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Purification and Properties of γ-Butyrobetaine Hydroxylase from *Pseudomonas* sp AK 1†

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ABSTRACT: γ-Butyrobetaine hydroxylase (4-trimethylaminobutyrate,2-oxoglutarate:oxygen oxidoreductase (3-hydroxylating), EC 1.14.11.1) has been isolated from *Pseudomonas* sp AK 1 by ion-exchange, adsorption, and molecular-sieving chromatography. The preparation was homogeneous as judged from electrophoresis in agarose and polyacrylamide gels, isoelectric focusing, and equilibrium sedimentation. The molecular mass was 95 kdaltons as determined by sedimentation equilibrium centrifugation. From electrophoresis in polyacrylamide gel the molecular mass was estimated to 92 kdaltons, from gel filtration through columns of Sephadex G-200 to 86 kdaltons, and from gel filtration through thin layers of Sephadex G-150 and G-200 to 82 kdaltons. Calculation of molecular mass from Stokes radius, sedimentation coefficient, and partial specific volume gave a value of 96 kdaltons, and from the sedimentation coefficient, 93 kdaltons. Gel filtration through Sephadex G-200 in 6 M guanidinium chloride and electrophoresis in polyacrylamide gel containing 3.5 mM sodium dodecyl sulfate resulted in single bands with mobilities corresponding to molecular masses of 39 and 37 kdaltons, respectively, indicating that the enzyme is composed of two polypeptide chains with similar size. NH_2-terminal amino acid sequencing in three cycles resulted in two amino acids in each cycle (Ala + Asn, Ala + Ile, Ala + Ile). The Stokes radius was 3.8 nm, corresponding to a diffusion coefficient of 5.7×10^{-7} cm^2/s. A sedimentation coefficient of 5.8×10^{-13} s and a frictional ratio of 1.26 was found. The partial specific volume was 0.729 mL/g at 20 °C as calculated from amino acid analysis. The isoelectric point was 5.1, as determined by isoelectric focusing analysis. The light absorption in the ultraviolet and visible regions was that of a protein without light-absorbing prosthetic groups. The absorption coefficient at 280 nm of a 1.0% solution at pH 6.5 was 12.6. Amino acid analysis by ion-exchange chromatography showed a half-cystine content of 19 mol per 95 kg of protein (23 residues/1000). Thirteen sulfhydryl groups were found by colorimetric analysis before as well as after reduction with $NaBH_4$, indicating absence of disulfide bonds. Less than 0.1 mol of iron was found per mol of enzyme.

The hydroxylation of γ-butyrobetaine to carnitine was first studied in crude preparations from rat liver (Lindstedt and Lindstedt, 1962, 1970; Lindstedt, 1967a,b). A *Pseudomonas* strain which had been isolated by enrichment culture in γ-butyrobetaine-containing media was then used as the source of γ-butyrobetaine hydroxylase (4-trimethylaminobutyrate, 2-oxoglutarate:oxygen oxidoreductase (3-hydroxylating), EC 1.14.11.1) (Lindstedt et al., 1967, 1970a,b). With crude preparations of this enzyme, it could be demonstrated that 2-oxoglutarate is decarboxylated in stoichiometric amounts with the formation of carnitine, that succinic semialdehyde is not an intermediate in succinate formation, and that molecular oxygen is incorporated into succinate (Lindstedt et al., 1968; Holme et al., 1968; Lindblad et al., 1969). This novel type of oxygenase reaction was then demonstrated for two other enzymes, i.e., prolyl hydroxylase (EC 1.14.11.2), and thymine 7-hydroxylase (EC 1.14.11.6) (for a review, see Hayaishi et al., 1975). There is evidence that thymidine 2′-hydroxylase and lysyl hydroxylase catalyze the same type of reaction, although it has not so far been demonstrated that molecular oxygen is incorporated into succinate formed during the reaction. Another 2-oxoacid-dependent hydroxylation—the formation of homogentisate from 4-hydroxyphenylpyruvate—probably occurs with a similar mechanism (Goodwin and Witkop, 1957; Lindblad et al., 1970).

Prolyl hydroxylase has been isolated from various animal sources by several groups (for a review, see Hayaishi et al., 1975). 4-Hydroxyphenylpyruvate dioxygenase has been isolated from human liver (Lindblad et al., 1970; to be published) and from a *Pseudomonas* strain (Lindstedt et al., to be published) as well as from several animal species (Fellman et al., 1972; Nakai et al., 1975).

We now report the isolation and characterization of γ-butyrobetaine hydroxylase from *Pseudomonas* sp AK 1.

Experimental Procedures

Materials. Compounds were obtained from the following sources: γ-butyrobetaine chloride from E. Merck AG, Darmstadt, West Germany; human transferrin from Kabi AB, Stockholm, Sweden; polyamide thin-layer plates from Cheng Chin Trading Co., Ltd., Tapei, Taiwan: BDC-OH[1] and

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[1] Abbreviations used are: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); BDC-OH, bis[4-(dimethylamino)phenyl]carbinol; Pth, phenylthiohydantoin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl.

Pth-amino acid reference collection from Pierce Chemical Co., Rockford, Ill.; hydroxylapatite from Bio-Rad Laboratories Inc., Richmond, Calif.; and 2-oxo[1-^{14}C]glutaric acid (14.2 Ci/mol) from New England Nuclear, Boston, Mass. Centrally deionized water was further purified by passage through granular activated carbon, mixed ion-exchange resin and a Millipore 0.45 μm filter (Super Q System of Millipore). The deionized water had a resistivity above 18 Mohm × cm.

