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Rat Liver γ-Butyrobetaine Hydroxylase Catalyzed Reaction: Influence of Potassium, Substrates, and Substrate Analogues on Hydroxylation and Decarboxylation†

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Received September 23, 1987; Revised Manuscript Received November 9, 1987

ABSTRACT: Interaction of rat liver γ-butyrobetaine hydroxylase (EC 1.14.11.1) with various ligands was studied by following the decarboxylation of α-ketoglutarate, formation of L-carnitine, or both. Potassium ion stimulates rat liver γ-butyrobetaine hydroxylase catalyzed L-carnitine synthesis and α-ketoglutarate decarboxylation by 630% and 240%, respectively, and optimizes the coupling efficiency of these two activities. Affinities for α-ketoglutarate and γ-butyrobetaine are increased in the presence of potassium. γ-Butyrobetaine hydroxylase catalyzed decarboxylation of α-ketoglutarate was dependent on the presence of γ-butyrobetaine, L-carnitine, or D-carnitine in the reaction and exhibited $K_{m(app)}$ values of 29, 52, and 470 μM, respectively. γ-Butyrobetaine saturation of the enzyme indicated a substrate inhibition pattern in both the assays. Omission of potassium decreased the apparent maximum velocity of decarboxylation supported by all three compounds by a similar percent. β-Bromo-α-ketoglutarate supported γ-butyrobetaine hydroxylation, although less effectively than α-ketoglutarate. The rat liver enzyme was rapidly inactivated by 1 mM β-bromo-α-ketoglutarate at pH 7.0. This inactivation reaction did not show a rate saturation with increasing concentrations of β-bromo-α-ketoglutarate. None of the substrates or cofactors, including α-ketoglutarate, protected the enzyme against this inactivation. Unlike β-bromo-α-ketoglutarate, β-mercapto-α-ketoglutarate did not replace α-ketoglutarate as a cosubstrate. Both β-mercapto-α-ketoglutarate and β-glutathione-α-ketoglutarate were noncompetitive inhibitors with respect to α-ketoglutarate.

The enzyme γ-butyrobetaine hydroxylase [4-trimethylammoniobutyrate,α-oxoglutarate:oxygen oxidoreductase (3-hydroxylating), EC 1.14.11.1] catalyzes the crucial, final step in the biosynthesis of L-carnitine (eq 1). It belongs to a unique

$$\gamma\text{-butyrobetaine} + \alpha\text{-ketoglutarate} + O_2 \xrightarrow{Fe^{2+}} \text{L-carnitine} + CO_2 + \text{succinate} \quad (1)$$

class of non-heme ferrous iron dioxygenases in which the hydroxylation of substrate is linked to the oxidative decarboxylation of α-ketoglutarate (Abbott & Udenfriend, 1974; Hayaishi et al., 1976). A partial reaction, namely, α-ketoglutarate decarboxylation uncoupled from hydroxylation, occurs with all α-ketoglutarate-coupled dioxygenases, especially in the presence of substrate analogues (Counts et al., 1978; Rao & Adams, 1978; Hsu et al., 1981; Holme & Lindstedt, 1982). Such a decarboxylation in the absence of γ-butyrobetaine hydroxylation was demonstrated for human liver and

† This work was supported by NIH Grant AM 10334 and by the Mobil Foundation.

0006-2960/88/0427-2222$01.50/0

bacterial enzymes (Holme et al., 1982). Hydroxylation of γ-butyrobetaine is significantly stimulated in the presence of a reductant such as ascorbate, and inclusion of catalase in the assay increases L-carnitine formation (Lindstedt & Lindstedt, 1970; Blanchard et al., 1982). The nature of stereochemistry at the active site and the chemical mechanism by which the α-ketoglutarate-coupled hydroxylations take place have been the object of much study and speculation [cf. Siegel (1979), Holme and Lindstedt (1982), and Blanchard and Englard (1983)].

Recently, we demonstrated that glutathione peroxidase plus reduced glutathione (GSH) was more effective than catalase in rat liver γ-butyrobetaine hydroxylase catalyzed L-carnitine synthesis, and we reported an apparent augmentation of L-carnitine synthesis by inorganic phosphate (potassium phosphate) (Punekar et al., 1987). In the present work we demonstrate that potassium is the species responsible for this activation and investigate the coupling of hydroxylation with decarboxylation reactions by using substrates and substrate analogues.

Materials and Methods

Materials. Materials were from the following sources: Aquasol and [1-^{14}C]acetyl coenzyme A, New England Nuclear; [1-^{14}C]-α-ketoglutarate, Amersham; bovine liver catalase (20000 units/mg), carnitine acetyltransferase (94 units/mg), glutathione, HEPES, *N*-ethylmaleimide, sodium α-ketoglutarate, sodium ascorbate, γ-butyrobetaine, and Dowex 1-X8 (200–400 mesh), Sigma; desferal mesylate (deferoxamine mesylate USP), CIBA. Authentic β-bromo-α-ketoglutaric acid was a generous gift of Dr. F. C. Hartman, Oak Ridge National Laboratory. All other materials were of the highest purity available from commercial sources.

