From Alcohol Dehydrogenase to a “One-way” Carbonyl Reductase by Active-site Redesign

A MECHANISTIC STUDY OF MANNITOL 2-DEHYDROGENASE FROM PSEUDOMONAS FLUORESCENS

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Directional preference in catalysis is often used to distinguish alcohol dehydrogenases from carbonyl reductases. However, the mechanistic basis underpinning this discrimination is weak. In mannitol 2-dehydrogenase from Pseudomonas fluorescens, stabilization of (partial) negative charge on the substrate oxygen by the side chains of Asn-191 and Asn-300 is a key feature of catalysis in the direction of alcohol oxidation. We have disrupted this ability through individual and combined substitutions of the two asparagines by aspartic acid. Kinetic data and their thermodynamic analysis show that the internal equilibrium of enzyme-NADH-fructose and enzyme-NAD\(^+\)-mannitol \((K_{\text{int}})\) was altered dramatically \((10^4 \text{ to } 10^5\)-fold) from being balanced in the wild-type enzyme \((K_{\text{int}} = 3)\) to favoring enzyme-NAD\(^+\)-mannitol in the single site mutants, N191D and N300D. The change in \(K_{\text{int}}\) reflects a selective slowing down of the mannitol oxidation rate, resulting because Asn \(\rightarrow\) Asp replacement (i) disfavors partial abstraction of alcohol proton by Lys-295 in a step preceding catalytic hydride transfer, and (ii) causes stabilization of a nonproductive enzyme-NAD\(^+\)-mannitol complex. N191D and N300D appear to lose fructose binding affinity due to deprotonation of the respective Asp above apparent \(pK\) values of 5.3 \(\pm\) 0.1 and 6.3 \(\pm\) 0.2, respectively. The mutant incorporating both Asn-Asp substitutions behaved as a slow “fructose reductase” at pH 5.2, lacking measurable activity for mannitol oxidation in the pH range 6.8–10. A mechanism is suggested in which polarization of the substrate carbonyl by a doubly protonated diad of Asp and Lys-295 facilitates NADH-dependent reduction of fructose by N191D and N300D under optimum pH conditions. Creation of an effectively “one-way” reductase by active-site redesign of a parent dehydrogenase has not been previously reported and holds promise in the development of carbonyl reductases for application in organic synthesis.

Enzymatic NAD or NADP-dependent interconversion of alcohol and carbonyl groups is a key chemical transformation in biology. The thermodynamic equilibrium constant of the reaction usually favors alcohol under a wide range of external conditions. However, the directional preference of biological catalysis, expressed as the ratio of reaction rate constants in direction of carbonyl reduction \((k_R)\) and alcohol oxidation \((k_O)\), is not uniform among natural enzymes and underpins a widely used classification that considers “alcohol dehydrogenases” \((k_R/k_O \leq 1)\) and “carbonyl reductases” \((k_R/k_O \geq 1)\). Horse liver alcohol dehydrogenase and human aldose reductase are representative members of each class, having \(k_R/k_O\) values of \(\sim 1\) (1) and 217 (2), respectively. Work on lactate dehydrogenase has shown that \(k_R/k_O\) is sensitive to active-site structural changes resulting from site-directed mutagenesis (3). However, a clear mechanistic basis for the distinction between dehydrogenases and reductases is currently lacking.

The oxyanion binding pocket of PfM2DH \((Pseudomonas fluorescens)\) mannitol 2-dehydrogenase\(^2\) constitutes a distinct structural motif of proficient catalytic function as an alcohol dehydrogenase (EC 1.1.1) (4). The enzyme is a well characterized member of the large and diverse superfamily of long-chain dehydrogenases and reductases (5, 6). It utilizes NAD\(^+\) to oxidize the C-2 alcohol of mannitol into the keto-group of the open-chain free-carbonyl form of \(\beta\)-fructose (Fig. 1A). Active-site preorganization in PfM2DH resulting from the side chains of Asn-191 and Asn-300 strongly resembles the well known catalytic “oxyanion holes” in serine proteases (4, 7–10). The x-ray crystal structure of PfM2DH bound with mannitol and NAD(H) shows that the oxyanion binding site belongs to a strikingly symmetric arrangement of groups, which include the reactive centers on the substrates and the catalytic residues on the enzyme, as depicted in Fig. 1B (11).

Mutational analysis has delineated catalytic roles for Asn-191 and Asn-300 during NAD(H)-dependent interconversion of mannitol and fructose by PfM2DH (4, 12). Positioning effects and electrostatic stabilization are exploited to facilitate formation of the reactive conformers in each direction of the reaction and to provide selective rate enhancement to the hydride transfer step. For mannitol oxidation, the “oxyanion hole” brings \(pK_a\) depression in both the \(\epsilon-NH_2\) group of the catalytic base Lys-295 and the reactive hydroxyl group of the substrate. Deprotonation of the lysine is required to “activate” the dehydrogenase function of PfM2DH at neutral pH where Lys-295

\(^2\) The abbreviations used are: PfM2DH, mannitol 2-dehydrogenase from \(P.\) fluorescens; KIE, kinetic isotope effect; MD, molecular dynamics; MES, 4-morpholineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; NADD, 4S-[\(^3\)H]NADH.

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\(^{\text{b}}\) The on-line version of this article (available at http://www.jbc.org) contains supplemental text, equations, Tables S1 and S2, Figs. S1–S4, and references.

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From Dehydrogenase to Reductase by Active-site Redesign

**EXPERIMENTAL PROCEDURES**

Chemicals and other materials were described elsewhere (4, 12–14). Reported methods were used for preparation of wild-type PjM2DH (18).

