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Synthesis of arabinitol 1-phosphate and its use for characterization of arabinitol–phosphate dehydrogenase

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In memoriam of Dr. Kirill N. Neustroev

Abstract—D-Arabinitol 1-phosphate (Ara-ol1-P), a substrate for D-arabinitol–phosphate dehydrogenase (APDH), was chemically synthesized from D-arabinonic acid in five steps (*O*-acetylation, chlorination, reduction, phosphorylation, and de-*O*-acetylation). Ara-ol1-P was used as a substrate for the characterization of APDH from *Bacillus halodurans*. APDH converts Ara-ol1-P to xylulose 5-phosphate in the oxidative reaction; both NAD^+ and $NADP^+$ were accepted as co-factors. Kinetic parameters for the oxidative and reductive reactions are consistent with a ternary complex mechanism.

Keywords: Pentitol metabolism; D-Arabinitol–phosphate dehydrogenase; Arabinitol 1-phosphate; Xylulose 5-phosphate

1. Introduction

Metabolic pathways of polyols, which are widespread in nature, have been investigated extensively in fungi, bacteria, and higher organisms. One reason is their therapeutic significance. In the last decade, it was shown that xylitol is an anti-cariogenic agent.[1] This pentitol can be transported into caries-causing oral bacteria, and inhibits bacterial growth due to its influence on pentitol phosphate metabolism.[2,3] Likewise, D-arabinitol was discovered to be a quantitative diagnostic marker for invasive candidiasis.[4] L-Arabinosuria, an abnormality of human polyol metabolism, has been reported to be related to the deficiency of L-arabinitol dehydrogenase.[5] In general, metabolism of pentitols in both prokaryotes and eukaryotes involves polyol transportation into the cell by permease, where a dehydrogenase oxidizes the five-carbon sugar alcohol to its corresponding keto-pentose. Then keto-pentoses are phosphorylated by specific kinases, and enter the pentose–phosphate pathway.[6,7] More recently, an alternative metabolic pathway of polyols was discovered, in which the alcohols are first phosphorylated by the PTS before their oxidation by polyol phosphate dehydrogenases. Xylitol and ribitol were reported to be metabolized by this route in several species of *Lactobacilli*.[8] The xylitol 5-phosphate dehydrogenase responsible for assimilation of xylitol via this pathway was isolated and described.[9] Earlier, we had purified and characterized a new polyol–phosphate dehydrogenase (E.C. 1.1.1.X) from the cell lysate of *Enterococcus avium* involved in a similar catabolic pathway for D-arabinitol. It exhibited a new type of specificity.[10] This NADH-dependent arabinitol–phosphate dehydrogenase (APDH) catalyzes the oxidation of D-arabinitol 1-phosphate and D-arabinitol 5-phosphate into D-xylulose 5-phosphate

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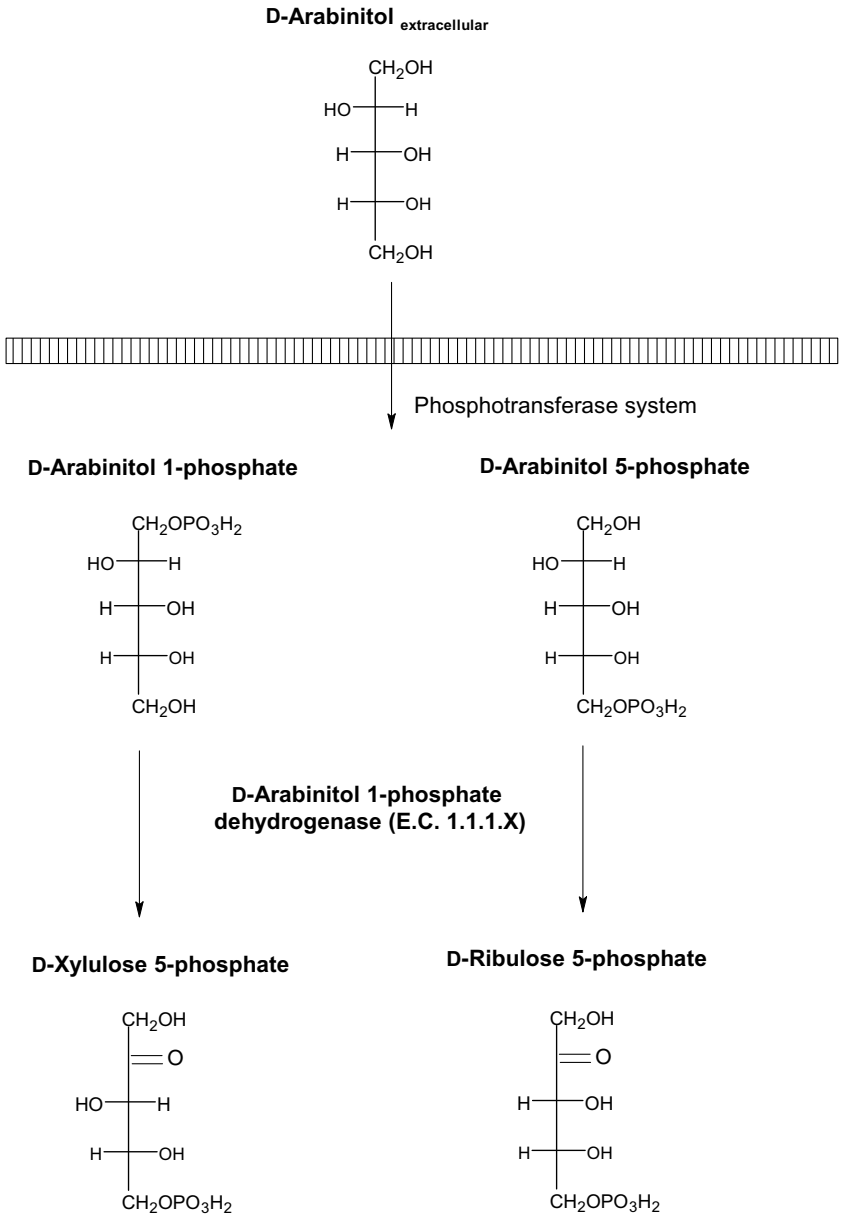

