \pm 30\ mM^{-1}\ s^{-1}$
NADH association			
wild-type[c]	4	9.2 ± 0.2	$k_a = 11 \pm 1\ \mu M^{-1}\ s^{-1}$
K228R	4	10.0 ± 0.2	$k_a = 4.3 \pm 0.6\ \mu M^{-1}\ s^{-1}$
H51Q	4	9.5 ± 0.1	$k_a = 8 \pm 1\ \mu M^{-1}\ s^{-1}$
Q51R228	4	9.9 ± 0.1	$k_a = 4.9 \pm 0.4\ \mu M^{-1}\ s^{-1}$

[a] The data were fitted to logarithmic forms of equations derived for various mechanisms of pH dependence (*36*) by using the nonlinear least-squares fitting program NONLIN (C. M. Metzler, The Upjohn Co., Kalamazoo, MI). A general mechanism with two ionizable groups that can describe the pH dependencies is shown in Scheme 1, and the equations for the simplest mechanism required to explain each set of results are listed below. Equation 1 describes the mechanism where only the monoprotonated form is reactive: $k_{obs} = k_a/(1 + [H^+]/K_1 + K_2/[H^+])$. Equation 2 describes a mechanism with a single ionizable group where only the unprotonated form is active: $k_{obs} = k_a/(1 + [H^+]/K_1)$. Equation 3 describes a mechanism with one ionizable group where both forms of the enzyme are reactive: $k_{obs} = k_a + k_bK_2[H^+]/(1 + K_2/[H^+])$. Equation 4 describes a mechanism with one ionizable group and only the protonated form of the enzyme reacts: $k_{obs} = k_a/(1 + K_2/[H^+])$. [b] From ref *2*. Standard errors are less than 5%. [c] From ref *3*.

Scheme 2



The isomerization of the E−NAD^+ complex is supported by kinetic studies (*2*, *17*−*21*) and crystallographic results where the apoenzyme is in the open form and the holo form is closed (*16*, *22*). The rate constants for the isomerization step were estimated by using transient data and kinetic simulations of the reaction. A limiting rate constant (when saturating concentrations of NAD^+ are used) of 500−600 s^{-1} was determined and assigned to the forward isomerization step, k_2 (*2*, *23*, *24*). The kinetic simulation programs KINSIM and FITSIM (*11*) were used to fit the progress curves for the formation of the E−NAD^+−pyrazole complex for the mutant enzymes and to estimate the microscopic rate constants for the formation of the ternary complex (Figure 4 and Table 3).

From simulations of NAD^+ association with the Q51R228 enzyme, it is apparent that the overall rate constant for NAD^+ binding is controlled by the forward isomerization step, k_2. The overall rate constant for NAD^+ association is defined by the equation $k_{on,NAD^+} = k_1k_2/(k_2 + k_{-1})$. When k_2 is small relative to k_{-1}, the equation simplifies to $k_{on,NAD^+} = k_1k_2/k_{-1}$. The forward isomerization rate constants for the Q51R228 enzyme are much smaller than the estimated values of k_{-1} over the observed pH range (Table 3). The microscopic step that is most affected by the double mutation is the forward isomerization step. At pH 8, the forward isomer-