A polyvalent antiserum was prepared by immunizing a rabbit with a 100 000g supernatant fraction of sonified bacterial cells. Half a milliliter of the 100 000g supernatant (3–4 mg of protein) was emulsified with 0.5 mL of Freund's complete adjuvant and injected into the foot pads. The procedure was repeated after 2 weeks. Blood was then obtained from an ear vein every second week for 2 months. The antiserum was tested by crossed immunoelectrophoresis against the material used for immunization. At least 30 peaks were obtained when the cell extract was analyzed, which demonstrates the polyvalent nature of the antiserum. The serum was stored at −20 °C.

Cell Cultures. Stock cultures of *Pseudomonas* sp AK 1 (Lindstedt et al., 1967, 1970a) were maintained on blood agar plates. The composition of the medium was: γ-butyrobetaine (3.6 g/L), KH_2PO_4 (5.3 g/L), $Na_2HPO_4 \cdot 2H_2O$ (10.9 g/L), $MgSO_4 \cdot 7H_2O$ (75 mg/L), and $MnCl_2 \cdot 4H_2O$ (4.5 mg/L). The pH was adjusted to 7.0. The temperature was 25 °C. Cultures were started by growing first for 30 h in 100-mL erlenmeyer flasks, and then in 2-L flasks for 30 h. The flasks were agitated on a gyratory shaker at 60 rpm. The main cultures were performed in 20-L flasks containing 16 L of medium. Purified air was bubbled through the medium at a rate of 1.6 L/min. The medium was stirred with a propeller (2000 rpm). The turbidity at 525 nm was measured on samples of the culture and the cells were harvested at the end of the logarithmic growth phase by centrifugation in a continuous-action rotor at 21 000g. The cells were washed three times with 0.15 M NaCl. The bacterial paste was kept frozen at −20 °C.

Enzyme Purification Procedures. All steps in the purification of the enzyme were carried out in a cold room at a temperature of 4 °C. Enzyme solutions were concentrated by ultrafiltration on PM-10 Diaflo ultrafiltration membranes or by vacuum dialysis in Sartorius Membranfilter 13200 collodion bags.

Enzyme Assay. The enzyme activity was determined by measurement of $^{14}CO_2$ from 2-oxo[1-^{14}C]glutarate in the presence of γ-butyrobetaine and cofactors. The composition of the incubation mixture was: enzyme (2–72 μg of protein), γ-butyrobetaine (29 mM), 2-oxo[1-^{14}C]glutarate (2.9 mM, 0.05 μCi/incubation), $FeSO_4$ (0.6 mM), sodium ascorbate (14 mM), catalase (1.4 g/L), and potassium phosphate buffer at pH 7.0 (14 mM). The total volume was 0.35 mL and the incubations were carried out at 37 °C for 30 min in 10-mL stoppered centrifuge tubes. The incubations were ended by addition of 0.3 mL of 0.6 M trichloroacetic acid. Labeled CO_2 was trapped on a piece of filter paper attached to a wire in the rubber stopper of the test tube. Twenty microliters of a 1 M solution of Hyamine-10 X in methanol had been pipetted onto the filter paper. After diffusion of $^{14}CO_2$ for 60 min at 37 °C, the filter papers were transferred to a scintillation mixture: 2,5-diphenyloxazole (10 g), 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (0.3 g), toluene (1000 mL), and methoxyethanol (600 mL). Radioactivity was measured in a Tri-Carb liquid scintillation spectrometer.

Protein Determinations. Protein was determined according to Lowry et al. (1951) with standard curves prepared from bovine serum albumin. With pure enzyme, the same value was obtained by dry weight as by the Lowry method. Reference proteins in the determinations of molecular mass, Stokes radius, and sedimentation coefficient were usually assayed by photometry at 280 nm. Catalase was assayed by following disappearance of H_2O_2 at 240 nm (Martin and Ames, 1961). Human albumin and transferrin were assayed immunochemically according to Laurell (1966).

Light Absorption Spectrum. For determination of the absorption coefficient, the enzyme solution was equilibrated with 2 mM potassium phosphate buffer, pH 6.5, by passage through a column of Sephadex G-25. The protein solution was then centrifuged for 60 min at 100 000g. The absorbance was determined after dilution of small samples with 2 mM potassium phosphate buffer at pH 6.5, which had been collected from the column used for equilibration. Background absorption at 280 nm was estimated by linear extrapolation of the spectrum between 340 and 380 nm. Three 0.2-mL samples containing about 0.5 mg of protein were also taken for dry-weight determination at 10 Pa to constant weight over P_2O_5. Dry weight determinations were also performed on the side fractions from the Sephadex column. The dried samples were weighed over a period of 1 week.

Analytical Ultracentrifugation. Analyses were made with a Spinco Model E analytical ultracentrifuge equipped with electronic speed and rotor temperature control units and with Rayleigh interference optics. Determinations of molecular mass of native enzyme were performed by sedimentation equilibrium centrifugation with the long-column meniscus depletion technique (Chervenka, 1970). The protein in 50 mM potassium phosphate buffer at pH 6.5 was centrifuged at 4 °C for 19 h at 15 000 rpm which allowed measurement close to the bottom of the tube. Protein concentrations were 0.6–0.7 g/L. The photographic plates were red in a Nikon profile projector. The slope in the plot of the logarithm of fringe displacement vs. the radial distance squared was calculated by least-squares fit to a straight line.

Sucrose Gradient Sedimentation. A 0.2-mL sample of an enzyme solution (0.6 g/L) with human albumin (1 g/L) and catalase (0.4 g/L) was layered onto 10 mL of a sucrose solution, the concentration of which was a linear gradient of 0.15 to 0.67 M sucrose in 50 mM Tris-HCl, pH 7.5. The tubes were centrifuged in a swing-out rotor, 6 × 14 mL, at 150 000g for 15 h at 4 °C. The centrifuge tubes were emptied by pressing out the solution with 1 M sucrose in 50 mM Tris-HCl at pH 7.5. Fractions of 5 drops equal to a total of about 100 μL were collected and weighed. The sedimentation coefficient for γ-butyrobetaine hydroxylase was calculated according to Martin and Ames (1961) using values for the reference proteins from Sober (1973).

