Complete Reversal of Coenzyme Specificity by Concerted Mutation of Three Consecutive Residues in Alcohol Dehydrogenase*

Received for publication, July 10, 2003, and in revised form, August 4, 2003 Published, JBC Papers in Press, August 4, 2003, DOI 10.1074/jbc.M307384200

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Gastric tissues from amphibian Rana perezi express the only vertebrate alcohol dehydrogenase (ADH) that is specific for NADP(H) instead of NAD(H). In the crystallographic ADH8-NADP+ complex, a binding pocket for the extra phosphate group of coenzyme is formed by ADH8-specific residues Gly223-Thr224-His225, and the highly conserved Leu200 and Lys228. To investigate the minimal structural determinants for coenzyme specificity, several ADH8 mutants involving residues 223 to 225 were engineered and kinetically characterized. Computer-assisted modeling of the docked coenzymes was also performed with the mutant enzymes and compared with the wild-type crystallographic binary complex. The G223D mutant, having a negative charge in the phosphate-binding site, still preferred NADP(H) over NAD(H), as did the T224I and H225N mutants. Catalytic efficiency with NADP(H) dropped dramatically in the double mutants, G223D/T224I and T224I/H225N, and in the triple mutant, G223D/T224I/H225N (kcat/Km,NADPH = 760 mM−1 min−1), as compared with the wild-type enzyme (kcat/Km,NADPH = 133,330 mM−1 min−1). This was associated with a lower binding affinity for NADP+ and a change in the rate-limiting step. Conversely, in the triple mutant, catalytic efficiency with NAD(H) increased, reaching values (kcat/Km,NADH = 155,000 mol−1 min−1) similar to those of the wild-type enzyme with NADP(H). The complete reversal of ADH8 coenzyme specificity was therefore attained by the substitution of only three consecutive residues in the phosphate-binding site, an unprecedented achievement within the ADH family.

Coenzyme specificity is an important property of NAD(P)-dependent oxidoreductases that is linked to their metabolic function. Thus the type of coenzyme, NAD+ or NADP+, often distinguishes between enzymes involved in alternative pathways (e.g. oxidative versus reductive or degradative versus biosynthetic). Because NAD+ and NADP+ only differ structurally in the phosphate group esterified at the 2’ position of adenosine ribose, dehydrogenases must possess a limited number of residues to discriminate between the two coenzyme types. Moreover, among dehydrogenases from a given enzyme family, the same protein fold is often used to bind either coenzyme type and even some enzymes show dual activity, meaning that they can use both coenzymes with similar efficiency (1).

A rather unique NADP-dependent alcohol dehydrogenase (ADH8)1 was discovered in the gastric tissues of amphibians (2). ADH8 belongs to the medium chain dehydrogenase/reductase (MDR) superfamily and is phylogenetically related to the NAD-dependent vertebrate ADH family. This enzyme is active with ethanol and functionally may participate in the reduction of retinal to retinol (kcat/Km,all-trans-retinal = 33,750 mM−1 min−1). Recently, the three-dimensional structure of the ADH8-NADP+ binary complex was determined at 1.8-Å resolution (3). Structural data suggested that the preference for NADP(H) depends on the segment Gly223-Thr224-His225 (Asp222-Ile/Leu224-Asn225 in most vertebrate NAD-dependent ADHs; Ref. 4), which together with Leu200 and Lys228, define a binding pocket for the terminal phosphate group of NADP(H). Henceforth residue numbering will correspond to that of horse ADH1 with the Swiss Prot entry P00327. Interestingly, NADP-dependent ADHs from distantly related microorganisms (5–7), also have a glycine and two more hydrophilic residues at the positions corresponding to 223, 224, and 225, respectively. In ADHs, residue 223 is located at the C-terminal end of the second β-strand of the Rossmann fold (8) and classically is considered as determinant for coenzyme specificity. The substitution D223G, as found in ADH8, would avoid the possible steric and electrostatic hindrances because of the extra phosphate group of NADP(H). In fact, different attempts to switch the coenzyme specificity in medium chain ADHs have been focused on mutations involving residue 223 (9–12). However, full reversal of coenzyme specificity, in terms of having a mutant enzyme as catalytically efficient as the wild type, has been rarely achieved. This implies that conversion of coenzyme specificity may require multiple substitutions in the coenzyme-binding domain. Other residues found in ADH8, such as Thr224 and His225, which are making hydrogen bonds with the oxygen atoms from the terminal phosphate group (3), could also be important in defining coenzyme specificity of ADH8.

In the present work, we have investigated, by means of site-directed mutagenesis, steady-state kinetics, and computer modeling, the individual and combined contribution of the adjacent residues Gly223-Thr224-His225 of ADH8 to coenzyme specificity. Moreover, because the ADH8 sequence is only 30% divergent from the closest NAD-dependent ADH structure (Rana perezi ADH1, Ref. 13), it was a suitable candidate for attempting the redesign of coenzyme specificity.

* This work was supported by grants from the Dirección General de Investigación Científica: BMC2000-0132 (to J. F.), BMC2002-02659 (to X. P.), and BIO2002-04419-C02-01 (to I. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: ADH, alcohol dehydrogenase; Kcat, catalytic efficiency with coenzyme; Kd for coenzyme; Ktrans for ethanol; MDR, medium chain dehydrogenase/reductase.
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The degree of purity for each protein was assessed by means of using the conditions previously described for the wild-type enzyme (14). The purification was monitored by Coomassie Blue staining. The protein concentration was determined using the Bio-Rad assay, based on the method of Bradford (16).

