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ARTICLE *in* CARBOHYDRATE RESEARCH · JULY 2008

Impact Factor: 1.93 · DOI: 10.1016/j.carres.2008.04.011 · Source: PubMed

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Carbohydrate Research 343 (2008) 1414–1423
Carbohydrate RESEARCH

Characterization of recombinant *Aspergillus fumigatus* mannitol-1-phosphate 5-dehydrogenase and its application for the stereoselective synthesis of *protio* and *deuterio* forms of D-mannitol 1-phosphate

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Received 1 February 2008; received in revised form 2 April 2008; accepted 4 April 2008
Available online 10 April 2008

Abstract—A putative long-chain mannitol-1-phosphate 5-dehydrogenase from *Aspergillus fumigatus* (*Af*M1PDH) was overexpressed in *Escherichia coli* to a level of about 50% of total intracellular protein. The purified recombinant protein was a ≈40-kDa monomer in solution and displayed the predicted enzymatic function, catalyzing NAD(H)-dependent interconversion of D-mannitol 1-phosphate and D-fructose 6-phosphate with a specific reductase activity of 170 U/mg at pH 7.1 and 25 °C. NADP(H) showed a marginal activity. Hydrogen transfer from formate to D-fructose 6-phosphate, mediated by NAD(H) and catalyzed by a coupled enzyme system of purified *Candida boidinii* formate dehydrogenase and *Af*M1PDH, was used for the preparative synthesis of D-mannitol 1-phosphate or, by applying an analogous procedure using *deuterio* formate, the 5-[^{2}H] derivative thereof. Following the precipitation of D-mannitol 1-phosphate as barium salt, pure product (>95% by HPLC and NMR) was obtained in isolated yields of about 90%, based on 200 mM of D-fructose 6-phosphate employed in the reaction. In situ proton NMR studies of enzymatic oxidation of D-5-[^{2}H]-mannitol 1-phosphate demonstrated that *Af*M1PDH was stereospecific for transferring the deuterium to NAD^+, producing (4*S*)-[^{2}H]-NADH. Comparison of maximum initial rates for NAD^+-dependent oxidation of *protio* and *deuterio* forms of D-mannitol 1-phosphate at pH 7.1 and 25 °C revealed a primary kinetic isotope effect of 2.9 ± 0.2, suggesting that the hydride transfer was strongly rate-determining for the overall enzymatic reaction under these conditions.

Keywords: D-Mannitol 1-phosphate; D-5-[^{2}H]-Mannitol 1-phosphate; Mannitol-1-phosphate 5-dehydrogenase; *Aspergillus fumigatus*; Biocatalysis; Stereoselective synthesis

1. Introduction

D-Mannitol is one of the most abundant sugar alcohols in nature and ubiquitous throughout the fungal kingdom. Recent evidence supporting a role of mannitol as stress metabolite in the parasitic lifestyle of different fungi has rekindled the interest in the physiological functions fulfilled by mannitol in lower and higher eukaryotes.[1] *Aspergillus niger* utilizes mannitol to protect its conidiospores against the exogenous stress resulting from high temperatures and an oxidative environment.[2] The ability of mannitol to scavenge reactive oxygen species (ROS) is exploited by the human pathogen *Cryptococcus neoformans* and the tobacco pathogen *Alternaria alternata* to suppress the host defense strategies that are based on the generation of ROS against the microbial parasite.[3,4] The wheat pathogen *Stagonospora nodorum* requires mannitol for asexual sporulation.[5] The human pathogen *Aspergillus fumigatus*, which is the most common etiologic agent

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doi:10.1016/j.carres.2008.04.011

for invasive aspergillosis in immunosuppressed hosts, produces and releases sufficient amounts of mannitol to raise serum mannitol levels of infected animals.[6,7] Therefore, these findings suggest that the metabolism of mannitol might be a viable target for the development of novel antifungal strategies. The primary biosynthetic route towards mannitol in *Aspergilli* and other fungi is the reduction of D-fructose 6-phosphate (Fru6*P*) to D-mannitol 1-phosphate (Man-ol1*P*) catalyzed by mannitol-1-phosphate 5-dehydrogenase (M1PDH). Man-ol1*P* is subsequently dephosphorylated to mannitol.

According to similarity at the level of the amino acid sequence, almost all the known fungal M1PDHs are classified as polyol-specific long-chain dehydrogenases and reductases (PSLDRs).[8] The PSLDRs constitute a distinct evolutionary lineage of $NAD(P)^+$-dependent secondary alcohol dehydrogenases. These do not require a metal cofactor such as Zn^{2+} for catalysis and are usually active as monomers.[9] The molecular size of PSLDRs varies between 380 and 550 residues, whereby M1PDHs constitute the smallest members of the family. Structure-based sequence analysis has revealed that the currently classified PSLDRs share a common structural organization where the active site is located in a cleft formed by an N-terminal Rossmann-fold coenzyme binding domain and a largely α-helical domain that provides key elements of substrate binding recognition and catalysis. With the exception of the early studies of the enzyme from *A. niger*, little is currently known about the relationships of structure and function of M1PDHs from fungi.[10]

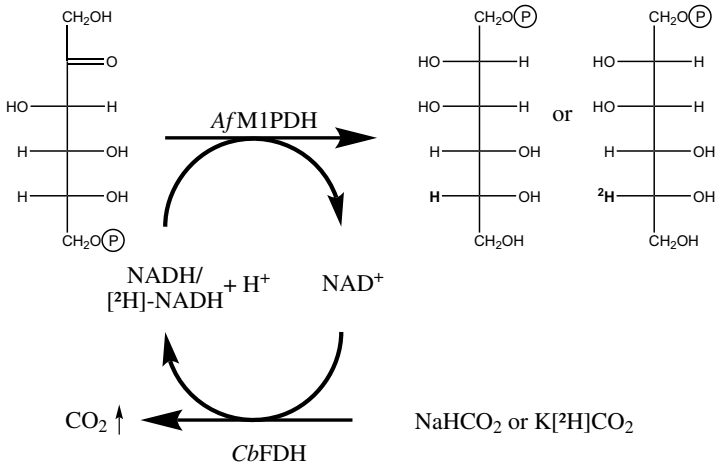
In this paper, we report on the biochemical characterization of a recombinant M1PDH from the human pathogenic mold *A. fumigatus* (*Af*M1PDH, EC 1.1.1.17). Assuming that mannitol constitutes a relevant pathogenic factor of *A. fumigatus*, the biosynthetic M1PDH is a potential drug target. M1PDH-directed inhibition of the microbial parasite should be selective considering that there are no PSLDR orthologues in the human genome. Detailed study of *Af*M1PDH is therefore not only of physiological interest but could also eventually gain a clinical importance.

