Purification and Properties of 2-Furoyl-Coenzyme A Hydroxylase from *Pseudomonas putida* F2

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1. 2-Furoyl-CoA hydroxylase of *Pseudomonas putida* F2 has been purified 60-fold by a combination of $(\text{NH}_4)_2\text{SO}_4$ fractionation, DEAE-cellulose chromatography and agarose chromatography. 2. The purified enzyme catalyses the formation of 5-hydroxy-2-furoyl-CoA, which tautomerizes to form 5-oxo- Δ^2 -dihydro-2-furoyl-CoA. 3. The enzyme has a requirement for an electron acceptor that can be satisfied by a membrane preparation from 2-furoate-grown *Ps. putida* F2 or by artificial electron acceptors, and so presumably the incorporated oxygen atom is derived from water rather than molecular oxygen. 4. The enzyme is a large protein with a molecular weight of 3.27×10^6 and is disrupted to form inactive subunits in the presence of 0.2% (w/v) sodium dodecyl sulphate. It has a pH optimum of 8.5–9.5, a K_m for 2-furoyl-CoA of $20.2 \mu\text{M}$ and an absorption spectrum with a trough at 265 nm and a single peak at 273 nm. No absorption peaks are detectable in the visible region of the spectrum. 5. The enzyme is resistant to the effects of a wide range of potential inhibitors, but is inhibited by the copper-chelating agents bathocuproin and cuprizone, though not by sodium diethyldithiocarbamate. 6. Flavins are absent and the iron content does not show a sustained increase during purification. The copper content of the protein increases in close correlation with the increase in specific activity during purification. 7. A catalytic sequence for the hydroxylation of 2-furoyl-CoA by a copper protein is proposed.

In previous work (Jones & Trudgill, 1967; Trudgill, 1968, 1969) the growth of *Pseudomonas putida* F2 on 2-furoic acid has been reported and the following induced pathway for the conversion of the compound into 2-oxoglutarate proposed: 2-furoate $\rightarrow$ 2-furoyl-CoA $\rightarrow$ 5-hydroxy-2-furoyl-CoA $\rightarrow$ 5-oxo- Δ^2 -dihydro-2-furoyl-CoA $\rightarrow$ 2-oxoglutaryl-CoA $\rightarrow$ 2-oxoglutarate.

Some indirect evidence has been obtained to support the view that the hydroxylation step draws its oxygen from water. The resulting obligatory requirement for an electron acceptor may be satisfied by Methylene Blue, Nitro Blue Tetrazolium or the membrane fraction from 2-furoate-grown cells. Other hydroxylases utilizing oxygen from water all catalyse the initial reactions in the bacterial degradation of pyridine-ring compounds and all display an electron-acceptor requirement (Hunt *et al.*, 1958; Dagley & Johnson, 1963; Hochstein & Dalton, 1965; Hayaishi, 1969; Hirschberg & Ensign, 1971*a,b*).

A partial purification of 2-furoyl-CoA hydroxylase, and separation of it from other enzyme activities of the pathway, has been reported (Kitcher & Trudgill, 1970; Griffin *et al.*, 1971) and some provisional physical measurements have been made.

In the present paper we report the results of a more rigorous purification of this hydroxylase, its relationship to the other induced enzymes of the catalytic sequence and its more unusual properties and characteristics.

Materials and Methods

Organism

Pseudomonas putida F2 was maintained on nutrient-agar slopes at 2°C. Cells were grown at 30°C in medium containing (per litre): KH_2PO_4 , 6 g; $(\text{NH}_4)_2\text{SO}_4$, 2 g; 2-furoic acid, 2 g; yeast extract, 0.1 g. The pH was adjusted to 7.1 by the addition of 5 M-NaOH and after sterilization 0.4 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ /l was added aseptically as a sterile 10% (w/v) solution.

Batches of cells were grown as previously described (Trudgill, 1969), but for larger quantities a 40-litre batch of medium was used in a 45-litre polythene aspirator vessel, modified to fit a New Brunswick Microferm stand (New Brunswick Scientific Co., New Brunswick, N. J., U.S.A.) and to accept the 14-litre fermenter unit. It was inoculated with 3 litres of freshly grown shake culture, aerated by the passage of sterile air at 6 litres/min and stirred at 250 rev./min. Cells in the late exponential phase of growth were harvested in a Sharples Super centrifuge and washed

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once by resuspension in phosphate buffer (KH_2PO_4 , 1 g/l; Na_2HPO_4 , 2 g/l), pH 7.1, followed by centrifugation at 13000g for 15 min at 3°C.

Disruption of cells

Washed cells, resuspended in phosphate buffer ($1.5 \times \text{cell vol.}$), were disrupted by passage through a French pressure cell (Milner *et al.*, 1950) with a pressure difference at the orifice of 140 MN/m^2 (20000 lbf/in^2).

Preparation of cell extracts and membrane fractions

Broken-cell suspensions were clarified by centrifugation at 39000g for 15 min at 2°C. Further centrifugation at 70000g for 60 min in a Martin Christ Omega II preparative ultracentrifuge (Martin Christ, 3360 Osterode/Harz, W. Germany) yielded a clear supernatant and a pink pellet fraction that, after being washed by resuspension in 23 mM-sodium-potassium phosphate buffer containing 5 mM- MgCl_2 and further centrifugation, was used as a crude membrane preparation.