Enzyme Activity Assays—Alcohol dehydrogenase activity was determined at 25 °C by monitoring the change in absorbance at 340 nm, using a Cary 400 Bio (Varian) spectrophotometer. One unit of activity corresponds to 1 μmol of reduced coenzyme formed or utilized per min, based on an absorption coefficient of 6220 m⁻¹ cm⁻¹ at 340 nm for NADH or NADPH. Cuvette path length was 1 cm in oxidation reactions and 0.2 cm in reduction reactions. Alcohol oxidation was performed in 0.1 M glycine/NaOH, pH 10.0, or in 0.1 M sodium phosphate/NaOH, pH 7.5, using 1 mM ethanol or 1.5 mM octanol as a substrate, respectively. For aldehyde reduction, 0.1 M sodium phosphate/NaOH, pH 7.5, and 0.2 mM nitrobenzaldehyde were used. When saturation could be reached, kinetic parameters were obtained from activity measurements with substrate or coenzyme concentrations that ranged from 0.1 Xₐ to 10 Xₐ. Each individual rate measurement was run in duplicate. Three determinations were performed for each kinetic constant. Kinetic constants were obtained with the non-linear regression program Grafit 5.0 (Erithacus Software Ltd.) and expressed as the mean ± S.D. Bisubstrate kinetic constants were calculated by fitting data to a Bi-Bi sequential mechanism (17). The standard assays were performed using 1 mM ethanol as a substrate in 0.1 M glycine/NaOH, pH 10.0, and 1.2 mM NADP⁺ for wild-type and G223D mutants; 2.4 mM NADP⁺ for T224I and H225N mutants; 0.4 mM NAD⁺ for G223D/T224I and H225N mutants; and 2.4 mM NAD⁺ for G223D/T224I and H225N mutants.

Deuterium Kinetic Isotope Effect—Kinetic constants for NAD⁺ and NADP⁺ were determined in 0.1 M Gly/NaOH, pH 10.0, with 1 mM ethanol-d₂ (Merck) or ethanol as a substrate at different coenzyme concentrations, depending on the enzyme assayed. For the G223D/T224I and G223D/T224I/H225N mutants, saturation with NADP⁺ could not be achieved, and thus a single concentration of 4.8 mM NADP⁺ was used to measure enzyme activity. Assay buffer and ethanol-d₂ solution were prepared in deuterated water.

Structural Modeling of the Variants—The structures of all the variants were modeled using the available coordinates of the binary complex ADHs-NAD⁺ (Protein Data Bank entry 1POF, Ref. 3), where the corresponding substituted residues were introduced with the graphics program O (18). Conformations of the replaced residues were initially assumed to be the rotamers that presented less steric constraints. The geometry of the models was then regularized and refined throughout cycles of energy minimization with the program XPLOR (19). The best fit was given by the models containing the mutations.

RESULTS

Our study focused on residues 223 to 225 of ADH8, with the aim of testing their role in the phosphate-binding site and attempting the switch of coenzyme specificity. The obvious choice was to mutate Gly²²³, Thr²²⁴, and His²²⁵ to Asp, Ile, and Asn, respectively, because the sequence Asp²²³/Ile/Leu²²⁴, Asn²²⁵ is well conserved among vertebrate NAD-dependent ADHs (4). Therefore, single ADH8 mutants, G223D, T224I, and H225N, double mutants, G223D/T224I and T224I/H225N, and the triple mutant, G223D/T224I/H225N, were generated by site-directed mutagenesis. Site-directed Mutagenesis—G223D, T224I, H225N, G223D/T224I, and T224I/H225N mutants were obtained using the wild-type ADH8 cDNA cloned into pGEX 4T-2 as a template. The number of mutations for each protein was assessed by means of using the conditions previously described for the wild-type enzyme (14). The purification was monitored by Coomassie Blue staining. The protein concentration was determined using the Bio-Rad assay, based on the method of Bradford (16).

Experimental Procedures

Site-directed Mutagenesis—G223D, T224I, H225N, G223D/T224I, and T224I/H225N mutants were obtained using the wild-type ADH8 cDNA cloned into pGEX 4T-2 as a template. The mutations were introduced by sequential steps of PCR. In a first round, two reactions, 1 and 2, were performed with the following primers: 1) RanaL primer, 5'-CTTATAGGATCATGTGACCTCGGGGAAAGAT-3'; and one of the antisense primers containing the mutations (underlined): 223R, 5'-GGCGTGACCTCCCTATAATACG-3'; 224R, 5'-GTCTTTAGGATATCAACCCCTATAATAC-3'; 225R, 5'-GTCTTTAGGATATCAACCCCTATAATAC-3'; 2) RanaR primer, 5'-CCACCTGAATTCTTAGTATA-3', and one of the antisense primers containing the mutations (underlined): 223R, 5'-GGCGTGACCTCCCTATAATACG-3'; 224R, 5'-GTCTTTAGGATATCAACCCCTATAATAC-3'; 225R, 5'-GTCTTTAGGATATCAACCCCTATAATAC-3'; 2) RanaR primer, 5'-CCACCTGAATTCTTAGTATA-3', and one of the antisense primers containing the mutations. In a final amplification step, purified overlapping PCR products were used as templates, using RanaL and RanaR primers. All reactions were performed in a DNA thermal cycler (MJ Research) with High Fidelity DNA polymerase (Roche Diagnostics) under the following conditions: hot start at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 68 °C for 12 min (11 cycles to step 2), and a final extension step. All PCR products were purified and cloned into pGEX 4T-2 as for wild-type cDNA (14).