Figure 1. Catabolic routes for D-arabinitol catalyzed by D-arabinitol 1-phosphate dehydrogenase.

(Xul5P) and D-ribulose 5-phosphate, respectively (Fig. 1).

To analyze the specificity and kinetic properties of *Enterococcus* APDH, we originally used D-arabinitol 1-phosphate produced by $NaBH_4$ reduction of commercial Xul5P followed by chromatographic separation of the stereoisomeric products. However, this method is expensive, tedious, and low yielding. We now report a new, five-step chemical synthesis of Ara-ol1-P from D-arabinonic acid, which can be carried out on the 10 g scale. The Ara-ol1-P so produced was used for detailed characterization of recombinant *Bacillus halodurans* APDH expressed in *Escherichia coli*.

2. Results and discussion

2.1. Synthesis of Ara-ol1-P

In spite of extensive interest in enzymes of polyol metabolism, there are only a few methods for the production of pentitol phosphate substrates for dehydrogenases involved into sugar metabolic pathways. The approaches have been mainly enzymatic, and use a selection of kinases, isomerases, epimerases, transketolases etc.[11,12] The advantages provided by the selectivity of enzymatic synthesis is offset, though, by the need for chromatographic separation of products

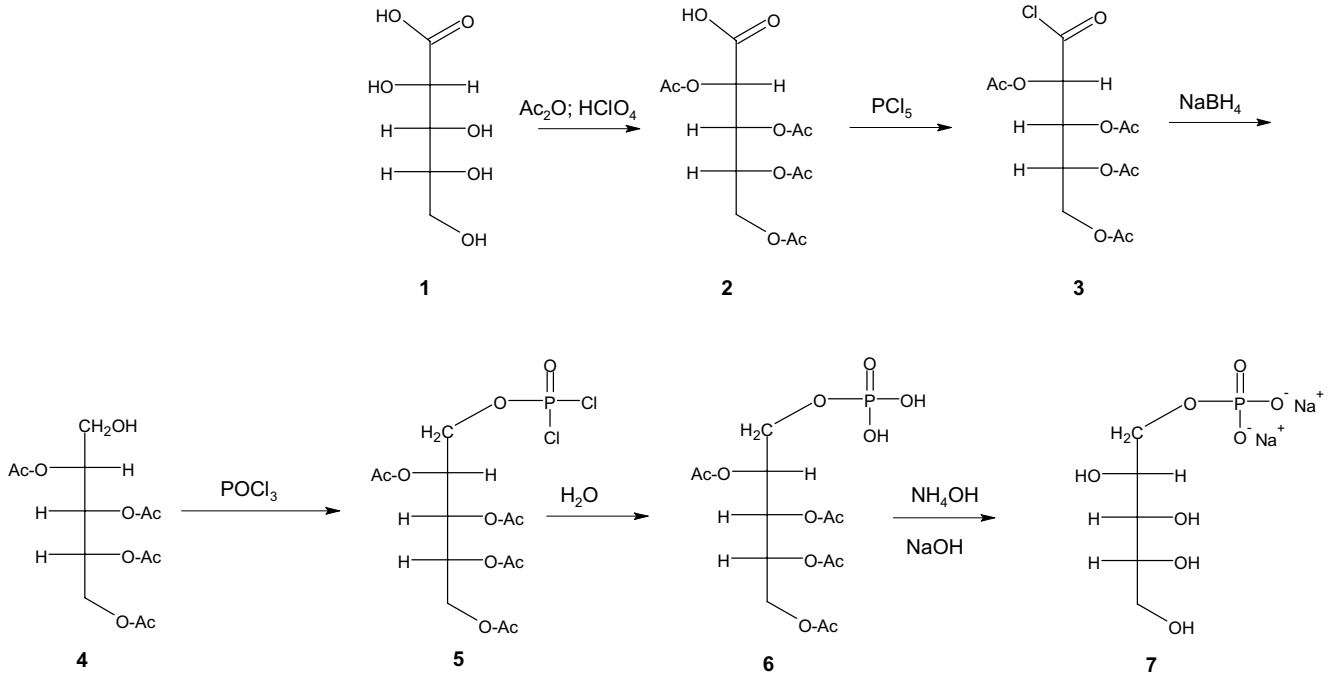

Figure 2. General scheme of Ara-ol1-P synthesis.

after each step, which also limits the scale of the reaction.

