Amann, R., Ludwig, W., & Schleifer, K. H. (1988b) *J. Gen. Microbiol. 134*, 2815–2821.
Boyer, P. D. (1975) *FEBS Lett. 58*, 1–6.
Boyer, P. D. (1988) *Trends Biochem. Sci. 13*, 5–7.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Davis, B. D., & Mingioli, E. S. (1950) *J. Bacteriol. 60*, 17–28.
Deckers-Hebestreit, G., & Altendorf, K. (1986) *Eur. J. Biochem. 161*, 225–231.
Dimroth, P. (1986) *Methods Enzymol. 125*, 530–540.
Dimroth, P. (1987) *Microbiol. Rev. 51*, 320–340.
Douglas, M., Finkelstein, D., & Butow, R. A. (1979) *Methods Enzymol. 56*, 58–66.
Dulley, J. R., & Grieve, P. A. (1975) *Anal. Biochem. 64*, 136–141.
Foster, D. L., Mosher, M. E., Futai, M., & Fillingame, R. H. (1980) *J. Biol. Chem. 255*, 12037–12041.
Friedl, P., Friedl, C., & Schairer, H. U. (1979) *Eur. J. Biochem. 100*, 173–180.
Hilpert, W., & Dimroth, P. (1986) *Methods Enzymol. 125*, 540–546.
Hilpert, W., Schink, B., & Dimroth, P. (1984) *EMBO J. 3*, 1665–1670.
Kauffer, S., Schmid, R., Steffens, K., Deckers-Hebestreit, G., & Altendorf, K. (1987) *Arch. Microbiol. 148*, 187–192.
Krämer, R., & Heberger, C. (1986) *Biochim. Biophys. Acta 863*, 289–296.
Laubinger, W. (1987) Thesis, Technische Universität München.
Laubinger, W., & Dimroth, P. (1987) *Eur. J. Biochem. 168*, 475–480.
Laubinger, W., & Dimroth, P. (1988) *Biochemistry 27*, 7531–7537.
Laubinger, W., & Dimroth, P. (1989) *Biochemistry 28*, 7194–7198.
Lübben, M., Lünsdorf, H., & Schäfer, G. (1987) *Eur. J. Biochem. 167*, 211–219.
Lübben, M., Lünsdorf, H., & Schäfer, G. (1988) *Biol. Chem. Hoppe-Seyler 369*, 1259–1266.
Mitchell, P. (1974) *FEBS Lett. 43*, 189–194.
Schink, B., & Pfennig, N. (1982) *Arch. Microbiol. 133*, 209–216.
Schneider, E., & Altendorf, K. H. (1987) *Microbiol. Rev. 51*, 477–497.
Steffens, K., Schneider, E., Deckers-Hebestreit, G., & Altendorf, K. (1987) *J. Biol. Chem. 262*, 5866–5869.
Volgel, G., & Steinhart, R. (1976) *Biochemistry 15*, 206–216.

Substitution of Arginine for Histidine-47 in the Coenzyme Binding Site of Yeast Alcohol Dehydrogenase I†

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ABSTRACT: Molecular modeling of alcohol dehydrogenases suggests that His-47 in the yeast enzyme (His-44 in the protein sequence, corresponding to Arg-47 in the horse liver enzyme) binds the pyrophosphate of the NAD coenzyme. His-47 in the *Saccharomyces cerevisiae* isoenzyme I was substituted with an arginine by a directed mutation. Steady-state kinetic results at pH 7.3 and 30 °C of the mutant and wild-type enzymes were consistent with an ordered Bi-Bi mechanism. The substitution decreased dissociation constants by 4-fold for NAD^+ and 2-fold for NADH while turnover numbers were decreased by 4-fold for ethanol oxidation and 6-fold for acetaldehyde reduction. The magnitudes of these effects are smaller than those found for the same mutation in the human liver β enzyme, suggesting that other amino acid residues in the active site modulate the effects of the substitution. The pH dependencies of dissociation constants and other kinetic constants were similar in the two yeast enzymes. Thus, it appears that His-47 is not solely responsible for a pK value near 7 that controls activity and coenzyme binding rates in the wild-type enzyme. The small substrate deuterium isotope effect above pH 7 and the single exponential phase of NADH production during the transient oxidation of ethanol by the Arg-47 enzyme suggest that the mutation makes an isomerization of the enzyme–NAD^+ complex limiting for turnover with ethanol.

Alignment of the amino acid sequences of the homologous horse liver and yeast alcohol dehydrogenases (EC 1.1.1.1) suggests that His-47 in the yeast enzyme (Arg-47 in the liver enzyme) binds the phosphate of the AMP moiety of coenzyme (Jörnvall et al., 1978; Eklund et al., 1976, 1981). Molecular modeling shows that Nδ of the imidazole ring can form a hydrogen bond with a phosphate oxygen (Eklund et al., 1987; Plapp et al., 1987). Histidine might interact more weakly than arginine and allow faster release of coenzymes, increasing turnover numbers and altering pH dependencies.

