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An Aspartate Residue in Yeast Alcohol Dehydrogenase I Determines the Specificity for Coenzyme†

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ABSTRACT: In the three-dimensional structures of enzymes that bind NAD or FAD, there is an acidic residue that interacts with the 2′- and 3′-hydroxyl groups of the adenosine ribose of the coenzyme. The size and charge of the carboxylate might repel the binding of the 2′-phosphate group of NADP and explain the specificity for NAD. In the NAD-dependent alcohol dehydrogenases, Asp-223 (horse liver alcohol dehydrogenase sequence) appears to have this role. The homologous residue in yeast alcohol dehydrogenase I (residue 201 in the protein sequence) was substituted with Gly, and the D223G enzyme was expressed in yeast, purified, and characterized. The wild-type enzyme is specific for NAD. In contrast, the D223G enzyme bound and reduced NAD^+ and $NADP^+$ equally well, but, relative to wild-type enzyme, the dissociation constant for NAD^+ was increased 17-fold, and the reactivity (V/K) on ethanol was decreased to 1%. Even though catalytic efficiency was reduced, yeast expressing the altered or wild-type enzyme grew at comparable rates, suggesting that equilibration of NAD and NADP pools is not lethal. Asp-223 participates in binding NAD and in excluding NADP, but it is not the only residue important for determining specificity for coenzyme.

The structure and mechanism of alcohol dehydrogenase (EC 1.1.1.1, ADH)[1] have been extensively studied (Pettersson, 1987; Eklund & Brändén, 1987). Most alcohol dehydrogenases from different sources use NAD rather than NADP as coenzyme. The three-dimensional structure of horse liver ADH suggests that Asp-223 is important in determining

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[1] Abbreviations: ADH, alcohol dehydrogenase; *Sc*ADH, alcohol dehydrogenase I from *Saccharomyces cerevisiae*; D223G, substitution of Asp-223 with Gly-223; L187A, substitution of Leu-187 with Ala-187 etc.

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this specificity (Ohlsson et al., 1974; Brändén et al., 1975; Eklund et al., 1984). The carboxylate of Asp-223 forms hydrogen bonds with the 2′- and 3′-hydroxyl groups of adenosine ribose and should electrostatically and sterically prevent the binding of NADP with its 2′-phosphate. Horse liver ADH binds NAD^+ 40 times better and NADH 7100 times better than the respective NADP coenzymes (Dalziel & Dickinson, 1965). Dogfish lactate dehydrogenase and lobster glyceraldehyde-3-phosphate dehydrogenase also use NAD and have Asp-53 or Asp-32, respectively, in the position corresponding to Asp-223 in horse liver ADH (Ohlsson et al., 1974). Amino acid sequence alignments of 23 different alcohol dehydrogenases show that Asp-223 is conserved in all NAD-dependent ADHs but is replaced by Gly-223 in the NADP-dependent ADH from *Thermoanaerobium brockii* (Lamed et al., 1981; Peretz & Burstein, 1989).

To investigate the contribution of Asp-223 in determining the specificity for coenzyme, we used site-directed mutagenesis to substitute glycine for the aspartate residue in yeast ADH that corresponds to Asp-223 in the liver enzyme. A three-dimensional model (Ganzhorn et al., 1987; Plapp et al., 1987) of the *Sc*ADH–NADP complex based on the homologous horse liver ADH suggests that this substitution would allow binding of NADP.

Experimental Procedures

Materials. LiNAD, NADH, KNADP, Na_4NADPH, and the Klenow fragment of DNA polymerase I were purchased from Boehringer Mannheim Biochemicals; T4 polynucleotide kinase and all restriction enzymes were from New England Biolabs. Deoxynucleotides, DEAE-Sepharose CL-6B, and octyl-Sepharose CL-4B came from Pharmacia P-L Biochemicals; Amersham supplied radioactive nucleotides, and ethanol-d_6 was from MSD Isotopes. Aldrich provided 2,2,2-trifluoroethanol. Ethanol and acetaldehyde were redistilled before use.

