RESEARCH COMMUNICATION

A catalytic consensus motif for D-mannitol 2-dehydrogenase, a member of a polyol-specific long-chain dehydrogenase family, revealed by kinetic characterization of site-directed mutants of the enzyme from *Pseudomonas fluorescens*

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Lys-295, Asn-300 and His-303 of D-mannitol 2-dehydrogenase from *Pseudomonas fluorescens* were mutated individually into alanine (K295A, N300A and H303A respectively). Purified mutants displayed catalytic efficiencies for NAD^+-dependent oxidation of D-mannitol 300-fold (H303A), 1000-fold (N300A) and approx. 400000-fold (K295A) below the wild-type level. Comparison of primary kinetic isotope effects on kinetic parameters for D-fructose reduction by wild-type and mutants at pH 10.0 demonstrate that Asn-300 has an auxiliary role in stabilization of the transition state of hydride transfer, and His-303 contributes to substrate positioning. The large solvent isotope effect of 11 ± 1 on k_{cat} for mannitol oxidation by K295A at pH(^{2}H) 10.5 suggests a role for Lys-295 in general base enzymic catalysis. Positional conservation of Lys-295, Asn-300 and His-303 across a family of polyol-specific long-chain dehydrogenases suggests a unique catalytic signature: Lys-Xaa_4-Asn-Xaa_2-His (where 'Xaa' denotes 'any amino acid').

Key words: alcohol dehydrogenase, chemical mechanism, polyol-specific long-chain dehydrogenase.

INTRODUCTION

The regiospecific $NAD(P)^+$-dependent oxidation of polyalcohols has a key role in all forms of metabolism and finds many applications in biotechnology. It is catalysed by a class of primary and secondary alcohol dehydrogenases (ADHs), which are diverse not only in regard to three-dimensional fold and substrate specificity, but also enzyme chemistry. In a commonly used sequence-based classification, the ADHs fall into three main groups that are generally referred to as short-chain dehydrogenases/reductases (SDRs), medium-chain dehydrogenases/reductases (MDRs) and long-chain dehydrogenases [1–3]. Note that the terms short-, medium- and long-chain refer to the length of the amino acid sequence and have nothing to do with the size of the preferred alcohol substrate! The primary structures of long-chain dehydrogenases are typically between 350 and 560 amino acids long [3,4]. The mannitol 2-dehydrogenases (D-mannitol:NAD^+ 2-oxido-reductases; EC 1.1.1.67; M2DHs) are part of an emerging family of secondary ADHs (and carbonyl reductases) within the heterogeneous group of long-chain dehydrogenases. Sixty six members of this novel family have been classified so far on the basis of similarities at the amino acid sequence level (M. Klimacek and B. Nidetzky, unpublished work). This is a mainly prokaryotic dehydrogenase/reductase family and includes, in addition to M2DHs, D-mannitol-1-phosphate 5-dehydrogenases (EC 1.1.1.17), D-mannonate 5-oxidoreductases (EC 1.1.1.57), D-fructuronate 5-reductases (EC 1.1.1.57), D-altronate 5-oxidoreductases (EC 1.1.1.58), and D-arabinitol 4-dehydrogenases (EC 1.1.1.11). Taking into account substrate stereochemical and structural requirements for the above family members, we would like to propose the name polyol-specific long-chain dehydrogenases/reductases (PSLDRs) and use this name throughout the manuscript. We emphasize, however, that assignment to the PSLDR family is unambiguous on the basis of sequence similarity, and is not compromised by substrate specificity. For example, PSLDRs are distinguished unequivocally from functionally related ADHs that take part in the metabolism of D-mannitol in fungi [5] and plants [6,7]. A M2DH from *Agaricus bisporus* and a D-mannitol 1-dehydrogenase (EC 1.1.1.255) from celery (*Apium graveolens*) are characterized by membership to the SDR and MDR superfamilies respectively. Also, PSLDRs are different from the family of metal-dependent long-chain dehydrogenases [8], often referred to as 'iron-containing' ADHs. Unlike MDRs, the M2DHs, and most probably other PSLDRs, do not use Zn^{2+} in catalysis [9]. PSLDR primary structures lack the catalytic consensus motif of SDR enzymes, which features a triad of positionally conserved serine, tyrosine and lysine residues [1]. Therefore PSLDRs are expected to differ in mechanistic terms from their counterpart secondary ADHs in the SDR and MDR superfamilies, for which detailed chemical reaction schemes have been proposed on the basis of kinetic evidence and active-site structures at atomic resolution [10,11].