γ-Butyrobetaine Hydroxylase. Partially purified γ-butyrobetaine hydroxylase (devoid of catalase and glutathione peroxidase activities) was prepared according to the combined methods of Lindstedt (1967) and Kondo et al. (1981) and modifications described previously (Punekar et al., 1987). This partially purified enzyme had a γ-butyrobetaine hydroxylase specific activity of 34.8 nmol of L-carnitine min^{-1} (mg of protein)$^{-1}$ in the presence of catalase and 100 mM KCl.

γ-Butyrobetaine Hydroxylase Assay (L-Carnitine Production). γ-Butyrobetaine hydroxylase catalyzed L-carnitine synthesis was assayed as described previously (Punekar et al., 1987) by using a L-carnitine assay modified from the combined procedures of Parvin and Pande (1977) and Noel et al. (1984). Unless indicated otherwise, assays were performed for 5 min at 37 °C in a final volume of 250 μL with the following components: 200 μM γ-butyrobetaine, 1.5 mM sodium α-ketoglutarate, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–NaOH (pH 7.0), 3.0 mM sodium ascorbate, 255 μM $Fe(NH_4)_2(SO_4)_2$, 76 μg of bovine liver catalase (1500 units), and 5 μg of γ-butyrobetaine hydroxylase. Reactions were initiated by the addition of Fe^{2+} and terminated by adding 50 μL of 6 mM deferoxamine mesylate.

γ-Butyrobetaine Hydroxylase Assay (CO_2 Production). γ-Butyrobetaine hydroxylase catalyzed α-ketoglutarate decarboxylating activity was assayed by following the release of $^{14}CO_2$ from [1-^{14}C]-α-ketoglutarate (Lindstedt et al., 1970). Unless indicated otherwise, reaction conditions were identical with those employed for the measurement of L-carnitine production but were performed in a total volume of 500 μL and included 0.2–0.5 μCi of [1-^{14}C]-α-ketoglutarate per assay. Incubations were performed in 20-mL glass vials fitted with plastic center wells (Kontes Glass Co.) and sealed with rubber serum vial stoppers. Each center well contained a 2.5-cm^2 section of filter paper and 50 μL of 1 M hyamine hydroxide in methanol. Incubations were initiated by injection of 100 μL of 1.25 mM $Fe(NH_4)_2(SO_4)_2$ and terminated by injection of 1.0 mL of 10% trichloroacetic acid (TCA) into the vials. Following acidification, the vials were incubated for 1 h at 37 °C. The center wells were then cut from their supporting stem and placed in 10 mL of tritosol scintillation mixture (Pande, 1976) and the ^{14}C contents determined. Degrees of decarboxylation in the absence of γ-butyrobetaine were identical with those obtained in the absence of the partially purified enzyme. Specific activity values for CO_2 production were corrected for nonenzymatic α-ketoglutarate decarboxylation by subtracting values obtained in the absence of enzyme.

Synthesis of (R,S)-β-Bromo-α-ketoglutaric Acid. β-Bromo-α-ketoglutarate was synthesized and assayed as described by Hartman (1981). The yellow, solidified material obtained following desiccation was washed with cold $CHCl_3$ to yield a white powder.

Chemical purity was assessed with high-performance liquid chromatography (HPLC) and proton NMR. Reverse-phase HPLC was monitored at 215 nm and performed by using a C_8 column with 0.4 M H_3PO_4 as the mobile phase. This system was able to resolve authentic β-bromo-α-ketoglutaric acid from α-ketoglutarate. Chromatography of the material obtained from the above synthesis yielded a single peak whose retention time corresponded to that of the authentic material. In addition, proton NMR data were consistent with the identity of this material as β-bromo-α-ketoglutaric acid.

Synthesis of (R,S)-β-Mercapto-α-ketoglutarate. β-Mercapto-α-ketoglutarate was prepared from β-bromo-α-ketoglutaric acid by the method of Plaut et al. (1986) and quantitated with 5,5′-dithiobis(2-nitrobenzoate) (Ellman, 1959). Identity of the synthesized material as β-mercapto-α-ketoglutarate was confirmed with proton NMR. Solutions of β-mercapto-α-ketoglutarate were prepared on the day of use and stored on ice.

Synthesis of (R,S)-β-Glutathione-α-ketoglutarate. The condensation product of β-bromo-α-ketoglutaric acid and GSH was prepared by incubating 10 μmol of each compound together in 1.0 mL of 50 mM sodium phosphate buffer (pH 7.0) for 20 min at room temperature and in the dark. Completion of the reaction at 20 min was verified by the absence of material reactive with 5,5′-dithiobis(2-nitrobenzoate) (Ellman, 1959).

Analysis of Kinetic Data. Kinetic data were analyzed by using a regression analysis program (RAGASSEK) developed and kindly provided by Professor Dexter B. Northrop, University of Wisconsin. RAGASSEK incorporates the nonlinear regression routine of Duggleby (1984). Computation was performed on a North Star-Horizon computer.

Results

Unless otherwise mentioned, all data presented are representative of two or four experiments.

