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Enzymatic properties, renaturation and metabolic role of mannitol-1-phosphate dehydrogenase from *Escherichia coli*

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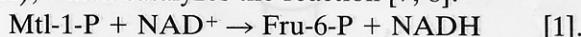
Summary — Enzymatic properties, renaturation and metabolic role of mannitol-1-phosphate dehydrogenase from *Escherichia coli*. D-mannitol-1-phosphate dehydrogenase was purified to homogeneity from *Escherichia coli*, and its physicochemical and enzymatic properties were investigated. The molecular weight of the polypeptide chain is 45 000 as determined by polyacrylamide gel electrophoresis in denaturing conditions. High performance size exclusion chromatography gives an apparent molecular weight of 47 000 for the native enzyme, showing that D-mannitol-1-phosphate dehydrogenase is a monomeric NAD-dependent dehydrogenase. D-mannitol-1-phosphate dehydrogenase is rapidly denatured by 6 M guanidine hydrochloride. Non-superimposable transition curves for the loss of activity and the changes in fluorescence suggest the existence of a partially folded inactive intermediate. The protein can be fully renatured after complete unfolding, and the regain of both native fluorescence and activity occurs rapidly within a few seconds at pH 7.5 and 20°C. Such a high rate of reactivation is unusual for a protein of this size. D-mannitol-1-phosphate dehydrogenase is specific for mannitol-1-phosphate (or fructose-6-phosphate) as a substrate and NAD⁺ (or NADH) as a cofactor. Zinc is not required for the activity. The affinity of D-mannitol-1-phosphate dehydrogenase for the reduced or oxidized form of its substrate or cofactor remains constant with pH. The affinity for NADH is 20-fold higher than for NAD⁺. The forward and reverse catalytic rate constants of the reaction: mannitol-1-phosphate + NAD⁺ ⇌ fructose-6-phosphate + NADH have different pH dependences. The oxidation of mannitol-1-phosphate has an optimum pH of 9.5, while the reduction of fructose-6-phosphate has its maximum rate at pH 7.0. At pH values around neutrality the maximum rate of reduction of fructose-6-phosphate is much higher than that of oxidation of mannitol-1-phosphate. The enzymatic properties of isolated D-mannitol-1-phosphate dehydrogenase are discussed in relation to the role of this enzyme in the intracellular metabolism.

mannitol-1-phosphate dehydrogenase / enzymatic activity / protein structure / sugar metabolism

Introduction

In *Escherichia coli*, the catabolism of hexoses and hexitols to pyruvate proceeds mainly through the direct glycolytic pathways via Fru-6-P and Fru-1,6-P₂, and to a lesser extent through 6-phosphogluconate (Entner-Doudoroff) and the pentosephosphate pathways [1, 2]. The flux of metabolites through the glycolytic pathway is probably controlled at the key step catalyzed by the enzyme phosphofructokinase, which shows a highly regulated allosteric behavior [3]. D-mannitol can be used as a unique carbon-energy source by *Escherichia coli* by entering glycolysis (fig 1). The presence of mannitol induces the biosynthesis of a specific phosphotransferase system for the entry of mannitol and its

phosphorylation into mannitol-1-P (Mtl-1-P) [4, 5, 6], and of an enzyme, Mtl-1-P dehydrogenase (Mtl-1P-DH), which catalyzes the reaction [7, 8]:



Two different enzymes have also been purified as Mtl-1-P dehydrogenase (Mtl-1P-DH): a monomeric protein of apparent molecular weight 40 000 [9], and a dimeric protein composed of 2 chains of apparent molecular weight 22 000 each [10].

This article reports the purification from *E. coli* K12 of a protein associated with the Mtl-1P-DH activity and describes some of its structural and functional properties. The possible metabolic role of this enzyme is also discussed.

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Abbreviations: Fru-6-P, D-fructose-6-phosphate; Fru-1,6-P₂, D-fructose-1,6-biphosphate; Mtl-1-P, D-mannitol-1-phosphate; Mtl-1P-DH, D-mannitol-1-phosphate dehydrogenase (EC 1.1.1.17); Glc-6-P, D-glucose-6-phosphate; Gu-HCl, guanidine hydrochloride; DTT, dithiothreitol; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; ACES, N-(2-acetamido)-2-aminoethanesulfonic acid; EDTA, ethylene-diamine-tetra-acetate

Materials and Methods

Materials

All chemical reagents used for buffers, activity measurements and Mtl-1P-DH purification were of analytical grade and obtained from Boehringer (Mannheim), Merck (Darmstadt) or Sigma (Saint Louis). Ultrapure guanidine hydrochloride (GU-HCl) was obtained from Schwarz-Mann (Orangeburg) and ultrapure urea from Sigma (Saint Louis). Compositions of liquid and solid media used were as described by Miller [13]. Thiamine and mannitol were added to the minimal medium YM9 to a final concentration of 1 µg/ml and 0.2% respectively. When necessary, liquid and solid media were supplemented with ampicillin (100 µg/ml).