The D223G mutation was produced in the *Sph*I fragment of the ADH I gene (Bennetzen & Hall, 1982) in M13mp18RF phage (Ganzhorn & Plapp, 1988) by the two-primer method of Zoller and Smith (1987) using phenotypic selection against the wild-type DNA containing uracil in place of thymine (Kunkel et al., 1987). The deoxyoligoribonucleotide mutamer was synthesized on a Beckman DNA synthesizer: TGGGTATTGGTGGTGGTGAA (underlines mark the sites of mutation). The mutation was confirmed by sequencing the entire gene (Sanger et al., 1977) in the single-stranded M13 phage. The *Sph*I fragment of the mutated gene was subcloned into the yeast shuttle vector YEp13 (Broach et al., 1979) as described by Ganzhorn and Plapp (1988). Alcohol dehydrogenase was expressed in the yeast strain 302-21#2 (MATa *adh1-11 adr2 leu2 trp1*) after transformation using the lithium acetate procedure (Ito et al., 1983) and selection for growth on media lacking leucine. The purification procedure for the mutant enzyme was the same as that described previously for the wild-type enzyme (Gould & Plapp, 1990). Molecular modeling used FRODO (Jones, 1985) on an Evans and Sutherland PS300 graphics terminal.

Steady-State Kinetics. The general procedures have been described (Ganzhorn et al., 1987). Enzyme activity was determined by measuring the change in absorbance at 340 nm with a Cary 118C spectrophotometer interfaced to an IBM PC/XT computer equipped with a Data Translation 2805 A/D board or with a Beckman DU-7 interfaced to an IBM AT. A FORTRAN program was used to estimate initial velocities by a linear or parabolic fit of the data. A lag phase (up to 0.4 min) was often observed in the initial velocity studies with D223G enzyme, and the velocity was obtained by fitting the portion beyond the lag time.

Table I: Kinetic Constants of Wild-Type and D223G Yeast Alcohol Dehydrogenases[a]

kinetic constants	wild type[b] NAD	D223G NAD	D223G NADP
K_a (mM)	0.16	18	20
K_b (mM)	17	190	120
K_p (mM)	0.74	4[c]	ND[d]
K_q (mM)	0.094	5[c]	ND
K_{ia} (mM)	0.95	16	15
K_{iq} (mM)	0.031	2[c]	ND
V_1 (s^{-1})	360	38	54
V_1/K_a (mM^{-1} s^{-1})	2300	2.1	2.7
V_2 (s^{-1})	1800	300[c]	ND
V_2/K_q (mM^{-1} s^{-1})	19000	60	110[d]
K_{eq} (pM)[e]	12	16	ND
activity (s^{-1})[f]	400	0.75	

[a] Initial velocity studies were performed at pH 7.3 and 30 °C in a physiological buffer of 83 mM potassium phosphate and 40 mM KCl (Cornell, 1983). K_a, K_b, K_p, and K_q are the Michaelis constants for $NAD(P)^+$, ethanol, acetaldehyde, and NAD(P)H, respectively. K_i values are inhibition constants. V_1 and V_2 are the turnover numbers for ethanol oxidation and acetaldehyde reduction. The $NAD(P)^+$ concentrations were varied in a range from one-eleventh to the K_m value in initial velocity studies and from one-eighth to the K_m value in product inhibition studies. Standard errors of fits were less than 20%, except for the values marked with c, which were 30–80%. [b] Data from Gould and Plapp (1990). [c] The kinetic constants for acetaldehyde reduction were determined from assays at 366 nm using a 5-mm path-length cuvette. The highest NADH concentration was 0.85 mM. Standard errors ranged from 30 to 80%. Three experiments gave comparable values. [d] K_p and K_q could not be determined because of the high K_m for NADPH. The attempts gave imprecise estimates of K_p, K_q, and K_{iq} values in the millimolar range. Nevertheless, V_2/K_q could be estimated with an error less than 10%. [e] Equilibrium constant calculated from $K_{eq} = V_1K_pK_{iq}[H^+]/V_2K_bK_{ia}$. [f] Turnover number in standard assay at 30 °C (Plapp, 1970). Enzyme concentration for D223G *Sc*ADH was calculated from A_{280} by using 1.26 mg^{-1} mL cm^{-1} as the extinction coefficient.

