Mannitol-1-phosphate dehydrogenase of *Escherichia coli*

Chemical properties and binding of substrates

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Mannitol-1-phosphate dehydrogenase was purified to homogeneity, and some chemical and physical properties were examined. The isoelectric point is 4.19. Amino acid analysis and polyacrylamide-gel electrophoresis in presence of SDS indicate a subunit $M_r$ of about 22000, whereas gel filtration and electrophoresis of the native enzyme indicate an $M_r$ of 45000. Thus the enzyme is a dimer. Amino acid analysis showed cysteine, tyrosine, histidine and tryptophan to be present in low quantities, one, three, four and four residues per subunit respectively. The zinc content is not significant to activity. The enzyme is inactivated (> 99%) by reaction of 5,5'-dithiobis-(2-nitrobenzoate) with the single thiol group; the inactivation rate depends hyperbolically on reagent concentration, indicating non-covalent binding of the reagent before covalent modification. The pH-dependence indicated a pK_a greater than 10.5 for the thiol group. Coenzymes (NAD$^+$ and NADH) at saturating concentrations protect completely against reaction with 5,5'-dithiobis-(2-nitrobenzoate), and substrates (mannitol 1-phosphate, fructose 6-phosphate) protect strongly but not completely. These results suggest that the thiol group is near the catalytic site, and indicate that substrates as well as coenzymes bind to free enzyme. Dissociation constants were determined from these protective effects: 0.6 ± 0.1 $\mu$M for NADH, 0.2 ± 0.03 $\mu$M for NAD$^+$, 9 ± 3 $\mu$M for mannitol 1-phosphate, 0.06 ± 0.03 $\mu$M for fructose 6-phosphate. The binding order for reaction thus may be random for mannitol 1-phosphate oxidation, though ordered for fructose 6-phosphate reduction. Coenzyme and substrate binding in the E'NADH–mannitol 1-phosphate complex is weaker than in the binary complexes, though in the E'NADH$^+$–fructose 6-phosphate complex binding is stronger.

INTRODUCTION

Mannitol-1-phosphate dehydrogenase (d-mannitol 1-phosphate: NAD$^+$ oxidoreductase, EC 1.1.1.17) was first reported from *Escherichia coli* by Wolff & Kaplan (1956a,b), and has also been purified from *Aerobacter aerogenes* (Liss et al., 1962), *Bacillus subtilis* (Horwitz & Kaplan, 1964) and *Streptococcus mutans* (Brown & Banks, 1977), as well as *Aspergillus niger* (Niehaus & Kiser, 1981) and reported from other fungi (Ueng et al., 1976; Hult et al., 1980), where it participates in the mannitol cycle for NADPH regeneration. The enzyme catalyses the reversible oxidation of mannitol 1-phosphate to fructose 6-phosphate (mannitol being a symmetrical molecule, the primary phosphate is properly a 1-phosphate). The enzyme is distinct from sorbitol-6-phosphate dehydrogenase also found in enterobacteria (Wolff & Kaplan, 1956b; Liss et al., 1962). The *Aero. aerogenes* and *E. coli* enzymes are B-specific in hydride transfer (do Nascimento & Davies, 1975; Alizadeh et al., 1976).

Klungsøyr (1966) has purified the *E. coli* enzyme, which he found to comprise up to 2% of the soluble protein of the organism when grown on mannitol, and has studied (Klungsøyr, 1967) the inhibition of the reaction by adenosine nucleotides. He reported the enzyme to have an $M_r$ (determined by analytical ultracentrifugation) of only 25000. Other known NAD-linked dehydrogenases generally have subunit $M_r$ values in the range 35000–40000, though *Neurosopora* malate dehydrogenase has been reported to have subunits of $M_r$ 13500 (Munkres, 1965). Furthermore, they are almost always dimers or tetramers, and some observations have suggested that only one-half the bound substrate and coenzyme may be converted into products at once (Harada & Wolfe, 1968; Bernhard et al., 1970). If mannitol-1-phosphate dehydrogenase is a monomer of $M_r$ 25000, the essential features of nicotinamide nucleotide-linked oxidation and reduction could be studied without interference from site–site interactions; if it is a dimer of monomer $M_r$ 12500, it would be even more attractively simple for studies of the participation of individual residues in catalysis. A small bacterial enzyme is particularly attractive for study of the effects of biosynthetically incorporated amino acid analogues (Browne et al., 1970; Chase, 1972).

The present paper reports studies on the chemical properties of the enzyme, and on the binding of substrates to it, as measured by protection against reaction with DTNB. Preliminary versions of some aspects have been reported (Chase & Lucek, 1974).

