Kinetic mechanism of *Escherichia coli* isocitrate dehydrogenase and its inhibition by glyoxylate and oxaloacetate

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1. The inhibition of *Escherichia coli* isocitrate dehydrogenase by glyoxylate and oxaloacetate was examined. The shapes of the progress curves in the presence of the inhibitors depended on the order of addition of the assay components. When isocitrate dehydrogenase or NADP⁺ was added last, the rate slowly decreased until a new, inhibited, steady state was obtained. When isocitrate was added last, the initial rate was almost zero, but the rate increased slowly until the same steady-state value was obtained. 2. Glyoxylate and oxaloacetate gave competitive inhibition against isocitrate and uncompetitive inhibition against NADP⁺. Product-inhibition studies showed that isocitrate dehydrogenase obeys a compulsory-order mechanism, with coenzyme binding first. Glyoxylate and oxaloacetate bind to and dissociate from isocitrate dehydrogenase slowly. These observations can account for the shapes of the progress curves observed in the presence of the inhibitors. 3. Condensation of glyoxylate and oxaloacetate produced an extremely potent inhibitor of isocitrate dehydrogenase. Analysis of the reaction by h.p.l.c. showed that this correlated with the formation of oxalomalate. This compound decomposed spontaneously in assay mixtures, giving 4-hydroxy-2-oxoglutarate, which was a much less potent inhibitor of the enzyme. Oxalomalate inhibited isocitrate dehydrogenase competitively with respect to isocitrate and was a very poor substrate for the enzyme. 4. The data suggest that the inhibition of isocitrate dehydrogenase by glyoxylate and oxaloacetate is not physiologically significant.

INTRODUCTION

There has recently been great interest in the control of the isocitrate dehydrogenase (NADP-linked, EC 1.1.1.42) from *Escherichia coli*. The activity of this enzyme is regulated by a phosphorylation/dephosphorylation mechanism whose role is to apportion flux between the citric acid cycle and the glyoxylate bypass during growth on acetate (reviewed by Nimmo, 1984). Many NADP-dependent isocitrate dehydrogenases can also be inhibited in a concerned fashion by glyoxylate and oxaloacetate (Illingworth & Tipton, 1970; Johanson & Reeves, 1977; Marr & Weber, 1969*a,b*; Ozaki & Shiiro, 1968; Shiiro & Ozaki, 1968), and it has been suggested that this may be physiologically significant in terms of the regulation of the branch-point at isocitrate (Marr & Weber, 1969*a,b*; Ozaki & Shiiro, 1968). However, this inhibition is observed with most or all NADP-dependent isocitrate dehydrogenases, including those from mammalian sources, but not with NAD-dependent isocitrate dehydrogenases, even from organisms such as yeast that use the glyoxylate bypass. The physiological relevance of the inhibition is therefore highly questionable.

It is, however, clear that the physiological significance of the inhibition can only be assessed accurately when its mechanism is understood. This mechanism has been a subject of considerable controversy. Ruffo *et al.* (1962, 1967) showed that glyoxylate and oxaloacetate can condense to form oxalomalate and that the condensation product inhibits isocitrate dehydrogenase. However, several groups have reported that the condensation of glyoxylate and oxaloacetate is too slow to account for the inhibition of isocitrate dehydrogenase by these com-

pounds (Johanson & Reeves, 1977; Marr & Weber, 1969*a*; Shiiro & Ozaki, 1968). Both Johanson & Reeves (1977) and Marr & Weber (1969*a*) concluded that the condensation product was not responsible for the inhibition. In addition, the nature of the condensation product itself has been questioned. Although Ruffo *et al.* (1962) reported that oxalomalate was stable at neutral pH, Johanson & Reeves (1977) and Payes & Laties (1963) showed that it decarboxylated readily under these conditions to form 4-hydroxy-2-oxoglutarate.

There is thus still some uncertainty as to the nature of the products formed during the condensation of glyoxylate and oxaloacetate and whether these are responsible for the inhibition of isocitrate dehydrogenase. I therefore decided to study the inhibition of homogeneous *E. coli* isocitrate dehydrogenase by glyoxylate and oxaloacetate, using h.p.l.c. to analyse the intermediates that are produced during the condensation. The results show that oxalomalate is an extremely potent competitive inhibitor of isocitrate dehydrogenase. Glyoxylate and oxaloacetate can inhibit isocitrate dehydrogenase less well without condensation. The results also show that *E. coli* isocitrate dehydrogenase obeys a compulsory-order mechanism.