Buffers

Sodium-potassium phosphate buffer, pH 7.1 (23 mM with respect to phosphate), was obtained by dissolving 2 g of anhydrous Na_2HPO_4 and 1 g of anhydrous KH_2PO_4 in deionized water to a final volume of 1 litre. This buffer was used as a routine in the enzyme-purification procedure.

Other phosphate buffers of different pH or ionic strength were prepared by adjusting solutions of KH_2PO_4 to the appropriate pH with NaOH.

Determination of enzyme activities

All enzyme assays were conducted at a temperature of 30°C.

(1) *Assay of 2-furoate $\rightarrow$ 2-oxoglutarate and 2-furoyl-CoA $\rightarrow$ 2-oxoglutarate conversions.* The ability of crude extracts or purified enzyme preparations to catalyse the conversion of 2-furoate or 2-furoyl-CoA into 2-oxoglutarate was measured by following the formation of 2-oxoglutarate phenylhydrazine as an increase in E_{320} at pH 6.5. The basal reaction mixture contained in 6.4 ml, 1.9 mmol of phosphate buffer, 40 μmol of ATP, 0.26 μmol of CoA, 0.2 μmol of Methylene Blue, 50 μmol of phenylhydrazine and a suitable amount of protein; 3 ml of this reaction mixture was transferred to each of two cuvettes, one of which served as a blank, the reaction being started in the other by addition of 2 μmol of 2-furoate. This procedure ensured that any endogenous increases in E_{320} were identical in both cuvettes (Trudgill, 1969). The increase in extinction was recorded at 1 min

intervals on a Unicam SP.800 spectrophotometer fitted with an SP.825 programme controller. Cuvettes were withdrawn from the spectrophotometer between readings and agitated to maintain aerobic conditions. When 2-furoyl-CoA was used as substrate the ATP and CoA were omitted from the reaction mixture.

(2) *Assay of 2-furoyl-CoA hydroxylase activity.* No single totally satisfactory assay for the enzyme has been devised: various disadvantages are slowness, low sensitivity, expense or lack of applicability to all stages of the purification. Taken in combination, however, they can be successfully used to monitor 2-furoyl-CoA hydroxylase purification and to investigate the properties of the enzyme.

(a) Anaerobic reduction of Methylene Blue. Thunberg cuvettes contained, in a volume of 3 ml, 0.56 mmol of phosphate buffer, pH 7.5, 0.1 μmol of Methylene Blue and 5–50 units of 2-furoyl-CoA hydroxylase. Cuvettes were made anaerobic by first being evacuated with a Speedivac two-stage high-vacuum pump (Edwards High-Vacuum Ltd., Manor Royal, Sussex, U.K.) followed by threefold repeated flushing with N_2 and re-evacuation. Reactions were then started by addition of 0.12 μmol of 2-furoyl-CoA from the Thunberg side arm, and reduction of Methylene Blue was monitored continuously at 665 nm. For assays of 2-furoyl-CoA preparations additions of the compound were restricted to less than 50 nmol and the purity was estimated from the total change in E_{665} and an ϵ value of $4.18 \times 10^4 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Schubert & Levine, 1955).

(b) Polarographic measurement of oxygen consumption by using Methylene Blue or a membrane preparation as intermediate electron carrier and an oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.). The assay mixture contained, in a volume of 2.5 ml, 0.2 mmol of phosphate buffer, pH 7.5, 0.5 μmol of Methylene Blue or a saturating amount of crude membrane preparation and 125 nmol of 2-furoyl-CoA. Amounts of hydroxylase protein used stimulated the initial consumption of 0.012–0.06 μg -atom of O_2/min .

(c) Rapid qualitative test for chromatography fractions. This test was used to check large numbers of column fractions for activity with minimum wastage of expensive substrate and at maximum speed. With minor modification it was also used to detect hydroxylase on Sephadex and polyacrylamide-gel electrophoresis systems. Nitro Blue Tetrazolium (2 mg) was dissolved in 1 ml of 0.1 M-phosphate buffer, pH 7.5, and 0.5 μmol of 2-furoyl-CoA was added. Samples of the reagent (0.05 ml) were transferred to a series of test tubes (75 mm $\times$ 10 mm) and incubated at 30°C. After the addition of 0.1 ml samples of column fractions the rate of formation of the purple formazan was followed visually.

(d) Assay of reaction-product formation. Reaction

mixtures in 1 cm-light-path micro-cuvettes contained, in a volume of 0.5 ml, 40 μ mol of phosphate buffer, pH 7.5, 0.1 μ mol of Methylene Blue and 0.004–0.02 units of 2-furoyl-CoA hydroxylase. Reactions were started by the addition of 50 nmol of 2-furoyl-CoA and the increase in extinction at 373 nm was followed. This 373 nm absorption is attributed to the tautomeric keto form of the reaction product (5-hydroxy-2-furoyl-CoA $\rightarrow$ 5-oxo- Δ^2 -dihydro-2-furoyl-CoA) by comparison with the properties of esters of 5-hydroxy-thiophen-2-carboxylic acid (Jakobsen *et al.*, 1963).

(3) *Assay of 2-furoyl-CoA synthetase activity.* Though it was not possible to assay this enzyme in isolation, its presence could be detected by substituting 2-furoate, CoA and ATP for the 2-furoyl-CoA in assay methods described under (2). Availability of purified 2-furoyl-CoA hydroxylase allowed the addition of an excess of this enzyme to such assay systems so that the rate-limiting 2-furoyl-CoA synthetase activity could then be assayed.

