BBA 65868

MALATE UTILIZATION BY A GROUP D *STREPTOCOCCUS*

II. EVIDENCE FOR ALLOSTERIC INHIBITION OF AN INDUCIBLE MALATE DEHYDROGENASE (DECARBOXYLATING) BY ATP AND GLYCOLYTIC INTERMEDIATE PRODUCTS

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(Received November 20th, 1968)

SUMMARY

1. An inducible malate dehydrogenase (decarboxylating) (L-malate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.39) from *Streptococcus faecalis* was shown to be subject to a product inhibition by nucleoside triphosphates and several glycolytic intermediates.

2. The glycolytic intermediate products, fructose 1,6-diphosphate, 3-phosphoglyceric acid and 6-phosphogluconate acted as competitive inhibitors for the substrate while ATP was an apparent competitive inhibitor for both substrate and cofactor.

3. Kinetic and thermal inactivation studies indicated the four inhibitors could be classified as negative allosteric effectors; under appropriate conditions fructose 1,6-diphosphate and 3-phosphoglyceric acid enhanced the reaction rate of the malate dehydrogenase (decarboxylating).

4. The physiological importance of the malate dehydrogenase (decarboxylating) feedback control to malate utilization by the organism is discussed.

INTRODUCTION

A physiological characterization of an inducible malate dehydrogenase (decarboxylating) (L-malate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.39) from *Streptococcus faecalis* was presented in a previous publication[1]. The synthesis of the malate dehydrogenase (decarboxylating) was not subject to catabolite repression by intermediate products of glucose or fructose dissimilation. However, malate utilization was inhibited following the addition of either hexose to a culture which had been growing at the expense of the dicarboxylic acid. The evidence suggested that essentially all regulation of malate dissimilation occurred at the level of enzyme function. This report describes the allosteric properties of several low molecular weight inhibitors of the malate dehydrogenase (decarboxylating).

METHODS AND MATERIALS

Enzyme assay

Malate dehydrogenase (decarboxylating) activity was measured in the direction of malate → pyruvate + CO_2 following reduction of NAD^+ at 340 nm according to a previously published procedure[1].

Purification of the malate dehydrogenase (decarboxylating)

The method of cultivation of *S. faecalis*, preparation of crude cell-free extracts and purification scheme are described elsewhere[1]. For the kinetic studies reported here, the purification procedure was modified to include DEAE-cellulose chromatography. Following the $(NH_4)_2SO_4$-fractionation step, the dialyzed enzyme preparation was absorbed to a DEAE-cellulose column and eluted with a linear gradient of 0.1–0.5 M NaCl in 0.1 M Tris–HCl buffer (pH 8.1) containing 0.1 mM $MnCl_2$. The fractions collected between a salt concentration of 0.18 and 0.22 M contained the major portion of the enzyme activity. The peak fractions were pooled, concentrated by $(NH_4)_2SO_4$ precipitation and chromatographed on a Sephadex-G-200 column as described previously. The additional step increased the specific activity of the preparation from 37 to 100 (defined as μmoles NAD^+ reduced per min per mg protein), however, the enzyme was not completely free of contaminating material as judged by polyacrylamide-gel electrophoresis.

Thermal-inactivation studies

Thermal-inactivation experiments were performed in the following manner: 0.4 ml of 0.1 M Tris–HCl buffer (pH 8.1) and 0.01–0.02 ml of an appropriate protective agent were brought to a volume of 0.45 ml with distilled water and heated to 65°. An amount of enzyme was added in a volume of 0.05 ml buffer which would give an absorbance change of 1.0 *A* unit per min per 0.05 ml of diluted solution when measured at 340 nm. At specific time intervals during incubation at 65°, 0.05 ml of the reaction mixture was removed, added to 0.05 ml of chilled 0.1 M Tris–HCl buffer (pH 8.1) and stored at 4°; subsequently, 0.05 ml of this solution was assayed for remaining activity. When inhibitors were used as protective agents, the concentrations employed were well below the inhibitory level upon dilution.