The analysis of the primary kinetic isotope effect (KIE) resulting from the deuteration of the substrate has provided a valuable insight into the kinetic and catalytic mechanisms utilized by several $NAD(P)^+$-dependent dehydrogenases.[11] The application of a similar KIE strategy to examine *Af*M1PDH requires the synthesis of *protio* and *deuterio* forms of Man-ol1*P*. We describe an enzymatic method (Scheme 1) that allows the preparation of both substrates in high purity and yield. To our knowledge, 5-[^{2}H]-Man-ol1*P* has not been reported in the literature so far.

2. Results and discussion

2.1. Cloning and expression of a putative M1PDH from *A. fumigatus*

The whole genome shotgun sequence of *A. fumigatus* shows an open-reading frame that encodes a putative M1PDH (UniProt/TrEMBL entry Q4X1A4).[12] Because the coding region of the *Af*M1PDH gene is not interrupted by introns, it was possible to amplify the entire gene directly from genomic DNA of *A. fumigatus*. The PCR product thus obtained had the expected size of 1187 bp and was cloned into a pQE-70 plasmid expression vector. The recombinant protein contained at its N-terminus an additional leucine after the initiator methionine that was introduced with the oligonucleotide primer used for PCR. Furthermore, a peptide of eight amino acids (-Arg-Ser-His_6) was fused to the C-terminal end of the protein to facilitate purification. Heterologous



Scheme 1. Enzymatic synthesis of Man-ol1*P* and 5-[^{2}H]-Man-ol1*P* using the coupled enzyme system *Af*M1PDH and *Candida boidinii* formate dehydrogenase (*Cb*FDH).

expression of the *Af*M1PDH gene was carried out in *E. coli* JM109. SDS PAGE of crude bacterial cell extracts revealed an abundant protein band at the position corresponding to the expected molecular mass of ≈44 kDa for the recombinant protein (data not shown). The comparison of specific M1PDH activities in the cell extract (80 U/mg) and the purified enzyme (170 U/mg) indicated that the *Af*M1PDH accounted for about 50% of the total intracellular protein in *E. coli*.

2.2. Purification, and molecular and kinetic characterization of *Af*M1PDH

*Af*M1PDH was purified to apparent homogeneity by a single step of column chromatography, using copper-loaded Chelating Sepharose. A balance of the purification with respect to protein and enzyme activity is given in Table 1. The isolated enzyme migrated as a single protein band in SDS PAGE (Fig. 1). In size exclusion chromatography, it was eluted in a single peak that corresponded to the molecular mass of about 40 kDa and contained all the applied protein and enzyme activity. Therefore, this result suggests that *Af*M1PDH is a functional monomer, like other PSLDRs that have been characterized at the protein level.

The purified *Af*M1PDH displayed the predicted enzymatic function. It showed a specific activity of 169 ± 4 U/mg for the reduction of Fru6*P* by NADH at pH 7.1. In the direction of NAD^+-dependent oxidation of Man-ol1*P* (100 mM glycine/NaOH buffer, pH 10.0), the specific activity was 17.6 ± 1.4 U/mg. Note that the pH values of measurement were chosen in a suitable pH range for mannitol oxidation and fructose reduction (data not shown). Using the same reaction conditions, the specific enzyme activities for Fru6*P* reduction by 0.2 mM NADPH and Man-ol1*P* oxidation by 2.0 mM $NADP^+$ were 0.58 ± 0.05 U/mg and 0.08 ± 0.01 U/mg, respectively. However, it must be emphasized that *Af*M1PDH could not be saturated with reduced and oxidized phosphorylated coenzyme in the steady state, suggesting that the presence of the 2′-phosphate of NADP(H) strongly decreased the binding affinity for the coenzyme.

Michaelis–Menten constants for the reduction of Fru6*P* by NADH and the oxidation of Man-ol1*P* by NAD^+ were determined at neutral pH and are summarized in Table 2. Under the conditions used, the turnover number (k_{cat}) for the direction of Fru6*P* reduction was about 10 times that for the direction of Man-ol1*P* oxidation. The K_m value for NADH was less than one tenth that for NAD^+. By contrast, apparent binding affinity ($1/K_m$) for Man-ol1*P* was higher than that for Fru6*P*. The catalytic efficiencies (k_{cat}/K_m) for reactions with Fru6*P* ($5.9 \times 10^4\ M^{-1}\ s^{-1}$) and Man-ol1*P* ($3.8 \times 10^4\ M^{-1}\ s^{-1}$) in the presence of a saturating concentration of coenzyme were therefore in the same range.

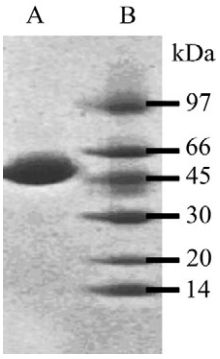


Figure 1. SDS PAGE of purified *Af*M1PDH: (A) *Af*M1PDH purified by metal affinity chromatography; (B) Low molecular weight standard (GE Healthcare).

