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Homocitrate synthase from *Penicillium chrysogenum*

Localization, purification of the cytosolic isoenzyme, and sensitivity to lysine

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Subcellular fractionation of cell-free extracts obtained by nitrogen cavitation showed that *Penicillium chrysogenum* Q176 contains a cytosolic as well as a mitochondrial homocitrate synthase activity. The cytosolic isoenzyme was purified about 500-fold, and its kinetic and molecular properties were investigated. Native homocitrate synthase shows a molecular mass of 155±10 kDa as determined by gel filtration and a pH of 4.9±0.1 as determined by chromatofocusing. The kinetic behaviour towards 2-oxoglutarate is hyperbolic, with $K_m = 2.2$ mM; with respect to acetyl-CoA the enzyme shows sigmoidal saturation kinetics, with $[S]_{0.5} = 41$ μM and $h = 2.6$. The enzyme was inhibited strongly by L-lysine ($K_i = 8 \pm 2$ μM; 50% inhibition by 53 μM at 6 mM-2-oxoglutarate), competitively with 2-oxoglutarate, in protamine sulphate-treated and desalted cell-free extracts and in partially purified preparations. The extent of this inhibition was strongly pH-dependent. Both isoenzymes are equally susceptible to inhibition by lysine. The same inhibition pattern is shown by the enzyme from strain D6/1014A, which is a better producer of penicillin than strain Q176.

INTRODUCTION

Homocitrate synthase [3-hydroxy-3-carboxyadipate 2-oxoglutarate-lyase (CoA-acetylating), EC 4.1.3.21] catalyses the first committed reaction of the α-aminoadipate pathway of lysine biosynthesis, which specifically occurs in yeast, higher fungi and euglenids (Bhattacharjee, 1985). The enzyme is considered to be regulatory in the pathway: in *Saccharomyces cerevisiae* two isoenzymes have been reported, both of which are subject to feedback inhibition by lysine, but only one appears to be repressed by lysine (Tucci & Ceci, 1972). Both inhibition and repression by lysine have also been reported, preliminarily for the *Neurospora crassa* enzyme (Hogg & Broquist, 1968). Tracy & Kohlhaw (1975) reported a regulation of homocitrate synthase from *S. cerevisiae* by CoA in the presence of bivalent metal ions such as Zn^{2+}. However, all these data have been obtained from studies using cell-free extracts, and homocitrate synthase has so far not been purified from any organism investigated, except for a small enrichment (12-fold) from yeast as described by Tucci & Ceci (1972).

Regulation of homocitrate synthase from the penicillin-producing fungus *Penicillium chrysogenum* has been investigated with respect to the role of α-aminoadipate as the starting amino acid for biosynthesis of this antibiotic (Demain, 1983), but no clear evidence has been obtained. Masurekar & Demain (1974) were unable to demonstrate lysine inhibition of *P. chrysogenum* homocitrate synthase *in vitro*, but Demain & Masurekar (1974) provided evidence that the enzyme *in vivo* is inhibited rather than repressed by lysine. In contrast, Luengo *et al.* (1980) reported the occurrence of both inhibition as well as repression, but they used very high concentrations of lysine (50 mM). Homocitrate synthase from high-penicillin-producing strains of *P. chrysogenum* has been reported to be less sensitive to lysine inhibition and/or repression (Luengo *et al.*, 1979).

Here we describe the preparation of highly purified homocitrate synthase from *P. chrysogenum* and some of the properties of this enzyme. We also demonstrate that the enzymes from two strains differing in their penicillin-producing capacity exhibit similar high sensitivity to inhibition by lysine.

MATERIALS AND METHODS

Organisms

The *P. chrysogenum* strain Q176 was used throughout this study. Strain D6/1014A was included only to determine the sensitivity of its homocitrate synthase towards L-lysine. Both strains have been described previously (Jaklitsch *et al.*, 1985, 1986, 1987).

Buffers used in this study

Buffer A: 100 mM-Tris/HCl, pH 7.8, containing 10% (v/v) glycerol. Buffer B: 50 mM-Tris/HCl, pH 8. P/M-buffer: 200 mM-potassium phosphate, pH 6.4, containing 0.6 M-mannitol. Tris/S-buffer: 10 mM-Tris/HCl, pH 7.3, containing 0.25 M-sucrose, 2 mM-$MgCl_2$, 1 mM-EDTA and 4 μM-phenylmethanesulphonyl fluoride.

Conditions of cultivation and preparation of cell-free extracts

The fungi were grown as described by Jaklitsch *et al.* (1987) but the second stage of cultivation was omitted. Cell-free extracts were prepared by sonication as described by Jaklitsch *et al.* (1985), with buffer A, which additionally contained 1 mM-phenylmethanesulphonyl fluoride, as suspending buffer. Centrifugation of homogenates at 11500 *g* for 20 min yielded cell-free extracts containing 12–15 mg of protein/ml (for volumes up to 20 ml) or 6–7 mg of protein/ml (for volumes of 60–80 ml) in larger-scale preparations designed for enzyme purification.

For subcellular-fractionation studies strain Q176 was grown to a mycelium density of 7–10 g wet wt./litre and treated as described below.

