Unidirectional Inhibition and Activation of 'Malic' Enzyme of *Solanum tuberosum* by *meso*-Tartrate

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A kinetic study of 'malic' enzyme (EC 1.1.1.40) from potato suggests that the mechanism is Ordered Bi Ter with NADP⁺ binding before malate, and NADPH binding before pyruvate and HCO₃⁻. The analysis is complicated by the non-linearity that occurs in some of the plots. *meso*-Tartrate is shown to inhibit the oxidative decarboxylation of malate but to activate the reductive carboxylation of pyruvate. To explain these unidirectional effects it is suggested that the control site of 'malic' enzyme binds organic acids (including *meso*-tartrate) which activate the enzyme. *meso*-Tartrate, however, competes with malate for the active site and thus inhibits the oxidative decarboxylation of malate. Because *meso*-tartrate does not compete effectively with pyruvate for enzyme-NADPH, its binding at the control site leads to a stimulation of the carboxylation of pyruvate. A similar explanation is advanced for the observation that malic acid stimulates its own synthesis.

Lé John (1967) has shown that certain metabolites inhibit the oxidative deamination of glutamate catalysed by glutamate dehydrogenase from *Blastocladiella emersonii* but do not affect the reductive amination of α -oxoglutarate. This phenomenon, which Lé John (1967) has called unidirectional inhibition, has also been reported for malate dehydrogenase (Kuramitsu, 1966) and may be compared with the unidirectional catalysis reported for NAD⁺-specific isocitrate dehydrogenase (Kornberg & Pricer, 1951; Barnes *et al.*, 1972). Since initial velocities only were studied, these effects are of course not at variance with the Onsager principle of microreversibility (Onsager, 1931; Denbigh, 1951).

Newsholme & Crabtree (1973) have suggested that fast (equilibrium) reactions may be more effective in metabolic control than relatively slow reactions. This suggestion makes arithmetic sense if the action of an effector on the enzyme is unidirectional; thus if the forward reaction is activated but the reverse reaction is unchanged, there will be an increased flux through the system. However, unidirectional activation of a reaction at equilibrium is at variance with the Onsager (1931) principle, unless the effector contributes energy to change the equilibrium. The effectiveness of the control mechanism proposed by Newsholme & Crabtree (1973) increases as the reaction becomes closer to equilibrium. However, unidirectional activation of reactions close to equilibrium has not yet been demonstrated.

In the present paper we report initial-rate studies and show that *meso*-tartrate inhibits the oxidative decarboxylation of malate and activates the reductive

decarboxylation of pyruvate catalysed by potato 'malic' enzyme (L-malate-NADP⁺ oxidoreductase, EC 1.1.1.40) and offer an explanation based on the allosteric properties of the enzyme.

Experimental

Materials

Buffers. All buffers were prepared with glass-distilled water at 25°C. Tris, under the trade name Trizma, and Mops [3-*N*-(morpholino)propanesulphonic acid] were obtained from Sigma (London) Chemical Co., London S.W.6, U.K.

Other chemicals. NADP⁺ and NADPH were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K., L-malic acid, sodium pyruvate, succinic acid and dithiothreitol from Sigma (London) Chemical Co.; 2-mercaptoethanol, D(+)-, L(-)- and *meso*-tartaric acid were from BDH Chemicals Ltd., Poole, Dorset, U.K.; DE-52 DEAE-cellulose was from Whatman Biochemicals Ltd., Springfield Mill, Maidstone, Kent, U.K., and Sephadex G-25 and G-200 were from Pharmacia, Uppsala, Sweden. Other chemicals were of the best reagent grade obtainable.

Methods

Purification of 'malic' enzyme. The method of Davies & Patil (1974) was used except that the last step in their purification scheme, electrofocusing, was replaced by molecular sieving on a column of Sephadex G-200. The peak fractions from the DEAE-cellulose purification step were placed on the top of a column (5 cm × 42 cm) of Sephadex G-200 previously equilibrated with Tris buffer (pH 8.6,

0.05M) containing 2-mercaptoethanol (5mM) and eluted with the same buffer. Fractions (10ml) were collected and after assay, dithiothreitol and sucrose were added to tubes 20–27 to give concentrations of 2mM and 40% (w/v) respectively. When stored at -20°C the enzyme retains full activity for at least 6 months and when kept at 1°C full activity is retained for 2–3 days.

