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Multiple forms of γ-butyrobetaine hydroxylase (EC 1.14.11.1)

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γ-Butyrobetaine hydroxylase [4-trimethylaminobutyrate,2-oxoglutarate:oxygen oxidoreductase (3-hydroxylating), EC 1.14.11.1] from human kidney was resolved into three forms by chromatofocusing. After further chromatography on an anion-exchanger, each form appeared as a single band on electrophoresis in polyacrylamide gel containing sodium dodecyl sulphate. The isoelectric points of isoenzymes 1, 2 and 3 were 5.6, 5.7 and 5.8 respectively, as estimated by isoelectric focusing. Their specific activities were 17–29 μkat/g of protein. The concentrations of the three isoenzymes were about equal, possibly slightly lower for isoenzyme 1. The requirement for Fe^{2+} and the K_m values for γ-butyrobetaine and 2-oxoglutarate were about the same for the different enzyme forms. L- and D-Carnitine caused decarboxylation of 2-oxoglutarate to the same extent (8 and 29%) with the three forms. The enzyme forms had the same mass, 64kDa, as determined by gel filtration in nondenaturing media. The same subunit mass, 42kDa, was obtained for the multiple forms by electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate. Isoenzyme 2 was resolved into two protein bands by isoelectric focusing in polyacrylamide gels containing urea. Isoenzyme 1 contained only one of these bands and isoenzyme 3 the other. The three enzyme forms of γ-butyrobetaine hydroxylase thus appear to be dimeric combinations of two subunits differing in charge but not in size. γ-Butyrobetaine hydroxylase from crude extracts of human, rat and calf liver was also separated into multiple forms by a chromatofocusing technique. The isoenzyme pattern was the same in human liver and kidney. The technique used to resolve the mammalian enzymes gave no evidence for the presence of multiple forms of the bacterial enzyme from *Pseudomonas* sp. AK 1.

γ-Butyrobetaine hydroxylase [4-trimethylaminobutyrate,2-oxoglutarate:oxygen oxidoreductase (3-hydroxylating), EC 1.14.11.1] catalyses the hydroxylation of γ-butyrobetaine to carnitine (Lindstedt & Lindstedt, 1970). This is the final step in the biosynthesis of L-carnitine from 6-*N*-trimethyl-L-lysine (Cox & Hoppel, 1973; Tanphaichitr & Broquist, 1973). The hydroxylase belongs to the class of enzymes, known as 2-oxoglutarate-dependent dioxygenases, that catalyse the conversion of 2-oxoglutarate into succinate and CO_2 concomitantly with the oxygenation of the other substrate (Lindstedt *et al.*, 1967, 1968). These enzymes generally require Fe^{2+} and a reducing cofactor, e.g. ascorbate, and are stimulated by catalase (for reviews see Hayaishi *et al.*, 1975; Udenfriend & Cardinale, 1982). The subcellular localization of the enzyme and its cofactor requirements were first studied in rat liver (Lindstedt & Lindstedt, 1970). Homogeneous preparations of the enzyme were later obtained from *Pseudomonas* sp. AK 1 (G. Lindstedt *et al.*, 1977) and used for a study of the physicochemical properties of this enzyme (G. Lindstedt *et al.*, 1977) and of the role of different stimulating factors in the hydroxylation reaction (Lindstedt *et al.*, 1980; Blanchard *et al.*, 1982). In man, the main sites for conversion of γ-butyrobetaine into carnitine are kidney and liver (Englard, 1979; Lindstedt *et al.*, 1980; Rebouche & Engel, 1980). The activity of γ-butyrobetaine hydroxylase per g of protein in the soluble fractions of kidney homogenates is about 4 times higher than in homogenates of liver (Lindstedt *et al.*, 1980; Rebouche & Engel, 1980). With a partially purified enzyme preparation from human kidney (Lindstedt *et al.*, 1982), it was found that during

normal catalysis the formation of CO_2 exceeds the hydroxylation of γ-butyrobetaine by about 20% (Holme *et al.*, 1982). With the bacterial enzyme this type of uncoupling is less than 2%. L-Carnitine is an uncoupler for the human enzyme. D-Carnitine is an uncoupler for both the human and the bacterial enzymes, but much more efficient for the human enzyme. To study these effects in more detail we wished to pursue the purification of the human enzyme, and in the course of this work also found evidence for the presence of three multiple forms.

Experimental

Materials

The materials used were obtained from the following sources: γ-butyrobetaine chloride from E. Merck, Darmstadt, Germany; 2-oxo[1-^{14}C]-glutarate from New England Nuclear, Boston, MA, U.S.A.; ultra-thimbles from Schleicher und Schüll, Dassel, Germany; Mono Q™ (prepacked anion-exchanger), Mono P™ (prepacked Polybuffer™ exchanger), Polybuffer 74 and Low pI calibration kit from Pharmacia Fine Chemicals, Uppsala, Sweden. Other compounds were commercially available of analytical grade.