Purification of Mtl-1P-DH

For purification of Mtl-1P-DH, *E. coli* MS37 (strain HE1, pro82, δ pfkB201, thi, δ (rha-pfkA)200, recA56, endA1, hsdR17, supE44, F': proA^{B+}, lacI^q, lacZ δ M15 [14], transformed with the pYOC280 plasmid, Ap^R, pfkAYOC280, constructed in our laboratory (to be published)), cells were grown in 2 \times TY medium, supplemented with ampicillin, for 20 h. Cells were then harvested by centrifugation at 9 000 g for 10 min at 4°C. The pellet was washed with 20 mM sodium phosphate buffer, containing 1 mM MgCl₂, 2 mM dithiothreitol (DTT), at pH 7.6 and centrifuged again. Cells were disrupted with alumina; broken cells were resuspended in T buffer composed of 50 mM Tris-HCl, 1 mM MgCl₂, 2 mM DTT, at pH 8.3 and centrifuged at 45 000 g for 45 min at 4°C. The crude extract was treated with DNase I and RNase A for 30 min at room temperature and then loaded onto an ion-exchange column (3 \times 32 cm) of TSK-DEAE (Merck, Darmstadt) equilibrated with T buffer. The column was first washed extensively with T buffer and then a gradient of NaCl from 0 to 0.5 M in T buffer was applied. The fractions containing the Mtl-1P-DH activity were pooled and dialysed against T

buffer. The enzyme was further purified by affinity chromatography using an Amicon Matrix Red-A column equilibrated with T buffer. Mtl-1P-DH was adsorbed in T buffer and eluted in a single step by 1 mM NADH in T buffer. Mtl-1P-DH was finally concentrated using an Amicon Diaflo cell (PM 10 membrane) and stored at 4°C. The homogeneity of the enzyme was verified by SDS-PAGE performed according to Laemmli [15].

Determination of protein concentration

The protein concentration was determined by the Bradford method [16] using immunoglobulins as a standard.

Determination of Mtl-1P-DH molecular weight

The molecular weight of the polypeptide chain was determined by SDS-PAGE, using phosphorylase b (MW: 94 000), albumin (MW: 67 000), ovalbumin (MW: 43 000), carbonic anhydrase (MW: 30 000) trypsin inhibitor (MW: 20 100) and α -lactalbumin (MW: 14 400) as standard proteins. Electrophoresis was carried out on slab gels containing 10% acrylamide in the presence of 0.2% SDS. Gels were stained with Coomassie blue type R and destained by a mixture of 7.5% acetic acid and 30% methanol (v/v).

The molecular weight of native Mtl-1P-DH was determined by HPLC gel filtration analysis, using a TSK G3000SW size exclusion column (7.5×300 mm), equilibrated with a 0.1 M sodium phosphate buffer containing 0.1 M NaCl at pH 6.8. Prior to use, the buffer was degassed and filtered through a Millipore 0.22 μm GSWP filter. The HPLC setting was composed of the following equipment: a LDB 2150 HPLC pump, a HPLC 2138 Uvicord S spectrophotometric detector, a Merck D 2000 Chromato-Integrator, and a Rheodyne 7125 injection valve equipped with a 20 μl sample loop. Ferritin (MW: 466 000), aldolase (MW: 140 000), phosphofructokinase (MW: 130 000), albumin (MW: 67 000) and ribonuclease (MW: 14 000) were used as standard proteins.

