On the role of Brønsted catalysis in *Pseudomonas fluorescens* mannitol 2-dehydrogenase

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X-ray structure of the *Pseudomonas fluorescens* mannitol 2-dehydrogenase ternary complex with NAD⁺ and D-mannitol suggests that Lys-295 provides catalytic base assistance to secondary alcohol group oxidation. We have replaced Lys-295 by site-directed mutagenesis with alanine or methionine and evaluated the catalytic significance of side-chain substitution by kinetic analysis of restoration of activity with external amines, and from pH and solvent isotope effects on the reaction catalysed by K295A (Lys-295 → Ala mutant). K295A and K295M (Lys-295 → Met mutants) show 3×10^4 - and 2×10^6 -fold lower turnover numbers respectively for D-mannitol oxidation (k_{catO}) at pH 10.0 than the wild-type. The second-order rate constant for non-covalent rescue of activity (k_B) by free methylamine base is $31 \text{ M}^{-1} \cdot \text{s}^{-1}$ for K295A, but only $0.021 \text{ M}^{-1} \cdot \text{s}^{-1}$ for K295M. A Brønsted relationship of $\log k_B$ (corrected for molecular size

effects) and $\text{p}K_a$ of the external amine is linear (slope $\beta = 0.66 \pm 0.16$; $r^2 = 0.99$) for K295A-catalysed D-mannitol oxidation at pH 10.0. The k_{catO} values of K295A in H₂O and ²H₂O are linearly dependent on [OL[−]] in the pL range 7.5–10.5 (where L is ¹H or ²H). The solvent isotope effect on k_{catO} is 0.69. The time course of D-fructose reduction by K295A at pH 8.2 displays a pre-steady-state burst of NADH consumption. These data support a mechanism in which the ϵ -NH₂ group of Lys-295 participates in an obligatory pH-dependent, pre-catalytic equilibrium which may control alcohol/alkoxide equilibration of enzyme-bound D-mannitol and activates the C2 atom for subsequent catalytic oxidation by NAD⁺.

Key words: alkoxide mechanism, catalytic base, long-chain dehydrogenases, stepwise hydrogen transfer.

INTRODUCTION

PsM2DH (*Pseudomonas fluorescens* mannitol 2-dehydrogenase) catalyses the regioselective oxidation of the *R*-configured alcohol group at C2 of the six-carbon polyol D-mannitol by NAD⁺. The enzyme is composed of two domains that form a cleft in which the active site is located. The N-terminal domain contains the residues involved in binding of NAD⁺, the C-terminal domain contributes the active-site groups and residues conferring substrate specificity. PsM2DH belongs to a family of long-chain dehydrogenases [1], which, in spite of highly divergent amino acid sequences, all share a structurally conserved active-site lysine residue. X-ray structures of several long-chain dehydrogenases differing in substrate specificity and reaction chemistry ([2,3] and references therein) now show that this lysine may be optimally positioned for acid/base catalytic function in each enzyme, probably indicating conserved mechanistic components of NAD(P)⁺-dependent dehydrogenation. We therefore considered elucidation of the exact role of the catalytic lysine in PsM2DH to be a biochemical problem of a reasonably broad relevance.

The structure of a non-productive ternary PsM2DH complex with NAD⁺ and D-mannitol bound at pH 5 has revealed a hypothetical chemical mechanism of alcohol dehydrogenation, as shown in Scheme 1. Structural and kinetic evidence suggest that the timing of hydrogen abstraction steps from the reactive alcohol group could be such that proton transfer to the ϵ -NH₂ group of Lys-295 occurs before hydride transfer to NAD⁺. Neighbouring the lysine in the structure, the side-chain amide groups of Asn-191 and

Asn-300 form an oxyanion hole. They are brought into positions that would seem to enable their function in the stabilization of a near full negative charge that is expected to develop at the C2 oxygen atom in a stepwise proton/hydride transfer mechanism. A proton-relay system is present in PsM2DH through which protons may be shuttled to bulk solvent [2]. Interpretation of the ternary complex structure supports a pH-dependent kinetic mechanism of wild-type PsM2DH, possibly involving an alkoxide intermediate [4]. The kinetic significance of replacing Lys-295 by alanine is in excellent agreement with a function of the lysine in general base catalysis to alcohol oxidation.

The hypothetical reaction chemistry for PsM2DH in Scheme 1 [4,5] represents an interesting new twist of NAD(P)⁺-dependent alcohol dehydrogenase mechanism; namely, the stepwise hydrogen abstraction without the participation of an active-site metal ion, as in the Zn²⁺-containing medium-chain dehydrogenases [6–8], that would stabilize the canonical alkoxide intermediate. The PsM2DH mechanism is plausible in light of enzymic precedent [4], indicating that an obligatory deprotonation step of the enzyme-bound alcohol before hydride transfer takes place should activate the reactive carbon for subsequent catalytic oxidation by NAD(P)⁺. In this paper, we report production of K295A (Lys-295 → Ala) and K295M (Lys-295 → Met) mutants of PsM2DH and kinetic analysis of the Lys → Ala mutant using pH and solvent isotope effects and chemical rescue by external amines. The results provide supporting evidence for a stepwise chemical mechanism of PsM2DH with Lys-295 functioning as the general acid/base catalyst.

Abbreviations used: ADH, alcohol dehydrogenase; K295A, Lys-295 → Ala mutant; K295M, Lys-295 → Met mutant; KIE, kinetic isotope effect; PsM2DH, recombinant *Pseudomonas fluorescens* mannitol 2-dehydrogenase harbouring a C-terminal oligohistidine fusion peptide; 6PGDH, 6-phosphogluconate dehydrogenase. The following nomenclature was used to describe KIEs: superscript 2H reports a primary deuterium KIE, for example ^{2H} k_{cat} is a primary deuterium KIE on k_{cat} , the turnover number.

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Scheme 1 Hypothetical sequence of proton and hydride transfer during PsM2DH-catalysed oxidation of D-mannitol at high pH (≥ 9.3) [2,4]

RS, reactant state; TS, transition state; PS, product state.