Activation by Potassium. Previously we reported an apparent stimulatory effect of inorganic phosphate (potassium phosphate) on L-carnitine synthesis by rat liver γ-butyrobetaine hydroxylase (Punekar et al., 1987). In the course of subsequent work it was discovered that sodium phosphate was unable to reproduce this effect (data not shown); this indicated that potassium may be the active species. As shown in Figure 1, KCl has a marked stimulatory effect on the γ-butyrobetaine hydroxylase catalyzed reaction. L-Carnitine synthesis and CO_2 production are increased 630% and 240%, respectively, with half-maximal stimulation for both activities occurring at 10

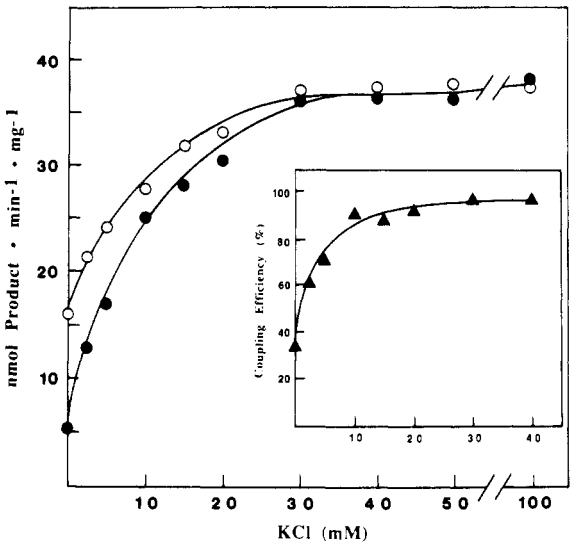

FIGURE 1: Activation of L-carnitine synthesis and CO_2 production by potassium. γ-Butyrobetaine hydroxylase catalyzed L-carnitine synthesis (●) and α-ketoglutarate decarboxylation (○) were assayed as described under Materials and Methods at increasing KCl concentrations. Assays for the carnitine produced were not affected by the KCl concentrations employed (data not shown). Coupling efficiency is the percent ratio of hydroxylation to decarboxylation at a given KCl concentration.

Table I: Effect of Monovalent Cations on γ-Butyrobetaine Hydroxylase Catalyzed L-Carnitine Synthesis[a]

cation	% of control[b]	ionic radius[c] (Å)
Li^+	76	0.68
Na^+	146	0.97
K^+	635	1.33
NH_4^+	302	1.43
Rb^+	377	1.47
Cs^+	109	1.67

[a] Assays were performed as described under Materials and Methods with 50 mM of the chloride salt of the cation indicated. α-Ketoglutarate and γ-butyrobetaine were present at saturating concentrations of 1.5 mM and 200 μM, respectively. A background Na^+ of approximately 15 mM was present in the assay medium. Reactions were of 5-min duration. The enzymatic assay for carnitine was not affected by any of the cations employed in this study (data not shown). [b] Control value is the enzyme activity without added cation: 6.02 nmol of carnitine min^{-1} (mg of protein)$^{-1}$. [c] Nonhydrated ionic radii (*Handbook of Chemistry and Physics*, 1972).

mM KCl. Though both activities are stimulated to the same maximal level, in the absence of potassium CO_2 production exceeds L-carnitine synthesis by a factor of 3. At a KCl concentration of approximately 30 mM the coupling efficiency approaches 100% (Figure 1, insert).

As with other potassium-activated enzymes (Suelter, 1970), γ-butyrobetaine hydroxylase is also activated by ammonium and rubidium ions (Table I). Alkali metals of significantly smaller or larger ionic radii either slightly inhibit (Li^+) or are without effect. Clearly, this activation is cation specific and not a general ionic strength effect.

Effect of Phosphate. Phosphate is known to enhance the damage to enzymes produced by iron salts (Lambeth et al., 1982) and to increase the need for protective enzymes (Punekar & Lardy, 1987; Punekar et al., 1987). In the absence of phosphate, optimal activity is achieved without addition of the protective enzymes catalase and glutathione peroxidase (Figure 2). With increasing phosphate concentration a progressive decrease in activity results, which is prevented to only a slight degree by the inclusion of catalase. In contrast, little decrease in the rate of L-carnitine synthesis occurs in the presence of glutathione peroxidase.

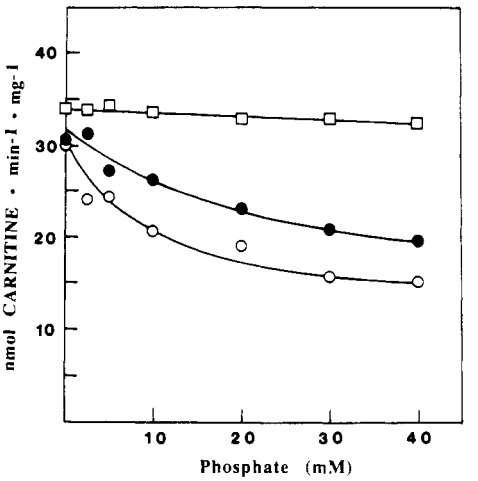

FIGURE 2: Inhibition of γ-butyrobetaine hydroxylase by phosphate. L-Carnitine synthesis was measured at increasing phosphate concentrations, a constant potassium concentration (30 mM), and with 3.5 μg (2 units) of glutathione peroxidase/5 mM reduced glutathione (□), 76 μg of catalase (●), or with neither protective enzyme (○). By use of the data of Green and Hughes (1955), K_2HPO_4, KH_2PO_4, and KCl were combined to achieve increasing phosphate concentrations with the same total concentration of potassium.