Table 2. Kinetic constants for purified *Af*M1PDH at 25 °C

	$K_{substrate}$ (mM)	$K_{cofactor}$ (mM)	k_{cat} (s^{-1})
Man-ol1*P*	0.23 ± 0.02	0.75 ± 0.09	8.5 ± 0.4
Fru6*P*	2.1 ± 0.2	0.016 ± 0.001	125 ± 3

Man-ol1*P* oxidation and Fru6*P* reduction were measured in 100 mM Tris/HCl buffer, pH 7.1. See Section 3 for the concentrations of substrates and coenzymes used.

Kinetic data obtained with *Af*M1PDH are well comparable with those for the M1PDH from *A. niger* reported by Kiser and Niehaus.[10] Like *Af*M1PDH, the enzyme from *A. niger* shows a large preference (⩾1000-fold) for the reaction with NAD(H) as compared to NADP(H). The K_m values for substrates and coenzymes are similar for both fungal M1PDHs. The M1PDH from *Escherichia coli* is also a monomer in solution and like the fungal enzymes, it has a higher affinity for NADH than for NAD^+ at neutral pH.[13] It prefers NAD^+ more than 100-fold over $NADP^+$, and its activity appeared to be independent of a metal cofactor.[14]

Table 1. Purification of recombinant *Af*M1PDH expressed in *E. coli*

Purification stage	Total activity (U)	Protein (mg/mL)	Specific activity (U/mg)	Total yield (%)	Purification factor
Crude extract	28 000	50	80	100	1
Affinity chromatography	24 000	4	135	86	1.7
Desalting	21 600	11	169	77	2.1

Results are based on processing 2 g moist bacterial biomass. The *Af*M1PDH activity was assayed in the direction of Fru6*P* reduction at 25 °C using 100 mM Tris/HCl buffer, pH 7.1. The concentrations of Fru6*P* and NADH were 50 mM and 0.2 mM, respectively.

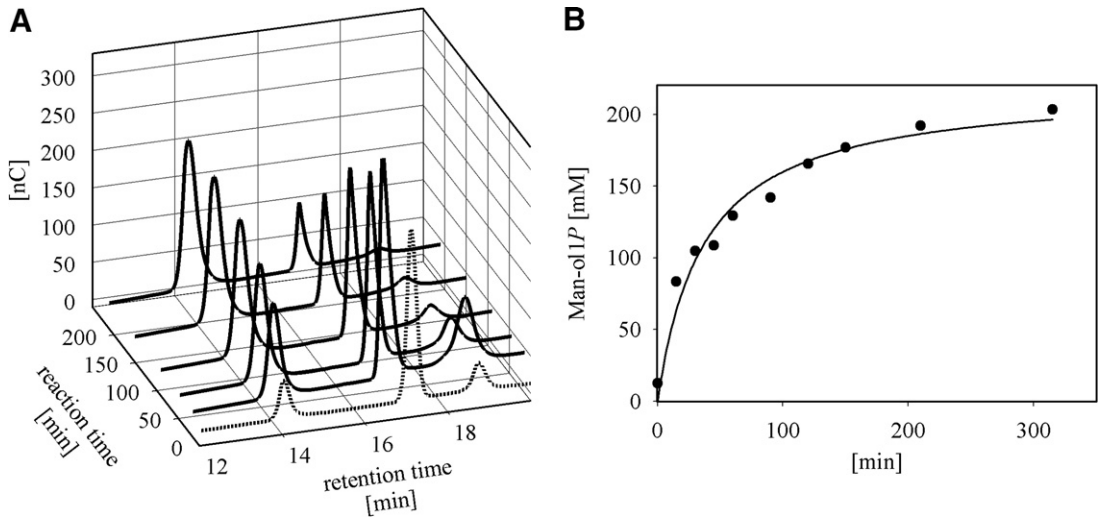


Figure 2. Enzymatic synthesis of Man-ol1*P* monitored by HPAE-PAD: (A) Dashed line: Superimposed traces of authentic standards of Man-ol1*P* (13.7 min), Glc6*P* (16.7 min) and Fru6*P* (18.4 min); Solid lines: Chromatograms for the first 210 min of Man-ol1*P* production from Fru6*P* using *Cb*FDH fraction I for cofactor recycling; (B) time course of Man-ol1*P* production from 200 mM Fru6*P*. The solid line indicates the trend of the data.

Membership of *Af*M1PDH to the PSLDR protein family leads to the suggestion that the catalytic activity of the enzyme is not dependent on a metal cofactor. To verify the structure-derived implication, we incubated purified *Af*M1PDH (0.27 μg/mL) in the presence of 100 mM EDTA for 1 h at room temperature and assayed at various times the residual activity of the enzyme in the direction of Fru6*P* reduction. No loss of activity was observed relative to a control in which EDTA was lacking, as expected for a metal-independent enzyme.

2.3. Enzymatic synthesis of D-mannitol 1-phosphate and 5-[^{2}H]-D-mannitol 1-phosphate

Previously reported routes for the chemical synthesis of Man-ol1*P* started from mannose 6-phosphate or mannitol.[10,15,16] A major drawback of using mannose 6-phosphate is the high cost of the substrate. Phosphorylation of mannitol was performed with phosphorylchloride in pyridinic solution. It required harsh reaction conditions and consumed large amounts of organic solvents. A heterogeneous mixture of mannitol-phosphates was obtained from which 90% pure Man-ol1*P* was recovered through a laborious re-crystallization procedure. The overall yield was therefore only ≈9%.[17] None of the described methods provide a direct access to the deuterated analogue of Man-ol1*P*.

We therefore designed a new synthetic route that is summarized in Scheme 1 and can be flexibly applied for the preparation of Man-ol1*P* or 5-[^{2}H]-Man-ol1*P*. A bi-enzymatic system constituted of *Af*M1PDH and formate dehydrogenase from *Candida boidinii* (*Cb*FDH) was employed. *Af*M1PDH catalyzes the desired synthetic reaction transforming Fru6*P* into Man-ol1*P* via NADH-dependent reduction, and the regeneration of NADH is achieved via NAD^+-dependent oxidation of formate into CO_2 catalyzed by *Cb*FDH.

