REDUCTION OF OXALOACETATE BY PIG LIVER ISOCITRATE DEHYDROGENASE

J.A.ILLINGWORTH and K.F.TIPTON
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW, England

Received 13 February 1970

Pure isocitrate dehydrogenase from pig liver cytoplasm catalyses the reduction of oxaloacetate by NADPH at a rate comparable with that observed for the usual substrates. The products are NADP and D-malate, the 'unatural' isomer. High concentrations of magnesium (25 mM) are necessary for maximal activity, and the reaction is not appreciably reversible. These results are discussed in connection with the inhibition of the enzyme by mixtures of glyoxylate and oxaloacetate. The reduction is not thought to be of physiological importance.

1. Introduction

A number of workers have studied the inhibition of isocitrate dehydrogenases from a variety of sources by mixtures of glyoxylate and oxaloacetate [1–3]. During the course of fluorimetric studies of this phenomenon it became apparent that oxaloacetate is a substrate for highly purified pig liver cytoplasmic isocitrate dehydrogenase. This enzyme was recently obtained by us in a pure form [4] and satisfies all the usual criteria for homogeneity.

2. Methods

Tritiated L-malic acid was prepared by the action of fumarase (0.2 mg) on 2 millimoles of sodium malate dissolved in 2 ml of tritiated water (100 mCi/ml) at pH 7.4 overnight. Excess tritium was removed by freeze drying, then 2 ml of 2 N HCl was added to destroy fumarase activity. Sodium was removed by passage through a column of sulphonated polystyrene ('Zeo-Karb' 225) in the hydrogen form, and HCl by rotary evaporation. No attempt was made to remove the 20% unlabelled fumarate present in the product.

^{14}C-Oxaloacetate was generated in the spectrophotometer cuvette from 0.5 mM oxaloacetate and 0.5 mM ^{14}C-aspartate using 1 mg of glutamate/oxaloacetic transaminase in 2 ml of 50 mM pyridine acetate buffer pH 6.0 containing 25 mM $MgCl_2$. After 5 min the reduction of the oxaloacetic acid was started by the addition of NADPH to 0.6 mM final concentration and 0.1 mg pure isocitrate dehydrogenase, and the reaction followed to completion in the spectrophotometer. Approximately 4 μCi of ^{14}C-D-malate were obtained in this manner from 10 μCi of aspartate. Excess aspartate was removed by passage through a column of 'Zeo-Karb' 225 in the hydrogen form and the non-retarded material was mixed with ^{3}H-L-malic acid to give a tritium carbon ratio suitable for double label counting. This mixture was chromatographed on a 10 × 1 cm column of 'Permutit' De-acidite FFIP (strong base resin) in 0.5 M trimethylammonium bicarbonate buffer, pH 8, and 3 ml fractions collected.

Assay systems were as follows:

1) Isocitrate oxidation: 1 mM *dl*-sodium isocitrate, 1 mM $MgCl_2$ and 250 μM NADP in 50 mM triethanolammonium chloride, pH 8.1 at 25°.

2) Oxaloacetate reduction: 1 mM oxaloacetic acid, 25 mM $MgCl_2$ and 125 μM NADPH in either 50 mM pyridine acetate or 100 mM sodium phosphate, pH 6.0 at 25°.

3) D-malate oxidation: 33 mM D-malic acid, 5 mM $MgCl_2$ and 250 μM NADP in 0.2 M hydrazine chloride pH 9.0 at 25°. (Semicarbazide inhibits isocitrate dehydrogenase, so hydrazine, which is non inhibitory, was used both as buffer and as a trap for oxaloacetate.)

3. Results

In the assay systems described pure isocitrate dehydrogenase has a specific activity of 46 international units per mg protein for isocitrate oxidation, and 9 international units per mg for oxaloacetate reduction. The pH optimum actually lies somewhat below 6.0 for oxaloacetate reduction, but the instability of both enzyme and substrates at this pH makes it unsuitable for general use. The reaction was scarcely reversible under the conditions tested, the rate of D-malate oxidation was only 0.1% of the rate of isocitrate oxidation in the same system. D-malate oxidation is 90% inhibited by 3 mM glyoxylate.