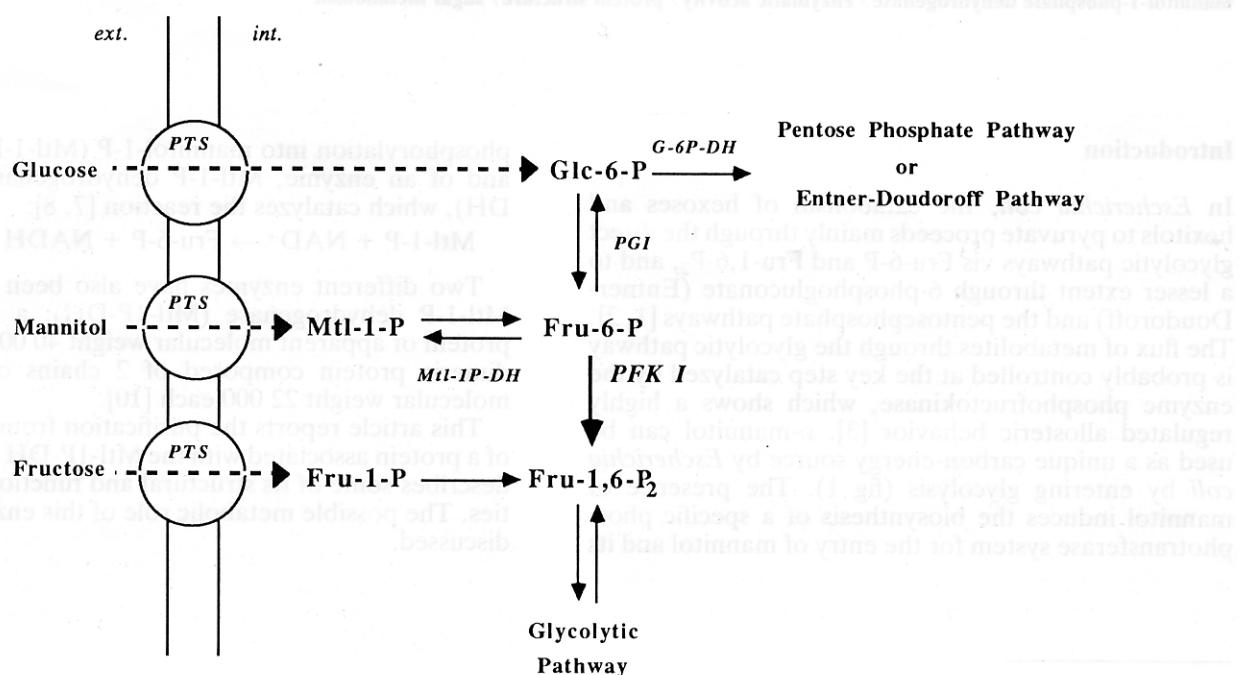


Fig 1. The metabolism of mannitol *E. coli*. Not all products are shown. Enzyme abbreviations: G-6P-DH: glucose-6-phosphate dehydrogenase, Mtl-1P-DH: mannitol-1-phosphate dehydrogenase, PFK I: phosphofructokinase I, PGI: phosphoglucone isomerase, PTS: phosphoenolpyruvate: carbohydrate phosphotransferase system.

Enzyme assay

Standard conditions for measuring Mtl-1P-DH activity made use of an assay mixture of 0.05 M Tris-HCl buffer, 1 mM NAD⁺ and 2 mM Mtl-1-P, at pH 9.0. The reaction was initiated by addition of the enzyme, and the change in absorbance at 340 nm was monitored at room temperature with a Perkin-Elmer Lambda 5 UV/VIS spectrophotometer. The pH dependence of Mtl-1P-DH activity and the values of the Michaelis constants for its substrates were determined using the system of 3 buffers described by Ellis and Morrison [17] which minimizes the changes in ionic strength with pH. This tribuffer is composed of 0.1 M ACES, 0.052 M Tris, and 0.052 M ethanolamine and can be used in the pH range from 5.5 to 10.5 by adjusting the pH with HCl or NaOH.

Denaturation and renaturation of Mtl-1P-DH

The transition curves for the denaturation of Mtl-1P-DH were measured in 0.1 M Tris-HCl buffer, 2 mM DTT, Gu-HCl at a given concentration, at pH 7.5 and 20°C. The residual activity remaining after 24 h was determined by transferring the protein into a standard assay mixture containing the same amount of Gu-HCl in order to avoid fast reactivation. Protein fluorescence was followed with a Spex Fluorolog 2 fluorometer equipped with a Spex DM1B Spectroscopy Laboratory Coordinator. Denaturation of Mtl-1P-DH was achieved by diluting the native enzyme with 8 M Gu-HCl in 0.1 M Tris-HCl, 2 mM DTT, at pH 7.5, so that the final Gu-HCl concentration was 6 M. Renaturation was carried out by diluting denatured Mtl-1P-DH at least 60-fold into 0.1 M Tris-HCl buffer, 2 mM DTT, at pH 7.5 and 20°C.

Automatic determination of N-terminal amino acid sequence

The N-terminal amino acid sequence of Mtl-1P-DH was determined using an Applied Biosystems 470A Protein Sequencer and an Applied Biosystems 120A Analyser coupled with a Spectra Physics SP4290 Integrator. The result was confirmed by specific sequencing of the single band obtained by SDS-PAGE, after electroblotting this band on an Immobilon membrane [18].