(4) *Assay of 5-oxo- Δ^2 -dihydro-2-furoyl-CoA hydroxylase activity.* Purified 2-furoyl-CoA hydroxylase was used to accumulate the 5-oxo- Δ^2 -dihydro-2-furoyl-CoA as described in assay method 2(d). When only 10 nmol of 2-furoyl-CoA was added the 5-oxo- Δ^2 -dihydro-2-furoyl-CoA formed gave E_{373} 1.2. Addition of protein fractions suspected of containing the hydroxylase at this point enabled the rate of product hydrolysis to be followed as a decrease in E_{373} .

Preparation and assay of 2-furoyl-CoA

N-Hydroxysuccinimide, a gift from Dr. K. Bartlett, was prepared according to the procedure of Anderson *et al.* (1964) and recrystallized from hot ethyl acetate. Condensation of 2-furoic acid with *N*-hydroxysuccinimide to form the ester was achieved by the general procedure of Lapidot *et al.* (1967). The ester was recrystallized from hot ethanol and gave a single spot (R_F 0.49) when chromatographed on Kieselgel G (E. Merck A.-G., Darmstadt, Germany) developed with chloroform.

2-Furoyl-CoA was prepared from the *N*-hydroxysuccinimide ester of 2-furoic acid by using a modification of the procedure of Al-Arif & Blecher (1969). A solution of 100 μ mol of thioglycollic acid and 50 μ mol of reduced CoA in 1.4 ml of deionized water was adjusted to pH 7.5 by gradual addition of solid NaHCO₃. To this solution, agitated by a continuous stream of O₂-free N₂, 50 μ mol of the ester dissolved in 3 ml of freshly distilled tetrahydrofuran was added. The reaction was allowed to proceed at room temperature and small samples were monitored for residual free thiol groups with the ammonium nitroprusside reagent of Toennies & Kolb (1951) at suitable time-intervals. When no free thiol groups remained, typically after a reaction period of 2 h, the flow of N₂ was increased to drive off the tetrahydro-

furan. Insoluble material was removed by centrifugation, and the supernatant was adjusted to pH 5 with 0.2 M-HCl and made up to 5 ml with deionized water.

This crude preparation of 2-furoyl-CoA exhibited absorption maxima at 234 and 258 nm. An unexpected peak at 310 nm may be due to an impurity. T.l.c. on cellulose developed with 0.1 M-sodium acetate buffer (pH 4.5)–ethanol (1:1, v/v) (Stadtman, 1957) revealed two spots when the chromatogram was sprayed with 0.2% (w/v) Rhodamine 6G in acetone, and examined under u.v. light. Though both spots reacted as thioesters when sprayed with the ammonium nitroprusside reagent of Toennies & Kolb (1951) followed by 2% (w/v) NaOH in aq. 90% methanol, only one of them (R_F 0.67) was active as a substrate for 2-furoyl-CoA hydroxylase. 2-Furoyl-CoA preparations were assayed by measuring the decrease in E_{665} catalysed by purified 2-furoyl-CoA hydroxylase under anaerobic conditions in the presence of 33 μ M-Methylene Blue. Typically a 50–60% yield was obtained from the *N*-hydroxysuccinimide ester of 2-furoic acid.

Purification of 2-furoyl-CoA hydroxylase

All purification steps were done at 2–3°C with 23 mM-phosphate buffer, pH 7.1.

(1) *Fractionation with (NH₄)₂SO₄.* Neutral saturated (NH₄)₂SO₄ was added to the 70000g supernatant to give 30% saturation and then stirred slowly for 30 min. The precipitated protein was harvested by centrifuging at 70000g for 30 min and redissolved in phosphate buffer to a final volume equal to about one-third of that of the original 70000g supernatant. This solution was dialysed for 4 h against 60 vol. of phosphate buffer to remove (NH₄)₂SO₄. The tubing for dialysis (Visking) was washed before use in boiling aqueous 1 mM-EDTA followed by repeated washing in deionized water.

(2) *Chromatography on DEAE-cellulose.* The dialysed protein solution was loaded on to a column (25–30 cm $\times$ 5 cm) of Whatman DE52 microgranular DEAE-cellulose and the 2-furoyl-CoA hydroxylase was eluted by the passage of a linear buffer gradient derived from 1100 ml of phosphate buffer and 1100 ml of the buffer containing 0.5 M-KCl. A flow rate of 60 ml/h was used and the column effluent collected in 15-ml fractions. The E_{260} and E_{280} values of each fraction were measured. Fractions collected between 0.2 M- and 0.3 M-KCl were tested for activity by the Nitro Blue Tetrazolium spot-test. Active fractions were then assayed by the E_{373} method, peak fractions were pooled and saturated (NH₄)₂SO₄ was added to give 35% saturation. After the mixture had been stirred slowly for 30 min the precipitated protein was collected by centrifuging at 28000g for 30 min and redissolved in 10 ml of phosphate buffer.