**Preparation of N191D, N300D, and N191D-N300D—Inverse PCR using suitable oligonucleotide primer pairs (N191D, [5’-TTGCGATGACCTGCCCCACAAT-3’] and [5’-GGCATCAGCTGGACCCGCGC-GACCTG-3’] and [5’-GGCATTTCCATCTTCCTCATA-3’]) was employed to introduce the desired mutations. In the shown primers, the underlined codons produce the respective site-directed substitution, and the mismatched bases are indicated in bold. Plasmid pETR241-PjM2DH encoding C-terminally His-tagged wild-type enzyme was used as template for PCR according to a reported protocol (12). To obtain the gene encoding N191D-N300D, we subjected the plasmids for N191D and N300D to a double digestion by NcoI and BamHI. The Ncol-BamHI fragment containing the relevant mutations for N300D was subcloned into the vector for N191D. The full sequence of each mutated gene was confirmed. All mutants were produced in Escherichia coli JM109 and purified by known methods (18). Supplemental Fig. S2 shows that each mutant was isolated as a full-length protein having the expected molecular mass of 55 kDa (analysis by SDS-PAGE; supplemental Fig. S2A) and that the overall native fold of PjM2DH was not altered because of the site-directed substitutions (CD spectroscopic analysis; supplemental Fig. S2B).

**Kinetic Characterization—**Methods for steady-state kinetic characterization were described in previous reports (12–14, 18). The buffers used (each 100 mM) were MES/NaOH (pH 5.2–6.8), Tris/HCl (pH 7.1–9.0), and glycine/NaOH (pH 9.0–

with a $pK_a$ of 9.2 in the enzyme-NAD$^+$ complex would otherwise be ionized (13). Partial abstraction of the mannitol proton by Lys-295 is thought to be a key catalytic event that primes the ternary complex for cleavage of the substrate C–H bond (13, 14). In the direction of fructose reduction, the oxyanion hole appears to go along with gain of carbonyl group polarization ability. The double mutant provided affirmation, because it lacked measurable activity for mannitol oxidation and behaved as a slow fructose reductase at low pH where the introduced Asp residues should be partially protonated. Conversion of an alcohol dehydrogenase into a carbonyl reductase by rational redesign of the enzyme active site has to our knowledge not been previously reported.

**FIGURE 1.** The reaction catalyzed by PjM2DH (A) and close-up structure of the enzyme active site (B). X-ray coordinates for enzyme bound to NAD$^+$ and mannitol were used in B (PDB entry: 1M2W). The symmetric arrangement of the catalytic groups and the reactive substrate hydroxyl is indicated in gray. Dashed lines show distances $\approx 4.1$ Å.

**TABLE 1.** The ternary complexes in PjM2DH comprising a highly acidic diad of Asp-19 (or Asp-300) and Lys-295 might be suitable for function as “fructose reductase.” This design concept was strongly supported by precedence within the aldo-keto-reductase protein superfamily where some enzymes (family 1D) utilize the (protonated) side chain of a Glu for strong carbonyl group polarization during double-bond reduction in $\alpha,\beta$-unsaturated ketone substrates (15–17).

Herein, we report on a detailed kinetic and mechanistic characterization of three mutants of PjM2DH: N191D, N300D, and a double mutant incorporating both Asn $\rightarrow$ Asp substitutions (N191D-N300D). Using free-energy profile analysis, we compared reactions catalyzed by N191D and N300D to the reaction catalyzed by the wild-type enzyme. The results reveal that $K_{int}$ for both single-site mutants decreased by about 5 orders of magnitude relative to the corresponding internal equilibrium for native PjM2DH. Experimental findings are combined with evidence from molecular dynamics (MD) simulations to suggest that the dramatic change in directional preference toward fructose reduction results, because replacement of either Asn by Asp (i) disfavors precatalytic “activation” of enzyme-bound mannitol by partial deprotonation and, furthermore, (ii) appears to go along with gain of carbonyl group polarization ability. The double mutant provided affirmation, because it lacked measurable activity for mannitol oxidation and behaved as a slow fructose reductase at low pH where the introduced Asp residues should be partially protonated. Conversion of an alcohol dehydrogenase into a carbonyl reductase by rational redesign of the enzyme active site has to our knowledge not been previously reported.
From Dehydrogenase to Reductase by Active-site Redesign

pH dependences of kinetic parameters ($k_{cat}$, $k_{cat}/K_{\text{substrate}}$) for oxidation and reduction were determined from initial rates recorded at varied concentrations of mannitol and fructose while using a constant, saturating ($\geq 3 \times K_{\text{coenzyme}}$) concentration of NAD$^+$ (2.0 mM at pH 8.0–10.5; 10.0 mM at pH 6.8–7.5) and NADH (200 mM), respectively. Kinetic isotope effects (KIEs) (resulting from deuteration of substrate (2-2H-mannitol) or coenzyme (4S-[2H]NADH, NADD) were measured using reported methods (4, 13). A nomenclature is used where subscripts O and R in $k_{cat}$ indicate mannitol oxidation and fructose reduction, respectively. Superscript D in a kinetic parameter (e.g. $^{1}\text{D}k_{\text{cat}}$) indicates a primary deuterium KIE (19).

Supplementary Table S1 summarizes the conditions applied. Time courses of formation and consumption of NADH were recorded from absorbance at 340 nm. The dead-time of the stopped-flow instrument was 1.5–6.0 ms depending on the substrate concentration used. KIEs on kinetic parameters (KIEs) (resulting from deuteration of substrate (2-2H-mannitol) or coenzyme (4S-[2H]NADH, NADD) were measured using reported methods (4, 13). A nomenclature is used where subscripts O and R in $k_{cat}$ indicate mannitol oxidation and fructose reduction, respectively. Superscript D in a kinetic parameter (e.g. $^{1}\text{D}k_{\text{cat}}$) indicates a primary deuterium KIE (19).

Supplementary Table S1 summarizes the conditions applied. Time courses of formation and consumption of NADH were recorded from absorbance at 340 nm. The dead-time of the stopped-flow instrument was 1.5–6.0 ms depending on the substrate concentration used. KIEs on kinetic parameters (KIEs) (resulting from deuteration of substrate (2-2H-mannitol) or coenzyme (4S-[2H]NADH, NADD) were measured using reported methods (4, 13). A nomenclature is used where subscripts O and R in $k_{cat}$ indicate mannitol oxidation and fructose reduction, respectively. Superscript D in a kinetic parameter (e.g. $^{1}\text{D}k_{\text{cat}}$) indicates a primary deuterium KIE (19).