Results

Protein Properties. The wild-type and D223G enzymes behaved similarly during purification. Final preparations appeared nearly homogeneous by polyacrylamide gel electrophoresis in the presence (Laemmli, 1970) or absence of sodium dodecyl sulfate. Electrophoresis under nondenaturing conditions showed that the D223G enzyme migrated more slowly toward the anode than did the wild-type enzyme, as would be expected because of the loss of a negative charge. The D223G enzyme retained more than 70% of its activity after 1 month at 4 °C.

Steady-State Kinetics. The initial velocity patterns for the D223G enzyme fit a sequential Bi mechanism for both ethanol oxidation and acetaldehyde reduction using NAD or NADP as coenzymes. The kinetic parameters (Table I) showed significant effects on the activity with NAD(H) as coenzyme. The turnover numbers were decreased 10-fold for ethanol oxidation and 6-fold for acetaldehyde reduction. The D223G substitution also decreased the affinities for NAD(H); K_{ia} and K_{iq}, which are dissociation constants for NAD^+ and NADH, increased 17- and about 65-fold, respectively; Michaelis constants for NAD^+ (K_a) and ethanol (K_b) increased by 100- and 10-fold, respectively. The kinetic parameters with NAD^+ are self-consistent since the equilibrium constant calculated from the Haldane relationship agreed well with the experimentally determined value of 10 pM (Sund & Theorell, 1963).

The D223G substitution also changed the specificity for the coenzyme so that NADP and NAD were used equally well. Kinetic parameters obtained for ethanol oxidation with