Subcellular fractionation

A modification of the procedure described by Osmani & Scrutton (1985) was used. Mycelium was harvested by filtration

Abbreviation used: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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through muslin cloth and washed with distilled water. Then 5 g of squeezed mycelium was suspended in 40 ml of P/M-buffer containing 80 mg of Novozyme 234 (Novo Industri, Bagsvaerd, Denmark), and shaken at 30 °C for 30 min. The mycelium was recovered by filtration through muslin, washed with 100 ml of P/M-buffer and 300 ml of Tris/S-buffer, resuspended in 30 ml of Tris/S-buffer, and subjected to nitrogen cavitation at 5.2 MPa and 4 °C with an incubation time of 35 min. Cell debris was removed by filtration through muslin and subsequent centrifugation at 800 *g* for 15 min at 0–4 °C. The resulting cell-free extract, which contained 1.0–1.9 mg of protein/ml, was centrifuged at 45000 *g* for 25 min, yielding a supernatant which is designated the cytosolic fraction. The pellet was resuspended in 3 ml of Tris/S-buffer and sonicated for 10–20 s, yielding the mitochondrial fraction.

Preparation of extracts for electrophoresis

Cell-free extracts and subcellular fractions were prepared as described under 'Subcellular fractionation', but the mitochondrial pellet was suspended in 3 ml of Tris/S-buffer, recentrifuged at 45000 *g* for 15 min, and resuspended in 200 μl of buffer A. Cell-free extract and the mitochondrial preparation were sonicated for 5 × 20 s at 0–5 °C. Disruption of mitochondria was assessed by the determination of citrate synthase activity in the absence and presence of 0.02% (v/v) Triton X-100. The sonicated mitochondrial preparation was used directly for electrophoresis. Samples (2.5 ml) of sonicated extract and of the cytosolic fraction were desalted with buffer A on PD-10 columns (Pharmacia, Uppsala, Sweden) and centrifuged at 34000 *g* for 10 min. Portions (2 ml) of the supernatants were concentrated to approx. 0.5 ml with Centricon 30 micro-concentrators (Amicon, Danvers, MA, U.S.A.) at 4000 *g* and 0–4 °C for 1 h. Samples (1–2 μl) of these preparations were loaded on the cellulose acetate strips.

Assay conditions for homocitrate synthase

Since homocitrate synthase is inhibited by an unidentified substance of high molecular mass present in the cell-free extract, assays were performed after protamine sulphate precipitation, followed by desalting the supernatant fraction on PD-10 columns.

Two assay procedures were used throughout these studies.

(1) Radioactive assay using [1-^{14}C]acetyl-CoA. A reaction mixture consisting of 0.05 μCi of [^{14}C]acetyl-CoA (equivalent to 40 μM-acetyl-CoA), 4 mM-2-oxoglutarate, cell-free extract (equivalent to 0.1 mg of protein) and buffer B was incubated in a total volume of 250 μl for 30 min at room temperature. The reaction was stopped by adding 30 μl of conc. HCl, and the protein was removed by centrifugation (Eppendorf). Unlabelled homocitric lactone (40 nmol/μl of supernatant) was added as carrier, and homocitrate was separated from the assay mixture by t.l.c. on silica gel, with diethyl ether/benzene/formic acid/water (21:9:7:2, by vol.; solvent 1) or butan-1-ol/formic acid/water (5:1:1, by vol.; solvent 2) as developing solvents. The spots were detected by spraying with Bromocresol Green, removed from the plates with a spatula, and eluted with 0.7 ml of 50 mM-sodium phosphate, pH 7, for 10 min. After addition of 9 ml of scintillation cocktail to the resulting suspension, radioactivity was counted in a liquid-scintillation counter (LKB). Enzyme activity is expressed as c.p.m. of [^{14}C]homocitrate formed/min under these conditions.

Fluorography of the t.l.c. plates showed that no additional radioactive products are formed under the conditions described; blanks lacking 2-oxoglutarate showed no radioactive products. This assay is suitable only for semi-quantitative detection of homocitrate synthase, since, owing to use of a non-saturating concentration of acetyl-CoA, homocitrate synthesis is not linear with time over the incubation period used. The assay is not suitable for initial-rate studies.

For fractionation studies, this assay was modified by using 0.1 μCi of [^{14}C]acetyl-CoA (80 μM-acetyl-CoA), 24 mM-2-oxoglutarate and 0.02% Triton X-100 in the assay mixture, and an incubation time of 120 min. Protamine sulphate precipitation was omitted. Owing to the low levels of enzyme activity after nitrogen cavitation, this assay was suitable for the determination of homocitrate synthase activity (see the Results section).

(2) Discontinuous 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) assay. The procedure is a modification of that used by Dr. J. K. Bhattacharjee (personal communication): cell-free extract (equivalent to 0.15 mg of protein) was incubated with 0.5 mM-acetyl-CoA, 50 mM-2-oxoglutarate and buffer B in a total volume of 0.5 ml for 10 min at room temperature. The reaction was terminated by the addition of 0.4 ml of 95% (v/v) ethanol; after addition of 0.45 ml of 5 mM-DTNB (dissolved in buffer B), the mixture was centrifuged for 15 min in an Eppendorf centrifuge. The A_{412} of the supernatant was read against a blank lacking 2-oxoglutarate. Removal of protein before addition of DTNB is not necessary for the reproducible measurement of enzyme activity. Activities are expressed in m-units/ml of enzyme preparation (1 unit = 1 μmol of CoA formed/min) by using a molar absorption coefficient (ϵ) of 13600 $M^{-1} \cdot cm^{-1}$. Kinetic measurements were performed with shorter times of incubation (2–7 min) after recording time-dependences at different substrate concentrations. Addition of concentrations of acetyl-CoA in the assay mixture higher than 1 mM caused inhibition of homocitrate synthase, owing to the presence of residual CoA in the acetyl-CoA preparations used.