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Results and Discussion—SigmaPlot 2004 version 9.0 was used for data fitting by linear or non-linear least squares regression. Equation 1 describes a pH $-\log(k_{cat}/K_{m})$ profile that shows a decrease with $+1$ slope below pH $K$ and is level at high pH (C). Equation 2 describes a wave-like pH dependence where $\log(k_{cat}/K_{m})$ decreases with $-1$ slope above pH $K$ from a constant value at low pH ($C_{pK}$) to a lower constant value at high pH ($C_{pK}$). $K$ is the proton dissociation constant, and [$H^+]$ is the proton concentration. Equation 3 is the rate formula for an Ordered Bi Bi kinetic mechanism where substrate A binds to the enzyme prior to substrate B (20). $K_{A}$ is the apparent dissociation constant for A. $K_{A}$ and $K_{B}$ are Michaelis constants for A and B, respectively. $[E]$ is the molar enzyme concentration, calculated from protein absorbance at 280 nm using a value of 55.4 mM$^{-1}$cm$^{-1}$ for $\epsilon_{280}$ of wild-type and mutated forms of PfM2DH (21). Equation 4 was used to fit stopped-flow time courses displaying a biphasic (“burst” kinetic) character. [$NADH]$ is the concentration of NADH at time t, $\Pi$ is [NADH] consumed in the pre-steady state, $k_{obsu}$ is the transient rate constant, $k_{ss}$ is the steady-state rate, and $Y_0$ is the initially applied [NADH] minus II. Reactions using NADD instead of NADH were analyzed identically.

$$\log(k_{cat}/K_{m}) = \log[C/(1 + K[H^+])] \quad (\text{Eq. 1})$$

$$\log(k_{cat}/K_{m}) = \log[(C_L + C_R[H^+]/K)]/(1 + [H^+]/K) \quad (\text{Eq. 2})$$

$$v = k_{cat}[E][A][B]/(K_{A}K_{B} + K_{A}[B] + K_{B}[A] + [A][B]) \quad (\text{Eq. 3})$$

$$[NADH]_t = [\Pi]e^{-k_{cat}t} - k_{cat}t + Y_0 \quad (\text{Eq. 4})$$

MD Simulations—These were performed with the program GROMACS 4.0.7 (22) using the GROMOS 53A6 force field (23). Crystallographic coordinates for PfM2DH having NADH and mannitol bound (chain A, PDB entry: 1M2W) were used. All selenocysteines present in the experimental structure were replaced by cysteines, and crystal waters were removed. Individual substitutions of Asn-191 and Asn-300 by Asp were built in this starting model. Protein structures were fully hydrated using the simple point charge water model in a cubic box. The pH was adjusted to a value of 7. System charges were neutralized assuming 0.1 mM NaCl, resulting in a total model size of $\approx 22,000$ atoms. Energy minimization was done using the steep-descent method such that the maximum of force on any atom was less than 1000 kJ, followed by position-restrained simulation at 300 K for 40 ps to equilibrate the water molecules. MD simulations were then done for minimally 15 ns at constant temperature (300 K, using the velocity rescaling temperature-coupling method) and pressure (1 bar, using the Parrinello-Rahman temperature-coupling method). An integration time step of 2 fs was used, and the van der Waals radius and Coulomb interactions (calculated using particle mesh Ewald method) were computed within cutoffs of 9 Å each. Periodic boundary conditions were applied. Simulation trajectories were analyzed with GROMACS to compute the root mean square deviation of atomic positions and atom–atom distances, respectively. Visual molecular dynamics (24) was used to generate simulation movies. Representative structure snapshots were saved and visualized using the PyMOL molecular graphics system.

Results—Structural Characterization of Mutants of PfM2DH, by Experiments and MD Simulation—N191D, N300D, and N191D-N300D were obtained as electrophoretically pure protein preparations that appeared structurally intact and properly folded (supplemental Fig. S2). The CD spectroscopic signature of each mutant was identical with limits of error to that of the wild-type enzyme (supplemental Fig. S28), suggesting a highly similar distribution of secondary structural elements in the proteins as isolated. To obtain more detailed information on possible perturbations in native protein structure caused by individual substitution of Asn-191 and Asn-300, we compared wild-type PfM2DH, N191D, and N300D in MD simulations of the (nonproductive) ternary complex between enzyme, NADH, and mannitol. The structural trajectory for each protein was analyzed using the experimental crystal structure of the wild-type enzyme as the reference. Supplementary Fig. S3 shows variation of the calculated root mean square deviation values with time. The simulation results are interpreted to imply that no significant change in the overall fold of each enzyme occurred over a total simulation time of 25 ns. Moreover, the sampled simulated structures of each mutant were highly similar to those obtained for wild-type PfM2DH. Except for conformational rearrangements of catalytic side chains (Asn-191, Asp-230, and Asn-300) that are relevant mechanically and will be described later, individual Asp replacements of Asn-191 and Asn-300 did not result in a global structural disruption of the mannitol binding site as simulated. Supplemental Fig. S4 (A–C) depicts simulation time courses for relevant atom–atom distances in the active sites of native PfM2DH, N191D, and N300D. According to the results of MD simulation, the site-directed substitutions of Asn-191 and Asn-300 were furthermore fully compatible with accommodation of the nicotinamide moiety of coenzyme in a productive orientation (data not shown). The apparent dissociation constant for enzyme-NAD$^+$...
From Dehydrogenase to Reductase by Active-site Redesign

(Kd), as obtained from fluorescence titration analysis at pH 10.0, was ~1 mM for all mutants and unchanged as compared with the corresponding Kd of the wild-type enzyme (data not shown). The results from MD simulation and experiments therefore support the suggestion that functional consequences in N191D and N300D can be interpreted to arise from locally disruptive effects of the respective site-directed substitution.