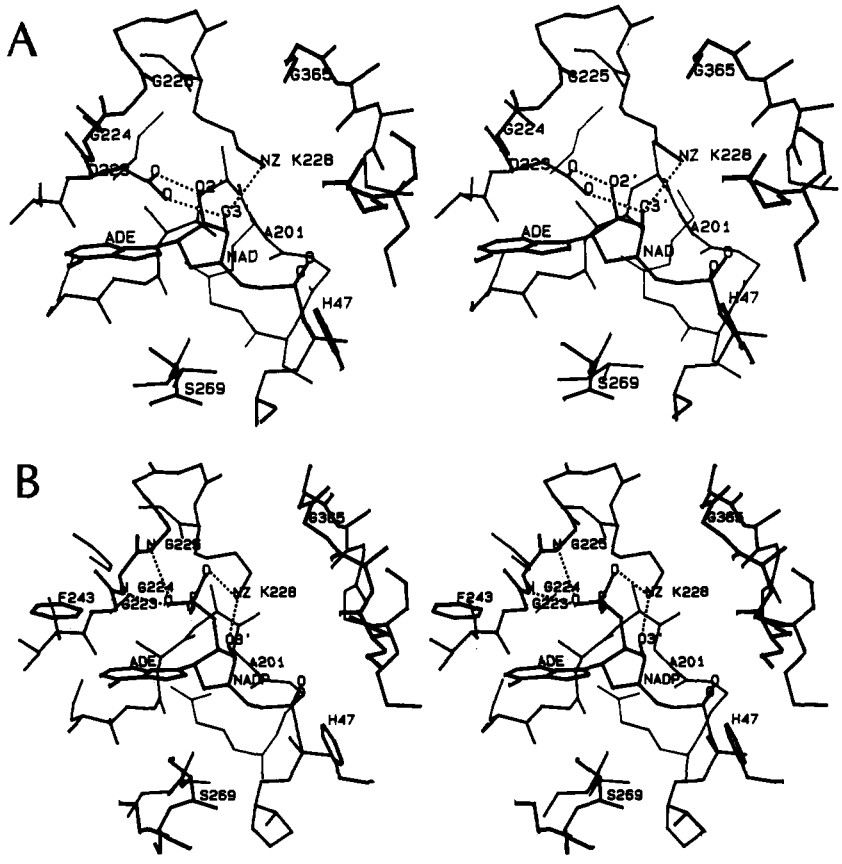

FIGURE 1: Coenzyme binding site of wild-type and D223G enzyme in a model of yeast alcohol dehydrogenase I. The model was based on the structure of horse liver enzyme complexed with NAD^+ and *p*-bromobenzyl alcohol (Eklund et al., 1982). Panel A shows the ADP moiety of NAD interacting with wild-type *Sc*ADH. Asp-223 and Lys-228 form hydrogen bonds with 2′- or 3′-hydroxyl groups (dashed lines). Panel B presents the proposed model of D223G enzyme complexed with NADP. The 2′-phosphate fits into the coenzyme binding pocket. Lys-228 interacts ionically with the negatively charged phosphate group. Hydrogen bonds could form between the 2′-phosphate and peptide backbone amides.

$NADP^+$ were similar to those with NAD^+. Wild-type *Sc*ADH is specific for NAD^+ (Dalziel & Dickinson, 1965; Tsai et al., 1989). We found that wild-type enzyme gave less than 1% of the activity with $NADP^+$ as it did with NAD^+ when assayed with 10 mM NAD^+ or $NADP^+$ and 500 mM ethanol at pH 7.3.

The mechanism of the D223G enzyme was investigated by use of kinetic isotope effects and by product and dead-end inhibition studies. Wild-type *Sc*ADH has a preferred ordered mechanism with NAD^+ binding before ethanol (Ganzhorn et al., 1987), and this is reflected in the deuterium isotope effects (Table II), where ${}^DV_1/K_a$ is smaller than ${}^DV_1/K_b$ (Northrop, 1982). The D223G enzyme, using NAD^+, had isotope effects similar to those for wild-type enzyme, but ${}^DV_1/K_a$ was more similar to ${}^DV_1/K_b$, indicating a somewhat more random mechanism. Acetaldehyde appeared to be a competitive inhibitor against varied concentrations of ethanol with fixed concentrations of NAD^+ or $NADP^+$, trifluoroethanol was a noncompetitive inhibitor against NAD^+, and acetaldehyde was noncompetitive against NAD^+ or $NADP^+$. [Kinetic studies with NAD(P)H were not feasible, due to the high K_m for reduced coenzyme.] Thus, the mechanism is not strictly ordered or simple rapid equilibrium random, but appears to be more random than is the mechanism for wild-type enzyme. The relatively small isotope effect on V_1 could indicate that the enzyme–coenzyme–ethanol ternary complex isomerizes before hydride transfer, as suggested previously for the D49N enzyme (Ganzhorn & Plapp, 1988).

Growth of Transformed Yeast. Although the D223G *Sc*ADH has dual coenzyme specificity and is less active on ethanol with NAD^+ than wild-type enzyme, it still allows transformed yeast to grow well anaerobically on glucose. The transformed yeast with D223G enzyme had the same doubling time as did yeast with wild-type enzyme when grown aerobically in YPD medium (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose) or in a minimal medium containing 0.67% yeast nitrogen base, 2% glucose, and 20 mg/L of tryptophan; growth was a little slower in YPD with antimycin A (1 mg/L). Since the enzyme is being expressed from a multicopy plasmid, the growth rate does not directly test for a critical role for Asp-223. Nevertheless, the D223G enzyme is active enough to support fermentation. The effect of D223G *Sc*ADH on the equilibration between cytosolic NAD and NADP pools does not seem to influence greatly the yeast growth.