In the present paper, we report identification of a catalytic signature for PSLDRs. Using site-directed mutagenesis of M2DH from *Pseudomonas fluorescens* (*Ps*M2DH) and detailed kinetic characterization of purified single-site mutants, we provide clear evidence for the functional importance of three amino acids present in a Lys-X_4-Asn-X_2-His motif (where 'X' is 'any amino acid') that is highly conserved among all PSLDR members. On the basis of kinetic isotope effects, we have dissected differential

Abbreviations used: ADH, alcohol dehydrogenase; KIE, primary ^{2}H kinetic isotope effect; K295A, etc., mutated enzyme bearing a Lys-295 → Ala replacement, etc.; M2DH, D-mannitol 2-dehydrogenase; MDR, zinc-dependent medium-chain dehydrogenase/reductase; NADD, 4-*S*-4-[^{2}H]NADH; PSLDR, polyol-specific long-chain dehydrogenase/reductase; (*Ps*)M2DH, M2DH (from *Pseudomonas fluorescens*); SDR, short-chain dehydrogenase/reductase; S-KIE, solvent ^{2}H kinetic isotope effect.

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contributions from the constituent groups of the motif during substrate binding, hydride transfer and proton transfer. The data provide strong evidence in support of the stepwise chemical mechanism of reaction for wild-type *Ps*M2DH that has been proposed relatively recently [9,12].

EXPERIMENTAL

Materials

Purified recombinant wild-type *Ps*M2DH containing a C-terminal metal-affinity-fusion peptide was prepared by a previously published protocol [9]. All other chemicals used were as described in recent publications on *Ps*M2DH [9,12]. T4 polynucleotide kinase, *Pfu* DNA polymerase, T4 DNA ligase and dNTPs were from Promega. Synergy DNA polymerase was from Genecraft (Műnster, Germany); ^2H_2O with a ^2H content $\geqslant 99\,\%$ was from Chemotrade (Dűsseldorf, Germany).

Site-directed mutagenesis and preparation of mutagenized *Ps*M2DH

The plasmid vector pETR241 harbouring the entire structural gene of *Ps*M2DH [9] was used as the template for PCR-based mutagenesis and expression of mutagenized genes. The replacement of Lys-295 by Ala (K295A) was achieved by using the overlap extension method, in which three rounds of amplification were performed. Briefly, two fragments of the target gene sequence were amplified in separate PCRs using Synergy DNA polymerase. Each reaction used one flanking primer that hybridized at one end of the target sequence (with the terminal restriction site shown below in bold) and one internal primer that hybridised at the site of the mutation and contained the mismatched bases (shown underlined).

K295A: 5′-GAAGAGATGGCGATCGG-3′—
5′-GATGATG**GGATCC**AACCG-3′
5′-CAACGGC**GAATTC**ATGGC-3′—
5′-CCGATCGCCATCTCTTC-3′

After extension and amplification of the overlapping fragments, the mutant construct was digested with *Eco*RI and *Bam*HI, and the desired band was gel-purified and subcloned into the pETR241 vector from which the respective fragment had been excised.

The inverse PCR protocol was used to generate the N300A and H303A mutants. The mutant constructs were obtained by directly amplifying the plasmid vector using *Pfu* DNA polymerase and two oligonucleotide primers, which are shown below with the mismatched bases underlined. Prior to the amplification by PCR, the primers were phosphorylated at the 5′ end using T4 polynucleotide kinase.

N300A: 5′-ATCGGCTTGCTCGCCGGCAGCCAC-3′
5′-CTTCATCTCTTCATAGGGTGTCAC-3′

H303A: 5′-AACGGCAGCGCCCTGGCCCT-3′
5′-GAGCAAGCCGATCTTCATCTCTTC-3′

The blunt-end amplification products obtained from PCR were then linked using T4 DNA ligase. Plasmid mini-prep DNA was subjected to dideoxy sequencing to verify that the desired mutations had been introduced, and that no misincorporation of nucleotides had occurred because of DNA polymerase errors.

The expression vectors for wild-type and mutated *Ps*M2DH were used to transform competent *Escherichia coli* JM 109 cells using electroporation. The production and purification of recombinant proteins followed exactly the protocol published for wild-type *Ps*M2DH [9].