EXPERIMENTAL

Materials

Non-substituted and substituted alkyl amines were of the highest purity available from Sigma. All materials for protein chromatography and electrophoresis were from Pharmacia. NAD^+ and NADH (sodium salts; 99.5% purity) were obtained from Seppim (France). Other materials, including 2- ^2H -D-mannitol, are described elsewhere [4,9].

Site-directed mutagenesis, and purification and structural characterization of mutants

The K295A mutant has been reported previously [9]. Replacement of Lys-295 by methionine was achieved with an inverse-PCR protocol [9], using 5'-TTGCTCAACGGATCCACCTG-3' and 5'-GCCGATCATCATCTCTTCATA-3' as oligonucleotide primers. The mismatched bases are underlined. To facilitate colony screening, a silent restriction site for *Bam*HI was engineered into one primer (marked in bold) using the programme Webcutter 2.0 (<http://www.firstmarket.com/cutter/cut2.html>). Plasmid miniprep DNA from positive clones was subjected to dideoxy sequencing to verify that the desired mutations had been introduced and no misincorporation of nucleotides had occurred because of DNA polymerase errors. The expression vector for K295M was transformed into electrocompetent cells of *Escherichia coli* JM109.

Production and purification of K295M were carried out according to published procedures [5]. Note, however, that careful regeneration of the metal affinity gel was required before each new purification run. The minimum procedure included dissociation of ligated Cu^{2+} with EDTA, washing the gel with 1 M NaCl, and recharging the metal sites with fresh CuSO_4 . Purity of PsM2DH and the K295A and K295M mutants was checked by SDS/PAGE and non-denaturing anionic PAGE, staining with Coomassie Brilliant Blue for the visualization of protein bands. CD spectra were recorded at 25 °C with a Jasco J-715 spectropolarimeter, as described previously [9]. Apparent dissociation constants of binary complexes with NAD^+ and NADH were calculated from fluorescence titration data obtained at 25 °C and pH 10.0, as reported elsewhere [9].

Kinetic measurements

Using a Beckman DU 650 spectrophotometer, initial rates of D-mannitol oxidation and D-fructose reduction were measured by recording the increase and decrease respectively in NADH absorbance at 340 nm, over a reaction time of 5 min (wild-type PsM2DH) and up to 30 min (K295M, K295A) at 25 °C. Each

assay for mutant activity contained a concentration of pure protein of approx. 0.38 to 0.68 mg/ml. Apparent kinetic parameters were obtained from initial rate measurements under conditions in which $[\text{NADH}]$ (200 μM) or $[\text{NAD}^+]$ (2 mM) was constant and saturating, and the substrate concentration was varied in the range indicated. pH studies were carried out in the pH range 8.2–10.5 using 50 mM Tris/HCl buffer (pH 8.2–9.0) or 50 mM glycine/NaOH buffer (pH 8.8–10.5). Initial rate measurements in $^2\text{H}_2\text{O}$ were performed as described previously [4,5]. Stopped-flow kinetic experiments were carried out under conditions as reported elsewhere [4].

Chemical rescue of activity in PsM2DH mutants

A representative series of primary amines was selected, which included ammonia, methylamine, ethylamine, propylamine, ethanolamine, propargylamine and 2,2,2-trifluoroethylamine, which differ in pK_a of the $-\text{NH}_2$ group from values 8.2 to 10.6 and in molecular volume from 32 $\text{\AA}^3$ to 84.7 $\text{\AA}^3$ [10]. Initial rate measurements for NAD^+ -dependent oxidation of D-mannitol were carried out at pH 10.0 (100 mM glycine/NaOH buffer) or pH 7.1 (100 mM Tris/HCl buffer) in the absence and presence of the respective amine whose concentration was varied in the range 60–3000 mM. The concentrations of D-mannitol (100 mM) and NAD^+ (1 mM) were constant and saturating. Control experiments showed that the effect of external amines on activity of wild-type PsM2DH was not significant under exactly comparable conditions.

Data processing and simulation

Data were fitted to eqns (1)–(5) using the least-squares method implemented in the programme SigmaPlot 2001 for Windows version 6.1 (Jandel):

$$v = k_{\text{cat}}[\text{E}][\text{A}]/(K_A + [\text{A}]) \quad (1)$$

$$v = k_{\text{cat}}[\text{E}][\text{A}]/\{K_A(1 + F_i E_{\text{V/K}}) + [\text{A}](1 + F_i E_v)\} \quad (2)$$

$$k_{\text{obs}} = k_{\text{solvent}} + (k_B[\text{amine}]_{\text{tot}})/(1 + ([\text{H}^+]/K_a)) \quad (3)$$

$$\log k_B = \beta \text{p}K_a + V(\text{molecular volume}) + C \quad (4)$$

$$A_t = A_0 - (\Pi + kt) \quad (5)$$

Initial rate data from experiments in which a single substrate was varied were fitted to eqn (1), where v is the initial rate, k_{cat} is the turnover number, $[\text{E}]$ and $[\text{A}]$ are enzyme and substrate concentrations, and K_A is an apparent Michaelis constant for A. Initial rate measurements in H_2O and $^2\text{H}_2\text{O}$ solvents were fitted to eqn (2), where F_i is the fraction of ^2H in the solvent and $E_{\text{V/K}}$ and

E_V represent the isotope effects minus 1 on kinetic parameters k_{cat}/K_A and k_{cat} respectively. Likewise, initial rate measurements with D-mannitol and 2-[^2H]-D-mannitol were fitted to eqn (2). Experimental turnover numbers (k_{obs}) recorded in the presence of an activity-restoring primary amine were fitted to eqn (3), where k_{solvent} is the observable turnover number in the absence of external amine, k_B is the second-order rate constant for activity rescue, $[\text{amine}]_{\text{tot}}$ is the total concentration of unprotonated and protonated amine, and K_a is the acid dissociation constant of the respective amine. Eqn (4) describes a linear free-energy relationship in which the observed value of k_B (in the log form) reflects the contributions from the electronic properties ($\text{p}K_a$) and molecular volume of the amine. In eqn (4), β is the Brønsted slope coefficient, V is a molecular volume correction coefficient, and C is a constant term. Eqn (5) was used to describe an absorbance time course in which an initial burst (Π) of absorbance change is followed by a steady-state rate of decrease in absorbance. A_0 and A_t are the absorbances at zero time and at time t , and k is the steady state rate. The program KINSIM (www.biochem.uiowa.edu/faculty/plapp, [11]) was used for kinetic simulations.