MATERIALS AND METHODS

Materials

Bovine serum albumin (fraction V), $\beta$-lactoglobulin, Coomassie Blue G, (bovine) haemoglobin, NAD$^+$ (grade III), NADH, 2-amino-2-methylpropane-1,3-diol, piperazone hexahydrate, phenazine methosulphate, $p$-
Nitrotetrazolium Blue, αα′-bipyridyl, 1,10-phenanthroline monohydrate, protamine sulphate and sodium diethyl-dithiocarbamate were obtained from Sigma Chemical Co. Tris and DTNB were products of Calbiochem. (NH₄)₂SO₄ (special enzyme grade), cytochrome c and ovalbumin were purchased from Mann Research Laboratories (now Schwarz–Mann); 4-mer - (4,7-)phenanthroline was purchased from K & K Chemicals. Chymotrypsinogen, chymotrypsin, deoxyribonuclease and ribonuclease A were obtained from Worthington Biochemical Corp. Glycolyl-1-tryptophan and 3-(2-aminoethyl)indole hydrochloride were purchased from Nutritional Biochemical Co.; the latter was converted into the free base as described by Liu & Chang (1971). Ninhydrin and methanesulphonic acid [4 M solution containing 0.2% 3-(2-aminoethyl)indole, packaged under N₂] were obtained from Pierce Chemical Co. Sephadex G-75 and QAE-(quaternary aminomethyl)-Sephadex A-50 were products of Pharmacia Fine Chemicals, and microgranular DEAE-cellulose DE-52 was a Whatman product. Coomassie Blue R and Amido Black were obtained from Colab Laboratories. Other chemical were reagent grade. Distilled water was deionized by passage through a mixed-bed ion-exchange resin.

Mannitol 1-phosphate for routine assays was prepared from mannitol and POCl₃ as described by Klungsøy (1966, 1967); for assay, the barium salt was converted into the sodium salt with Na₂SO₄, BaSO₄ being removed by centrifugation. Mannitol 1-phosphate for binding studies was a product of Sigma Chemical Co. (prepared by reduction of mannose 6-phosphate).

**Growth of organism**

A tryptophan-requiring strain of E. coli (lacking tryptophan synthetase and tryptophanase; Browne et al., 1970) was used as source of the enzyme, since it was desired eventually to study the effects of incorporation of tryptophan analogues into the enzyme (Chase, 1972). This strain (AAC9) was generously supplied by Dr G. D. Hegeman. Cells were grown in a New Brunswick Scientific Co. fermenter at 35 °C on the medium of Mayers & Spizizen (1954), with substitution of mannitol (6 g/l) for glucose and with supplementation with ferric ammonium citrate (1.14 mg/l), CaCl₂,2H₂O (0.4 mg/l), l-tryptophan (20 mg/l) [or DL-tryptophan (40 mg/l)] and yeast extract (0.5 g/l). This strain required glycine, as well as tryptophan, for rapid growth. Cells were harvested with a Sharples continuous centrifuge and stored at −20 °C.

**Assay of enzyme activity**

The enzyme was assayed essentially as described by Klungsøy (1966). The assay mixture contained 2-amino-2-methylpropane-1,3-diol/HCl buffer, pH 9.0 (0.05 M), NAD⁺ (1.33 mM) and mannitol 1-phosphate (2.0 mM), and bovine serum albumin (0.1 mg/ml) for stability of the enzyme when it was very dilute. The reaction was initiated by addition of enzyme, and increase of A₅₅₀ was monitored in either a Gilford recording spectrophotometer (assay volume 1.0 ml) or a Calibrometer photometer with Heath recorder (assay volume 2.0 ml). Both instruments were thermostatically maintained at 30 °C. The unit is formation of 1 μmol of NADH/min under these conditions, assuming ε₅₆₀ = 6200 M⁻¹·cm⁻¹.

**Determination of protein**

Protein concentrations were determined by the methods of Lowry et al. (1951) or Bradford (1976), standardized with bovine serum albumin (concentration determined by weight after drying in a vacuum oven). For absolute molar concentrations either amino acid analysis or the method of Klungsøy (1969), based on Cu²⁺ binding to peptide bonds, was used.

**Enzyme purification**

Mannitol 1-phosphate dehydrogenase was purified by a modification of the procedure of Klungsøy (1966), necessitated by the lower specific activity and larger volume of crude extracts. Cells were broken by ultrasonic oscillation, and cell debris was removed by centrifugation to yield crude extract. Nucleic acids were removed with MnCl₂ (0.05 M), and the pH was adjusted to 4.25 to precipitate other proteins. After centrifugation, the supernatant solution was re-adjusted to pH 7, and the enzyme was precipitated with (NH₄)₂SO₄ (75% saturation) and further fractionated by extraction of the precipitate with 61% saturated and 49% saturated (NH₄)₂SO₄ solutions. Alternatively, other proteins were precipitated at 52.5% saturation in (NH₄)₂SO₄, and the enzyme was precipitated at 67.5% saturation. Precipitated enzyme was redissolved in a minimum volume of 0.03 M-imidazole/HCl buffer, pH 7.3, dialysed briefly to lower the ionic strength, and filtered upward through a column (5 cm x 89 cm) of Sephadex G-75 equilibrated with the same buffer. Active fractions were adsorbed directly on a column of QAE-Sephadex A-50, and the enzyme was eluted with a pH gradient (7.3–5.3) in 0.03 M-imidazole hydrochloride/0.04 M-piperazine dihydrochloride (adjusted to pH 3 with HCl and back to pH 5.3 and 7.3 with 0.08 M Na₂CO₃ to keep [Cl⁻] constant). In some purifications, the active fractions were diluted with an equal volume of water, the pH was adjusted to 7.3, and the enzyme was adsorbed on a small column of DEAE-cellulose DE-52 and eluted with an NH₄Cl gradient (0.03–0.20 M) in 0.03 M-imidazole/HCl buffer, pH 7.3. The enzyme is stable for months in solution at 4 °C, and for years as an (NH₄)₂SO₄ precipitate.