Structural and functional characterization of recombinant *Ps*M2DH, and site-directed mutants thereof

The purity of recombinant proteins was checked by using SDS/PAGE and non-denaturating anionic PAGE, with Coomassie Blue staining for the visualization of protein bands. CD spectra of the wild-type and the mutant enzymes were recorded at 25 °C using a Jasco J-715 spectropolarimeter. Data acquisition and analysis were achieved with Jasco software. The proteins were dissolved at a concentration of $\approx 10\ \mu$M in 10 mM Tris/HCl buffer, pH 7.1. A cuvette with a light path of 0.1 cm was used for CD measurements, and spectra were recorded in the wavelength range of 250 nm to 190 nm. Fluorescence measurements were carried out with a Hitachi F-2000 spectrofluorimeter equipped with a thermostatically controlled cell holder. Apparent dissociation constants for binary complexes with NAD^+ and NADH were determined by using fluorescence titration at 25 ± 1 °C. Binding of NAD^+ was measured by adding 2 μl aliquots from a concentrated coenzyme solution in water (60 mM) to 1 ml of protein solution (2 μM) in 50 mM glycine/NaOH buffer, pH 10.0. Quenching of protein intrinsic tryptophan fluorescence (excitation 290 nm; emission 335 nm) upon binding of NAD^+ served as reporter of the binding event. Measurement of the binding of NADH was performed in the same way, except that the coenzyme addition took place from a 4 mM stock solution of NADH to a 6 μM solution of the protein, and the increase in NADH fluorescence was recorded (excitation 360 nm; emission 450 nm). Appropriate controls were determined in all cases, and corrections of the fluorescence signals were done as required. Coenzyme dissociation constants (K_0) were obtained from non-linear fits of the data to eqn (1) using Sigmaplot 2001 (SPSS Inc., Chicago, IL, U.S.A.):

$$\Delta F = \Delta F_{max}[\text{coenzyme}]/(K_d + [\text{coenzyme}]) \quad (1)$$

where ΔF is the observed difference in the fluorescence signal for the protein in the absence or presence of NAD^+, or NADH fluorescence in the presence or absence of protein. ΔF_{max} is the difference at apparent saturation of the protein with NAD^+ or NADH. Initial rates of D-mannitol oxidation and D-fructose reduction by wild-type enzyme and each point mutant of *Ps*M2DH were measured in spectrophotometric assays that monitored the increase and decrease of NADH absorbance at 340 nm. Reactions took place in 50 mM glycine/NaOH buffer at pH 10.0 and 25 °C using conditions in which one substrate was varied at a constant concentration of the second substrate, which was saturating unless indicated otherwise (2 mM NAD^+; 200 μM NADH; $\leqslant 1$ M D-mannitol; $\leqslant 2$ M D-fructose). The time of the assay was varied between 5 and 30 min, depending on the extent to which the original activity level had been decreased in the enzyme variant. Likewise, protein concentration in the assay was varied between 0.4 and 440 μg/ml. The kinetic parameters were obtained from non-linear fits of the data to the Michaelis–Menten equation. In the case when saturation of the enzyme with substrate could not be achieved, the catalytic efficiency was calculated from the part of the Michaelis–Menten curve where the initial rate is linearly dependent on the concentration of the varied substrate. The slope of the observed straight line corresponds to the expression $k_{cat}[E]/K_A$, where k_{cat} is the turnover number, [E] is the molar enzyme concentration and K_A is the apparent Michaelis constant for the substrate. A molar absorption coefficient of 54000 $M^{-1} \cdot cm^{-1}$ (at 280 nm and 25 °C) was assumed for *Ps*M2DH and point mutants thereof.

Kinetic isotope effects

Primary kinetic isotope effects (KIEs) were determined at pH 10.0 in the direction of D-fructose reduction by using a direct comparison of initial rates observed with NADH and enzymically synthesized 4-*S*-4-[^{2}H]NADH (NADD) containing $\geqslant 98\,\%$ ^{2}H at this position on the nicotinamide ring. Reactions took place under conditions in which coenzyme was constant and saturating at 200 μM, and D-fructose was varied in a suitable concentration range. Solvent KIEs (S-KIEs) on kinetic parameters were determined at pH(^{2}H) 10.0 in the direction of D-mannitol oxidation using polyol as the varied substrate, and NAD^+ at a constant and saturating concentration of 2 mM. Initial rates in ^1H_2O or ^2H_2O solvent were compared. KIEs and S-KIEs on kinetic parameters were obtained from non-linear fits of the data to eqn (2):

$$v = k_{cat}[E][A]/\{K_A(1+F_iE_{V/K})+[A](1+F_iE_V)\} \qquad (2)$$

where v is the initial rate, [E] is the enzyme concentration, [A] is the substrate concentration and $E_{V/K}$ and E_V are the isotope effects -1 on k_{cat}/K_A and k_{cat} respectively. The fraction of deuterium in NADD or the solvent is given by F_i.