Results**Purification of Mtl-1P-DH**

Mtl-1P-DH has been purified to homogeneity by 2 successive chromatographic steps (table I). The crude extract is first fractionated by ion-exchange chromatography on a TSK-DEAE column, eluting with a salt-gradient (fig 2). All the Mtl-1P-DH activity present in the initial crude extract is found in a single peak (table I), and no activity can be detected outside this peak (fig 2), which suggests that this activity is borne by only one protein. The active fractions are then pooled and submitted to an affinity chromatography on an Amicon Matrex Red-A column, eluting with NADH. This two-step procedure yields homogeneous Mtl-1P-DH as shown by a unique band in SDS-PAGE (fig 3). This purification differs from the procedures described earlier [9, 10] and does not involve any step of protein precipitation by ammonium sulfate [10] and/or acid [9].

Molecular weight and subunit structure of Mtl-1P-DH

Figure 3 shows that the apparent molecular weight of Mtl-1P-DH in denaturing conditions is about 45 000.

This is much closer to the value of 40 000 obtained by Novotny *et al* [9] than to that of 22 000 reported by Chase [10]. The N-terminal amino acid sequence of Mtl-1P-DH was:

?-K-A-L-H-F-G-A-G-N-I-G-R-G-F-I-G-K-L-L-A-D-A-G-I-Q-L-T-F-A-D-V-N-Q-V-V-L-D-A-L-N-A-

The first 25 residues are identical to those reported by Novotny *et al* [9], confirming that the 2 proteins are very similar. There could however be a small difference in their SDS-PAGE mobilities: Novotny *et al* found that Mtl-1P-DH migrates slightly faster than ovalbumin, the marker protein of molecular weight 43 000 (see fig 1 of reference [9]), whereas we found that it migrates slightly more slowly than the same marker (fig 3). If real, such a difference would indicate that the protein described by Novotny *et al* [9] misses 30 to 50 residues on its C-terminal end, probably because of limited proteolysis. Limited proteolysis could also explain the molecular weight of 22 000 found by Chase [10].

The apparent molecular weight of Mtl-1P-DH in non-denaturing conditions was determined by size-exclusion chromatography on a calibrated HPLC column; a value of 47 000 was found (fig 4), in agreement with both earlier reports which gave 40 000 [9] or 45 000 [10]. The native form of Mtl-1P-DH thus corresponds to a monomeric species with a single chain; such a monomeric native state is rather unusual among the NAD-dependent dehydrogenases [19].

Fluorescence of Mtl-1P-DH

The intrinsic fluorescence of native Mtl-1P-DH is characterized by a maximum excitation wavelength of 280 nm and a maximum emission wavelength of 330 nm. The shape of the emission spectrum of the enzyme does not depend on the excitation wavelength, indicating that the fluorescence is mainly due to tryptophan residues [20]. Complete denaturation of Mtl-1P-DH by 6 M Gu-HCl results in a decrease of about 60% of the fluorescence intensity emitted at 330 nm (upon excitation at 280 nm), and in an increase of the maximum emission wavelength to 352 nm. These changes indicate that (part of) the tryptophan residues become exposed to the aqueous solvent upon unfolding of the protein [20]. The fluorescence emission spectrum of completely denatured Mtl-1P-DH is very similar to that of a mixture of free tyrosine and tryptophan in 6 M Gu-HCl, in a molar ratio of 3 Tyr to 4 Trp. This relative proportion of 3 Tyr to 4 Trp is the same as that reported by Chase [10].

Denaturation and renaturation of Mtl-1P-DH

The fluorescence and enzymatic activity of Mtl-1P-DH were measured after an incubation of 24 h in various