The G223D/T224I and G223D/T224I/H225N mutants were obtained using a method based on the QuikChange XL site-directed mutagenesis kit (Stratagene). Full-length pGEX 4T-2, containing G223D and T224I/H225N cDNAs, was used as templates to obtain the double and triple mutants, respectively. Mutagenesis was performed by means of a single PCR using an antisense primer containing the mutations (22222222R, 5'-GTCTTTATATTGATATCAACCCCTATAATAC-3' or TMR, 5'-GTCTTTATATTGATATCAACCCCTATAATAC-3') and primers containing either sense or antisense PCR conditions were the same as above except for the use of Expand Long Template polymerase (Roche). PCR products were incubated with DpnI at 37 °C for 60 min. This treatment ensured the digestion of the dam-methylated parental strand (15). The resulting nicked circular mutagenic strands were transformed into Escherichia coli BL21, where bacterial DNA ligase repaired the nick and allowed normal replication to occur. Prior to expression, all mutated DNAs were completely sequenced, to ensure that unwanted mutations were absent.

Protein Expression and Purification—All mutants were expressed using the conditions previously described for the wild-type enzyme (14). Batch-wise purification of wild-type and T224I enzymes followed identical procedures (14). For G223D, H225N, G223D/T224I, T224I/H225N, and G223D/T224I/H225N mutants, the only difference was the matrix used in the last affinity chromatography step (Cibacron Blue 3-GA; Sigma). The degree of purity for each protein was assessed by means of SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. Protein concentration was determined using the Bio-Rad assay, based on the method of Bradford (16).

Enzyme Activity Assays—Alcohol dehydrogenase activity was determined at 25 °C by monitoring the change in absorbance at 340 nm, using a Cary 400 Bio (Varian) spectrophotometer. One unit of activity corresponds to 1 μmol of reduced coenzyme formed or utilized per min, based on an absorption coefficient of 6220 m⁻¹ cm⁻¹ at 340 nm for NADH or NADPH. Cuvette path length was 1 cm in oxidation reactions and 0.2 cm in reduction reactions. Alcohol oxidation was performed in 0.1 M glycine/NaOH, pH 10.0, or in 0.1 M sodium phosphate/NaOH, pH 7.5, using 1 mM ethanol or 1.5 mM octanol as a substrate, respectively. For aldehyde reduction, 0.1 M sodium phosphate/NaOH, pH 7.5, and 0.2 mM nitrobenzaldehyde were used. When saturation could be reached, kinetic parameters were obtained from activity measurements with substrate or coenzyme concentrations that ranged from 0.1 Xₐ to 10 Xₐ. Each individual rate measurement was run in duplicate. Three determinations were performed for each kinetic constant. Kinetic constants were obtained with the non-linear regression program Grafit 5.0 (Erithacus Software Ltd.) and expressed as the mean ± S.D. Bisubstrate kinetic constants were calculated by fitting data to a Bi-Bi sequential mechanism (17). The standard assays were performed using 1 mM ethanol as a substrate in 0.1 M glycine/NaOH, pH 10.0, and 1.2 mM NADP⁺ for wild-type and G223D mutants; 2.4 mM NADP⁺ for T224I and H225N mutants; 0.4 mM NAD⁺ for G223D/T224I and H225N mutants; and 2.4 mM NAD⁺ for G223D/T224I and T224I/H225N mutants.

Deuterium Kinetic Isotope Effect—Kinetic constants for NAD⁺ and NADP⁺ were determined in 0.1 M Gly/NaOH, pH 10.0, with 1 mM ethanol-d₂ (Merck) or ethanol as a substrate at different coenzyme concentrations, depending on the enzyme assayed. For the G223D/T224I and G223D/T224I/H225N mutants, saturation with NADP⁺ could not be achieved, and thus a single concentration of 4.8 mM NADP⁺ was used to measure enzyme activity. Assay buffer and ethanol-d₂ solution were prepared in deuterated water.

Structural Modeling of the Variants—The structures of all the variants were modeled using the available coordinates of the binary complex ADHs-NAD⁺ (Protein Data Bank entry 1POF, Ref. 3), where the corresponding substituted residues were introduced with the graphics program O (18). Conformations of the replaced residues were initially assumed to be the rotamers that presented less steric constraints. The geometry of the models was then regularized and refined throughout cycles of energy minimization with the program XPLOR (19).
site-directed mutagenesis. The expression level for the wild-type enzyme and mutants was ~30 mg/liter of culture. All enzymes were purified to homogeneity with a 10% yield, and they were stable for several weeks when stored at 4 °C.