pH Dependences of Kinetic Parameters for the Mutants—Individual site-directed replacements of Asn-191 and Asn-300 by Asp introduce an extra ionizable group in the active site of PjM2DH, and this was expected to bring about a change in pH-activity dependence for the resulting mutants as compared with the wild-type enzyme. The pH profiles of log kcat and log(kcat/Km) for N191D and N300D are displayed in Fig. 2, A and B, respectively, where they are compared with the corresponding pH profiles for native PjM2DH that were taken from our previous reports (13, 18). kcat was independent of pH (N300D) or showed only a slight linear increase (N191D) as the pH was raised from 6.8 to 10.5. With both mutants, activity for oxidation of mannitol was below limit of detection at a pH smaller than 6.8. kcat of N191D and N300D was likewise independent of pH in the range 5.2–7.0. Neither single-site mutant could be saturated with fructose under conditions of pH ≈ 8, precluding determination of a kcat value in this pH range (see Fig. 2A). Fig. 2B shows that the pH profiles of log(kcat/Km) for N191D and N300D were level at high pH and decreased with a slope of +1 as the pH was decreased below apparent pK. The pH profiles of log(kcat/Km) for N191D and N300D decreased from a constant value at low pH to a lower constant value at high pH.

Combined substitution of Asn-191 and Asn-300 by Asp caused complete disruption of “mannitol dehydrogenase” activity below detection limit at pH 10.0. No activity toward mannitol was measured across the entire pH range studied in Fig. 2. However, N191D-N300D displayed significant fructose reductase activity, and this was maximal at the lower end of the experimental pH range. As shown in Fig. 2B, log(kcat/Km) of the double mutant decreased linearly, with a slope of ~−1, in response to an increase in pH from 5.2 to 7.0. At each point of measurement in this pH range, the rate of substrate reduction by N191D-N300D was linearly dependent on the fructose concentration (data not shown), indicating that apparent binding of fructose by the double mutant was very weak. No kcat value was therefore obtained for N191D-N300D.

To derive relevant parameters from the experimental pH dependences in Fig. 2, we used non-linear fitting of data with a suitable equation (Equations 1 and 2). Results are summarized in Table 1. The apparent pK in the pH profile of log(kcat/Km) was changed from a value of 9.2 for wild-type PjM2DH to much lower values of 5.3 and 6.3 for N191D and N300D, respectively. The magnitude of the pH shift caused by each site-directed substitution is remarkable. Displacement of the pH dependence for kcat/Km into the low pH region was further enhanced in the double mutant. It is therefore interesting that the apparent pK observed in the pH profiles of log(kcat/Km) for the mutants was not (N191D) or only slightly changed (N300D) in comparison to the wild-type reference.

At the optimum protonation state for mannitol oxidation (high pH), N191D and N300D displayed similar catalytic efficiencies that were lowered by four orders of magnitude as compared with the corresponding kcat/Km Value for the wild-type enzyme. At low pH where fructose reduction proceeds optimally, N191D was 90-times more efficient, in kcat/Km terms, than N300D. Considering the large loss of enzyme efficiency in mannitol oxidation resulting from substitution of Asn-191 or Asn-300 by Asp, it was striking that ciency in mannitol oxidation resulting from substitution of Asn-191 or Asn-300 by Asp, it was striking that...
From Dehydrogenase to Reductase by Active-site Redesign

### Steady-state Kinetic Characterization of the Mutants—Apparent kinetic parameters for single-site mutants were obtained at the optimum pH for mannitol oxidation (pH 10.0) and fructose reduction (pH 5.2). For the double mutant, kinetic parameters in fructose reduction were obtained at pH 6.8. For the wild-type enzyme and N191D was performed at pH 7.1. Because saturation of N300D with fructose could not be achieved at this pH, the full kinetic study for N300D was carried out at pH 6.8. All results are summarized in Table 2. Due to the low activity of N191D and N300D, it was difficult to acquire rates at NAD\(^+\) concentrations smaller than 60 \(\mu\)M. The parameters \(K_{\text{NAD}}\) (apparent dissociation constant for enzyme-NAD\(^+\)) and \(K_{\text{fructose}}\) (Michaelis-Menten constant) are therefore statistically not well determined for N191D and N300D, respectively. However, the internal consistency of each set of kinetic parameters at pH 7.1 (or pH 6.8) was confirmed using Haldane relationship analysis (Table 2) in which the kinetically determined equilibrium constant is compared with the thermodynamic equilibrium constant of the reaction (20).

N191D and N300D showed large losses (~2 \(\times\) 10\(^4\)-fold) in efficiency, in terms of both \(k_{\text{catO}}/K_{\text{fructose}}\) and \(k_{\text{catO}}/K_{\text{NAD}}\) at pH 10.0, as compared with wild-type enzyme. These losses reflect almost exclusively the corresponding decreases in \(k_{\text{catO}}\) for the mutants in comparison to \(k_{\text{catO}}\) for native PfPM2DH. In fructose reduction at pH 5.2, losses in \(k_{\text{catO}}/K_{\text{fructose}}\) for N191D (17-fold) and N300D (1200-fold) were mainly due to analogous decreases in \(k_{\text{catO}}\) as compared with the corresponding parameters of wild-type PfPM2DH. In N300D, apparent binding of fructose was weakened 16-fold as compared with native enzyme, \(k_{\text{catO}}/K_{\text{fructose}}\) was relatively insensitive (\(~\)10-fold change) to substitution of Asn-191 and Asn-300. The observed 3.4 \(\times\) 10\(^4\)-fold loss in \(k_{\text{catO}}/K_{\text{fructose}}\) for the double mutant is reasonably explained as an additive effect of the individual site-directed substitutions. The clear asymmetry of the effects, replacement of Asn-300 being ~70-fold more strongly disruptive on \(k_{\text{catO}}/K_{\text{fructose}}\) than is replacement of Asn-191, was not expected from the balanced structural arrangement of active-site groups in the native enzyme (Fig. 1B). However, considering the uncertainty level associated with current enzyme active-site redesign, the difference between N191D and N300D in terms of \(k_{\text{catO}}/K_{\text{fructose}}\) was accepted as observed and not further pursued.