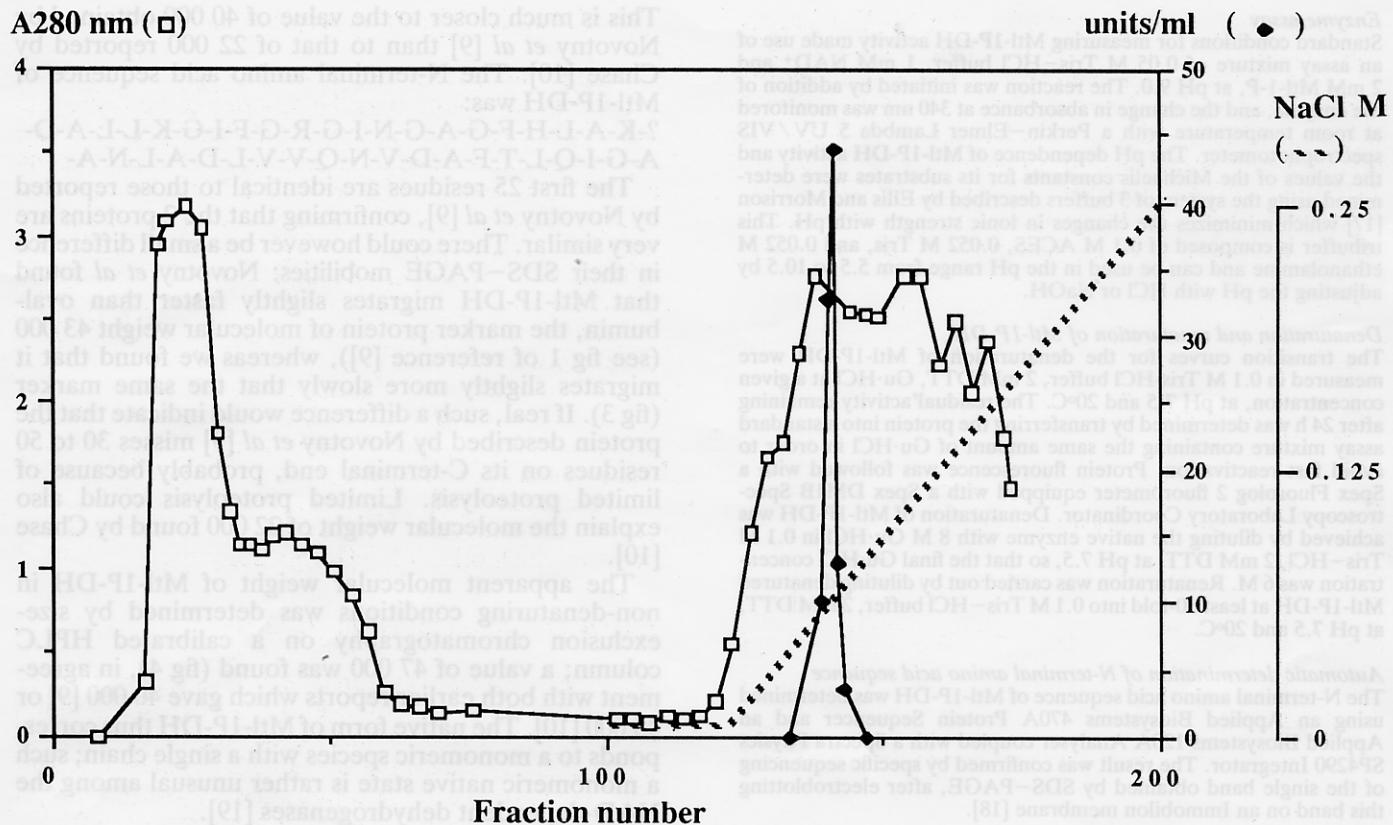


Fig. 2. Fractionation of Mtl-1P-DH by ion-exchange chromatography on a TSK-DEAE column. Elution is followed either by activity (◆) (expressed in arbitrary units) or by absorbance at 280 nm (□).



Table I. Purification of mannitol-1-phosphate dehydrogenase

Sample	Total activity ($\mu\text{mol}/\text{min}$)	Specific activity ($\mu\text{mol}/\text{min}$ per mg of protein)	Yield (%)
Crude extract	137	0.3	100
TSK-DEAE	138	100	100
Amicon-RedA	47	500	34

Fig. 3. SDS-PAGE of Mtl-1P-DH during its purification. Lane 1: crude extract. Lane 2: TSK-DEAE eluate. Lane 3: Amicon Red-A matrix eluate. Lane M: molecular weight markers: a: phosphorylase b, b: albumin, c: ovalbumin, d: carbonic anhydrase, e: trypsin inhibitor, f: α -lactalbumin. The molecular weights of these proteins are mentioned in Materials and Methods.

concentrations of Gu-HCl at 20°C (fig 5). The activity disappears in 1 step between 0 M and 0.6 M Gu-HCl, while the fluorescence seems to change in 2 steps: the first one takes place between 0 M and 0.6 M Gu-HCl together with inactivation and involves 80% of the amplitude, and the second occurs between 0.8 M and 1.2 M Gu-HCl and involves the remaining 20% of the amplitude (fig 5). The first step could correspond to a partial unfolding into an inactive species, while the