**Enzyme characterization**

Isoelectric focusing was carried out in an 110 ml apparatus according to the instructions of the manufacturer, LKB Produkter. Polyacrylamide-gel electrophoresis was carried out at 4 °C in the standard Davis (1964) system, with 7.5% polyacrylamide gels. Protein bands were located by staining with Coomassie Blue R [1% in 7% (v/v) acetic acid], Amido Black [1% in 15% (v/v) acetic acid] or Coomassie Blue G [0.04% in 3.5% (v/v) HClO₄]. Mannitol 1-phosphate dehydrogenase activity was located by incubating fixed gels in the standard assay mix (without bovine serum albumin) supplemented with 0.018% phenazine methosulphate and 0.08% p-Nitrotetrazolium Blue (Schachter et al., 1969) until satisfactory bands of formazan appeared (generally 2–3 min), then replacing the assay mixture with 7% acetic acid to fix the gels. Gel electrophoresis for determination of Ms (Hedrick & Smith, 1968) was carried out in 6%, 9% and 12% gels (acylamide/NN'-methylenebisacylamide ratio 30:0.8) in the Davis (1964) buffer system, with as standards β-lactoglobulin (Mₛ 17500), deoxyribonuclease (Mₛ 31000), ovalbumin (Mₛ 45000) and haemo-
Properties of mannnitol-1-phosphate dehydrogenase

globin ($M_r$ 64,500). Polyacrylamide-gel electrophoresis in the presence of SDS was carried out in the system of Weber & Osborn (1969), with as standards bovine serum albumin ($M_r$ 68,000), ovalbumin, chymotrypsinogen ($M_r$ 25,000) and chymotrypsin (reduced, $M_r$ 12,000; non-reduced, $M_r$ 25,000).

Determination of $M_r$ by gel filtration (Andrews, 1964) was carried out on a column (2.5 cm x 89 cm) of Sephadex G-75 equilibrated with 0.05 M-imidazole/HCl buffer, pH 7.3. Standard proteins used were cytochrome $c$ ($M_r$ 12,500), ribonuclease A ($M_r$ 13,700), $\beta$-lactoglobulin, chymotrypsinogen, ovalbumin and bovine serum albumin. The void volume was determined as the volume at which Blue Dextran or bovine serum albumin dimer was eluted.

Automatic amino acid analysis was carried out with a modified Phoenix P.I. model VG-6200B analyzer or a Durrum Instruments model D-500 analyzer. Protein samples were hydrolysed under vacuum at 110 °C after being flushed with $N_2$, in 6 M-HCl/3 M-toluene-p-sulphonic acid containing 0.20% 3-(2-aminoethyl)indole (Liu & Chang, 1971), in 4 M-methanesulphonic acid containing 0.20% 3-(2-aminoethyl)indole (Moore, 1975) or in Ba(OH)$_2$ (Pon et al., 1970). Performic acid oxidation was as described by Hirs (1967). Thiol groups were determined with DTNB (Ellman, 1959) (0.24 mM in 0.1 M-Tris/HCl buffer, pH 8.25), readings being taken against a blank cuvette containing the reagent. Tryptophan was also determined colorimetrically with p-dimethylaminobenzaldehyde (Spies & Chambers, 1949) and ninyhdrin (Gaitonde & Dovey, 1970), with glycyl-L-tryptophan as standard.

Zinc was determined by atomic absorption spectrometry. Glassware used in diluting the zinc standard and the sample had been stored in 50% (v/v) conc. HNO$_3$. In attempting to remove zinc from the enzyme, samples precipitated with (NH$_4$)$_2$SO$_4$ were diluted with 0.025 M-piperazine/HCl buffer, pH 5.1; the concentrated buffer and the dialysis flasks had been extracted with diphenylthiocarbazone in chloroform to remove zinc and with chloroform to remove residual diphenylthiocarbazone.

The rate of reaction of the enzyme with DTNB was typically determined by preparing a sample of the enzyme (usually 0.27 μM) in 0.05 M-Tris/HCl buffer, pH 8.55, total volume 0.108 or 0.109 ml. A 10 μl portion was removed with an Eppendorf micropipette for determination of zero-time activity. Then 1 μl or 2 μl of DTNB was added (to concentration 0.1, 0.2 or 2 mM, except when [DTNB] was being varied). The mixture was incubated at 30 °C, and at appropriate times after the addition of DTNB samples, usually 10 μl (a total of five to seven), were removed and added to assay mixture for determination of activity remaining. The rate of inactivation was determined by a linear least-squares treatment of ln $v$ or ln(%) of zero-time activity) versus time; rates of inactivation were then fitted to appropriate equations (see the Results section) for dependence on [DTNB] or concentration of competing ligand by non-linear least-squares procedures (Wilkinson, 1961; Fraser & Suzuki, 1973, modified for the equations in this paper by Dr. Peter C. Kahn of this department).

RESULTS
Purification of the enzyme

The progress of a purification is shown in Table 1. The highest specific activity obtained is comparable with that found by Klungsøyr (1966) (516 units/mg, after conversion from his unit). In polyacrylamide-gel electrophoresis, the major band of stained protein corresponded to the single band of activity detected by formazan precipitation; other minor bands, some apparently active by formazan precipitation, were sometimes seen. Material showing only one band of stained protein in gel electrophoresis was used for amino acid analysis and other chemical studies.

Isoelectric focusing in the pH range 3–6 separated the enzyme from minor contaminants; the pH of the three fractions of highest enzyme activity was 4.19, indicating the approximate isoelectric point. However, all amphotolyte and sucrose could not be removed, so that amino acid analysis on the combined fractions was not carried out. Recovery of activity was 56% of that applied; the enzyme is at the verge of inactivation at this pH, and indeed some precipitation during the run was generally observed. Attempted preparative gel electrophoresis also resulted in poor recovery (24%) of activity applied, perhaps owing to the high pH (9.4) of the running gel (Jovin, 1973).