Inactivation studies using diethyl pyrocarbonate implicated one histidine residue in the mechanism of *Saccharomyces cerevisiae* alcohol dehydrogenase I, although two histidines (His-47 and His-51) are in the active site. The free enzyme showed a pK value of 7.1 for inactivation (Dickenson & Dickinson, 1975a). The reacting residue could not be identified due to the lability of ethoxyformylated histidine.

A mutant yeast alcohol dehydrogenase with Arg-47 was generated previously by selecting for mutants that could grow in the presence of allyl alcohol (Wills, 1976; Wills et al., 1981). Wills proposed that the mutants survived because the redox

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state of the cell was altered and the equilibrium between allyl alcohol and the toxic acrolein was shifted toward the alcohol.

We created the Arg-47 enzyme by site-directed mutagenesis of the cloned yeast alcohol dehydrogenase gene to determine the effect of the substitution on coenzyme binding and the reaction mechanism, to identify the histidine residue involved in catalysis, and to explain the phenotype of yeast cells harboring this mutation.

Experimental Procedures

Materials. LiNAD$^+$, NADH, and the Klenow fragment of DNA polymerase I were purchased from Boehringer Mannheim Biochemicals; T4 DNA ligase was from International Biotechnologies Inc.; exonuclease III, T4 polynucleotide kinase, and restriction enzymes were from New England Biolabs; deoxynucleotides, DEAE-Sepharose CL-6B, and octyl-Sepharose CL-4B resin were from Pharmacia P-L Biochemicals; pyrazole was from Eastman Kodak; radioactive nucleotides were from Amersham; and ethanol-d_5 was from Fluka Chemical Corp.

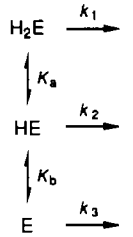
The mutamer (*muta*genic oligo*mer*) sequence GTGTCTGTCGCACTGACTTG (the underline marks the point of mutation) was used for mutagenesis in a double-stranded plasmid as described by Ganzhorn et al. (1987). The mutation was sequenced after being subcloned into M13mp18RF phage by procedures described in the manual from New England Biolabs. Transformation and expression of mutant and wild-type alcohol dehydrogenases in the yeast strain 302-21#2 were described by Ganzhorn et al. (1987) with the exception that 10 μg/mL antimycin A was used to maintain selective pressure for alcohol dehydrogenase activity.

The wild-type and mutant enzymes were purified from yeast cells grown anaerobically in 2% Bacto-peptone, 1% Bacto yeast extract, and 2% glucose. Frozen yeast cells (about 25 g) were thawed and lysed in a 350-mL bead beater with about 170 mL of 20 mM Tris-HCl, pH 8.0, containing 1 mM 2-mercaptoethanol and 0.5 mM EDTA (lysis buffer). The lysate was filtered through cheesecloth to remove cellular debris and beads and was brought to a final volume of 250 mL with rinses of the beads. The lysate was brought to 0.2% (w/v) in protamine sulfate, stirred for 20 min, and centrifuged at 10000*g* for 20 min to remove the precipitate. The enzyme was fractionated and concentrated by precipitation with poly(ethylene glycol) 4000 (Fluka) in the range from 8 to 22%. The precipitate was redissolved in 50 mL of lysis buffer, and the solution was loaded onto a 2.5 × 15 cm column of DEAE-Sepharose CL-6B equilibrated with the lysis buffer. The column was developed with 150 mL of lysis buffer and a 500-mL gradient from 20 to 500 mM Tris-HCl, pH 8.0. Pooled enzyme was made 0.9 M in ammonium sulfate and loaded onto a 50-mL column of octyl-Sepharose CL-4B equilibrated with 50 mM Tris-HCl, pH 8.0, and 0.9 M ammonium sulfate. About half of the protein flowed through the column while washing with buffer in 0.9 M ammonium sulfate, and the enzyme was eluted stepwise with buffer not containing ammonium sulfate and concentrated by ultrafiltration to at least 5 mg/mL. In 24 h, 200 mg of enzyme was isolated with a 14-fold purification. Peptide mapping and sequencing were as described by Ganzhorn and Plapp (1988).

Steady-State Kinetics. Procedures for product and dead-end inhibition experiments were described by Ganzhorn et al. (1987). Data were fitted by the appropriate equations (Cleland, 1979).

The pH dependencies of the Michaelis constant for NAD$^+$, K_a, and the dissociation constant for NADH, K_{iq}, were determined by inhibition experiments at 200 mM ethanol with 0.4–2 mM NAD$^+$ and 0–0.4 mM NADH throughout the pH range. Concentrations of NADH above 0.2 mM required cuvettes with a path length of 0.5 cm. At low pH, initial velocity experiments for the Arg-47 enzyme used ethanol concentrations from 20 to 200 mM while NAD$^+$ concentrations were varied from 0.2 to 2 mM. Product inhibition experiments for the reverse reaction used 4 mM acetaldehyde with 40–200 μM NADH and 0–4 mM NAD$^+$. The buffers were 0.1 ionic strength. Buffers in the pH range 5.5–9.2 contained 10 mM sodium pyrophosphate and were brought to the final pH with concentrated phosphoric acid. Sodium phosphate buffers were added in the correct ratio to maintain the pH and adjust the ionic strength. Pyrophosphate and phosphate buffers were chosen because of their lack of interaction with the general anion binding site of horse liver alcohol dehydrogenase (Oldén & Pettersson, 1982). Carbonate buffers were used above pH 9.2 to ensure good buffering capacity.