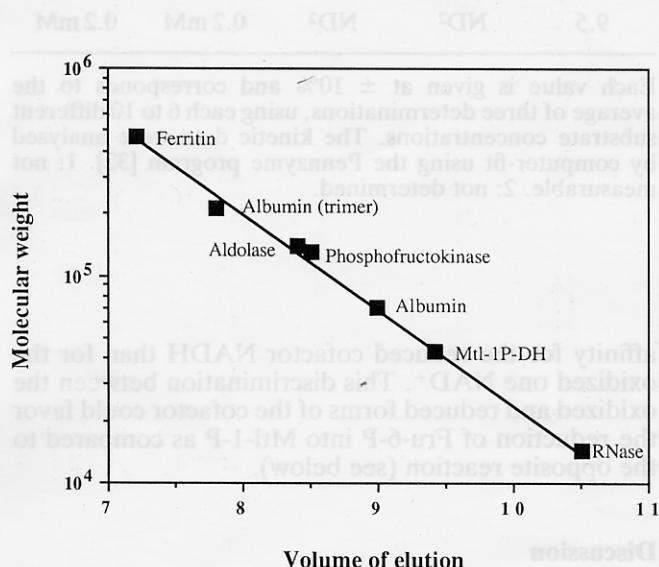


Fig 4. Determination of the apparent molecular weight of native Mtl-1P-DH by gel filtration on a HPLC TSK G3000 SW column. The molecular weights of the standard proteins are indicated in *Materials and Methods*.

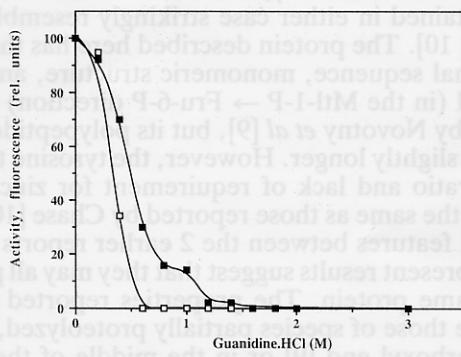


Fig 5. Guanidine-HCl dependent inactivation and denaturation of Mtl-1P-DH at 20°C. The enzyme at a concentration of 800 nM has been incubated for 24 h in 0.1 M Tris, 2 mM DTT, Gu-HCl at the indicated concentration, pH 7.5 (■): Fluorescence emitted at 352 nm upon excitation at 280 nm (the fluorescence of fully denatured enzyme is taken as zero). (□): Residual activity.

second step leads to the complete unfolded polypeptide chain.

When transferred into 6 M Gu-HCl, Mtl-1P-DH denatures so rapidly that the kinetics of both the loss of activity and of the fluorescence change are too fast to be measured by manual mixing. After completely unfolding by 6 M Gu-HCl, Mtl-1P-DH can be quantitatively renatured: all the native fluorescence and more than 90% of the enzymatic activity are recovered upon dilution into 0.1 M Tris-HCl, 2 mM DTT, less than 0.1 M residual Gu-HCl, at pH 7.5 and 20°C. This renaturation is also a fast process, whether monitored by fluorescence or activity, and is complete within the dead-time of manual mixing, 15–30 s.

The behavior of Mtl-1P-DH in urea solutions has also been investigated. The mid-point of the unfolding transition is about 1.8 M urea, for the changes in both fluorescence and activity. Refolding after urea-induced denaturation is fully reversible and is also a rapid process which takes place within seconds.

Enzymatic properties of Mtl-1P-DH

With NADH as a cofactor, Mtl-1P-DH seems to have a strict specificity for Fru-6-P as a substrate and shows no detectable activity with other sugars such as glucose-6-phosphate, fructose-1-phosphate, fructose-1,6-diphosphate, or arabinose-5-phosphate. Novotny *et al* [9] have found that, with NAD⁺ as a coenzyme, Mtl-1P-DH was active only with Mtl-1P-DH as a substrate and not with glucitol-6-phosphate.

We have found that the enzyme is unable to utilize NADPH as a cofactor with Fru-6-P as a substrate, and it has been reported that NADP⁺ cannot serve as a coenzyme with Mtl-1-P as a substrate [9]. Mtl-1P-DH seems to have a strict specificity for Mtl-1-P/Fru-6-P and NAD⁺/NADH. Under all conditions studied, the saturation of Mtl-1P-DH by its substrate or coenzyme follows a hyperbolic curve, as expected in view of its monomeric structure.

Zinc is apparently not required for the activity of Mtl-1P-DH as it is for other dehydrogenases [21]. Exhaustive dialysis against buffer containing 2 mM EDTA does not affect the activity of Mtl-1P-DH. Also, Mtl-1P-DH can be quantitatively reactivated after unfolding, independent of the presence of Zn⁺⁺ ions in the renaturation buffer. That no correlation exists between the activity of Mtl-1P-DH and its zinc content has also been noted by Chase [10].