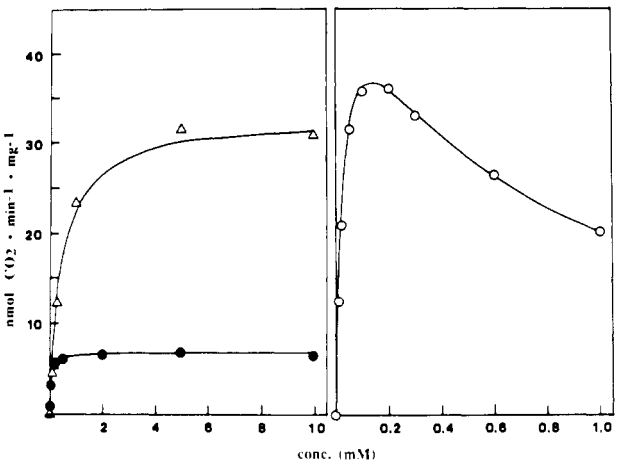

FIGURE 3: γ-Butyrobetaine, L-carnitine, and D-carnitine saturations. Decarboxylation of α-ketoglutarate catalyzed by γ-butyrobetaine hydroxylase was assayed as described under Materials and Methods with saturating α-ketoglutarate (1.5 mM), 100 mM KCl, and varying concentrations of γ-butyrobetaine (○), L-carnitine (●), or D-carnitine (△). No enzymatic decarboxylation of α-ketoglutarate occurred in the absence of a second substrate.

Decarboxylation and Uncoupling. Rat liver γ-butyrobetaine hydroxylase catalyzed α-ketoglutarate decarboxylation occurs in the presence of γ-butyrobetaine, L-carnitine, or D-carnitine (Figure 3), as previously demonstrated with the human kidney enzyme (Holme et al., 1982). As shown in Figure 3, the maximal velocity of α-ketoglutarate decarboxylation supported by γ-butyrobetaine and D-carnitine was significantly greater than that with L-carnitine. However, the $K_{a(app)}$ values of both γ-butyrobetaine and L-carnitine were lower than that of D-carnitine (Table II). Excessive concentrations of γ-butyrobetaine inhibit the decarboxylation of α-ketoglutarate (Figure 3). Kinetic constants obtained from the assay of carnitine production and CO_2 release were similar (Table II).

With the omission of potassium, γ-butyrobetaine kinetic constants ($K_{m(app)}$ and K_i), as determined from assay of L-carnitine production, are increased 4–7-fold and thus parallel the decrease of the apparent maximal velocity (Table II). In the absence of potassium rates of CO_2 production by the hydroxylase at saturating γ-butyrobetaine (200 μM), D-

Table II: γ-Butyrobetaine Hydroxylase Kinetic Constants[a]

varied substrate	product assayed[b]	addition[c]	$K_{m(app)}$	V_{app}	K_i
γ-butyrobetaine	L-carnitine	KCl	16	51.4	820
	CO_2	KCl	29	50.5	750
	L-carnitine		66	6.9	6300
α-ketoglutarate	L-carnitine	KCl	50	39.0	
	L-carnitine		290	7.6	
β-bromo-α-ketoglutarate	L-carnitine	KCl	200	5.4	
D-carnitine	CO_2	KCl	470[d]	32.8	
L-carnitine	CO_2	KCl	52	6.9	

[a] Analysis of kinetic data performed as described under Materials and Methods. [b] Assays were performed with saturating concentrations of the alternative substrate as denoted under Materials and Methods and figure legends. K_{app} and V_{app} values are μM and nmol of product min^{-1} (mg of protein)$^{-1}$, respectively. [c] 100 mM KCl. [d] Values for D- and L-carnitine are apparent activation constants ($K_{a(app)}$).

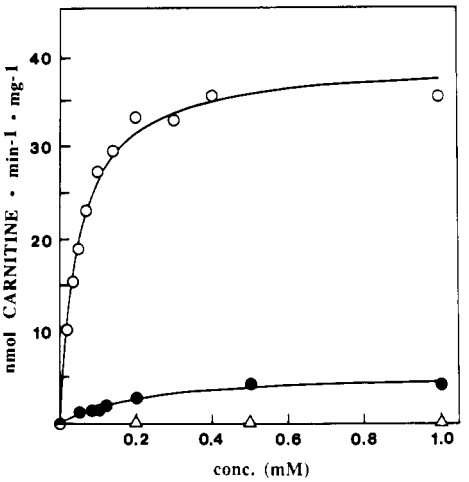

FIGURE 4: Support of L-carnitine synthesis by α-ketoglutarate and α-ketoglutarate analogues. γ-Butyrobetaine hydroxylase catalyzed L-carnitine synthesis was assayed as described under Materials and Methods at 200 μM γ-butyrobetaine, 100 mM KCl, and with varying concentrations of α-ketoglutarate (○), β-bromo-α-ketoglutarate (●), or β-mercapto-α-ketoglutarate (△). The coupled assay system (carnitine acetyltransferase) was not affected by β-bromo-α-ketoglutarate or β-mercapto-α-ketoglutarate (data not shown).

carnitine (10 mM), or L-carnitine (5 mM) were all decreased by roughly a factor of 3 to 16.2, 8.6, and 1.7 nmol CO_2 min^{-1} mg^{-1}, respectively.