RESULTS

K295M mutant

Figure 1(A) shows an SDS/polyacrylamide gel of purified K295M mutant along with wild-type PsM2DH and K295A mutant. CD spectra of the three proteins are compared in Figure 1(B). The spectrum of the wild-type and the spectra of the mutants are almost superimposable. The observed small differences in minimum ellipticity values at 209 nm and 221 nm are readily explained by slight variations in the protein concentrations used.

Apparent K_d values of K295M complexes with NADH and NAD^+ were determined at pH 10.0 and are $27 \pm 12 \mu\text{M}$ and $1200 \pm 60 \mu\text{M}$ respectively. The data agree well with corresponding K_d values of the wild-type and the K295A mutant [9]. Significant enzyme activity of K295M mutant was detectable only when extended reaction times of up to 24 h were used and about $12.5 \mu\text{M}$ protein was present. At pH 10.0, the turnover number for NAD^+ -dependent oxidation of D-mannitol was 2×10^6 -fold lower than that of the wild-type. K295M was 150-fold less active than K295A, and this large catalytic defect precluded determination of steady-state kinetic parameters for D-mannitol oxidation by K295M. D-Fructose solutions in 50 mM glycine/NaOH buffer, pH 10.0, were obviously not stable enough to allow reasonably precise activity measurement for K295M over many hours of reaction. Time-dependent formation of yellow-coloured reaction products was observed, and this occurred at about the same rate in the absence and presence of enzyme.

pH and solvent isotope effects

Initial rates of NAD^+ -dependent oxidation of D-mannitol catalysed by K295A mutant were recorded in H_2O and $^2\text{H}_2\text{O}$ solvent in the pH(^2H) range 8.2–10.5. Data were obtained under conditions of varied [D-mannitol] and constant $[\text{NAD}^+]$ (2 mM), and kinetic parameters at each pH (^2H) value are from fits of the experimental rates to eqn (1). Results are shown in Figures 2 and 3, and the insets display published pH profiles of $\log k_{\text{catO}}$ (where the subscript 'O' denotes oxidation) (Figure 2) and $\log (k_{\text{catO}}/K_{\text{mannitol}})$ (Figure 3) for the wild-type. The comparison of wild-type and mutant pH profiles in H_2O solvent serves to emphasize the large effect of the mutation on the pH dependence of PsM2DH catalysis. Both pH profiles for K295A

Figure 1 Structural characterization of K295A and K295M mutants compared with wild-type PsM2DH

(A) SDS/PAGE of purified wild-type PsM2DH (WT), K295A and K295M using Coomassie Blue staining for visualization of protein bands. About $8 \mu\text{g}$ of each protein sample was loaded on to the gel. A commercial low-molecular-mass standard from Amersham Biosciences was used (Std.). (B) CD spectra of wild-type PsM2DH, K295A and K295M mutants at 25°C . The spectra were recorded with a Jasco 715 spectropolarimeter using a light path of 0.1 cm. Solutions contained $10 \mu\text{M}$ of protein dissolved in 10 mM Tris/HCl buffer, pH 7.1. θ is the measured ellipticity.

are linear in the measured pH range ($r^2 \geq 0.98$), and fits of the data for $\log k_{\text{catO}}$ and $\log (k_{\text{catO}}/K_{\text{mannitol}})$ gave slope values of 1.25 ± 0.04 and 0.99 ± 0.3 respectively. Figures 2 and 3 also show p^2H effects on kinetic parameters of the K295A mutant. The catalytic efficiency increases about 3.5-fold as p^2H is increased from a value of 10.5 to 10.9. In spite of a limited number of data points available, linearity of the relationship of $\log k_{\text{catO}}$ against p^2H appears and is suggested by the data in Figure 2, and a slope value of 1.07 ± 0.2 has been calculated.

The pH(^2H) profiles in Figures 2 and 3 can be converted into plots of k_{catO} and $k_{\text{catO}}/K_{\text{mannitol}}$ versus the concentration of OH^- (or O^{2-}). These plots are linear (results not shown), and solvent isotope effects on k_{catO} and $k_{\text{catO}}/K_{\text{mannitol}}$ are obtained from the ratio of slope values recorded in H_2O and $^2\text{H}_2\text{O}$ solvent. The isotope effects are 0.69 ± 0.10 for k_{catO} and 0.50 ± 0.10 for $k_{\text{catO}}/K_{\text{mannitol}}$. Isotope effects calculated by comparing kinetic parameters at a constant concentration of OL^- (where L is ^1H or ^2H) agree well with these estimates.

In the direction of D-fructose reduction, initial rates were recorded in the pH range 8.2–10.0. A constant NADH concentration of $200 \mu\text{M}$ was used, and [D-fructose] was varied as indicated ($\leq 2 \text{ M}$). Kinetic parameters were calculated using eqn (1), and the pH profiles of $\log k_{\text{catR}}$ and $\log (k_{\text{catR}}/K_{\text{fructose}})$ (where the subscript

Figure 2 pH(²H) profiles for turnover numbers of K295A mutant

The graph shows $\log k_{\text{catO}}$ versus pH (closed circles), $\log k_{\text{catR}}$ versus pH (closed triangles) and $\log k_{\text{catO}}$ versus $p^2\text{H}$ (open circles). The inset displays pH profiles of wild-type PsM2DH in the directions of D-mannitol oxidation (closed circles) and D-fructose reduction (closed triangles) [4,5].

