Metabolic regulation of malic enzyme activity from Paracoccus denitrificans by glyoxylate and acetylCoA

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SUMMARY: Paracoccus denitrificans contains both NAD^+- and $NADP^+$-linked malic enzyme activities when grown on malate/nitrate. The enzyme is inactive in the absence of NH_4^+. AcetylCoA inhibits both activities competitively with respect to L-malate. Glyoxylate (0.5 mM) causes 60% inhibition of the $NADP^+$-linked activity but has little effect on the NAD^+-linked activity. Citrate, aspartate, AMP, ADP, and ATP, at 0.5mM, have little effect on either of the two activities. The results are discussed with regards to the control of malic enzyme activity within the cell.

Malic enzyme (L-malate:NAD oxidoreductase (decarboxylating) EC1.1.1.39) oxidatively decarboxylates malate to pyruvate and carbon dioxide. Under conditions where P.denitrificans is grown on malate as the sole source of carbon malic enzyme activity is essential for operation of the Krebs cycle (1). Malate is converted to oxaloacetate via malate dehydrogenase and the oxaloacetate then combines with acetylCoA to form citrate under the action of the citrate condensing enzyme. In order for the cycle to operate there must be a constant input of acetylCoA. The acetylCoA is formed from malate via malic enzyme and pyruvate dehydrogenase activity. It thus seemed likely that malic enzyme activity in P.denitrificans would be regulated by acetylCoA as has been found in Escherichia coli (2) and Halobacterium cutirubrum (3). ActylCoA is not the only metabolite which would be expected to regulate malic enzyme activity - glyoxylate is yet

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another candidate. Under conditions where malate synthetase activity is required to replenish the Krebs cycle of intermediates utilized for biosynthesis (see Kornberg, H.L. (4)) it could well be expected that malic enzyme activity would be regulated by glyoxylate in order to prevent the generation of a futile cycle. With these views in mind we have studied the effects of acetylCoA and glyoxylate on the malic enzyme activity from P.denitrificans.

MATERIALS AND METHODS

P.denitrificans (N.C.I.B. 8944) was grown on a medium similar to the glucose/NO_3^- medium previously described (5) except that Na_2CO_3 was omitted from the medium and DL-malate was the carbon source. The medium contained in addition 0.5 g of yeast extract per litre. Cells were harvested in the exponential phase ($OD_{550\ nM}$=4.0) and washed with 0.1 volumes of Tris/H_2SO_4, pH 8.0; resuspended to 0.01 of the original volume in the same buffer and stored at -18°C until required.

All enzyme assays were performed at 30°C. One unit of enzyme activity is defined as that amount catalyzing the conversion of 1 µmole of substrate per minute. Malic enzyme unless otherwise specified, was assayed by following the increase in absorbance at 340nm in a mixture (final volume 1 ml) containing 5 µmoles L-malate, 20 µmoles NH_4Cl, 0.2µmoles $MnCl_2$, 100 µmoles Tris/H_2SO_4, pH 8.0, and 5 µmoles of $NAD(P)^+$. Citrate synthetase was assayed by following the decrease in absorbance at 232nm due to the disappearance of the thio-ester bond in a reaction mixture (final volume 1 ml) containing the following: 100 µmoles Tris/HCl, pH 8.0, 0.16 µmoles oxaloacetate and 0.2 µmoles acetylCoA. The reaction was initiated by the addition of enzyme and if no activity was detected the validity of the assay system was confirmed by the addition of authentic citrate synthetase. Protein was estimated by the method of Lowry, as modified by Bailey (6), after precipitation in 5% trichloracetic acid and resuspension in 5% NaOH. Bovine serum albumin, fraction 5, previously dried in a dissicator was used as standard. Malic enzyme was inactivated by progressive heat treatment, isoelectric focussing and chromatography on hydroxylapatite or DEAE-cellulose. No significant purification was achieved by $(NH_4)_2SO_4$ fractionation or chromatography on ECTEOLA-cellulose or CM-cellulose. The specific activities were increased by acetone fractionation using the following procedure: 100 ml of cells were thawed out and the pH was adjusted to pH 8.0 with NaOH. The sample was degassed for 10 minutes and sonicated at maximum output for 10 minutes, in 15 second bursts using a Soniprobe (Dawe Instruments Ltd.) with ice/acetone as coolant. The temperature of the sonicate never exceeded 5°C. The sonicate was centrifuged at 25,000 X g for 15 minutes to remove intact bacteria and the supernatant further centrifuged at 40,000X g for 1 hour to remove bacterial membranes. The preparation was made 0.1 M with respect to both $MnCl_2$ and L-malate and chilled to 1°C. All remaining steps, including centrifugation, were performed at temparatures between -15° and -20°C. Acetone at -20°C was slowly added to the preparation to bring