RESULTS

A conserved motif in PSLDR members

Multiple alignment of PSLDR sequences revealed that Lys-295, Asn-300 and His-303 of *Ps*M2DH are highly conserved among all 66 known family members. Except for D-mannonate oxidoreductase from *Rhizobium loti* (Q98JL8), in which His-303 is replaced by a glutamine residue, D-mannitol-1-phosphate 5-dehydrogenase from *Enterococcus faecalis* (P27543), in which Asn-300 is replaced by a threonine residue, and D-mannonate 5-dehydrogenase (D-fructuronate reductase) from *Lactococcus lactis* (Q9CF49) and D-mannitol-1-phosphate 5-dehydrogenase from *Thermoanaerobacter tengcongensis* (AAM25119), in which the Lys/Asn couple is separated by five instead of four amino acids, the positional conservation of the three constituent amino acids of the motif is universal. This is emphasized in Figure 1, which shows the alignment of ten representative PSLDR sequences in the segment of the primary structure that contains the motif. Note that overall amino acid sequence identity among these PSLDR members may be as low as 10 %. Interestingly, Lys-295 was always followed by a hydrophobic amino acid (Val, Leu, Ile or Met) and Asn-300 was always preceded by a hydrophobic (Leu or Val) residue. Lys-295, Asn-300 and His-303 of *Ps*M2DH were, therefore, clear candidates for site-directed mutagenesis. The replacement with alanine was each mutation chosen to remove all polar interactions at the respective position.

Structural and functional characterization of mutants K295A, N300A and H303A

Point mutants of *Ps*M2DH were prepared successfully by using reported conditions for production of the wild-type enzyme in *E. coli* [9]. Analysis by SDS/PAGE (results not shown) indicated that a protein species with the expected molecular mass of 54 kDa for the full-length *Ps*M2DH monomer was the predominant component in induced bacterial cell extracts.

Electrophoretically pure preparations of individual mutants were obtained by using copper-loaded metal-affinity chromatography. Isolated mutants co-migrated with wild-type enzyme in denaturing and non-denaturing anionic PAGE, and co-eluted with monomeric wild-type in sizing-chromatography experiments using a Superdex 75 gel-filtration column. Far-UV CD spectra of wild-type and mutant enzymes were almost superimposable (results not shown), suggesting that the relative content of α-