EXPERIMENTAL

Materials

The active form of isocitrate dehydrogenase (specific activity 202 $\mu\text{mol/min per mg}$ of protein) was purified from *E. coli* ML308 (Borthwick *et al.*, 1984). Glutamate dehydrogenase, malate dehydrogenase and oxaloacetic

acid were obtained from Boehringer Corp. (London), Lewes, Sussex, U.K. Oxalomalate and glyoxylic acid were from Sigma (London) Chemical Co., Poole, Dorset, U.K. Other materials were obtained as described previously (Borthwick *et al.*, 1984; Nimmo *et al.*, 1984; Nimmo & Nimmo, 1984).

Methods

Assay procedures. Isocitrate dehydrogenase was routinely assayed spectrophotometrically at 37 °C in the presence of 0.5 mM-MnCl₂, 0.4 mM-NADP⁺ and 2.5 mM-DL-isocitrate. The buffers used were either 50 mM-Mes/NaOH, pH 6.2, or 50 mM-Mops/NaOH, pH 7.3. Product-inhibition studies were carried out at 25 °C and pH 6.2, by using a Hitachi-Perkin/Elmer MPF2A spectrofluorimeter (excitation 340 nm, emission 460 nm) to detect NADPH formation, at a full-scale sensitivity of 5 μ M-NADPH. Assays were conducted in duplicate; where only a single point is shown, the results obtained were identical. Line fitting was as described by Meek & Nimmo (1983).

Oxaloacetate and pyruvate were assayed spectrophotometrically by measuring oxidation of NADH in the presence of malate dehydrogenase and lactate dehydrogenase respectively. 4-Hydroxy-2-oxoglutarate was assayed by reductive amination catalysed by glutamate dehydrogenase (Johanson & Reeves, 1977).

Condensation of glyoxylate with oxaloacetate. Solutions of oxaloacetic acid and glyoxylic acid were prepared immediately before use. The oxaloacetic acid was contaminated with some 1% pyruvic acid, as judged by enzymic assay. The glyoxylic acid (5 μ mol) was analysed by h.p.l.c. and no impurities were detected. Glyoxylic acid and oxaloacetic acid (each 20 mM) were incubated at pH 7.5 and 0 °C in 0.1 M-potassium phosphate. To monitor the production of inhibitory material, isocitrate dehydrogenase (0.1 μ g/ml) was assayed in the standard conditions at pH 6.2. A sample of the incubation was added to an assay that was in progress, and the initial and inhibited rates were measured (cf. Fig. 1, trace f). At the concentrations used here (5–50 μ M), freshly prepared glyoxylate and oxaloacetate do not inhibit isocitrate dehydrogenase significantly. One unit of inhibitor is defined as the amount that gives 50% inhibition in these conditions. Plots of percentage inhibition against amount of inhibitor gave a rectangular hyperbola (not shown), and a double-reciprocal plot of the data was used as a standard curve. The amounts of inhibitor in different samples were estimated from assays that gave 25–75% inhibition.

H.p.l.c. This was carried out on an Aminex HPX-87H organic acids column (Bio-Rad Laboratories, Watford, Herts, U.K.) equilibrated in 3.5 mM-H₂SO₄, at a flow rate of 1 ml/min. Sample detection was at 215 nm. Data were collected and peaks were integrated by using a Gilson Data Master model 620 interfaced with an Apple II microcomputer.

RESULTS

Inhibition of glyoxylate and oxaloacetate

In preliminary experiments, the effects of altering the order of addition of the assay components (isocitrate

Fig. 1. Progress curves of isocitrate dehydrogenase assays

The standard assay conditions given in the Experimental section were used. Traces (a)–(e): pH 7.3, 0.5 μ g of enzyme/ml. Trace (a): the assay was started by addition of enzyme. Trace (b): as (a), glyoxylate and oxaloacetate (each 0.15 mM) were added at the points marked G and O respectively. Trace (c): as (a), but glyoxylate and oxaloacetate were added to the cuvette before the enzyme. Trace (d): as (c), but the assay was started by the addition of isocitrate. Trace (e): as (a), but at the point marked GO 1 μ l of a mixture of glyoxylate and oxaloacetate incubated together for 16 h (see the Experimental section) was added to the cuvette (final volume 1 ml). Trace (f): as (e), but at pH 6.2 with 0.1 μ g of enzyme/ml.

dehydrogenase, isocitrate, NADP⁺, glyoxylate and oxaloacetate) were monitored. The results were very striking, and typical progress curves are shown in Fig. 1. In the absence of the inhibitors, the order of addition of isocitrate dehydrogenase, isocitrate and NADP⁺ was immaterial; addition of the last component resulted in the immediate establishment of a linear rate of increase of A_{340} (e.g. trace a). In the standard assay conditions this rate was maintained until some 90% of the coenzyme had been reduced, indicating that the enzyme was stable in these assay conditions.