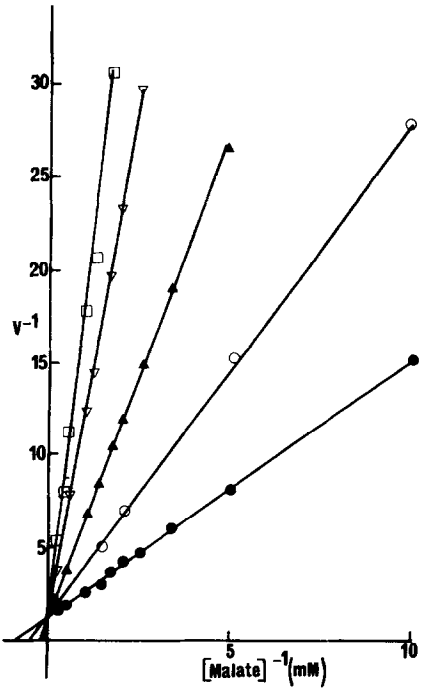

Fig. 1a

Lineweaver-Burk plot for the NAD^+-linked malic enzyme with L-malate as the variable substrate. Conditions are as described in materials and methods except that the assays were performed in the presence of ● zero, ○ 0.05, ▲ 0.1 ▽ 0.2 and □ 0.3 mM Acetyl-CoA.

the acetone concentration to 30% (v/v), the solution was then allowed to stand for 20 minutes, centrifuged at 27,000 X g for 25 minutes and the supernatant was retained. The acetone concentration of the supernatant was then increased to 45%, again using acetone at -20°C, and the solution was left to stand for 25 minutes and then centrifuged at 27,000 X g for 25 minutes. The preparation was resuspended in 50 mM Tris/HCl, pH 7.0, containing 0.01 M L-malate and 0.01 M $MnCl_2$. This preparation was stored at -18°C and was routinely dialysed against 50 mM Tris/HCl, pH 8.0, prior to use. A threefold purification was thereby accomplished. No further purification was obtained on reapplication to this preparation of any of the techniques previously mentioned. The partially purified preparation had a specific activity of 0.44 for NAD^+-linked malic enzyme and 0.41 for the $NADP^+$-linked enzyme. Between 60-70% of the activity present in the crude extract was recovered. The preparation contained appreciable malate dehydrogenase activity but completely lacked citrate synthetase activity so allowing a valid appraisal of the effect of AcetylCoA on malic enzyme activity. AcetylCoA (sodium salt), glyoxylate, oxaloacetate, aspartate, citrate, L-malate and buffers were obtained

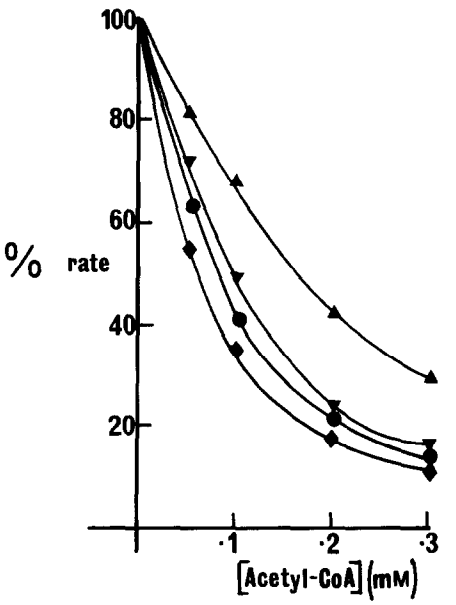

Fig. 1b

Effect of varying concentrations of Acetyl-CoA on the NAD^+-linked malic enzyme. The conditions are as described in materials and methods except that the concentrations of L-malate employed were ▲ 5.0, ▼ 2.0, ● 0.5 and ◆ 0.1 mM.

from Sigma Chemical Co. Ltd., remaining biochemicals were purchased from The Boehringer Corporation London Ltd. All remaining reagents were of the highest purity available.