**Steady-state Kinetics of Wild-type and Mutant Enzymes**—Steady-state kinetic constants of wild-type and substituted ADH8 with NAD⁺ and NADP⁺, at pH 10.0 and 7.5, determined under saturating concentrations of substrate, are presented in Tables I and II, respectively. Kinetic constants in the reductive direction for NADH and NADPH, at pH 7.5, are shown in Table III.

In the wild-type recombinant ADH8, the kinetic constants were similar to those previously reported for the native enzyme purified from frog stomach tissue (2). At a given pH and with the same substrate, the \( k_{\text{cat}}/K_m \) values attained with NADP(H) and NAD(H) were similar, suggesting a common rate-limiting step, independent of the cofactor used. By contrast, \( K_m \) values for NADP(H) were 10–40-fold lower than those for NAD(H). This resulted in an enzyme much more specific for NADP(H), especially at pH 7.5.

Regarding the effect of substitutions on coenzyme kinetics, most of the observations made at pH 10.0 (Table I) could be confirmed at pH 7.5, for both the oxidation (Table II) and the reduction (Table III) reactions. This suggests that the introduced mutations had a similar effect on each coenzyme kinetics, independently of pH and whether the cofactor used was in its oxidized or reduced form. However, the substitutions did have a different effect on whether the cofactor used was NADP(H) or NAD(H).

Single mutations provided a positive effect on NAD(H) kinetics, with a moderate increase in \( k_{\text{cat}}/K_m \) values. T224I showed the best catalytic efficiency for a single mutant, whereas H225N displayed the lowest catalytic efficiency, mostly due to its high \( K_m \) values for this coenzyme. In all single mutants, \( k_{\text{cat}} \) values for NAD(H) remained essentially constant, which is suggestive of a common rate-limiting step. At any pH employed, none of the three mutants showed a reversal of coenzyme specificity. Only the mutant T224I showed a very similar catalytic efficiency with the two cofactors at pH 10.0 (Table I).

A double mutation, G223D/T224I or T224I/H225N, was the minimal change to show a clear-cut effect on coenzyme specificity, both at pH 7.5 and 10.0. The effect was most marked on NADP(H) kinetic constants, especially in the G223D/T224I mutant. This mutant could not be saturated with NADP(H) and the catalytic efficiency with NADP(H) decreased to about the same order of magnitude as those of the double mutants. Overall, the triple substitution produced a complete reversal of coenzyme specificity, both at pH 7.5 and 10.0. In comparison with the double mutants, the effect was most marked on NADP(H) increased 30–50-fold with respect to that of the wild-type enzyme, and it was even higher than the \( k_{\text{cat}}/K_m \) value of the wild-type enzyme with NADP(H). This was mainly provoked by a decrease in \( K_m \) values for NAD(H), which paralleled an increase in the affinity for the coenzyme (see below). On the other hand, the triple mutant could not be saturated by NADP(H) and the catalytic efficiency with NADP(H) decreased to about the same order of magnitude as those of the double mutants. Overall, the triple
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**Table III**

Kinetic constants of wild-type and mutant ADH8 with NADH and NADPH at pH 7.5

Activities were measured with 0.2 mM m-nitrobenzaldehyde and 0.1 mM sodium phosphate/NaOH at pH 7.5.

| Enzyme          | Coenzyme | \(K_m\) | \(k_{cat}\) | \(k_{cat}/K_m\) |
|-----------------|----------|---------|-------------|-----------------|
|                 |          | [mM]    | [min\(^{-1}\)] | [min\(^{-1}\) [mM\(^{-1}\)]] |
| Wild-type       | NADPH    | 0.030 ± 0.006 | 4.000 ± 10 | 133.3 ± 26.670 |
|                 | NADH     | 0.44 ± 0.07  | 2.455 ± 190 | 5.58 ± 990      |
| G223D           | NADPH    | 0.110 ± 0.008 | 1.765 ± 120 | 16.05 ± 1.590   |
|                 | NADH     | 0.150 ± 0.006 | 1.610 ± 50  | 10.73 ± 550     |
| T224I           | NADPH    | 0.050 ± 0.001 | 1.920 ± 80  | 38.40 ± 1.720   |
|                 | NADH     | 0.110 ± 0.008 | 1.920 ± 10  | 17.45 ± 1.260   |
| H225N           | NADPH    | 0.080 ± 0.003 | 4.605 ± 110 | 57.56 ± 2.535   |
|                 | NADH     | 0.660 ± 0.016 | 2.350 ± 70  | 3.56 ± 140      |
| G223D/T224I     | NADPH    | NS\(^a\)   | NS          | 89 ± 7\(^b\)     |
|                 | NADH     | 0.040 ± 0.002 | 1.500 ± 190 | 37.55 ± 5.100   |
| T224I/H225N     | NADPH    | NS          | NS          | 1.250 ± 50\(^b\)  |
|                 | NADH     | 0.150 ± 0.016 | 2.805 ± 100 | 18.70 ± 2.100   |
| G223D/T224I/H225N | NADPH  | NS          | NS          | 76 ± 21\(^b\)    |
|                 | NADH     | 0.040 ± 0.001 | 6.200 ± 150 | 155.0 ± 4.100   |

\(^a\) NS, no saturation up to 1.2 mM NADPH.

\(^b\) \(k_{cat}/K_m\) was calculated as the slope of the linear plot of \(V\) versus [S].