Figure 3 pH(²H) profiles for catalytic efficiencies of K295A mutant

The graph shows $\log (k_{\text{catO}}/K_{\text{mannitol}})$ versus pH (closed circles), $\log (k_{\text{catR}}/K_{\text{fructose}})$ versus pH (closed triangles) and $\log (k_{\text{catO}}/K_{\text{mannitol}})$ versus $p^2\text{H}$ (open circles). The inset displays pH profiles of wild-type PsM2DH in the directions of D-mannitol oxidation (closed circles) and D-fructose reduction (closed triangles) [4,5].

'R' denotes reduction) are shown in Figures 2 and 3 respectively. Both pH profiles show linear increases with increasing pH, and fits of the data gave slope values of 0.72 ± 0.13 ($\log k_{\text{catR}}$) and 0.55 ± 0.002 [$\log (k_{\text{catR}}/K_{\text{fructose}})$]. The insets in Figures 2 and 3 show the reported pH profiles of $\log k_{\text{catR}}$ and $\log (k_{\text{catR}}/K_{\text{fructose}})$ for the wild-type. The high-pH decrease which is displayed in the pH profiles of the wild-type is completely eliminated from the pH profiles of the mutant.

Chemical rescue studies

Non-covalent chemical rescue of activity lost in the K295A and K295M mutants, compared with wild-type, was employed to

Table 1 Chemical rescue of activity in K295A by external amines at pH 10.0

n.a., not applicable; -, no significant activation observed.

Amines	pK_a^*	Molecular volume ($\text{\AA}^3$)*	k_B ($\text{M}^{-1} \cdot \text{s}^{-1}$)	Molarity-equivalent (M)†
Without amine (OH^-)‡	(15.7)‡	0.003 s ⁻¹	n.a.	
Ammonia	9.2	32.0	0.075	533
Ethanolamine	9.5	84.7	0.25	160
Methylamine	10.6	53.2	31.0	1.3
Ethylamine	10.6	73.8	7.34	5.5
Propylamine	10.6	94.2	—	—
Propargylamine	8.2	82.3	0.077	519
2,2,2-Trifluoroethylamine	5.7	94.2	—	—

* pK_a and molecular volume values are from [10].

† Values are related to the wild-type k_{catO} of 40 s⁻¹.

‡ Assuming specific base catalysis in K295A mutant when no external amine is present.

characterize the contribution of the mutated side chain on the catalytic rate enhancement provided by wild-type enzyme. It was also used to ultimately confirm retained active-site integrity in PsM2DH variants. About 45 % of wild-type activity could be restored in K295A mutant upon inclusion of 3 M methylamine in the assay for D-mannitol oxidation at pH 10.0. Under identical conditions, external methylamine did not stimulate or inhibit the activity of wild-type PsM2DH, arguing strongly against a non-specific activation of K295A mutant. Restoration of activity by 3 M methylamine was approx. 1480-fold less efficient in K295M than the K295A mutant. The rescued enzyme activity in the K295A mutant obeyed Michaelis–Menten kinetics with respect to D-mannitol as the varied substrate, and kinetic parameters in the absence ($k_{\text{catO}} = 2.9 \times 10^{-3} \cdot \text{s}^{-1}$; $K_{\text{mannitol}} = 11 \text{ mM}$) and presence of 2 M methylamine ($k_{\text{catO}} = 12.9 \text{ s}^{-1}$; $K_{\text{mannitol}} = 6.9 \text{ mM}$) indicate no substantial change in apparent affinity for substrate in response to external amine. Within the time of the chosen assays and limits of detection of the analytical methods, non-enzymic reactions of D-mannitol did not occur in the presence of external amines. No evidence appeared that would have suggested a change in product identity (D-fructose) when enzymic D-mannitol oxidation took place in assays including a high molarity of primary amine.

Ammonia and a number of primary amines, which differed in their $-\text{NH}_2$ group pK_a and molecular volume of the side chain, were tested for their ability to restore activity in the K295A mutant. Complementation of the catalytic defect (in terms of fold increases in k_{catO}) was found to display a linear dependence on the concentration of each amine (up to approx. 3.0 M). We thus exclude the possibility that saturation in amine (which would have suggested a two-step catalytic process composed of reversible amine binding and reaction) could have escaped our detection. The slope value of each linear relationship yields a bimolecular rate constant (k_B) for the amine-specific restoration of enzyme activity and can be related to the structural and electronic properties of the respective amine (see below). Comparison of experiments conducted with ethanolamine at pH 7.1 ($k_B = 0.27 \text{ M}^{-1} \cdot \text{s}^{-1}$) and pH 10.0 ($k_B = 0.25 \text{ M}^{-1} \cdot \text{s}^{-1}$) showed that the relative stimulation of k_{catO} for K295A is not pH-dependent if appropriate correction is made for the protonation equilibrium of the rescuing amine at each pH and the concentration of free amine base thus available for catalytic reaction.

Corrected k_B values for all amines tested are summarized in Table 1. The results reveal that k_B is dependent on the pK_a of the amine, as would be expected if the external amine was involved in enzymic base catalysis. However, a simple Brønsted plot of

Figure 4 Brønsted relationship of bimolecular rate constants (k_B) for rate enhancement of D-mannitol oxidation by external primary amines, after correction for effect of molecular volume

Multiple regression analysis was performed using eqn (4) and k_B values from Table 2 (denoted by full circles), except that of ammonia (denoted by an open circle).

$\log k_B$ versus pK_a does not show a clear correlation (results not shown), indicating that molecular factors other than amine pK_a are important for restoration of activity in K295A mutant. k_B values for methyl-, ethyl-, and propyl-amine can be compared on the basis of effectively constant pK_a to gauge the effect of amine side chain size. The decrease in k_B as the side chain increases in length indicates a strongly negative steric discrimination of the K295A active site. In search of an interpretable overall structure-activity correlation for the kinetic substituent effects in Table 1, we fitted values of $\log k_B$ to a multiple linear model (eqn 4) in which pK_a and molecular volume were the independent variables. The k_B value for chemical rescue of K295A by ammonia was excluded from the fit. Estimates from multiple linear least-squares regression analysis are: $\beta = +0.66 \pm 0.16$; $V = -0.04 \pm 0.01$, $C = -3.3 \pm 2.3$; $r^2 = 0.99$; $F = 42$; $P = 0.1083$. A Brønsted plot of $[\log k_B - V(\text{molecular volume})]$ versus pK_a is linear, as shown in Figure 4.