The feeble rate with D-malate makes it desirable to have a positive identification of the products of oxaloacetate reduction. If oxaloacetate reduction is allowed to proceed to completion all of the NADPH consumed is subsequently regenerated on addition of excess isocitrate, so one product must almost certainly be NADP. By following oxaloacetate disappearance at 283 nm (the isosbestic point for the NADP/NADPH pair) and NADPH oxidation at 340 nm it is easy to establish that there is a one to one correspondence between NADPH oxidation and oxaloacetate reduction after allowing for a slight non-enzymic blank rate. As can be seen from fig. 1, the reaction product co-chromatographs with genuine malic acid. The reaction product must be D-malate as it is not a substrate for malate dehydrogenase. This is easily shown by adding NAD, hydrazine hydrate, and malate dehydrogenase to the normal assay cocktail after oxaloacetate reduction is complete. No NADH is produced although only 5 nmoles of genuine malate are needed to produce a detectable rise in absorbance.

In the absence of CO_2 2-oxoglutarate is a potent competitive inhibitor of oxaloacetate reduction. Succinate and fumarate, but not maleate are also somewhat inhibitory. Oxaloacetate samples themselves contain traces of an inhibitor (perhaps a condensation product) which is rapidly destroyed in the presence of $MgCl_2$. In consequence an accelerating initial rate is observed unless the assay cocktail is incubated for three min before addition of enzyme.

The optimal magnesium concentration for oxaloacetate reduction is some 25 times higher than the optimal concentration for isocitrate oxidation or 2-oxoglutarate reductive carboxylation. We suspect that the

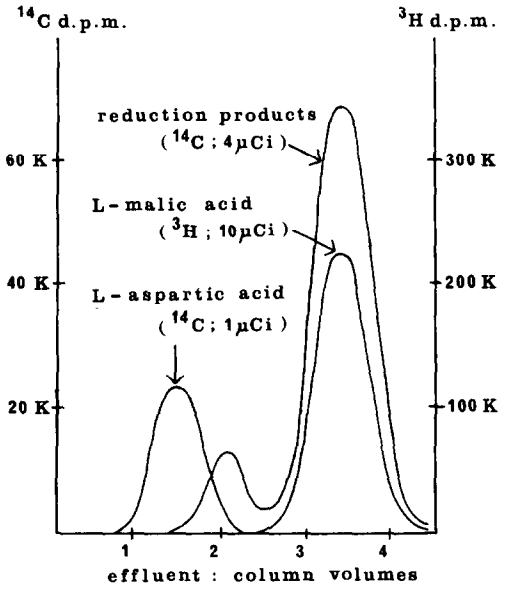


Fig. 1. Chromatography of oxaloacetate reduction products on a strong base ion exchange resin.

(The elution positions of aspartic acid and oxaloacetate acid were determined in separate experiments. Oxaloacetic acid is very strongly retarded in this system and is not shown. The minor peak is probably pyruvate.)

true substrate may be the magnesium complex of the enol form of oxaloacetate. Formation of such a complex may be observed if the difference spectrum of oxaloacetate + $MgCl_2$ is observed relative to an oxaloacetate blank in the region of 290 nm at pH values around neutrality. In alkali this difference spectrum is greatly intensified. A similar result is obtained with phenylpyruvate at 320 nm, which suggests that these complexes involve a five membered ring chelate rather than the six membered structure which is only possible with oxaloacetate. The high dissociation constant (25 mM) which may be calculated from these spectra agrees well with the high magnesium requirement for the enzymic reaction. Isocitrate dehydrogenase does not appear to decarboxylate oxaloacetate to any extent. This again might be expected if the enol form of oxaloacetate were the true substrate.

Oxaloacetate reduction is progressively inhibited by 0.1 mM glyoxylate. This inhibition has an absolute requirement for NADP, as is illustrated by the results in

table 1. NADPH will not replace NADP, and in systems containing no NADP no inhibition is observed until a small amount of NADPH has been oxidised by the oxaloacetate present.

