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THE BINDING OF ADENINE NUCLEOTIDES TO THE MANNITOL-1-PHOSPHATE DEHYDROGENASE OF *ESCHERICHIA COLI*

LEIV KLUNGSÖYR

Department of Biochemistry, University of Bergen, Bergen (Norway)

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SUMMARY

The binding of adenine nucleotides to mannitol-1-phosphate dehydrogenase (D-mannitol-1-phosphate:NAD^+ oxidoreductase EC 1.1.1.17) from *Escherichia coli* was studied with kinetic methods.

1. When the enzyme catalyzes the reaction between Fru-6-*P* and NADH, in experiments with varying [NADH], the K_i of ATP was approx. 0.06 mM, and the K_i of AMP was approx. 0.8 mM. ATP and AMP were apparently bound to the same site on the enzyme.

2. When the enzyme catalyzes the reaction between mannitol-1-*P* and NAD^+, in experiments with varying [NAD^+], the K_i of AMP was independent of the mannitol-1-*P* concentration and nearly the same as in the other direction of the reaction, *i.e.* 1 mM. The K_i for ATP was 1.5 mM at high concentrations of mannitol-1-*P*, but decreased with decreasing mannitol-1-*P* concentrations.

3. At high concentrations of substrate, Fru-6-*P* inhibited the reaction between itself and NADH, while mannitol-1-*P* stimulated the reaction between itself and NAD^+.

4. At 62° the dissociation constants of the enzyme–ATP and enzyme–NADH complexes seem to be similar, and near 0.03 mM.

5. It is suggested that the enzyme–mannitol-1-*P* binary complex has low affinity for ATP, NAD^+ and NADH, compared with the affinity of the free enzyme or the enzyme–Fru-6-*P* binary complex for these substances. The binding of AMP is not influenced by the binding of Fru-6-*P* or mannitol-1-*P*.

INTRODUCTION

In a previous paper[1] the purification of mannitol-1-phosphate dehydrogenase (EC 1.1.1.17) from mannitol-grown *Escherichia coli* was described. The enzyme seems to be a globular protein with a mol. wt. of only about 25 000. Adenosine phosphates inhibited the enzyme, and in the present work the studies of this inhibition have been extended. The results of these kinetic experiments are discussed, and hypotheses

concerning the mechanism of action of mannitol-1-phosphate dehydrogenase and its function in the carbohydrate metabolism of *E. coli* are formulated.

A preliminary report of this work was given at the 4th Meeting of the Federation of European Biochem. Soc. in Oslo, 1967.

MATERIALS AND METHODS

The enzyme was prepared as described previously[1], except that mercaptoethanol was omitted from reagents and solutions.

The method for preparation of mannitol-1-*P* (ref. 1) has been slightly modified. It was found essential to stir well during the addition of phosphoryl chloride, and to add this reagent slowly. The pyridine is removed in a rotary evaporator shortly after completion of the reaction, and dilute acid is added to the reaction mixture until the concentration of free HCl is 1 M. The solution is heated at 100° for 90 min, and the product, which is isolated as described previously, is more than 90% pure mannitol-1-*P*.

NAD^+, ATP, and AMP were obtained from Sigma Chemical Company, St. Louis, Mo. NADH was prepared from NAD^+ as described by DALZIEL[2]. Fru-6-*P* was obtained from Boeringer, Mannheim, Germany.

Duplicate determinations of initial reaction rates were made at 25°. The reduction of NAD^+ was measured in a Beckman DB spectrophotometer equipped with a Beckman recorder. The rates are expressed as the change in absorbance at 340 mμ per min ($\Delta A \cdot \text{min}^{-1}$). Oxidation of NADH was usually measured in an Aminco-Chance dual-wavelength spectrophotometer. The wavelengths were set at 340 and 370 mμ (which give about 60% of maximal sensitivity). The range was set at 5% transmission. The rates are expressed as per cent change in transmission difference per min ($\%\Delta T \cdot \text{min}^{-1}$).