Enzyme assay

The activity of γ-butyrobetaine hydroxylase was determined by measuring the release of $^{14}CO_2$ from 2-oxo[1-^{14}C]glutarate (Lindstedt *et al.*, 1970). The composition of the incubation mixture was: γ-butyrobetaine (1.4mM), 2-oxo[1-^{14}C]glutarate (0.30–1.4mM, 0.14mCi/l), $FeSO_4$ (0.6mM), sodium ascorbate (14mM), catalase (1.4g/l), potassium phosphate buffer, pH6.5 (14mM), and variable amounts of enzyme. Separate incubations were carried out to measure γ-butyrobetaine-independent activity. The total volume was 0.35ml and the incubations were carried out at 37°C for 10 to 30min.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Enzyme sources

Homogenates of human kidney and liver and of rat and calf liver were prepared in 0.25M-sucrose (2ml/g of tissue) in a Potter–Elvehjem homogenizer. The homogenates were centrifuged at 100000*g* for 1h. A fraction precipitated with $(NH_4)_2SO_4$ at 0–70% saturation was used in the fractionation of the calf liver enzyme. γ-Butyrobetaine hydroxylase from human kidney was purified through the four steps of the procedure described previously (Lindstedt *et al.*, 1982). γ-Butyrobetaine hydroxylase from *Pseudomonas* sp. AK 1 was a homogeneous preparation (G. Lindstedt *et al.*, 1977).

Chromatographic procedures

All steps were performed at 4°C. Enzyme solutions were concentrated by vacuum dialysis in ultra-thimbles. Portions (15–20mg of protein) of the purified human kidney enzyme were applied on to a column (1.6cm × 25cm, 50ml) of Sephadex G-25 (fine grade) equilibrated with 25mM-piperazine/HCl buffer, pH6.0. The protein fractions were applied on to a Mono P™ chromatofocusing column with the use of the fast protein liquid-chromatographic system supplied by Pharmacia. The pH gradient was formed by equilibrating the column with 25mM-piperazine/HCl buffer, pH6.0, and eluting with 10% (v/v) Polybuffer 74, pH4.8. Fractions of volume 0.5ml were collected at a flow rate of 0.5ml/min. The peaks of γ-butyrobetaine hydroxylase activity were concentrated separately and filtered through columns (0.9cm × 11cm, 7ml) of Sephadex G-25 (fine grade) in 20mM-Bistris {2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol}/HCl buffer, pH6.5. Each sample was then applied on to a Mono Q™ column in 20mM-Bistris/HCl buffer, pH6.5, and eluted with a 35ml linear gradient of 0–0.18M-KCl in 20mM-Bistris/HCl buffer, pH6.5. Fractions of volume 0.5ml were collected at a flow rate of 1ml/min. Pooled fractions were concentrated by vacuum dialysis against 10mM-potassium phosphate buffer, pH6.5.

Gel filtration

A column (1.6cm × 85cm, 170ml) of Sephadex G-200 (superfine grade) was prepared in 50mM-potassium phosphate buffer, pH6.5, containing 0.1M-NaCl. The column was equilibrated at 4°C to a flow rate of 3ml/h. Different preparations of γ-butyrobetaine hydroxylase were run together with Blue Dextran 2000, human immunoglobulin G (153kDa; Sober, 1973, p. C-11), human transferrin (76.6kDa; Fish *et al.*, 1969), human albumin (68.5kDa; Sober, 1973, p. C-10), ovalbumin (43.5kDa; Sober, 1973, p. C-15) and horse myoglobin (16.9kDa; Sober, 1973, p. C-10). Immunoglobulin G, transferrin and albumin were identified in the eluate by immunochemical methods (Mancini *et al.*, 1965; Laurell, 1966), and ovalbumin and myoglobin were identified by absorbance at 280nm and 405nm. $K_{av.}$ was calculated from $K_{av.} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume, V_0 is the void volume determined by Blue Dextran and V_t is the total volume (Reiland, 1971).

Electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate

Polyacrylamide-gel electrophoresis was carried out in 0.1% (w/v) sodium dodecyl sulphate with slab gels or cylindrical gels, 7.5% (w/v) in acrylamide, according to the procedure of Weber *et al.* (1972). The reference proteins used were: human transferrin (76.6kDa; Fish *et al.*, 1969), human albumin (68.5kDa; Sober, 1973, p. C-10), bovine catalase subunit (60.0kDa; Weber *et al.*, 1972), ovalbumin (43.5kDa; Sober, 1973, p. C-15), rabbit fructose bisphosphate aldolase subunit (40.0kDa; Weber *et al.*, 1972), bovine chymotrypsinogen (23.7kDa; Sober, 1973, p. C-10) and horse myoglobin (16.9kDa; Sober, 1973, p. C-10). The samples were denatured and reduced in 10mM-sodium phosphate buffer, pH7.0, containing sodium dodecyl sulphate (1%, w/v) and 2-mercaptoethanol (1%, v/v) for 3min at 100°C. The gels were stained with Coomassie Brilliant Blue. Molecular masses were determined by the procedure of Weber *et al.* (1972).