The pH dependence of the kinetic constants obtained in these experiments was evaluated by reference to the general model



where E represents a form of enzyme with or without substrate, K_a and K_b are acid dissociation constants, and the k's are rate or dissociation constants. Depending upon the kinetic significance of the various steps, the pH dependencies could be fitted by one of the following equations, which describe the form of dependence and do not imply that the same groups are ionizing in each reaction. In eqs 2 and 3, for instance, K_b could be substituted for K_a, and k_1 could be incremented to k_2, and k_2 to k_3. The data were fitted to logarithmic forms of the equations by using the nonlinear least-squares fitting program NONLIN (C. M. Metzler, The Upjohn Co., Kalamazoo, MI).

$$k_{obsd} = k_2/(1 + [H^+]/K_a) \quad (1)$$

$$k_{obsd} = k_1/(1 + K_a/[H^+]) \quad (2)$$

$$k_{obsd} = \frac{k_1 + k_2K_a/[H^+]}{1 + K_a/[H^+]} \quad (3)$$

$$k_{obsd} = \frac{k_2 + k_3K_a/[H^+]}{1 + K_a/[H^+] + [H^+]/K_b} \quad (4)$$

$$k_{obsd} = \frac{k_2}{1 + [H^+]/K_a + K_b/[H^+]} \quad (5)$$

Transient Kinetics. NAD$^+$ binding was studied by mixing 4 μM Arg-47 enzyme in 0.2 ionic strength, pH 7.0 pyrophosphate–phosphate buffer at 30 °C with unbuffered NAD$^+$, at concentrations varied from 50 to 800 μM, and 6 to 100 mM pyrazole. The reaction was followed by the increase in absorbance at 292 nm. For wild-type enzyme, 83 mM potassium phosphate and 40 mM KCl buffer pH 7.3, was used, with NAD$^+$ concentrations varied from 75 to 500 μM and pyrazole concentrations varied from 5 to 75 mM. A Kinetic Instruments rapid mixing device and an On-Line Instruments

Systems program were used for collection and analysis of the data, as previously described (Sekhar & Plapp, 1988).

Results

Protein Properties. The wild-type and Arg-47 enzymes behaved identically during purification. Final preparations appeared nearly homogeneous in polyacrylamide gel electrophoresis in the presence or absence of sodium dodecyl sulfate. Electrophoresis under nondenaturing conditions showed that the Arg-47 enzyme migrated more slowly toward the anode than did the wild-type enzyme, as would be expected from an extra positive charge. Protein and activity comigrated, providing evidence that the mutant enzyme is being produced. Furthermore, the presence of an arginine residue at position 47 was supported by the isolation and sequencing of the tryptic peptide starting with Thr-48 (residues 45–59 in the yeast sequence). The peptide maps for wild-type and mutant enzymes were similar except for the position of this peptide, which eluted 4 min earlier than the peptide containing residues 39–59 in wild-type enzyme.

Enzyme Mechanism. Product and dead-end inhibition patterns for both wild-type and Arg-47 enzymes were consistent with the ordered Bi-Bi mechanism. Coenzymes inhibited competitively against one another, and ethanol inhibited noncompetitively against varied acetaldehyde concentrations. Acetaldehyde inhibited noncompetitively against varied ethanol concentrations with wild-type enzyme, since there were small but significant intercept effects as determined by Student's t test. However, the Arg-47 enzyme did not give significant intercept effects. Because of the small intercept effects with the wild-type enzyme, the apparent difference probably is not significant. An ordered Bi-Bi mechanism should present a noncompetitive pattern in this experiment but may appear competitive if the intercept inhibition constant is much larger than the slope inhibition constant. [Similar results were obtained for the wild-type alcohol dehydrogenase isoenzyme II (Ganzhorn et al., 1987).] NADH inhibited noncompetitively against varied ethanol concentrations. Low concentrations of trifluoroethanol, a dead-end inhibitor (competitive against varied concentrations of ethanol), inhibited uncompetitively against varied NAD^+ concentrations, but there were small slope effects at higher concentrations. These results may indicate a random mechanism at higher alcohol concentrations. Moreover, concentrations of ethanol above 200 mM produced substrate activation; this could be due to the formation of an enzyme–NADH–ethanol complex, which could release NADH faster than the enzyme–NADH complex does. It appears that the overall mechanism has not been altered significantly by substitution of an arginine residue and that it is best described as predominantly ordered Bi-Bi at lower concentrations of ethanol. This mechanism was found previously for wild-type enzyme (Wratten & Cleland, 1963; Dickinson & Monger, 1973; Dickenson & Dickinson, 1978; Cook & Cleland, 1981; Ganzhorn et al., 1987).