RESULTS

Survey for malate dehydrogenase (decarboxylating) inhibitors

Growth experiments described previously[1] suggested that glucose or an intermediate product of glucose metabolism was capable of inhibiting the malate dehydrogenase (decarboxylating). These results prompted a survey for enzyme inhibitors. A variety of low molecular weight organic compounds including glycolytic intermediate products, amino acids of the aspartate family and nucleoside mono-, di- and triphosphates, were tested as potential inhibitors of the malate dehydrogenase (decarboxylating) at concentrations of 1 and 10 mM. In addition to the four nucleoside triphosphates tested (ATP, CTP, GTP and ITP), fructose 1,6-diphosphate, 6-phosphogluconate and 3-phosphoglycerate were effective inhibitors of the malate dehydrogenase (decarboxylating). The compounds were screened with crude cell-free extracts

and the purified enzyme to determine whether the purification procedure destroyed or altered the sensitivity toward specific inhibitory substances. No differences between crude extracts and the purified enzyme were noted.

Inhibition by glycolytic intermediate products

A study of the kinetic properties of the inhibitions produced by fructose 1,6-diphosphate, 6-phosphogluconate and 3-phosphoglycerate respectively, appeared necessary to understand the mode and site of action of these compounds. Lineweaver–Burk plots indicated that fructose 1,6-diphosphate was a competitive inhibitor for malate altering the apparent K_m of the substrate (Fig. 1A). The K_m for NAD^+ was not affected (Fig. 1B), however, a slight decrease in v_{max} was observed. Qualitatively, 6-phosphogluconate and 3-phosphoglycerate affected the enzyme in the same fashion. The three inhibitors were effective over the same concentration range, between 0.5 and 20 mM.

Attempts to confirm the competitive nature of the respective inhibitions using an alternate method suggested by DIXON AND WEBB[2] were unsuccessful; plotting $1/v$

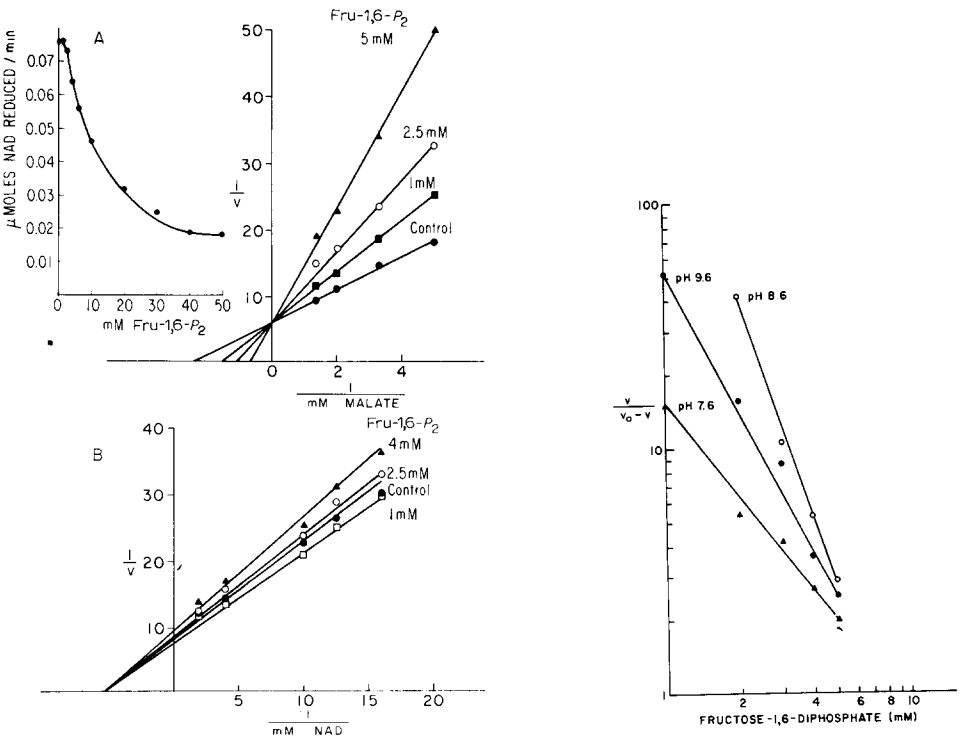

Fig. 1. Inhibition of malate dehydrogenase (decarboxylating) by fructose 1,6-diphosphate. Effect of various concentrations of fructose 1,6-diphosphate on the K_m of (A) malate and (B) NAD^+. Insert: determination of maximum inhibition produced by fructose 1,6-diphosphate at saturating concentrations of malate. Protein 3.6 μg.