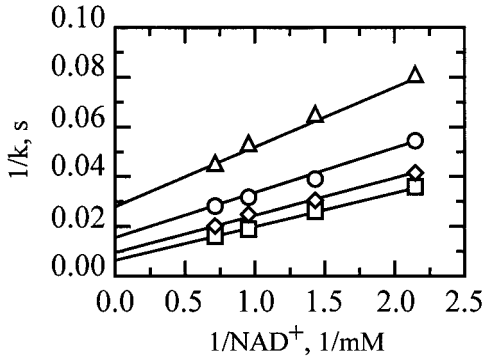


FIGURE 2: Dependence of the observed rate constants for the formation of the Q51R228−NAD^+−pyrazole complex on the concentrations of NAD^+ and pyrazole. The concentrations of pyrazole were 2.5 (△), 5 (○), 10 (◇), and 20 mM (□). Each point represents the average of three or more determinations. The buffer was 0.1 ionic strength sodium pyrophosphate/phosphate buffer at pH 9.43 and 25 °C. Lines were calculated from the fit to the SEQUEN equation $k_{obs} = k_{lim}[NAD^+][pyrazole]/(K_{ia}K_{Pyz} + K_{NAD^+}[pyrazole] + K_{Pyz}[NAD^+] + [NAD^+][pyrazole])$, with a K_{NAD^+} of 3.6 mM, a K_{ia} of 540 μM, a K_{Pyz} of 2.7 mM, and a k_{lim} of 300 s^{-1}.

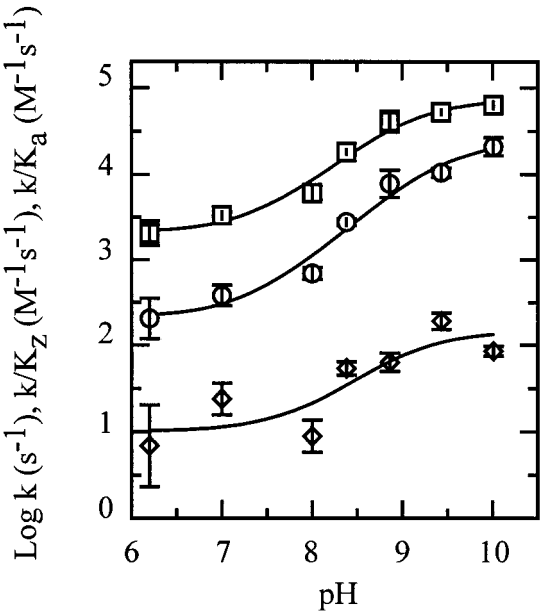


FIGURE 3: pH dependence of the kinetic constants for the reaction of Q51R228 with NAD^+ and pyrazole: k_{lim} (◇), k/K_{Pyr} (○), and k/K_{NAD^+} (□). The pK values were best fit to eq 3 in Table 2 which describes a mechanism with one ionizable group where both forms react and the unprotonated from is most active (see Scheme 1). The pK value for k/K_{NAD^+} was 9.0 ± 0.2 with a k_a of 3.3 ± 0.7 mM^{-1} s^{-1} and a k_b of 120 ± 30 mM^{-1} s^{-1}. The pK value for k/K_{Pyz} was 9.4 ± 0.2 with a k_a of 0.3 ± 0.1 mM^{-1} s^{-1} and a k_b of 40 ± 20 mM^{-1} s^{-1}. The pK value for k_{lim} was 9.0 ± 0.6 with a k_a of 16 ± 9 s^{-1} and a k_b of 200 ± 200 s^{-1}.

ization rate constant decreases by 170-fold compared to that of wild-type ADH. The forward isomerization step is pH-dependent, and the rate constants increase with increasing pH. The overall rate constant for NAD^+ dissociation is defined by the equation $k_{off,NAD^+} = k_{-1}k_2k_{-2}/(k_{-1} + k_2)(k_2 + k_{-2})$. When k_2 is less than k_{-2} and k_{-1}, the equation simplifies to $k_{off,NAD^+} = k_2$, and k_{off} is approximated by the forward isomerization step k_2, as shown by the correlation in Table 3.

At pH 8, the apparent NAD^+ association rate constant for the H51Q enzyme decreases by 110-fold compared to that