Kinetic Constants. The steady-state kinetic constants (Table I) show that the substitution with arginine caused small changes. Coenzyme dissociation constants were decreased by 2-fold for NADH and 4-fold for NAD^+, presumably as a result of the additional stabilizing ionic interaction of Arg-47 with the pyrophosphate of the coenzyme. Turnover numbers were decreased by 6-fold for ethanol oxidation and 4-fold for acetaldehyde reduction, which could be explained by slower rates of release of coenzyme. However, as discussed below, the slower turnover number for ethanol oxidation may be partly due to a new slow step, an isomerization of the enzyme–NAD^+ complex.

Table I: Kinetic Constants for Wild-Type and Arg-47 Mutant Yeast Alcohol Dehydrogenases

constant[a]	wild type[b]	Arg-47	constant[a]	wild type[b]	Arg-47
K_a (μM)	160	150	V_2 (s^{-1})	1800	460
K_b (mM)	21	66	$K_{eq}I$ (pM)[d]	12	29
K_p (mM)	0.74	4.7	$K_{eq}II$ (pM)	15	[c]
K_q (μM)	94	10	R[e]	0.35	0.42
K_{ia} (μM)	950	260	activity (s^{-1})[f]	400	60
K_{ib} (mM)	120	1600	K_d, CF_3CH_2OH (mM)	2.5	19
K_{ip} (mM)	1.5	[c]	k_1 (μM^{-1} s^{-1})[g]	2.3	0.4
K_{iq} (μM)	31	16	k_2 (s^{-1})[g]	2100	100
$K_{ia}K_b/K_a$ (mM)	96	180	k_7 (s^{-1})[g]	590	740
$K_{iq}K_p/K_q$ (mM)	0.21	1.8	k_8 (μM^{-1} s^{-1})[g]	19	46
V_1 (s^{-1})	360	60			

[a] K_a, K_b, K_p, and K_q are the Michaelis constants for NAD^+, ethanol, acetaldehyde, and NADH, respectively. K_i values are product inhibition constants. V_1 is the turnover number for ethanol oxidation and V_2 the turnover number for acetaldehyde reduction in 83 mM potassium phosphate buffer with 40 mM KCl and 0.25 mM EDTA at pH 7.3 and 30 °C. Standard errors of fits were 10–20% of the listed values. [b] These constants were determined from multiple experiments, producing small changes in the values reported previously (Ganzhorn et al., 1987). [c] Undefined. [d] Equilibrium constants calculated from Haldane equations: $K_{eq}I = V_1K_pK_{iq}[H^+]/V_2K_bK_{ia}$, where $[H^+] = 5 \times 10^{-8}$ M at pH 7.3, and $K_{eq}II = (V_1/V_2)^2K_{ip}K_q[H^+]/K_{ib}K_a$. [e] R = fraction of enzyme in the ternary complex, calculated from $[(1 - K_a/K_{ia})/V_1 + (1 - K_q/K_{iq})/V_2]/(1/V_1 + 1/V_2)$ (Janson & Cleland, 1974). [f] Turnover numbers in standard assay (Plapp, 1970) at 30 °C, based on titration of active sites. [g] Rate constants refer to the mechanism shown below, where A is NAD^+, B is ethanol, P is acetaldehyde, Q is NADH, and E is enzyme, and were calculated from $k_1 = V_1/K_a$, $k_2 = k_1K_{ia}$, $k_7 = k_8K_{iq}$, and $k_8 = V_2/K_q$.

$$E \underset{k_2}{\overset{k_1[A]}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3[B]}{\rightleftharpoons}} EAB \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EPQ \underset{k_6[P]}{\overset{k_5}{\rightleftharpoons}} EQ \underset{k_8[Q]}{\overset{k_7}{\rightleftharpoons}} E$$

Table II: Substrate Isotope Effects with Wild-Type and Arg-47 Enzymes[a]

pH	$^DV_1/K_b$		$^DV_1$	
	wild type	Arg-47	wild type	Arg-47
6.0	3.6 ± 0.2	3.5 ± 0.2	2.0 ± 0.2	3.5 ± 0.2
7.0	3.2 ± 0.2	2.4 ± 0.2	1.8 ± 0.1	1.7 ± 0.1
8.0	3.0 ± 0.2	1.5 ± 0.1	1.8 ± 0.1	1.2 ± 0.1
9.0	2.6 ± 0.1	1.1 ± 0.2	1.4 ± 0.1	1.1 ± 0.1
10.0	2.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.1 ± 0.1

[a] Initial velocity studies used concentrations of ethanol or ethanol-d_5 that varied from 15 to 200 mM while the NAD^+ concentration was fixed at 10 mM.