Assays of other enzymes

Citrate synthase, NAD-dependent isocitrate dehydrogenase, NADP-dependent isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase were determined as described by Osmani & Scrutton (1983, 1985), but Triton X-100 was used instead of Nonidet P-40.

Purification of homocitrate synthase

Step 1: protamine sulphate precipitation. To 65–75 ml of cell-free extract containing 450 mg of protein, 100 mg of solid protamine sulphate was added. After stirring at room temperature for 5–10 min, the precipitate was removed by centrifugation (15 min, 11500 *g*).

Step 2: $(NH_4)_2SO_4$ precipitation. To 65 ml of supernatant obtained in step 1, 13.5 g of $(NH_4)_2SO_4$ was slowly added under magnetic stirring at 0–4 °C, giving 35% saturation. The precipitate was removed by centrifugation for 20 min at 11500 *g*. Homocitrate synthase activity was then precipitated by addition of 11.4 g of $(NH_4)_2SO_4$ to 70 ml of the supernatant fraction (giving 60% saturation). The precipitate was collected by centrifugation, dissolved in buffer A, desalted on three PD-10 columns (2.5 ml per column), and diluted with buffer A to give a final protein concentration of 7–10 mg/ml.

Step 3: Q-Sepharose Fast Flow ion-exchange chromatography. The desalted $(NH_4)_2SO_4$-precipitated material was applied to an XK16/20 column connected to an f.p.l.c. system (Pharmacia) and containing 30 ml of Q-Sepharose Fast Flow gel which had been previously equilibrated with buffer A. Proteins were eluted with a NaCl step gradient in buffer A. Most of the protein was eluted at approx. 0.12 M-NaCl, but homocitrate synthase activity was eluted at 0.16 M-NaCl. Active fractions (30–35 ml) were combined and concentrated by addition of $(NH_4)_2SO_4$ to 70%

saturation. The precipitate was dissolved in buffer A and clarified by centrifugation at 12000 *g* for 2 min. The final volume was 0.5–1 ml. It was noted that at this step, when glycerol was omitted from buffer A and assay method 1 was used for enzyme detection, an activity totally dependent on acetyl-CoA became apparent, the product of which co-migrated with homocitrate in solvent 1, but not in solvent 2. The addition of glycerol was essential for a good separation of this enzyme from homocitrate synthase.

Step 4: gel filtration on Superose 12. The preparation from step 3 was applied to 100 ml of Superose 12 in an HR16/50 column which was connected to the f.p.l.c. system. Equilibration and elution were performed with buffer A at a flow rate of 1 ml/min; 2 ml fractions were collected. Activity of homocitrate synthase was detected between 44 and 50 ml, with the peak of activity at 47–48 ml.

Step 5: hydroxyapatite chromatography. The preparation obtained in step 4 (5–6 ml; about 2 mg of protein) was loaded directly on to a 1.5 cm × 8 cm column containing 1.4 ml of Bio-Gel HTP (Bio-Rad, Richmond, CA, U.S.A.) at 4–6 °C. Homocitrate synthase was eluted by addition of 3–5 ml of 30 mM-sodium phosphate in buffer A and re-equilibrated with buffer A by using PD-10 columns.

Step 6: MonoQ anion-exchange chromatography. The preparation from step 5 was subsequently applied to a MonoQ column (f.p.l.c. system), which had been equilibrated with buffer A. Proteins were eluted by application of a linear gradient (60 ml) of 0–0.4 M-NaCl in buffer A. Two peaks of homocitrate synthase activity were detected, eluted between 0.18 M and 0.22 M-NaCl (peak A) and at approx. 0.24 M-NaCl (peak B).

Analytical procedures

Cellulose acetate electrophoresis was carried out as described by Osmani & Scrutton (1983), with 19 mM-potassium phosphate, pH 7.4, as running buffer. Homocitrate synthase activity was detected as described by those authors for citrate synthase. The activity stain contained 0.5% (w/v) Agar Noble (Difco) in buffer B, 0.3 mM-acetyl-CoA, 30 mM-2-oxoglutarate, 0.1 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide/ml and 0.1 mg of phenazine methosulphate/ml. The staining system was incubated at 20 °C in the dark for 20–40 min, or overnight. No activity bands appeared when 2-oxoglutarate was omitted.

SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970), with 10%-acrylamide gels and low-molecular-mass markers from Bio-Rad. Reducing bands were detected by silver staining (Merril *et al.*, 1981).

For chromatofocusing, protamine sulphate-treated and desalted extract (equivalent to 20 mg of protein) was applied to a MonoP column (f.p.l.c. system), which had been equilibrated with 40 mM-Bistris, pH 6.55, containing 10% (v/v) glycerol. A linear pH gradient was achieved by elution at 1 ml/min with Polybuffer 74 which was diluted 1:10 with distilled water containing 10% glycerol and adjusted to pH 3.9; 1.5 ml fractions were collected and their pH and enzyme activity measured.