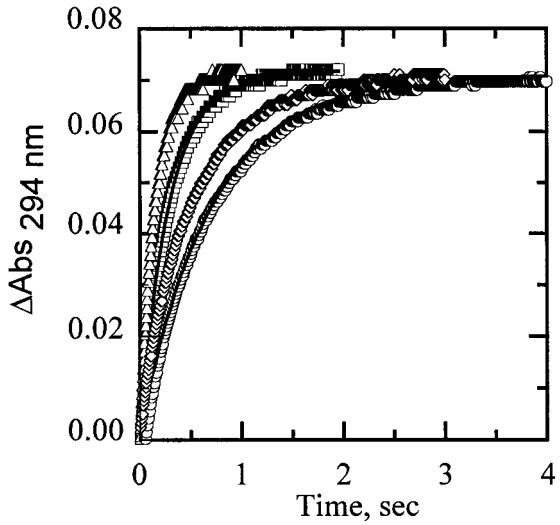


FIGURE 4: Transient and simulated reactions for association of NAD^+ with the Q51R228 enzyme. Points are experimental, and the lines are simulated progress curves obtained by fitting with the FITSIM program. The Q51R228 enzyme (9.3 μN) at pH 7 reacted with 1.97 mM NAD^+ and 70 mM pyrazole (△), 0.88 mM NAD^+ and 30 mM pyrazole (□), 1.32 mM NAD^+ and 10 mM pyrazole (◇), and 0.88 mM NAD^+ and 10 mM pyrazole (○).

of the wild-type enzyme. The simulation of the NAD^+ association with the H51Q enzyme at pH 8 shows that the largest effect is on the isomerization steps. Compared to those of wild-type ADH, the forward isomerization rate constant, k_2, decreases by 33-fold and the reverse isomerization rate constant, k_{-2}, increases by 33-fold. The removal of His-51 in the H51Q and Q51R228 mutant enzymes causes a decrease in k_2 and an increase in k_{-2}. The equilibrium constant for the isomerization step favors the open form in the H51Q and Q51R228 enzymes as opposed to the wild-type enzyme where the closed form is favored. The equilibrium constant (k_2/k_{-2}) for the isomerization step decreases by 22-, 1000-, and 5000-fold for the K228R, H51Q, and Q51R228 enzymes, respectively, compared to that of the wild-type enzyme.

NADH Association. The association rate constants for NADH binding were determined for H51Q, K228R, and Q51R228 enzymes within a pH range of about 6−10.5 (Figure 5). The association rate constants were determined by following the quenching of protein fluorescence with the stopped-flow instrument. The rate constants for the H51Q, K228R, and Q51R228 enzymes were similar to the association rate constants that were observed for wild-type *Eq*ADH, which has a pK value of 9.2 (*3*). The pH dependencies for NADH association for wild-type, H51Q, K228R, and Q51R228 enzymes were best fit to a mechanism (eq 4 in Table 2) with one ionizable group where only the protonated form binds NADH. H51Q, K228R, and Q51R228 enzymes have pK values similar to that of wild-type ADH with pK values of 9.5, 10.0, and 9.9, respectively (Table 2).

DISCUSSION

Coenzyme binding to horse liver alcohol dehydrogenase has been extensively studied, but interpretation of the pH dependencies in terms of the protein structure remains a subject of debate. The pK value of 9 for NAD^+ association has been assigned to the ionization of the catalytic zinc-

Table 3: pH Dependence of the Microscopic Rate Constants for the Binding of NAD^+ to Wild-Type, H51Q, K228R, and Q51R228 Enzymes[a]

enzyme	pH	k_1 (μM^{-1} s^{-1})	k_{-1} (s^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})	k_3 (mM^{-1} s^{-1})	k_{on,NAD^+}[b] (mM^{-1} s^{-1})	k_{off,NAD^+}[b] (s^{-1})
WT[c]	8.0	45	23000	620	65	120	1180	57
H51Q	8.0	4.9	1000	19	2100	95	90	18
K228R	8.7	8.2	1800	23	54	380	100	16
Q51R228	6.2	1.0	1200	4.2	78	6	3.3	3.9
Q51R228	7.0	3.1	8800	19	4000	180	6.5	18
Q51R228	8.0	8.6	2800	3.6	1800	600	11	3.6
Q51R228	8.4	5.9	4900	33	4500	690	39	33
Q51R228	10.0	6.1	8000	160	1900	470	120	140