Analytical isoelectric focusing

The isoelectric focusing in polyacrylamide gel was carried out essentially as described by Wrigley (1971), with slab gels, 5% (w/v) in acrylamide. The electrolyte solutions contained 0.1M-NaOH at the cathode and 40mM-aspartic acid at the anode. Carrier ampholyte solution in the pH range 3.5–10 was used. The pH gradient profile was determined with a calibration kit of proteins with pI values between 2.80 and 6.55.

Isoelectric focusing in 9M-urea was performed in cylindrical gels as described by Anderson & Anderson (1978) but without the second-dimension electrophoresis. A 20μl portion of each of the protein solutions (about 0.4g/l) was mixed with 10μl of sodium dodecyl sulphate (4%, w/v)/2-mercaptoethanol (10%, v/v)/glycerol (20%, v/v), and the mixtures were heated at 95°C for 5min, After cooling, 30μl of 9M-urea was added to each. Prefocusing of the gels was carried out for 1h at 200V. Focusing was then carried out at 300V for about 18h. After electrophoresis the proteins were fixed in trichloroacetic acid (10%, w/v)/ethanol (33%, v/v) for 30min. The gels were washed in trichloroacetic acid (5%, w/v)/ethanol (33%, v/v) until the precipitates were dissolved. Before staining in Coomassie Brilliant Blue (0.2%, w/v) for about 4h, the gels were placed in the destaining solution, methanol/acetic acid/water (3:1:6, by vol.) for 30min.

Results

Separation into multiple forms

In one series of experiments we used as starting material enzyme from human kidney that had been partially purified in a four-step procedure. The enzyme could be separated into three fractions (peaks I, II and III) with enzymic activity either by using a chromatofocusing technique (Fig. 1) or by anion-exchange chromatography (Fig. 2). Protein from each enzyme peak obtained in the chromatofocusing experiment was then chromatographed on an anion-exchanger under strictly identical conditions. The peaks of enzyme activity obtained in these experiments were essentially symmetrical and appeared at increasingly larger elution volumes (Fig. 3). Material from each peak was subjected to electrophoresis in polyacrylamide gels

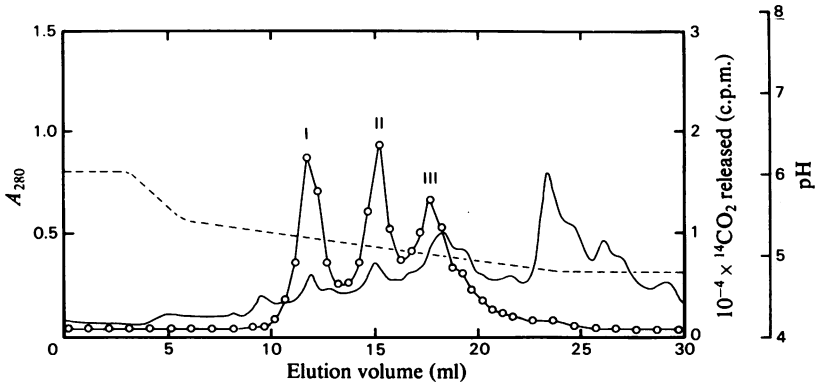

Fig. 1. *Separation on a Mono P™ chromatofocusing column of a partially purified γ-butyrobetaine hydroxylase from human kidney*

The protein applied was 16mg. The column was eluted with a pH gradient (----) between pH5.4 and 4.8. Fractions of volume 0.5ml were collected at a flow rate of 0.5ml/min. Enzyme activities (○) were determined on 10μl of the fractions. ——, A_{280}. Incubations were carried out for 15min with 0.6mM-2-oxo[1-^{14}C]glutarate.