**Table IV**

Bisubstrate kinetics of wild-type and mutant ADH8 with NAD\(^+\) and NADPH\(^+\) at pH 10.0

Activities were measured in 0.1 M glycine/NaOH at pH 10.0, with ethanol as a substrate. Standard errors of fits were less than 20%, except for the indicated values.

| Enzyme          | Coenzyme | \(K_m\) | \(K_s\) | \(k_{cat}\) | \(k_{cat}/K_m\) |
|-----------------|----------|---------|---------|-------------|-----------------|
|                 |          | [mM]    | [mM]    | [min\(^{-1}\)] | [min\(^{-1}\) [mM\(^{-1}\)]] |
| Wild-type       | NADP\(^+\) | 0.02    | 0.42    | 25          | 1,520           | 3,040           |
|                 | NAD\(^+\)  | 0.56    | 0.83    | 31          | 1,790           | 103             |
| G223D           | NADP\(^+\) | 0.037\(^a\) | 0.14    | 109         | 570             | 141             |
|                 | NAD\(^+\)  | 0.22\(^a\) | 0.33    | 35          | 1,100           | 143             |
| T224I           | NADP\(^+\) | 0.028   | 0.12    | 98          | 900             | 328             |
|                 | NAD\(^+\)  | 0.12\(^a\) | 0.20    | 41          | 2,250           | 457             |
| H225N           | NADP\(^+\) | 0.16    | 0.10    | 40          | 1,700           | 265             |
|                 | NAD\(^+\)  | 0.71    | 0.88    | 39          | 1,795           | 65              |
| T224I/H225N     | NADP\(^+\) | 1.22    | 1.16    | 56          | 76              | 1               |
|                 | NAD\(^+\)  | 0.12    | 0.23    | 68          | 2,090           | 256             |
| G223D/T224I/H225N | NADP\(^+\) | NS\(^a\) | NS      | NS          | NS              | NS              |
|                 | NAD\(^+\)  | 0.017\(^a\) | 0.04    | 45          | 2,450           | 3,200           |

\(^a\) Standard errors of fits were 20-30%.

\(^b\) NS, no saturation up to 9.6 mM NADP\(^+\).

**Table V**

Deuterium kinetic isotope effects of wild-type and mutant ADH8 at pH 10.0

Activities were measured with 1 mM ethanol-d\(_6\) or ethanol as a substrate and 0.1 M glycine/NaOH at pH 10.0.

| Enzyme          | NADP\(^+\) | NAD\(^+\) |
|-----------------|------------|-----------|
|                 | \(k_{cat}/k_{cat}^D\) | \(k_{cat}(K_m/K_m^D)^{1/2}\) | \(k_{cat}/k_{cat}^D\) | \(k_{cat}(K_m/K_m^D)^{1/2}\) |
| Wild-type       | 1.99       | 1.99      | 1.85      | 2.02            |
| G223D           | 2.04       | 1.95      | 1.72      | 1.86            |
| T224I           | 1.94       | 2.04      | 1.88      | 2.04            |
| H225N           | 2.11       | 2.04      | 1.87      | 1.81            |
| G223D/T224I     | 1.31\(^a\) | 1.15\(^a\) | 1.78      | 1.57            |
| T224I/H225N     | 1.03       | 1.08      | 1.60      | 1.80            |
| G223D/T224I/H225N | 0.90\(^a\) | 1.02\(^a\) | 2.08      | 1.66            |

\(^a\) Activity ratio was calculated from a single concentration of coenzyme (4.8 mM NADP\(^+\)) because saturation could not be reached.

\(^b\) \(k_{cat}/K_m\) was calculated as the slope of the linear plot of \(V\) versus [S], since saturation could not be reached.

The mutant was much more NAD(H) specific than wild-type ADH8 was with NAD(H).

Bisubstrate kinetics were performed by covarying the concentrations of ethanol and either coenzyme, and were compatible with a Bi-Bi sequential kinetic mechanism (Table IV). In the enzymatic forms tested, \(K_m\) values for ethanol (\(K_s\)) ranged between 25 and 110 mM. Single substitutions had modest effects on the \(k_{cat}\) value with NADP\(^+\) but this dropped ~20-fold in the T224I/H225N double mutant and could not be determined in the triple mutant because saturation could not be reached. For the double mutant, this dramatic change in the \(k_{cat}\) value with NADP\(^+\) paralleled an increase in the dissociation (\(K_m\)) and Michaelis constant (\(K_s\)) values for NADP\(^+\) by 60- and 30-fold, respectively. In contrast, the \(k_{cat}\) value with NAD\(^+\) remained relatively unchanged in all the substituted forms, suggesting a common rate-limiting step. Catalytic efficiency for NAD\(^+\), as measured by \(k_{cat}/K_mK_s\) (20), increased by 30-fold,
reaching the same level as that of the wild-type enzyme for NADP$.^+$

The kinetic isotope effect was studied with deuterated ethanol in the presence of either coenzyme. A substrate kinetic isotope effect of $\sim 2$-fold was observed with either NADP$^+$ or NAD$^+$ in the wild-type ADH8 (Table V), suggesting that hydride transfer may be the rate-limiting step during catalysis. When using NADP$^+$ as a cofactor, the same increase was only seen in the single mutant forms. In contrast, the double and triple mutants did not display any significant isotope effect, indicating that hydride transfer was no longer rate-limiting. With NAD$^+$ as a coenzyme, the 2-fold isotope effect was obtained for all the mutant forms.