Determination of the molecular mass of the native enzyme was performed as described above (see Step 4 of enzyme purification) after calibration of Superose 12 with citrate lyase (316 kDa), pyruvate kinase (237 kDa), alcohol dehydrogenase (150 kDa), hexokinase (100 kDa), isocitrate dehydrogenase (60 kDa) (all from Boehringer, Mannheim, Germany), Blue Dextran (2 MDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and cytochrome *c* (13 kDa) (Pharmacia).

Protein was determined as described by Bradford (1976).

RESULTS

Stability of homocitrate synthase in cell-free extracts of *P. chrysogenum*

As a prerequisite for purification, the stability of homocitrate synthase was investigated. Owing to the low levels and the high instability of the mitochondrial isoenzyme in the cell-free extract (see below under 'Cellulose acetate Electrophoresis'), all properties of homocitrate synthase given in this and the following sections refer to the cytosolic enzyme, unless stated otherwise.

As reported by Masurekar & Demain (1974), the enzyme activity was very unstable in crude and protamine sulphate-treated desalted extracts, particularly if glycerol was not present during cell disruption. Addition of 2 mM-2-oxoglutarate, 1 mM-L-lysine, dithiothreitol or EDTA to the extracts did not prevent this loss in activity. Glycerol stabilized the activity to some extent. After storage at 4–6 °C for 48 h in the presence of 20% glycerol, 35% of the initial activity remained. Freezing and thawing resulted in 50% loss of initial activity, although preparations obtained by gel filtration on Superose 12 were somewhat more stable.

Effects of assay conditions

The following findings were obtained with protamine sulphate-treated and desalted cell-free extracts.

The effect of pH was determined in succinate, malonate, Bistris, Mes, phosphate, imidazole, Tris and ethanolamine buffers (all at 50 mM) by the radioactive assay method. To rule out interference by other acetyl-CoA-dependent activities (see Step 3 of the purification procedure in the Materials and methods section), two developing solvents were applied to separate homocitrate from other components of the assay mixture. The reaction proceeds optimally at pH 7.5–8.0. At pH 7, the activity was decreased in phosphate buffer as compared with imidazole buffer, but addition of phosphate up to 50 mM to buffer B had little effect on the activity. Effects exerted by the following substances were studied by the DTNB assay: Mg^{2+}, which is usually included in the assay (Gaillardin *et al.*, 1976; Tucci & Ceci, 1972), was inhibitory rather than stimulatory (about 30% inhibition at 10 mM-$MgSO_4$); also EDTA was slightly inhibitory (30% inhibition by 5 mM-EDTA). Substances containing thiol groups, e.g. dithiothreitol, dithioerythritol, cysteine, 2-mercaptoethanol and GSH, were either ineffective or stimulatory (100–220% activity with thiol groups at 0.2 mM), the extent of stimulation being more pronounced at lower acetyl-CoA concentrations.

A continuous spectrophotometric assay as used for citrate synthase (Srere, 1969) is not applicable to determine homocitrate synthase activity, since the latter enzyme is inactivated by DTNB. Activities determined in crude extracts by a continuous spectrophotometric procedure appear to result from the combined action of glutamate–oxaloacetate transaminase and citrate synthase in the presence of aspartate. This activity is not detectable after desalting of crude extracts, but can be restored by addition of aspartate to the assay mixture.

Subcellular localization of homocitrate synthase

In order to assess the quality of the fractionation procedure, typical marker enzymes were included in the study. Fig. 1 shows that NAD–isocitrate dehydrogenase is exclusively found in the mitochondrial fraction, whereas a small amount of citrate synthase is released from the mitochondria in the course of the fractionation procedure. On the other hand, only a negligible amount (approx. 1%) of the cytosolic marker enzyme glucose-6-phosphate dehydrogenase contaminated the mitochondrial frac-