[a] The rate constants were obtained from KINSIM and FITSIM computer simulation programs with data collected from the stopped-flow instrument for the mechanism shown in Scheme 2. Twelve to sixteen traces with varied concentrations of NAD^+ and pyrazole were simulated to obtain estimates of the microscopic rate constants. [The rate constants for the dissociation of pyrazole (k_{-3}) were determined experimentally by diluting enzyme that was incubated with NAD^+ and pyrazole into an assay mixture with NAD^+ and ethanol. The reaction observed at 340 nm due to NADH formation exhibited an initial lag phase followed by a linear increase in absorbance. By extrapolating the linear portion of the reaction back to the time axis, we could determine the relaxation time. During the fitting, k_{-3} was fixed between 0.01 and 0.03 s^{-1}, while the other rate constants were allowed to vary.] The data at pH 8.0 were for the reaction with 33 mM sodium phosphate buffer and 0.25 mM EDTA. The buffers for the other pH values are described in Experimental Procedures. The standard errors for the estimated rate constants were less than 13%. R^2 values of ≥ 0.99 were obtained for the fits. [b] The overall rate constants for NAD^+ binding were calculated from the rate constants that were determined from the simulation of the full mechanism. The following equations were used: $k_{on,NAD^+} = k_1k_2/(k_2 + k_{-1})$ and $k_{off,NAD^+} = k_{-1}k_2k_{-2}/(k_{-1} + k_2)(k_2 + k_{-2})$. [c] From ref *23*.

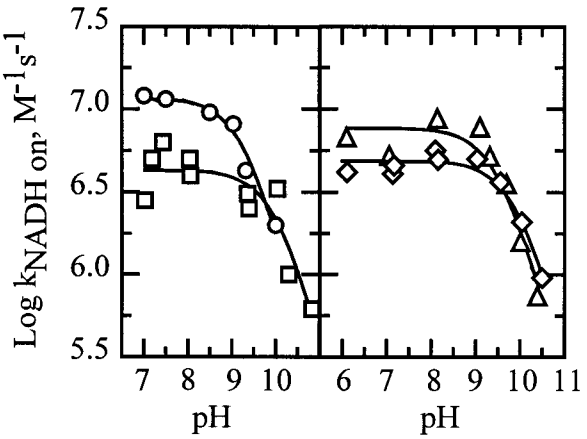


FIGURE 5: pH dependence of the NADH association with wild-type, H51Q, K282R, and Q51R2228 enzymes. The rate constants were determined from transient kinetic analysis: wild-type (○), K228R (□), H51Q (△), and Q51R228 (◇). The data for the pH dependence of NADH association with the wild-type enzyme are from ref *3*.

bound water (*25*−*27*) or to Lys-228 (*2*). The p*K* value of 6.9 has been assigned to the macroscopic p*K* value of the proton relay system through the linkage of the two ionizable groups, His-51 and the catalytic zinc-bound water (*2*). Others have suggested that the ionization of the zinc-bound water does not contribute to the observed pH dependence for coenzyme association (*28*). Site-directed mutagenesis of His-51 and Lys-228 was used to prepare enzymes that could be used to resolve these discrepancies.

pH Dependence of NAD$^+$ *Association.* The substitution of Lys-228 with an arginine abolishes the p*K* value of 9 that is observed for NAD^+ association with wild-type ADH. This result is similar to the results of previous chemical modification studies (*2*) where the acetimidylated enzyme abolishes or shifts the p*K* value of 9 to a higher (nonobservable) value. The pH dependencies of K228R and acetimidylated enzymes are consistent with the identification of Lys-228 as the ionizable group that controls the p*K* value of 9 for NAD^+ association and are not consistent with the predictions for the role of the catalytic zinc-bound water. The pH dependence for the wild-type enzyme can be explained by electrostatic effects; below the p*K* value of 9, the protonated Lys-228 attracts the negatively charged pyrophosphate of NAD^+. When Lys-228 is deprotonated, the rate of NAD^+ association decreases.