O08355 NGAVTRKALLAFAALHN-AE-LHDWIKAHVSFPNAMVDRITEMTSTAHRLQLHDEHGIDDAWPVVCEPFVQWVLED-KFVNGRPAWEK--VGVQFTDDVTPYEEMK 295
Q9I1D6 NGEVARKALLAFAERLD-PG-LARWIATHVSFPNAMVDRITEMTSPAHRRQLAQRHDVEDAWPVVCEPFVQWVLED-RFSAGRPAWEK--VGVQFTDDVTPYEEMK 295
Q8YCQ0 NGETCKNVIVQFAGCTD-TD-LAHYIEETVAFPSTMVDRIVEATTDEDRISISRTLKVLDQWPITTEPFSQWVIED-RFPTGRPAWEI--AGATFVQDVAAFELMK 301
Q9KWR5 NGNVARKAFLGYAKARD-PE-LAKWIEENATFPNGMVDRITETVSAEIAKKLNAASGLDDDLPLVAEDFHQWVLED-QFADGRPPLEK--AGVQMVGDVTDWEYVK 294
Q8VUU5 NGNVARKAFLGYAKARD-PE-LAKWIEENATFPNGMVDRITETVSADIAKKLNEASGLHDDLPLVAEDFHQWVLED-SFADGRPALEK--AGVQFVGDVTDYEHVK 294
P58708 NGARFRAGMRAFLALRG-DAALLAWFDANVSCPSAMVDRITERPTDDVRTRVHAATGVDDRCPVMGESFIQWVIED-NFIAGRPAWEI--AGAEIVADVHPYEEAK 277
P24214 NGDALRELVLRYAQEWALPEAFIQWLDQANSFCSTLVDRIVTGYPRDEVAKLEEELGYHDGFLDTAEHFYLFVIGGPKSLATELRLDKYPLNVLIVDDIKPYKERK 281
Q9K681 ASSMLQEYTKAS-----LSEEEWSKVDRVTGFPNATVDRIVE--AQDH----------ADPLTVSVEPFYEWVIETKSMKGEPPTID----GVTYVEDLTPYIERK 208
Q97SH1 GSQFLYQEVKKY-----LSPEGLTEADNYIGFPNAAVDRIVE--AQSH----------EDSLFVVVEPFNEWVVETKRLKNPDLRLK----DVHYEEDLEPFIERK 213
P39941 NGVTVKTMLVAFAKLKK-DEKFAAWIEDKVTSPNSMVDRVTERCTDKERKYVADTWGIKDQCPVVAEPFIQWVLED-NFSDGRPPWEL--VGVQVVKDVDSYELMK 304
O08355 IGLLNGSHLALTYLGFLKGYRFVHETWNDPLFVAYMRAYMDLDVTPNLAP--VPGIDLTDYKQTLVDRFSNQAIADQLERVCSDGSSKFPKFTVPTI-NRLIADGR 397
Q9I1D6 IGLLNGSHLALTYLGFLRGYRFVHETLGDPLLRRYVRAFMDRDVAPLLAP--VPGIDLERYKDSLVERFANRAIADQLERVCSDGSSKFPKFIVPTA-NRLIAAGR 397
Q8YCQ0 LRLLNGSHSTLAYLGFLAGHRTVSQTTAAPGFKQLIKALMEIEIAPTLPK--LEGFDLSSYQAALIHRFENHALHHLTSQIAMDGSQKLPQRLLNTIQERL-DGGG 403
Q9KWR5 IRMLNAGHVMLCYPGILVGYENVDDAIEDSELLGNLKNYLNKDVIPTLKA--PSGMTLEGYRDSVISRFSNKAMSDQTLRIASDGCSKVQVFWTETV-RRAIEDKR 396
Q8VUU5 IRMLNAGHIMLCYPAVLAGFENVDHAFADPDLRRILENFLNKDVIPTLKA--PPGMTLEGYRDSVISRFSNPAMADQTLRISGDGSSKIQVFWTETV-RKAFEGKR 396
P58708 IRILNATHSCIAYAGTLAGLTYIHEGMRDAAIYRFAYDYVTDDVIPCLTP--SP-LDLERYRDVVLERFGNPYVLDTNQRVAADGFSKIPGFIAPTLAECFARGAD 379
P24214 VAILNGAHTALVPVAFQAGLDTVGEAMNDAEICAFVEKAIYEEIIPVLD---LPRDELESFASAVTGRFRNPYIKHQLLSIALNGMTKFRTRILPQLLAGQKANGT 383
Q9K681 LFTVNTGHAMVAYLGFQKGLMTIKEAISDQTIAEKTRQALAETKGLLVHKYNFSPEAHDEYIEKIFKRYNNPYLSDRVERVGRNPIRKL------GYNERLVKPAR 307
Q97SH1 LFSVNSGHATSAYIGAHYGAKTILEAMQNPNIKSRIESVLAEIRSLLIAKWNFDKKELENYHKVIIERLENPFIVDEVSRVARTPIRKL------GYNERFIRPIR 312
P39941 LRLLNGGHSAMGYLGYLAGYTYIHEVVNDPTINKYIRVLMREEVIPLLPK--VPGVDFEEYTASVLERFSNPAIQDTVARICLMGSGKMPKYVLPSIYEQLRKPDG 407

Figure 1 Identification of a conserved Lys-X_4-His-X_2-His motif through multiple alignment of PSLDR sequences

Ten representative PSLDR members are shown out of 66 aligned protein sequences. Alignment was done by searching the SWISS-PROT, TrEMBL and TrEMBL_new databases at the Swiss Institute of Bioinformatics server using the BLAST network service and the NCBI BLAST 2 software [13]. O08355, D-mannitol 2-dehydrogenase from *Ps. fluorescens*; Q9I1D6, D-mannitol 2-dehydrogenase from *Ps. aeruginosa*; Q8YCQ0, D-mannonate oxidoreductase from *Brucella melitensis*; Q9KWR5, D-sorbitol dehydrogenase from *Gluconobacter oxydans*; Q8VUU5, L-sorbose reductase from *Gluconobacter oxydans*; P58708, D-arabinitol 4-dehydrogenase from *Ralstonia solanacearum*; P24214, D-altronate oxidoreductase from *E. coli*; Q9K681, D-mannitol-1-phosphate 5-dehydrogenase from *Bacillus halodurans*; Q97SH1, D-mannitol-1-phosphate 5-dehydrogenase from *Streptococcus. mutans*; P39941, D-mannitol 2-dehydrogenase from *S. cerevisiae*. Mutated residues are marked with *. Conservation mode: strictly conserved residues are shown in black boxes, and conservations at the 90% level are shown in grey boxes.