Amino acid analysis

The results of amino acid analysis of purified mannitol-1-phosphate dehydrogenase, showing only one

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Activity (units/ml)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>174</td>
<td>26.23</td>
<td>4564</td>
<td>129.2</td>
<td>22480</td>
<td>4.93</td>
<td>100</td>
</tr>
<tr>
<td>Acid supernatant</td>
<td>172</td>
<td>22.20</td>
<td>3822</td>
<td>129.2</td>
<td>22200</td>
<td>5.82</td>
<td>99</td>
</tr>
<tr>
<td>75% –satn.-(NH$_4$)$_2$SO$_4$ precipitate</td>
<td>108</td>
<td>11.90</td>
<td>1285</td>
<td>121.5</td>
<td>13122</td>
<td>10.2</td>
<td>58</td>
</tr>
<tr>
<td>49% –satn.-(NH$_4$)$_2$SO$_4$, supernatant</td>
<td>101</td>
<td>4.74</td>
<td>479</td>
<td>120.0</td>
<td>12120</td>
<td>25.3</td>
<td>54</td>
</tr>
<tr>
<td>80% –satn.-(NH$_4$)$_2$SO$_4$, precipitate, dialysed</td>
<td>8.0</td>
<td>44.7</td>
<td>358</td>
<td>1263</td>
<td>10100</td>
<td>8.2</td>
<td>45</td>
</tr>
<tr>
<td>Sephadex G-75 eluate</td>
<td>35</td>
<td>1.6</td>
<td>56</td>
<td>248</td>
<td>8673</td>
<td>156</td>
<td>39</td>
</tr>
<tr>
<td>QAE-Sephadex eluate</td>
<td>14.4</td>
<td>0.67</td>
<td>9.7</td>
<td>338</td>
<td>4865</td>
<td>502</td>
<td>22</td>
</tr>
</tbody>
</table>

Table 1. Purification E. coli mannitol-1-phosphate dehydrogenase

For experimental details see the text.
Table 2. Amino acid analysis of \emph{E. coli} mannitol-1-phosphate dehydrogenase

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Found</th>
<th>Integral value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine*</td>
<td>1.25 ± 0.14</td>
<td>1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>(19)</td>
<td>19</td>
</tr>
<tr>
<td>Threonine</td>
<td>8.43 ± 0.57†</td>
<td>9</td>
</tr>
<tr>
<td>Serine</td>
<td>8.51 ± 0.81†</td>
<td>9</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>20.77 ± 1.44</td>
<td>21</td>
</tr>
<tr>
<td>Proline</td>
<td>8.76 ± 1.32</td>
<td>9</td>
</tr>
<tr>
<td>Glycine</td>
<td>16.93 ± 1.53</td>
<td>16</td>
</tr>
<tr>
<td>Alanine</td>
<td>22.35 ± 1.12</td>
<td>22</td>
</tr>
<tr>
<td>Valine</td>
<td>16.61 ± 1.00</td>
<td>17</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.85 ± 0.70</td>
<td>4</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>10.85 ± 0.38</td>
<td>11</td>
</tr>
<tr>
<td>Leucine</td>
<td>17.69 ± 1.20</td>
<td>18</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.52 ± 0.42</td>
<td>6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.11 ± 0.43</td>
<td>3</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.99 ± 0.82</td>
<td>4</td>
</tr>
<tr>
<td>Lysine</td>
<td>10.74 ± 0.38</td>
<td>11</td>
</tr>
<tr>
<td>Arginine†</td>
<td>8.85 ± 0.24‡</td>
<td>9</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>4.02 ± 0.45</td>
<td>4</td>
</tr>
<tr>
<td>By p-dimethylamino-benzaldehyde</td>
<td>2.62 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>By ninhydrin</td>
<td>3.35 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>

* As cysteic acid (not corrected for incomplete recovery; Hirs, 1967).
† Two peaks in some analyses (see the text). Includes determination as ornithine after Ba(OH)$_2$ hydrolysis.
‡ Extrapolated to zero time.

The most notable observation is that the enzyme contains only a single cysteine residue. Tyrosine and histidine contents are also low, facilitating investigations of the possible role of these residues in dehydrogenase catalysis.

Polyacrylamide-gel electrophoresis in the presence of SDS

A sample of purified mannitol-1-phosphate dehydrogenase was subjected to gel electrophoresis in the presence of 0.2% SDS. The mobility of the enzyme was unaffected by inclusion of 2-mercaptoethanol in the preincubation mixture, indicating that it does not contain chains held together by a disulfide bond (cf. chymotrypsin). The logarithm of the $M_r$ values of the standard proteins was plotted versus distance (cm) migrated; the location of the mannitol-1-phosphate dehydrogenase band on this line indicated a protomer $M_r$ of about 22,500, in reasonable agreement with the minimum $M_r$ suggested by amino acid analysis and the value (25000) reported by Klungsøyr (1966).