Enzyme assays. The standard assay was carried out at pH 7.0 by measuring the increase in E_{340} associated with NADP^+ reduction. The assay mixture contained Mops buffer (pH 7.0 or 7.6, 0.1M), MnCl_2 or MgCl_2 (1.67mM), NADP^+ (0.4mg), sodium L-malate (3.3mM) and enzyme in a final volume of 3ml. The reaction was started by the addition of 0.1ml of enzyme containing less than 0.1 unit.

The reductive carboxylation of pyruvate was measured by following the decrease in E_{340} when the enzyme was added to an assay mixture containing Mops buffer (pH 7.0, 0.1M), MnCl_2 or MgCl_2

Fig. 1. Effect of various organic acids on the rate of decarboxylation of malate catalysed by partially purified 'malic' enzyme

Enzyme activity was assayed at 340nm in cells of 1cm light-path containing Mops buffer (0.1M, pH 7.6), MnCl_2 (1.67mM), L-malate (0.33mM), NADP^+ (0.4mg) and enzyme (40 μg of protein) in a final volume of 3ml. Δ — Δ , Itaconic acid (C_5); $\bullet$ — $\bullet$, glutaric acid (C_5); $\circ$ — $\circ$, L(+)-Tartrate (C_4); $\bullet$ — $\bullet$, D(—)-tartrate (C_4); $\square$ — $\square$, adipic acid (C_6); Δ — Δ , pimelic acid (C_7); $\triangle$ — $\triangle$, meso-tartrate (C_4); $\blacksquare$ — $\blacksquare$, suberic acid (C_8); $\circ$ — $\circ$, sebamic acid (C_{10}).

Fig. 2. Double-reciprocal plots of rate against malate concentration in the presence and the absence of meso-tartrate

Enzyme activity was assayed at pH 7.0 with MnCl_2 (1.67mM) as described in the text. $\circ$, Control (absence of meso-tartrate); $\square$, 1.7mM-meso-tartrate; Δ , 3.3mM-meso-tartrate.

(1.67mM), NADPH (0.4mg), NaHCO_3 (10mM) and pyruvate (concentration varied) in a final volume of 3ml.

Assays were carried out at 30°C in cells of light-path 1cm in a Unicam SP.500 spectrophotometer equipped with a Vitatron recorder.

Unit of enzyme activity. A unit of enzyme activity is defined as the formation of 1 μmol of NADPH/min .

Results

Effect of meso-tartrate on the oxidative decarboxylation of malate

Davies & Patil (1974) noted that a number of dicarboxylic acids activate potato 'malic' enzyme. We have now shown that when the chain length of the dicarboxylic acid is increased from C_4 to C_5 , the capacity to activate the enzyme is retained, whereas increasing the chain length beyond C_6 leads to a loss in the capacity to activate the enzyme. We have confirmed that D(—) and D(+)-tartaric acid activate the enzyme but meso-tartaric acid inhibits (Fig. 1).

The type of inhibition produced by meso-tartrate was examined with respect to malate and the results are shown in Fig. 2 as a double-reciprocal plot.

K_i was obtained from the equation:

$$K_i = K_m[I]/(K'_m - K_m)$$

where K'_m is the Michaelis constant in the presence of inhibitor. $K_i = 1.2$ mM, which was also evaluated from Dixon (1953) plots.

The kinetic analysis of 'malic' enzyme is complicated by its allosteric properties. We have examined the enzyme at pH 7.0, where the plot of velocity against malate concentration approximates to Michaelis-Menten kinetics. To clarify the situation we have examined the effect of *meso*-tartrate on the oxidative decarboxylation of malate in the presence of an acti-

vator (succinate) and also in the presence of an inhibitor (phosphate). The results in Fig. 3 suggest that *meso*-tartrate acts with respect to malate as a competitive inhibitor of 'malic' enzyme.