Table 1 Comparison of steady-state kinetic parameters for wild-type and mutated *Ps*M2DH at pH 10.0

Parameter	Enzyme ...	N300A	H303A	K295A	Wild-type
k_{cato} (s^{-1})		2.61 ± 0.02	62.5 ± 1.6	$3 \times 10^{-3} \pm 3 \times 10^{-5}$	40 ± 0.5†
$K_{mannitol}$ (mM)		26 ± 1	213 ± 17	11 ± 2	0.40 ± 0.02†
$k_{cato}/K_{mannitol}$ ($M^{-1} \cdot s^{-1}$)		100	293	0.27	1×10^5†
K_{NAD} (μM)		25 ± 3	115 ± 6	22 ± 3	93 ± 8†
K_{dNAD} (μM)*		850 ± 25	850 ± 35	920 ± 33	1200 ± 45
k_{catr} (s^{-1})		0.28 ± 0.01	n.a.§	n.a.	20 ± 0.7†
$K_{fructose}$ (mM)		537 ± 26	> 2000‡	$\geqslant 1000$‡	44 ± 0.1†
$k_{catr}/K_{fructose}$ ($M^{-1} \cdot s^{-1}$)		0.52	1.5‡	5.5×10^{-3}‡	455†
K_{NADH} (μM)		20 ± 3	56 ± 4	34 ± 5	10 ± 2†
K_{dNADH} (μM)*		5.3 ± 0.9	5.9 ± 0.8	12 ± 1	17 ± 2

* From fluorescence titrations.
† data from [12].
‡ saturation of enzyme in substrate not achieved.
§ n.a., not applicable.

helical and β-sheet secondary structures in the wild-type protein had not been changed as result of the individual point mutations. Apparent dissociation constants for binary complexes of each individual mutant with NADH and NAD^+ were determined at pH 10.0 and compared with the K_d value for the corresponding wild-type complex under otherwise identical conditions, as shown in Table 1. Observed K_d values for complexes of wild-type and mutant enzymes with NAD^+ (Table 1) were identical within the experimental error of $\pm 25\%$ ($K_d \approx 850$–1200 μM). K_d values for binary complexes with NADH revealed differences, however minor, between the wild-type and the mutant proteins in terms of NADH-binding affinity. In good agreement with data published recently for the wild-type [9], Scatchard analysis of data from fluorescence titrations yielded a value of 1 ($\pm 15\%$) for the stoichiometry of binding of NADH to each *Ps*M2DH mutant. In summary, identity in secondary-structure composition and similarity in coenzyme-binding properties serve to emphasize that global structural features of wild-type *Ps*M2DH have been maintained in the point mutants.

Contributions of mutated residues to catalytic properties of *Ps*M2DH

Steady-state kinetic parameters for D-mannitol oxidation and D-fructose reduction catalysed by wild-type enzyme and each point mutant were determined, and are summarized in Table 1. Compared with wild-type, all three mutants caused dramatic decreases in k_{cat}/K values for reactions with polyol and ketose substrates. Catalytic efficiencies observed with K295A were reduced by nearly six orders of magnitude, relative to the corresponding wild-type levels. H303A and N300A showed comparably smaller decreases in k_{cat}/K, by factors of about 300 and 1000 respectively. Large changes in $k_{cato}/K_{mannitol}$ (where k_{cato} is the k_{cat} value in the direction of D-mannitol oxidation) for K295A reflect $\approx$ 28-fold increases in $K_{mannitol}$, and mainly a $>$ 13000-fold decrease in k_{cato}. By contrast, the observed decreases in k_{cat}/K for H303A are almost entirely the result of dramatically weakened apparent substrate binding. The observed 1000-fold decrease in $k_{cato}/K_{mannitol}$ caused by the replacement of Asn-300 are distributed between a 15-fold reduction in k_{cato} and a 66-fold increase in $K_{mannitol}$. By contrast, the change in $k_{cato}/K_{fructose}$ for N300A by approximately the same factor reflects a significantly larger decrease in k_{catr} (approx. 70-fold; k_{catr} is the k_{cat} value in the direction of D-fructose reduction) and, therefore, a correspondingly smaller increase in $K_{fructose}$ (approx. 15-fold). Apparent Michaelis constants for NAD^+ and NADH are reported in Table 1, and reveal only minor differences among wild-type and the point mutants in terms of coenzyme 'affinity'. Interestingly, the mutations caused an increase in K_{NADH} by a factor of 3–6 compared with the wild-type, whereas an opposite effect was observed on K_{NAD}. KIEs on k_{catr} and $k_{catr}/K_{fructose}$ were measured for each mutant, and the results are compared with published data for the wild-type in Table 2. S-KIEs on kinetic parameters for oxidation of D-mannitol were determined for all three mutants, as shown in Table 2. Compared with the wild-type, significant alterations in KIEs are caused by each mutation, suggesting that Lys-295, Asn-300 and His-303 are involved in the chemical mechanism of *Ps*M2DH (as will be discussed below).