(3) *Agarose chromatography*. The pooled protein fractions from DEAE-cellulose chromatography were loaded on to a column (55cm × 2.5cm) of Bio-Gel A-5m (200–400 mesh) or, in later preparations, a column (85cm × 2.5cm) of Bio-Gel A-15m (100–200 mesh) (Bio-Rad Laboratories, Richmond, Calif. U.S.A.) and the 2-furoyl-CoA hydroxylase was eluted by upward flow of phosphate buffer at 20ml/h. Fractions (5ml) were collected, E_{260}/E_{280} was measured for each fraction and the 2-furoyl-CoA hydroxylase activity measured by the Nitro Blue Tetrazolium spot-test. Active fractions were assayed by the E_{373} method and those of adequate specific activity pooled.

Pooled enzyme was brought to 30% saturation by addition of saturated aq. $(\text{NH}_4)_2\text{SO}_4$ and the enzyme harvested by centrifuging at 44000g for 30min. The pellet was dissolved in 2ml of phosphate buffer and dialysed for 3h against 100vol. of buffer.

Purified enzyme was stored at 2°C under an atmosphere of N_2 until required for use.

(4) *Routine assays*. The 70000g supernatant from the broken-cell suspension, the dialysed 30%-saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction, the concentrated pool from the DEAE-cellulose chromatography and the final enzyme preparation were assayed for 2-furoyl-CoA hydroxylase activity by the polarographic procedure with 2-furoyl-CoA as substrate in the presence of Methylene Blue. Protein was measured by the biuret method of Gornall *et al.* (1949).

Enzyme units

Units of 2-furoyl-CoA hydroxylase activity are expressed as μg -atoms of O_2 consumed/min in the polarographic assay 2(b). In the absence of a pure sample of the hydroxylation product 5-oxo- Δ^2 -dihydro-2-furoyl-CoA and an ϵ value for the compound, activities in assay methods 2(d) and 4 are expressed simply as a rate of change of E_{373} .

Ultracentrifugal analysis

Sedimentation coefficients and molecular weights were determined in a Beckman-Spinco model E analytical ultracentrifuge equipped with a schlieren phase-plate diaphragm and Rayleigh interference optics. Photographic plates were measured on an optical comparator (Precision Grinding Ltd., Mitcham, Surrey, U.K.). Sedimentation coefficients were determined at 20°C and rotor speeds were as stated. Meniscus-depletion sedimentation equilibrium runs were performed by the method of Yphantis (1964). Graphs of $\log c$ plotted versus x^2 (in which c is the protein concentration, measured as fringe displacement, and x the distance from the

centre of rotation) were constructed and the apparent molecular weights obtained from the equation:

$$\text{Mol.wt.} = 2RT(d \log c / dx^2) / (1 - \bar{v}\rho)$$

a value of 0.72 being assumed for $\bar{v}$.

Electrophoresis

Electrophoresis of 2-furoyl-CoA hydroxylase preparations was performed on 0.6mm-thick layers of superfine Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with barbitone-acetate buffer, pH 8.6 (Oxoid Ltd., London S.E.1, U.K.), and supported on glass plates (20cm × 20cm). 2-Furoyl-CoA hydroxylase typically migrated 7–8cm during a 5h electrophoresis period at a potential of 10V/cm, and was detected by overlaying the Sephadex with strips of Whatman 3MM chromatography paper soaked in the Nitro Blue Tetrazolium–2-furoyl-CoA mixture described under 'Determination of enzyme activities' followed by observation of the formation of the purple formazan band. Protein was detected by the indirect replica technique on Whatman 3MM paper with Amido Black 10B or Coomassie Brilliant Blue as staining agent (Radola, 1968).

Determination of copper and iron

The copper content of protein fractions was measured directly on suitable dilutions with an EEL 240 Atomic Absorption Spectrophotometer (Evans Electroselenium Ltd., Halstead, Essex, U.K.) fitted with the appropriate hollow-cathode lamp and an acetylene–air burner. The iron content of protein fractions was determined directly by using the appropriate lamp or on protein fractions that had been ashed at 450°C, further oxidized with conc. HNO_3 , dried, treated with 5M-HCl, dried again and finally dissolved in 0.1M-HCl for analysis. Calibration curves were obtained with standard solutions of hydrated CuSO_4 and FeCl_3 .

Preparation of CO

The gas was prepared by the controlled addition of formic acid to conc. H_2SO_4 and scrubbed by passage through water.

Other materials

Apyrase (EC 3.6.1.5), hexokinase (EC 2.7.1.1.), CoA and glutamate dehydrogenase (EC 1.4.1.2) were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. $\alpha\alpha'$ -Bipyridyl, *p*-chloromercuribenzoic acid, iodoacetamide, Methylene Blue, sodium arsenite, sodium azide, phenylhydrazine hydrochloride and deoxyribonuclease (EC 3.1.4.5)

were from BDH Chemicals Ltd., Poole, Dorset, U.K. Amido Black 10B, 2-furoic acid, Rhodamine 6G (c.l. 45160), thioglycolic acid and Tiron (disodium 1,2-dihydrobenzene-3,5-disulphonate) were from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. Menadione was obtained from K & K Laboratories Inc., Plainview, N.Y., U.S.A. Cuprizone [bis(cyclohexanone)oxaldihydrazone] was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Coomassie Brilliant Blue G 250 (c.l. 42655) was obtained from Serva G.m.b.H., Heidelberg, Germany. Bathocuproin (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), Blue Dextran, glycylglycine, 2-heptyl-4-hydroxyquinoline *N*-oxide, Nitro Blue Tetrazolium [2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethyl-4,4'-diphenylene)-tetrazolium chloride] and sodium diethyldithiocarbamate were from Sigma Chemical Co., St. Louis, Mo., U.S.A. *N*-Hydroxysuccinimide was a much-appreciated gift from Dr. K. Bartlett and tobacco mosaic virus was kindly given by Professor R. Markham.