It has been suggested [2] that the inhibition due to glyoxylate plus oxaloacetate results from their condensation to form the substrate analogue, oxalomalate. It is also possible that this condensation is catalysed by the enzyme itself [5]. Isocitrate dehydrogenase is clearly able to bind oxaloacetate to the active site, and a subsequent condensation to form oxalomalate would be closely analogous to the normal fixation of CO_2 during the reductive carboxylation of 2-oxoglutarate. It is clear from the experiments in which only glyoxylate and oxaloacetate were preincubated together before addition of the enzyme that the concentration of inhibitor does not increase with time in the absence of the enzyme, since both the initial rate and the subsequent time course of inhibition were the same in all cases.

There is a second possible explanation. Oxaloacetate is not stable in the reaction buffer, and at 283 nm a slow fall in absorbance results from a magnesium dependent non-enzymic decarboxylation. Oxalomalate [2] is reported to be even more unstable than oxaloacetate, and undergoes an extremely rapid decarboxylation in the presence of magnesium. When no enzyme is present the addition of glyoxylate to solutions of oxaloacetate in the assay buffer accelerates the fall in absorbance at 283 nm. The decarboxylation rate of 1 mM oxaloacetate is doubled by 5 mM glyoxylate in the presence of 25 mM $MgCl_2$.

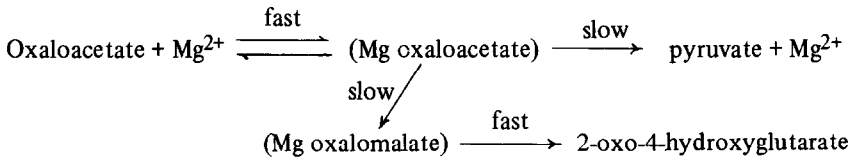
The decomposition product of oxalomalate has not been positively identified. There are a number of possible products, however the end result is the same in all cases - a steady state level of oxalomalate is estab-

Table 1

Inhibition experiments in 100 mM sodium phosphate pH 6.0 containing 25 mM $MgCl_2$ at 25°. The following reagent concentrations were employed: 50 μM NADP, 125 μM NADPH, 1.0 mM oxaloacetate, 0.1 mM glyoxylate. Unless otherwise stated the preincubations were of 3 min duration. 0.005 mg enzyme was added per assay and the total reaction volume was 2 ml.

Mixture preincubated in cuvette	Additions at start of reaction	Initial rate and time for 50% inhibition
Oxaloacetate, NADPH,	Enzyme	40 nmoles/min no inhibition
Oxaloacetate, NADPH, NADP	Enzyme	40 nmoles/min no inhibition
Oxaloacetate, NADPH, NADP, glyoxylate	Enzyme	40 nmoles/min 20 sec
Oxaloacetate, NADPH, glyoxylate	Enzyme	40 nmoles/min 60 sec approximately*
Enzyme, oxaloacetate, NADP, glyoxylate	NADPH	12 nmoles/min 20 sec
Enzyme, oxaloacetate, glyoxylate	NADPH	40 nmoles/min 60 sec approximately*
Oxaloacetate and glyoxylate for times ranging from 3 to 30 min	NADP, NADPH, and Enzyme	40 nmoles/min 20 sec approximately

* in these cases the inhibition is not truly first order.



lished in a few sec after adding glyoxylate, and this extremely low level thereafter changes only slowly with time as the oxaloacetate and glyoxylate are gradually depleted.

It is not possible to distinguish between these two cases at present, and indeed this will be extremely difficult. It is also unclear why oxalomalate should inhibit the enzyme and this problem is now under active study.

Acknowledgements

We are grateful to Dr. C.J.R.Thorne for a gift of pure malate dehydrogenase, and to Dr. H.B.F.Dixon who supplied the glutamate/oxaloacetic transaminase used in this work. We are indebted to Dr. A.R.Hunter for his assistance with the double label experiments. J.A.I. thanks the Science Research Council for a research studentship.

References

[1] B.Payes and G.G.Laties, Biochem. Biophys. Res. Commun. 10 (1963) 460.
[2] A.Ruffo, E.Testa, A.Adinolfi, G.Pelizza and R.Moratti, Biochem. J. 103 (1967) 19.
[3] J.J.Marr and M.W.Weber, J. Biol. Chem. 244 (1969) 5709.
[4] J.A.Illingworth and K.F.Tipton, Biochem. J., in press.
[5] P.K.Tubbs, J.F.A.Chase, B.Middleton and J.A.Illingworth, in preparation.