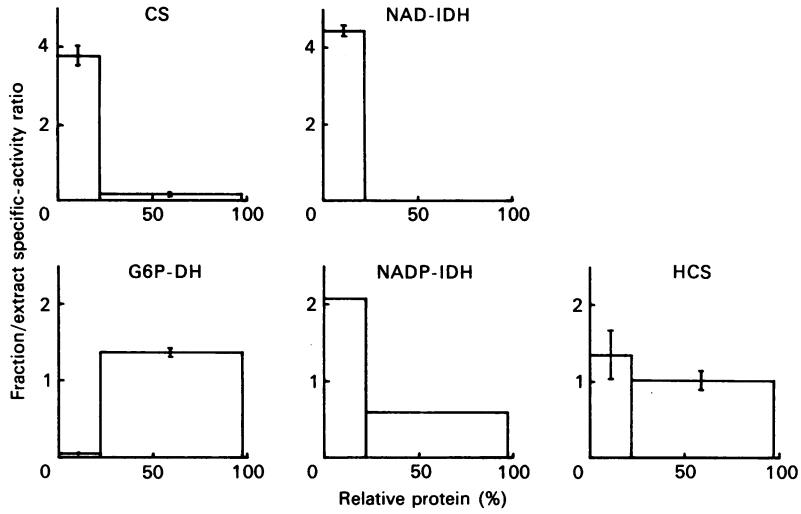

Fig. 1. Subcellular localization of some enzymes in *P. chrysogenum* Q176

Cell-free extract and subcellular fractions were prepared and enzyme activities determined as described in the Materials and methods section in the absence (glucose-6-phosphate dehydrogenase) or presence (all other enzymes) of 0.02% Triton X-100. The left bar of each plot represents the mitochondrial activity, and the right bar shows the cytosolic activity. Specific activities in the cell-free extract (800 *g* supernatant) and the extent of recoveries in the two fractions were: citrate synthase (CS), 0.184±0.014 unit/mg, 100±10%; NAD-isocitrate dehydrogenase (NAD-IDH), 0.030±0.003 unit/mg, 104±6%; glucose-6-phosphate dehydrogenase (G6P-DH), 0.430±0.023 unit/mg, 101±1%; NADP-isocitrate dehydrogenase (NADP-IDH), 0.061 unit/mg, 92%; homocitrate synthase (HCS), 480±180 c.p.m./min per mg, 101±2%. Except for NADP-IDH, the data are means±S.E.M. of at least four experiments.

tion. NADP-dependent isocitrate dehydrogenase was chosen as an example for dual localization.

The results obtained for homocitrate synthase clearly demonstrate that the main activity is located in the cytosol. This result is corroborated by the fact that no activity was found in the microsomal pellet prepared by centrifugation of the cytosolic fraction at 100000 *g* and 4 °C for 60 min. On the other hand, approx. 25% of total activity is present in the mitochondrial fraction. The evidence for the presence of a mitochondrial isoenzyme is further confirmed by the high extent of latency of homocitrate synthase activity in the mitochondrial fraction: only 16±8% of activity (mean ±S.E.M., $n = 4$) was detected when Triton X-100 was omitted from the assay mixture. Cell-free extracts used for fractionation showed a latency of 24±6% (mean±S.E.M., $n = 4$), which reflects the mitochondrial portion of homocitrate synthase activity. Latent activities for NAD-isocitrate dehydrogenase, citrate synthase, NADP-isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase in cell free extracts prepared by nitrogen cavitation were 100, 82±6 (mean±S.E.M., $n = 3$), 50 and 0% respectively.

Cellulose acetate electrophoresis

Electrophoretic analysis of subcellular fractions confirmed the presence of two homocitrate synthase isoenzymes in *P. chrysogenum* Q176.

The cytosolic isoenzyme was easily detectable in the cell-free extract, in the cytosolic fraction, and also in the mitochondrial fraction when it was prepared as described in the Materials and methods section under 'Subcellular fractionation' and treated for electrophoresis as described for cell-free extracts. It moved approx. 2.5 cm from the loading point when the cellulose acetate strip was run for 45 min as described above. The main problem encountered in obtaining proper electrophoretograms was a serious diffusion of this band, which could not be prevented by using different buffers or pH values.

The mitochondrial isoenzyme, however, moved up to 3 mm from the point of sample application, or remained there when electrophoresis was run at pH 6 or 6.5, without showing considerable diffusion. This activity band was absent from the cytosolic fraction, and appeared after an incubation time of 20–30 min only with highly concentrated mitochondrial preparations. In cell-free extracts, obtained by nitrogen cavitation and treated as described above, or desalted cell-free extracts prepared by sonication, the mitochondrial band was only visible after prolonged (overnight) incubation of the staining system. Several cm of the strip were covered by the zone of cytosolic activity after such incubation periods.

Additionally, all preparations apart from purified mitochondrial fractions showed a faint band positioned 1.5 cm from the loading point. This band was strongly enhanced in intensity within a few minutes by addition of oxaloacetate or L-aspartate to the staining system, and was thus shown to be a glutamate–oxaloacetate transaminase/citrate synthase complex.

Purification of cytosolic homocitrate synthase

Purification of homocitrate synthase from cell harvest to gel filtration on Superose 12 was usually carried out within 1 day; the preparation obtained at step 4 was kept frozen until further purification. Active homocitrate synthase was purified 180–500-fold by the procedure described, but, owing to the lability of the enzyme, the overall yield is poor. The actual enrichment of total homocitrate synthase protein is presumably much higher. A typical result is given in Table 1. Since cell-free desalted extracts displayed only about 40% of the activity present in protamine sulphate-treated extracts, values for crude extract are given in parentheses in Table 1.

Peak A and B obtained in this last step (see the Materials and methods section) were submitted to SDS/polyacrylamide-gel electrophoresis. The result of this analysis is shown in Fig. 2, and indicates marked enrichment in a polypeptide of molecular mass 54 kDa. The elution of two peaks of homocitrate synthase activity is thought to be an artifact, which could be explained by the formation of an aggregate between homocitrate synthase and other proteins differing from the pure enzyme in its pI.

Table 1. Purification of cytosolic homocitrate synthase from *P. chrysogenum* Q176

Step	Total protein (mg)	Total activity (m-units)	Specific activity (m-units/mg)	Purification* (fold)	Yield* (%)
Crude extract	575	(1044)	(2.05)		
Protamine sulphate precipitation	345	2594	7.52	1.67	100
$(NH_4)_2SO_4$ precipitation	173	2092	12.1	2.68	81
Q-Sepharose Fast Flow	11.4	552	48.4	10.7	21
Superose 12	2.23	285	128	28	11
Bio-Gel HTP/MonoQ	0.040	41.7	1042	231	1.6
MonoQ; distribution among peaks A/B	0.034/0.006	28.6/13.1	830/2300	185/510	1.1/0.5

* Relative to uninhibited enzyme activity in desalted crude extracts.