Investigations of pentitol metabolic pathways require pentitol 1-phosphates and pentitol 5-phosphates. D-Xylitol 5-phosphate and D-arabinitol 5-phoshate can be obtained by $NaBH_4$ reduction[10] of D-xylofuranose 5-phosphate and D-arabinopyranose 5-phosphate, respectively, as reported earlier.[13] However, chemical approaches for production of pentitol 1-phosphates, particularly Ara-ol1-P, have not been reported so far. The method used for synthesis of arabinitol 1-phosphate is set out in Figure 2. D-Arabinonic acid (readily obtained from D-arabinose) was *O*-acetylated and converted to the acid chloride. Reduction with $NaBH_4$ afforded *O*-acetylated D-arabinitol selectively unprotected at position 1. Phosphorylation with $POCl_3$ in pyridine, just above the freezing point of the solvent gave the *O*-acetylated D-arabinitol 1-phosphate, which could be de-*O*-acetylated by 20% aqueous ammonia without the phosphoryl migration observed under acid and neutral conditions.[14–16] Chromatography was required only for the final product.

2.2. Enzymatic properties of APDH and kinetic parameters

APDH activity was assayed by NADH reduction of xylulose 5-phosphate. The enzyme used in this study was a recombinant APDH from *B. halodurans* produced in *E. coli*[10] and partly purified. The preparation of APDH was essentially homogeneous on a SDS-PAGE revealing a single polypeptide band with an estimated molecular mass of 36 ± 2 kDa. Analytical gel-filtration on a Superose 12 and Sephacryl S200 columns revealed a single symmetrical APDH peak corresponding to 140 ± 5 kDa. Thus, in its native state, the *B. halodurans* arabinitol 1-phosphate dehydrogenase is a tetramer similarly to the APDH from *E. avium*. The specificity of the enzyme and its enzymatic properties were investigated using the oxidative reaction of Ara-ol1-P as described in Experimental section. The products of Ara-ol1-P oxidation by APDH with NAD^+ were dephosphorylated by phosphatase digestion and analyzed by HPLC. Only xylulose and trace quantities of arabinitol were detected demonstrating that the product of the reaction was D-xylulose 5-phosphate (see scheme given in Fig. 1). Analysis of dephosphorylated products of the APDH-mediated D-xylulose 5-phosphate reduction using NADH as a co-factor revealed only D-arabinitol as a product after the dephosphorylation. As expected, the only product of the APDH-catalyzed oxidation of Ara-ol5-P was D-ribulose 5-phosphate. Thus, substrate specificity of D-arabinitol phosphate dehydrogenase from *Bacillus halodurans* seems to be limited to the oxidation of D-arabinitol 1-phosphate to D-xylulose 5-phosphate and D-arabinitol 5-phosphate to D-ribulose 5-phosphate using either NAD^+ or NADH as co-factors (Fig. 1).

Maximal rates of the APDH in the forward (oxidative) direction were found at pH 8.1–8.5 and in the reverse (reductive) direction—at pH 6.8–7.2. Experiments on pH—and temperature stability were performed using Ara-ol1-P as a substrate. The enzyme

Table 1. Kinetic data obtained for the purified APDH from *B. halodurans*

Polyol substrate, reaction direction	Substrate K_m (mM)		Co-factor K_m (mM)		Ternary complex K_m (mM^2)		V_{max} (μmol/min per mg)	
Ara-ol1-P, oxidative	$K_m^{Ara\text{-}ol1\text{-}P}$	4.0 ± 0.1	$K_m^{NAD^+}$	0.5 ± 0.02	$K_m^{Ara\text{-}ol1\text{-}P,NAD^+}$	3.8 ± 0.1	$V_{max}^{Ara\text{-}ol1\text{-}P,NAD^+}$	5.9 ± 0.15
Xul5P, reductive	K_m^{Xul5P}	0.22 ± 0.004	K_m^{NADH}	0.02 ± 0.0004	$K_m^{Xul5P,NADH}$	0.01 ± 0.002	$V_{max}^{Xul5P,NADH}$	27.0 ± 0.5
Rul5P, reductive	K_m^{Rul5P}	4.0 ± 0.051	K_m^{NADH}	0.02 ± 0.001	$K_m^{Rul5P,NADH}$	0.4 ± 0.005	$V_{max}^{Rul5P,NADH}$	3.6 ± 0.1
Fru5P, reductive	K_m^{Fru5P}	0.27 ± 0.005	K_m^{NADH}	0.02 ± 0.001	$K_m^{Fru5P,NADH}$	0.02 ± 0.002	$V_{max}^{Fru5P,NADH}$	5.0 ± 0.15
Rib5P, reductive	K_m^{Rib5P}	40.0 ± 1.2	K_m^{NADH}	2.8 ± 0.08	$K_m^{Rib5P,NADH}$	ND	$V_{max}^{Rib5P,NADH}$	1.2 ± 0.1