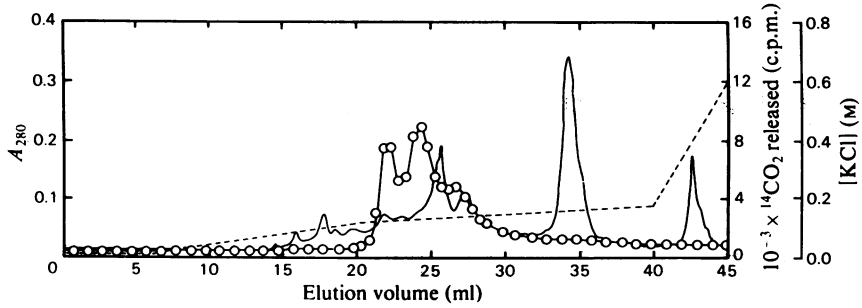

Fig. 2. *Separation on a Mono Q™ anion-exchanger of a partially purified γ-butyrobetaine hydroxylase from human kidney*
The protein applied was 1.5mg. The column was eluted with increasing concentration of KCl (----) in 20mm-Bis-tris/HCl buffer, pH6.5. Fractions of volume 0.5ml were collected at a flow rate of 1ml/min. Enzyme activities (○) were determined on 25μl of the fractions. ——, A_{280}. Incubations were carried out for 15min with 0.7mM-2-oxo-[1-^{14}C]glutarate.

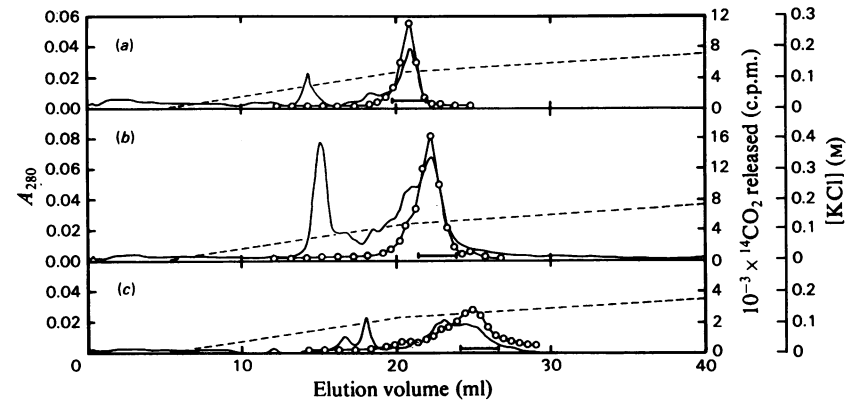

Fig. 3. *Chromatography on a Mono Q™ anion-exchanger of peak I (a), peak II (b) and peak III (c) from the chromatofocusing on Mono P™*
The column was eluted with increasing concentration of KCl (----) in 20mM-Bistris/HCl buffer, pH6.5. Fractions of volume 0.5ml were collected at a flow rate of 1ml/min. Enzyme activities (○) were determined on 10μl of the fractions. ——, A_{280}. Incubations were carried out for 15min with 0.3mM-2-oxo[1-^{14}C]glutarate. The transverse bars indicate the fractions that were pooled.

after denaturing in sodium dodecyl sulphate. The protein from each peak appeared essentially as one main band (Fig. 4*a*). Table 1 gives a summary of the results from a typical purification experiment, which started with 100g of human kidney. In this experiment the purification was about 600-fold as compared with the activity of the initial 100000*g* supernatant. The recovery of enzyme activity in the final chromatography, measured on pooled peak fractions, was 15–40%, which included losses in a concentration step and the buffer exchange on Sephadex G-25. There was, however, an increase in specific activity of 1.3–1.8-fold due to the removal of inactive protein from the enzyme.

When a crude kidney extract (100000*g* supernatant fraction) was applied on to a chromatofocusing column and eluted with a pH gradient between pH5.5 and 4.5 a similar pattern of three enzyme-containing peaks was found (Fig. 5*a*).

γ-Butyrobetaine hydroxylase in human, rat and calf liver and in Pseudomonas sp. AK 1

The supernatants after centrifugation at 100000*g* of homogenates of human, rat and calf liver were separated on a chromatofocusing column. The γ-butyrobetaine hydroxylase activity was resolved into three major forms (Figs. 5*b*, 5*c* and 5*d*) as in human kidney. In rat liver the amount of enzyme in peak I appeared to be significantly higher than in peaks II and III, whereas in calf liver peak II was the largest. The pure enzyme from *Pseudomonas* sp. AK 1 was run under various conditions on both the chromatofocusing column and on the anion-exchanger. We