The substitution of His-51 drastically affects the pH dependence of NAD^+ association compared to that of wild-type ADH, indicating an important role for His-51 (Figure 1). The macroscopic p*K* value of 6.9 in the wild-type enzyme is probably due to the proton relay system. When His-51 in the proton relay system is substituted, the p*K* value is shifted up from 6.9 to 8. This is similar to the results that were observed for carbonic anhydrase II; when a hydrogen bonding system was disrupted by site-directed mutagenesis, the p*K* value of the catalytic zinc-bound water also increased (*29*).

When both Lys-228 and His-51 are changed, a p*K* value of 9 is observed (Figure 1). This p*K* value is probably due to the ionization of the catalytic zinc-bound water, which is the only group remaining in the active site of the Q51R228 enzyme that could interact closely with the coenzyme and ionize in the pH range of 6−10. The pH dependence of the Q51R228 enzyme is the opposite of what was previously predicted for the zinc-bound water, where a decrease in the rate of binding above the p*K* value of 9 was assigned to ionization of the zinc-bound water (*5*, *25*). In studies with the Q51R228 enzyme, the coenzyme binds faster above the p*K* of 9, where the zinc-bound water could be deprotonated. The observed p*K* value in the Q51R228 enzyme is similar to the p*K* value observed for hydrated zinc [$Zn^{2+}(H_2O)_6$], which ionizes with a p*K* value of 9.1 (*30*), and for models of the active site with macrocyclic ligands that yield a p*K* value of about 9 for the ligated zinc (*31*).

Thus, the p*K* values of 8 and 9 for the H51Q and Q51R228 enzymes, respectively, appear to result from the deprotonation of the zinc-bound water where the zinc hydroxide form of the enzyme binds NAD^+ most rapidly. In the wild-type enzyme, the p*K* for the corresponding region of the pH dependence is about 7. This lower value may be due to facilitation of proton release by His-51. Nevertheless, the binding process and the explanation in structural terms are more complex.

Isomerization. The binding of NAD^+ is a two-step process (Scheme 2). X-ray crystallography demonstrates that when NAD^+ or NADH (and usually a substrate or substrate

analogue) is bound, the catalytic domains of the enzyme rotate about 10° with respect to the coenzyme binding domains (*4*, *22*, *32*). The forward isomerization (rate constant k_2) is relatively fast in wild-type ADH (*2*, *23*, *24*), and significantly slower in the mutated enzymes that have decreased apparent association rate constants. The mutations at positions 51 and 228 have large effects on the rate constants for the forward isomerization step as determined by KINSIM and FITSIM analysis (Table 3). For H51Q and K228R enzymes, the rate of forward isomerization decreases 33- and 27-fold, respectively. In the Q51R228 enzyme, the isomerization rate constant, k_2, varies from 4.2 to 160 s^{-1} over the pH range of 6.2−10, respectively. The decrease in the rate of the forward isomerization can explain the decrease in the overall association rate constant.

We suggest that the disruption of the proton relay system in H51Q and Q51R228 enzymes slows the forward isomerization (rate constant k_2) because His-51 is not present to facilitate the removal of the proton from the water bound to the catalytic zinc. The p*K* values of about 9 that are observed for the limiting rates of NAD^+ and pyrazole association in the Q51R228 enzyme may be due to the ionization of the catalytic zinc-bound water. The forward isomerization could be faster when the catalytic zinc-bound water is deprotonated and the $Zn-OH^-$ electrostatically attracts the positive charge of the nicotinamide ring, which are juxtaposed in the holoenzyme complex (*4*).

An alternative explanation for the decrease in the isomerization rate constant may be that the enzymes with Gln-51 interact less well with the nicotinamide ribose. During the conformational change, the helix containing residues 47−53 moves closer to the bound coenzyme (*16*). This movement allows His-51 to form a hydrogen bond with the 2′-hydroxyl group of the nicotinamide ribose of NAD. It is possible that the Gln substitution alters this interaction even though a hydrogen bond from the H51Q enzyme is feasible. However, the dissociation constants for NAD^+ and NADH are about the same with wild-type and H51Q enzymes at pH 8.