Abbreviations: Ara-ol1-P, arabinitol 1-phosphate; Fru5P, fructose 5-phosphate; Rib5P, ribose 5-phosphate; Rul5P, ribulose 5-phosphate; Xul5P, xylulose 5-phosphate.

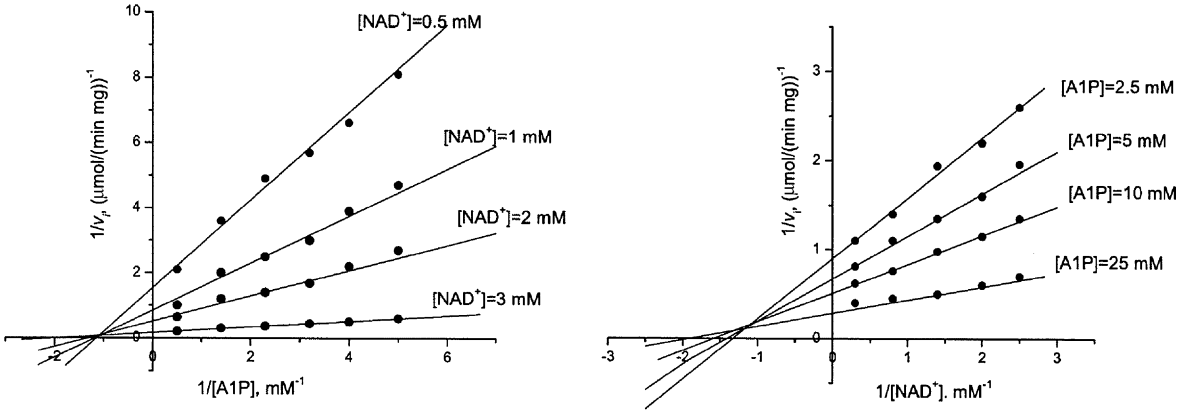

Figure 3. Initial velocities of APDH-catalyzed reactions with Ara-ol1-P as a substrate.

was stable at pH 7.0 during 48 h at 4 °C (data not shown). APDH activity was apparently optimal at 50 °C; however between 40 and 50 °C, 50% of the enzyme activity was retained for 5 min. Above 55 °C activity decreased drastically. Like *Enterococcus* APDH,[10] the enzyme accepted both $NADP^+$ and NADHP as co-factors. NADH was about 12 times more effective over the tested concentration range (up to 3.5 mM).

Table 1 summarizes kinetic parameters obtained for the forward and reverse directions of *B. halodurans* APDH catalysis. Analysis of the double-reciprocal primary plots of velocity against Ara-ol1-P and NAD^+ shown in Figure 3 indicates the expected ternary complex mechanism.[17,18] As can be seen from Table 1, the values for ternary K_m for both substrates are of the same order of magnitude while values for V_{max} in the reverse and forward directions differ about 4.5 times. Comparison of arabinitol–phosphate dehydrogenases from both *E. avium* and *B. halodurans* reveals that the two enzymes have rather similar kinetic characteristics in the reactions with Ara-ol1-P or xylulose 5-phosphate. However, *B. halodurans* APDH has a wider substrate specificity than the *Enterococcus* enzyme and can use fructose 5-phosphate and ribose 5-phosphate in the reductive reaction (kinetic parameters are given in Table 1).

The enzymatic synthesis of D-xylulose 5-phosphate described earlier comprises two sequential enzymatic reactions. Firstly, D-xylulose was prepared by oxidation of D-arabinitol by D-arabinitol dehydrogenase from *Acetobacter suboxidans*. Secondly, D-xylulose 5-phosphate was obtained by phosphorylation with ATP and xylulose kinase; the final product was isolated as its hydrazone.[12] The suggested synthesis of Ara-ol1-P enables its gram-scale production and therefore, synthesis of xylulose 5-phosphate in a one-stage enzymatic reaction using *Bacilus* APDH and synthetic Ara-ol1-P makes such an approach quite attractive.