Fig. 3 shows free energy profiles for NADH-dependent reduction of fructose catalyzed by wild-type enzyme (pH 7.1), N191D (pH 7.1), and N300D (pH 6.8), constructed from the

### TABLE 2

| Parameter | N191D-N300D | N300D | Wildtype |
|-----------|-------------|-------|----------|
| \(k_{\text{cat}}\) (s\(^{-1}\)) | pH 7.1 | pH 7.1 | pH 7.1 |
| 0.0015 ± 3 \(\times\) 10\(^{-5}\) | 0.0014 ± 3 \(\times\) 10\(^{-4}\) | 0.0011 ± 2 \(\times\) 10\(^{-4}\) | 40 ± 0.5 |
| \(K_{\text{m,s}}\) (mM) | pH 7.1 | pH 7.1 | pH 7.1 |
| 0.32 ± 0.03 | 3.2 ± 0.01 | 9.1 ± 0.1 | 1483 ± 80\(^{a}\) |
| \(K_{\text{m,b}}\) (mM) | pH 7.1 | pH 7.1 | pH 7.1 |
| 0.30 ± 0.02 | 9.3 ± 0.2 | 9.1 ± 0.1 | 40 ± 0.5 |
| \(K_{\text{catO}}\) | pH 7.1 | pH 7.1 | pH 7.1 |
| 4.9 | 0.015 | 0.061 | 20 ± 0.2 |
| \(k_{\text{catO}}/K_{\text{NAD}}\) (M\(^{-1}\)s\(^{-1}\)) | pH 7.1 | pH 7.1 | pH 7.1 |
| 20 | 20 | 20 | 1.0 ± 0.02 |

\(^a\) From fits of Equation 3 to initial-rate data for oxidation and reduction. Equilibrium constants \(K_{\text{i}}\) for fructose reduction by NADH of 3.4 (N191D), 31 (N300D), and 11,000 (wild-type enzyme) were calculated from the Haldane relationship \(K_{\text{eq}} = K_{\text{catO}}K_{\text{NAD}}/K_{\text{catO}}K_{\text{NAD}}\) where \(K_{\text{fructose}}\) was not corrected. The kinetically determined \(K_{\text{eq}}\) values are in useful agreement with a \(K_{\text{eq}}\) of 9 (± 25) determined experimentally at pH 7.1 (6.8) and 25 °C.

\(^b\) Data from Ref. 13.

\(^c\) Not measurable.

\(^d\) ND, not determined.

\(^e\) \(K_{\text{NAD}}\) is the dissociation constant of enzyme-NAD\(^+\). However, this does not imply ordered binding, NAD\(^+\) prior to mannitol, in N191D or N300D at pH 7.1. Note that Equation 3 is applicable to both ordered and random kinetic mechanisms.

\(^f\) Data from Ref. 4.

\(^g\) Values were determined at non-saturating concentrations of fructose (2.0 m < \(K_{\text{fructose}}\)).

\(^h\) Values are corrected for the proportion of free carbonyl form (1%) present in aqueous solution of fructose (29).

### FIGURE 3

Free energy profiles for fructose reduction catalyzed by wild-type enzyme (solid line), N191D (dashed lines), and N300D (dotted line). ΔG values were calculated as described under supplemental Results, assuming a 1 m standard state for all reactants. E, A, B, P, and Q are enzyme, NADH, fructose, mannitol, and NAD\(^+\), respectively. EA, EAB, EPQ, and EQ are the corresponding enzyme complexes. \(E^\text{TS}\) is the transition state of reaction.
corresponding kinetic parameters in Table 2. By comparing the energy levels for central ternary complexes in reduction (EAB) and oxidation (EPQ), it is immediately recognized from Fig. 3 that each Asn → Asp substitution resulted in very large perturbation of the internal equilibrium of the enzymatic conversion, as will be discussed later. Another clear consequence of the mutations is destabilization of the transition state of the reaction. The two mutants can be distinguished according to the effect of the site-directed substitution on the ratio $K_{iNAD^{+}}/K_{NAD^{+}}$, which increased ~700-fold for N300D but decreased 10-fold for N191D as compared with the corresponding wild-type ratio of 0.10 at neutral pH (Table 2). The presence of mannitol thus enhanced the apparent affinity of N191D for NAD$^{+}$, whereas it had the opposite effect on NAD$^{+}$ binding in N300D. Binding of NAD$^{+}$ to free enzyme was 27-fold tighter in N191D, whereas it was unchanged in N300D as compared with wild-type PfM2DH.

Transcript Kinetic Analysis of Reactions Catalyzed by Wild-type PfM2DH and Mutants Thereof—To examine changes in the location of the rate-determining step(s) for reactions catalyzed by N191D and N300D as compared with the corresponding reactions catalyzed by the wild-type enzyme that have been well characterized (4, 13, 18), we performed rapid-mixing, stopped-flow experiments with these two mutants. Fig. 4 shows time courses of NADH consumption during reduction of fructose by N191D and N300D at pH 5.2 (Fig. 4A) and by N191D at pH 7.1 (Fig. 4B). In each case, the reaction proceeded with a fast pre-steady-state “burst” that was followed by a linear steady-state phase. Data were fitted with Equation 4, and relevant transient rate constants ($k_{obsR}$) are summarized in Table 2 along with published $k_{catR}$ values for the wild-type enzyme. The full set of constants is given in supplemental Table S2. $k_{obsR}$ of N191D was not dependent on pH in the range 5.2–7.1. Each site-directed replacement caused a relatively modest decrease in $k_{obsR}$ (N191D: 4.3-fold; N300D: 21-fold) as compared with the wild-type reference. In the stopped-flow time courses shown in Fig. 4A, the amount of NADH (per enzyme equivalent) consumed in the “burst” phase was 4.5-fold higher for N191D as compared with N300D (see also supplemental Table S2). This observation appears to be inconsistent with a ratio of rate constants $k_{obsR}/k_{catR}$ that is higher for N300D than it is for N191D. However, in terms of $K_{fructose}$ at the steady state (Table 2), N300D binds fructose 7.2-times more weakly than does N191D. The portion of enzyme tied in the reactive ternary complex under stopped-flow reaction conditions should thus be smaller for N300D than N191D. Additionally, the relative amount of productively bound enzyme could be different for the two mutants.