A k_{catO} value of 40 s^{-1} for wild-type PsM2DH at pH 10.0 has been used to calculate effective molarity equivalent of the $\epsilon\text{-NH}_2$ group of Lys-295 to the free base form of each of the chosen amines in Table 1. (Note that because proton transfer is not rate-determining in the wild-type, molarity equivalents are apparent only). Comparison of k_B values for methylamine-dependent chemical rescue of activity in K295A ($31 \text{ M}^{-1} \cdot \text{s}^{-1}$) and K295M ($0.021 \text{ M}^{-1} \cdot \text{s}^{-1}$) mutants indicates that complementation of the catalytic defect is much more efficient in the case of the methyl side chain, compared with the long and non-polar side chain of methionine.

Pre-steady state kinetics

Typical time courses of NADH formation and consumption during D-mannitol oxidation at pH 10.0 and D-fructose reduction at pH 8.2 by the K295A mutant are displayed in Figure 5. In the forward reaction, absorbance traces obtained under conditions of [D-mannitol] of 2 mM displayed a time lag of NADH production of approx. 5 min, as shown in Figure 5.

Figure 5 Time courses for K295A catalysed D-mannitol oxidation (open circles) and D-fructose reduction (full circles)

Absorbance traces were recorded with 2 mM D-mannitol at pH 10.0 and 80 mM fructose at pH 8.2. The concentrations of NAD^+ (2 mM) and NADH (200 μM) were saturating at the steady state.

Figure 6 Substrate-dependent burst of NADH consumption during K295A-catalysed reduction of fructose at pH 8.2

Reactions were carried out using 4.8 μM of K295A and a saturating concentration of NADH (200 μM). Burst (Π) values were calculated using eqn (5). Linear regression of all data points in this Figure gave a slope of $4.10 \cdot 10^{-3} \pm 3.10 \cdot 10^{-4}$ and a correlation coefficient $r^2 = 0.99$.

The time lag became shorter as [D-mannitol] increased. It was not detectable at saturating D-mannitol levels ($\geq 20 \text{ mM}$) in both H_2O and $^2\text{H}_2\text{O}$ solvent, even when rapid mixing in a stopped-flow instrument was employed (results not shown). In marked contrast, time courses in the reverse direction displayed a burst of NADH consumption. At high D-fructose levels, the magnitude of the burst corresponded to the molarity of enzyme active sites (4.8 μM) present, as shown in Figure 6. Experimental absorbance traces at different [D-fructose] were fitted to eqn (5), and Figure 6 shows an analysis of the dependence of the burst magnitude on the D-fructose concentration present.

Table 2 pH-dependent primary isotope effects on kinetic parameters for K295A catalysed D-mannitol oxidation

pH	$^2\text{H } k_{\text{catO}}$	$^2\text{H } k_{\text{cat}}/K_{\text{mannitol}}$
9.5	1.00 ± 0.07	1.54 ± 0.20
10.0	1.07 ± 0.06	1.76 ± 0.14
10.5	1.06 ± 0.07	1.70 ± 0.20

Primary deuterium isotope effects on steady-state kinetic parameters for D-mannitol oxidation by K295A mutant

Isotope effects were determined in the range pH 8.2–10.0 by comparing directly the initial rates of polyol oxidation using *protio* and *deuterio* D-mannitol. Data were fitted to eqn (2), and the isotope effects on k_{catO} and $k_{\text{catO}}/K_{\text{mannitol}}$ are summarized in Table 2. The isotope effects were constant within experimental error in the studied pH range, and the effect on k_{catO} was not significantly different from a value of 1 (no isotope effect).

DISCUSSION

The pH dependence of catalytic efficiencies for D-mannitol oxidation and D-fructose reduction by wild-type PsM2DH are consistent with a chemical mechanism involving a general acid–base catalyst with a macroscopic pK_a of approx. 9.3. Lys-295 is a likely candidate to fulfil this catalytic role, as suggested by the X-ray structure of a non-productive PsM2DH ternary complex [2]. The positional conservation of Lys-295 in different long-chain dehydrogenases [1,9] that show a very diverse structural relationship to PsM2DH made this lysine a particularly interesting candidate for site-directed mutagenesis to explore what its exact function in PsM2DH might be. A working hypothesis of this study was that Lys-295 of wild-type PsM2DH determines an experimentally observable pre-catalytic internal equilibrium, which is pH-dependent, involves the D-mannitol-bound enzyme, and may reflect alcohol/alkoxide equilibration in the ternary reactant complex [2,4]. Replacement of the side chain of Lys-295 by the non-ionizable methyl side chain of alanine was undertaken to probe the relationship between the pH-independent catalytic step of hydride transfer and the pH-sensitive step preceding it.

Non-covalent rescue of PsM2DH activity in K295A mutant

Mutation of Lys-295 to alanine results in a marked loss of catalytic activity, compared with wild-type PsM2DH. The effect of the mutation is almost exclusively on the catalytic rate and not on the apparent binding of D-mannitol, supporting the notion that the main function of the ϵ -amino group of Lys-295 is to facilitate the chemical step of the reaction. The restoration of up to 45 % of wild-type activity in this mutant upon addition of a series of primary amines strongly suggests that exogenous $-\text{NH}_2$ groups can compensate for the loss of the mutated side chain. Analysis of the pH-dependence of chemical rescue has clearly proven that the unprotonated form of the exogenous amine is responsible for enhancement of activity in K295A. Using amines of varying sizes, chemical rescue of activity in the K295A mutant displays a strong correlation with molecular volume of the exogenous amine. The decrease in k_B value from $31 \text{ M}^{-1} \cdot \text{s}^{-1}$ to $7.3 \text{ M}^{-1} \cdot \text{s}^{-1}$ as the molecular volume increases from $53 \text{ \AA}^3$ (methylamine) to $74 \text{ \AA}^3$ (ethylamine) indicates considerable steric restraint for larger-sized amines within the volume vacated by replacement of the side

chain of Lys-295 by the side chain of alanine. A possible reason for the limited capability of small-sized ammonia to rescue activity in the K295A mutant might be strong hydration of this simplest amine in aqueous solution, probably indicating that a free $-\text{NH}_2$ group must be available for efficient rescue. It should be noted that pH 10.0 solutions of ammonia were prepared with required caution to ensure that substantial vaporization of ammonia did not interfere with the measurements. All these data are in excellent agreement with the suggestion that Lys-295 functions as a general base in D-mannitol oxidation by PsM2DH.