Fig. 2. The pH dependency of fructose 1,6-diphosphate interaction strength. v_0 = reaction rate in absence of fructose 1,6-diphosphate, v = fructose 1,6-diphosphate inhibited reaction rate. Buffers, 0.1 M Tris–HCl, pH as indicated; protein, 3.6 μg. Curve slopes (n values) cited in text.

against $[I]$ at several substrate concentrations yielded non-linear slopes (data not shown). Replotting the data in another fashion, activity against fructose 1,6-diphosphate concentration, a curve with a distinctly sigmoidal character rather than an hyperbola was obtained (see insert in Fig. 1). Within the same concentration range, similar curves were obtained with 6-phosphogluconate and 3-phosphoglycerate. At saturating concentrations, a 75–80% loss of activity was observed with each of the three inhibitors.

A sigmoidal saturation curve is generally thought to be characteristic of an enzyme with multiple binding sites for a ligand, in this instance the respective inhibitors, which interact with one another in a cooperative manner[3,4]. The degree of cooperativity can be estimated by plotting the inhibition data as $\log v/(v_0 - v)$, against $\log [I]$; the slope of the line is defined as the cooperativity coefficient (n). Fig. 2 shows that the fructose 1,6-diphosphate interaction strength is dependent upon H^+ concentration. At the optimal pH, 8.6, a n value of 3 is obtained; increasing the pH to 9.6 decreased n to 2, while decreasing the pH to 7.6 resulted in a value of 1. The decrease in the n value produced by lowering the pH from 8.6 to 7.6 was accompanied

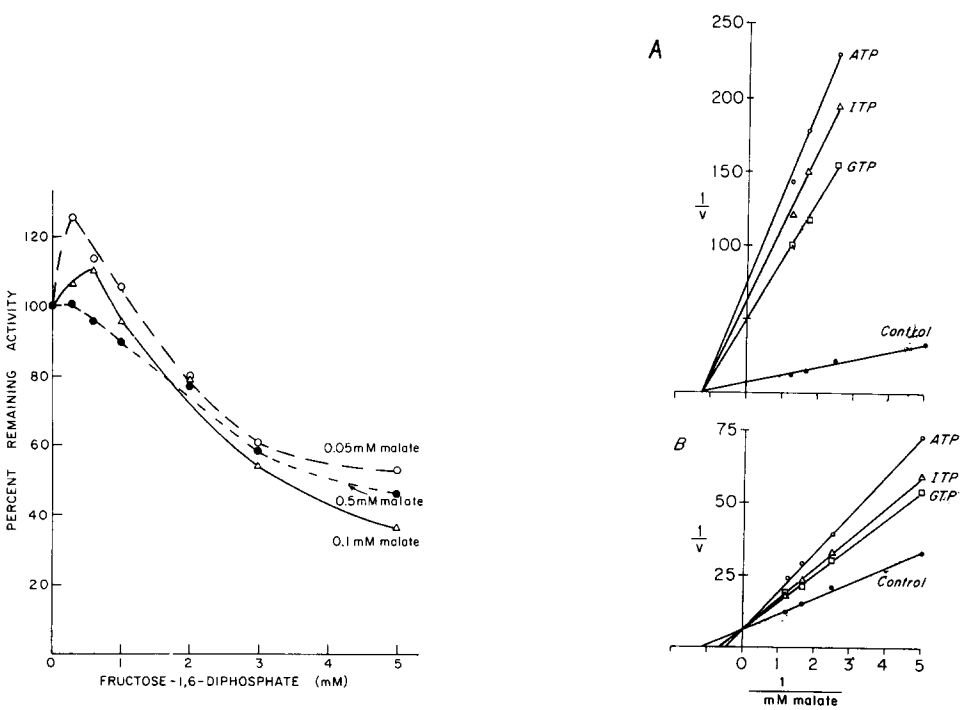

Fig. 3. Enhancement of malate dehydrogenase (decarboxylating) activity by non-inhibitory concentrations of fructose 1,6-diphosphate. Percent remaining activity was calculated using the reaction rate obtained in the absence of fructose 1,6-diphosphate at 0.5, 0.1 or 0.05 mM malate respectively as the control. Protein 3.6 μg.