The pH dependence of Mtl-1P-DH activity was studied in the presence of 1 mM NAD⁺ and 2 mM Mtl-1-P for the "direct" reaction from Mtl-1-P to Fru-6-P, and in the presence of 1 mM NADH and 2 mM Fru-6-P for the "reverse" one from Fru-6-P to Mtl-1-P. The measurements were made in a buffer which minimizes the variations in ionic strength and the results are

shown in figure 6. The rate of the forward reaction increases with pH according to the ionization of a group with a pK of 7.9 ± 0.2 , reaches a maximum value at pH 9.5, and decreases sharply as pH becomes more alkaline. The rate of the reverse shows a bell-shaped pH dependence, with an optimum as pH 7.0; at pH increases, the activity of Mtl-1P-DH first increases according to the ionization of a group with a pK of 5.2 ± 0.3 and then decreases following the ionization of a group with a pK of 8.8 ± 0.2 . The catalytic rate constants for the 2 reactions at their optimum pH are slightly different: k_{cat} for the forward reaction at pH 9.5 is 190 s^{-1} , and k_{cat} for the reverse reaction at pH 7.0 is 240 s^{-1} . The values of the Michaelis constants K_M relative to one substrate were measured at saturating concentration of the other substrate, in either direction, and at different pH values; the values are given in table II. The affinity of Mtl-1P-DH for the reduced or oxidized form of its substrate or cofactor remains constant with pH, and only the maximum velocity depends on the pH value.

Mtl-1P-DH does not have a strong preference for the oxidized or reduced state of its substrate, as seen from the very similar value of K_M for Mtl-1P and Fru-6-P (table II). The enzymes has however a 20-fold higher

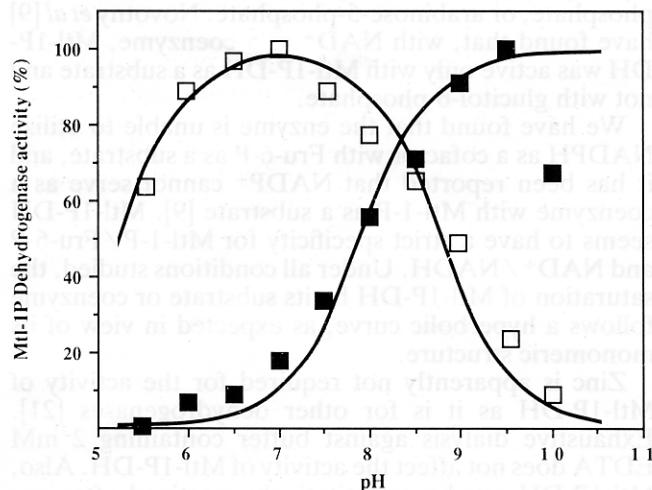


Fig 6. pH dependence of the activity of Mtl-1P-DH. Activity was measured as described in methods, in the presence of 1 mM NAD⁺ and 2 mM Mtl-1P-DH for the direct reaction (■), or 1 mM NADH and 2 mM F-6P for the reverse one (□). Activities are expressed relative to the maximal velocity of direct ($k_{cat} = 190 \text{ sec}^{-1}$) and reverse ($k_{cat} = 240 \text{ sec}^{-1}$) reactions. The solid lines correspond to theoretical titration curves of a unique ionizing group with a pK of 7.9 ± 0.2 and of 2 ionizing groups with pK 's of 5.2 ± 0.3 and 8.8 ± 0.2 . These pK values were determined by computer-fit using a modification of the Pennzyme program [32].

Table II. Values of K_M at different pH.

pH	Fru-6-P	NADH	Mtl-1P	NAD ⁺
5.4	0.5 mM	0.01 mM	NM ¹	NM ¹
6.9	0.2 mM	0.01 mM	0.2 mM	0.2 mM
8.9	0.3 mM	0.01 mM	0.2 mM	0.2 mM
9.5	ND ²	ND ²	0.2 mM	0.2 mM

Each value is given at $\pm 10\%$ and corresponds to the average of three determinations, using each 6 to 10 different substrate concentrations. The kinetic data were analyzed by computer-fit using the Pennzyme program [32]. 1: not measurable. 2: not determined.

affinity for the reduced cofactor NADH than for the oxidized one NAD⁺. This discrimination between the oxidized and reduced forms of the cofactor could favor the reduction of Fru-6-P into Mtl-1-P as compared to the opposite reaction (see below).

Discussion

Comparison with previously reported properties of Mtl-1P-DH

The two Mtl-1P-DH isolated earlier catalyze the same reaction; they have similar molecular weights when native (40 000 vs 45 000), and the amino acid compositions obtained in either case strikingly resemble each other [9, 10]. The protein described here has the same N-terminal sequence, monomeric structure, and optimum pH (in the Mtl-1-P \rightarrow Fru-6-P direction) as that isolated by Novotny *et al* [9], but its polypeptide chain could be slightly longer. However, the tyrosine to tryptophan ratio and lack of requirement for zinc found here are the same as those reported by Chase [10]. The common features between the 2 earlier reports [9, 10] and the present results suggest that they may all pertain to the same protein. The properties reported earlier would be those of species partially proteolyzed, either at the carboxyl end [9] or in the middle of the chain [10].