Interpretation of Brønsted correlation for restoration of activity by primary amines

A Brønsted relationship of the form $\log k_B$ (corrected for molecular size effects) versus pK_a of external amine is linear for the K295A-catalysed D-mannitol oxidation at pH 10.0. The slope of this correlation is 0.66. For the discussion that follows we assume that proton abstraction from the 2-OH determines alcohol/alkoxide equilibration of enzyme-bound D-mannitol before the catalytic step of hydride transfer takes place. A requirement for this mechanism is that the pK_a of the 2-O(H) of D-mannitol in the ternary complex is effectively depressed from a value of 13.5 [12] in solution to a value close to the pK_a of 9.3 for Lys-295, clearly a substantial pK_a change. The conformation of the oxyanion hole in PsM2DH can be compared with oxyanion holes seen in other enzymes, serine protease [13] and aldehyde dehydrogenase [14] for example. Interactions with oxyanion hole residues, which, like in PsM2DH, often are main-chain NH groups or amide side chains, appear to be capable of lowering the pK_a of bound oxyanions by as much as 5 pH units [13]. Hydrogen-bond distances of 2.9 Å and 3.1 Å between O2 and Nδ2 groups of Asn-300 and Asn-191 respectively, are in good agreement with enzyme–substrate interactions measured in other oxyanion holes. In light of precedent in the literature, a significant δpK_a ($\text{pK}_{a,\text{free}} - \text{pK}_{a,\text{bound}}$) for the substrate 2-OH in the PsM2DH ternary complex would thus seem to be conceivable, such that a near matching of pK_a values for Lys-295 and the 2-OH would provide facile proton transfer. However, we emphasize that, based on the experimental evidence, we have no way of distinguishing alcohol/alkoxide equilibration from a pre-catalytic conformational change that is pH-dependent.

Therefore our mechanistic interpretation of the Brønsted β value of 0.66 is that the ground state of the reaction has 66 % of a full positive charge on the amine nitrogen that accepts the proton (and likewise, 66 % of a full negative charge on the oxygen atom from which the proton is abstracted). In this scenario, efficient base catalysis from an external amine is expected to facilitate the process of D-mannitol oxidation by facilitating formation of the reactive alkoxide. Therefore although in the proposed scenario this is not a direct component of the rate-limiting transition state of C–H bond cleavage/formation, (partial) alcohol deprotonation will have its catalytic function effectively expressed in the observed rate enhancement by the K295A mutant. The structure–activity relationship analysis of chemical rescue data thus provides evidence in good support of the widely held notion that alkoxide formation affords a significant catalytic advantage to the enzymic oxidation of alcohol substrates by NAD^+ . Such evidence is hard to come by and to the best of our knowledge, very sparse in the mechanistic literature on alcohol dehydrogenases. Several researchers have utilized homologous series of alcohol substrates in which different side chain substituents were introduced to systematically perturb the electronic properties of the alcohol group. Extensive kinetic studies have

Scheme 2 Proposed reaction mechanism of K295A mutant

$\text{E}_{\text{OH}}^{\text{NAD}^+}$ and $\text{E}_{\text{H}_2\text{O}}^{\text{NAD}^+}$ are enzyme forms in which bound OH^- and H_2O serve as specific base and acid respectively. Binary enzyme–coenzyme complexes are indicated. B, D-mannitol; X^- , the C2-alkoxide of D-mannitol; P, D-fructose. The rate constants k_3 and k_4 describe the proton transfer steps; the catalytic steps of hydride transfer are given by rate constants k_5 and k_6 . In the mutant, specific acid/base catalysis by $\text{OH}^-/\text{H}_2\text{O}$ is thought to take place, whereas in the wild-type, Lys-295 is the general acid/base catalyst with an apparent $\text{p}K_a$ of approx. 9.3 [4]. The mechanism assumes that, because in the mutant dissociation of the alkoxide can occur, D-fructose reduction is not limited by the effective rate of protonation of the alkoxide. In the wild-type, the alkoxide dissociates only after it has become protonated by the enzyme, which above $\text{p}K_a$ 9.3 takes place at a very slow rate. Note that the loss of a proton (as H_2O at high pH) may occur upon conversion of $\text{E}_{\text{H}_2\text{O}}^{\text{NADH}}$ into $\text{E}_{\text{OH}}^{\text{NAD}^+}$, which makes the Scheme stoichiometrically complete. However, it is not known at which step exactly this proton exchange takes place.

been carried out with modified substrates of liver ADH (alcohol dehydrogenase) coupled with thorough searches for interpretable quantitative structure–reactivity correlations of observable kinetic substituent effects. Ambiguity inherent to the substrate–modification approach is that nearby electron-withdrawing substituents have a dual effect on the reactive alcohol: a decrease in the electron density at carbon which will lower the rate of the hydride transfer step, and an increase in electron density at the hydroxyl oxygen which will facilitate proton transfer. Therefore functional replacement of the catalytic base group at the wild-type active site through incorporation of a series of small molecules in a suitable mutant is of value because it allows systematic examination of the proton transfer step ideally uncoupled from the hydride transfer step.

In light of the above interpretation, we think that the poor restoration of activity in the K295M mutant by external methylamine is not only a result of greater steric restraint induced by a methionine side chain, compared with an alanine side chain. Alkoxide formation would be expected to be energetically more demanding if positioning of the reactive 2-OH in the ternary complex occurred in the relatively more hydrophobic micro-environment of the K295M mutant, compared with the K295A mutant.