Agarose Gel Electrophoresis. Electrophoresis in 1% (w/v) agarose gel in 67 mM sodium phosphate buffer at pH 6.3 was performed as described by Johansson (1972). Crossed immunoelectrophoresis in agarose gel was performed as described by Ganrot (1972).

Polyacrylamide Gel Electrophoresis. The electrophoresis was performed according to a modification of the system originally described by Ornstein (1964) and by Davis (1964). No sample and spacer gels were used. The cathode buffer was Tris–glycine (0.02 M), pH 8.3, and the anode buffer was Tris-HCl (0.12 M), pH 8.1. The gel buffer was 0.38 M Tris-HCl at pH 8.9. Gels with different acrylamide concentration (50, 75, and 100 g/L) but with constant ratio of methylenebisacrylamide to acrylamide (5%, w/w) were used. About 15–50 μg of protein were dissolved in sucrose solution (80 g/L)

containing bromophenol blue (0.5 g/L) and layered onto the gel surface under the buffer. Electrophoresis was performed at 4 °C. Gels which were to be assayed for enzyme activity were cut into segments, which were eluted with 10 mM potassium phosphate buffer at pH 6.5 at 4 °C overnight.

Determination of molecular mass was performed by electrophoresis in gels with different concentrations of polyacrylamide according to Hedrick and Smith (1968) with bovine albumin, human transferrin, yeast hexokinase, and bovine catalase as references using molecular mass values from Sober (1973) and Fish et al. (1969). No spacer gel was used. Acrylamide concentrations were 50, 75, 100, and 125 g/L. About 20 μg of protein was added to a solution with sucrose (100 g/L) and bromophenol blue (0.5 g/L), which was applied to the gel.

Electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate was performed by a method similar to that described by Shapiro et al. (1967), modified according to Weber and Osborn (1969) with human immunoglobulin L chain, rabbit aldolase, bovine catalase, and human transferrin as reference proteins. Samples of 100–400 μg of protein were incubated for 3 h at 37 °C in 200 μL of 10 mM sodium phosphate buffer, pH 7.1, containing sodium dodecyl sulfate (35 mM), with or without mercaptoethanol (140 mM). Before electrophoresis, 100 μL of the samples were equilibrated with 10 mM sodium phosphate buffer, pH 7.1, containing sodium dodecyl sulfate (3.5 mM) with or without mercaptoethanol (14 mM) by filtration through 1-mL columns of Sephadex G-25 (medium). To 100 μL of the samples were then added 30 μL of glycerol and 3 μL of bromophenol blue solution (10 g/L). Fifty microliters of the mixtures were applied to the gels. Electrophoresis was performed at 8 V/cm for about 3 h.

Analytical Isoelectric Focusing in Polyacrylamide Gel. Isoelectric focusing in polyacrylamide gel was performed essentially as described by Wrigley (1968). Gels were polymerized chemically at room temperature. Carrier ampholyte solutions of pH range 3–10, 3–5, 4–6, and 5.0–5.5 were used. The gels were cut lengthwise. One half was washed several times with 0.3 M trichloroacetic acid to remove carrier ampholytes before protein staining. Gel strips which were to be assayed for enzyme activity were kept at 4 °C. They were cut into 3-mm pieces which were eluted with 0.2 mL of 10 mM potassium phosphate buffer, pH 6.5, at 4 °C overnight. The eluates (0.1 mL) were analyzed for γ-butyrobetaine hydroxylase activity. One gel strip was cut into 3-mm pieces, which were eluted with 1 mL of water before pH measurements.

Stokes Radius Determination. The Stokes radius was measured by gel chromatography in a Sephadex G-200 column (2.5 × 94 cm) using 50 mM potassium phosphate buffer at pH 6.5 for elution (Ackers, 1964; Siegel and Monty, 1966). The effective pore radius (r) of the batch of Sephadex G-200 used was determined by chromatography of bovine catalase, human transferrin, bovine and human albumin, and ovalbumin (Ackers, 1964). Using the figures for the Stokes radius of these proteins (5.22, 4.00, 3.65, 3.53, and 2.76 nm, respectively, calculated from values in Sober, 1973), an r value of 20.8 ± 1.6 (SD) nm (n = 5) was obtained. Stokes radius for γ-butyrobetaine hydroxylase was calculated from its distribution coefficient using this value of r. Stokes radius for the enzyme was also determined by plotting $(-\log K_{av})^{1/2}$ vs. Stokes radius of the reference proteins (Siegel and Monty, 1966).

Thin-Layer Gel Filtration. The molecular mass of the enzyme was determined by thin-layer gel filtration (Johansson and Rymo, 1962) in a TLG apparatus (Pharmacia Fine Chemicals, Uppsala, Sweden) as recommended by the manufacturer. Sephadex G-150 and G-200 (superfine) in 50 mM potassium phosphate buffer at pH 6.5 were used for the native enzyme. Sephadex G-200 (superfine) in 6 M guanidinium chloride with 0.1 M 2-mercaptoethanol at pH 6.5 was used for the denatured enzyme. The enzyme was denatured according to Fish et al. (1969). Human immunoglobulin G, rabbit creatine kinase, human transferrin, bovine albumin, ovalbumin, bovine chymotrypsinogen, and equine myoglobin were used as references (Fish et al., 1969; Noda et al., 1954; Sober, 1973). Cytochrome *c* was used as chromatographic reference.