A typical time course of synthesis of Man-ol1*P* by the coupled action of dehydrogenases is summarized in Figure 2. We used a 2.5-fold excess of *Cb*FDH activity (5 U/mL) over *Af*M1PDH activity (2 U/mL) to ensure that a substantial portion of the total coenzyme, which was added as NAD^+ at the start of the reaction (0.5 mM), was present in the required reduced form. Furthermore, a slight molar excess of formate (250 mM) over Fru6*P* (200 mM) was chosen to ensure the complete conversion of ketose substrate into product. A partially purified preparation of *Cb*FDH with a specific activity of 1.4 U/mg was used in the reaction. High performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) was employed for monitoring the progress of the reaction, as shown in panel A of Figure 2. Under the conditions used, more than 99% of the initial Fru6*P* was converted into Man-ol1*P*. Panel B of Figure 2 shows the time course of Man-ol1*P* production. The workup of Man-ol1*P* included the removal of the enzymes by ultrafiltration as the first step. Further purification targeted the elimination of NAD(H) because the contamination of the product with coenzyme could interfere with the planned enzyme kinetic measurements. Because anion exchange chromatography on a MonoQ 5/50 GL (GE Healthcare) column was not successful, we focused on selective precipitation of Man-ol1*P* as barium salt. A screening of conditions for the recovery of product in the highest possible purity revealed that the addition of an equal volume of EtOH to the reaction mixture facilitated precipitation of Man-ol1*P* while NAD(H) and residual formate were retained in the supernatant. After washing and drying the precipitate, Man-ol1*P* was obtained in an isolated yield of 90% and a purity

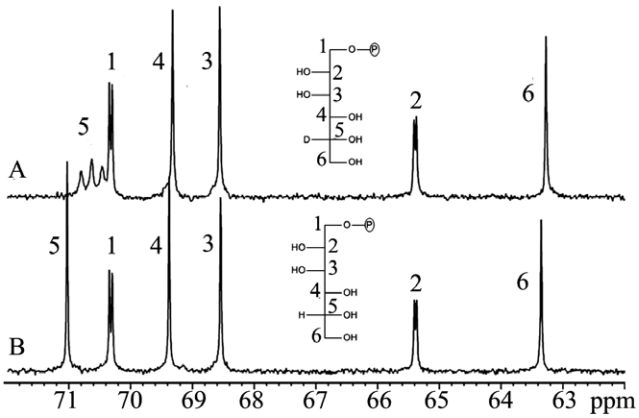


Figure 3. ^{13}C spectra of D-5-[^{2}H]-mannitol 1-phosphate (A) and D-mannitol 1-phosphate (B).

of >95% (determined by HPAE-PAD and NMR). Using NMR analysis, neither NAD^{+} nor NADH could be detected in the isolated product. A possible contamination of the product by Fru6*P*, D-glucose 6-phosphate (Glc6*P*), formate, mannitol, and fructose was ruled out within the limits of detection of the used HPAE-PAD and NMR methods. A ^{13}C NMR spectrum of the final product is shown in Figure 3.

Inspection of the HPAE-PAD traces in Figure 2A shows that immediately after the start of the reaction, a new compound was formed which according to its retention time (16.7 min) could be clearly distinguished from Man-ol1*P* and was identified as Glc6*P*. The Glc6*P* gradually disappeared as the production of Man-ol1*P* progressed. Because no Glc6*P* was added with the substrate, the results imply the presence of an isomerase activity that reversibly interconverts Fru6*P* and Glc6*P*. The only possible source for this activity was the partially purified *Cb*FDH. We therefore measured the activity of phosphoglucose isomerase (PGI) in the used preparation of *Cb*FDH and the *E. coli* cell extract from which it was obtained. The results summarized in Table 3 show that the heat treatment employed in the purification of *Cb*FDH only partially eliminated the PGI activity present in the starting material. A useful consequence of the PGI activity carried over with the *Cb*FDH preparation was that Glc6*P*, which is cheaper than Fru6*P*, could be employed as a substrate for the synthesis of Man-ol1*P* without affecting negatively the space-time yield of the reaction as well as the yield and purity of the final product.

Substitution of formate by [^{2}H]-formate as reductant for the enzymatic conversion of Fru6*P* was expected to provide convenient access to the deuterated analogue of Man-ol1*P*, namely 5-[^{2}H]-D-mannitol 1-phosphate (5-[^{2}H]-Man-ol1*P*). Using reaction conditions otherwise identical to those for Man-ol1*P* production, we observed the complete conversion of ketose substrate but only partial (≈2/3) deuteration of position C–5 in the isolated Man-ol1*P* product. Because the deuterium label in the commercial [^{2}H]-formate had been verified by NMR spectroscopy. We examined rigorously the role of the purity of *Cb*FDH, using for 5-[^{2}H]-Man-ol1*P* synthesis each of the different enzyme preparations shown in Table 3. The highly purified *Cb*FDH obtained after anion exchange chromatography (Fig. 4) was required to synthesize 5-[^{2}H]-Man-ol1*P* with a high degree of deuterium labeling. The isotopic purity of the isolated product was estimated by NMR to be >95% ^{2}H (Fig. 3). To our knowledge, the synthesis of 5-[^{2}H]-Man-ol1*P* has not been reported so far.

We asked the question of whether a causal relationship exists between the removal of PGI activity from the *Cb*FDH preparation and the enhancement of deuterium label in the 5-[^{2}H]-Man-ol1*P* product. A deuterium

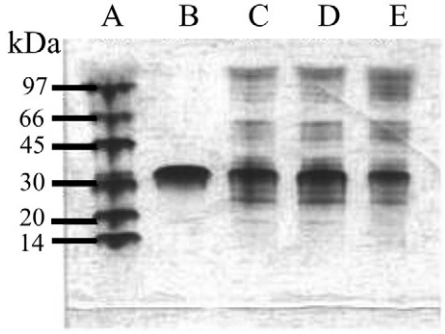


Figure 4. *Cb*FDH purification documented by SDS PAGE analysis: (A) low molecular weight standard (GE Healthcare); (B) purified *Cb*FDH obtained after anion exchange chromatography (fraction III); (C) preparation after heat treatment and desalting (fraction II); (D) preparation after heat treatment (fraction I); (E) crude *E. coli* cell extract.