Results

Purification of 2-furoyl-CoA hydroxylase

This enzyme, induced in *Ps. putida* F2 by growth with 2-furoate, has been purified 60-fold in our most successful preparations (Table 1). The effective selection of high-molecular-weight proteins by 30% saturation of the 70000g supernatant with $(\text{NH}_4)_2\text{SO}_4$ can clearly be seen if both the supernatant and the redissolved pellet fraction are chromatographed on Bio-Gel A-5m (Fig. 1). These purification steps effect a complete separation of the hydroxylase from the lower-molecular-weight enzymes that catalyse the conversion of its reaction product into 2-oxoglutarate. This is illustrated by the progressive separation of 2-furoyl-CoA hydroxylase from 5-oxo- Δ^2 -dihydro-2-furoyl-CoA hydrolase that occurs during these purification steps (Fig. 2).

Introduction of a DEAE-cellulose chromatography step between the $(\text{NH}_4)_2\text{SO}_4$ fractionation and the

Bio-Gel A-5m chromatography greatly increased the capacity of the purification sequence and the degree of purification obtained. The selection of the hydroxylase in the high-molecular-weight fraction by $(\text{NH}_4)_2\text{SO}_4$ fractionation results in a simple protein profile on the DEAE-cellulose (Fig. 3). Purification at

Fig. 1. Chromatography of the products of $(\text{NH}_4)_2\text{SO}_4$ fractionation on Bio-Gel A-5m

The redissolved pellet (a) 60mg of protein from $(\text{NH}_4)_2\text{SO}_4$ fractionation (0–30% saturation), and (b) 60mg of the supernatant protein were chromatographed on a column (55 cm $\times$ 2.5 cm) of Bio-Gel A-5m. $\circ$, 2-Furoyl-CoA hydroxylase activity (assay method 2d); $\bullet$, 5-oxo- Δ^2 -dihydro-2-furoyl-CoA hydrolase activity (assay method 4); ----, E_{280} .

Table 1. Summary of the purification of 2-furoyl-CoA hydroxylase from *Ps. putida* F2

Details of the purification stages are given in the Materials and Methods section.

Step	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)
(1) 70000g supernatant from broken cells (136g wet wt.)	170	628	11 560	0.054	100
(2) $(\text{NH}_4)_2\text{SO}_4$ fractionation	60	612	1500	0.41	97
* (3) Chromatography on DEAE-cellulose	—	296	110	2.68	48
* (4) Chromatography on Bio-Gel A-5m	—	177	55	3.22	21

* Only the most active fractions were taken at steps (3) and (4).

Fig. 2. Separation of 2-furoyl-CoA hydroxylase from 5-oxo- Δ^2 -dihydro-2-furoyl-CoA hydrolase by $(\text{NH}_4)_2\text{SO}_4$ fractionation and Bio-Gel A-5 m chromatography

Formation and disappearance of 5-oxo- Δ^2 -dihydro-2-furoyl-CoA was followed at 373 nm (Materials and Methods section, assay methods 2d and 4): (a) 5.2 mg of 70000g supernatant; (b) 1.25 mg of dialysed 0–30%-saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction; (c) 8 μg of fraction I from Fig. 1 followed (as shown by the arrow) by 0.13 mg of fraction II from Fig. 1 at 4 min after the initiation of reaction. Initial reactions were, in all cases, started by the addition of 10 nmol of 2-furoyl-CoA after 1 min equilibration at 30°C.

Fig. 3. Purification of 2-furoyl-CoA hydroxylase on DEAE-cellulose

The dialysed 0–30%-saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction (990 mg of protein) was loaded on to a column (28 cm $\times$ 5 cm) of DEAE-cellulose equilibrated with 23 mM-phosphate buffer, pH 7.1. 2-Furoyl-CoA hydroxylase was eluted with an increasing concentration of KCl in the same buffer and fractions (15 ml) were collected. o, 2-Furoyl-CoA hydroxylase activity; ----, protein measured by $E_{260}^{1\text{cm}}/E_{280}^{1\text{cm}}$ ratio; ·····, calculated molarity of KCl gradient.

this stage is mainly achieved by the retention of a large part of the protein by the column when a 0–0.5 M-KCl gradient is used.

Chromatography of pooled active fractions from DEAE-cellulose on Bio-Gel A-5 m or Bio-Gel A-15 m that reportedly have exclusion limits for globular proteins of 5×10^6 and 15×10^6 daltons respectively yield single hydroxylase activity peaks that correlate

closely with the major protein peaks and indicate the enzyme to be of very large size (Fig. 4).