Kinetic constants were used to calculate rate constants for the ordered Bi-Bi mechanism (Cleland, 1963). The substitution with Arg-47 decreased k_1 by 6-fold and k_2 by 21-fold (Table I). However, the maximum velocity for aldehyde reduction by the Arg-47 enzyme is 4.5-fold faster than the calculated k_2. This discrepancy could be explained by an isomerization of the enzyme–NAD^+ complex (Wratten & Cleland, 1963).

Isotope Effects. Substrate isotope effects with ethanol-d_5 were studied to further identify rate-limiting steps in the reaction. Large isotope effects were observed on V_1/K_b and smaller effects on V_1 for the wild-type enzyme, but the effects were diminished at high pH (Table II). This agrees with the results of Dickenson and Dickinson (1975b), who determined that the rate of coenzyme dissociation limits turnover at high pH while hydrogen transfer limits V_1/K_b. The isotope effects on both V_1/K_b and V_1 for the Arg-47 enzyme were generally smaller than those for wild-type enzyme and insignificant at high pH. The similar pH dependencies of the isotope effects suggest that the same step limits V_1 and V_1/K_b in the Arg-47 enzyme. This step could be an isomerization of the enzyme–NAD^+ complex.

Table III: Macroscopic pK Values and pH Independent Rate Constants for Wild-Type and Arg-47 Mutant Yeast Alcohol Dehydrogenases[a]

	wild-type enzyme			Arg-47 enzyme		
	eq	pK values	limiting constant	eq	pK values	limiting constant
V_1 (s^{-1})	3	7.0 ± 0.03	250 ± 3	4	6.5 ± 0.1	40 ± 2
			500 ± 4		9.3 ± 0.5	100 ± 10
V_1/K_b (mM^{-1} s^{-1})	3	7.7 ± 0.1	3.2 ± 0.1	4	6.6 ± 0.1	0.52 ± 0.02
			25 ± 1		10.0 ± 0.7	5 ± 3
V_2 (s^{-1})	3	7.0 ± 0.3	4000 ± 300	3	6.6 ± 0.1	1260 ± 60
			1300 ± 100			50 ± 2
V_2/K_p (mM^{-1} s^{-1})	3	7.8 ± 0.2	2900 ± 200	3	7.6 ± 0.1	63 ± 2
			450 ± 50			6.6 ± 0.4
K_{ia} (μM)	5[b]	5.8 ± 0.4	690 ± 10	2	9.8 ± 0.1	590 ± 10
		8.7 ± 0.3				
K_{iq} (μM)	4	6.0 ± 0.3	25 ± 2	3	9.0 ± 0.2	8 ± 5
		9.2 ± 0.4	600 ± 100			100 ± 10
k_1 (μM^{-1} s^{-1})	5	7.0 ± 0.3	8 ± 1	4	6.9 ± 0.2	0.25 ± 0.02
		8.7 ± 0.3			8.9 ± 0.9	0.5 ± 0.1
k_2 (s^{-1})	5	7.0 ± 0.3	6300 ± 1200	1	7.1 ± 0.1	200 ± 10
		9.6 ± 1				
k_7 (s^{-1})	pH independent		500 ± 100	pH independent		50 ± 10
k_8 (μM^{-1} s^{-1})	2	7.8 ± 0.1	32 ± 1	3	7.6 ± 0.2	10 ± 1
						0.5 ± 0.1

[a] The pK values and rate constants are from fits to the logarithmic forms of the equations listed under Experimental Procedures. Constants are defined in the footnotes to Table I. [b] pK_{ia} was fitted.

Transient Reactions. The reaction of the Arg-47 enzyme with saturating concentrations of NAD^+ (2 mM) and ethanol (250 mM), at pH 7.3, showed a single exponential phase of NADH production in the stopped-flow spectrophotometer, consistent with a rate-limiting isomerization occurring before hydride transfer. The binding of NAD^+ was also investigated with the yeast enzymes. With the horse liver enzyme, increasing the concentrations of NAD^+ and pyrazole to saturation led to a limiting rate, ascribed to an isomerization of the enzyme–NAD^+ complex (Sekhar & Plapp, 1988). Both yeast enzymes showed similar bimolecular binding constants for NAD^+ [7.0 (±0.6) × 10^5 M^{-1} s^{-1}] and for pyrazole [2.0 (±0.3) × 10^4 M^{-1} s^{-1}], but the Arg-47 enzyme showed a limiting reaction rate of 130 ± 5 s^{-1}, whereas the wild-type enzyme showed none. Rates of 750 s^{-1} have been measured on this instrument (Sekhar & Plapp, 1988). It appears that the Arg-47 enzyme has a kinetically significant rate of isomerization, which can explain the lack of a transient phase in ethanol oxidation and the small isotope effects.

pH Dependence of Kinetic Constants. The role of residue 47 in ionic interactions was investigated by examining the pH dependence of kinetic constants. Product inhibition experiments gave estimates of K_a, K_{iq}, K_q, and K_{ia}, whereas initial velocity experiments were used to obtain estimates of K_b and K_p. The equilibrium constants calculated from kinetic constants by using the Haldane equation agreed within a factor of 2 at each pH with the known value of 1 × 10^{-11} M at 30 °C (Bäcklin, 1958). The kinetic constants were fitted to the equation for the simplest model describing the pH dependencies (Figure 1, Table III). The pH-dependence profiles for the two enzymes were surprisingly similar to one another and were also similar to those described previously for wild-type enzyme (Dickenson & Dickinson, 1975b). For most kinetic constants, the differences were in the magnitudes of the kinetic constants rather than in the pK values. Most significantly for this study, the pK value near 7 was not eliminated.