Table 3. Purification of recombinant *Cb*FDH

Purification stage	Total activity FDH (U)	Protein (mg/mL)	Specific activity FDH (U/mg)	Total yield FDH (%)	Purification factor FDH	Total activity PGI (U)	Specific activity PGI (U/mg)
Crude extract	249	40.6	1.0	100	1.0	317	1.2
Heat denaturation	185	6.4	1.4	74	1.5	232	1.8
Desalting	153	9.4	1.4	62	1.5	203	1.9
Q-Sepharose FF	131	5.22	4.4	53	4.5	<0.06	<0.002

Results are based on processing 3.3 g wet cells of *E. coli*. Desalting was performed by separating the total amount of protein in two batches which was necessary due to the high concentration of formate carried over from the heat treatment. The activities of *Cb*FDH and PGI were assayed as described under Section3.

wash-out experiment was performed in which the completely deuterated 5-[^{2}H]-Man-ol1*P* (75 mM) was incubated at room temperature and pH 7.5 in the presence of the PGI-containing standard preparation of *Cb*FDH (obtained after heat treatment) or a commercial PGI from baker's yeast. Incubation with 2.3 U PGI/mL was done for 22 h and the product was isolated as described above. No deuterium wash-out was observed within the limits of experimental error of ^{13}C NMR analysis. Further examination of the interesting reaction that leads to partial deuteration of Man-ol1*P* was beyond the remit of this study and is left for consideration in the future.

2.4. Stereochemical course of hydrogen transfer catalyzed by *Af*M1PDH

NAD(P)$^+$-dependent dehydrogenases are usually highly stereoselective with regard to transfer of hydrogen from the prochiral C–4 of the nicotinamide moiety of NADH or NADPH. These are classified according to stereochemical preference as pro-*R* or pro-*S* specific. To determine the stereochemical course of the reaction catalyzed by *Af*M1PDH, we incubated the purified enzyme in the presence of 5-[^{2}H]-Man-ol1*P* and NAD$^+$ and monitored by using in situ ^{1}H NMR analysis the formation of the deuterated, hence chiral NADH product. The results in Figure 5 reveal that (4*S*)-[^{2}H]-NADH was produced upon the enzymatic oxidation of 5-[^{2}H]-Man-ol1*P*. The stereoselectivity of the reduction of NAD$^+$ was absolute within the limits of detection of the chosen analytical method. Therefore, these results imply that the *Af*M1PDH belongs to the group of pro-*S* specific dehydrogenases, a functional classification that the fungal enzyme shares with M1PDHs from the bacteria *Aerobacter aerogenes* and *E. coli*.[9,18,19] The stereoselectivity of biocatalytic hydrogen transfer to NAD$^+$ reflects the relative orientation of substrate and coenzyme upon their binding to the enzyme. It is dictated by the protein structure and therefore expected to be conserved among evolutionary related dehydrogenases. The pro-*S* stereoselectivity of *Af*M1PDH is consistent with the proposed membership of the enzyme to the family of PSLDRs.[20,21]

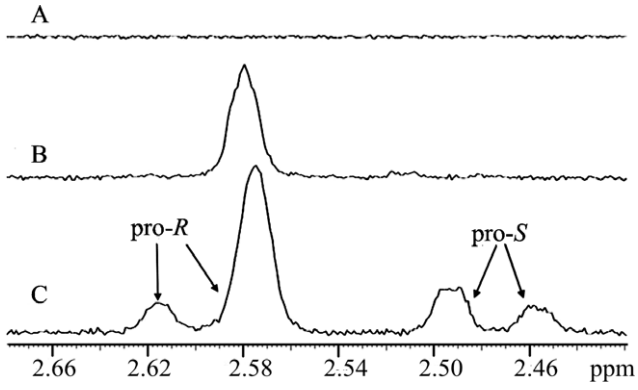


Figure 5. Stereospecificity of hydrogen transfer to the nicotinamide moiety of NAD(H) catalyzed by *Af*M1PDH: (A) 5-[^{2}H]-Man-ol1*P* and NAD$^+$ before enzymatic conversion; (B) formation of (4*S*)-[^{2}H]-NADH after enzymatic reaction for 30 min; incubations in (A) and (B) were carried out using 2 mM NAD$^+$, 5 mM 5-[^{2}H]-Man-ol1*P* in 30 mM Tris/HCl, p^{2}H 7.1; (C) formation of NADH and (4*S*)-[^{2}H]-NADH after 15 min of enzymatic reaction using 5 mM NAD$^+$ and 5 mM of a 2:1 mixture of 5-[^{2}H]-Man-ol1*P*/Man-ol1*P* in 50 mM Tris/HCl, p^{2}H 9.8.

2.5. KIE on NAD$^+$-dependent enzymatic oxidation of D-mannitol 1-phosphate

Figure 6 compares time courses of the enzymatic oxidation of Man-ol1*P* and 5-[^{2}H]-Man-ol1*P* obtained under conditions in which the concentrations of the substrate (6 mM) and NAD$^+$ (6 mM) were saturating in the steady state and therefore maximum initial rates (V_{max}) are measured. It shows that substrate deuteration caused a significant slowing down of the reaction catalyzed by *Af*M1PDH. Because the molar enzyme concentration was identical in the two experiments, the slopes of linear plots of the concentration of reduced coenzyme against the reaction time could be directly used to calculate the KIE, which is the ratio of the slopes measured with *protio* and *deuterio* substrate and had a value of 2.9 ± 0.2. The degree of rate reduction expressed in the value of KIE depends on how much the isotope-sensitive step of hydride transfer contributes to the overall reaction. Under the V_{max} conditions used, the substrate binding steps are not relevant kinetically. However, microscopic reaction steps comprised in the value of V_{max} include kinetic isomerizations of substrate-bound enzyme forms, the chemical conversion steps, and the release of product and NADH to regenerate the free enzyme. The dissociation of the NAD(P)H product constitutes the rate-determining step in reactions catalyzed by many