If indeed the protein described by Chase [10] is a proteolyzed derivative of Mtl-1P-DH, then it is possible to cleave the chain into only 2 fragments of equal size. These 2 fragments remain associated into an active species and could correspond to the NAD-binding and substrate-binding domains. We are now searching for

a specific cleavage in the middle of the Mtl-1P-DH chain, which will generate such complementary fragments, a result which has not yet been obtained for NAD-dependent dehydrogenases, to the best of our knowledge.

Structural and catalytic properties of Mtl-1P-DH

The conclusion drawn from this and earlier works [9, 10] is that there is a Mtl-1P-DH which has a polypeptide chain with a molecular weight of 45 000 and is active under a monomeric state. Mtl-1P-DH is thus another example of monomeric NAD-dependent dehydrogenase, beside octopine dehydrogenases from molluscs [22, 23]. The activity of this enzyme does not require zinc and is specific for the pairs (NAD⁺/Mtl-1-P) and (NADH/Fru-6-P). A mechanism similar to that of lactate dehydrogenase [21] could explain the pH dependences of both the "direct" and "reverse" reactions of Mtl-1P-DH. This mechanism involves an ionizable group acting as an acid-base catalyst which interacts with the hydroxyl/keto group of the substrate through a hydrogen-bond. Oxidation of Mtl-1-P would be more efficiently catalyzed by the basic form (X) of this group (through a -CH-OH...X-interaction), while reduction of Fru-6-P would be more efficiently catalyzed by the acidic form (XH) (through a -C=O...HX-interaction). Because of these different hydrogen bonds, the ionization of this group: X + H⁺ XH, would occur with a lower pK of 7.9 in the ternary complex between the enzyme, NAD⁺ and Mtl-1-P, and with a higher pK of 8.8 in the complex with NADH and Fru-6-P. The two pK's of 7.9 and 8.8 which control the "direct" and the "reverse" reactions could then be related to the same ionizable group of Mtl-1P-DH. No specific assignment can yet be proposed for this (or these) group(s) or for that with a pK of 5.2.

Renaturation of Mtl-1P-DH

Mtl-1P-DH can be fully renatured after complete unfolding. The reappearance of enzymatic activity or native fluorescence occurs within seconds and is too fast to measure by manual dilution. Such a rapid reactivation is quite unusual for a protein; the aldolase from *Staphylococcus aureus* is the only other example of such fast reactivation of a protein of this size (reference 24 and personal communication from RKE Rudolph). For most monomeric proteins, reactivation is limited by either cis-trans isomerization of X-Pro peptide bonds [24, 25, 26] or the pairing of already folded domains [24, 27, 28], and both take minutes or longer. It is thus surprising that Mtl-1P-DH renatures so rapidly with a chain of molecular weight 45 000 which has 16 prolines [9, 10] and is certainly folded in (at least) 2 domains.

Importance of Mtl-1P-DH for the in vivo metabolism of sugars

The importance of one particular enzyme in the overall metabolism depends on the actual conditions inside the cell. In *Escherichia coli*, the intracellular pH is maintained around a neutral value [29, 30], where the maximum velocity of Mtl-1P-DH is 10–20 times higher for the reduction of Fru-6-P than for the oxidation of Mtl-1-P (fig 6). The reduction of Fru-6-P will also be favored over the oxidation of Mtl-1-P by the 20-fold higher affinity of Mtl-1P-DH for NADH as compared to NAD⁺ (table II).

In *E. coli* cells growing on glucose, Mtl-1P-DH will compete with phosphofructokinase for the utilization of Fru-6-P, while it will compete with phosphoglucose isomerase during growth on lactate (fig 1). It is likely that these competitions between Mtl-1P-DH and other enzymes using Fru-6-P as substrate are responsible for the significant formation of Mtl-1-P observed during growth of *E. coli* on a variety of carbon sources [11, 12]. Mtl-1P-DH could produce enough Mtl-1-P and/or Mtl inside the cell to partially induce the mtl operon and thus maintain its activity at an appreciable level, although its biosynthesis has not been induced by added external mannitol.

During growth on mannitol, Fru-6-P is continuously utilized by the irreversible reaction catalyzed by phosphofructokinase (fig 1). Therefore, despite the unfavorable balance of Fru-6-P as compared to Mtl-1-P, there will be a steady-state flux through the step catalyzed by Mtl-1P-DH because of the permanent displacement towards Fru-6-P. In this case, the cell will adjust the rate of sugar breakdown according to its needs by exploiting the sophisticated regulatory properties of phosphofructokinase [31].

Acknowledgments

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