Determination of $M_r$ of the native protein

The first method used was gel-filtration chromatography (Andrews, 1964), on a calibrated Sephadex G-75 column equilibrated with 0.03 M-imidazole/HCl buffer, pH 7.3. The $M_r$ values found in three experiments were 48300, 45100 and 44300, clearly consistent with a dimer of monomer $M_r$ about 22,500, rather than a monomer. Essentially the same $M_r$ was found if the buffer was 0.05 M-Tris/HCl buffer, pH 7.3, containing 0.05 M-NaCl and 1 mM-2-mercaptoethanol, as used by Klungsøyr (1966) in ultracentrifugation, or 0.05 M-sodium acetate buffer, pH 5.3, containing 0.05 M-NaCl.

Similarly, gel electrophoresis of the enzyme, along with standard proteins, at several polyacrylamide concentrations (Hedrick & Smith, 1968) gave an $M_r$ value of 45500.

Zinc content

Initial zinc assays of purified mannitol-1-phosphate dehydrogenase found 1.14 Zn atoms/monomer, suggesting the presence of catalytically important zinc, as in yeast and liver alcohol dehydrogenases (Theorell \textit{et al.}, 1955; Drum \textit{et al.}, 1967). However, dialysis against phosphate buffers, which inactivates liver alcohol dehydrogenase by removal of zinc at pH 5.0 (Drum \textit{et al.}, 1967), did not inactivate mannitol-1-phosphate dehydrogenase significantly at pH values above 4.2, nor was zinc consistently removed even when the enzyme was inactivated. Incubation in 0.1 M-diethylidithiocarbamate, pH 7.0, which inactivates liver alcohol dehydrogenase completely in 24 h (Drum \textit{et al.}, 1967), did not inactivate the enzyme, by 95% in 100 h, but the inactivation was prevented by the presence of 0.14 M-2-mercaptoethanol. This suggests that diethylidithiocarbamate reacts with an enzyme thiol group rather than with Zn$^{2+}$, 2-mercaptoethanol preventing inactivation by reducing any mixed disulfide formed with the reagent, α,β-Bipyridyl and 1,10-phenanthroline, which bind tightly to the catalytic zinc of liver alcohol dehydrogenase ($K_i$ values 0.4 mM and 0.008 mM; Sigman, 1967), are only very weak inhibitors of mannitol-1-phosphate dehydrogenase [$K_i$ values, by the method of Dixon (1953), 11 ± 4 mM and 8 ± 4 mM respectively]. The non-chelating isomer 4,7-phenanthroline was almost equally inhibitory ($K_i$ 12 ± 6 mM). Finally, zinc determinations on other
Fig. 1. Reaction of E. coli mannitol-1-phosphate dehydrogenase with DTNB

Enzyme (0.473 mg, by amino acid analysis) was incubated at 25 °C in 1.0 ml of 0.1 m-Tris/HCl buffer, pH 8.25. After addition of DTNB (0.24 mM), A_{412} was monitored, and at the indicated times samples (1 µl) were removed and assayed for activity. A control incubation without DTNB showed no loss of activity over this period of time. ●, Activity remaining at time t, as percentage of initial activity; ○, the comparable value for A_{412}, 100 × [1 - (A_{412} at time t/ final A_{412})]; the 'final A_{412}' is a theoretical value chosen to linearize the plot over the first 17 min (92% of inactivation), since actual A_{412} decreased after that time. □, Percentage activity remaining after time t, under the same conditions (0.1 ml of incubation mixture) + 5.0 mM-NAD+; △, percentage activity remaining after time t, under the same conditions (0.1 ml of incubation mixture) + 1.0 mM-mannitol 1-phosphate.

Fig. 2. Dependence on [DTNB] of rate of inactivation of E. coli mannitol-1-phosphate dehydrogenase

The reaction as carried out in 0.05 m-Tris/HCl buffer, pH 8.6, as described in the text, at various values of [DTNB].

81% of the subunit concentration indicated by protein determination, showing that the two active sites react independently with DTNB. A similar result was obtained with the less bulky 2-nitro-5-thiocyanatobenzoate (Degani & Patchornik, 1974). If only DTNB-reactive subunits are enzymically active, their catalytic-centre activity is 265 s⁻¹, or the specific activity is 702 µmol/min per mg.

The rate of inactivation depends hyperbolically on the DTNB concentration (Fig. 2), indicating that a non-covalent E:DTNB complex is formed before the covalent modification reaction. The observed rates were fitted to a version of the Michaelis–Menten equation:

\[
b = \frac{k[DTNB]}{K_d + [DTNB]}
\]

where b is the observed rate of inactivation, k is the limiting rate of inactivation at this pH, and K_d is the dissociation constant of the E:DTNB non-covalent complex. The fit gave \( k = 0.723 \pm 0.034 \text{ min}^{-1} \) and \( K_d = 0.0551 \pm 0.00765 \text{ mM} \).

The variation of the rate of inactivation with pH was studied over the range 7–11 (Fig. 3). The logarithm of the rate of inactivation increased linearly with pH up to about pH 10, then levelled off. The data from pH 8.75 to 11.0 were fitted to the equation:

\[
b = \frac{k[DTNB]}{K_d(1 + [L]/K_L) + [DTNB]}
\]

(the hyperbolic form of eqn. (7) of Chase & Shaw (1969)); [L] is the concentration of some protecting ligand, and \( K_L \) is its dissociation constant from the enzyme. In this case L is \( H^+ \), \( K_d = 0.0551 \text{ mM} \), and \( K_L \) is the dissociation constant of the reacting thiol group. In two experiments values of \( K_L \) found were 27.1 ± 13.5 pm and 13.1 ± 4.5 pm (pK_L = 10.57 and 10.88); but, considering the limited data in this pH region, it can only be asserted that the pK_L is greater than pH 10.5. The constancy of \( K_d \) in the pH range 7–10.5 is indicated by the fit of the data to a single straight line (Fig. 3); a change in \( K_d \), at a non-saturating [DTNB], would shift the line up or down as the rate changed with degree of saturation.