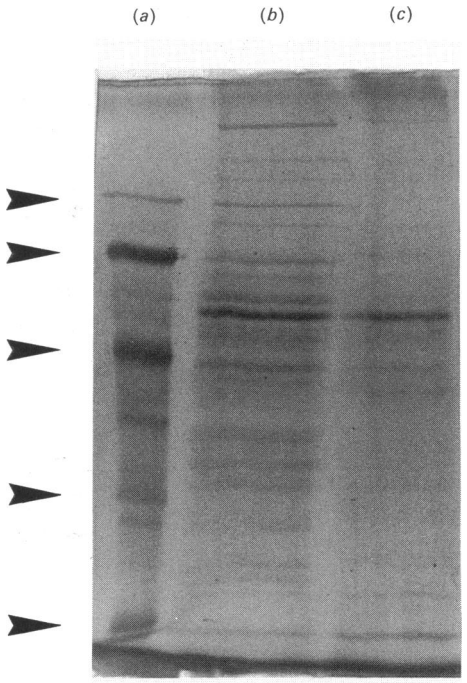

Fig. 2. SDS/polyacrylamide-gel electrophoresis of purified homocitrate synthase

Lanes: (*a*) molecular-mass markers (indicated by arrows; from top to bottom, 97.4, 66.2, 42.7, 31 and 21.5 kDa); (*b*) peak A and (*c*) peak B as obtained by MonoQ ion-exchange chromatography.

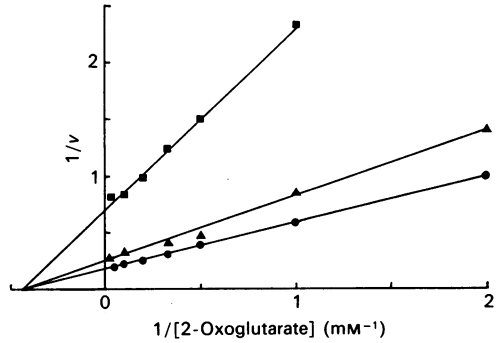

Fig. 3. Effect of 2-oxoglutarate on the activity of homocitrate synthase at different acetyl-CoA levels

[Acetyl-CoA]: ●, 0.52 mM; ▲, 0.155 mM; ■, 0.062 mM. For assay conditions, see the Materials and methods section (assay method 2); v is given in m-units/ml.

Molecular properties of cytosolic homocitrate synthase

The molecular mass of native homocitrate synthase was estimated at 155 ± 10 kDa (mean $\pm$ S.D., $n = 3$) by gel filtration on Superose 12 as described above. The pI of the cytosolic isoenzyme is 4.9 ± 0.1 (mean $\pm$ S.D., $n = 2$) as determined by chromatofocusing (see the Materials and methods section), which is in close agreement with that determined by Tucci & Ceci (1972) in cell-free extracts from yeast. The instability of the mitochondrial isoenzyme did not permit a determination of its pI by chromatofocusing. Its electrophoretic behaviour, however, suggests a pI which corresponds to or is higher than that reported by Tucci & Ceci (1972) for the second isoenzyme (pI 5.8).

Kinetic properties

Kinetic data were recorded for protamine sulphate-treated desalted cell-free extracts and preparations obtained from Step 4 of the purification procedure. No significant differences between these preparations were observed.

The relationship between initial rate and [2-oxoglutarate] is hyperbolic, with $K_m = 2.19 \pm 0.13$ mM (mean $\pm$ S.E.M., $n = 4$), which is not affected by alterations in the concentration of the second substrate, acetyl-CoA (Fig. 3). On the other hand, the relationship between initial rate and [acetyl-CoA] is sigmoidal, with $[S]_{0.5} = 41 \pm 5$ μM (mean $\pm$ S.E.M., $n = 8$) and Hill coefficient, $h = 2.6 \pm 0.2$ (mean $\pm$ S.E.M., $n = 4$). Freezing and thawing of the enzyme preparation strongly decreased co-operativity to a value of $h = 1.0$–1.4 without affecting $[S]_{0.5}$. As shown in Fig. 4, a decrease in the concentration of 2-oxoglutarate from 30 to 2.5 mM resulted in a decrease in V, whereas $[S]_{0.5}$ remained almost constant.

The enzyme was not inhibited by addition of up to 50 mM-homocitrate, but is sensitive to inhibition by CoA. This latter effect is markedly dependent on the time of incubation before starting the reaction with acetyl-CoA. When the reaction was started immediately after the addition of the inhibitor, half-maximal inhibition was achieved by addition of 0.2 mM-CoA. However, if the enzyme was incubated for 10 min with CoA before addition of acetyl-CoA, half-maximal inhibition was observed in the presence of 0.04 mM-CoA. The inhibition by CoA is specific, since no similar effect is observed on addition of other thiols (see above).