Fig. 4. Effect of ATP concentration on the nature of the inhibition of the malate dehydrogenase (decarboxylating). A. System containing 3 mM nucleoside triphosphates. B. System containing 1 mM nucleoside triphosphates. Conventional assay system employed substituting 1 mM $MgCl_2$ for 0.1 mM $MnCl_2$, 3.6 μg enzyme protein used per cuvette.

by a change in shape of the inhibitor saturation curve from sigmoidal to hyperbolic.

Despite qualitative similarities in the mode of inhibition, a n value of unity was obtained with 3-phosphoglycerate at the three H^+ concentrations tested above, while values of 1.0 and 1.6 were obtained with 6-phosphogluconate at pH 7.6 and 8.6 respectively. In contrast to fructose 1,6-diphosphate, relatively little cooperativity could be demonstrated with 6-phosphogluconate and none with 3-phosphoglycerate.

At below saturating concentrations of substrate, non-inhibitory levels of fructose 1,6-diphosphate and 3-phosphoglycerate (between 0.1 and 0.3 mM) enhanced the reaction rate. Reproducible stimulations of enzyme activity between 25–30% above the control value have been observed at malate concentrations of 0.05 mM. One such experiment with fructose 1,6-diphosphate is depicted in Fig. 3. Increasing the concentration of substrate decreased the degree of enhancement produced by fructose 1,6-diphosphate and 3-phosphoglycerate. It is not unusual for structural analogues of substrates which act as competitive inhibitors to stimulate reaction rates of enzymes possessing more than one substrate site[5,6]. Although a competitive inhibition between malate and each of the inhibitors was observed, the situation may not be strictly analogous since it is unlikely that either fructose 1,6-diphosphate or 3-phosphoglycerate is bound at the substrate site.

Inhibition by nucleoside triphosphates

Although the enzyme was sensitive to all four nucleoside triphosphates tested, CTP, GTP, ITP and ATP; the most effective of these was ATP. ADP was only one-third as inhibitory as ATP on an equimolar basis and AMP had little or no effect on the rate of catalysis. The nature of the inhibition could be altered by varying the concentration of the nucleoside triphosphates. At a concentration of 3 mM or greater, a non-competitive inhibition with respect to malate was observed (Fig. 4A), while levels of 1 mM or less produced a competitive inhibition (Fig. 4B). The effect of nucleoside triphosphate concentration was observed with both Mn^{2+} and Mg^{2+} activated systems. The non-competitive character of the inhibition produced by higher concentrations of ATP, ITP and GTP is similar to that produced by reducing the amount of metal ion from saturating to non-saturating levels. These compounds are excellent metal sequestering agents and since they were present in excess as compared to the concentration of metal ion, the resultant non-competitive inhibition is probably a consequence of chelation. Recently, the inhibition of a Mg^{2+} activated rabbit muscle pyruvate kinase by ATP was attributed to the formation of a metal-nucleoside triphosphate complex[7].

Over the range of 0.1–1.0 mM, however, ATP becomes a competitive inhibitor for malate (Fig. 5A) and NAD^+ (Fig. 5B). Plotting the percent inhibition of enzyme activity against ATP concentration in this relatively low range, a sigmoidal curve similar to those observed with the glycolytic intermediate products was obtained. The onset of the inhibition occurred at a concentration of 0.05 mM and began to plateau at 0.2 mM; a 3-fold increase of the ATP level only increased the extent of inhibition by an additional 10–15%. Replotting the data as $\log v/(v_0 - v)$ against $\log$ [ATP] a n value of 2 is obtained. Chelation effects cannot be invoked to explain the action of ATP in this instance since the increase in degree of inhibition is not proportional to the concentration of nucleoside triphosphate. Other data will be presented shortly which support this interpretation of the results and essentially