**Computer Modeling of Binary Complexes—Docking simulations of NADP$^+$ and NAD$^+$ to the wild-type and mutant enzymes were performed based on the three-dimensional structure of the binary complex ADH8-NADP$^+$ (3). In this structure, the oxygen atoms from the extra phosphate group interact through five hydrogen bonds with the side chains of Thr$^{224}$, His$^{225}$, Lys$^{228}$, and the nitrogen main chain atoms of Leu$^{200}$ and Thr$^{224}$ (Fig. 1A). In the G223D-NADP$^+$ complex, steric and electrostatic hindrances by the side chain of Asp$^{223}$ force the coenzyme phosphate to relocate in the binding site, moving away from Lys$^{228}$ (compare Fig. 1, B and A). Thus, as a result of the G223D mutation, hydrogen bonds with Leu$^{200}$, Lys$^{228}$, and the nitrogen main chain atom of Thr$^{224}$ are missing. But these losses are partially compensated by the existence of two new hydrogen bonds between the same oxygen atom of the terminal phosphate and the carbonyl oxygen atom of Leu$^{202}$ and the oxygen atom of Ser$^{364}$. Thus, one oxygen atom of the extra phosphate group is left without interacting, although it could do so through a water molecule. Small differences are observed in T224I-NADP$^+$ and H225N-NADP$^+$ complexes with respect to NADP$^+$ docked to the wild-type enzyme. In both cases, two hydrogen bond interactions involving the extra phosphate are lost, but a new hydrogen bond is formed in such a way that all the oxygen atoms of the extra phosphate group are interacting with some residue (data not shown). When comparing NADP$^+$ binding to the T224I/H225N mutant with respect to the wild-type enzyme, an oxygen atom of the extra phos-

![Fig. 1. Stereo views of the adenosine-phosphate moiety of NADP$^+$ bound to wild-type and mutant forms of ADH8. Interaction through hydrogen bonds is represented with dotted lines. A, crystallographic binary complex of wild-type ADH8 (Protein Data Bank code 1P0F, Ref. 3). Docking of NADP$^+$ inside the coenzyme-binding pocket of ADH8 mutants: B, G223D; C, G223D/T224I/H225N.](image-url)
phosphate group would not interact with any residue, but it is conceivable that it could do so through a water molecule (data not shown).

In the simulation of docking NADP$^+$ to the G223DT224I/H225N mutant, as it was previously observed for the G223D mutant, there is a relocation of the coenzyme. In this case, although, several protein-phosphate interactions (three hydrogen bonds) are lost and they are not compensated by new hydrogen bond formation. As a result, an oxygen atom of the extra phosphate group does not interact with any residue, but it is conceivable that it could do so through a water molecule (data not shown).

FIG. 2. Stereo views of the adenosine moiety of NAD$^+$ docked inside the coenzyme-binding pocket of wild-type ADH8 and triple mutant. Interaction through hydrogen bonds is indicated with dotted lines. A, wild-type ADH8; B, G223D/T224I/H225N.

In ADH8 single mutants, $k_{cat}$ values for NADP(H) decreased slightly, whereas a moderate increase in $K_i$ values for NADP$^+$ was observed. Hydride transfer appeared to be the rate-limiting step. In contrast, in the T224I/H225N mutant, $k_{cat}$ values for NADP$^+$ suffered a sharp decrease, which was associated with a marked increase in the $K_i$ value for NADP$^+$. In this regard, in double and triple mutants, the catalytic efficiency dropped dramatically, reflecting the fact that $K_i$ values for NADP(H) increased, and even in most cases saturation could not be reached, and apparent $k_{cat}$ values likely decreased. In concordance with this observation, the kinetic isotope effect was lost, indicating a change in the rate-limiting step, no longer being hydride transfer. A slower limiting step, such as a conformational change associated with coenzyme binding could be involved. However, at this time, we have no evidence for a conformational change upon coenzyme binding, because the crystal structure of the apoenzyme showed a closed conformation (3). On the other hand, it is likely that coenzyme dissociation was not the new rate-limiting step because the increase in $K_i$ values was not followed by an increase in $k_{cat}$ values.

The $k_{cat}$ values for NAD(H) remained relatively unchanged for the mutant enzymes with respect to the wild-type recombinant ADH8, indicating that the rate-limiting step was likely the same for all the enzymatic forms when NAD(H) was the coenzyme. This result was consistent with the finding of a slight deuteration kinetic isotope effect whenever NAD$^+$ was the coenzyme, suggesting that hydride transfer was rate-limiting. Hydride transfer has also been found limiting in other ADHs using ethanol as a substrate, such as human ADH3 (26) and horse liver I269S (27). The finding in ADH8 differs from what has been described for horse liver ADH and other ADH forms, with ethanol and other aliphatic alcohols as substrates, where the rate-limiting step is NADH dissociation (28, 29). In those cases, an increase in $K_i$ and $K_{iq}$ (dissociation constants for NAD and NADH, respectively) often parallels an increase in $k_{cat}$ values, which is not observed in ADH8.