Amino Acid Analysis. The enzyme was desalted on columns of Sephadex G-25 equilibrated with 10 mM NH_4HCO_3 adjusted to pH 7.0 with acetic acid. The protein concentration of the eluate was determined according to Lowry et al. (1951). About 1 mg of protein was then lyophilized in borosilicate-glass tubes. The hydrolysis was performed at 7 Pa at 110 ± 2 °C for 20 and 70 h in an oil bath (Moore and Stein, 1963). The HCl was removed under reduced pressure on a rotary evaporator. The dry samples were dissolved in 0.3 M lithium citrate buffer at pH 2.2. Norleucine and α-amino-γ-guanidinobutyric acid were used as internal standards. A Jeol Model JLC-5AH amino acid analyzer was used.

Cystine *plus* cysteine were determined as cysteic acid and methionine as methionine sulfone after performic acid oxidation (Hirs, 1967). Tryptophan and tyrosine were determined by spectrophotometry in unhydrolyzed samples in 6 M guanidinium chloride by the method of Edelhoch (1967) and in 0.1 M NaOH by the method of Goodwin and Morton (1946).

Partial Specific Volume. The partial specific volume was calculated from the amino acid composition of the protein (Cohn and Edsall, 1943).

Carbohydrate Analysis. Neutral sugars were assayed by the phenol–sulfuric acid procedure of Dubois et al. (1956) using D-glucose as standard.

NH_2-Terminal Amino Acids. The NH_2-terminal amino acids were determined as phenylthiohydantoin derivatives as described by Iwanaga et al. (1969). Lyophilized samples of the enzyme (3–15 mg) which had been desalted on Sephadex G-25 columns were analyzed in parallel with an insulin preparation. The phenylthiohydantoins were identified by chromatography on polyamide sheets (Summers et al., 1973) or on silica gel plates with a fluorescence indicator (Jeppson and Sjöquist, 1967). The phenylthiohydantoins of isoleucine and leucine were separated by thin-layer chromatography in system V of Jeppson and Sjöquist (1967). Quantitative determinations were performed as described by Sjöquist (1960) by spectrophotometry of the phenylthiohydantoins after paper chromatography.

Determination of Thiol Groups. Thiol groups were determined on denatured protein with Nbs_2 (Habeeb, 1972). About 0.2–0.5 μg of protein was analyzed in a final volume of 1.5 mL. A value for the molar absorptivity at 410 nm of 13 600 M^{-1} cm^{-1} was used in the calculations. Determination of thiol groups was also performed on denatured protein with BDC-OH (Rohrbach et al., 1973). Samples of 0.06–0.3 μg of protein were analyzed in a final volume of 2.5 mL. The molar absorptivity of the carbonium–immonium ion from BDC-OH was 55 400 to 58 000 M^{-1} cm^{-1}.

Determination of Disulfide Bonds. The protein was first reduced with $NaBH_4$ and then assayed for thiol groups as described by Habeeb (1972). About 0.2–0.5 μg of the protein was analyzed in a final volume of 3 mL. A molar absorptivity for the 2-nitro-5-thiobenzoate ion at 412 nm of 12 000 M^{-1} cm^{-1} was used.

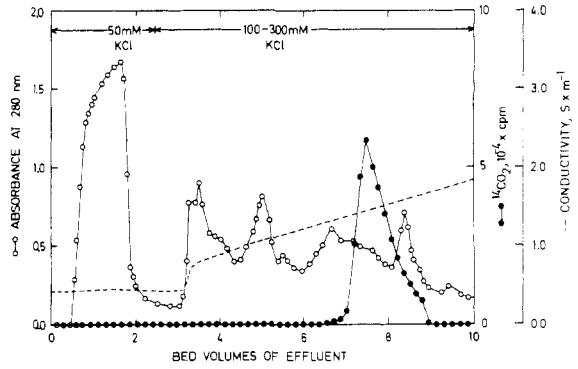

FIGURE 1: DE52 cellulose chromatography of the preparation of γ-butyrobetaine hydroxylase obtained after streptomycin treatment. The column (800 mL) was eluted with increasing concentrations of KCl in 10 mM potassium phosphate buffer, pH 6.5. Fractions of 12 mL were collected at a flow rate of 250 mL/h. Enzyme activities (●) were determined on 10 μL of the fractions. Absorbance at 280 nm (○). Conductivity (- - -).

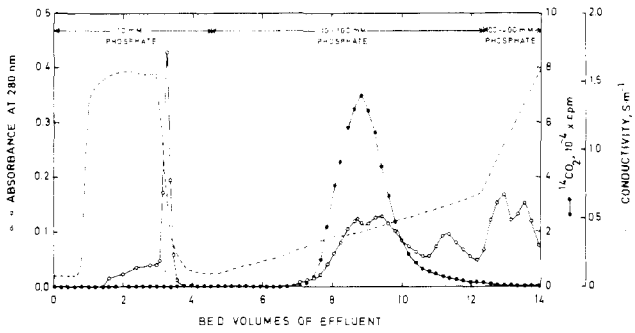

FIGURE 2: Hydroxylapatite chromatography of the pooled enzyme fractions from the DE52 cellulose chromatography. The column (580 mL) was eluted with linear gradients of potassium phosphate buffer, pH 6.5. Fractions of 12 mL were collected at a flow rate of 150 mL/h. Enzyme activities (●) were determined on 10 μL of the fractions. Absorbance at 280 nm (○). Conductivity (- - -).

Iron Determination. The ferroin method of Massey (1957) was used with the exception that 1,10-phenanthroline was replaced by an equivalent concentration of sodium bathophenanthrolinesulfonate to achieve greater sensitivity. The trichloroacetic acid was a low-iron product. Iron wire, >99.999% pure, which had been dissolved in HNO_3 (Brumby and Massey, 1967) was used as the standard. About 80–150 μg of protein was analyzed in a final volume of 255 μL. With this method, a mean value of 2.0 (n = 6, range 1.9–2.2) mol of iron per 76.6 kg of protein was obtained for transferrin.