Fig. 4C depicts stopped-flow progress curves for mannitol oxidation by N191D and N300D at pH 10.0. After normalization for the total concentration of enzyme used ($[E]$), slopes of the linear time courses of NADH formation corresponded to $k_{catO}$ (Table 2) within limits of the experimental error (±10%). The transient rate constant for mannitol oxidation ($k_{obsO}$) is therefore approximately equal to $k_{catO}$ (Table 2). Comparison between $k_{obsO}$ for native PfM2DH and $k_{catO}$ for N191D and N300D reveals that both mutants have lost massively, by ~6 orders of magnitude, their wild-type catalytic ability to promote oxidation of mannitol. The large difference in disruptive effect of each Asn → Asp mutation on rate constants for oxidation ($k_{obsO}$ or $k_{catO}$) and reduction ($k_{obsR}$) is striking.
Primary Deuterium Kinetic Isotope Effects (D-KIEs)—We performed steady-state and transient kinetic experiments to measure D-KIEs on the relevant parameters ($D_k_{cat}$, $k_{cat}/K_{substrate}$ and $D_k_{obs}$) for N191D and N300D. The analysis of D-KIEs can provide important information on the contribution of the isotope-sensitive step of hydride transfer to rate limitation in the overall reaction of the mutants. $D_k_{cat}$ and $D_k_{cat}/K_{substrate}$ were determined at different pH values, including for each direction of reaction the respective optimum pH as well as a pH value above or below the pK seen in the pH-log($k_{cat}/K_{substrate}$) profile (Table 1). A D-KIE on $k_{cat}/K_{NAD^+}$ for N191D and N300D was also determined at pH 10.0. Results are shown in Table 3 along with relevant D-KIEs for the wild-type enzyme that were taken from literature. The D-KIEs on kinetic parameters for mannitol oxidation by N191D and N300D were hardly different from unity (no D-KIE). In fructose reduction by the two mutants, by contrast, a substantial D-KIE of ~2 or greater was measured on $k_{cat}/K_{fructose}$ across the examined pH range. The corresponding $D_k_{cat}$ was smaller than $D_k_{cat}/K_{fructose}$. The pH dependence of $D_k_{cat}/K_{fructose}$ for the mutants was different from that for the wild-type enzyme (Table 3).

Considering the presence of a pre-steady-state burst of NADH consumption in stopped-flow progress curves of fructose reduction by N191D and N300D (Fig. 4), it was also of interest to obtain a D-KIE on $k_{obs}$ for each mutant. $k_{obs}$ is a kinetic parameter that includes all steps of the reaction up to the one that is rate-limiting in the steady state, and the value of $D_k_{ob}$ informs about the role of hydride transfer in being rate-determining for this particular series of steps. We determined $D_k_{ob}$ for N191D and N300D at pH 5.2. A reference value of $D_k_{ob}$ was obtained for wild-type PfM2DH at pH 7.1. Note: comparison of reactions catalyzed by native enzyme and mutants thereof using $D_k_{ob}$ values at a single pH was not possible because of very small “burst phases” at pH 5.2 (wild-type PfM2DH) or 7.1 (mutants; see Fig. 4B). However, the shown $D_k_{ob}$ values (Table 3) are for near-optimum pH conditions for fructose reduction by each enzyme. They are similar for all three enzymes studied and are comparable to the corresponding value of $D_k_{cat}/K_{fructose}$.

DISCUSSION

Interpretation of pH-rate Dependences for the Mutants of PfM2DH—In accordance with previous studies of the wild-type enzyme (12–14, 18), Lys-295 is the most likely group in enzyme-NAD$^+$ complexes of N191D and N300D to which the pK value in the respective pH profile of log($k_{cat}/K_{mammal}$) can be assigned. Catalytic function of the lysine as a Bronsted base thus appears to have been retained in the mutants. However, considering the perturbation of active-site electrostatics expected from an Asn → Asp substitution, displacement of the pK of Lys-295 from where it was in native PfM2DH (pK$_D$ = 9.2) was surprisingly small in N191D (ΔpK$_D$ ≈ 0) and N300D (ΔpK$_D$ ≈ +0.8). The absence of a transient lag or a burst phase in multiple turnover stopped-flow progress curves of mannitol oxidation implies that $k_{cat}$ of the mutants was limited by ternary complex conversion. The observation, that D-KIEs on $k_{cat}$/ $k_{cat}/K_{mammal}$ and $k_{cat}/K_{NAD^+}$ had similar values, likewise supports this notion (for the general case see Ref. 25). Lack of pH dependence in $k_{cat}$ for N191D and N300D therefore suggests that, as in wild-type enzyme (4), binding of mannitol causes substantial depression of the pK$_D$ of Lys-295.