Effect of meso-tartrate on the reductive carboxylation of pyruvate

The effect of *meso*-tartrate on the reductive carboxylation of pyruvate was studied and the results are presented in Fig. 4. Recognizing the very marked stimulation of malate formation by organic acids, we have examined the effect of *meso*-tartrate in the presence of phosphate as an inhibitor (Fig. 4a) and

Fig. 3. Double-reciprocal plots showing the effect of *meso*-tartrate on the rate of malate decarboxylation

Enzyme was assayed at pH 7.0 in the presence of MgCl_2 (1.67 mM) as described in the text. (a) In the presence of an inhibitor (phosphate, 8.3 mM). $\circ$, Absence of phosphate and *meso*-tartrate; $\blacksquare$, phosphate (8.3 mM); $\triangle$, phosphate (8.3 mM) + *meso*-tartrate (1.0 mM); $\square$, phosphate (8.3 mM) + *meso*-tartrate (1.7 mM); $\bullet$, phosphate (8.3 mM) + *meso*-tartrate (3.3 mM). (b) In the presence of an activator (succinate, 1.67 mM). $\circ$, Absence of succinate and *meso*-tartrate; $\blacksquare$, succinate (1.67 mM); $\triangle$, succinate (1.67 mM) + *meso*-tartrate (1.0 mM); $\square$, succinate (1.67 mM) + *meso*-tartrate (1.7 mM); $\bullet$, succinate (1.67 mM) + *meso*-tartrate (3.3 mM).

Fig. 4. Double-reciprocal plots showing the effect of *meso*-tartrate on the reductive carboxylation of pyruvate

Enzyme was assayed in the presence of MgCl_2 (1.67 mM) as described in the text. (a) In the presence of an inhibitor (phosphate, 8.3 mM). $\circ$, In the absence of phosphate; $\bullet$, phosphate (8.3 mM); $\triangle$, phosphate (8.3 mM) + *meso*-tartrate (1.7 mM); $\square$, phosphate (8.3 mM) + *meso*-tartrate (3.3 mM). (b) In the presence of an activator (succinate, 1.67 mM). $\circ$, In the absence of succinate; $\bullet$, succinate (1.67 mM); $\triangle$, succinate (1.67 mM) + *meso*-tartrate (1.7 mM); $\square$, succinate (1.67 mM) + *meso*-tartrate (3.3 mM).

also with succinate as an activator (Fig. 4b). The results show that in the absence of succinate, *meso*-tartrate behaves as an activator which does not affect the K_m (apparent) for pyruvate. In the presence of succinate, *meso*-tartrate behaves as a non-competitive inhibitor with respect to pyruvate. $K_i = 1.65$ mM was calculated from the equation:

$$K_i = V'_{\max} [I] / (V'_{\max} - V'_{\max})$$

where $V'_{\max}$ is the maximum velocity in the presence of the inhibitor.

Fig. 5. Effect of malate on the reductive carboxylation of pyruvate

Enzyme activity was assayed in the presence of $MgCl_2$ (1.67 mM) as described in the text except that potassium phosphate (6.6 mM) was present, the pyruvate concentration was 2.6 mM and malate concentration was varied as indicated.

Effect of malate on the reductive carboxylation of pyruvate

Since organic acids activate 'malic' enzyme, malate might be expected to activate its own synthesis. The effect of malate on the reductive carboxylation of pyruvate is shown in Fig. 5, and confirms the existence of autocatalysis in this reaction.

Determination of Michaelis constants

Initial-velocity studies of the oxidative decarboxylation of malate were carried out, by varying the concentration of one substrate while holding the concentration of the second substrate constant at a number of values. The double-reciprocal velocity concentration plots are shown in Fig. 6.

The double-reciprocal plots are linear and intersect to the left of the $1/v$ axis. According to Cleland (1963a) this indicates that the substrates $NADP^+$ and malate must bind to the enzyme before any product is released. The precise order of substrate addition requires product-inhibition studies.