The maximum pH-independent rate constant for the association of NAD^+ with the K228R enzyme decreases by 13-fold compared to that of the wild-type enzyme (Table 2). The arginine substitution also decreased the affinity for NAD^+ binding by 7-fold. From the KINSIM and FITSIM analysis of the K228R enzyme, the rate of forward isomerization, k_2, decreases 27-fold compared to that of wild-type ADH. In the structure of the activated picolinimidylated ADH, the modified Lys-228 fits between the cleft of the two domains that come closer together during the conformational change (*33*). An arginine at position 228 may also be accommodated in the cleft, but the interactions of the guanidino group interfere with the closing of the cleft and thereby slow the conformational change.

pH Dependence of NADH Association. The pH dependence and association rate constants for NADH binding to H51Q, K228R, Q51R228, and wild-type enzymes are very similar, in contrast to the large effects that are observed for NAD^+ binding (Figure 5). The substitution of His-51 has a very small effect. The arginine substitution in K228R and Q51R228 enzymes causes a ca. 2-fold decrease in the maximum association rate constant and a p*K* value shift of about 0.8 unit. The results from the NAD^+ binding studies show that the K228R substitution affects the conformational change of the enzyme. An effect of the substitution on NADH binding may not be observed when the forward isomerization rate constant is fast. For NADH association, the rate of forward isomerization is faster than 1000 s^{-1} for wild-type ADH (*23*, *24*). Microscopic rate constants for NADH association ($k_1 = 30\ \mu M^{-1}\ s^{-1}$, $k_{-1} = 390\ s^{-1}$, $k_2 = 1400\ s^{-1}$, and $k_{-2} = 17\ s^{-1}$) were estimated by Adolph and co-workers (*24*) at pH 8.5 for wild-type horse ADH. If the rate constant of the forward isomerization step were to decrease 20-fold as was observed for NAD^+ association with the K228R enzyme, then the calculated apparent association rate constant would be 4.5 $\mu M^{-1}\ s^{-1}$, which is very close to the observed rate constant of 4.3 $\mu M^{-1}\ s^{-1}$ for the K228R enzyme.

These results indicate that the binding of NAD^+ and the binding of NADH are controlled differently, and the contributions of the amino acid residues differ. The removal of His-51 has drastic effects on NAD^+ binding but little or no effect on NADH association. The reason for the observed difference may simply be due to the difference in charge on the nicotinamide ring. When NAD^+ binds to the enzyme, the nicotinamide ring becomes buried in a hydrophobic pocket. The positive charge of the nicotinamide ring is most likely stabilized by the negative charge on the catalytic $Zn-OH^-$. His-51 helps facilitate formation of the hydroxide by shuttling a proton through the proton relay system. Rapid binding of NADH, however, should not require deprotonation of the zinc-bound water. Rather, the p*K* value for NADH association may be controlled by electrostatic interactions between the pyrophosphate group and the positively charged protein at pH values above the isoelectric point (*34*). Results with a mutant human enzyme are consistent with this suggestion. The p*K* for NADH binding shifted from 8.1 to 6.8 when Arg-369, which interacts with the pyrophosphate in the β_1 isozyme, was substituted in the β_3 isozyme with a cysteine residue (*35*). Further research is required to confirm the electrostatic effects on NADH binding to the horse liver enzyme.

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REFERENCES

1. Dalziel, K. (1963) *J. Biol. Chem. 238*, 2850−2858.
2. Sekhar, V. C., and Plapp, B. V. (1988) *Biochemistry 27*, 5082−5088.
3. Kvassman, J., and Pettersson, G. (1979) *Eur. J. Biochem. 100*, 115−123.
4. Ramaswamy, S., Eklund, H., and Plapp, B. V. (1994) *Biochemistry 33*, 5230−5327.
5. Pettersson, G. (1987) *CRC Crit. Rev. Biochem. 21*, 349−389.
6. Eklund, H., Plapp, B. V., Samama, J.-P., and Brändén, C.-I. (1982) *J. Biol. Chem. 257*, 14349−14358.
7. Plapp, B. V., Brooks, R. L., and Shore, J. D. (1973) *J. Biol. Chem. 245*, 3470−3475.
8. Park, D.-H., and Plapp, B. V. (1991) *J. Biol. Chem. 266*, 13296−13320.
9. Theorell, H., and Yonetani, T. (1963) *Biochem. Z. 338*, 537−555.
10. Cleland, W. W. (1979) *Methods Enzymol. 63*, 103−138.
11. Frieden, C. (1994) *Methods Enzymol. 240*, 311−322.
12. Gould, R. M., and Plapp, B. V. (1990) *Biochemistry 29*, 5463−5468.