Substitution with Arg-47 led to a shift in the pK value from 7.0 to 6.5 for V_1 and from 7.7 to 6.6 for V_1/K_b (Figure 1A). Since the substrate isotope effects (Table II) show that hydride transfer is partially rate-limiting only for V_1/K_b for wild-type enzyme, the shifts in pK values for V_1 and V_1/K_b can result from alterations in rate-limiting steps (Cleland, 1979). For

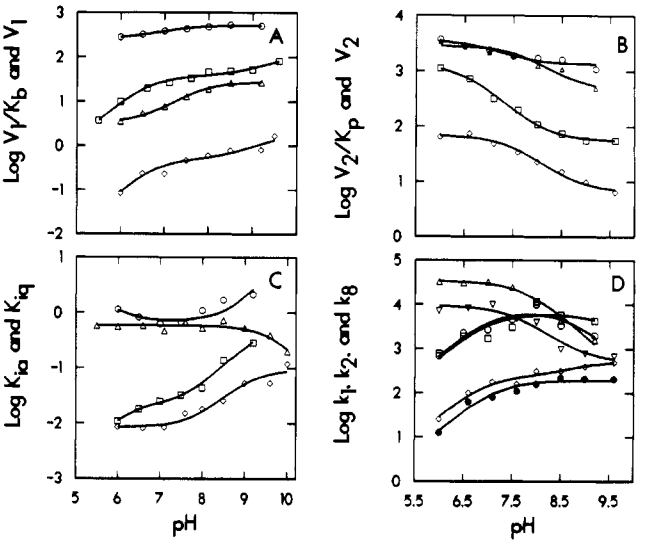


FIGURE 1: pH dependence of kinetic constants for wild-type and Arg-47 enzymes. Values of V and k_2 are s^{-1}; V/K, k_1, and k_8 are in mM^{-1} s^{-1}. (A) V_1 for wild-type (○) and Arg-47 (□) enzymes and V_1/K_b for wild-type (△) and Arg-47 (◇) enzymes. (B) V_2 for wild-type (○) and Arg-47 (□) enzymes and V_2/K_p for wild-type (△) and Arg-47 (◇) enzymes. (C) Dissociation constants for coenzymes are K_{ia} for NAD^+ with wild-type (○) and Arg-47 (△) enzymes and K_{iq} for NADH with the wild-type (□) and Arg-47 (◇) enzymes. (D) k_8, V_2/K_q, is the association rate constant for NADH for wild-type (△) and Arg-47 ((▽) enzymes; k_2, $V_1/K_{ia}/K_a$, is the dissociation rate for NAD^+ for wild-type (□) and Arg-47 (●) enzymes; and k_1, V_1/K_a, is the NAD^+ association rate for wild-type (○) and Arg-47 (◇) enzymes.

these kinetic parameters, the fitting for the mutant required an additional pK value, which was, however, not well determined by the data. (We judge that pK values with standard errors of ±0.3 or larger are not well determined. However, models that gave pK values with errors up to ±1 are listed in Table III since the data showed evidence of the pK value.) The pK values for V_2 and V_2/K_p were essentially unchanged (Figure 1B). NAD^+ binding was weaker above a pK value of about 8.7 with the wild-type enzyme, whereas with the Arg-47 enzyme, K_{ia} was pH independent until above a pK of 9.8, where coenzyme bound more tightly (Figure 1C). The pH dependencies for the NADH dissociation constant, K_{iq}, were similar, but the pK value of 6 for wild-type enzyme seemed to be

Table IV: Comparison of Kinetic Data on His47Arg Enzymes

kinetic constant	this study[a]		Wills[b]		human isozymes[c]	
	His-47	Arg-47	His-47	Arg-47	His-47	Arg-47
K_a (μM)	160	160	240	60	180	7.4
K_b (mM)	20	66	24	24	0.94	0.049
K_p (mM)	1.5	5.1	3.4	2.2	0.24	0.085
K_q (μM)	370	65	140	60	110	6.4
K_{ia} (μM)	1700	720	410	75	340	90
K_{iq} (μM)	140	26	150	50	9.7	0.19
V_1 (s^{-1})	530	50	350[d]	79[d]	6.7	0.15
V_2 (s^{-1})	1700	74	2500[d]	650[d]	65	4.0

[a] In pyrophosphate–phosphate buffer, pH 8.5 and 30 °C. [b] From Wills (1976); 32 mM sodium pyrophosphate buffer, pH 8.8, and 25 °C. [c] Data for the His-47 enzyme are from Yin et al. (1984); for Arg-47, from Bosron et al. (1983); kinetics were determined in 0.1 M sodium phosphate buffer at pH 7.5 and 25 °C. [d] Units/mg.

eliminated. These differences might reflect the substitution, since the wild-type enzyme with an unprotonated His-47 is expected to bind coenzyme more weakly than the Arg-47 enzyme does. However, it is not clear why the pK values should be 8.7 for NAD^+ binding and 6.0 for NADH binding. Furthermore, the rate constants for coenzyme binding and dissociation (Figure 1D) showed no differences in pK values for the predominant effect near pH 7.