Discussion

The detailed interactions of NAD with horse liver alcohol dehydrogenase have been described (Eklund et al., 1984). Molecular modeling of the yeast enzyme was readily accomplished because of the homology of the enzymes. Figure 1 compares the binding of NAD to wild-type *Sc*ADH and of NADP to mutant D223G *Sc*ADH. In the wild-type *Sc*ADH (Figure 1A), the carboxylate of Asp-223 forms hydrogen bonds with the 2′- and 3′-hydroxyl groups of the adenosine ribose of NAD. The amino group of Lys-228 could also form a hydrogen bond with the 3′-hydroxyl. It is clear in Figure 1A that the 2′-phosphate group in NADP would prevent by steric hindrance the formation of hydrogen bonds between Asp-223 and the hydroxyl group and would generate charge repulsion

Table II: Deuterium Isotoped Effects for Ethanol Oxidation[a]

enzyme	coenzyme	isotope effect[b] $^DV_1$	$^DV_1/K_a$	$^DV_1/K_b$
wild type[c]	NAD^+	1.8	1.8	3.2
D223G	NAD^+	1.5	2.2	2.5
D223G	$NADP^+$	1.1	1.6	2.2

[a] Initial velocity studies were performed at pH 7.3, 30 °C, in 83 mM potassium phosphate and 40 mM KCl (Cornell, 1983). The concentration of ethanol or ethanol-d_5 was varied from 56 to 500 mM and that of $NAD(P)^+$ from 2.2 to 20 mM. V_1, K_a, and K_b were obtained from a fit to the SEQUEN program (Cleland, 1979). Errors for the isotope effects were <10%. [b] Nomenclature of Northrop (1982). K_a and K_b are Michaelis constants for $NAD(P)^+$ and ethanol, respectively. [c] Data from Ganzhorn and Plapp (1987).

between the phosphate and Asp-223.

In D223G *Sc*ADH (Figure 1B), on the other hand, NADP could fit into the coenzyme binding pocket, with the 2′-phosphate group in the space that was originally occupied by the side chain of Asp-223. The amino group of Lys-228 could interact ionically with the 2′-phosphate. Bound NADP could be further stabilized by hydrogen bonding with the amide groups of Gly-224 and Gly-225 in the peptide backbone. From this molecular modeling study, we expected the D223G substitution of *Sc*ADH to decrease the affinity and specificity for NAD, and to increase the specificity for NADP, as compared to wild-type *Sc*ADH. The expected result was obtained. The observed 17–65-fold increase in K_{ia} and K_{iq} for the NAD-linked reaction with the D223G substitution corresponds to a loss of 1.7–2.5 kcal/mol in the free energy of binding. The magnitude correlates with the loss of two hydrogen bonds between Asp-223 and the 2′- and 3′-hydroxyl groups of adenosine ribose. Asp-223 clearly contributes to the determination of coenzyme specificity.

The D223G substitution also substantially decreased activity with NAD, whereas it increased the activity with NADP. The mechanism changed from preferred ordered to a more random one for the reaction with NAD^+. Horse liver alcohol dehydrogenase changes conformation upon coenzyme binding (Eklund et al., 1984). This rearrangement may account for the ordered mechanism. Although there is no direct evidence that coenzyme binding in the yeast enzyme causes similar conformational changes, previous studies suggested that the ternary complexes of the enzyme are in the "closed" form (Ganzhorn & Plapp, 1988). The D223G substitution could make the transition from the open to the closed conformation less favored. Therefore, NAD^+ and ethanol may bind randomly to the open form, but with lower affinity. The resulting ternary complex may have to undergo further rearrangements before hydride transfer occurs. This additional isomerization step produces an internal commitment factor that could reduce the magnitude of the observed isotope effect on turnover (Cook & Cleland, 1981). Thus, the D223G substitution also appears to affect the enzyme dynamics.

Since the D223G enzyme binds $NADP^+$ relatively weakly and reacts less efficiently with $NADP^+$ than the wild-type enzyme does with NAD^+, other amino acid residues are also involved in determining the specificity for coenzyme. In NADP-dependent enzymes, one or more positively charged amino acid residues (usually arginine) bind the 2′-phosphate (Huang et al., 1990; Scrutton et al., 1990). The NADP-dependent ADH from *Thermoanaerobium brockii* binds $NADP^+$ (Tsai et al., 1989) about 300-fold better than D223G *Sc*ADH does, and the amino acid sequence alignment places Gly-223 and Arg-225 near the 2′-phosphate (Peretz & Burstein, 1989).