3. Experimental

3.1. Materials

Xylulose 5-phosphate (Xul5P), arabinose 5-phosphate, ribulose 5-phosphate (Rul5P), fructose 5-phosphate (Fru5P), and ribose 5-phosphate (Rib5P) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Fluka Chemie Gmbh (Buchs, Switzerland). Xylitol 5-phosphate (Xyl-ol5-P) was synthesized according to the protocol described earlier.[13] Arabinitol 5-phosphate (Ara-ol5-P) was produced by $NaBH_4$ reduction of arabinose 5-phosphate[19] followed by a chromatographic separation on a Dextro-Pak™ cartridge column (8 × 100 mm) WATO85650 from Waters Co.

(Milford, USA) using isocratic elution in H_2O.[10] D-Arabinonic acid was obtained by oxidation of D-arabinose and isolated as Ca-salt form according to the procedure previously described.[20]

3.2. 2,3,4,5-Tetra-*O*-acetyl-D-arabinonic acid (2)

Calcium arabinonate (10 g) was added with stirring portion wise to a solution of $HClO_4$ (5% v/v) in Ac_2O (100 mL) cooled to 0 °C (~1 h). The reaction mixture was heated to 40 °C and stirring was continued until a clear solution was obtained. After keeping the reaction mixture at 40 °C for 1 h, anhyd NaOAc (8 g) was added. The solution was stirred for 5 min and cooled to 0 °C; H_2O (400 mL) was poured into the reaction mixture, which was subsequently allowed to warm to room temperature (rt, 25 °C) and was kept at rt for 1 h to complete the reaction. The mixture was cooled to 0 °C, 10 M HCl (20 mL) was added and extraction by $CHCl_3$ was initiated immediately (4×100 mL). The organic fractions were combined, dried (Na_2SO_4), filtered, and the solvent was removed by rotary evaporation. The residue was dissolved in toluene (120 mL), heated to 50 °C and cooled to rt several times with stirring until crystals were formed. The precipitate was filtered off and dried under vacuum to give 15 g (80%) of compound **2**: ^{1}H NMR ($CDCl_3$): δ 5.68 (dd, 1H, $J_{2,3}$ 2.2, $J_{3,4}$ 9.2 Hz, H-3), 5.32 (d, 1H, H-2), 5.22 (ddd, 1H, $J_{4,5a}$ 2.6, $J_{4,5b}$ 4.4 Hz, H-4), 4.28 (1H, dd, $J_{5a,5b}$ 12.6 Hz, H-5a), 4.13 (dd, 1 H, H-5b); ^{13}C NMR ($CDCl_3$): δ 170.82, 170.77, 170.07, 169.67 (s, 1C, CO in OAc), 69.32 (s, 1C, C-2), 68.60 (s, 1C, C-3), 68.06 (s, 1C, C-4), 61.62 (s, 1C, C-5), 20.68, 20.63, 20.43, 20.29 (s, 1C, Me in OAc); ESI$^+$ MS $[M+Na]^+$ *m/z* 379.0617 calcd for $C_{13}H_{17}Na_2O_{10}$, observed 379.0632.

3.3. 2,3,4,5-Tetra-*O*-acetyl-D-arabinoyl chloride (3)

Diethyl ether (120 mL) was stirred with PCl_5 (7.8 g) for 10 min and tetra-*O*-acetyl-D-arabinonic acid **2** (11 g) was added. Stirring was continued until the solution became clear. After additional stirring for 1.5 h, 400 mL of hexane was added and a gel formed. The gel was crystallized by repeated cycles of stirring, evaporation of small amounts of ether and addition of hexane, yielding 10 g (70%) of compound **3**: ^{1}H NMR ($CDCl_3$): δ 5.83 (dd, 1H, $J_{2,3}$ 2.1, $J_{3,4}$ 9.1 Hz, H-3), 5.44 (d, 1H, H-2), 5.21 (ddd, 1H, $J_{4,5a}$ 2.7, $J_{4,5b}$ 4.7 Hz, H-4), 4.28 (dd, 1H, $J_{5a,5b}$ 12.7 Hz, H-5a), 4.11 (dd, 1H, H-5b), 2.18, 2.09, 2.06, 2.06 (1H, Me in OAc); ^{13}C NMR ($CDCl_3$): δ 170.44 (s, 1C, C-1), 169.62, 169.42, 169.39, 168.93 (s, 1C, CO in OAc), 76.18 (s, 1C, C-2), 68.09 (s, 1C, C-3), 67.62 (s, 1C, C-4), 61.39 (s, 1C, C-5), 20.63, 20.59, 20.27, 20.09 (s, 1C, Me in OAc); ESI$^+$MS $[M+Na]^+$ *m/z* 375.0459 calcd for $C_{13}H_{17}NaO_9Cl$, observed 375.0471.