Discussion

A Bradykinetic (Slow) Enzyme. A major effect of the substitution of arginine for His-47 in the *S. cerevisiae* alcohol dehydrogenase I enzyme is the decrease in the turnover numbers and catalytic efficiencies in the forward and reverse reactions. The kinetic characteristics can explain the observation of Wills (1976) that yeast harboring the mutant Arg-47 alcohol dehydrogenase resist poisoning by allyl alcohol by maintaining a redox state altered to favor NADH over NAD^+. This mutant was selected from petite yeast cells forced to grow in the presence of allyl alcohol, which is converted to the toxic acrolein by alcohol dehydrogenase (Wills, 1976). We find that the substitution produces an enzyme with a decreased V_2 and a higher K_p in a physiological buffer, at pH 7.3 and 30 °C. Thus, acetaldehyde reduction would be slower, and if anaerobic metabolism is limited by the rate of NAD^+ regeneration, the NADH/NAD^+ ratio would increase. The decrease in NAD^+ should decrease the oxidation of allyl alcohol and allow cells to survive.

Another effect of the substitution is the increased affinity of the Arg-47 enzyme for coenzymes. Wills (1976) reported that the dissociation constants for NAD^+ and NADH with the Arg-47 enzyme decreased about 4-fold at pH 8.8. Our studies at pH 8.5 confirm this observation (Table IV). The magnitudes depend upon pH and buffer and were 2–4-fold lower near pH 7. These changes in kinetic constants for the yeast enzyme are not as large as those for the natural variants of human liver β isozymes, which have Arg-47 in the β_1 enzyme and His in the β_2 enzyme (Bosron et al., 1983; Yin et al., 1984). The effects of the Arg to His substitution on the yeast and liver enzymes illustrate the principle that the same mutation in enzymes with different sequences can yield quantitatively different effects. Since the magnitude of a kinetic constant is determined by the cooperative interactions of the many amino acid residues that participate in binding of substrates, the relative contribution of one residue can vary depending upon the background of other interactions. Some of the energy gained in the presumed stronger ionic interaction with arginine can be lost by a weakening of other enzyme–coenzyme interactions. Local electrostatic effects can modulate the strength of the ionic interaction.

Furthermore, substitution of a histidine residue with an arginine could affect the orientation of coenzyme in the active site and the binding of the alcohol. The dissociation constant for trifluoroethanol is increased 8-fold. Alcohol binds to the hydrogen-bonded network that includes the hydroxyl group of Thr-48, the 2′-hydroxyl group of the nicotinamide ribose, and the imidazole group of His-51. The weaker binding of alcohol could reduce the rates of hydride transfer.

Essential Histidines. Substitution of a critical histidine with an arginine residue should have inactivated the enzyme or at least altered the pH dependencies for some steps in the mechanism. However, the pH dependencies were only slightly affected by the His47Arg substitution, and the pK value of about 7 was still apparent. Some small changes would be expected when a residue at the active site is substituted, since pH profiles result from the interaction of several ionizing groups. Thus, it appears that His-47, by itself, does not determine the pH dependencies, and another ionizing group or system must be involved. His-51 is a good candidate. Indeed, diethyl pyrocarbonate inactivates both the wild-type yeast and the horse liver enzymes about 10 times faster than it reacts with free histidine, and it has been suggested that the enhanced reactivity is due to the hydrogen-bonded system linking His-51 to the catalytic zinc (Hennecke & Plapp, 1983). Furthermore, substitution of His-51 with glutamine decreased activity with ethanol by 10-fold and produced a nearly linear pH dependence for V_1 and V_1/K_b with no obvious pK of 7 (Plapp et al., 1987).

Altered Mechanism. Although the substitution with arginine did not greatly alter pK values, the magnitudes of several rate constants were affected. The isotope effects, the limiting rate for NAD^+ binding, and the magnitudes of the rate constants suggest that the changes could be due to a slow isomerization of the enzyme–NAD^+ complex, a step that appears to be fast in the wild-type enzyme. Transient studies also suggest a relatively slow isomerization for the horse enzyme and show that a group or system (probably including His-51) with a pK of about 7 must be unprotonated for most rapid reaction (Sekhar & Plapp, 1988). The wild-type and Arg-47 mutant yeast enzymes also show pK values of about 7 in the pH dependencies for k_1 and k_2, the rate constants for NAD^+ binding. Arg-47 (and by implication His-47) could not be responsible for this pH dependence, but the substitution could affect the rates of the reactions.