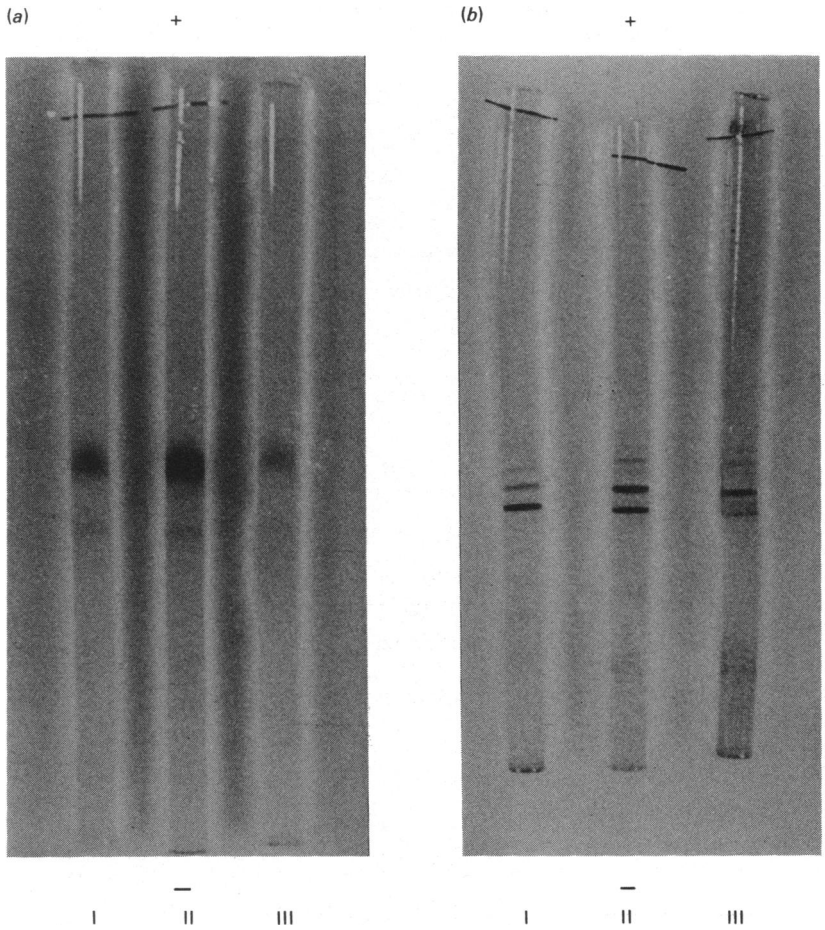

Fig. 4. *Separation of the multiple forms of human kidney γ-butyrobetaine hydroxylase according to size and to charge*
(*a*) Electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate. Protein from peak I (10 μg), peak II (20 μg) and peak III (6 μg) was denatured and reduced in buffer containing sodium dodecyl sulphate and mecaptoethanol for 3 min at 100°C. The metal wires indicate the migrations of Bromophenol Blue. (*b*) Analytical isoelectric focusing in polyacrylamide gels containing 9 M-urea. The samples were denatured and reduced in sodium dodecyl sulphate and mercaptoethanol for 5 min at 95°C. After cooling, urea was added to the samples. About 5 μg of protein was applied on to the gels.

Table 1. *Fractionation of γ-butyrobetaine hydroxylase from human kidney into multiple forms*
Results represent yields obtained from 100 g of human kidney.

Purification step	Protein (mg)	Total activity (nkat)	Specific activity (μkat/g)
Hydroxyapatite chromatography*	36	94	2.6
Separation on Mono P™ (chromatofocusing medium)			
Peak I	0.98	21	21
Peak II	1.6	25	16
Peak III	2.4	23	9.7
Separation on Mono Q™ (anion-exchanger) Peak I	0.25	7.3	29
Separation on Mono Q™ (anion-exchanger) Peak II	0.36	8.6	24
Separation on Mono Q™ (anion-exchanger) Peak III	0.18	3.1	17

* Step 4 in the purification procedure described previously (Lindstedt *et al.*, 1982).