Preparations of mannitol-1-phosphate dehydrogenase gave values well below 1 Zn atom/subunit; typically 0.20–0.25 Zn atom/subunit, and as low as 0.10 Zn atom/subunit.

Reaction of the thiol group with DTNB

This reagent reacts relatively slowly with mannitol-1-phosphate dehydrogenase at pH 8.25; the rates of appearance of A_{412} (formation of 2-nitro-5-mercaptobenzoate dianion) and disappearance of enzymic activity could readily be monitored (Fig. 1), and are essentially identical (0.231 ± 0.006 min⁻¹ and 0.230 ± 0.005 min⁻¹ respectively). In another experiment, with enzyme reduced with 1,4-dithiothreitol under N₂ and filtered through Sephadex G-25 to remove the reductant, the molarity of 2-nitro-5-mercaptobenzoate produced was...
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The scheme following in enzyme independently, to free enzyme and protect the thiol group. NADH and fructose 6-phosphate act similarly.

where TNB represents 2-nitro-5-mercaptobenzoate. Reaction may occur either from the binary complex $E\cdot DTNB$, at rate $k$, or from the ternary complex $E\cdot L\cdot DTNB$, at rate $k'$. The observed rate of reaction $b$ is a function of $[DTNB]$, $[L]$, $k$, $k'$ and the dissociation constants; $K_dK'_L = K'_dK_L$, so that only three of the dissociation constants need be determined independently.

The observed rates of reaction (b) at various concentrations of the substrates were initially fitted to the equation:

$$b = \frac{k'[L][DTNB]}{K_d + [L]}$$

(3)

which is the equation for direct reaction of DTNB (at a fixed concentration) with the enzyme without a non-covalent intermediate $E\cdot DTNB$ complex. For $L = NAD^+$, NADH (and $H^+$) this gave values of $k' \leq 0$, indicating that the $E\cdot L\cdot DTNB$ complex is not formed or does not react. The appropriate equation is then eqn. (2), and fits of the data for $L = NAD^+$ gave values of $K_L = 0.18 \pm 0.03$ mM with two different enzyme preparations. Two experiments at enzyme concentrations 0.27 $\mu$M and 0.029 $\mu$M gave $K_L$ for NADH = 0.605 $\pm$ 0.12 $\mu$M and 0.60 $\pm$ 0.06 $\mu$M respectively. This indicates that the enzyme does not, as has been suggested for bovine heart cytoplasmic malate dehydrogenase (Koren & Hammes, 1975), dissociate with a change of $K_L$ in this concentration range.

Fits of the data with mannitol 1-phosphate and fructose 6-phosphate to eqn. (3) indicated appreciable values of $k'$, i.e. these ligands do not completely protect against inactivation by DTNB even at saturating concentrations (see Fig. 4). The proper equation for the above scheme with $k' > 0$ is:

$$b = \frac{kK'_L[DTNB]}{K_dK'_L + K'_d[L] + K_L[DTNB] + (K'_d/K_d)[L][DTNB] + K_d'K'_L + K'_d[L] + K_L(K'_d/K_d)[DTNB] + [L][DTNB] + [L][DTNB]}$$

(4)

or the equivalent:


(5)
Table 3. Substrate dissociation constants from binary and abortive ternary complexes

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Binding to</th>
<th>Eqn. no.</th>
<th>[DTNB] (mm)</th>
<th>$K_f$ found (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTNB</td>
<td>E</td>
<td>(1)</td>
<td>0.01–0.5</td>
<td>55.1 ± 7.65</td>
</tr>
<tr>
<td></td>
<td>E:Fru-6-P</td>
<td>(1), (4)</td>
<td>0.01–2.0</td>
<td>876 ± 113*</td>
</tr>
<tr>
<td></td>
<td>E:Man-1-P</td>
<td>(1), (4)</td>
<td>0.05–4.0</td>
<td>805 ± 111†</td>
</tr>
<tr>
<td>NAD+</td>
<td>E</td>
<td>(2)</td>
<td>0.1</td>
<td>184 ± 36</td>
</tr>
<tr>
<td></td>
<td>E:Fru-6-P</td>
<td>(2)</td>
<td>0.1</td>
<td>177 ± 23</td>
</tr>
<tr>
<td></td>
<td>E:Man-1-P</td>
<td>(2)</td>
<td>2.0</td>
<td>15.98 ± 2.27†</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>E</td>
<td>(4)</td>
<td>0.1</td>
<td>598 ± 33</td>
</tr>
<tr>
<td></td>
<td>E:NAD+</td>
<td></td>
<td></td>
<td>53.8 ± 9.3</td>
</tr>
<tr>
<td>Mannitol 1-phosphate</td>
<td>E</td>
<td>(4)</td>
<td>0.1</td>
<td>9.86 ± 1.11</td>
</tr>
<tr>
<td></td>
<td>E:NADH</td>
<td></td>
<td>0.2</td>
<td>8.15 ± 4.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>267 ± 68</td>
</tr>
</tbody>
</table>

* Determined at 30 mM-fructose 6-phosphate, corrected to infinite [fructose 6-phosphate].
† Determined at 15 mM-mannitol 1-phosphate, corrected to infinite [mannitol 1-phosphate].
‡ Determined at 0.028 μM-enzyme.
§ Determined at 0.269 μM-enzyme.
|| Calculated from the other dissociation constants (see the text).