3.4. 2,3,4,5-Tetra-*O*-acetyl-D-arabinitol (4)

Acyl chloride **3** (9 g) was dissolved in 20 mL of dry tetrahydrofuran and added to a stirred solution of $NaBH_4$ (3 g) in water (100 mL) at 0 °C. After stirring the mixture for 1 min, a solution of 4 M HOAc (30 mL) in water was added. After extraction with $CHCl_3$ (3×200 mL), the organic phase was separated, washed with $KHCO_3$ (15 g in 100 mL of water) and water (100 mL) and dried (anhyd Na_2SO_4). The solvent was removed by evaporation to yield an oil (7.5 g, 60%): ^{1}H NMR ($CDCl_3$): δ 5.37 (dd, 1H, $J_{2,3}$ 2.3,$J_{3,4}$ 8.9 Hz, H-3), 5.22 (ddd, 1H, $J_{2,1a}$ 5.1, $J_{2,1b}$ 2.1 Hz, H-2), 5.19 (ddd, 1H, $J_{4,5a}$ 2.5, $J_{4,5b}$ 4.5 Hz, H-4), 4.41 (dd, 1H, $J_{1a,1b}$ 11.7 Hz, H-1a), 4.25 (dd, 1H, $J_{5a,5b}$ 12.5 Hz, H-5a), 4.22 (dd, 1H, H-5b), 4.16 (dd, 1H, H-1b); ^{13}C NMR ($CDCl_3$): δ 171.11, 170.67, 170.60, 170.42 (s, 1C, CO in OAc), 70.50 (s, 1C, C-2), 70.14 (s, 1C, C-3), 69.7 (s, 1C, C-4), 64.89 (s, 1C, C-5), 62.01 (s, 1C, C-1), 20.88 (s, 1C, Me in OAc), 20.75 (s, 3C, Me in OAc); ESI$^+$ MS $[M+Na]^+$ *m/z* 343.1005 calcd for $C_{13}H_{20}NaO_9$, observed 343.1051.

3.5. Phosphorylation of 4

Oily compound **4** (1.7 g) was diluted in tetrahydrofuran (36 mL) and diisopropylethylamine (7.2 mL) was added. After the solution was cooled to −10 °C, $POCl_3$ (3.6 mL) was added; the resulting mixture was warmed to rt and kept for 5 min. The reaction was cooled to incipient solidification in liquid nitrogen and H_2O (54 mL) was poured in. The solution was adjusted to pH 5 by gradual addition of water solution of $KHCO_3$ (3 M, total volume 38 mL) at rt and the solvent (35 g of tetrahydrofuran) was removed by rotary evaporation. The impurities were separated by $CHCl_3$ extraction (2×30 mL). The water fraction containing 2,3,4,5-tetra-*O*-acetyl-D-arabinitol 1-phosphate (**6**) was acidified with 10 M HCl (4 mL) and extracted with EtOAc (3×140 mL). The EtOAc fractions were re-extracted with H_2O (40 mL) and a solution of $NaHCO_3$ (0.5 M, 5 mL) was slowly added to adjust the water phase to pH 4–5. The water phase containing **6** was concentrated by rotary evaporation to 7 mL and used for the synthesis of **7** without isolation. The purity of **6** was analyzed on an Agilent Zorbax Eclipse XDB-C8 column (4.6×150 mm) using 0.1 M triethylamine phosphate as a starting buffer (pH 6.7) and 90% MeOH as an eluting buffer. Elution was carried out with linear gradient (7–90%) of MeOH, flow rate was 0.8 mL/min. The HPLC analysis revealed the only chromatographic peak corresponding to compound **6**: ^{1}H NMR ($CDCl_3$): δ 5.42 (dd, 1H, $J_{2,3}$ 2.8, $J_{3,4}$ 7.5 Hz, H-3), 5.29 (dt, 1 H, $J_{1,2}$ 6.0 Hz, H-2), 5.21 (ddd, 1H, $J_{4,5a}$ 2.6, $J_{4,5b}$ 6.6 Hz, H-4), 4.27 (dd, 1H, $J_{5a,5b}$ 12.3 Hz, H-5a), 4.10 (dd, 1H, H-6b), 3.81 (dd, 2H, $J_{H,P}$ 6.3 Hz, CH_2-1); ^{13}C NMR

($CDCl_3$): δ 171.40, 171.09, 170.98, 170.40 (s, 1C, CO in OAc), 69.71 (d, 1C, $J_{C,P}$ 8.8 Hz, C-2), 68.81 (d, 1C, $J_{C,P}$ 3.4 Hz, C-3), 68.57 (s, 1C, C-4), 62.84 (d, 1C, $J_{C,P}$ 5.4 Hz, C-1), 58.24 (s, 1C, C-5), 20,73, 20.65, 20.54, 20,54 (s, 1C, Me in OAc). ESI^+ MS $[M+Na]^+$ *m/z* 467.0308 calcd for $C_{13}H_{19}Na_3O_{12}P$, observed 467.0341.

3.6. De-*O*-acetylation of 6

The solution **6** (7 mL) was treated with 20% (v/v) solution of NH_4OH (4 mL) for 3.5 h at rt and NH_3 was removed by rotary evaporation.