RESULTS

The experiments with adenosine phosphate inhibition of the reaction between NAD^+ and mannitol-1-*P* in the presence of mannitol-1-phosphate dehydrogenase (ref. 1) were made at pH 9. In order to compare the effects of ATP and AMP upon both directions of the reaction, all experiments were made at pH 7.3 (0.1 M imidazole–HCl buffer).

The plots of reciprocal reaction rates *versus* reciprocal NAD^+ concentrations, which are presented in Fig. 1, show the effect of mannitol-1-*P* concentration upon inhibitory effect of ATP and AMP. Whereas AMP inhibited the reaction to the same degree in the presence of 0.5 mM and 5 mM mannitol-1-*P* (the K_i in both being 1.1 mM), ATP inhibition depended upon mannitol-1-*P* concentration. The following K_i values for ATP were found: at 0.5 mM mannitol-1-*P*, $K_i = 0.49$ mM; at 5 mM mannitol-1-*P*, $K_i = 1$ mM; and at 10 mM mannitol-1-*P*, $K_i = 1.5$ mM. In Fig. 1 the points of the ATP-inhibited experiments fall on straight lines which do not intersect the ordinate at the intersection with the uninhibited plots. The inhibition by ATP is, therefore, probably not strictly competitive with NAD^+.

Fig. 2 shows the plots of the reciprocal initial rates *versus* the reciprocal mannitol-1-*P* concentration in the absence and presence of ATP.

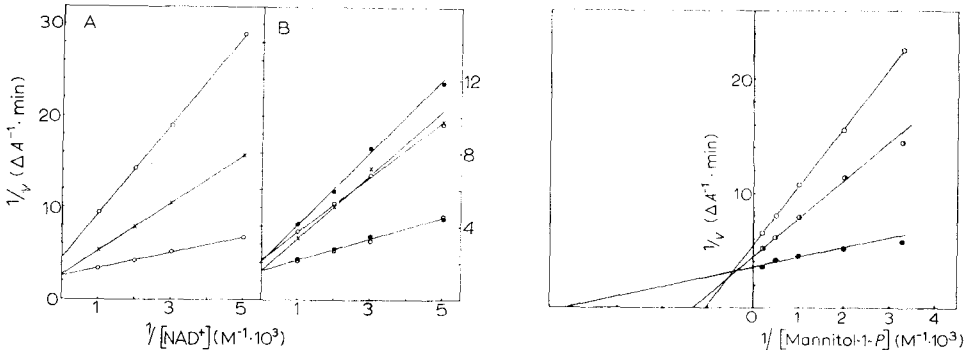


Fig. 1. $1/v$ plotted against $1/[NAD^+]$. A: 0.5 mM mannitol-1-*P*; ○, without inhibitor, and with 2.4 mM ATP; ×, with 2.25 mM AMP. B: ●, 5 mM mannitol-1-*P*, without inhibitor, and with 2.4 mM ATP; ×, 5 mM mannitol-1-*P*, and 2.25 mM AMP; ○, 10 mM mannitol-1-*P* without inhibitor, and with 2.4 mM ATP.

Fig. 2. $1/v$ plotted against $1/[\text{mannitol-1-}P]$. ●, without inhibitor; ◒, with 2.5 mM ATP; ○, with 5 mM ATP. The concentration of NAD^+ was 0.5 mM.

Fig. 3 gives the results from experiments with several concentrations of NAD^+ and mannitol-1-*P*. Such results may be used for determining the kinetic coefficients of the initial rate equation, when $1/v$ *versus* the reciprocal substrate concentrations yield linear plots[3]. Fig. 3 also includes the secondary plot of the intercepts with the $1/v$ axis corresponding to infinite NAD^+ concentration, *versus* reciprocal mannitol-1-*P* concentrations. A similar secondary plot of the intercepts with the $1/v$ axis corresponding to infinite mannitol-1-*P* concentration, *versus* $1/(NAD^+)$, cannot be constructed because of the curved form of the primary plots. Apparently the reciprocal initial rate equation is non-linear. From the data in Fig. 3, an approximate K_m for