To obtain $K_f$, estimates were first obtained by fitting to eqn. (3). Values of $K_a$ were then obtained by varying [DTNB] at very high concentrations of fructose 6-phosphate and mannitol 1-phosphate. These values were then used as fixed parameters, along with [DTNB], $K_a$ and $k$ (obtained from determinations of $b$ at [L] = 0 and eqn. 1), to fit values of $b$ at various values of [L] to eqn. (4). Finally, the values of $K_{a2}'$ were adjusted slightly by using the obtained values of $k'$ and $K_f$ as fixed parameters in eqn. (4). The results are shown in Table 3. The ratios $k'/k$, representing the degree of protection by substrate, were 0.44 ± 0.044 for fructose 6-phosphate, and 0.50 ± 0.09 and 0.51 ± 0.16 for two determinations with mannitol 1-phosphate.

Since protection is not complete at saturating concentrations of the sugar phosphate ligand, the dissociation constants of coenzymes from the abortive ternary complexes enzyme•NAD+•fructose 6-phosphate and enzyme•NADH•mannitol 1-phosphate can be determined similarly, by varying [coenzyme] at high sugar phosphate concentration (with DTNB this was performed at two concentrations of DTNB) and fitting to eqn. (2). The results are shown in Table 3. The dissociation constants for sugar phosphates from these complexes ($K_{a2}'$) can then be calculated from the dissociation constant for the coenzyme ($K_{a2}'$) and the dissociation constants of the binary complexes ($K_a$ and $K_c$), since $K_{a2}'/K_c = K_aK_{c2}'$.

**DISCUSSION**

The present results differ from those reported by Klungsøy (1966) in two respects. The level of activity in crude extracts, 2.5–4 units/mg of protein, is one-fifth to one-third that observed by Klungsøy (1966). Other strains of E. coli surveyed in this laboratory (P. Krevetski & T. Chase, Jr., unpublished work), including A.T.C.C. 8739 as used by Klungsøy (1966), had activity levels comparable with strain A6C9 used in this study, as did E. coli B surveyed by Liss et al. (1962) and strains surveyed by Solomon & Lin (1972).

More importantly, two methods dependent on the hydrodynamic radius of the native protein molecule indicated an $M_r$ of about 45000, in contrast with Klungsøy’s (1966) value of 25000. Liss et al. (1962) reported an $M_r$ of 40000 for the *Aero. aerogenes* enzyme, Brown & Banks (1977) reported an $M_r$ of 45000 for the *S. mutans* enzyme, and Niehaus & Kiser (1981) reported an $M_r$ of 40000 for the *Asp. niger* enzyme. Gel electrophoresis in the presence of SDS indicated an $M_r$ of 22500, suggesting that the native enzyme is a dimer. No reason for the discrepancy is readily apparent. Strain difference is not responsible, since the enzyme from A.T.C.C. 8739 also emerged from a Sephadex G-75 column at a position indicating an $M_r$ of 45000 (P. Krevetski & T. Chase, Jr., unpublished work). Presence of a large amount of non-amino-acid material attached to the peptide chain, with resultant increase in both hydrodynamic radius and partial specific volume, could reconcile the results from ultracentrifugation, Sephadex chromatography and gel electrophoresis of the native enzyme, but not the behaviour in gel electrophoresis in the presence of SDS. Monomeric dehydrogenases do exist [e.g. dihydrofolate reductase (Gundersen et al., 1972; Nagelschmidt & Jænicke, 1972), octopine dehydrogenase (Olomucki et al., 1972) and aldehyde reductase (Turner & Tipton, 1972)], but are not common, and typically use NADP+ as coenzyme rather than NAD+.

Although not as simple as was hoped, mannitol-1-phosphate dehydrogenase of *E. coli* remains a useful example for investigation of the mechanism of action of nicotinamide nucleotide-dependent dehydrogenases. The
low content of cysteine, tyrosine, histidine and tryptophan (one, three, four and four residues/subunit respectively) should facilitate examination of their possible roles in catalysis and conformational change, either by chemical modification or by biosynthetic incorporation of \( ^{13} \)C-labelled amino acids or fluorinated analogues for n.m.r. studies (Browne et al., 1973; Hull & Sykes, 1974).

Although totally zinc-free preparations of the enzyme have not been obtained, no experiment indicated zinc to be an essential component of it. It is difficult to rule out the presence of zinc-less inactive enzyme in even a highly purified preparation, but a zinc content of 0.10 mol/mol of subunit, for a preparation of specific activity 284 units/mg (55% of the highest observed), seems too low for a specific role. No combination of chelation and dialysis completely removed zinc from an enzyme preparation, and only prolonged incubation in diethyl-dithiocarbamate destroyed activity; this was prevented by the presence of 2-mercaptoethanol, suggesting a thiol reaction to be responsible for inactivation. Weak inhibition by 1,10-phenanthroline and \( \alpha\alpha' \)-bipyridyl was matched by non-chelating 4,7-phenanthroline, and probably is the result of non-specific binding at the coenzyme-binding site. It is concluded that the residual zinc content is without significance, and results from non-specific tight binding.