Asp-191 and Asp-300 introduce a pH dependence to $k_{cat}/K_{fructose}$ that was not present in the wild-type enzyme (13, 18). Each Asp arguably represents the ionizable group with apparent pK$_a$ of 5.3 ± 0.1 (N191D) and 6.3 ± 0.2 (N300D) that must be protonated in enzyme-NADH for optimum activity in fructose reduction (Table 1). Further depression of apparent pK$_a$ to a value outside of the measured range (<5.2) can be assumed for the double mutant. Retention of activity (in $k_{cat}/K_{fructose}$ terms) at pH ≥ 9 where either Asp of N191D and N300D would be completely ionized suggests that the pK$_a$ of Lys-295 in enzyme-NADH was increased in the two mutants to a value (>10.0) that was also out of the experimental pH range (Fig. 2B and Table 1). It would seem therefore that the negative charge on the nearby Asp locks Lys-295 in the required protonation state for reduction. It was shown in previous studies (13, 18) that $D_k_{cat}/K_{fructose}$ for the wild-type enzyme decreases to the equilibrium D-KIE at high pH due to deprotonation of Lys-295. By contrast, $D_k_{cat}/K_{fructose}$ for N191D and N300D did not display a similar pH dependence in the high pH region (see Table 3).

The presence of a pre-steady-state burst of NADH consumption in multiple turnover stopped-flow progress curves of fructose reduction by the mutants and the pattern of D-KIEs where $D_k_{cat}/K_{fructose} > D_k_{cat} ≈ 1$ indicates that $k_{fructose}$ of N191D and N300D at the optimum pH of 5.2 is limited by release of the second product, which could be mannitol or NAD$^+$ (see below). The observation, that D-KIEs on the pre-steady-state rate constant and on $k_{cat}/K_{fructose}$ were of comparable magnitude, is consistent with the proposed kinetic scenario. The absence of a pH dependence on $k_{cat}$ for the mutated enzymes in the pH range 5.2–7.1 (where substrate binding affinity was sufficiently high to allow determination of the $k_{cat}$) may therefore simply reflect the kinetic complexity of this rate parameter. An alternative explanation for the pH independence of $k_{cat}$ for N191D and N300D is that the pK$_a$ of Asp-191 and Asp-300 is
strongly increased upon changing from substrate-limited (\(k_{\text{catO}}/K_{\text{fructose}}\) enzyme-NADH) to substrate-saturated (\(k_{\text{catF}}\)) reaction conditions. Formation of a hydrogen bond between the protonated carboxyl group of Asp and the carbonyl oxygen of bound fructose could be responsible for this \(pK_a\) change. However, we must emphasize that ambiguity remains in the interpretation of the \(pH\) dependence of \(k_{\text{catF}}\) for the two mutants. The \(pH\) dependence of \(k_{\text{catF}}/K_{\text{fructose}}\) in the \(pH\) range 5.2–8.0, where Asp-191 or Asp-300 would undergo deprotonation while Lys-295 remained protonated, supports a mechanism in which the ionization state of aspartate affects the isotope-sensitive step of hydrate transfer. In summary, therefore, \(pH\)-profile analysis for the direction of fructose reduction provides clear evidence of a new ionizable group in both N191D and N300D, as compared with wild-type enzyme, which appears to contribute to a strongly acidic environment in the active sites of the two mutants.

**Reaction Coordinate Analysis for N191D and N300D:** The Mutants Behave as Fructose Reductases—Selective disruption of \(k_{\text{catO}}/K_{\text{NAD}}\) as compared with \(k_{\text{catR}}/K_{\text{NAD}}\) in N191D and, to a lesser extent, in N300D (Table 2) is difficult to reconcile with an Ordered Bi Bi kinetic mechanism where these catalytic efficiencies stand for coenzyme binding to free enzyme. However, in accordance with previous studies of PM2DH showing that individual substitutions of Asn-191 and Asn-300 by Ala caused loss of order in binding of NAD\(^{+}\) and mannitol (4), we believe that, under conditions of a saturating mannitol concentration, \(k_{\text{catO}}/K_{\text{NAD}}\) for N191D and N300D is really the second-order rate constant for reaction of enzyme-mannitol with NAD\(^{+}\). These details of the kinetic mechanism of N191D and N300D are, however, not essential for the conclusions drawn herein and were therefore not further pursued. Notwithstanding, Fig. 3 might show only one of two possible routes of formation of enzyme-NAD\(^{+}\)-mannitol in the two mutants.

Mutation of Asn-191 stabilized enzyme-NAD\(^{+}\)-mannitol by \(-20.6 \text{ kJ mol}^{-1}\), whereas it destabilized enzyme-NADH-fructose by \(+11.5 \text{ kJ mol}^{-1}\), relative to the corresponding complexes of wild-type PM2DH. The kinetic barrier for mannitol oxidation was strongly increased (\(\Delta \Delta G = +35.8 \text{ kJ mol}^{-1}\)) as a result of the mutation, whereas it was hardly affected for fructose reduction (\(\Delta \Delta G = +3.5 \text{ kJ mol}^{-1}\)). Loss of “transition state” stabilization energy in N191D as compared with wild-type enzyme was \(-15.2 \text{ kJ mol}^{-1}\). The overall pattern of changes in free energy profile resulting from site-directed substitution of Asn-300 by Asp was quite similar to the one just described for N191D. Differential stabilization of ternary complexes undergoing oxidation and reduction, as shown for the two mutants in Fig. 3, results in a dramatic change of internal equilibrium (\(K_{\text{int}} = k_{\text{obsR}}/k_{\text{obsO}}\)), from a value of 0.4 (403/1041) in the wild-type enzyme (4) to \(1.7 \times 10^5 (= 93/0.00055)\) in N191D and 1.3 \times 10^4 in N300D. The internal equilibrium for wild-type PM2DH can be compared within the same order of magnitude to the thermodynamic equilibrium constant of the reaction (Table 2).