Hydroxylation and Effect of α-Ketoglutarate Analogues. To further investigate the nature of the γ-butyrobetaine hydroxylase keto acid requirement, the ability of β-bromo-α-ketoglutarate, β-mercapto-α-ketoglutarate, and β-glutathione-α-ketoglutarate to support L-carnitine synthesis was studied (Figure 4). Only β-bromo-α-ketoglutarate was able to support L-carnitine synthesis; the two other analogues were completely ineffective. Relative to α-ketoglutarate, β-bromo-α-ketoglutarate is a poor substrate; its apparent K_m is 4-fold greater than, and its apparent maximum velocity one-seventh, that of α-ketoglutarate (Table II). In the absence of potassium, the α-ketoglutarate kinetic constants approximate those of β-bromo-α-ketoglutarate determined in the presence of potassium (Table II). Omission of potassium from the β-bromo-α-ketoglutarate-supported reaction resulted in rates of L-carnitine synthesis roughly one-sixth that of the rate with potassium, a level too low to permit reliable determination of kinetic constants under the conditions employed in this study (data not shown).

The alkylating properties of β-bromo-α-ketoglutarate are well documented (Hartman, 1981). To test the ability of this compound and β-mercapto-α-ketoglutarate to inactivate rat

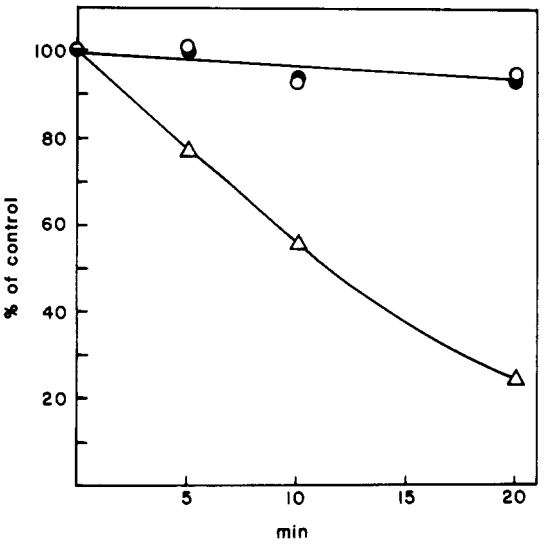

FIGURE 5: Inactivation of γ-butyrobetaine hydroxylase by β-bromo-α-ketoglutarate. γ-Butyrobetaine hydroxylase (0.5 mg/mL) was preincubated for varying lengths of time at 37 °C in 50 mM HEPES–NaOH (pH 7.0) (○) plus 1.0 mM β-bromo-α-ketoglutarate (△) or 1.0 mM β-mercapto-α-ketoglutarate (●). Aliquots of the preincubation solutions were diluted 20-fold (200-μL final volume) into a solution, on ice, containing glutathione (500 μM), potassium phosphate (25 mM), and all of the components necessary for assay of γ-butyrobetaine hydroxylase catalyzed L-carnitine synthesis (Materials and Methods) except Fe^{2+}. After 5 min on ice, the tubes were transferred to 37 °C simultaneous with the addition of Fe^{2+} to initiate the reaction. In a separate experiment, no inactivation of γ-butyrobetaine hydroxylase by 1.0 mM β-bromo-α-ketoglutarate was noted after 30 min at 0 °C. The 100% value represents a γ-butyrobetaine hydroxylase specific activity of 18.4 nmol of L-carnitine min^{-1} mg^{-1}.

liver γ-butyrobetaine hydroxylase, each of these compounds was incubated with γ-butyrobetaine hydroxylase for various lengths of time before assay (Figure 5). In these experiments, incubation of γ-butyrobetaine hydroxylase with β-mercapto-α-ketoglutarate for 20 min resulted in no loss of activity; however, this enzyme was inactivated in a time-dependent fashion by β-bromo-α-ketoglutarate. Neither desalting of β-bromo-α-ketoglutarate-inactivated γ-butyrobetaine hydroxylase nor treatment of the inactivated enzyme with thiols restored activity. In addition, both decarboxylation and hydroxylation were inactivated in parallel, and no substrate or cofactor (α-ketoglutarate, γ-butyrobetaine, Fe^{2+}, ascorbate, or potassium) or combination thereof protected against inactivation by β-bromo-α-ketoglutarate (data not shown).

In a series of experiments, the time course of γ-butyrobetaine hydroxylase inactivation by increasing concentrations of β-bromo-α-ketoglutarate (0–2 mM) was monitored. By use of these data, the order of inactivation and second-order rate constant were determined as described by Levy et al. (1963). From the first-order plots (data not shown), k_{app} (apparent first-order rate constant) values were determined and a plot of log k_{app} versus log [β-bromo-α-ketoglutarate] gave a reaction order of close to one (slope = 0.87), and a second-order rate constant of 125 M^{-1} min^{-1} was calculated. The linearity of this plot indicated lack of inactivation rate saturation with increasing β-bromo-α-ketoglutarate concentration, and a reaction order of 1 implies that inactivation by β-bromo-α-ketoglutarate is due to modification of one integral site per active unit of enzyme.