The inactivation of the enzyme by DTNB, and the protection against inactivation by substrates as well as coenzymes, suggests that the thiol group lies very near the active site, although a chemical role in catalysis is not thereby suggested. Either the protection by coenzymes or (more probably) that by substrates could result from an enzyme conformational change rather than direct steric interference, but it is unlikely that both protect merely by a conformational change. We have reported elsewhere (Cerione & Chase, 1983) evidence for different conformational changes caused by binding of NAD\(^+\) and NADH to the enzyme, and yet other, smaller, changes on binding of mannitol 1-phosphate and fructose 6-phosphate to pyridoxal phosphate-modified enzyme. However, DTNB might react with and stabilize a different, inactive, conformation of the enzyme whose formation is prevented by binding of either substrates or coenzymes. It is possible that a thiol group near the catalytic site is common in dehydrogenases; in one case the chemical non-essentiality of the thiol group was demonstrated by re-activation of DTNB-inactivated enzyme (isocitrate dehydrogenase from Azotobacter) by conversion into the thiocyanato-enzyme with CN\(^-\) (Chung et al., 1970). In the present case the location of the thiol group, and potentially of other reactive residues such as histidine, may be further mapped by observing the protective effect of substrate and coenzyme analogues.

The sugar phosphate substrates protect principally by increasing the \( K_{DL} \) of DTNB about 15-fold (Table 3). The rate of reaction at saturating [DTNB] is decreased only about 2-fold, suggesting DTNB to be slightly less well placed for reaction in the complex. Complete protection by coenzymes suggests that DTNB binds in the coenzyme site, possibly the nicotinamide portion (near the substrate site) rather than the adenine portion.

The slowness of the reaction with DTNB is due to the unusually high p\( K_a \) of the cysteine residue, which could result from proximity of a negatively charged group. Dividing the limiting rate of reaction at saturating [DTNB] and complete dissociation of the thiol group, 65 min\(^{-1} \) = 1.1 s\(^{-1} \), by the \( K_{DL} \), 55 \( \mu \)M (which is equivalent to substituting \([E][DTNB]\) for \([E\cdot DTNB]\) in the first-order rate equation, observed rate = \( k[E\cdot DTNB] \)) yields an apparent second-order rate constant 20000 s\(^{-1} \).M\(^{-1} \), in good agreement with the second-order rate constant for the reaction of DTNB with GSH, 21700 s\(^{-1} \).M\(^{-1} \) at complete dissociation (149 s\(^{-1} \).M\(^{-1} \) at pH 6.5; Degani & Patchornik, 1974). Thus the reaction rate does not appear to be affected by any factors other than those causing the high p\( K_a \) of the cysteine residue.

Protection against DTNB inactivation by substrates as well as coenzymes indicates that, unlike the substrates of lactate dehydrogenase, malate dehydrogenase and alcohol dehydrogenase, they bind to the enzyme in the absence of coenzyme. The slightly tighter binding of mannitol 1-phosphate, compared with NAD\(^+\), supports the earlier suggestion (Chase & Lucek, 1974) that the binding order may be random for this direction. A random, but not rapid-equilibrium, mechanism could explain the concave-downward plots of 1/\( v \) versus 1/[mannitol 1-phosphate] that have been observed (Klingsøyr, 1967). However, the dramatic increase of \( K_l \) for NADH in the presence of mannitol 1-phosphate supports Klingsøyr's (1967) suggestion that this apparent substrate activation results from more rapid dissociation of NADH from the abortive enzyme-\( NADH+\)mannitol 1-phosphate complex than from the normal enzyme-\( NADH \) complex, though such a kinetic argument cannot be proven by determination of a dissociation constant, the ratio of 'off' and 'on' rate constants. Dissociation of NADH might also be favoured in the enzyme-\( NADH+\)fructose 6-phosphate complex, as compared with enzyme-\( NADH \); but this cannot be investigated by the present technique.

The higher \( K_l \) for fructose 6-phosphate (as compared with that for mannitol 1-phosphate) is expected, since at most 4.1% is in the open chain (keto) form that presumably is the substrate (Midelfort et al., 1976). The true \( K_l \) for this form is thus about 24 \( \mu \)M, similar to that for mannitol 1-phosphate. However, the very much lower \( K_l \) for NADH, as well as the high practical \( K_l \) for fructose 6-phosphate, ensures that the reaction from this side will be essentially ordered (this does not necessarily hold for release of nascent fructose 6-phosphate product, since it is already in the open-chain form). The abortive ternary complex resulting from the mutual tightening of NAD\(^+\) and fructose 6-phosphate binding presumably is responsible for the substrate inhibition seen at high fructose 6-phosphate concentrations (Klingsøyr, 1966). The different effects on dissociation constants in the ternary complexes, namely increased in enzyme-\( NADH+\)mannitol 1-phosphate but decreased in enzyme-\( NADH+\)fructose 6-phosphate, presumably result from different conformational changes caused by coenzyme binding, rather than by sugar phosphate binding (since the two sugar phosphates have virtually the same effects on DTNB binding). Such different conformational changes have also been observed by the different effects of the two coenzymes on protein fluorescence (Cerione & Chase, 1983), though small differences in enzyme-mannitol 1-phosphate and enzyme-fructose 6-phosphate complexes are also visible in the fluorescence spectra of pyridoxal phosphate-modified enzyme. Mannitol-1-phosphate dehydrogenase remains a useful case for the study of the role of coenzyme- and
substitute-directed conformational changes in dehydro-
genase catalysis.

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