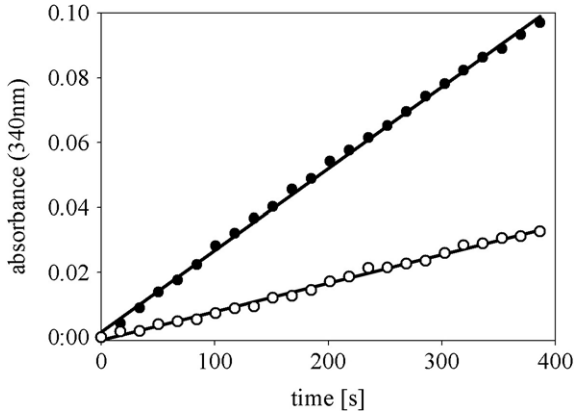


Figure 6. Primary deuterium isotope effect on k_{cat} of Man-ol1*P*/5-[^{2}H]-Man-ol1*P* oxidation: initial rates of Man-ol1*P* oxidation (●) and 5-[^{2}H]-Man-ol1*P* oxidation (○). The concentrations of substrate and NAD$^+$ were 6 mM each.

$NAD(P)^+$-dependent dehydrogenases, reflected by a KIE on V_{max} that is equal to or not much larger than unity.[11] In *Af*M1PDH, by contrast, the chemical step of hydride transfer strongly governs the maximum rate of Man-ol1*P* oxidation by NAD^+ at pH 7.1.

Summarizing, we have functionally expressed the M1PDH from *A. fumigatus* in *E. coli* and performed a biochemical characterization of the recombinant enzyme. A new method of synthesis of Man-ol1*P* and its *deuterio* analogue 5-[^{2}H]-Man-ol1*P* was established. Using the KIE approach thus made possible, we determined that hydride transfer to NAD^+ is a slow step in the overall oxidation of Man-ol1*P* and product release is not rate-determining.

3. Experimental

3.1. Materials

Fructose 6-phosphate (Fru6*P*), D-mannitol 1-phosphate (Man-ol1*P*), β-nicotinamide adenine dinucleotides ($NAD(P)^+$, NAD(P)H), sodium formate, potassium [^{2}H]-formate, salts and buffer reagents were from Sigma–Aldrich (St. Louis, MO, USA). T4-Ligase, restriction enzymes and *Taq* DNA polymerases were from Fermentas (Burlington, Canada). The pQE70-vector was obtained from Qiagen (Hilden, Germany). PWO-polymerase was a product of PEQLAB (Erlangen, Germany). Oligonucleotides were obtained from VBC-Biotech (Vienna, Austria). Genomic DNA of *A. fumigatus* was kindly provided by Dr. Hubertus Haas (Division of Molecular Biology, Medical University of Innsbruck, Austria).

3.2. D-Mannitol 1-phosphate

Enzymatic conversion of Fru6*P* or Glc6*P* (200 mM) to Man-ol1*P* was carried out in a 30 mM Tris/HCl buffer, pH 7.5, containing 250 mM sodium formate, 0.5 mM NAD^+, 5 U/mL partially purified *Cb*FDH (fraction I; see Section 3.6), and 2 U/mL purified *Af*M1PDH. Enzyme activities of *Cb*FDH (NAD^+-dependent oxidation of formate) and *Af*M1PDH (NADH-dependent reduction of Fru6*P*) were measured at 25 °C under conditions in which the concentrations of substrate and coenzyme were saturating (*Cb*FDH: 250 mM formate, 2 mM NAD^+; *Af*M1PDH: 50 mM Fru6*P*, 0.2 mM NADH). The enzyme assays were performed in 30 mM Tris/HCl, pH 7.5.

The synthesis of Man-ol1*P* was carried out in a batch reaction at room temperature, typically for 20 h. The working volume was 10 mL. The pH of the reaction was controlled at a value of 7.5 through the addition of diluted HCl. Gentle mixing with a magnetic stirrer was used before taking samples or while adjusting the pH. A pH of 7.5 was chosen because both *Af*M1PDH and *Cb*FDH show good activity and stability under these conditions. After the reaction, enzymes were removed by ultrafiltration using Vivaspin 6 MWCO 10 kDa microconcentrator tubes (Sartorius AG, Goettingen, Germany) at 4500 g. Man-ol1*P* was precipitated through the addition of an equimolar amount of $BaSO_4$ followed by the dilution of the reaction mixture with the same volume of absolute EtOH. Precipitation was allowed to proceed for 2 h at 4 °C. The precipitate was collected by filtration and washed exhaustively with absolute EtOH. The barium salt of Man-ol1*P* was dried over night at 40 °C. ^{1}H NMR (D_2O): ppm 3.81 (m, 2H), 3.71 (m, 2H), 3.63 (m, 3H), 3.49 (dd, 1H, J_1 11.7 Hz, J_2 5.3 Hz); ^{13}C NMR (D_2O): ppm 71.0 (C-5), 70.3 (C-1, d, J_{C-P} 5.8 Hz), 69.4 (C-4), 68.5 (C-3), 65.4 (C-2, d, J_{C-P} 4.8 Hz), 63.4 (C-6). The isolated product showed the same retention time in HPAE-PAD (13.7 min) as the authentic Man-ol1*P* standard obtained from Sigma–Aldrich.