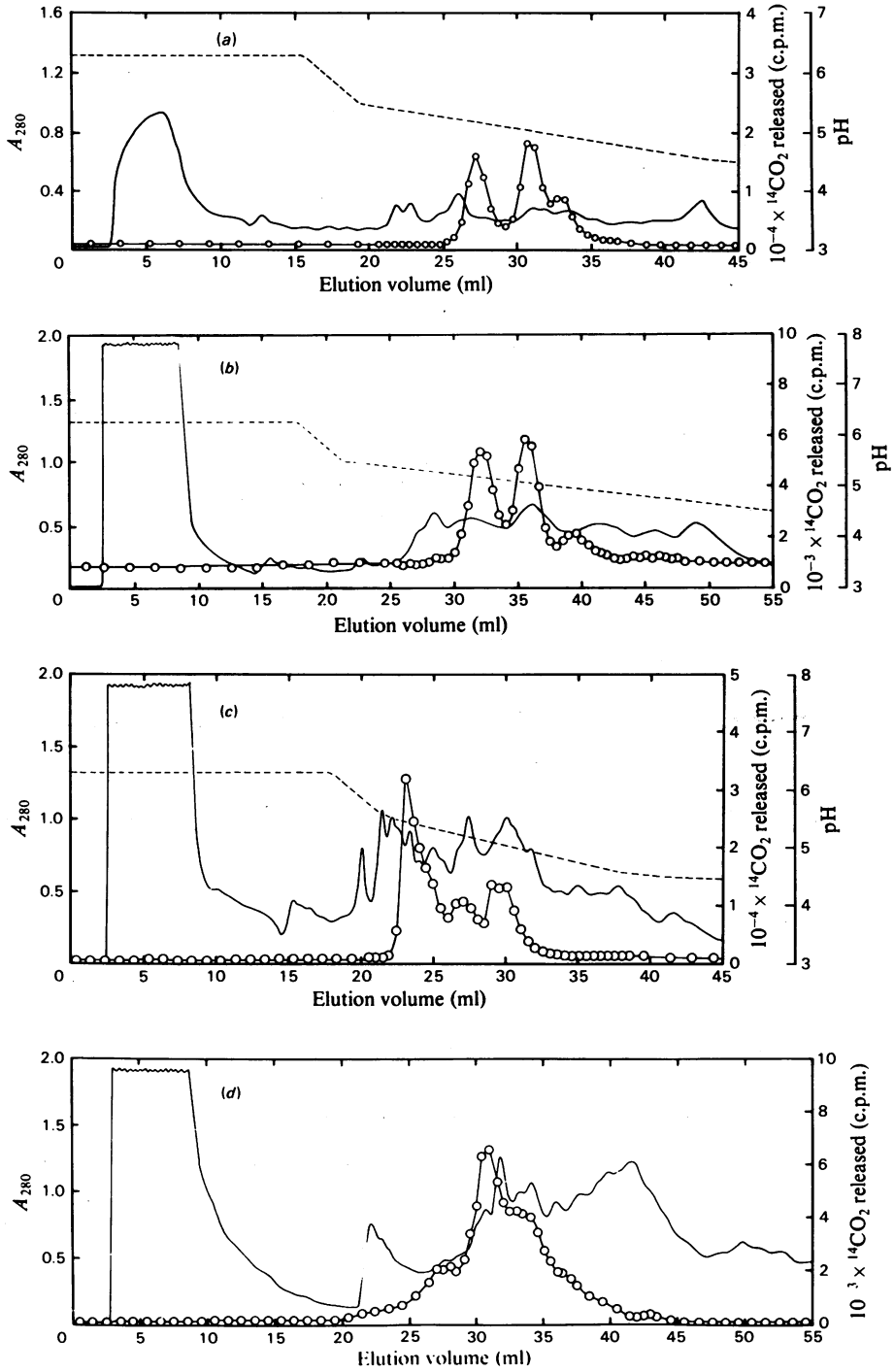

Fig. 5. *Separation on a Mono P™ chromatofocusing column of 100000g supernatants of human kidney (a), human liver (b), rat liver (c) and calf liver (d)*

After filtration of 2ml of a 100000*g* supernatant of each tissue homogenate (33%, w/v) through columns (1.6cm × 15cm, 30ml) of Sephadex G-25 (fine grade) in 25mM-piperazine/HCl buffer, pH6.3, the samples were applied on to the Mono P™ column. The column was eluted with a pH gradient between pH5.5 and 4.5 (----). Fractions of volume 0.5ml were collected at a flow rate of 0.5ml/min. Enzyme activities (○) were determined on 200μl of the fractions. ——, A_{280}. Incubations were carried out for 15min (*a*), 20min (*d*), 30min (*b*) or 45min (*c*) with 0.3mM-2-oxo[1-^{14}C]glutarate.

always obtained only one fully symmetrical peak and no evidence for the occurrence of multiple forms.

Isoelectric point

Isoelectric focusing was performed in slab gels of polyacrylamide. The pH gradient was determined with a calibration kit of proteins with pI values between 2.80 and 6.55. The isoelectric points of the three enzyme forms of human kidney enzyme were 5.6, 5.7 and 5.8. We name the enzyme forms isoenzyme 1 (peak III), 2 (peak II), and 3 (peak I), in increasing order of mobility towards the anode (IUPAC–IUB Commission on Biochemical Nomenclature, 1978).

Molecular mass

The molecular mass of the human kidney enzymes was estimated by gel filtration. Experimentally determined $K_{av.}$ values were plotted versus the logarithms of the molecular masses of the reference proteins. The mass obtained for the γ-butyrobetaine hydroxylase activity of an unresolved hydroxyapatite fraction and for a mixture of the purified multiple forms was 64kDa.

Subunits

The subunit masses of the reduced and denatured isoenzymes were determined by electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate. Identical subunit masses were obtained (see Fig. 4*a*). A subunit mass of 42kDa was obtained for the isoenzymes of γ-butyrobetaine hydroxylase, when calculated from a plot of relative mobilities versus the logarithms of the subunit masses of the reference proteins.