β-Mercapto-α-ketoglutarate was an effective noncompetitive inhibitor of γ-butyrobetaine hydroxylase when α-ketoglutarate was employed as the variable substrate. A Lineweaver–Burk plot yielding an intersecting pattern with both slope (K_{is} of

0.7 mM) and intercept (K_{ii} of 1.2 mM) effects was obtained (data not shown). A qualitatively similar reciprocal plot pattern was obtained when the Fe^{2+} concentration was decreased from 250 to 25 μM (figure not shown) with K_{is} and K_{ii} values of 1.1 mM and 1.8 mM, respectively. Similarly β-glutathione-α-ketoglutarate inhibited γ-butyrobetaine hydroxylase noncompetitively with respect to α-ketoglutarate. The data yielded an intersecting reciprocal plot pattern with K_{is} and K_{ii} values of 730 μM and 870 μM, respectively.

Discussion

Data presented here indicate that rat liver γ-butyrobetaine hydroxylase catalyzed L-carnitine synthesis and decarboxylation are activated by potassium. Similarly, Englard and Carnicero (1978) have noted an enhancing effect of KCl on rabbit kidney γ-butyrobetaine hydroxylase catalyzed detritiation of γ-[2,3-^{3}H]butyrobetaine. With rat liver γ-butyrobetaine hydroxylase, half-maximum activation of both decarboxylation and hydroxylation occurs at a potassium concentration of 10 mM—a concentration similar to that reported for other potassium-activated enzymes (Suelter, 1970). Like those enzymes, rat liver γ-butyrobetaine hydroxylase is also activated by NH_4^+ and Rb^+ (Table I).

The previously reported apparent stimulatory effect of inorganic phosphate (potassium phosphate) (Punekar et al., 1987) can now be seen as due to potassium. Phosphate is known to increase the rate of ferrous oxidation with concomitant generation of activated oxygen species (Lambeth et al., 1982) and was shown to inhibit pseudomonad γ-butyrobetaine hydroxylase (Lindstedt et al., 1970). A similar inhibitory effect of phosphate occurs with rat liver γ-butyrobetaine hydroxylase (Figure 2). The finding that glutathione peroxidase prevents this decreased activity is consistent with our previous report (Punekar et al., 1987).

In our previous report (Punekar et al., 1987) a potassium phosphate concentration of 20 mM was employed and thus included approximately 30 mM potassium. Therefore, the enzyme was assayed under conditions of near-saturating potassium (Figure 1) but at a phosphate concentration that results in an approximately 30% decrease in activity (Figure 2). Under these conditions the effect of potassium phosphate was on the maximal velocity of the catalyzed reaction, without a significant alteration of the concentration of Fe^{2+} ions required for half-maximal saturation (Punekar et al., 1987). In contrast, apparent K_m values for both α-ketoglutarate and γ-butyrobetaine are decreased approximately 5-fold by the addition of saturating potassium (Table II). Such a differential effect of activating monovalent cations on substrate affinity has been reported for several other enzymes [cf. Welch et al. (1968), McClure et al. (1971), and McGregor et al. (1974)].

The coupling efficiency of rat liver γ-butyrobetaine hydroxylase is dependent on the presence of potassium. Decarboxylation exceeds hydroxylation by approximately 2–3-fold in the absence of potassium but becomes fully coupled to hydroxylation at potassium concentrations of approximately 30 mM (Figure 1). A review of the literature regarding the coupling efficiency of α-ketoglutarate-coupled dioxygenases reveals that for human kidney (Holme et al., 1982), pseudomonad (Holme et al., 1982; Lindstedt et al., 1970; Lindstedt et al., 1968), and rat liver γ-butyrobetaine hydroxylase (Lindstedt & Lindstedt, 1970) experiments quantitating the coupling efficiency have in all cases yielded values of close to 100% and were all performed in at least 14 mM potassium phosphate. However, reported coupling efficiencies for neurospora thymine hydroxylase (Liu et al., 1972), rat skin prolyl hydroxylase (Rhoads & Udenfriend, 1968), and chick embryo lysyl hydroxylase (Kivirikko et al., 1972) were determined in the absence of potassium but were also close to 100%. Thus, not all α-ketoglutarate-coupled dioxygenases require potassium to couple hydroxylation to decarboxylation. Given the in vitro effect of potassium in maintaining coupling efficiency and stimulating the overall activity of the rat liver γ-butyrobetaine hydroxylase catalyzed reaction, it may be that for this enzyme, and possibly other α-ketoglutarate-coupled dioxygenases, optimal activity and coupling in vivo are ensured in part by the presence of potassium.

α-Ketoglutarate-coupled dioxygenases, including γ-butyrobetaine hydroxylases from several sources, are known to catalyze decarboxylation in the absence of a hydroxylatable substrate. With prolyl hydroxylase (Counts et al., 1978), lysyl hydroxylase (Puistola et al., 1980a), thymine hydroxylase (Hsu et al., 1981), and human kidney and pseudomonad (Holme et al., 1982) γ-butyrobetaine hydroxylase, a small but significant degree of decarboxylation occurs in the absence of the hydroxylatable substrate or nonhydroxylatable substrate analogue. In contrast, the decarboxylation catalyzed by rat liver (Lindstedt & Lindstedt, 1970, and Figure 3) and calf liver γ-butyrobetaine hydroxylase (Kondo et al., 1984) has not been observed in the absence of a substrate analogue.