3.3. 5-[^{2}H]-D-Mannitol 1-phosphate

The synthesis of 5-[^{2}H]-Man-ol1*P* used the analogous procedure employed for the production of Man-ol1*P* except that potassium [^{2}H]-formate served as the reductant and a purified preparation of *Cb*FDH (fraction III; see Section 3.6) was utilized. ^{1}H NMR (D_2O): ppm 3.81 (m, 2H), 3.71 (m, 2H), 3.63 (m, 2H), 3.49 (d, 1H, *J* 11.7 Hz); ^{13}C NMR (D_2O): 70.6 (C-5, t, J_{C-D} 22.5 Hz), 70.3 (C-1, d, J_{C-P} 5.8 Hz), 69.3 (C-4), 68.6 (C-3), 65.4 (C-2, d, J_{C-P} 4.8 Hz), 63.3 (C-6). The 5-[^{2}H]-Man-ol1*P* eluted in HPAE-PAD at the same retention time (13.7 min) as the authentic Man-ol1*P* standard.

3.4. Analytical methods

3.4.1. Protein analysis. Purification of *Cb*FDH and *Af*M1PDH was monitored by SDS PAGE using the Phast System (GE Healthcare, Chalfont St. Giles, United Kingdom). Staining of protein bands was done with Coomassie Brilliant Blue. Size exclusion chromatography of purified *Af*M1PDH was carried out on a Superdex 200 HR 10/30 gel filtration column (GE Healthcare). The column was calibrated with a gel filtration standard from Bio-Rad Laboratories (Hercules, USA).

3.4.2. Assays. Photometric measurements were carried out with a DU800 spectrophotometer from Beckman Coulter Inc. (Fullerton, CA, USA). The activities of *Cb*FDH and *Af*M1PDH were determined from initial rate measurements in which the production or consumption of NADH at a wavelength of 340 nm ($\varepsilon = 6.22\ cm^{-1}\ mM^{-1}$) was measured. Unless otherwise indicated, the activity of *Af*M1PDH was assayed at 25 °C either in the direction of NADH-dependent reduction of Fru6*P* using a 100 mM Tris/HCl buffer, pH 7.1,

or in the direction of NAD^+-dependent oxidation of Man-ol1*P* using a 100 mM glycine/NaOH buffer, pH 10.0. The concentrations of Fru6*P* and NADH were 50 mM and 0.2 mM, respectively. The concentrations of Man-ol1*P* and NAD^+ were 0.5 mM and 2 mM, respectively. The activity of *Cb*FDH was assayed in the direction of NAD^+-dependent oxidation of formate at 30 °C using 100 mM potassium phosphate buffer, pH 7.5. The concentrations of formate and NAD^+ were 162 mM and 2 mM, respectively.

PGI activity was measured in a coupled enzymatic assay at 30 °C essentially as described by Ruijter and Visser except that *Af*M1PDH was used instead of the M1PDH from *A. nidulans*.[22] The principle of the assay is that Glc6*P* is isomerised by PGI to Fru6*P*, which is in turn reduced by *Af*M1PDH. Therefore, PGI activity is measured as the decrease in the absorbance of NADH at 340 nm. The assay was performed in 100 mM potassium phosphate buffer, pH 7.5, and contained 20 mM Glc6*P*, ≈4 U/mL *Af*M1PDH, and 0.2 mM NADH.

Protein concentrations were measured with the Bio-Rad Protein Assay, which is based on the method of Bradford and was calibrated against BSA.[23] Man-ol1*P* and 5-[^{2}H]-Man-ol1*P* were quantified enzymatically using *Af*M1PDH in 100 mM glycine/NaOH buffer, pH 10.0, containing 2 mM NAD^+. Concentrations were calculated from the formed NADH using appropriate calibration of the assay with authentic Man-ol1*P* in the range of 0.04–0.20 mM.

3.4.3. Carbohydrate analysis. High performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) was utilized to measure the concentration of Fru6*P*, Glc6*P*, and Man-ol1*P* or 5-[^{2}H]-Man-ol1*P* in samples taken from enzymatic conversions. The analysis was performed using a Dionex BioLC system (Dionex Corporation, Sunnyvale, CA, USA) equipped with a CarboPac PA10 column (4 × 250 mm) and an Amino Trap guard column (4 × 50 mm) thermostated at 30 °C. Phosphorylated sugars were detected with an ED50A electrochemical detector using a gold working electrode and a silver/silver chloride reference electrode by applying the predefined waveform for carbohydrates. Elution was carried out at a flow rate of 1 mL/min based on a previously described method with a linear gradient from 50 to 275 mM NaOAc applied within 22 min in an isocratic background of 4 mM NaOH.[24] Column was flushed 5 min with 800 mM NaOAc (+4 mM NaOH) and 15 min with the starting conditions before the next sample was injected.

3.4.4. NMR. Measurements were carried out on a Varian INOVA 500 MHz spectrometer (Varian Inc., Palo Alto, CA, USA) at 22 °C in D_2O (99.9% D) using the VNMR 6.1c software. ^{1}H NMR spectra were measured at 499.98 MHz and ^{13}C NMR at 125.69 MHz. For ^{13}C NMR spectra of Man-ol1*P* and 5-[^{2}H]-Man-ol1*P* 15 000 scans were accumulated and 1 Hz line broadening was used for sensitivity enhancement prior to Fourier transformation. Enzymatic conversions of NAD^+ (2 mM or 5 mM) into [^{2}H]-NADH and NADH with *Af*M1PDH (≈1 μM) using Man-ol1*P*/5-[^{2}H]-Man-ol1*P* (5 mM) in 30 mM Tris/HCl, p^{2}H 7.1, or 50 mM Tris/HCl, p^{2}H 9.8, were performed directly in the NMR sample tube, which is placed in the magnet. Proton NMR spectra were recorded by acquiring 30272 data points and accumulation of 200 scans.