Molecular weight of 2-furoyl-CoA hydroxylase

Sedimentation velocity studies with purified hydroxylase performed at 20°C and 24630 rev./min in the analytical ultracentrifuge yielded a rather

Fig. 4. Chromatography of 2-furoyl-CoA hydroxylase and marker materials on Bio-Gel A-5m and Bio-Gel A-15m

A column (55 cm $\times$ 2.5 cm) of Bio-Gel A-5m (a) and a column (85 cm $\times$ 2.5 cm) of Bio-Gel A-15m (b) were used. The columns were equilibrated with 23 mM-phosphate buffer, pH 7.1, and run with upward flow at 2°C. Fractions (5 ml) were collected. $\circ$, 2-Furoyl-CoA hydroxylase activity was assayed by following reaction-product formation at 373 nm. Marker compounds used were as follows (molecular weights are given in parentheses on the Figure): (a) for the Bio-Gel A-5m column: 1, Blue Dextran; 2, glutamate dehydrogenase; 3, ATP disphosphohydrolase; 4, hexokinase; 5, ribonuclease; 6, $(\text{NH}_4)_2\text{SO}_4$; (b) for the Bio-Gel A-15m column: 1, tobacco-mosaic virus; 2, glutamate dehydrogenase; 3, $(\text{NH}_4)_2\text{SO}_4$. The Blue Dextran was detected by measuring $E_{625}^{1\text{cm}}$, the tobacco mosaic virus by measuring $E_{260}^{1\text{cm}}$ and the marker proteins either by measurement of $E_{280}^{1\text{cm}}$ or where applicable by measurement of the specific catalytic activity. $(\text{NH}_4)_2\text{SO}_4$ was detected by measuring the conductivity of the column effluent.

asymmetric sedimentation peak with $s_{20,w}$ 42.9S. Treatment of the enzyme with 0.2% (w/v) sodium dodecyl sulphate resulted in a loss of catalytic activity and the appearance of a single symmetrical sedimentation peak with $s_{20,w}$ 3.26S.

Meniscus-depletion sedimentation equilibrium runs performed on the native enzyme at 12°C and 3848 rev./min yielded a linear plot of interference-fringe displacement against the square of the distance from the centre of rotation (Fig. 5) and a molecular weight of 3.27×10^6 .

Purity of 2-furoyl-CoA hydroxylase preparations

The large size of the enzyme is reflected in our inability to persuade it to enter conventional polyacrylamide gels (Davis, 1964) with acrylamide concentrations that varied from 4–8% (w/v) and *NN'*-methylenebisacrylamide concentrations that varied from 2.5–5% (w/v). Electrophoresis at 3–4 mA per gel tube was performed for periods from 45 min to 4 h. Electrophoresis on thin layers of superfine Sephadex G-200 yielded a single band of 2-furoyl-CoA hydroxylase activity that was associated with the major

Fig. 5. Molecular-weight determination from sedimentation equilibrium data

A meniscus-depletion sedimentation equilibrium run was performed with 2-furoyl-CoA hydroxylase (0.42 mg of protein/ml) in 23 mM-phosphate buffer, pH 7.1. After centrifugation in the Beckman-Spinco model E analytical ultracentrifuge at 3840 rev./min and 12°C for 72 h, photographs were taken by using the Rayleigh interference optical system. The slope of the plotted line is a function of the molecular weight (see the Materials and Methods section).

protein band. A more rapidly migrating impurity was judged by visual observation to constitute about 30% of the protein.

Spectral characteristics

The absorption spectrum of the native enzyme represents a typical protein absorption pattern with a trough at 265 nm and a single absorption maximum at 273 nm. Our purest preparations displayed none of the absorption in the visible region that is characteristic of flavoproteins and enzymes containing copper or iron.

Effect of pH on enzyme activity

To explore the pH-dependence of 2-furoyl-CoA hydroxylase activity a series of buffers covering the pH range 5.5–10.5 was used. None of the buffers had any unique effect on enzyme activity and a pH optimum over the range 8.5–9.5 was obtained. With the exception of coupled assays in which 2-oxoglutarate was determined as its phenylhydrazone at slightly

acidic pH all assays were performed at pH 7.5 in concentrated phosphate buffer. This pH, as well as perhaps being more physiological than that at which optimum activity is observed, was considered more suitable for routine work since under these conditions the stability of alkali-labile 2-furoyl-CoA was assured.

Kinetic studies on 2-furoyl-CoA hydroxylase

In the presence of excess of Methylene Blue the double-reciprocal plot of initial reaction velocity against 2-furoyl-CoA concentration yielded a straight line and a K_m for 2-furoyl-CoA of 20.2 μ M. Though a markedly non-linear double-reciprocal plot was obtained when the Methylene Blue concentration was varied in the presence of excess of 2-furoyl-CoA, maximum reaction velocity was for practical purposes obtained at 5 mM-Methylene Blue.

Effects of inhibitors on enzyme activity

When Methylene Blue was used as electron acceptor hydroxylase activity, measured by observing the rate of product formation (assay method 2d in the Materials and Methods section) was markedly inhibited only by the copper-chelating agents bathocuproin and cuprizone; less than 20% inhibition was obtained in the presence of 10 mM-EDTA (Table 2) and the copper-chelating agent dithiodiethylcarbamate was without effect. The less marked inhibitions observed in parallel polarographic assays (assay method 2b) may simply be due to the relative insensitivity of the latter assay.

Generally accepted inhibitors of electron transport were without effect on 2-furoyl-CoA hydroxylase when Methylene Blue was used as electron acceptor. However, when a crude membrane preparation was substituted for the Methylene Blue in assay method 2(b) marked inhibitions were observed (Table 3) and were approximately equal to the inhibition of NADH oxidation obtained with the same membrane preparation under identical conditions. This evidence supports our view that the membrane fraction has a conventional electron-transporting role when functioning in conjunction with the 2-furoyl-CoA hydroxylase.