As with γ-butyrobetaine-supported decarboxylation, that supported by D- or L-carnitine is stimulated approximately 3-fold by the addition of saturating potassium. D-Carnitine-stimulated decarboxylation of α-ketoglutarate by rat liver γ-butyrobetaine hydroxylase was considerably higher than that reported for the pseudomonad and human kidney enzymes (Holme et al., 1982), and the substrate analogue uncoupled rates of other α-ketoglutarate-coupled dioxygenases (Rao & Adams, 1978; Hsu et al., 1981; Holme and Lindstedt, 1982). Although the $K_{a(app)}$ for D-carnitine in the decarboxylation reaction was found to be 1 order of magnitude higher than the $K_{m(app)}$ for γ-butyrobetaine, both compounds support comparable maximal velocities (Figure 3 and Table II). However, L-carnitine, the product of the normal hydroxylation reaction, supported a much smaller maximal velocity. The lower $K_{a(app)}$ for L-carnitine compared with that of D-carnitine is in keeping with the stereoselective hydroxylation by the enzyme. In contrast to the mammalian γ-butyrobetaine hydroxylases (Figure 3 and Holme et al., 1982), decarboxylation by the pseudomonad enzyme is not supported by L-carnitine (Holme et al., 1982).

Kinetic mechanisms derived for prolyl hydroxylase (Myllyla et al., 1977), lysyl hydroxylase (Puistola et al., 1980b), thymine hydroxylase (Holme & Lindstedt, 1982), and pseudomonad γ-butyrobetaine hydroxylase (Blanchard & Englard, 1983) all support an ordered sequential mechanism in which α-ketoglutarate binds prior to the substrate undergoing hydroxylation. Given the similarities among the α-ketoglutarate-coupled dioxygenases, such a kinetic mechanism most likely holds for rat liver γ-butyrobetaine hydroxylase as well. In this regard, our observation of substrate inhibition by γ-butyrobetaine (Figure 3 and Table II) is analogous with the inhibition of prolyl hydroxylase by polypeptide substrate (Myllyla et al., 1977). The fact that substrate inhibition is observed with both assays (Table II) rules out the possibility that high concentrations of γ-butyrobetaine uncouple the enzyme as do D- and L-carnitine. Additionally, rat liver cytosolic γ-butyrobetaine is present at concentrations of approximately 10 μM as calculated from the data of Noel et al. (1984) by using the value of 0.4 mL of cytosol/g of fresh liver (Zahlten et al., 1973; Bolender et al., 1973). Given that the γ-butyrobetaine K_i is approximately 1 mM, it is unlikely that inhibition of γ-bu-

tyrobetaine hydroxylase by γ-butyrobetaine is of physiological significance.

The ability of second-substrate analogues to allow γ-butyrobetaine hydroxylase catalyzed α-ketoglutarate decarboxylation in the absence of hydroxylation (Holme et al., 1982) indicates that a possible conformational change in the enzyme induced by occupancy at the second substrate site facilitates decarboxylation. As an example, yeast hexokinase has an extremely low intrinsic ATPase activity that is stimulated by certain nonphosphorylatable pentoses (Dela Fuente & Sols, 1963; Dela Fuente et al., 1970). Lyxose (0.1 M) causes an approximately 20-fold increase in the V_{max} and a 40-fold decrease in the K_m for ATP hydrolysis (Kaji & Colowick, 1965; Dela Fuente et al., 1970; Danenberg & Cleland, 1975). This effect has been attributed to a lyxose-induced conformational change of the protein (Colowick, 1973; Danenberg & Cleland, 1975).

A plausible mechanism for the α-ketoglutarate-coupled dioxygenases, including rat liver γ-butyrobetaine hydroxylase, proposes that α-ketoglutarate decarboxylation is concomitant with the formation of a ferryl–oxo complex, possibly through a ferrous persuccinate intermediate. This ferryl–oxo species is proposed to then react with the second substrate to hydroxylate it (Blanchard & Englard, 1983). With regard to decarboxylation, it appears that both second-substrate site occupancy, with the possible enzyme conformational change, and a suitable monovalent cation are required for optimal rates. However, a significant degree of decarboxylation occurs in the absence of potassium, and with γ-butyrobetaine it exceeds L-carnitine formation. Thus, while γ-butyrobetaine or one of its nonhydroxylatable congeners promotes decarboxylation, a suitable monovalent cation is required for efficient generation or effective reaction of the putative ferryl–oxo complex. Because our assay system contained approximately 15 mM Na^+, and the addition of an additional 50 mM Na^+ slightly stimulated L-carnitine formation (Table I), we do not know to what degree hydroxylation, or for that matter decarboxylation, would proceed in the absence of stimulating monovalent cation.

Several theories have been proposed to explain the mechanism by which monovalent cations stimulate activatable enzymes (Nakashima & Tuboi, 1976). Evidence for a role of monovalent cations in inducing a required enzyme conformational change (Boyer, 1962; Evans & Sorger, 1966), as a bridge between reactants and components of the enzyme active site (Lowenstein, 1960; Melchoir, 1965), or a combination of these roles (Nowak & Mildvan, 1972) strongly implicates these mechanisms as the mode of action for the given enzyme studied. At this stage, our data on the ability of potassium-like cations to activate γ-butyrobetaine hydroxylase would be consistent with either theory.

Several reports have demonstrated the usefulness of the α-ketoglutarate analogues β-bromo-α-ketoglutarate (Hartman, 1981; Mantsala & Zalkin, 1976) and β-mercapto-α-ketoglutarate (Plaut et al., 1986) in the investigation of α-ketoglutarate-requiring enzymes. However, none of these α-ketoglutarate analogues has been employed previously in the study of an α-ketoglutarate-coupled dioxygenase.