RESULTS AND DISCUSSION

Paracoccus denitrificans possesses both NAD^+- and $NADP^+$-linked malic enzyme activities and is unusual in that it has an absolute requirement for NH_4^+ (Km 8.0 mM). The effect of acetylCoA was investigated on both of these activities. Fig.1a shows the effect of acetylCoA on the NAD^+-linked malic reaction in the presence of varying concentrations of L-malate. The apparent Km for L-malate is altered in the presence of acetylCoA but the Vmax is unaffected. Fig.1b is a replot of the data from Fig.1a to show the relationship

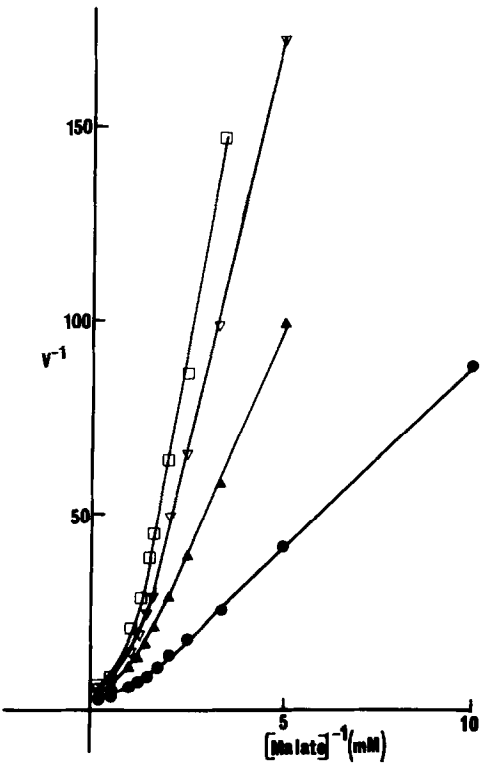

Fig. 2a

Lineweaver- Burk plot for the $NADP^+$-linked malic enzyme with L-malate as the variable substrate. Conditions are as described in materials and methods except that the assays were performed in the presence of ● zero, ▲ 0.1, ▽ 0.2 and □ 0.3 mM Acetyl-CoA.

between the concentration of acetylCoA and the degree of inhibition of the NAD^+-linked malic enzyme activity. This figure demonstrates that acetylCoA inhibits in a competitive manner with respect to L-malate. The effect of acetylCoA was also investigated on the $NADP^+$-linked malic enzyme. The enzyme exhibits kinetics consistent with homotropic cooperativity for L-malate and the apparent degree of homotropic cooperativity is increased in the presence of increasing concentrations of acetylCoA (Fig.2a). The data has been replotted

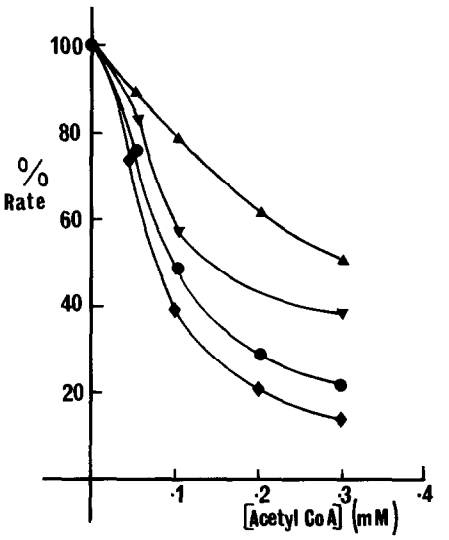

Fig. 2b

Effect of varying concentrations of Acetyl-CoA on the $NADP^+$-linked malic enzyme. The conditions are as described in materials and methods except that the concentrations of L-malate employed were ▲ 5.0, ▼ 2.0, ● 0.5 and ◆ 0.1 mM.

as % rate versus acetylCoA(Fig.2b); from this it can be seen that acetylCoA inhibits in a competitive manner with respect to L-malate but that the inhibition is not as marked as that found with the NAD^+-linked activity.