Copper and iron contents of hydroxylase preparations

Though the inhibition of 2-furoyl-CoA hydroxylase activity by bathocuproin and cuprizone may perhaps be considered as indicating the presence of copper with a catalytic function, this is to accredit these inhibitors with a restricted specificity that they do not possess. Yamamoto *et al.* (1969) demonstrated that,

Table 2. *Effects of inhibitors on 2-furoyl-CoA hydroxylase activity when Methylene Blue is used as electron acceptor*

The activity of purified 2-furoyl-CoA hydroxylase was measured by following reaction-product formation at 373 nm (assay method 2*d*). All potential inhibitors were preincubated with the enzyme in the presence of phosphate buffer, pH 7.5, for the time-periods indicated. When significant inhibition was observed the effect was checked by using the polarographic assay procedure (assay method 2*b*).

Inhibitor	Final concn. (mM)	Preincubation time (min)	Inhibition¶ (%)
<i>p</i> -Chloromercuribenzoate*	0.1	10	3
Sodium arsenite	10	15	0
Iodoacetamide	5	10	0
Potassium cyanide	1	5	0
2- <i>n</i> -Heptyl-4-hydroxyquinoline <i>N</i> -oxide	0.02	20	0
Sodium azide	10	15	0
CO†	—	—	0
EDTA	10	30	16 (0)§
αα'-Bipyridyl‡	5	15	0
Tiron	1	30	0
8-Hydroxyquinoline	1	30	0
Menadione	1	5	0
Bathocuproin	1	30	90 (56)§
	0.1	30	32
Cuprizone	1	30	73 (7)§
	0.1	30	32
Sodium diethyldithiocarbamate	10	15	0

* Assayed in 0.1 M-glycylglycine buffer, pH 7.5.

† See the Materials and Methods section for details.

‡ Inhibitor solution made up in 20% (v/v) ethanol.

§ Polarographic assay method (2*b*).

|| No inhibition observed over the pH range 6.3–8.0.

¶ Calculated by comparison with controls preincubated without inhibitor.

Table 3. *Effects of inhibitors of electron transport on 2-furoyl-CoA hydroxylase activity with a membrane preparation as electron acceptor*

The activity of purified 2-furoyl-CoA hydroxylase in the presence of a rate-limiting amount of membrane preparation was followed polarographically as described in the Materials and Methods section (assay method 2*b*). Expt. 1. The reaction vessel contained, in a volume of 2.5 ml, 0.2 mmol of phosphate buffer, pH 7.5, 80 µg of hydroxylase and 10 mg of membrane protein. Reactions at 30°C were started by the addition of 125 nmol of 2-furoyl-CoA. Inhibitors were preincubated with buffer and protein for the times shown. Expt. 2. NADH oxidase activity of the membrane preparation was measured polarographically. The reaction vessel contained, in a volume of 2.5 ml, 0.2 mmol of phosphate buffer, pH 7.5, and 0.4 mg of membrane protein. Reactions at 30°C were started by the addition of 1 µmol of NADH.

	Inhibitor	Final concn. (mM)	Preincubation time (min)	Inhibition* (%)
Expt. 1	Potassium cyanide	1	5	70
	Sodium azide	10	15	53
Expt. 2	Potassium cyanide	1	5	70
	Sodium azide	10	15	50

* Calculated by comparison with controls preincubated without inhibitor.

Fig. 6. Copper content of 2-furoyl-CoA hydroxylase

Copper determinations were performed at all stages of enzyme purification by the atomic absorption procedure described in the Materials and Methods section. All values are corrected for traces of copper present in the buffer systems.

although imidazole acetate monooxygenase could not be shown to contain any significant amount of copper, almost complete inhibition of activity could be obtained with 0.1 mM-bathocuproin or 0.1 mM-cuprizone.

However, the significance of inhibitions observed with 2-furoyl-CoA hydroxylase is reinforced by the increase in copper content of the protein, at a rate nearly proportional to the increase in specific activity, that occurs during purification (Fig. 6).

The 70000g supernatant contains 0.16 μg of iron/mg of protein. Though we have not performed iron assays on our purest hydroxylase preparations, the iron content increases eightfold during the initial purification steps, (NH₄)₂SO₄ fractionation and DEAE-cellulose chromatography but decreases again after Bio-Gel A-15m chromatography. We suspect that these changes in iron content, which do not correlate with the changes in enzyme specific activity during purification, represent contamination of our enzyme by iron proteins. This may be indicated by the small absorption peak at 411 nm observed with all but our purest preparations and is reinforced by the absence of effect on enzyme activity by iron-chelating compounds.

Discussion

Induced enzymes of bacteria that catalyse the hydroxylation of aromatic rings usually utilize molecular oxygen as a source of the hydroxyl oxygen (Gibson, 1968; Evans, 1970; Dagley, 1971). However, studies with ¹⁸O₂ and H₂¹⁸O and also by more indirect means have shown that the hydroxylation of nicotinic acid by *Pseudomonas fluorescens* (Hunt *et al.*, 1958), by a *Clostridium* sp. (Holcenberg & Stadtman, 1969) and by a *Bacillus* sp. (Hirschberg & Ensign, 1971b) involves the incorporation of the oxygen of water as the oxygen of the hydroxyl group at the six position.