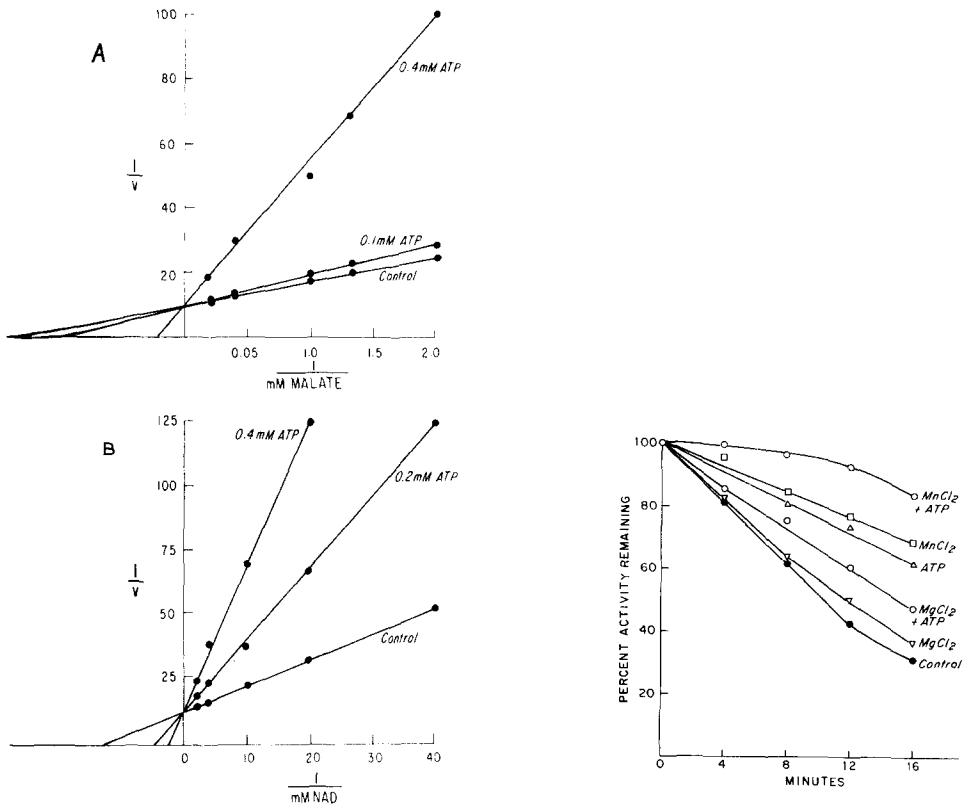

Fig. 5. Characterization of the inhibition by ATP on the malate dehydrogenase (decarboxylating). Effect of various concentrations of ATP on apparent K_m for (A) substrate and (B) cofactor. Protein 3.6 μg.

Fig. 6. Protection of the malate dehydrogenase (decarboxylating)against thermal inactivation by Mn^{2+} and ATP. Details of procedure presented in METHODS AND MATERIALS. Concentrations of ATP, Mn^{2+} and Mg^{2+} present in mixture during heating were 0.4, 1 and 1 mM respectively. Concentration of protein in assay, 3.6 μg.

preclude the possibility of a nonenzymatic ATP–Mn^{2+} interaction at the levels employed in these experiments.

Heat-inactivation studies

Thermal-inactivation studies were undertaken in an attempt to establish the existence of specific inhibition sites on the enzyme by determining whether the various inhibitors altered the catalytic properties of the enzyme towards heat. The heat lability of the purified malate dehydrogenase (decarboxylating) at 65° decreased markedly as the pH was lowered from 9.2 to 8.1. The rate of thermal inactivation at pH 8.1 was retarded by a variety of compounds. In general, the nucleoside triphosphates at concentrations of 0.4–0.8 mM were very effective as protective agents. CTP, GTP and ITP protected the malate dehydrogenase (decarboxylating) against thermal inactivation as effectively as ATP. However, of all the compounds tested, Mn^{2+} offered the enzyme the greatest degree of protection (Fig. 6); Mg^{2+} provided