3.7. D-Arabinitol 1-phosphate, disodium salt (7)

The solution was adjusted to pH 9 by step-by-step addition of NaOH (1 M, total volume 2.5 mL) and rotary evaporation of forming NH_3, and the residue was concentrated under vacuum to 3 mL. The resulting solution was mixed with MeOH (45 mL) and left for 1 h at rt to form a solid. The di-Na-salt of D-arabinitol 1-phosphate was centrifuged, washed with MeOH (5 mL) and freeze dried to give 0.5 g (34%) of pure title compound. ^1H NMR(D_2O): δ 3.95 (dt, 1H, $J_{1,2}$ 6.2, $J_{2,3}$ 1.9 Hz, H-2), 3.80 (ddd, 1H, $J_{1a,1b}$ 12.8, $J_{1a,P}$ 6.5 Hz, H-1a), 3.78 (ddd, 1H, $J_{1b,P}$ 6.5 Hz, H-1b), 3.57 (dd, 1H, $J_{4,5a}$ 3.0, $J_{5a,5b}$ 11.7 Hz, H-5a), 3.68 (ddd, 1H, $J_{4,5b}$ 5.9, $J_{3,4}$ 8.4 Hz, H-4), 3.61 (dd, 1H, H-3), 3.59 (dd, 1H, H-5b); ^{13}C NMR (D_2O): δ 76.12 (s, 1C, C-4), 75.46 (s, 1C, C-3), 74.44 (dd, 1C, $J_{C,P}$ 5.0 Hz, C-2), 70.36 (dd, 1C, $J_{C,P}$ 4.2 Hz, C-2), 68.28 (s, 1C, C-5). ESI^+MS $[M+Na]^+$ *m/z* 298.9885 calcd for $C_5H_{11}Na_3O_8P$, observed 298.9925.

3.8. Analytical methods

The molecular mass of the protein under denaturing conditions was estimated by SDS-PAGE on a 10% polyacrylamide gel according to the method of Laemmli[21] using molecular weight calibration standards kit LMW (14,400–94,000) from Pharmacia, Sweden. Isoelectric focusing was performed on Servalyt PRECOATES plates 3–10 (Serva Electrophoresis GmbH, Heidelberg, Germany). Protein concentrations were determined by the method of Lowry using BSA as a standard.[22] ^1H NMR spectra and ^{13}C NMR spectra were recorded with an AMX-500 Bruker spectrometer. Standard impulse sequence DEPT 135° from spectrometer library was used. Mass spectrometric analysis was performed with a Q-Tof™ 2 mass spectrometer (Waters Corporation, Micromass MS Technologies, Manchester, UK). Calibration of the TOF analyzer (single-reflectron mode, resolution >10,000 FWHM) was obtained over the *m/z* range 50–1000 using a solution of NaI (1.5 g/L) in 1:1 2-propanol–water. A scan time of 1.5 s with an interscan delay of 0.1 s was used in all MS modes. Solutions of substances under analysis (typical concentration 10–20 μmol in 1:1 MeCN–water containing 0.5 mM NaCl) were infused into the ion source at 2 μL/min (syringe pump). For accurate mass determinations in the TOF MS mode, centroid spectra were generated from continuum spectra using the $(M+Na)^+$ adduct of raffinose (α-Gal*p*-(1→6)-α-Glc*p*-(1↔2)-β-Fru*f*) as an internal standard (calcd mass 527.1588 u). The raffinose (raffinose pentahydrate, R-0250, Sigma–Aldrich, minimum purity 99%) concentration in each sample was adjusted empirically to give a peak intensity similar to that of the arabinitol analyte.

3.9. Purification of APDH

The coding sequence of APDH gene was amplified from *B. halodurans* (JCM 9153) genomic DNA using oligonucleotide primers oBHDH5 (5′-GAGGGAATTCATGAAAGCATTAGTAAAAACACAACATGGC-3′) and oBHDH3 (5′-CAAAGATCTAGAAGCTTCGTCCATACGGTCCTCCTTTTCCCTAAT-3′). The PCR product was digested with *Eco*RI and *Hin*dIII and ligated to *Eco*RI and *Hin*dIII digested vector pTAC. Construction of these vectors has been described earlier.[23] The resulting plasmid was designated pTAC(APDH). *E. coli* (pTAC(APDH)) strain was cultivated in Luria Bertani medium containing 100 mg/L ampicillin. The expression of APDH was induced by addition of IPTG (1 mM) when the cell density reached OD_{600} = 0.2. After induction, cultivation was continued overnight at 30 °C. The cells were collected by centrifugation and disrupted by sonication (by multiple 20 s pulses with intermediate cooling on ice bath) until essentially all cells were broken as judged by microscopic examination. Ammonium sulfate precipitate containing the APDH activity was dialyzed against 20 mM Tris HCl buffer, pH 7.8, supplemented with 3 mM DTT, applied to a DEAE 5PW (Pharmacia Biotech, Uppsala, Sweden) column (21.5 × 159 mm) equilibrated with the same buffer, and eluted with a linear gradient (0–1 M) of NaCl. Fractions with APDH activity were pooled, concentrated using an Amicon PM-30 membrane to 10 mL, and dialyzed against 20 mM Tris HCl buffer, pH 7.8, supplemented with 3 mM DTT. Obtained protein solution was chromatographed on a Mono Q HR (5/5) (Pharmacia) with a linear gradient (0–1 M) of NaCl in the same buffer. The APDH activity was applied on a Sepharose Blue CL6B (Pharmacia) column (10 × 50 mm) equilibrated with 50 mM Tris HCL buffer, pH 8.0, 100 mM NaCl, 3 mM DTT, and eluted with 3 mM NADH in the same buffer. Purified enzyme was stored in 50% glycerol–20 mM Tris HCl buffer solution at −20 °C. Aliquots of the enzyme were taken for all further investigations. All purification steps were carried out at 4 °C.