The γ-butyrobetaine hydroxylase requirement for α-ketoglutarate is highly specific. In a study by Lindstedt and Lindstedt (1970) none of a series of organic acids could substitute for α-ketoglutarate in the hydroxylation reaction, though some inhibited the enzyme. Rat liver γ-butyrobetaine hydroxylase was found to use β-bromo-α-ketoglutarate as a cosubstrate for L-carnitine synthesis (Figure 4). To date, this finding and that of the ability of α-ketoadipic acid to support hydroxylation by prolyl hydroxylase (Majamma et al., 1984) are the only reported cases of compounds that replaced α-ketoglutarate as a cosubstrate in an α-ketoglutarate-coupled dioxygenase reaction.

In addition to functioning as a substrate, β-bromo-α-ketoglutarate was found to inactivate the enzyme irreversibly. β-Bromo-α-ketoglutarate is highly reactive with thiols (Hartman, 1981), and rat liver γ-butyrobetaine hydroxylase is known to possess essential thiol group(s) that can be modified by organomercurials and *N*-ethylmaleimide with resulting loss of enzymatic activity (Linstedt & Linstedt, 1970). The inhibition of chick embryo prolyl hydroxylase by *N*-ethylmalemide is partially prevented in the presence of α-ketoglutarate (Popenoe et al., 1969). Thus, it appeared plausible that inactivation of rat liver γ-butyrobetaine hydroxylase by β-bromo-α-ketoglutarate could result from alkylation of a thiol or amino group within the α-ketoglutarate binding domain. However, the findings that no substrate, cosubstrate, or combination thereof protected against inactivation of this enzyme by β-bromo-α-ketoglutarate and that no saturation of the inactivation rate occurred with increasing β-bromo-α-ketoglutarate concentrations indicate that this inactivation most likely occurs outside the active site.

Plaut et al. (1986) have recently demonstrated that the β-sulfur-substituted α-ketoglutarates are potent inhibitors of NADP–isocitrate dehydrogenase from several sources and alternate substrates for pig heart NADP–isocitrate dehydrogenase. With γ-butyrobetaine hydroxylase, inhibition by β-mercapto-α-ketoglutarate or β-glutathione-α-ketoglutarate was best fit to a noncompetitive pattern with α-ketoglutarate as the varied substrate. However, unlike D- and L-carnitine, neither of these two substituted α-ketoglutarates allowed α-ketoglutarate decarboxylation (data not shown); indicating that, though both compounds inhibit noncompetitively with respect to α-ketoglutarate, neither interacts effectively with the second substrate site. Because of the possible iron chelating ability of β-mercapto-α-ketoglutarate, inhibition by this compound was studied at both low and high Fe^{2+} concentrations. β-Mercapto-α-ketoglutarate inhibition was not significantly affected by a 10-fold decrease in the Fe^{2+} concentration; this indicates a negligible competition for free iron between β-mercapto-α-ketoglutarate and the enzyme. Nonetheless, coordination of β-mercapto-α-ketoglutarate with the iron–enzyme complex could significantly alter the iron chemistry.

In contrast to β-bromo-α-ketoglutarate, neither β-mercapto-α-ketoglutarate (Figure 4) nor β-glutathione-α-ketoglutarate replaced α-ketoglutarate in the hydroxylation reaction. Thus, it appears that effective binding at the α-ketoglutarate site is maintained with C3-bromination but altered by C3-thiolation. In addition, further modification of the β-sulfur-substituted α-ketoglutarate by the addition of the bulky glutathione moiety does not alter the pattern of inhibition with respect to α-ketoglutarate. A possible explanation for these data can be derived from the work of Majamma et al. (1984). They demonstrated that the α-ketoglutarate binding site of chick embryo prolyl hydroxylase consists of three subsites: I interacts with the C5-carboxyl group, II involves chelation of the enzyme-bound ferrous ion by the C1–C2 oxygens, and subsite III consists of a hydrophobic binding domain in the C3–C4 region of α-ketoglutarate. If an analogous configuration exists in rat liver γ-butyrobetaine hydroxylase, one would expect interaction at subsite III to be less perturbed by the C3-bromo addition than by the relatively less hydrophobic C3-mercapto substitution and the bulky

C3-glutathione modification. Finally, though neither of the β-sulfur-substituted α-ketoglutarate analogues supported L-carnitine synthesis, we did not assess the possible decarboxylation of these analogues by γ-butyrobetaine hydroxylase nor the degree of β-bromo-α-ketoglutarate decarboxylation relative to hydroxylation. Future work employing 1-^{14}C analogues should clarify these points.

ACKNOWLEDGMENTS

We thank Dr. Patricio Riquelme and Professor W. W. Cleland for helpful discussions and advice.

Registry No. K, 7440-09-7; NH_4, 14798-03-9; Rb, 7440-17-7; γ-butyrobetaine hydroxylase, 9045-31-2; α-ketoglutaric acid, 328-50-7; β-bromo-α-ketoglutaric acid, 76444-16-1; β-mercapto-α-ketoglutaric acid, 112792-99-1; β-glutathione-α-ketoglutaric acid, 112793-00-7; L-carnitine, 541-15-1; D-carnitine, 541-14-0; γ-butyrobetaine, 407-64-7.

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