Results

Growth Conditions. The specific activity of the enzyme in the 100 000g supernatant fraction from sonically disrupted cells was maximal after 20–30 h of growth at 25 °C, i.e., at the end of the logarithmic growth phase. A growth time of 30 h was chosen. A moderate aeration of the culture was necessary to obtain satisfactory yield of enzyme. The yield of bacteria increased when aeration was increased from 70 to 200 mL min^{-1} (L of medium)$^{-1}$. The specific activity of the cell extract increased slightly when going from 70 to 100 mL of air min^{-1} (L of medium)$^{-1}$, but decreased when higher flow rates were used. An air flow of 100 mL min^{-1} L^{-1} was therefore chosen.

Purification of γ-Butyrobetaine Hydroxylase. Step 1. Extraction of Enzyme. About 85 g of bacterial paste was suspended in 850 mL of ice-cold 10 mM potassium phosphate buffer at pH 6.5. The cells were sonically disrupted for 4 × 5 min in a "Rosette" cell (Branson Instrument Co., Stamford, Conn.) with a Branson Type S 75 sonifier operating at 75 W and 20 kHz. The cell was kept in ice water during the procedure and the temperature of the content was not allowed to exceed 10 °C. The mixture was then centrifuged for 60 min at 100 000g.

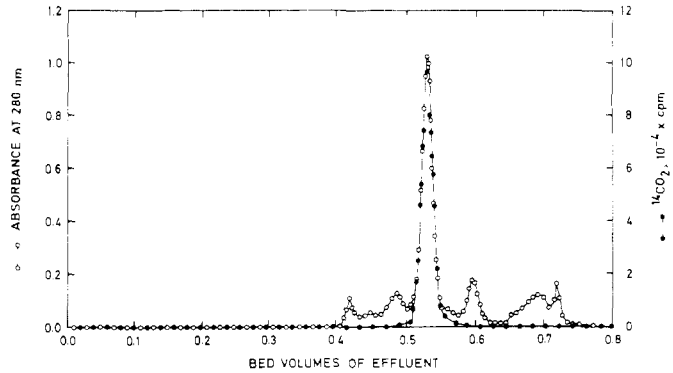

FIGURE 3: Filtration of the pooled hydroxylapatite fractions containing γ-butyrobetaine hydroxylase activity through Sephadex G-100 (superfine). Two serial columns (2 × 440 mL) were eluted with 50 mM potassium phosphate buffer, pH 6.5, with a flow rate of 5–6 mL/h. Portions (3 μL) of the 2-mL fractions were analyzed for enzyme activity (●). Absorbance at 280 nm (○).

Step 2. Streptomycin Treatment. A 0.18 M solution of streptomycin sulfate in water (a μL) was added dropwise to ernatant from step 1 (b mL) during 30 min of stirring × 0.75 × extract A_{260nm}). The precipitate was spun y centrifugation at 23 000g for 45 min. The pH value changed from 6.5 to 6.2 on treatment with streptomycin sulfate.

Step 3. DEAE-Cellulose Chromatography. The supernatant from step 2 (865 mL) was applied onto a DE52 column (5 × 41 cm, 800 mL) which was eluted first with 50 mM KCl in 10 mM potassium phosphate buffer at pH 6.5 and then with a linear gradient between 100 and 300 mM KCl in 10 mM potassium phosphate buffer at pH 6.5. The enzyme activity was eluted at about 200 mM KCl (Figure 1).

Step 4. Hydroxylapatite Chromatography. The pooled fractions from step 3 were applied onto a hydroxylapatite column (5 × 30 cm, 580 mL). The column was eluted first with 10 mM potassium phosphate buffer at pH 6.5, then with a gradient between 10 and 100 mM potassium phosphate at pH 6.5, and finally with a gradient between 100 and 400 mM potassium phosphate at pH 6.5. The enzyme activity was eluted between 40 and 50 mM phosphate buffer (Figure 2). The pooled fractions were concentrated by ultrafiltration to a protein concentration of about 1 g/L. Aliquots of the concentrate were kept frozen at −60 °C.

Step 5. Gel Filtration on Sephadex G-100. A sample with about 30 mg of protein from step 4 was further concentrated to about 6 g/L by vacuum dialysis in a collodion bag, surrounded by 50 mM potassium phosphate buffer at pH 6.5. Sucrose was then added to a concentration of 100 g/L. The sample was layered on top of the Sephadex G-100 gel bed underneath the eluant. Two serially connected columns (2 × 440 mL) were eluted with 50 mM potassium phosphate buffer at pH 6.5 at a flow rate of about 5 mL/h. The enzyme activity was eluted at 0.55 bed volume of effluent (Figure 3). The pooled fractions were concentrated by vacuum dialysis in a collodion bag. Portions of the concentrate were kept frozen at −60 °C.

TABLE I: Purification of γ-Butyrobetaine Hydroxylase from *Pseudomonas* sp AK 1.

Purification Step	Protein (mg)	Total Act. (μkat)	Specific Act. (μkat/g)
1. Cell-free extract		100	
2. Streptomycin treatment	6600	67	10
3. DEAE-cellulose chromatography	490	45	92
4. Hydroxylapatite chromatography	120	25	210
5. Sephadex G-100 filtration	38	14	360

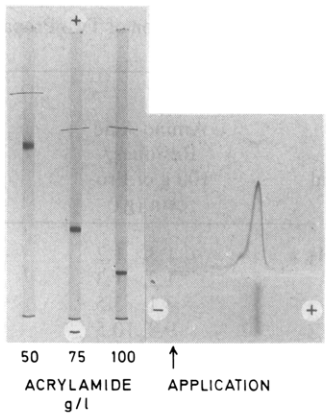

FIGURE 4: *Left panel*: Electrophoresis of γ-butyrobetaine hydroxylase (20 μg) in different concentrations of acrylamide at pH 8.9. The ratio of methylenebisacrylamide to acrylamide was 1:20. The electrophoresis was carried out for 3 h at 4 °C with a current of about 2 mA/gel. The metal wires indicate the migrations of bromophenol blue. *Right panel*: Crossed immunoelectrophoresis into an agarose gel, containing a rabbit antiserum to a 100 000*g* supernatant fraction from sonically disrupted cells of *Pseudomonas* sp AK 1.