Many enzymes that bind NAD or FAD have a similar protein folding pattern (Rossmann et al., 1975), which has been further described as an "ADP-binding, $\beta\alpha\beta$ fold" (Wierenga et al., 1985; Hanukoglu & Gutfinger, 1989). The NAD or FAD binding sites have a nearly universally conserved Gly-X-Gly-X-X-*Gly* sequence (199–204 in horse liver ADH numbering system) connecting the first β-strand to the α-helix and a conserved negatively charged residue at the COOH-terminal of the second β-strand (Asp-223 in ADH). In contrast, the binding sites for NADP may not have the $\beta\alpha\beta$ fold (Matthews et al., 1977; Fita & Rossmann, 1985; Karplus et al., 1991). However, for at least one family of enzymes, the flavoprotein disulfide oxidoreductases, the NADP binding site has the ADP-binding $\beta\alpha\beta$ fold with a conserved Gly-X-Gly-X-X-*Ala* sequence and one or more positively charged residues at the COOH-terminal of the second β-strand (Karplus & Schulz, 1987; Scrutton et al., 1990).

Some other mutagenesis studies have probed the specificity for nicotinamide coenzymes (Table III). The substitution of Asp-53 in lactate dehydrogenase with serine reduced the catalytic efficiency (k_{cat}/K_m) with NADH by a factor of 3 but did not increase the efficiency with NADPH (Feeney et al., 1990). The L187A and P188S substitutions in glyceraldehyde-3-phosphate dehydrogenase produced a mutant enzyme that had 100-fold less reactivity with NADP than with NAD (Corbier et al., 1990). Seven mutations in the ADP-binding $\beta\alpha\beta$ fold of NADP-dependent glutathione reductase produced an enzyme with comparable efficiency (k_{cat}/K_m) with NADH or NADPH (Scrutton et al., 1990). These results support the principles that (1) many residues contribute to binding and specificity for coenzyme and (2) a single mutation in different structures can produce effects with different magnitudes. However, the data presented in the cited studies are often insufficient to determine the affinity for coenzymes, and the limited knowledge about the enzyme mechanisms makes it difficult to interpret the V/K_m values.

Table III: Kinetic Parameters of Native and Mutant Enzymes with NAD and NADP

enzyme	K_m (mM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
coenzyme	NADH	NADPH	NADH	NADPH	NADH	NADPH
*Bs*LDH[a] wild type	0.015	0.06	170	30	11000	500
*Bs*LDH D53S	0.04	0.1	145	55	3600	550
*Hs*GSR[b] wild type	2.0	0.022	11	270	5.5	12000
*Hs*GSR Septuple	0.086	0.22	35	11	410	50
coenzyme	NAD^+	$NADP^+$	NAD^+	$NADP^+$	NAD^+	$NADP^+$
*Bs*GAPDH[c] wild type	0.15	NA[d]	280	NA	1900	NA
*Bs*GAPDH L187A/P188	0.35	7.1	280	58	800	8.2
*Sc*ADH wild type[e]	0.16	NA	360	NA	2300	NA
*Sc*ADH D223G	18	20	38	54	2.1	2.7

[a] Lactate dehydrogenase from *Bacillus stearothermophilus*, assayed in the presence of fructose 1,6-bisphosphate (Feeney et al., 1990). [b] Human glutathione reductase (Scrutton et al., 1990). The mutant enzyme had seven substitutions: A179G/A183G/V197E/R198M/K199F/H200D/R204P. [c] Glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* (Corbier et al., 1990). [d] Not available. [e] Data from Gould and Plapp (1990).

With yeast ADH, the single D223G mutation changed the coenzyme specificity of NAD-dependent *Sc*ADH so that NADP and NAD were used equally well. This study confirms that Asp-223 in alcohol dehydrogenase participates in determining the specificity for NAD, by facilitating binding of NAD and excluding NADP. In order to completely reverse the coenzyme specificity from NAD-dependent to NADP-dependent, further mutations are needed.

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