Neither glyoxylate alone nor oxaloacetate alone, at concentrations of 0.5 mM, affected the activity of isocitrate dehydrogenase in the standard assay conditions. However, inhibition was observed when both compounds were present. When the compounds were added sequentially to 0.15 mM to an assay of isocitrate dehydrogenase that was in progress, slowly increasing inhibition of isocitrate dehydrogenase was observed (Fig. 1, trace b). This developed only after the addition of the second inhibitor, irrespective of the order of the addition (results not shown). When assays were initiated by the addition of either isocitrate dehydrogenase (trace c) or NADP⁺ (results not shown) to cuvettes already containing glyoxylate and oxaloacetate, the initial rate of the reaction was unaffected by the inhibitors, but the rate decreased slowly until a new, inhibited, steady-state rate was established. However, when assays were initiated by the addition of isocitrate to cuvettes already containing isocitrate dehydrogenase, NADP⁺, glyoxylate and oxaloacetate, a completely different pattern was observed. The initial rate of NADPH formation was almost zero, but a slow acceleration was observed until a final steady-state rate was obtained (trace d). This was equal to the final steady-state rate observed when the reaction was initiated by addition of isocitrate dehydrogenase or NADP⁺.

To investigate the possible role of formation of an adduct between glyoxylate and oxaloacetate in the inhibition of isocitrate dehydrogenase, the two inhibitors were pre-mixed at 20 mM each and incubated at 0 °C and pH 7.5 for several hours. When samples of this mixture were added to an assay of isocitrate dehydrogenase that was in progress, the results shown in trace *e* (Fig. 1) were obtained. Isocitrate dehydrogenase activity decreased abruptly after a short lag, but then slowly recovered again over a period of 5–10 min. If the assay of isocitrate dehydrogenase was carried out at pH 6.2 rather than pH 7.3, the abrupt decline in isocitrate dehydrogenase activity was again observed, but there was no detectable increase in activity over the next 10 min (trace *f*).

It is shown below that the inhibition of isocitrate dehydrogenase illustrated in traces (*e*) and (*f*) is caused by the formation of an adduct between glyoxylate and oxaloacetate. However, the rate of formation of this adduct (see Fig. 6) cannot account for the inhibitions shown in traces (*b*)–(*d*). It therefore appears that isocitrate dehydrogenase can be inhibited by glyoxylate and oxaloacetate irrespective of the formation of an adduct between them. However, the concentrations of the inhibitors used in traces (*b*)–(*f*) (Fig. 1) show that inhibition by the adduct is much more potent than that by freshly prepared glyoxylate and oxaloacetate. An explanation of the shapes of the progress curves shown in traces (*b*)–(*d*) is given in the next section.

Kinetic mechanism of isocitrate dehydrogenase

The kinetic nature of the inhibition of isocitrate dehydrogenase by glyoxylate and oxaloacetate was examined in experiments in which the inhibitors were added separately to the cuvettes immediately before the assays to prevent the occurrence of any condensation (see Fig. 6). The assays were initiated by addition of either isocitrate dehydrogenase or isocitrate (see Fig. 1, traces *c* and *d*); the two methods gave essentially identical results. The inhibition observed was competitive with respect to isocitrate and uncompetitive with respect to NADP⁺ (Fig. 2).

These results suggested that isocitrate dehydrogenase may obey a compulsory-order mechanism with NADP⁺ bound first, and that glyoxylate and oxaloacetate can interact with the isocitrate-binding site. The kinetic mechanism of the enzyme was therefore investigated by using product inhibition. Double-reciprocal plots with respect to isocitrate at different concentrations of NADP⁺ gave a family of intersecting lines indicative of a sequential mechanism (not shown). NADPH gave competitive and mixed inhibition with respect to NADP⁺ and isocitrate respectively (Fig. 3). 2-Oxoglutarate gave uncompetitive and mixed inhibition with respect to NADP⁺ and isocitrate respectively (Fig. 4).