The results from the purification procedure are given in Table I. The specific activity was the same in all enzyme-containing fractions in step 5.

Stability. The stability of the enzyme was better in phosphate buffer than in Tris-HCl or Tris–glycine buffers. Preparations stored in 10 mM phosphate buffers at pH 6.5 at −60 or at −170 °C showed no appreciable loss of specific activity over a period of 6 months. A slight precipitate of amorphous material sometimes appeared on thawing. Storage at −20 or at 4 °C resulted in appreciable loss of activity, 40–50% in 1 week. This loss of activity could not be prevented by the addition of γ-butyrobetaine or 2-oxoglutarate. Dithiothreitol, 1 mM, could to some extent reactivate preparations stored at −20 °C. Freeze-dried preparations were difficult to dissolve.

Criteria of Purity. The preparation of γ-butyrobetaine hydroxylase from *Pseudomonas* sp AK 1 was homogeneous by the following criteria:

Polyacrylamide Gel Electrophoresis. One band was obtained after electrophoresis in gels of three different polyacrylamide concentrations but with the same cross-linking (Figure 4). The location of the gel segment containing the enzymatic activity corresponded to the location of the protein band.

Agarose Gel Electrophoresis. A single band was obtained after electrophoresis in agarose gel. One peak was obtained after crossed immunoelectrophoresis in agarose gel, using an antiserum prepared by immunizing rabbits with the 100 000*g* supernatant fractions of sonically disrupted bacterial cells (Figure 4).

Isoelectric Focusing in Polyacrylamide Gel. A single band was obtained after isoelectric focusing in polyacrylamide gels with ampholytes covering several pH intervals.

Analytical Ultracentrifugation. The plot of the logarithm of fringe displacement vs. the square of the radial distance was linear to the bottom of the cell.

Physical and Chemical Properties. Sedimentation Coefficient. The sedimentation coefficient was determined by centrifugation of γ-butyrobetaine hydroxylase in a sucrose gradient with and without human albumin and catalase as reference proteins. The sedimentation behavior of the enzyme was unchanged by the addition of the reference proteins. Calculations according to Martin and Ames (1961) gave a $s_{20,w}$ of $(5.8 \pm 0.12) \times 10^{-13}$ s (mean ± SD, $n = 7$).

Shape. The radius of the equivalent sphere of γ-butyrobetaine hydroxylase was determined by chromatography on Sephadex G-200. Calculations from the distribution coefficients of γ-butyrobetaine hydroxylase and the four reference proteins according to Ackers (1964) gave a value of Stokes radius of 3.8 ± 0.17 (SD) nm ($n = 5$). A value of 3.8 ± 0.15 (SD) nm ($n = 5$) was obtained from a plot of $(-\log K_{av})^{1/2}$ vs. Stokes radius according to Siegel and Monty (1966). This value corresponds to a diffusion coefficient ($D_{20,w}$) of 5.7×10^{-7} cm^2/s and to a frictional ratio, f/f_{min} of 1.26 (Siegel and Monty, 1966; Tanford, 1961). A frictional ratio of 1.26 was also calculated from the sedimentation coefficient and the partial specific volume (see below) using a value of 95 kdaltons for the molecular mass (Tanford, 1961).

Partial Specific Volume. The partial specific volume was calculated from the amino acid composition. The values 0.730 and 0.728 mL/g were found for two different enzyme preparations.

Molecular Mass. A molecular mass of 95 kdaltons was obtained by ultracentrifugation using a value of 0.729 mL/g at 20 °C for the partial specific volume. Calculation of molecular mass from data obtained with the Sephadex G-200 column used for Stokes radius determination gave a value of 86 ± 6.2 (SD) kdaltons ($n = 5$). Values of molecular mass of 79 and 84 kdaltons were obtained by thin-layer gel filtration of native enzyme. A mean value of 92 kdaltons (89, 95) was obtained by electrophoresis of the native enzyme in gels with different concentrations of polyacrylamide according to Hedrick and Smith (1968) using two reference proteins with higher mass (catalase and hexokinase) and two proteins with similar or lower mass (transferrin and albumin).

The molecular mass was calculated to 96 kdaltons from the sedimentation coefficient, the Stokes radius, and the partial specific volume (Siegel and Monty, 1966). An estimate of the molecular mass was obtained from the sedimentation coefficient determined by sucrose gradient centrifugation (Martin and Ames, 1961). With albumin as standard the molecular mass for γ-butyrobetaine hydroxylase was calculated to 98 kdaltons, and with catalase as standard to 88 kdaltons.