The effect of 0.5mM glyoxylate was investigated on the $NADP^+$-linked malic reaction using the four combinations of substrate presented in table 1. From this data it can be seen that both Vmax and Km are affected. The effect of 0.5 mM glyoxylate was also investigated on the NAD^+-linked malic reaction. In the presence of 0.5 mM NAD^+ and 0.5 mM L-malate the reaction was inhibited by 9%; since the inhibition was only slight in comparison with that found for the $NADP^+$-linked enzyme the effect of glyoxylate was not further investigated.

TABLE 1

	V	V^o	% inhibition
0.5 mM malate 0.5 mM $NADP^+$	.0469	.1244	62.3%
0.5 mM malate 5.0 mM $NADP^+$	.061	.152	60.0%
5.0 mM malate 0.5 mM $NADP^+$	.148	.204	27.0%
5.0 mM malate 5.0 mM $NADP^+$	.277	.335	17%

Effect of glyoxylate on $NADP^+$-linked malic enzyme activity. The conditions are as reported in the text. Initial velocities in presence (V) and absence (V^o) of glyoxylate are recorded as the rate of increase in absorbance at 340nm min^{-1}.

The effect of citrate, aspartate, AMP, ADP and ATP were also investigated on both malic enzyme activities. These metabolites at a concentration of 0.5 mM exerted little effect on either enzyme activity. In the presence of 0.5 mM L-malate and 0.5 mM $NAD(P)^+$ none of these metabolites exerted an effect greater than 8%.

AcetylCoA can be formed from pyruvate by the pyruvate dehydrogenase complex and hence is an eventual product of malate decarboxylation. Both malic enzyme activities are strongly inhibited by acetylCoA and the inhibition causes an increase in the apparent Km for L-malate as has been observed for the $NADP^+$-linked enzymes from H.cutirubrum (3) and E.coli (2). The acetylCoA thus exerts a competitive type of inhibition with respect to L-malate in P.denitrificans. AcetylCoA plays an important role in both lipogenesis

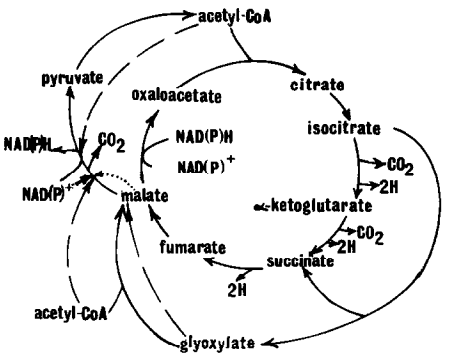

Fig. 3

Metabolic regulatory properties of malic enzyme from P.denitrificans ___ ___ ___ ___ inhibition of $NAD(P^+)$ enzyme, ---------- stimulation of $NADP^+$ enzyme.

and as an entrance point into the Krebs cycle; inhibition of malic enzyme by this metabolite can thus be envisaged as a mechanism for the control of both lipogenesis and Krebs cycle activity. Since acetylCoA may be considered to be a precursor of malate synthesis via malate synthetase inhibition of malate decarboxylation by acetylCoA is a mechanism whereby anaplerotic synthesis of malate would not become futile due to its subsequent decarboxylation.

Glyoxylate strongly inhibits the $NADP^+$-linked malic enzyme from P.denitrificans as has been found with the marine Pseudomonas described by Cazzulo and Massarini (7). If one consider glyoxylate as a product of isocitrate metabolism via the glyoxylate cycle and a precursor of malate synthesis via malate synthetase inhibition of malate decarboxylation via glyoxylate can be envisaged as a further mechanism whereby anaplerotic synthesis of malate would not become futile due to its subsequent decarboxylation. Fig.3 summarises the control properties of the malic enzyme from P.denitrificans and emphasises the important role played by acetylCoA and glyoxylate on the regulation of the metabolic activity of malic enzyme in this organism.

ACKNOWLEDGMENTS

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