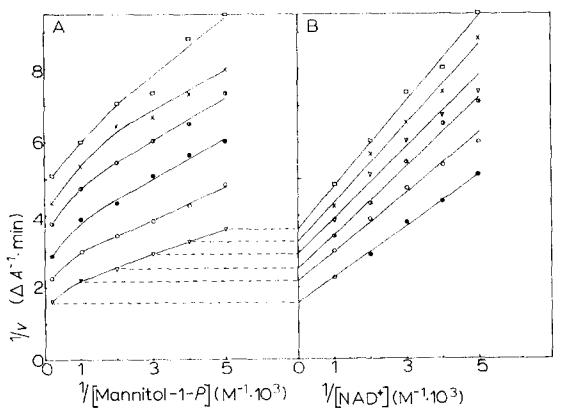
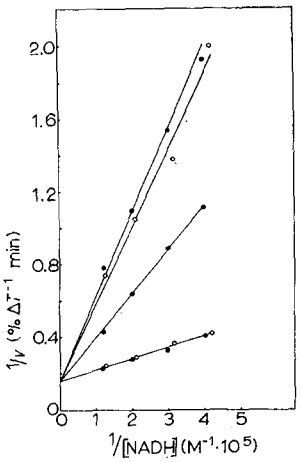


Fig. 3. A: $1/v$ plotted against $1/[\text{mannitol-1-}P]$. The concentrations of NAD^+ were: ○, 1 mM; ●, 0.5 mM; ◒, 0.333 mM; ×, 0.25 mM; and □, 0.2 mM; △, intercepts with the $1/v$ axis in B, corresponding to infinite NAD^+ concentration, plotted against $1/[\text{mannitol-1-}P]$. B: $1/v$ plotted against $1/[NAD^+]$. The concentrations of mannitol-1-*P* were: ●, 5 mM; ○, 1 mM; ◒, 0.5 mM; △, 0.333 mM; ×, 0.25 mM; and □, 0.2 mM.



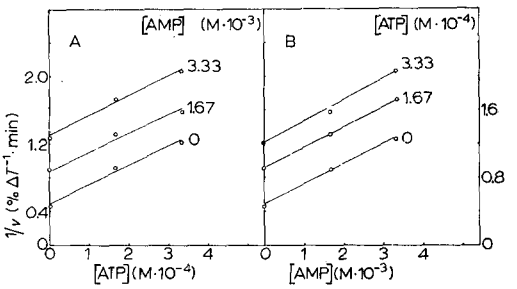
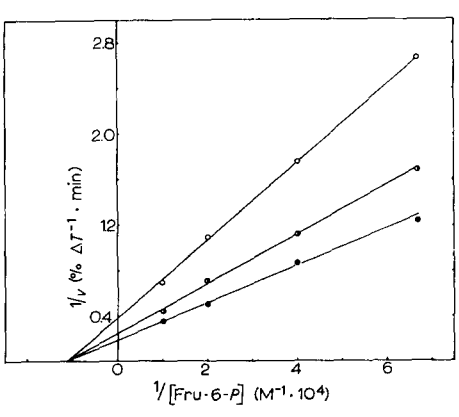


Fig. 4. $1/v$ plotted against $1/[NADH]$. ○, without inhibitor, and in the presence of 5 mM AMP. ●, without inhibitor, and in the presence of 0.15 and 0.45 mM ATP. The concentration of Fru-6-*P* was 0.15 mM.

Fig. 5. Simultaneous inhibition of mannitol-1-phosphate dehydrogenase by ATP and AMP. A: $1/v$ plotted against [ATP], without AMP, with 1.67 mM, and with 3.33 mM AMP. B: $1/v$ plotted against [AMP], without ATP, with 0.167 mM, and with 0.333 mM ATP. Fru-6-*P* concentration was 0.15 mM, and NADH concentration was 5 μM.

NAD^+ may be estimated by extrapolation to very high mannitol-1-*P* concentrations. This procedure yields a value of 0.5 mM.