Subunit Mass. One band was observed when denatured enzyme was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The subunit mass was 37 ± 1.9 (SD) kdaltons ($n = 7$) using three reference proteins with higher mass (transferrin, catalase, aldolase) and two with lower mass (immunoglobulin L chain and myoglobin). The same value was obtained with and without treatment of the enzyme with 0.1 M 2-mercaptoethanol. A mass of 39

TABLE II: Amino Acid Composition of Two Preparations of γ-Butyrobetaine Hydroxylase.[a]

Amino Acid Residue	Amino Acid Residues/100 g of Protein (g)	No. of Amino Acid Residues/95 kg of Protein
Amide-NH_3	1.3, 1.2	72, 66
Lys	1.2, 1.3	9, 10
His	3.1, 3.5	21, 24
Arg	9.8, 10.5	60, 64
Asp	10.1, 9.9	83, 82
Thr[b]	3.2, 3.1	30, 29
Ser[b]	3.2, 3.7	35, 41
Glu	9.2, 9.2	68, 68
Pro	4.9, 4.1	48, 40
Gly	2.6, 2.4	44, 41
Ala	6.1, 5.8	81, 77
Val[c]	5.3, 5.6	51, 54
½-cystine[d]	2.3, 2.2	20, 18
Met[d]	2.8, 2.4	21, 18
Ile[c]	3.0, 3.0	25, 25
Leu	9.7, 9.0	82, 76
Tyr[b]	4.1, 3.9	24, 23
Phe	5.7, 5.6	37, 37
Trp[e]	2.5, 2.4	13, 12
Total	90 89	

[a] The values given are the mean values obtained from duplicate analyses of each of two preparations of γ-butyrobetaine hydroxylase. The deviation from the mean values were not greater than ±2% for all amino acids, except for half-cystine and lysine in preparation 1 and for half-cystine, lysine, and proline in preparation 2. The deviation from the mean values for these amino acids were not greater than ±5%. [b] Extrapolated to zero time of hydrolysis assuming first-order kinetics (Moore and Stein, 1963). [c] Values from the 70-h hydrolysates. [d] Determined on 20-h hydrolysates of performic acid oxidized samples. [e] Determined by spectrophotometry on unhydrolyzed samples (Goodwin and Morton, 1946; Edelhoch, 1967).

kdaltons was obtained when denatured enzyme was analyzed by thin-layer gel filtration in a solution of 6 M guanidinium chloride and 0.1 M 2-mercaptoethanol, using eight reference proteins, four of which had higher mass.

Light-Absorption Spectrum. The light-absorption spectrum at pH 6.5 and 13 was that of a typical protein. No absorption peaks were noted in the visible region. The absorption coefficient, $A_{280nm}^{1\%,1cm}$, in 2 mM potassium phosphate buffer at pH 6.5 was 12.6. Identical values were obtained for the absorption coefficient when the protein concentration was determined on the basis of dry weight and by the Lowry method. A molar absorptivity of 1.2×10^5 M^{-1} cm^{-1} was obtained with a value of 95 kdaltons for the molecular mass. The molar absorptivity was also calculated according to Wetlaufer (1962) using the results from the amino acid analysis (see below). A value of 1.0×10^5 M^{-1} cm^{-1} was obtained assuming a contribution of 5500 M^{-1} cm^{-1} from each tryptophan and 1340 M^{-1} cm^{-1} from each tyrosine residue.

Isoelectric Point. The isoelectric point was 5.1 (range 5.0–5.3) as determined by analytical isoelectric focusing in polyacrylamide gels, using different pH intervals (3–5, 4–6, 5.0–5.5). Only one-fourth of the enzyme activity was recovered after isoelectric focusing, indicating that the enzyme is unstable at the isoelectric point. Similar results were obtained in experiments with preparative isoelectric focusing in sucrose gradients.

Amino Acid Composition. The results of the amino acid analyses of two different enzyme preparations of γ-butyrobetaine hydroxylase are given in Table II. A recovery of 90% (w/w) was obtained. It may be noted that the half-cystine content was 19 mol per 95 kg of protein (23 residues/1000).

TABLE III: NH_2-Terminal Amino Acids of γ-Butyrobetaine Hydroxylase.

Step	Amino Acid	Amount of Pth-Amino Acid Derivate/95 kg of Protein[a] (mol)	Recovery (%) of Residues from Insulin	
1	Ala	0.80	Phe	77
	Asn	0.29	Gly	62
	Asp	0.56		
2	Ala	0.66	Val	74
	Ile	0.70	Ile	69
3	Ala	0.57	Val	*b*
	Ile	0.46	Asn	

[a] The values are not corrected for recovery. [b] Not determined.

NH_2-Terminal Amino Acids. Quantitative NH_2-terminal amino acid analysis of γ-butyrobetaine hydroxylase was performed by a phenyl isothiocyanate method in three steps (Table III). Alanine, asparagine, and aspartic acid were found in the first step. The total amount of asparagine and aspartic acid was approximately the same as the amount of alanine, indicating that the NH_2-terminal amino acids of the two subunits are alanine and asparagine.

Thiol Groups. The thiol content was determined in denatured enzyme with BDC-OH and Nbs_2, and also with Nbs_2 after treatment of the denatured enzyme with $NaBH_4$. Four enzyme preparations were analyzed with Nbs_2 in 70 mM sodium dodecyl sulfate; a value of 13.1 ± 0.61 (SD) mol per 95 kg of protein was obtained (n = 10). The thiol content was also determined in two preparations with Nbs_2 in 8 M urea and 0.2 M $NaBH_4$ and with BDC-OH in 4 M guanidinium chloride. The figures obtained were 12.3 mol per 95 kg of protein (range 11.9–13.1, n = 4) and 12.7 mol per 95 kg of protein (range 11.5–13.9, n = 4).

Carbohydrate Content. Hexose determination by a phenol–sulfuric acid method gave a value for the hexose content of less than 0.6 g/100 g of protein. The same value was obtained for albumin, indicating the absence of significant amounts of hexose in the enzyme.

Iron Content. Less than 0.1 mol of Fe^{2+} was found per 95 kg of protein by a bathophenanthroline method.

Discussion

Pseudomonas sp AK 1 is a better source for preparation of γ-butyrobetaine hydroxylase than liver from rat, human, or sheep, as the enzyme activity per gram of protein in the starting material is several thousand times higher. In the present study, the search for optimum conditions for yield of enzyme from cells of *Pseudomonas* sp AK 1 resulted in severalfold higher enzyme activity than that obtained previously (Lindstedt et al., 1970b). A better yield is obtained at 25 than at 37 °C. The degree of aeration and vigorous stirring appear critical. The enzyme activity decreased if the aeration was increased much above 100 mL min^{-1} (L of medium)$^{-1}$, a phenomenon that has also been noted with the 2-oxoglutarate-dependent thymine 7-hydroxylase.