Effects of lysine and intermediates of lysine biosynthesis

As shown previously only for cell-free extracts of yeasts, homocitrate synthase is strongly inhibited by L-lysine. This effect was studied in protamine sulphate-treated and desalted cell-free extracts by the DTNB method after ensuring that it can also be detected by using the radioactive assay. Virtually no [^{14}C]homocitrate (only up to 6% of total activity) was formed at

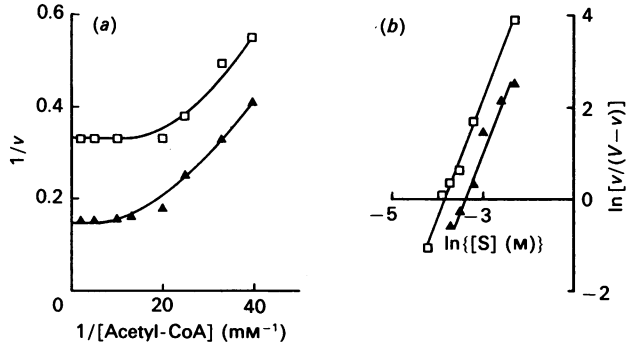

Fig. 4. Effect of acetyl-CoA on the initial reaction rates of homocitrate synthase at different 2-oxoglutarate levels

(*a*) Lineweaver–Burk plot; (*b*) Hill plot. [2-Oxoglutarate]: ▲, 30 mM; □, 2.5 mM; *v* is given in m-units/ml.

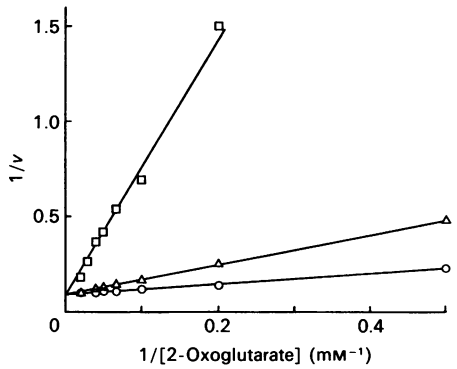

Fig. 5. Effect of 2-oxoglutarate on the initial reaction rates of homocitrate synthase at different concentrations of L-lysine

[Lysine]: ○, 0 mM; △, 0.02 mM; □, 0.2 mM; [acetyl-CoA] constant at 0.53 mM; *v* is given in m-units/ml.

0.48 mM-L-lysine. A similar result was obtained with subcellular fractions of *P. chrysogenum* Q176: both isoenzymes showed an inhibition of 80–100% at this lysine concentration.

When the concentration of 2-oxoglutarate is varied at three different lysine concentrations (Fig. 5), the data obtained show that lysine inhibits homocitrate synthase competitively with respect to 2-oxoglutarate, with $K_i = 8 \pm 2$ μM (mean ± S.E.M., $n = 2$) as calculated from the slopes of the Lineweaver–Burk plot.

In order to detect possible regulatory relationships between the sensitivity of homocitrate synthase towards lysine and the formation of penicillins, two strains (Q176 and D6/1014A) differing in their ability to produce penicillin (cf. Jaklitsch *et al.*, 1985) were compared with respect to lysine-sensitivity of homocitrate synthase *in vitro* by varying the concentration of lysine with 2-oxoglutarate constant at 6 mM (Fig. 6). The enzyme of both strains showed the same result: 50% inhibition at 53 ± 4 μM-lysine (mean ± S.E.M., $n = 4$) and non-linearity at lysine concentrations higher than 0.15 mM. The same result also was found with homocitrate synthase from strain Q176 purified through Stage 4 of the procedure described above. Peaks A and B from Step 6 were equally susceptible to lysine inhibition, but only 45% inhibition was observed at 0.5 mM-lysine.

The effect of pH on lysine inhibition, determined in protamine sulphate-treated and desalted extracts, was surprisingly marked, as there was no inhibition detectable by 0.5 mM-lysine at pH 6.6–7.0 (in 50 mM-Bistris or -imidazole), and only 20% inhibition was achieved by 1 mM-lysine.

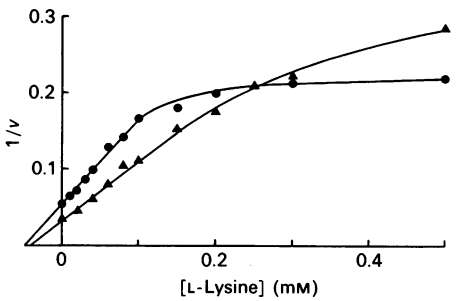

Fig. 6. Effect of L-lysine on the activity of homocitrate synthase from two different strains of *P. chrysogenum*

Strains used: ▲, Q176; ●, D6/1014A. Concentrations of substrates: 6 mM-2-oxoglutarate, 1 mM-acetyl-CoA; *v* is given in m-units/ml.

Several intermediates of the lysine biosynthetic pathway, including 2-oxoadipate, α-aminoadipate and saccharopine, did not influence homocitrate synthase activity when added at 0.5 mM final concentration.

Effects of nucleotides and inorganic cations

Nucleotides such as ADP (1 mM) and ATP/Mg^{2+} (5 mM/10 mM) did not influence homocitrate synthase activity. Slight inhibition (13–15%) was observed with AMP (1 mM) and GTP/Mg^{2+} (5 mM/10 mM). Inorganic cations such as K^+ (up to 50 mM) and Fe^{2+} (25 μM) did not affect enzyme activity, whereas Mn^{2+} and Zn^{2+} (both at 25 μM) showed 35 and 60% inhibition respectively.