Similar observations have been reported for the hydroxylation of picolinic acid by *Rhodotorula* MR (Dagley & Johnson, 1963) and the hydroxylation of nicotine by *Arthrobacter oxydans* (Hochstein & Dalton, 1965, 1967).

In contrast with the molecular-oxygen-utilizing hydroxylases, which normally require the provision of an electron donor, utilization of the oxygen of water for the formation of a hydroxyl group requires the presence of an electron acceptor. The nicotinate hydroxylase of *Ps. fluorescens* (Hunt *et al.*, 1958) is situated in the particulate fraction of broken cells and electrons are presumably fed directly into the electron-transport chain. The other nicotinate hydroxylases and the picolinate hydroxylase of *Rhodotorula* MR are soluble proteins. Though they may in most cases be assayed with artificial dyes as electron acceptors, there is evidence that the membrane electron-transport system is the natural acceptor in *A. oxydans* and the *Bacillus* sp. (Hirschberg & Ensign, 1971a).

The one clear exception is the nicotinate hydroxylase from the *Clostridium* (Holcenberg & Stadtman, 1969). In the absence of a functional electron-transport chain, NADP⁺ serves as the primary electron acceptor.

Though we have not performed the definitive experiments with ¹⁸O₂ and H₂¹⁸O because of our lack of success in isolating the reaction product, all the indirect evidence demonstrates 2-furoyl-CoA hydroxylase to be one of this group of water-oxygen-utilizing enzymes. It displays the expected requirement for an electron acceptor that can be satisfied by the membrane fraction from 2-furoate-grown cells or by Methylene Blue or Nitro Blue Tetrazolium; there is no requirement for NADH or NADPH (Trudgill, 1969). Hayaishi (1969) has suggested that hydroxylations in which the oxygen of water is incorporated in this manner are only encountered with substrates more polar than benzenoid compounds; certainly 2-furoyl-CoA is in this category.

The more detailed studies of hydroxylases of this group so far reported are those of Holcenberg & Stadtman (1969), who showed the nicotinate hydroxylase of a *Clostridium* sp. to be a non-haem-iron-containing flavoprotein with a molecular weight of

300000, determined from ultracentrifuge data. The nicotine hydroxylase of a *Bacillus* sp. is also a very large protein with a molecular weight of 400000–450000 as measured by chromatography on Bio-Gel A-5m, and contains 2 mol of flavin and 8 mol of iron/mol of protein (Hirschberg & Ensign, 1971a).

In common with these enzymes 2-furoyl-CoA hydroxylase is certainly a large protein. A molecular weight of 3.27×10^6 has been obtained by the meniscus-depletion sedimentation equilibrium technique, considerably more than the value of 900000 that was suggested by Griffin *et al.* (1971) on the basis of its behaviour on Bio-Gel A-5m (Fig. 4). Chromato-

graphy of the enzyme on Bio-Gel A-15m gives a different picture and suggests a much larger molecule. This curious behaviour of the enzyme relative to glutamate dehydrogenase on the two Bio-Gel columns was quite reproducible and inexplicable, though it serves to illustrate the dangers inherent in basing molecular weights on Bio-Gel column results.

2-Furoyl-CoA hydroxylase is undoubtedly composed of several subunits, as incubation with 0.2% (w/v) sodium dodecyl sulphate caused a decrease of the $s_{20,w}$ value from 42.9 to 3.26S.

Unlike the *Bacillus* and *Clostridium* nicotine hydroxylases and the nicotine hydroxylase of *A.*

Scheme 1. Proposed mechanism for the hydroxylation of 2-furoyl-CoA by *Ps. putida* F2

oxydans, the 2-furoyl-CoA hydroxylase shows none of the absorption in the visible region normally associated with the presence of flavin and our preparations have never displayed, the characteristic yellow colour of flavoproteins. Again, in contrast, we have not been able to find any convincing evidence for the enzyme being an iron protein, and this is reflected by the failure of $\alpha\alpha'$ -bipyridyl and tiron to inhibit enzyme activity. Instead we have produced rather equivocal evidence that the enzyme contains catalytically active copper. There is a close correlation between increase in specific activity and copper content of the protein during purification. The lack of any observable blue colour during purification and the absence of absorption in the 600 nm region makes contamination of the enzyme with such copper proteins as azurins (Brill *et al.*, 1968) unlikely. The purest preparation on which we have assayed copper content (specific activity 1.6 units/mg of protein), has 46 mol of copper/mol of enzyme. The copper-chelating agents bathocuproin and cuprizone both inhibit hydroxylase activity; however, in puzzling contrast, sodium diethyldithiocarbamate is without effect, though it clearly reacts with copper present as judged by the formation of a yellow chromophore and the increase in E_{440} . A complete resolution of this equivocal situation may require the use of electron-paramagnetic-resonance studies.

The lack of spectral characterization in the visible region of the absorption spectrum is not necessarily incompatible with the presence of a copper prosthetic group, since dopamine (3,4-dihydroxyphenylethylamine) β -hydroxylase of bovine adrenal medullary tissue has been shown to contain copper and yet displays no absorption peaks in the visible region of the spectrum (Friedman & Kaufman, 1965).

A catalytic sequence for 2-furoyl-CoA hydroxylation is suggested in Scheme 1. Though the copper is indicated as being only involved in electron transport it is also possible that it may bind the 2-furoyl-CoA and the postulated reaction intermediates to the catalytic centre(s) through the sulphur of the thioester group and the oxygen of the furan ring.

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