Experiments with the inhibition by ATP and AMP of the reaction between NADH and Fru-6-*P* in the presence of mannitol-1-phosphate dehydrogenase are presented in Fig. 4. The K_i for ATP was found to be 0.054, and 0.071 mM, at two ATP concentrations. The K_i for AMP was 0.89 mM. The apparent K_m value for



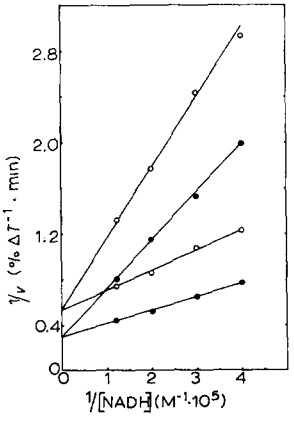


Fig. 6. $1/v$ plotted against $1/[Fru\text{-}6\text{-}P]$. ●, without inhibitor; ◒, with 0.8 mM AMP; ○, with 0.15 mM ATP. The concentration of NADH was 5 μM.

Fig. 7. $1/v$ plotted against $1/[NADH]$. ○, 50 μM Fru-6-*P* without inhibitor, and with 167 μM ATP. ●, 100 μM Fru-6-*P*, without inhibitor, and with 0.167 mM ATP.

NADH was approx. 4 μM at 100 μM Fru-6-*P* and 3.5 μM at 50 μM Fru-6-*P*.

An experiment was made with simultaneous inhibition of the reaction between NADH and Fru-6-*P* by ATP and AMP, and the results were plotted as described by YONETANI AND THEORELL[4] (Fig. 5). The reciprocal initial rates plotted *versus* the concentration of one inhibitor gave a straight line for each concentration of the other inhibitor, and these lines had the same slope. AMP and ATP therefore seem to be bound at the same site on the enzyme.

In Fig. 6 reciprocal initial rates are plotted against reciprocal concentrations of Fru-6-*P* at a constant concentration of NADH. The inhibition by ATP and AMP seems to be of the non-competitive type. The K_i for ATP with varying NADH concentration was found to be similar at two Fru-6-*P* concentrations (Fig. 7). With 50 μM Fru-6-*P*, K_i was 59 μM, and with 100 μM Fru-6-*P*, K_i was 66 μM. At much higher concentrations of Fru-6-*P* (5 mM and 2.5 mM) at which substrate inhibition occurs, the inhibitory effect of ATP was virtually absent.