3.5. Gene cloning, and production and purification of recombinant *Af*M1PDH

The intron-less open-reading frame encoding *Af*M1PDH was amplified from genomic DNA of *A. fumigatus Af*293 using PCR with PWO-polymerase and the following pair of oligonucleotide primers:

forward primer: 5′-GATCTAGCATGCTAGGAAAG-AAGGCTATCCAGTTTG-3′,
reverse primer: 5′-GATCTAAGATCTCTTGCTGTC-CTTCTGCACCTT-3′.[12]

The PCR product was digested with SphI and BglII and inserted into the plasmid vector pQE70 previously treated with the same restriction enzymes. The obtained construct was transformed into *E. coli* JM109. Cells harboring pQE70-*Af*M1PDH were cultivated at 37 °C in LB-Lennox-medium containing 115 mg/L ampicillin. Expression of recombinant *Af*M1PDH was induced by IPTG (125 μM) in mid-exponential growth phase after the temperature was decreased to 25 °C. After about 20 h post-induction, *E. coli* biomass was harvested by centrifugation (4400 g, 20 min, 4 °C) and disrupted twice with a French Press at 1500 psi cell pressure. Debris of bacterial cells were removed by ultracentrifugation at 80 000 g for 45 min at 4 °C. The crude cell extract was then applied on a column of copper-loaded Chelating Sepharose Fast Flow (XK 16 column from GE Healthcare; 1.6 × 6.2 cm) equilibrated with a 50 mM Tris/HCl buffer, pH 7.1, that contained 300 mM sodium chloride and glycerol (10% v/v). Competitive elution was achieved using a linear gradient of imidazole in the range of 0–220 mM which was applied over eight column volumes while employing a constant flow rate of 1.5 mL/min. Imidazole, sodium chloride and glycerol were afterwards removed using a HiPrep 26/10 prepacked desalting column (GE Healthcare).

3.6. Gene cloning, and production and purification of recombinant *Cb*FDH

C. boidinii ATCC 18810 was grown on YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L

glucose), and its genomic DNA was isolated using the Wizard® Genomic DNA Purification Kit from Promega (Madison, WI, USA). The gene encoding *Cb*FDH (EC 1.2.1.2) was amplified by PCR with the High Fidelity PCR Enzyme Mix from Fermentas (Burlington, Canada) and the following pair of oligonucleotide primers:

forward primer: 5′-GATCTAGAATTCATGAAGAT-CGTTTTAGTCTTATATGATGCTG-3′,
reverse primer: 5′-GATCTACTGCAGTTATTTCTTA-TCGTGTTTACCGTAAGCTTTAG-3′.

The PCR product was digested with EcoRI and PstI and inserted into the plasmid vector pBTac1 (Boehringer Mannheim, Germany) previously cleaved with the same restriction enzymes. Identity of the primary amino acid sequence with the *Cb*FDH described by Sakai et al. (UniProt/TrEMBL entry O93968) was confirmed by DNA sequencing.[25] Expression of recombinant *Cb*FDH was done in *E. coli* JM109, using the procedure described under Section 3.5. Purification of *Cb*FDH was performed using slight modifications of the protocol described by Slusarczyk et al. for the isolation of FDH1 from *C. boidinii* (UniProt/TrEMBL entry O13437).[26] Briefly, the crude *E. coli* extract (obtained as described under 3.5) was incubated in the presence of 10% w/v formate or [^{2}H]-formate at 60 °C for 5 min. Precipitated proteins were removed by centrifugation (11 200 g, 10 min, 4 °C). The supernatant (fraction I) was desalted in two batches using a pre-packed desalting column (see Section 3.5). The desalted protein solution (fraction II) was loaded on a column of Q-Sepharose Fast Flow (XK 16 column from GE Healthcare; 1.6 × 9.0 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.5. At a flow of 10 mL/min the column was flushed with 4.5 column volumes of the phosphate buffer before adding up to 150 mM NaCl in a linear gradient of 50 column volumes. Purified *Cb*FDH was stabilized by the addition of either 200 mM sodium formate or potassium [^{2}H]-formate (fraction III).

3.7. Characterization of *Af*M1PDH

To redissolve the barium salts of Man-ol1*P* and 5-[^{2}H]-Man-ol1*P*, we added an equimolar amount of Na_2SO_4 to the product. The formed $BaSO_4$ was removed by centrifugation (10 min at 11 200 g). The actual concentrations of dissolved Man-ol1*P* and 5-[^{2}H]-Man-ol1*P* were determined enzymatically as described under 3.4. Kinetic parameters of *Af*M1PDH for NAD^+-dependent oxidation of Man-ol1*P* were measured at 25 °C in 100 mM Tris/HCl buffer, pH 7.1, using varied concentrations of NAD^+ (0.04–8.8 mM) or Man-ol1*P* (0.03–3.4 mM) while keeping the concentration of the respective other substrate constant and saturating (Man-ol1*P*: 4.3 mM; NAD^+: 5.8 mM). Similarly, kinetic parameters for NADH-dependent reduction of Fru6*P* were obtained using concentrations of NADH between 0.002 and 0.2 mM or Fru6*P* between 0.5 and 40 mM, while keeping the other substrate constant and saturating (45 mM Fru6*P*; 0.2 mM NADH). The enzyme was appropriately diluted in the corresponding reaction buffer. Reactions were started with enzyme and initial rates were recorded photometrical as described in Section 3.4. Steady state kinetic parameters were determined by fitting the Michaelis–Menten equation to the experimental data using Sigma Plot 9.0 (SYSTAT Software Inc., San Jose, CA, USA). The kinetic turnover numbers (k_{cat}) were calculated from the maximal initial velocities using the molecular mass of 44.2 kDa as derived from the unprocessed amino acid sequence of recombinant *Af*M1PDH. Primary deuterium KIE on k_{cat} in the direction of alcohol oxidation was obtained by using saturating concentration of Man-ol1*P* or 5-[^{2}H]-Man-ol1*P* (6 mM) and NAD^+ (6 mM), employing otherwise identical reaction conditions as described above for Man-ol1*P* oxidation.

Acknowledgments

We are especially grateful to Dr. Hubertus Haas (Division of Molecular Biology, Medical University of Innsbruck, Austria) for providing genomic DNA of *A. fumigatus*. We thank Eva Glaumann for assistance in cloning of *Cb*FDH. Financial support from the Austrian Science Fund FWF (P18275-B09) is gratefully acknowledged.

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