These results give further strong support to the view that isocitrate dehydrogenase obeys a compulsory-order mechanism, with coenzyme binding first. The observation that 2-oxoglutarate was an uncompetitive inhibitor with respect to NADP⁺ at sub-saturating concentrations of isocitrate suggests that products are released in the order CO₂, 2-oxoglutarate, NADPH. With this order of release, one might expect 2-oxoglutarate to be an uncompetitive inhibitor against both NADP⁺ and isocitrate. The mixed inhibition against isocitrate can be accommodated by postulating formation of a dead-end E·NADP⁺·2-oxoglutarate complex. The probable exis-

Fig. 2. Inhibition of isocitrate dehydrogenase by glyoxylate and oxaloacetate

Double-reciprocal plots of velocity against (a) [isocitrate] at 0.05 mM-NADP⁺ and (b) [NADP⁺] at 0.02 mM-DL-isocitrate. The concentrations of glyoxylate and oxaloacetate were 0 (▲), 0.04 mM each (□) and 0.06 mM each (●).

tence of this complex and the difficulty in studying inhibition by CO₂ precluded calculation of all the individual kinetic constants.

The progress curves shown in Fig. 1, traces (*b*)–(*d*), can be explained on the basis of this ordered mechanism if the additional assumption is made that glyoxylate and oxaloacetate bind to and dissociate from isocitrate dehydrogenase relatively slowly. The inhibitors can only interact with isocitrate dehydrogenase in the presence of NADP⁺. Thus, in assays in which the reaction is initiated by addition of NADP⁺ or isocitrate dehydrogenase, or in which glyoxylate and oxaloacetate are added to an assay that is in progress, slow binding of the inhibitors occurs and the observed rate decreases until the steady state is reached (Fig. 1, traces *b* and *c*). In contrast, whenever the reaction is initiated by the addition of isocitrate, the enzyme is preincubated with NADP⁺, glyoxylate and oxaloacetate. Binding of the inhibitors can proceed to saturation, since isocitrate, which competes with the inhibitors, is absent. Thus the initial rate observed on addition of isocitrate is essentially zero. The inhibitors

Fig. 3. Inhibition of isocitrate dehydrogenase by NADPH

Double-reciprocal plots of velocity against (a) [isocitrate] at 0.05 mM- $NADP^+$ and (b) [$NADP^+$] at 0.05 mM-DL-isocitrate. The concentrations of NADPH were 0 ($\Delta, \blacktriangle$), 0.01 mM ($\square$) and 0.025 mM ($\bullet$).

then dissociate slowly until the final steady-state rate is obtained (trace *d*). This explanation implies that, when reactions are initiated by addition of isocitrate, the initial rate observed should depend on the time for which isocitrate dehydrogenase, $NADP^+$, glyoxylate and oxaloacetate were preincubated. This was indeed the case; a preincubation time of 60–90 s was required to give full inhibition. However, the dependence of initial rate on preincubation time was not studied in detail.

Condensation of glyoxylate and oxaloacetate

In view of the observation (Fig. 1, traces *e* and *f*) that preincubation of glyoxylate and oxaloacetate results in generation of a potent inhibitor of isocitrate dehydrogenase, the condensation of the two compounds was monitored by using h.p.l.c. to separate and quantify the reaction products. The products were stable under the

Fig. 4. Inhibition of isocitrate dehydrogenase by 2-oxoglutarate

Double-reciprocal plots of velocity against (a) [isocitrate] at 0.04 mM- $NADP^+$ and (b) [$NADP^+$] at 0.03 mM-DL-isocitrate. The 2-oxoglutarate concentrations were 0 ($\blacktriangle$), 1 mM ($\square$) and 2 mM ($\bullet$).

acid conditions used for the chromatography; addition of H_2SO_4 to pH 2, followed by incubation for several hours, did not alter the traces that were obtained. Representative traces for samples taken during a condensation are shown in Fig. 5. The peaks corresponding to glyoxylate and oxaloacetate are labelled G and O respectively. Three new peaks, labelled A, B and C, appear during the incubation. Peak C is co-eluted with pyruvate, and is derived from decarboxylation of oxaloacetate; it appeared at the same rate in control experiments from which glyoxylate was omitted (results not shown). However, the appearance of peaks A and B was dependent on the presence of both glyoxylate and oxaloacetate.

Fig. 5. Analysis of the condensation of glyoxylate and oxaloacetate

Incubations and h.p.l.c. were as described in the Experimental section. (a) Samples (0.01 ml) were taken at 0, 4, 10, 24 and 50 h (traces a–e respectively). (b) Trace a: sample taken after 7 days incubation. Trace b: commercial oxalomalate (0.01 ml of a 20 mM solution). Trace c: as trace d of Fig. 5(a).