Figs. 8 and 9 show the results of some experiments with heat-inactivation of

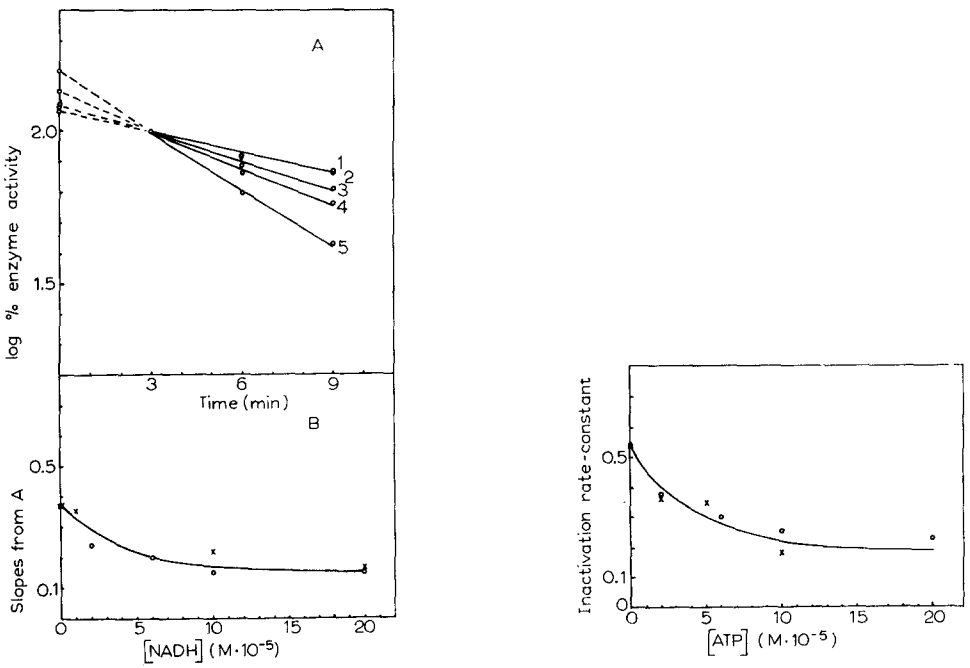


Fig. 8. Protection by NADH against heat inactivation of mannitol-1-phosphate dehydrogenase. The enzyme was dissolved in 0.01 M imidazole–HCl buffer (pH 7.0) containing 0.05 M neutralized $(NH_4)_2SO_4$. The test tubes with the enzyme solutions were placed in a water-bath at 62° and rotated during the first 3 min. Samples were withdrawn every 3 min and analyzed for dehydrogenase activity at pH 9. All values are averages from 3 different experiments. A. log per cent remaining activity plotted against time of heating. The activity at 3 min was set equal to 100%. Concentrations of NADH: 1, 200 μM; 2, 100 μM; 3, 60 μM; 4, 20 μM; 5, None. B. The slopes of the lines in A plotted against NADH concentration (○). The values from a different set of experiments are included (×).

Fig. 9. Protection by ATP against heat inactivation of mannitol-1-phosphate dehydrogenase. Conditions are described in the legend to Fig. 8. (○) and (×) represent different sets of experiments.

the enzyme. The enzyme was dissolved in 0.05 M $(NH_4)_2SO_4$ containing 0.01 M imidazole–HCl buffer at pH 7. Under these conditions the rate of heat-inactivation at 61–62° could be measured with reasonable accuracy. NADH and ATP were found to protect the enzyme from inactivation, whereas 0.1 mM NAD^+ and AMP had little effect.

When the inactivation rate constant is plotted against the concentration of the protecting agents, NADH (Fig. 8) and ATP (Fig. 9), a concentration of 'half efficiency' may be estimated. This concentration, which for both agents was close to 30 μM, is suggested to be an expression for K_s in the case of NADH, and for K_i in the case of ATP[5].

DISCUSSION

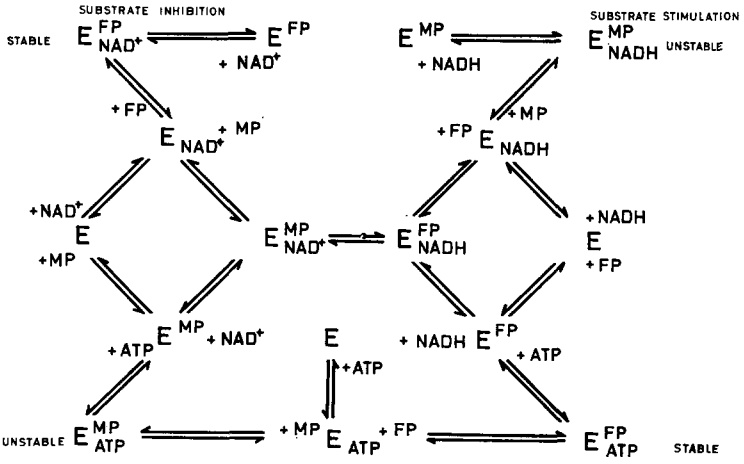
The conditions necessary for a random-order mechanism, which would approximate a reciprocally linear system[3], seem not to be fulfilled by mannitol-1-phosphate dehydrogenase. The mechanism is further complicated by the stimulatory and inhibitory effects of the substrates. (For discussion of such effects, see also DALZIEL[6].)

The experiments do not supply sufficient results to permit a quantitative treatment of the mannitol-1-phosphate dehydrogenase mechanism, but a qualitative interpretation of the findings is attempted.

The following hypothesis seems to explain most of these findings (see Scheme I). Enzyme, substrates and co-enzymes form ternary complexes by random order of addition. The enzyme–mannitol-1-*P* binary complex has low affinity for ATP, NAD^+ and NADH, compared with the affinity of the free enzyme or the enzyme–Fru-6-*P* binary complex for these substances. The binding of AMP is not influenced by the binding of Fru-6-*P* or mannitol-1-*P*. In addition to the two reactive ternary com-

SCHEME I

PROPOSED INTERMEDIARY COMPLEXES IN THE REACTION AND INHIBITION OF MANNITOL-1-*P* DEHYDROGENASE



Abbreviations: MP, mannitol-1-*P*. FP, Fru-6-*P*.

plexes, two abortive ternary complexes may also form. These complexes account for the substrate effects (inhibition and stimulation).