The present preparation of γ-butyrobetaine hydroxylase is homogeneous as judged by a number of criteria. We initially used preparative electrophoresis in polyacrylamide gels prepared from acrylamide (50 g/L) containing 3% of the cross-linking agent as step 5, i.e., after hydroxylapatite and DEAE-cellulose chromatography. The enzymically active protein fraction from this step gave a single band after agarose gel electrophoresis in 67 mM sodium phosphate buffer at pH 6.3. However, analytical polyacrylamide gel electrophoresis with higher concentrations of acrylamide and of the cross-linking agent (75 g/L, 5%) revealed two major and one minor band. Thus, the proteins from step 4 appeared to differ more in molecular size than in charge. Molecular sieving with high resolution was therefore chosen in the final step. Electrophoresis in less porous polyacrylamide gels would have been an alternative, but, due to the long electrophoresis time required, the recovery was low also when excess persulfate had been eliminated.

The molecular mass of the enzyme is 95 kdaltons, as determined by ultracentrifugation. Evidence that the enzyme is built up by subunits of similar size was obtained by analysis in dissociating media by gel filtration and by polyacrylamide gel electrophoresis. The subunit mass was found to be about 38 kdaltons. The results from analysis of the NH_2-terminal amino acids show the presence of two different subunits.

The 2-oxoacid-dependent hydroxylases so far studied in some detail have been reported to be dimers or tetramers. Rhoads and Udenfriend (1970) have reported that prolyl hydroxylase from skin of newborn rats has a molecular mass of 130 kdaltons and that the enzyme appears to consist of two dissimilar subunits of about 65 kdaltons. Prolyl hydroxylase from chick embryos has been reported to have a mass twice as large and to be a dimer (Halme et al., 1970; Pänkäläinen et al., 1970) or a tetramer (Berg and Prockop, 1973a) composed of two types of subunits. 4-Hydroxyphenylpyruvate dioxygenase from chicken and human appears to be a dimer (Wada et al., 1975; Lindblad et al., to be published) and from rabbit and *Pseudomonas* sp P.J. 874 to be a tetramer (Laskowska-Klita and Mochnacka, 1973; Lindstedt et al., to be published). In the case of the human enzyme, the subunits are dissimilar.

The same estimates of molecular mass of denatured enzyme were obtained before and after treatment with reductants, indicating the absence of interchain disulfide bonds. Amino-acid analysis by ion-exchange chromatography gave a higher value for the half-cystine content than colorimetric analysis, 19 mol vs. 13 per 95 kg of protein. Treatment of the enzyme with $NaBH_4$ did not increase the number of sulfhydryl groups in the colorimetric analysis. Thus, either there are disulfide bonds which were not reduced under the conditions used or some of the sulfhydryl groups are unavailable to the colorimetric reagent. The enzyme contained relatively large amounts of acidic amino acids, in good agreement with the finding of a low isoelectric point.

The most active preparations of γ-butyrobetaine hydroxylase had a specific activity of 360 μmol s^{-1} g^{-1} at 37 °C and pH 7.0, which corresponds to a turnover number of about 34 mol s^{-1} mol^{-1}. The turnover number for prolyl hydroxylase from chick embryo has been reported to be 4 mol s^{-1} mol^{-1} by Berg and Prockop (1973b). From data reported by Rhoads and Udenfriend (1970) and Halme et al. (1970), the turnover number for the rat skin and chick embryo enzyme may be calculated to be 0.2 mol s^{-1} mol^{-1} and 0.3 mol s^{-1} mol^{-1}, respectively.

The light-absorption spectrum of γ-butyrobetaine hydroxylase did not indicate the presence of prosthetic groups such as flavin or pyridine nucleotides. Neither was there any evidence for the presence of non-heme iron to sulfur linkages.

Ferrous iron is required for the activity of the 2-oxoglutarate-dependent hydroxylases. Less than 0.1 mol of Fe^{2+} per 95 kg of protein was found in the pure γ-butyrobetaine hydroxylase, indicating low affinity for the metal ion, as could be expected from the previous finding of a K_m in the micromolar range. Similar results have been reported for highly purified preparations of prolyl hydroxylase from chick embryo (Kivirikko et al., 1968; Pänkäläinen and Kivirikko, 1971). Significant amounts of iron have been found in our laboratory in 4-hydroxyphenylpyruvate dioxygenase from *Pseudomonas* sp P.J. 874 and from human liver. The content varied between 0.6 and 1.3 mol/mol for the pseudomonad enzyme and was about 0.4 mol/mol for the human enzyme. The pseudomonad, but not the human enzyme, is stimulated by ferrous iron. Significant amounts of iron have also been found in 4-hydroxyphenylpyruvate dioxygenase from rabbit (Laskowska-Klita and Mochnacka, 1973) as well as from chicken (Wada et al., 1975). The 2-oxoglutarate-dependent hydroxylases thus differ from the 2-oxoacid-dependent 4-hydroxyphenylpyruvate dioxygenase with respect to the metal binding.

The basic features of 2-oxoacid-dependent hydroxylation have been clarified (Lindblad et al., 1969; 1970; Cardinale et al., 1971; Holme et al., 1971) but the intermediates in the reaction have not been established. Kinetic studies of thymine 7-hydroxylase have established that the reaction is of the ordered sequential type (Holme, 1975), but the nature of the active site and the role of the metal ion have not been studied in detail. The availability of pure γ-butyrobetaine hydroxylase of high catalytic activity may help to solve some of the remaining questions concerning this class of enzymes.

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