3.10. Enzymatic properties and kinetic parameters

The measurement of the enzyme activity in the reductive (reverse) reaction of Xul5P was performed according to Ref. 10 in 50 mM Tris HCl buffer, pH 7.0, 1 mM DTT, with 0.07 mM NADH, 1 mM Xul5P. One unit of the enzyme activity was defined as an amount catalyzing the oxidation of 1 μmol of NADH in 1 min under the conditions described. Enzyme activity in the oxidative (forward) direction was measured in 20 mM Tris HCl, pH 8.5, 1 mM DTT, and 0.5 mM NAD^+ using Ara-ol1-P or Ara-ol5-P as a substrate. To determine the pH optimum, purified APDH was incubated in the following 100 mM buffer solutions: pH 3.0|5.5—NaOAc buffer; pH 5.5|7.0—MES buffer; pH 7.0|9.0—Tris HCl buffer. Each buffer contained 1 mM DTT. The dehydrogenase activity was measured under the standard assay conditions. For pH stability assays, purified enzyme (0.04 U) was incubated for 18 h, at 4 °C in the same buffer solutions as described above. After incubation the dehydrogenase activity was measured for the reverse reaction under the standard conditions. The effect of temperature on activity was measured in oxidative and reductive reactions at a temperature range 20–65 °C and at optimal pH values for both reaction directions, respectively. To evaluate temperature stability of the APDH, the enzyme was incubated without a substrate at various temperatures in 20 mM Tris HCl, pH 7.2, 1 mM DTT. Aliquots of the reaction mixture were withdrawn at 5 min intervals with subsequent cooling to 20 °C, and the residual activity of the enzyme was measured in the reductive reaction under standard assay conditions. $NADP^+$ and NADPH were tested as co-factors in the oxidative and reductive reactions under standard assay conditions and at optimal pH values (7.2 and 8.5) found for NAD^+ and NADH as co-factors, respectively.

Double-reciprocal primary plots of velocity against Ara-ol1-P and NAD^+ were obtained by fitting the experimental data to equations developed for a ternary complex mechanism.[15]

$$v_i = \frac{V_{\max}}{1 + K_M^{\text{Ara-ol1-P}}/[NAD^+] + K_M^{NAD^+}/[\text{Ara-ol1-P}] + K_M^{\text{Ara-ol1-P},NAD^+}/([\text{Ara-ol1-P}][NAD^+])}, \quad (1)$$

where v_i is the initial velocity, $V_{\max}$ is the maximum reductive velocity, $K_M^{\text{Ara-ol1-P},NAD^+}$ is the ternary complex constant, $K_M^{\text{Ara-ol1-P}}$, and $K_M^{NAD^+}$ are the Michaelis–Menten constants for Ara-ol1-P and NAD^+ at saturating NAD^+ and Ara-ol1-P concentrations, respectively. The ternary complex constant, $K_M^{\text{Ara-ol1-P},NAD^+}$ was calculated from the abscissa of the primary-plot common intersection point at fixed [NAD^+] or at fixed [Ara-ol1-P] according to Eq. 2.[8]

$$K_M^{\text{Ara-ol1-P},NAD^+} = \frac{-K_M^{NAD^+}}{(\text{abscissa}_1)} = \frac{-K_M^{\text{Ara-ol1-P}}}{(\text{abscissa}_2)}. \quad (2)$$

All kinetic experiments were performed in 20 mM Tris HCl, pH 7.2, at 20 °C. The same procedures were applied for the reductive reaction using Xul5P as a substrate and NADH as a co-factor at pH 7.0 and 20 °C. $NADP^+$ and NADPH were tested in the same manner in the forward and reverse reactions using Xul5P, Ara-ol1-P, and Ara-ol5-P at corresponding pH optima (20 mM Tris HCl, pH 7.1 and 8.3).

Products of the oxidative and reductive reactions were analyzed after the incubation of APDH (0.1–0.01 U) with different substrates under standard reaction conditions. The reaction mixture was then treated with a phosphatase (Sigma Chemical Co., cat. No P4252)[10] and the resulted dephosphorylated polyols were analyzed on a carbohydrate analysis column (size 3.9 × 300 mm, Waters W0191H005).

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