An isomerization of the horse liver enzyme has been well characterized by comparison of the structures of apoenzyme and enzyme complexed with coenzyme and a substrate or substrate analogue. The catalytic domain moves toward the coenzyme-binding domain, and the loop containing residues 292–298 rearranges to accommodate reisudes 46–60 (Eklund et al., 1981, 1984; Colonna-Cesari et al., 1986). Moreover, Arg-47, which is flexible in the free enzyme, becomes fixed in its interaction with the pyrophosphate of the coenzyme. Therefore, Arg-47 participates in the isomerization; His-47 should affect the conformational change differently.

Although there is no direct evidence for the isomerization in yeast alcohol dehydrogenase, fluorometric studies (Karlovič et al., 1976) and the substrate specificity of the Met294Leu mutant of the enzyme (Ganzhorn et al., 1987) suggest that the active enzyme is in the "closed" form. The isomerization of the enzyme–coenzyme complex could be responsible for the ordered mechanism and for creating a binding site for alcohol.

Although Arg-47 stabilizes coenzyme binding (Eklund et al., 1981, 1984; Bosron et al., 1983; Yin et al., 1984), alcohol

dehydrogenases with other amino acid residues at this position occur naturally. Of the 24 known sequences, Arg is found in 6, His in 12, Gly in 5, and Thr in 1. We have shown that the substitution of Arg for His in the yeast enzyme does not substantially strengthen coenzyme binding, even if there is a direct interaction, but rather slows the rate of a conformational change. It seems that amino acid residue 47 is not "essential" for coenzyme binding but is dynamically involved in the catalytic mechanism.

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REFERENCES

Bäcklin, K. I. (1958) *Acta Chem. Scand. 12*, 1279–1285.
Bosron, W. F., Magnes, L. J., & Li, T.-K. (1983) *Biochemistry 22*, 1852–1857.
Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104–137.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.
Colonna-Cessari, F., Perahia, D., Karplus, M., Eklund, H., Brändén, C.-I., & Tapia, O. (1986) *J. Biol. Chem. 261*, 15273–15280.
Cook, P. F., & Cleland, W. W. (1981) *Biochemistry 20*, 1790–1796.
Dickenson, C. J., & Dickinson, F. M. (1975a) *Eur. J. Biochem. 52*, 595–603.
Dickenson, C. J., & Dickinson, F. M. (1975b) *Biochem. J. 147*, 303–311.
Dickenson, C. J., & Dickinson, F. M. (1978) *Biochem. J. 171*, 629–637.
Dickinson, F. M., & Monger, G. P. (1973) *Biochem. J. 131*, 261–270.
Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T., Söderberg, B.-O., Tapia, O., Brändén, C.-I., & Åkeson, Å. (1976) *J. Mol. Biol. 102*, 27–59.
Eklund, H., Samama, J.-P., Wallén, L., Brändén, C.-I., Åkeson, Å., & Jones, T. A. (1981) *J. Mol. Biol. 146*, 561–587.
Eklund, H., Samama, J.-P., & Jones, T. A. (1984) *Biochemistry 23*, 5982–5996.
Eklund, H., Horjales, E., Vallee, B. L., & Jörnvall, H. (1987) *Eur. J. Biochem. 167*, 185–193.
Ganzhorn, A. J., & Plapp, B. V. (1988) *J. Biol. Chem. 249*, 5446–5454.
Ganzhorn, A. J., Green, D. W., Hershey, A. D., Gould, R. M., & Plapp, B. V. (1987) *J. Biol. Chem. 262*, 3754–3761.
Hennecke, M., & Plapp, B. V. (1983) *Biochemistry 22*, 3721–3728.
Janson, C. A., & Cleland, W. W. (1974) *J. Biol. Chem. 249*, 2562–2566.
Jörnvall, H., Eklund, H., & Brändén, C.-I. (1978) *J. Biol. Chem. 253*, 8414–8419.
Karlovič, D., Amiguet, P., Bonner, F. J., & Luisi, P. L. (1976) *Eur. J. Biochem. 66*, 277–284.
Oldén, B., & Pettersson, G. (1982) *Eur. J. Biochem. 125*, 311–315.
Plapp, B. V. (1970) *J. Biol. Chem. 245*, 1727–1735.
Plapp, B. V., Ganzhorn, A. J., Gould, R. M., Green, D. W., & Hershey, A. D. (1987) *Prog. Clin. Biol. Res. 232*, 227–236.
Sekhar, V. C., & Plapp, B. V. (1988) *Biochemistry 27*, 5082–5088.
Wills, C. (1976) *Nature 261*, 26–29.
Wills, C., Kratofil, P., & Martin, T. (1981) in *Genetic Engineering of Microorganisms for Chemicals* (Hollaender, A., Ed.) pp 305–329, Plenum, New York.
Wratten, C. C., & Cleland, W. W. (1963) *Biochemistry 2*, 935–941.
Yin, S.-J., Bosron, W. F., Magnes, L. J., & Li, T.-K. (1984) *Biochemistry 23*, 5847–5853.