Residue 223 Is Not the Only Discriminating Residue for Coenzyme Specificity—In the coenzyme-binding domain of NAD-dependent dehydrogenases with the Rossmann fold, an acidic residue (Asp$^{223}$ in NAD-dependent ADHs, Ref. 4) is located at between NAD(H) and NADP(H) remains unknown. This fundamental question and the challenge of reverting coenzyme specificity prompted us to perform the present site-directed mutagenesis study. Therefore adjacent residues 223, 224, and 225 were systematically mutated and checked, separately or in different combinations.

Effect of Substitutions on the Rate-limiting Step and Catalytic Constant—In ADH8 single mutants, $k_{cat}$ values for NADP(H) decreased slightly, whereas a moderate increase in $K_i$ values for NADP$^+$ was observed. Hydride transfer appeared to be the rate-limiting step. In contrast, in the T224I/H225N mutant, $k_{cat}$ values for NADP$^+$ suffered a sharp decrease, which was associated with a marked increase in the $K_i$ value for NADP$^+$. In this regard, in double and triple mutants, the catalytic efficiency dropped dramatically, reflecting the fact that $K_i$ values for NADP(H) increased, and even in most cases saturation could not be reached, and apparent $k_{cat}$ values likely decreased. In concordance with this observation, the kinetic isotope effect was lost, indicating a change in the rate-limiting step, no longer being hydride transfer. A slower limiting step, such as a conformational change associated with coenzyme binding could be involved. However, at this time, we have no evidence for a conformational change upon coenzyme binding, because the crystal structure of the apoenzyme showed a closed conformation (3). On the other hand, it is likely that coenzyme dissociation was not the new rate-limiting step because the increase in $K_i$ values was not followed by an increase in $k_{cat}$ values.

The $k_{cat}$ values for NAD(H) remained relatively unchanged for the mutant enzymes with respect to the wild-type recombinant ADH8, indicating that the rate-limiting step was likely the same for all the enzymatic forms when NAD(H) was the coenzyme. This result was consistent with the finding of a slight deuteration kinetic isotope effect whenever NAD$^+$ was the coenzyme, suggesting that hydride transfer was rate-limiting. Hydride transfer has also been found limiting in other ADHs using ethanol as a substrate, such as human ADH3 (26) and horse liver I269S (27). The finding in ADH8 differs from what has been described for horse liver ADH and other ADH forms, with ethanol and other aliphatic alcohols as substrates, where the rate-limiting step is NADH dissociation (28, 29). In those cases, an increase in $K_i$ and $K_{iq}$ (dissociation constants for NAD and NADH, respectively) often parallels an increase in $k_{cat}$ values, which is not observed in ADH8.

DISCUSSION

ADH8 is unique within the vertebrate ADH family in that it uses preferentially NADP(H) as a cofactor (2). ADH8 also binds NAD(H), but with $K_i$ values that are 10 to 40 times higher than those for NADP(H). Structural studies on the binary complex ADH8-NADP$^+$ suggested that a phosphate binding pocket is configured by non-conserved residues 223, 224, and 225, and the highly conserved Leu$^{200}$ and Lys$^{228}$ (3). However, the contribution of these residues to the discrimination be-
the C-terminal end of the second β-strand (30). Asp<sup>223</sup> is doubly hydrogen bonded to the adenine ribose diol in ADH-NAD<sup>+</sup> complexes (21–24). The significance of this acidic residue has been observed long ago and has been considered a fingerprint for NAD(H) binding pockets (8, 31). In ADHs, it has been proposed as the residue that discriminates against NADP(H) (9). NADP-dependent enzymes exhibit a smaller and uncharged residue, usually Gly, Ala, or Ser (3, 10, 32–34), that would reduce steric and electrostatic hindrances on binding of the extra phosphate group of NADP(H). Thus, to reverse coenzyme specificity, several studies have targeted this residue by site-directed mutagenesis. In one case, the homologous Asp residue in yeast ADH1 was substituted by Gly and the resulting D223G mutant utilized the two coenzymes with similar but very low efficiency (9). In cinnamyl alcohol dehydrogenase, an NADP-dependent enzyme from the MDR superfamily, the equivalent reverse mutation S212D did not change the coenzyme specificity either (10). Similarly, the substitution of the corresponding Asp in other NAD-dependent dehydrogenases having the Rossmann fold did not generate NADP-dependent enzymes, although substitution improved the catalytic efficiency for NAD<sup>+</sup> and made it worse for NADH (35, 36).

In the present paper, single mutations G223D, T224I, and H225N only provided small changes to coenzyme kinetics, which were slightly detrimental for NADPH and beneficial for NADH. For NADH, in general, there existed a good correlation between the loss of a few hydrogen bonds and electrostatic interactions, and a modest increase in $K_m$ and $K_v$ values, as predicted by molecular docking simulations. In particular, the substitution G223D did not greatly hamper NAD<sup>+</sup> binding as it would have been anticipated but resulted in an enzyme that still preferred NADP(H) over NADH (Fig. 3) although with a lower catalytic constant. The modeling results for the G223D mutant confirm the generation of unfavorable steric and electrostatic conditions on NAD(H) binding. But interestingly, the extra phosphate group of coenzyme can still be accommodated in the coenzyme binding pocket by moving away from Asp<sup>223</sup> and Lys<sup>228</sup>, at the expense of losing some hydrogen bonds and favorable electrostatic interactions. According to these results and confirming previous findings in other ADHs (9–12), Asp<sup>223</sup> alone cannot be considered the discriminating residue in the coenzyme specificity of ADH8, and thus the combined substitutions in other positions along with that of residue 223 would be important as well.