Table 2 Kinetic isotope effects for wild type and mutated *Ps*M2DH

	N300A	H303A	K295A	Wild-type¶
$^2H K_{catr}$*	1.98 ± 0.10	§n.a.	n.a.	0.90 ± 0.07
$^2H K_{catr}/K_{fructose}$*	2.43 ± 0.26	2.58 ± 0.20	1.02 ± 0.02	0.83 ± 0.15
$^2H_2O k_{cato}$†	1.30 ± 0.03	1.80 ± 0.10	11 ± 1‡	1.73 ± 0.10
$^2H_2O k_{cato}/K_{mannitol}$†	2.38 ± 0.16	2.02 ± 0.16	2.83 ± 0.8‡	2.36 ± 0.18

* KIEs on kinetic parameters for D-fructose reduction at pH 10.0.
† S-KIEs on kinetic parameters for D-mannitol oxidation at pH(^{2}H) 10.0.
‡ S-KIEs at pH(D) 10.5.
¶ data from [12].
§ n.a., not applicable.

DISCUSSION

On the basis of similarities at the level of primary structure, M2DHs have been classified into a family of distantly related long-chain dehydrogenases/reductases named PSLDRs, which can be distinguished clearly from well-known SDR [1] and MDR [2] superfamilies and from the iron-containing ADH family [8]. In spite of an overall sequence identity that can be as low as 10%, a Lys-Xaa_4-Asn-Xaa_2-His motif was found to be highly conserved among all members of the PSLDR family. Therefore

this result raised the significant question of a possible role of the Lys/Asn/His 'triad' in enzymic catalysis. In an effort to assign a potential catalytic function to each residue, we mutated Lys-295, Asn-300 and His-303 of *Ps*M2DH individually into alanine, and characterized the three enzyme variants in detailed kinetic experiments. The maintenance of native-like secondary structure and NAD(H)-binding properties of wild-type *Ps*M2DH in all mutants suggests that interpretations of the kinetic consequences of each mutation can be based on the assumption that the enzyme active site has not been globally disrupted by site-directed replacement of the original side chain with the side chain of alanine. The large reductions in enzymic rates caused by the individual mutations reveal clearly that the 'triad' of Lys/Asn/His has a major role in substrate binding and catalysis by *Ps*M2DH. Therefore the results strongly support the contention that the conserved Lys-Xaa$_4$-Asn-Xaa$_2$-His motif represents a catalytic consensus sequence of PSLDRs. This motif is novel among the ADH superfamilies. We emphasize, however, that in addition to the motif examined herein there are, of course, other residues in *Ps*M2DH that are highly conserved among PSLDRs whose functional role needs to be explored further. Asp-230 and Ile-232 are examples of universally conserved residues (M. Klimacek and B. Nidetzky, unpublished work).

Slide chains of Asn-300 and His-303 have auxiliary roles in catalysis

Mutation of Asn-300 and His-303 reduced $k_{cato}/K_{mannitol}$, and likewise $k_{catr}/K_{fructose}$, by 2.5 and 3 orders of magnitude respectively. These rate-decreasing factors are fully consistent with a secondary role for each amino acid in catalysis by *Ps*M2DH. KIEs served as indicators of major changes with respect to partly rate-limiting reaction steps as a result of the mutations, as follows. At pH 10.0, $^{2H}k_{catr}/K_{fructose}$ for the wild-type enzyme matches the value for the equilibrium isotope effect on NADH-dependent carbonyl group reduction. The fact that hydride transfer comes to equilibrium in the direction of D-fructose reduction at high pH was explained by the occurrence of a post-catalytic equilibrium, which involves the D-mannitol-bound enzyme and, unlike hydride transfer, is sensitive to pH [12]. Irrespective of whether this internal equilibrium implicates the actual proton-transfer step or is only conformational, release of D-mannitol from the ternary complex at pH values $\geqslant 10.0$ takes place at a low rate. KIE data for N300A reveal that, compared with wild-type, the rate of the isotope-sensitive step of hydride transfer has been decreased selectively such that sizeable normal isotope effects on $k_{catr}/K_{fructose}$ and k_{catr} are now observed. The large value of $^{2H}k_{catr}$ for N300A suggests that hydride transfer has become a major rate-limiting step for the overall reverse reaction catalysed by this mutant. An approx. 71-fold decrease in the k_{catr} value for N300A, compared with the wild-type value, is in good agreement with this notion. In the absence of KIE data for k_{catr} the value of 2.6 for $^{2H}k_{catr}/K_{fructose}$ must be interpreted with caution. A comparison of $^{2H}k_{catr}/K_{fructose}$ for wild-type and H303A shows clearly that the forward commitment to catalysis (which is the ratio of the hydride-transfer rate constant and the rate constant for the dissociation of D-fructose) has been decreased in the mutant, leading to a corresponding increase in the observable KIE. A decrease in forward commitment, relative to wild-type, could occur because the hydride transfer has been slowed and/or the dissociation rate of the substrate has been increased in the mutant. Considering the fact that turnover numbers in the direction of D-mannitol oxidation are closely similar for wild-type and H303A, we believe that replacement of the imidazole side chain of His-303 has a major effect on the substrate dissociation rate from the ternary complex, but does not substantially decrease the hydride-transfer rate constant.