Product-inhibition studies

To distinguish between various possible sequential mechanisms and to determine the order of binding, inhibition studies were carried out by using products as inhibitors. With $NADP^+$ as the variable substrate, $NADPH$ showed competitive inhibition (Fig. 7a), whereas HCO_3^- and pyruvate gave mixed inhibition, though non-competitive inhibition cannot be eliminated because of non-linearity (Fig. 7b or 7c).

With malate as the variable substrate pyruvate showed uncompetitive inhibition (Fig. 8a), whereas HCO_3^- and $NADPH$ showed non-competitive inhibition (Figs. 8b and 8c).

Fig. 6. Double-reciprocal plots of initial velocity against (a) $NADP^+$ concentration and (b) malate concentration

Enzyme was assayed at pH 7.0 in the presence of $MnCl_2$ (1.67 mM) as described in the text. (a) $\circ$, L-Malate (0.066 mM); $\bullet$, L-malate (0.10 mM); $\triangle$, L-malate (0.133 mM); $\square$, L-malate (0.167 mM). (b) $\circ$, $NADP^+$ (8.5 μ M); $\bullet$, $NADP^+$ (17 μ M); $\triangle$, $NADP^+$ (34 μ M); $\square$, $NADP^+$ (85 μ M).

Fig. 7. Double-reciprocal plots showing the effect of products (a) NADPH, (b) NaHCO_3 and (c) pyruvate on the rate of malate decarboxylation with NADP^+ as the variable substrate

Enzyme activity was assayed in the presence of MnCl_2 (1.67 mM) at pH 7.0 as described in the text, except for the presence of a product, and NADP^+ was varied as indicated in the Figure. (a) NADPH as product inhibitor. $\circ$, Control in the absence of NADPH; $\bullet$, NADPH (34 μM); Δ , NADPH (68 μM); $\square$, NADPH (102 μM). (b) NaHCO_3 as product inhibitor. $\circ$, Control in the absence of NaHCO_3 ; $\bullet$, NaHCO_3 (13 mM); Δ , NaHCO_3 (20 mM); $\square$, NaHCO_3 (26 mM). (c) Pyruvate as product inhibitor $\circ$, Control in the absence of pyruvate; $\bullet$, pyruvate (1.67 mM); Δ , pyruvate (3.3 mM); $\square$, pyruvate (6.6 mM).

Fig. 8. Double-reciprocal plots showing the effects of products (a) pyruvate, (b) NaHCO_3 and (c) NADPH on the rate of malate decarboxylation with L-malate as the variable substrate

Enzyme activity was assayed in the presence of MnCl_2 (1.67 mM) at pH 7.0, NADP^+ (34 μM), enzyme (40 μg of protein), product (pyruvate, NADPH or NaHCO_3) and L-malate varied as indicated. (a) Pyruvate as product inhibitor. $\circ$, Control in the absence of pyruvate; $\bullet$, pyruvate (1.67 mM); Δ , pyruvate (3.3 mM); $\square$, pyruvate (6.6 mM). (b) NaHCO_3 as product inhibitor. $\circ$, Control in the absence of NaHCO_3 ; $\bullet$, NaHCO_3 (13 mM); Δ , NaHCO_3 (20 mM); $\square$, NaHCO_3 (26 mM). (c) NADPH as product inhibitor. $\circ$, Control in the absence of NADPH; $\bullet$, NADPH (34 μM); Δ , NADPH (68 μM); $\square$, NADPH (102 μM).

Discussion

The possibility that an effector molecule might activate a reaction in one direction but inhibit the reaction in the reverse direction has been discussed by Frieden (1964). The principle of microreversibility requires that under equilibrium conditions any molecular process and the reverse of that process will take place at the same rate. However, the

kinetics analysed by Frieden (1964) are those of initial-velocity studies and are thus clearly not at variance with the principle of microreversibility. Lé John (1967) and Sanner (1972) have studied the glutamate dehydrogenase from *Blastocladiella emersonii* and shown that certain metabolites inhibit the oxidative deamination of glutamate without having an effect on the reverse reaction. We have examined potato 'malic' enzyme and shown that

meso-tartrate inhibits the oxidative decarboxylation of malate but activates the reverse reaction.