13. Sund, H., and Theorell, H. (1963) *Enzymes* (*2nd Ed.) 7*, 25−83.
14. Park, D.-H. (1991) Ph.D. Thesis, The University of Iowa, Iowa City, IA.
15. Fan, F., and Plapp, B. V. (1995) *Biochemistry 34*, 4709−4713.
16. Eklund, H., and Brändén, C.-I. (1979) *J. Biol. Chem. 254*, 33458−33461.
17. Wratten, C. C., and Cleland, W. W. (1963) *Biochemistry 2*, 935−941.
18. Coates, J. H., Hardman, M. J., Shore, J. D., and Gutfreund, H. (1977) *FEBS Lett. 84*, 25−28.
19. Parker, D. M., Hardman, M. J., Plapp, B. V., Holbrook, J. J., and Shore, J. D. (1978) *Biochem. J. 173*, 269−275.
20. Hardman, M. J. (1981) *Biochem. J. 195*, 773−774.
21. Plapp, B. V., Sogin, D. C., Dwoschack, R. T., Bohlken, D. P., Woenckhaus, C., and Jeck, R. (1986) *Biochemistry 25*, 5396−5402.
22. Eklund, H., Samama, J.-P., Wallén, L., Brändén, C.-I., Åkeson, Å., and Jones, T. A. (1981) *J. Mol. Biol. 146*, 561−587.
23. Sekhar, V. C., and Plapp, B. V. (1990) *Biochemistry 29*, 4289−4295.
24. Adolph, H. W., Kiefer, M., and Cedergren-Zeppezauer, E. (1997) *Biochemistry 36*, 8743−8754.
25. Andersson, P., Kvassman, J., Lindström, A., Oldén, B., and Pettersson, G. (1980) *Eur. J. Biochem. 108*, 303−312.
26. Hemmingsen, L., Bauer, R., Bjerrum, M. J., Zeppezauer, M., Adolph, H. W., Formicka, G., and Cedergren-Zeppezauer, E. (1995) *Biochemistry 34*, 7145−7153.
27. Theorell, H. (1961) *Fed. Proc. Am. Soc. Exp. Biol. 20*, 967−970.
28. Dietrich, H., MacGibbon, A. K. H., Dunn, M. F., and Zeppezauer, M. (1983) *Biochemistry 22*, 3432−3438.
29. Krebs, J. F., Ippolito, J. A., Christianson, D. W., and Fierke, C. A. (1993) *J. Biol. Chem. 268*, 27458−27466.
30. Dunn, M. F. (1975) *Struct. Bonding (Berlin) 23*, 61−62.
31. Woolley, P. (1975) *Nature 258*, 677−682.
32. Colonna-Cesari, F., Perahia, D., Karplus, M., Eklund, H., Brändén, C.-I., and Tapia, O. (1986) *J. Biol. Chem. 261*, 15273−15280.
33. Plapp, B. V., Eklund, H., Jones, T. A., and Brändén, C.-I. (1983) *J. Biol. Chem. 258*, 5537−5547.
34. Lively, C. R., Feinberg, B. A., and McFarland, J. T. (1987) *Biochemistry 26*, 5719−5735.
35. Stone, C. L., Jipping, M. B., Owus-Dekyi, K., Hurley, T. D., Li, T.-K., and Bosron, W. F. (1999) *Biochemistry 38*, 5829−5835.
36. Dworschack, R. T., and Plapp, B. V. (1977) *Biochemistry 16*, 111−116.
37. Plapp, B. V. (1970) *J. Biol. Chem. 245*, 1727−1735.

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