the least protection against thermal denaturation. In combination, Mn^{2+} and ATP exhibited a synergistic effect which afforded the enzyme nearly complete protection during incubation at 65°. The increase in the level of protection observed in the presence of ATP and $MnCl_2$ suggests that the respective affinities of these two compounds for the enzyme is greater than their affinity for each other as a chelate-complex at the concentrations employed in these experiments. In contrast, Mg^{2+} significantly reduced the efficacy of ATP to protect the enzyme against thermal inactivation. It is possible that Mg^{2+} preferentially interacts with ATP thereby reducing the amount of nucleoside triphosphate available to bind with and protect the enzyme. This conclusion is consistant with data presented earlier[1] which indicated that Mn^{2+} has a far greater affinity for the malate dehydrogenase (decarboxylating) than Mg^{2+}. NAD^+ and malate afforded little or no protection when tested at concentrations between 0.1–0.5 mM and 1–10 mM respectively. Fructose 1,6-diphosphate and 3-phosphoglycerate were ineffective at levels of 1 mM or less, however, concentrations of 10 mM provided approximately the same degree of protection as Mn^{2+}.

DISCUSSION

The inhibition of the malate dehydrogenase (decarboxylating) by ATP and other nucleoside triphosphates may serve as a regulatory mechanism providing the organism with an effective means of controlling malate catabolism during growth. A control mechanism of this sort would be necessary if the microorganism is to maintain a balance between energy production and biosynthesis. Although the levels at which ATP is an effective inhibitor, 0.1–1.0 mM, appear to be within the generally accepted physiological range, it is difficult to distinguish between a direct effect on the enzyme, namely, binding at an inhibitor site or an indirect effect produced by a non-enzymatic interaction between ATP and the metallic cofactor. While the latter possibility cannot be dismissed entirely, the thermal-inactivation studies and kinetic evidence strongly suggest that at concentrations between 0.1 and 1 mM, ATP binds directly with the enzyme at two or more sites.

In addition to the potential regulatory action of ATP, the streptococcal malate dehydrogenase (decarboxylating) was also inhibited by fructose 1,6-diphosphate, 3-phosphoglycerate and 6-phosphogluconate, intermediate products of glucose dissimilation. The inhibition of malate utilization resulting from the addition of glucose to growing cultures of *S. faecalis* may be attributed to a temporary accumulation of fructose 1,6-diphosphate, 3-phosphoglycerate, ATP or any combination thereof. However, the complex medium employed in these experiments probably provides the organism with essentially all of the preformed basic units required for the biosynthesis of its cellular components. Since it has been demonstrated that the generation time of the organism is dependent upon the nature and the concentration of the growth substrate[8], the rate limiting process appears to be energy production rather than biosynthesis. The failure to observe an increase in the growth rate following the addition of relatively low levels of glucose, while malate utilization was completely inhibited suggests that no significant change in the intracellular levels of ATP occurred. Hence fructose 1,6-diphosphate, 3-phosphoglycerate or both are probably the agents responsible for the cessation of malate catabolism. The inhibition of the enzyme activity by fructose 1,6-diphosphate and 3-phosphoglycerate probably

provides the organism with a means of regulating the rate of pyruvate formation from malate in the presence of a fermentable carbohydrate thereby maintaining a balanced condition of growth with efficient utilization of two energy rich substrates. At saturating levels of substrate, the concentration of fructose 1,6-diphosphate or 3-phosphoglycerate required to effectively inhibit the malate dehydrogenase (decarboxylating) is relatively high, between 5–20 mM. While these levels of intermediate product may seem inordinately high, MIZUSHIMA AND KITAHARA[9] have recently shown that the intracellular concentrations of fructose 1,6-diphosphate and 3-phosphoglycerate during exponential growth of *Lactobacillus plantarum* are 17 and 40 mM respectively. If the group D streptococci contain pools of fructose 1,6-diphosphate and 3-phosphoglycerate equivalent to those of lactobacilli, then these glycolytic intermediate products could indeed be effective regulators of the enzyme.

The role of 6-phosphogluconate inhibition cannot be rationalized as easily as fructose 1,6-diphosphate. However, the group D streptococci possess a functional hexose monophosphate shunt[10,11]; under conditions of growth which require the use of the phosphorylated pentose pathway, *i.e.*, during gluconate catabolism, a means of regulating the inducible malate dehydrogenase (decarboxylating) would be available. This alternate control could be important in those instances where the path of carbon is diverted away from glycolysis and production of fructose 1,6-diphosphate.

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