What is the catalytic principle underlying the observed “one-way catalysis” (26) by N191D and N300D as compared with the wild-type enzyme that provides effective catalysis to the reaction in both directions? Transition state complementarity for the native enzyme has been clearly lost as result of either site-directed substitution, as shown in Fig. 3. However, results in Fig. 3 also reveal that the active site containing Asp-191 or Asp-300 has acquired a substantial amount of complementarity with respect to mannitol, as compared with the wild-type enzyme. A maximum amount of binding energy will therefore be realized when mannitol binds to N191D and N300D while binding of fructose is expected to involve a substantial amount of destabilization energy, affecting substrate, the mutated enzyme or both. Selective rate acceleration to fructose reduction may be derived from this destabilization, providing an explanation for why loss of transition state stabilization in N191D and N300D as compared with wild-type PM2DH had almost no effect on decreasing \(k_{\text{obsR}}\). Stabilization of mannitol is expected to involve productive as well as nonproductive modes of binding. Evidence from MD simulation clearly supports non-productive binding of mannitol whereby two different orientations of the reactive C2-OH of the substrate appear to be preferred, as shown in Scheme 1A and, in more detail, in supplemental Fig. S4. One set of conformations frequently sampled in the simulation of either mutant involved a bidentate hydrogen bond from Asp-230 to C1 and C2 hydroxy groups of mannitol. This conformation was especially predominant in N300D. The other conformational ensemble featured a hydrogen bond between the C2-OH and Asp, especially Asp-191, whereas the C1-OH remained bonded to Asp-230. Scheme 1A shows representative structure snapshots from the MD simulation. Full time courses of simulated atom-atom distances are given in supplemental Fig. S4 (A–C). The experimental observation that \(k_{\text{catO}}\) was \(-10^3\)-fold more sensitive than \(k_{\text{catO}}/K_{\text{manoino}}\) to mutation of Asn-191 and Asn-300 is likewise consistent with nonproductive binding of mannitol (Scheme 1A), implying that a substantial fraction of each mutant will be tied in a ternary complex that is unable to yield product and can regenerate the active enzyme only by dissociation. In the productive complex at the optimum \(pH\) for mannitol oxidation (Scheme 1B), the negatively charged side chain of Asp-191 or Asp-300 might be oriented away from Lys-295. The proposed conformer provides a tentative explanation for why the \(pK_a\) of Lys-295 in enzyme-NAD\(^{+}\) is relatively insensitive to site-directed substitution Asn \(\rightarrow\) Asp. Nonetheless, the catalytically important “activation” of the reactive alcohol group in substrate through partial proton abstraction by Lys-295 (Scheme 1C) is expected to be disfavored electrostatically in both N191D and N300D.

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4 Kinetic data in Table 2 show that binding of mannitol enhances the affinity of N300D for NAD\(^{+}\) (\(K_{\text{NAD}} \gg K_{\text{NAD}}\)). By contrast, the affinity of N191D for NAD\(^{+}\) is weakened by binding of mannitol (\(K_{\text{NAD}} \ll K_{\text{NAD}}\)). In the crystal structure of wild-type PM2DH bound with NAD\(^{+}\) (PDB: 1LJ8), Asn-191 points towards the nicotinamide ring of coenzyme. We therefore believe that \(-30\)-fold tighter binding of NAD\(^{+}\) by free N191D as compared to NAD\(^{+}\) binding by wild-type enzyme and N300D reflects attraction between the negatively charged Asp and the positively charged nicotinamide moiety. From the crystal structure, similarly strong interactions are not expected for Asp-300.

5 The value of \(K_{\text{int}}\) for N300D (at pH 6.8) was calculated using the thermodynamic relationship, \(\Delta \Delta G = \Delta G_{\text{EPQ}} - \Delta G_{\text{AAB}} = -RT \ln K_{\text{int}}\). \(\Delta G_{\text{EPQ}}\) (\(= -38\) kJ/mol) and \(\Delta G_{\text{AAB}}\) (\(= -14.6\) kJ/mol) are from Fig. 3.
Proposed Catalytic Mechanism of N191D and N300D—The proposed catalytic function of Lys-295 and Asp-191 (or Asp-300) in facilitating reduction of fructose by NADH is shown in Scheme 2. Herein, the active site of the mutated mannitol dehydrogenases provides a “super-acidic” micro-environment, aiding in a very strong polarization of the substrate carbonyl, which in turn is expected to promote the hydride transfer from NADH. Lys-295 retains its native role (14) as catalytic Brønsted acid in N191D and N300D. The proposed arrangement of catalytic groups as well as their function in carbonyl group polarization have strong analogy to Δ4-3-ketosteroid 5β-reductase from the aldo-keto-reductase protein superfamily (15, 27). The 5β-reductase catalyzes the double bond reduction of the Δ4-ene by NADPH and uses a diad of glutamic acid and tyrosine for interaction with the substrate carbonyl. Δ4-3-Ketosteroid isomerase catalyzes isomerization of 3-oxo-Δ4-steroids to their Δ4-conjugated isomers. Using aspartic acid and tyrosine, this enzyme shows a similarly “super-acidic” binding pocket for the 3-keto group of the substrate as the 5β-reductase (28).

In conclusion, redesign of the oxyanion hole of PfM2DH through substitution of Asn-191 or Asn-300 by Asp brought about a drastic change in the directional preference of enzyme catalysis. A double mutant harboring both Asn → Asp substitutions was devoid of measurable activity in mannitol oxidation but performed as weakly active fructose reductase under low pH conditions. This is to our knowledge the first time that a parent dehydrogenase was converted into a reductase through targeted modification of the catalytic center. Optimal catalysis of the reaction in both directions is a feature of the wild-type enzyme, which seems to reflect complementarity of the active site to the transition state. By strongly stabilizing bound mannitol in productive as well as non-productive conformers,
N191D and N300D by contrast achieve essentially one-way catalysis of fructose reduction, characterized by a high maximal rate (not very different from that of the wild-type enzyme in $k_{\text{obsR}}$ as well as $k_{\text{catR}}$ terms) accompanied by weaker binding of the ketose substrate. Design of “super-acidic” catalytic centers capable of strong polarization of the reactive carbonyl group could be of general relevance in the development of essentially uni-directional reductases to be applied as biocatalysts for organic synthesis. These engineered reductases are expected to show optimum activity at low pH where as an additional advantage, oxidation of alcohol product is disfavored thermodynamically.

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