Two major bands appeared when reduced and denatured isoenzymes were subjected to analytical isoelectric focusing in the presence of 9M-urea (Fig. 4*b*). Isoenzyme 1 (peak III) gave rise to one dominant band (A) and isoenzyme 3 (peak I) to another dominant band (B), whereas isoenzyme 2 (peak II) gave rise to two bands with about the same intensity and with the same mobilities as bands A and B. As the isoelectric points of the three isoenzymes differed only by about 0.1 pH unit, it was difficult to separate them completely (see Fig. 3), which resulted in a faint B-band in isoenzyme 1 and a faint A-band in isoenzyme 3.

Kinetic properties

No activity of the different isoenzymes was obtained when Fe^{2+} was omitted from the assay medium. Sigmoid curves were obtained when the enzyme activity of the multiple forms were plotted versus the Fe^{2+} ion concentration (Fig. 6). The Figure shows the result obtained in incubations with isoenzyme 2. The results with the other isoenzymes were very similar. The minimum concentration of Fe^{2+} required for maximal enzyme activity was about 15μM. The Michaelis constants for 2-oxoglutarate were 0.10, 0.13 and 0.11mM for the three isoenzymes, when estimated from experiments performed in air at 1.4mM-γ-butyrobetaine. No decarboxylation of 2-oxoglutarate was found when γ-butyrobetaine was omitted from the assay medium. The Michaelis constants for γ-butyrobetaine were 0.13, 0.11 and 0.13mM for isoenzymes 1, 2 and 3 respectively, when estimated from experiments performed in air at 0.7mM-2-oxoglutarate. D-Carnitine was an effective uncoupler for all three forms, but uncoupling occurred also with L-carnitine (Table 2).

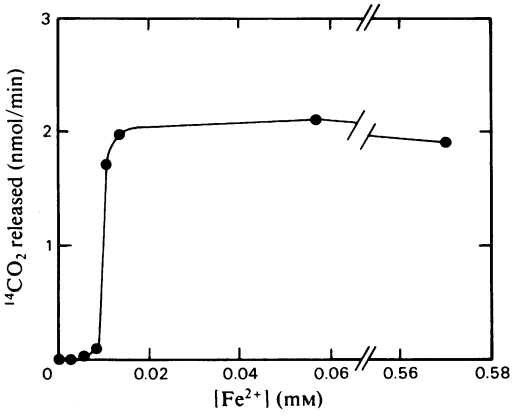

Fig. 6. *Effect of Fe^{2+} on the activity of human kidney γ-butyrobetaine hydroxylase*
The γ-butyrobetaine-dependent decarboxylation of 2-oxoglutarate was measured. Enzyme form 2 (4μg/ml) was used. Incubations were carried out for 30min with 1.4mM-2-oxo[1-^{14}C]glutarate.

Table 2. *Effect of* L- *and* D-*carnitine on the decarboxylation of 2-oxoglutarate catalysed by the multiple forms of γ-butyrobetaine hydroxylase from human kidney*

	Relative activity (%)		
Substrate	Enzyme form 1	Enzyme form 2	Enzyme form 3
γ-Butyrobetaine	100	100	100
D-Carnitine	28	25	29
L-Carnitine	9	8	9

Discussion

The presence of three enzyme forms in a partially purified human γ-butyrobetaine hydroxylase is not due to any preparation artifact since they appear in the same relative amounts also in 100000*g* supernatants of kidney homogenates. Also, the separation into isoenzymes may be achieved either by a chromatofocusing technique or by chromatography on an anion-exchanger. Both techniques separate the enzyme into a similar pattern, i.e. equal amounts of isoenzyme 2 and 3 and possibly a somewhat smaller amount of isoenzyme 1. Results based on the four human kidneys that have been available to us indicate that there is no significant interindividual difference in isoenzyme pattern. It also appears that the pattern is the same in liver and kidney, which are the organs with the highest concentration of γ-butyrobetaine hydroxylase in man. Rat liver, which has been the source of enzyme in several earlier studies on the enzyme properties, also contains three isoenzymes. The enzyme activity in calf liver may also be separated into multiple forms by chromatofocusing. It thus appears that the mammalian enzyme is composed of isoenzymes, in contrast with the bacterial enzyme from *Pseudomonas* sp. AK 1. Prolyl 4-hydroxylase and lysyl 5-hydroxylase, which are both 2-oxoglutarate dependent enzymes, have been obtained in homogeneous form from mammalian sources, but so far have not been reported to contain isoenzymes (Berg & Prockop, 1973; Kuutti *et al.*, 1975; Tuderman *et al.*, 1975; Turpeeniemi-Hujanen *et al.*, 1980; Kedersha & Berg, 1981). 4-Hydroxyphenylpyruvate dioxygenase is an internal 2-oxo acid-dependent enzyme, which has several properties in common with the 2-oxoglutarate-dependent enzymes. This enzyme can be resolved into multiple forms when human, chicken or hog liver is the source (Rundgren, 1977; Wada *et al.*, 1975; Roche *et al.*, 1982), but not when isolated from a bacterial source (S. Lindstedt *et al.*, 1977). The isoenzymes of human kidney γ-butyrobetaine hydroxylase are due to different combinations of A and B subunits, as is also the case for the human liver 4-hydroxyphenylpyruvate dioxygenase (Rundgren, 1977).