Proceeding with the reaction from left to right, it follows from what has been postulated that the ternary complex *E*–mannitol-1-*P*–NAD^+ is relatively unstable with respect to NAD^+ binding, whereas in the complex *E*–Fru-6-*P*–NADH, NADH is more stably bound. This is partly the reason for the relatively high value of K_m for NAD^+. It also accounts for the low inhibitory effect of ATP upon this direction of the reaction, since the ternary complex *E*–mannitol-1-*P*–ATP is also unstable. When the concentration of mannitol-1-*P* is low, however, more enzyme is free, and more of the relatively stable binary complex *E*–ATP is formed.

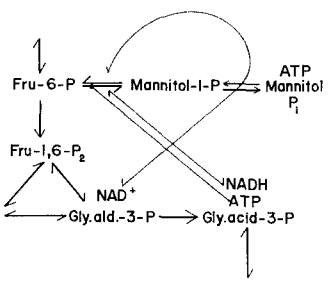
Substrate stimulation by mannitol-1-*P* may be explained by assuming that high concentrations of this substrate increase the probability for mannitol-1-*P* to replace Fru-6-*P* in the product ternary complex, and thus speed up its loss of NADH.

The same considerations apply when the reaction is regarded from right to left. Because of the stability properties of the ternary complexes, the K_m for NADH will be low. ATP will inhibit maximally, since the *E*–ATP and *E*–Fru-6-*P*–ATP complexes are stable. Substrate inhibition is explained by an increase with Fru-6-*P* concentration of the probability for Fru-6-*P* to replace mannitol-1-*P* in the product ternary complex, thus slowing down the dissociation of NAD^+ from the oxidized abortive complex. Since ATP is similarly bound by the free enzyme and by the binary *E*–Fru-6-*P* complex, Fru-6-*P* and ATP inhibitions will not be additive.

One may picture the enzyme surface as having a site which is specific for AMP and the AMP part of the other nucleotides. This site is not influenced by the binding of the substrates. In addition to the binding of the AMP moiety, ATP is bound by other groups. Since ATP differs from AMP by having additional charged phosphate groups, the extra binding of ATP may possibly be caused by a positively charged group on the enzyme. The NAD coenzymes are assumed to be bound in the same way as ATP. When mannitol-1-*P* is bound to the enzyme, this happens in such a way that the extra binding site is lost. The loss of the extra site (positive charge) is not caused by the phosphate group in mannitol-1-*P*, since Fru-6-*P* must be bound without causing loss of the extra site.

It is tempting to use the results from the experiments *in vitro* to elaborate a hypothesis as to how the adenylate inhibition of mannitol-1-phosphate dehydrogenase may act *in vivo*. Some of the pertinent reactions of glycolysis in *E. coli* are shown in Scheme II.

Under conditions of low ATP concentrations and high NADH concentrations,



SCHEME II

Fru-6-*P* will be reduced to mannitol-1-*P*. Assuming that hexose-6-*P* is available, the cell may in this way increase its ATP concentration. The wasteful process of producing mannitol-1-*P* and hydrolyzing it to mannitol is automatically turned off when the concentration of ATP increases. Further increase in ATP concentration will start a remobilization of mannitol by the mannitol-kinase reaction, which apparently is also ATP regulated[7].

Under aerobic conditions, with high ATP concentrations, the reduction of Fru-6-*P*, even if possible for equilibrium reasons, will be strongly inhibited.

It is suggested that the mannitol-1-phosphate dehydrogenase reaction (in co-operation with the mannitol kinase reaction) acts as an electron buffer which is coupled to the energy balance of the *E. coli* cell, and regulates the NADH/NAD^+ ratio. The mechanism may have its counterparts in other organisms, and it may be profitable to investigate 'side line' dehydrogenases for inhibition effects similar to those described here.

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