DISCUSSION

This paper reports for the first time the purification of a cytosolic homocitrate synthase, yielding a maximally 500-fold purified preparation. The enzyme preparation is still not homogeneous, but its instability at this stage prevented further purification steps. The strongest band found in SDS/polyacrylamide-gel electrophoresis was at 54 kDa, but a second band at 21 kDa was clearly present. Traces of other proteins seen upon prolonged silver staining did not account for more than 2% of the final protein present.

We have shown here that homocitrate synthase from *P. chrysogenum* occurs in the form of two isoenzymes, located in different compartments, i.e. cytosol and mitochondrion. Two isoenzymes have also been found in *S. cerevisiae* (Tucci & Ceci, 1972), showing the same pI values as those from *P. chrysogenum*, which in the case of the mitochondrial isoenzyme remains to be proved directly. However, their location has not been assessed. Betterton *et al.* (1968) described only a mitochondrial location of homocitrate synthase in yeast. Considering the high instability of homocitrate synthase isoenzymes, this clearly remains to be re-evaluated. In *P. chrysogenum* the mitochondrial isoenzyme was highly unstable; therefore only the cytosolic isoenzyme was purified in the present work. The findings of a dual location of homocitrate synthases in this fungus raises the question of its physiological role. Further studies are needed to demonstrate whether the other enzymes of the first part of the α-aminoadipate pathway may also occur as compartmented isoenzymes. Since in *P. chrysogenum* the biosynthesis of α-aminoadipate is involved in the initiation of β-lactam biosynthesis (Jaklitsch *et al.*, 1986; Hönlinger & Kubicek, 1989*a*), it is tempting to speculate on a role of the cytosolically located α-aminoadipate-synthesizing pathway in penicillin biosynthesis.

The kinetic properties of the *P. chrysogenum* homocitrate synthase are similar to those reported for the enzyme from cell-

free extracts of *Yarrowia lipolytica* (Gaillardin *et al.*, 1976). However, the *P. chrysogenum* enzyme exhibits greater affinity for acetyl-CoA, and inhibition by lysine is competitive with respect to 2-oxoglutarate without inducing co-operativity in the relationship between v and [2-oxoglutarate]. In contrast with the enzyme from *S. cerevisiae* (Tracy & Kohlhaw, 1975), no CoA/bivalent-metal-ion-mediated inhibition was observed.

Regulation of homocitrate synthase activity in *P. chrysogenum* by lysine has been known for many years, but the mechanism underlying this regulation has remained controversial. Although Demain & Masurekar (1974) provided indirect evidence for an inhibitory effect, attempts to demonstrate this effect directly had been unsuccessful (Masurekar & Demain, 1974; Luengo *et al.*, 1980). We have shown here that homocitrate synthase from two strains of *P. chrysogenum* is highly sensitive to inhibition by L-lysine *in vitro*, using either a cell-free extract or the purified preparation as an enzyme source. Inhibition by lysine is only observed within a narrow range of pH. It is possible that the failure of Masurekar & Demain (1974) to demonstrate sensitivity to lysine *in vitro* is due to use of an inappropriate pH. On the other hand, Luengo *et al.* (1980) used extended incubation times, which may have led to a non-linear progress curve. Under such conditions, the effect of an inhibitor may be strongly underestimated. It should be noted that all kinetic data reported in the present study were obtained by using appropriate precautions to maintain linear time/activity relationships. The K_i so determined indicates a very high sensitivity of homocitrate synthase to L-lysine. In view of the cytosolic lysine concentration in *P. chrysogenum* (0.2–0.5 mM; Affenzeller *et al.*, 1989), strong inhibition should predominate *in vivo*. It should be borne in mind that the cytosolic pH is a function of the proton gradient maintained by the cytoplasmic membrane-bound ATPase. Hence a shortage of ATP, owing to a lack of sufficient cellular energy supply (e.g. carbon-source limitation), will lead to a decreased gradient, and consequently to a higher cytosolic proton concentration, i.e. lower pH. Therefore, a lack of feedback inhibition by lysine of homocitrate synthase may be expected when the fungus enters the stationary phase of growth rates. This mechanism could explain why this fungus produces secondary metabolites from intermediates of the lysine-biosynthetic pathway. The finding that the cytosolic homocitrate synthase from two *P. chrysogenum* strains, which differ in their penicillin-producing capacity, exhibited a comparably high sensitivity to inhibition by lysine is in agreement with this hypothesis, because it makes the postulation of a lysine-insensitive homocitrate synthase in high-producing mutants unnecessary. Although it is possible that strains used for industrial production of penicillin contain a lysine-insensitive homocitrate synthase, it should be noted that α-aminoadipate is in part recycled during penicillin biosynthesis (Friedrich & Demain, 1978). Hence the intracellular concentration of this intermediate rather than its rate of formation may be more relevant to control of penicillin biosynthesis.

High pool concentrations of α-aminoadipate are only observed under carbon-catabolite-derepressed conditions of cultivation (Martin *et al.*, 1984; Jaklitsch *et al.*, 1986), which are characterized by a much decreased flux through the α-aminoadipate pathway (Hönlinger & Kubicek, 1989*b*). The present findings suggest that pH-dependent regulation of homocitrate synthase by lysine may be another important factor influencing the α-aminoadipate pool concentration in this fungus.

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