We suggest that an explanation of these unidirectional effects is to be found in the allosteric properties of 'malic' enzyme. The control site is relatively unspecific, so that a wide range of dicarboxylic acids from C₄ to C₆ can combine with and activate the enzyme. The active site, however, is highly specific so that in general dicarboxylic acids that can combine with the control site cannot combine with the active site. The obvious exception is L-malate, and this capacity to bind at both sites leads to the sigmoid kinetics observed in plots of rate versus malate concentration. *meso*-Tartrate resembles L-malate and we suggest that

it competes for the active site of 'malic' enzyme. A comparison of the structure of L-malate, *meso*-tartrate, D(-)- and L(+)-tartrate suggests why *meso*-tartrate is an effective inhibitor.

In Fig. 9, L-malic acid is shown making a three-point attachment to 'malic' enzyme. D(-)-Tartaric acid cannot bind, since it lacks the L-configuration required for the three-point attachment. L(+)-Tartaric acid has the required L-configuration at either end of the molecule, but when one end binds to the enzyme the other end cannot combine owing to steric hindrance produced by the hydroxyl group. *meso*-Tartaric acid has the L-configuration at one end of the molecule and the D-configuration at the other, so that when the L-configuration combines with the active site the hydroxyl group at the other end projects away from the active site and does not interfere with the three-point attachment. *meso*-Tartrate may

combine at the control site and so put the enzyme into an 'active' configuration, but by combining at the active site it serves to inhibit the oxidative decarboxylation of malate.

In the reverse direction, *meso*-tartrate activates the enzyme, presumably by combining at the control site, but is a poor competitor with pyruvate for the active site. The kinetics of the enzyme are consistent with this interpretation. The initial-velocity patterns suggest that NADP⁺ and malate bind sequentially, without specifying the order. The product inhibition studies resemble those reported for pigeon liver 'malic' enzyme, which have been interpreted as an Order Bi Ter (Hsu *et al.*, 1967):

This sequence predicts that pyruvate should show uncompetitive inhibition with respect to malate and NADP⁺. In the case of the potato enzyme, pyruvate shows uncompetitive inhibition with respect to malate, but the inhibition is non-competitive with respect to NADP⁺. This raises some uncertainty about the binding of pyruvate and may indicate the formation of a dead-end complex. However, the data show clear competitive inhibition by NADPH with respect to NADP⁺, so that NADP⁺ is the first substrate to bind and NADPH the last product to leave. Thus in the direction of malate decarboxylation, the active site for malate is E-NADP⁺ and *meso*-tartrate is a good competitive inhibition (Fig. 3). In the direction of pyruvate carboxylation, the active site for pyruvate is E-NADPH, and *meso*-tartrate does not compete so effectively with pyruvate. In fact *meso*-tartrate activates pyruvate carboxylation,

Fig. 9. Model showing the proposed association between hydroxy dicarboxylic acids and the active site of 'malic' enzyme

(a) D(-)-Tartaric acid; (b) L(+)-tartaric acid; (c) L-malic acid; (d) *meso*-tartaric acid.

presumably because it binds to the control site. To demonstrate inhibition by *meso*-tartrate, it is necessary to activate the enzyme with succinate and then *meso*-tartrate behaves as a non-competitive inhibitor (Fig. 4).

It is virtually certain that the unidirectional effects of *meso*-tartrate on 'malic' enzyme have no physiological significance. However, the mechanism proposed for these effects may prove to be involved in other examples of unidirectional activation or inhibition. The effectiveness of *meso*-tartrate as an inhibitor of 'malic' enzyme is paralleled by its serving as a substrate for malate dehydrogenase (Davies & Kun, 1957) and by being the most potent inhibitor of fumarase (Frieden, 1956), suggesting that the active sites of the three enzymes are structurally related.

It is always tempting to ascribe physiological significance to allosteric effects, and the observation that malate stimulates its own synthesis, presumably by binding at the control site, provides a positive feedback model for autocatalysis. However, it is likely that 'malic' enzyme catalyses the decarboxylation of malate and the observed kinetics of autocatalysis are unlikely to have physiological significance.

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