In Table 3 are summarized the characteristics of the γ-butyrobetaine hydroxylases, which have been extensively purified. The enzymes are quite similar and are apparently dimers of equally sized subunits. The native molecular mass of the human enzyme as estimated by gel filtration was less than the sum of the subunit molecular masses determined by gel electrophoresis in sodium dodecyl sulphate. Possibly, the low value could be due to reversible binding of the enzyme to the column material or to an elongated form of the enzyme. A lower molecular mass was also estimated for the pseudomonad enzyme from gel-filtration experiments than from equilibrium sedimentation. The specific activity reached by this purification is 20–30-fold higher than that reported for the calf liver enzyme, 0.9 μkat/g of protein (Kondo *et al.*, 1981). It is, however, in the same range as that reported for homogeneous preparations of prolyl 4-hydroxylase and lysyl 5-hydroxylase from mammalian sources, 20–43 μkat/g of protein (Berg & Prockop, 1973; Kuutti *et al.*, 1975; Tuderman *et al.*, 1975; Nietfeld & Kemp, 1980; Turpeeniemi-Hujanen *et al.*, 1980; Kedersha & Berg, 1981). The specific activity of the bacterial γ-butyrobetaine hydroxylase is much higher, 360 μkat/g of protein (G. Lindstedt *et al.*, 1977). The isoenzymes are very similar in their catalytic properties. Both enantiomers of carnitine are uncouplers for the three isoenzymes, as was the case with an unresolved preparation (Holme *et al.*, 1982). The stimulation with Fe^{2+} does not follow Michaelis–Menten kinetics. A sigmoid relationship between the Fe^{2+} concentration and the enzyme activity has also been obtained with the pseudomonad enzyme in phosphate buffer as well as in Tris/HCl, Mes (4-morpholine-ethanesulphonic acid) and Mops (4-morpholine-propanesulphonic acid) buffers (results not shown). Sigmoid curves are obtained with enzyme preparations at different degrees of purity both from *Pseudomonas* sp. AK 1 and from human

Table 3. *Comparison of γ-butyrobetaine hydroxylase from various sources*
Key to references: [a] present work; [b] G. Lindstedt *et al.* (1977); [c] Kondo *et al.* (1981).

	Human kidney enzyme[a]	*Pseudomonas* enzyme[b]	Calf liver enzyme[c]
Multiple forms	3	None	None[c] 3[a]
Molecular mass (kDa)	64*	86* 95†	80
Subunit mass (kDa)	42	37	46
Subunit pattern	AA, AB, BB	AB	–
Isoelectric point	5.6, 5.7, 5.8	5.1	–

* Gel filtration.
† Equilibrium sedimentation.

kidney. The profile seems to be independent of protein concentration. In studies of the stimulation with Fe^{2+} on the 2-oxoglutarate-dependent thymine 7-hydroxylase and thymidine 2'-hydroxylase in our laboratory, no indications of sigmoidicity in the Fe^{2+} binding have been obtained. The assay mixtures used for these dioxygenases are very similar to that used for γ-butyrobetaine hydroxylase. Thus the sigmoidicity in the Fe^{2+} binding to γ-butyrobetaine hydroxylase is probably not due to the presence of a chelator in the assay mixture, but may possibly be explained by a co-operative effect in the binding of 2mol of iron/mol of enzyme. Thymine 7-hydroxylase and thymidine 2'-hydroxylase are monomers (Warn-Cramer *et al.*, 1983; Holme, 1983) and are supposed to have only one iron-binding site. Cumming *et al.* (1978) have shown that the binding of Fe^{2+} to prolyl 4-hydroxylase follows non-Michaelis–Menten kinetics, and suggest that prolyl hydroxylase is an allosteric enzyme under positive heterotropic control. They used a model assuming 4mol of iron/mol of prolyl 4-hydroxylase. It appears, however, that this enzyme, consisting of four subunits (AABB), reaches maximal activity with 2mol of Fe^{2+}/mol of enzyme (De Jong & Kemp, 1982). We have not been able to obtain a native iron-containing enzyme, either bacterial or mammalian. The results obtained so far are well compatible with the presence of two active centres, each containing one Fe^{2+} ion, per molecule of enzyme.

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