Proton inventory analysis of steady-state kinetic parameters for D-mannitol oxidation by wild-type *Ps*M2DH has suggested that a value of 2.4 for $^{2H_2O}k_{cato}/K_{mannitol}$ at pH(^{2}H) 10.0 probably reflects the contribution of a single solvation catalytic proton bridge to the stabilization of the transition state of hydride transfer [12]. Rapid-mixing stopped-flow kinetic experiments at the same pH(^{2}H) have shown that $^{2H_2O}k_{cato}$ is a manifestation of a normal S-KIE on the NADH-release rate [12]. $^{2H_2O}k_{cato}/K_{mannitol}$ values for N300A, H303A and the wild-type are similar, indicating no or little changes in the fractionation factor of approx. 0.43 ($= 1/2.4$) for $k_{cato}/K_{mannitol}$ as a result of the mutations. The change in $^{2H_2O}k_{cato}$ for N300A and H303A, relative to the wild-type, appears to mirror the corresponding change in k_{cato} (see Tables 1 and 2), which is substantial for N300A, but is almost insignificant for H303A. In conclusion, these results provide good evidence in favour of the suggestion that Asn-300 has an important auxiliary role in catalysis by facilitating the hydride-transfer step. His-303, in turn, seems to be needed for precise positioning of the substrate. The differential rate-reducing effect on turnover numbers for D-mannitol oxidation (15-fold) and D-fructose reduction (71-fold) by N300A needs to be explored further. It could reflect either a non-productive reactant-state stabilization by the enzyme variant specifically in the reverse direction of reaction, or that the enzyme-bound internal equilibrium has been shifted as result of the mutation.

Lys-295 has a primary catalytic role in *Ps*M2DH

Mutation of Lys-295 caused a >100000-fold reduction in catalytic efficiencies, expressed as either k_{cat}/K or $k_{cat}/K_{iNAD(H)}K$, for D-mannitol oxidation and D-fructose reduction by *Ps*M2DH. The magnitude of the observed effect is consistent with a primary role for this residue in enzymic catalysis. The large S-KIE on k_{cato} for D-mannitol oxidation by the mutant leads to the suggestion that Lys-295 could serve as the general acid–base catalyst of the reaction catalysed by *Ps*M2DH, or function in a proton-relay mechanism [12]. S-KIEs on $k_{cato}/K_{mannitol}$ for wild-type and K295A have similar values, indicating that only minor, if any, changes in the transition-state fractionation factor occur as a consequence of the mutation. The value of 1.0 for the KIE on $k_{catr}/K_{fructose}$ by K295A brings to light a marked difference between the wild-type and this 'catalytically crippled' mutant. In K295A, a large forward commitment to catalysis appears to lead to a complete masking of an otherwise sizeable isotope effect (cf. values of $^{2H}k_{catr}/K_{fructose}$ for N300A and H303A). If, as in the wild-type [12], a large reverse commitment was the dominant factor, the equilibrium isotope effect would be seen. The observed high value of >2 M for $K_{fructose}$ seems to be in agreement with a large forward commitment factor. Considering the stepwise chemical mechanism of *Ps*M2DH [12], a plausible scenario would thus be that Lys-295 assists proton removal from the reactive hydroxy group of the polyol, which facilitates catalysis because the resulting alkoxide is expected to undergo faster hydride transfer than would the protonated alcohol. In light of the catalytic acid–base function of tyrosine in SDR enzymes [1], we also searched for candidate tyrosine residues in the *Ps*M2DH sequence. Unlike Lys-295, no tyrosine residue is strictly conserved throughout the PSLDR family. Tyr-291, Tyr-308, Tyr-354 and Tyr-413 of *Ps*M2DH are replaced individually by phenylalanine in many PSLDR members, and phenylalanine clearly cannot serve a general acid–base function.

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