Kvassman et al. [15] have established a linear free-energy relationship for the dissociation constant of the liver ADH ternary complex (K_d) and $\text{p}K_a$ of the free alcohol. A correlation of $\text{p}K_d$ versus alcohol $\text{p}K_a$ yielded a Brønsted α value of 0.61. The similar sensitivity to acid–base strength of alcohol binding by liver ADH and efficiency of chemical rescue in the K295A mutant is interesting because liver ADH uses Zn^{2+} to ligate the alcohol, whereas PsM2DH lacks a metal cofactor. However, we note that the linear free-energy relationships are not on the same steps in the two enzymes.

Interpretation of pH and solvent isotope effects on the reaction of K295A mutant

The pH profiles of $\log k_{\text{catR}}$ and $\log (k_{\text{catR}}/K_{\text{fructose}})$ for wild-type PsM2DH decrease at high pH and are very much different from the corresponding pH profiles of the K295A mutant. Therefore this supports the suggestion that the $\text{p}K_a$ of the general acid/base, the side chain of Lys-295, is observed in the pH profiles of the wild-type. The pH profiles of the mutant display similar and roughly linear increases with increasing pH. The dependence upon pH of $\log k_{\text{catR}}$ and $\log (k_{\text{catR}}/K_{\text{fructose}})$ of K295A are obtained under conditions of saturating and limiting substrate concentration respectively. In addition, steps outside the sequence of steps from

D-fructose binding to D-mannitol release are unlikely to be rate-determining in a mutant catalytically as defective as K295A. Therefore the observed pH profiles for the K295A mutant must reflect the pH-dependence of the catalytic step of hydride transfer or a later step, which could be proton transfer or alcohol product release. The pre-steady-state burst of NADH oxidation at pH 8.2 shows that a step after hydride transfer must be rate-limiting under these conditions. The absence of ‘burst kinetics’ at pH 10.0 suggests that the ratio of the effective rates of hydride transfer and product release (including proton transfer) decreases at high pH. The absence of a pH effect on primary kinetic isotope effects on kinetic parameters for D-mannitol oxidation indicates that, as in the wild-type [4], hydride transfer is not pH-dependent in the K295A mutant, or it has the same pH-dependence as another reaction step, so that the ratio of the two steps remains effectively unchanged over the studied pH range of 8.2–10.5. We think that protonation of an alkoxide intermediate (by H_2O) cannot be faster at pH 10.0 than pH 8.2. Therefore possible explanations of the observed pH effects on k_{catR} and $k_{\text{catR}}/K_{\text{fructose}}$ for K295A would be that relatively rapid dissociation of the unprotonated alkoxide occurs at the high-pH conditions, but not at the low-pH conditions (Scheme 2); an enzyme conformational change which is required for alcohol product release has a higher rate at pH 10.0 than at pH 8.2. In the latter case, however, the forward commitment to catalysis of D-mannitol oxidation (which is a reverse commitment in the direction of D-fructose reduction) is expected to decrease at high pH, and so isotope effects on $k_{\text{catO}}/K_{\text{mannitol}}$ should increase, which is not observed.

pH effects on $\log k_{\text{catO}}$ reveal a direct dependence of K295A activity on the concentration of LO^- , suggesting that specific base catalysis may be operable in this mutant. The observed correlation appears to be far too pronounced to reflect secondary effects on other active site groups. The solvent isotope effect of 0.69 on k_{catO} reflects the ratio of ground state (Φ_{RS}) and transition state (Φ_{TS}) fractionation factors, $\Phi_{\text{RS}}/\Phi_{\text{TS}}$. The Φ values describe exchange equilibrium constants in $^2\text{H}_2\text{O}$ solvent. In the same way, the solvent isotope effect of approx. 0.5 on $k_{\text{catO}}/K_{\text{mannitol}}$ can be expressed in terms of a ratio of fractionation factors, considering that the ground state at a limiting D-mannitol level reflects the K295A holoenzyme ($\Phi_{\text{RS, binary}}$) and free D-mannitol ($\Phi \approx 1$). If we assume a $\Phi_{\text{RS, binary}}$ value of 0.43 (on the basis of the fractionation factor of the LO^- group in the holoenzyme– LO^- complex), the $k_{\text{catO}}/K_{\text{mannitol}}$ solvent isotope effect can be used to estimate Φ_{TS} to be 0.86 (0.43/0.5). This Φ_{TS} value in turn leads to a value of 0.59 (0.69×0.86) for $\Phi_{\text{RS, ternary}}$ in the K295A– NAD^+ –alkoxide– L_2O complex, which corresponds reasonably to the fractionation factor of a protonic site hydrogen-bonded with an alkoxide ion in water,

Scheme 3 Hypothetical reactant state and transition state structures for reactions catalysed by K295A mutant and wild-type PsM2DH as deduced from solvent isotope effects

^aThe transition-state structure of wild-type is not known, and the Φ_{TS} value of 0.43 may contain contributions from the proton relay [2,3], as described in the text. ^bProduct states for wild-type and mutant are assumed to be similar, i.e., a hydrogen bond to the carbonyl group of D-fructose, and hence they should have identical fractionation factors.

$\Phi = 0.5$ [16]. The isotope effects for the reaction in the ternary complex of the K295A mutant could be described by a mechanism in which the charge on the alkoxide present in the reactant state diminishes in the transition state as the carbonyl group forms. Therefore the environment of proton that is hydrogen-bonded to the alkoxide will change synchronously as hydride transfer to NAD^+ occurs (Scheme 3).