Complete Reversal of Coenzyme Specificity in the Triple Mutant—In the triple mutant, the attained $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values for NADH and $K_m$ for NAD<sup>+</sup> are in the same order of magnitude as those for NAD(P)H in the wild-type enzyme. Thus, complete reversal of coenzyme specificity has been achieved (Fig. 3). Moreover, the $k_{cat}/K_m$ values with NADH obtained in the triple mutant are similar to those of typical NAD-dependent ADHs (37–42). It is conceivable that some kind of threshold in catalytic power has been attained in many ADHs regarding coenzyme usage. Overall, the triple mutant is much more “coenzyme specific” than the wild-type enzyme, as illustrated by the ratio $k_{cat}/K_m$NADH/$k_{cat}/K_m$NADPH (Fig. 3). This behavior is typical of NAD-dependent vertebrate ADHs, which frequently are highly specific for NAD(H) although some have residual activity with NAD(P)H. In the triple mutant, the extra phosphate group poses steric and electrostatic problems for NAD(H) binding, especially because of the presence of both Asp<sup>223</sup> and a less hydrophilic environment at positions 224 and 225. In contrast, NAD(H) can easily be accommodated in the vacant phosphate-binding site (with Gly) and interact with His<sup>225</sup> and Lys<sup>228</sup> in the NADP-dependent wild-type enzyme.

Synergistic Effects between Adjacent Residues Determine Coenzyme Specificity—Our results highlight the importance of cooperative or synergistic effects between two or more adjacent amino acid residues. Single amino acid substitutions had only very limited effects, whereas double and triple mutations involving the same residues caused great changes on coenzyme binding and kinetics. In ADH8, it is the combined contribution of these groups, interacting with one another and with the coenzyme, that provides alternative binding sites for the adenine/adenosine-phosphate moiety of NAD(H)/NADP(H). NAD(H) requires the simultaneous presence of Asp<sup>223</sup>, Ile<sup>224</sup>, and Asn<sup>225</sup>, although it does not interact with Asn<sup>225</sup> in ADH8 nor in other ADH structures. NADP(H) needs Gly<sup>223</sup>, Thr<sup>224</sup>, and His<sup>225</sup>, but it does not interact with Gly<sup>223</sup>. Apparently, in ADH8, the small side chain of Gly<sup>223</sup> provides additional space for the phosphate group of NADP(H) to interact with Lys<sup>228</sup>, while in the triple mutant Asn<sup>225</sup> leaves more space than His, for the adenine ring of NAD(H) to interact hydrophobically with Ile<sup>224</sup>. Asp<sup>223</sup> helps discriminate against NADP(H) and provides hydrogen bonding at the precise distance for NAD(H) to interact properly with Ile<sup>224</sup> and Lys<sup>228</sup>. Lys<sup>228</sup> is a versatile highly conserved residue that performs a dual function, i.e. the electrostatic interaction with the extra phosphate of NADP(H) or the hydrogen bond interaction with the 3′ oxygen of NAD(H) ribose. It is clear that each of these residues has in ADH8 a different function from that in NAD-dependent ADHs. As expected, ionic interactions play a predominant role in NAD(P)H binding, whereas hydrogen bonding and hydrophobic interactions are more prevalent for NAD(H) binding. The present results are consistent with the previous finding that NAD<sup>+</sup> binds to ADH8 in a similar conformation to that of NAD<sup>+</sup> found in ADH binary complexes, showing small differences only in the adenosine moiety of coenzyme (3).

The most effective switches in coenzyme specificities have come from multiple mutations in different regions of the primary structure of dehydrogenases (43–46). In contrast, the complete reversal of coenzyme specificity in ADH8 has been obtained by means of substitution of only three consecutive amino acid residues. The absence of an acidic residue together with the presence of polar residues around the extra phosphate group contribute to the NADP(H) specificity of ADH8. ADH architecture appears to be flexible enough to satisfy changes in coenzyme requirements and thus NADP-dependent enzymes have convergently evolved within the MDR superfamily at different times using seemingly different strategies and independent pathways: e.g. secondary ADH in bacteria (5–7), cinnamyl ADH in plants and fungi (10), quinone oxidoreductase in animals and bacteria (33), sorbitol dehydrogenase in insects (34), and ADH8 in amphibians (3). Our data support that concerted evolution of adjacent amino acid residues (223 to 225) within a short sequence segment, requiring minimally four point mutations in the gene sequence, has allowed coenzyme specificity in amphibian ADH to switch. This may reflect ADH8 late enzymogenesis, with a relatively recent evolutionary origin of this NADP-dependent structure from an ancestral NAD(H)-specific structure (class I ADH, 70% identity), as well as a fast functional adaptation to different metabolic needs (i.e. reduction of aldehydes, such as retinal, instead of oxidation of alcohols) (2). Undoubtedly, the simplicity of these evolutionary changes has contributed to facilitate the redesign of the coenzyme-binding site and to achieve the complete reversal of coenzyme specificity reported here.

Acknowledgment—We thank A. A. Santos for performing site-directed mutagenesis, expression, and purification of T224I and H225N mutants.
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