Fig. 6. Condensation of glyoxylate and oxaloacetate

Areas under peak A ($\blacktriangle$) and peak B ($\blacksquare$) were taken from traces similar to those shown in Fig. 5. The amounts of inhibitory material ($\square$) and 4-hydroxy-2-oxoglutarate ($\circ$) were determined as described in the Experimental section. Data from three separate experiments are shown.

Progress of the reaction was monitored by measurement of peak areas, and the results are shown in Fig. 6. The amount of peak A rose over the first 6 h of the incubation, remained at a steady-state value over the period 8–25 h and then fell slowly. In contrast, little peak B was produced in the first 6–8 h of the incubation. There was then approximately linear accumulation of peak B with

time over the next 30 h. The data suggest strongly that peak B is derived from peak A.

The amount of inhibitory material generated during the incubation of glyoxylate and oxaloacetate was assessed as described in the Experimental section, and the results are also shown in Fig. 6. The effects of warming the condensation products to 37 °C are shown in Fig. 7.

oxaloacetate can inhibit isocitrate dehydrogenase in a concerted fashion, as suggested by other groups (see the Introduction). The reaction progress curves for isocitrate dehydrogenase in the presence of glyoxylate and oxaloacetate are very unusual (Fig. 1); qualitatively similar data were presented, but not explained, by Johanson & Reeves (1977). The shapes of the curves can in fact be explained on the basis of three assumptions. These are that the enzyme obeys a compulsory-order mechanism with coenzyme binding first, that glyoxylate and oxaloacetate bind at the isocitrate-binding site, and that binding and dissociation of glyoxylate and oxaloacetate are slow. Good kinetic evidence for the first two points is given above. There seems to be no obvious explanation for the third point, and the data presented here do not indicate whether the binding of glyoxylate and oxaloacetate to the enzyme is random or ordered.

Previous workers have suggested that the inhibition of isocitrate dehydrogenase by glyoxylate and oxaloacetate may be physiologically significant (see the Introduction). However, this inhibition is not restricted to enzymes from organisms that can synthesize glyoxylate. To my knowledge, there are no reliable data available on the intracellular concentrations of glyoxylate and oxaloacetate in *E. coli*. However, by analogy with mammalian cells, the intracellular concentration of oxaloacetate is probably very low, perhaps in the micromolar range. The intracellular concentration of *threo*-D₅-isocitrate in *E. coli* growing on acetate is some 0.5 mM (El-Mansi *et al.*, 1985). It can be calculated from the data in Fig. 2 that at this concentration of isocitrate the presence of 0.06 mM of each of glyoxylate and oxaloacetate would inhibit isocitrate dehydrogenase by only some 3%. This clearly suggests that the inhibition is not physiologically significant. It is more likely that all NADP-dependent isocitrate dehydrogenases, but not the NAD-dependent enzymes, share some common structural feature that allows inhibition by glyoxylate and oxaloacetate; it is already known that one inhibitor, 3-hydroxybutane-1,2,3-tricarboxylate, can discriminate between the enzymes in this way (Plaut *et al.*, 1975).

It has been reported that mammalian NADP-dependent isocitrate dehydrogenases obey a random-order kinetic mechanism (e.g. Londesbough & Dalziel, 1970; Uhr *et al.*, 1974). In contrast, the data for the *E. coli* enzyme reported here strongly suggest an ordered mechanism. This is supported by the inhibition patterns given by NADPH, 2-oxoglutarate, oxalomalate, glyoxylate and oxaloacetate. It is also consistent with the observation that NADP⁺ causes a marked conformational change in isocitrate dehydrogenase (Garland & Nimmo, 1984).

Aitken & Brown (1972) proposed a compulsory order mechanism for the isocitrate dehydrogenase of *Halobacterium salinarium*. However, Marr & Weber (1973) concluded that the isocitrate dehydrogenase of *Salmonella*

typhimurium obeyed a random-order mechanism, since all the product-inhibition patterns were competitive. This enzyme resembles the *E. coli* enzyme in many ways, for example size, subunit organization and control by phosphorylation, and there is therefore no obvious explanation for this discrepancy. However, it should be noted that it is unusual for all the product-inhibition patterns given by a random-order mechanism to be competitive, because of the formation of abortive complexes (e.g. Dixon & Webb, 1979), and the patterns for the *Salmonella* enzyme should perhaps be re-investigated by using the sensitive fluorimetric assay described here.

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