Proton inventory data for $k_{\text{catO}}/K_{\text{mannitol}}$ of wild-type PsM2DH have suggested a transition-state fractionation factor of 0.43 [4]. If Lys-295 is assumed to be deprotonated in the transition state of hydride transfer at pH 10.0, its $\epsilon\text{-NH}_2$ group may hydrogen-bond with the alkoxide ($\Phi \approx 1$), but will not be able to form a strong catalytic proton bridge, which is required to explain the observed solvent isotope effect. The proton relay system in PsM2DH could give rise to a Φ_{TS} value of 0.43, but its function is not understood well enough at the present time to warrant further discussion. Unfortunately, the solvent isotope effect on k_{catO} for the wild-type at pH 10.0 is not useful because it reflects mainly the NADH dissociation event [4]. However, the occurrence of an inverse solvent isotope effect of approx. 0.9 on the pre-steady-state 'burst' rate of NADH formation by the wild-type at saturating D-mannitol levels contains relevant information [4]. It suggests a reactant state fractionation factor of approx. 0.39 (0.9×0.43) for the wild-type, and this value would be consistent with a strong proton bridge from the 2-OH to the catalytic base Lys-295, perhaps a low-barrier hydrogen bond if the $\text{p}K_a$ values of the two groups were truly near matching. It is interesting to compare values of Φ_{TS} and $\Phi_{RS, \text{ternary}}$ for PsM2DH with corresponding fractionation factors for liver ADH. Φ_{TS} of liver ADH is 0.73, from which an estimate of 0.37 for $\Phi_{RS, \text{ternary}}$ has been derived [8].

Interpretation of primary deuterium kinetic isotope effects

The KIE (kinetic isotope effect) on k_{catO} is approx. 1, suggesting that hydride transfer is not rate-limiting for the overall enzymic reaction at saturating D-mannitol concentrations. A high ratio of catalysis, which may completely depress the KIE on k_{catO} , could arguably result from slow steps that precede the catalytic step. Observation of a pre-steady-state time lag during D-mannitol oxidation by K295A at substrate levels in the range of K_{mannitol} indicates that a step prior to hydride transfer is actually rate-determining under these conditions (pH 10.0). The decrease of the time lag as [D-mannitol] is increased reveals that substrate

binding is indeed slow. At enzyme saturation with D-mannitol, the situation is not so clear and a full characterization of the kinetic scheme of the K295A mutant may thus be required. If, as in Scheme 2, the K295A- NAD^+ -alkoxide complex is formed exclusively via the K295A- NAD^+ -alcohol complex under all pH conditions, the dissociation of it occurs via release of alkoxide at pH 10.0. KINSIM simulations (results not shown) indicate that the time lag could escape detection because even at a saturating D-mannitol concentration, the fraction of reactive complex would be much smaller than the total molarity of active sites present.

The KIE on $k_{\text{catO}}/K_{\text{mannitol}}$ for the K295 mutant has a small, however finite, value of 1.8, which is identical within limits of experimental error with the corresponding wild-type values in the pH range 8–9. Unlike $^2\text{H}(k_{\text{catO}}/K_{\text{mannitol}})$ for the wild-type, which decreases at pH values above 9.5 [4], the KIE on $k_{\text{catO}}/K_{\text{mannitol}}$ is constant up to a pH value of 10.5. It would seem therefore that for pH values below $\text{p}K_a \approx 9.3$, which is the macroscopic $\text{p}K_a$ of the general acid–base catalyst of wild-type enzyme [4], commitment factors have not changed significantly as result of the mutation. Elimination of the high-pH decrease in the pH profile of $^2\text{H}(k_{\text{catO}}/K_{\text{mannitol}})$ for the mutant is exactly what one would expect if the original enzyme catalytic acid–base were removed by mutation and another acid–base (e.g., $\text{H}_2\text{O}/\text{HO}^-$) of higher $\text{p}K_a$ (15.74) replaced it.

Proposed function of Lys-295 in the chemical mechanism of PsM2DH

The data are consistent with a chemical mechanism of PsM2DH in which Lys-295 abstracts a proton from the 2-OH of D-mannitol in an internal equilibrium prior to hydride transfer. This is the simplest explanation for the observed pH-dependence of the pre-catalytic step, but it clearly cannot prove the mechanism. The proposed alkoxide is thought to be stabilized and precisely positioned for catalysis through both a hydrogen bond with the protonated or unprotonated side chain of Lys-295 and hydrogen bonds with the side chains of Asn-191 and Asn-300, which are the components of the oxyanion hole. With the assumption of an effect on depression of the $\text{p}K_a$ of the reactive hydroxyl, the function of this oxyanion hole in PsM2DH can be compared with the established role of Zn^{2+} in medium-chain ADHs [7,8,15]. The active-site triad of Lys-295, Asn-191 and Asn-300 facilitate hydride transfer to C4 of NAD^+ by a combination of propinquity

effects which enable efficient Brønsted catalysis by the well-aimed side chain of Lys-295 and support C–H bond cleavage in the substrate. Loss of the lysine side chain in the K295A mutant will thus not only cause a substantial increase in the internal alcohol/alkoxide equilibrium, but likely has an additional non-favourable influence on substrate orientation proximal to the nicotinamide ring and charge development on oxygen in the transition state.

The proposed role of Lys-295 in PsM2DH can be compared with the function of the structurally analogous Lys-183 in 6-phosphogluconate dehydrogenase (6PGDH), which catalyses the NAD(P)⁺-dependent oxidative decarboxylation of a 6-phosphogluconate substrate. Zhang et al. [17] have shown that Lys-183 is the probable general acid/base catalyst for the oxidoreductive chemistry of the reaction, and that the kinetic mechanism of wild-type 6PGDH involves a pH-dependent internal equilibrium at the ternary complex [18,19]. However, Cook and co-workers consider it unlikely that the internal equilibrium involves the actual proton transfer from the reactive hydroxyl, essentially on account of the high pK_a of this OH group in solution and observations made in multiple isotope effect measurements [17–20]. Whatever the exact roles of the pre-catalytic equilibria in the two enzymes may be, structural relationships between PsM2DH and 6PGDH at the levels of global fold and active-site architecture [2,3,21] can clearly be expanded to include the overall kinetic mechanism and acid–base catalytic chemistry. Similar relationships may prevail among PsM2DH and other long-chain dehydrogenases, such as ketopantoate reductase [10,22] and UDP-glucose dehydrogenase [23] for example.

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