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UNCOUPLING IN THE γ-BUTYROBETAINE HYDROXYLASE REACTION BY D- AND L-CARNITINE

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In the presence of D-carnitine significant decarboxylation of 2-oxoglutarate occurs with γ-butyrobetaine hydroxylase (EC 1.14.11.1) both from Pseudomonas sp AK 1 and from human kidney. No product was formed from carnitine when DL-carnitine was incubated with either enzyme but succinate was formed in 1:1 stoichiometry to decarboxylation using D-carnitine and the human enzyme. L-Carnitine is also an uncoupler for the human enzyme. There is no significant decarboxylation of 2-oxoglutarate in the absence of a substrate, but during normal catalysis in the presence of γ-butyrobetaine the formation of CO_2 from 2-oxoglutarate exceeds carnitine formation with 20% for the human enzyme.

INTRODUCTION

γ-Butyrobetaine hydroxylase (4-trimethylaminobutyrate:oxygen oxidoreductase (3-hydroxylating), EC 1.14.11.1) catalyzes the final step in carnitine biosynthesis in a 2-oxoglutarate-dependent dioxygenase reaction (1). This enzyme has been purified to homogeneity from a pseudomonad strain (2), and recently from calf liver (3).

A partial reaction, i.e. 2-oxoglutarate decarboxylation uncoupled from hydroxylation, occurs with several 2-oxoglutarate-dependent dioxygenases, both in the absence but more efficiently in the presence of substrate analogues (4,5,6,7,8). In this communication we show that with γ-butyrobetaine hydroxylase both the product of the enzymic reaction and its enantiomer are effective uncouplers and that with a human enzyme preparation there is a significant uncoupling during hydroxylation of γ-butyrobetaine.

MATERIALS AND METHODS

Chemicals and enzyme preparations

Compounds were obtained from the following sources: γ-butyrobetaine chloride and DL-carnitine chloride from E. Merck AG, Darmstadt, FRG; D-carnitine chloride from Nutritional Biochemicals Corporation, Cleveland, OH; L-carnitine chloride from Mann Research Laboratories, New York, NY; 2-oxo[1-^{14}C]glutarate and 2-oxo[U-^{14}C]glutarate from New England Nuclear, Boston, MA;

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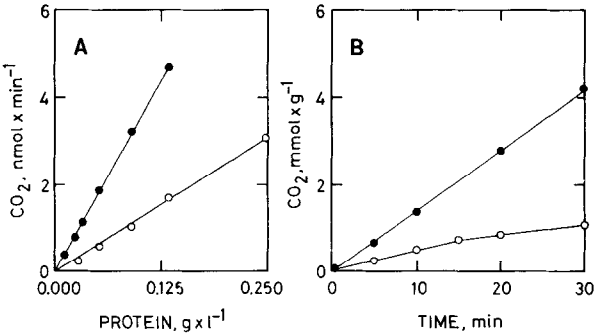


Figure 1. (A) Relation of 2-oxo [1-^{14}C]glutarate decarboxylation to concentration of human γ-butyrobetaine hydroxylase in the presence of γ-butyrobetaine (●) and of D-carnitine (○). (B) Time course of decarboxylation of 2-oxo[1-^{14}C]glutarate in the presence of γ-butyrobetaine (●) and D-carnitine (○). The enzyme concentration was 0.09 g/l.

DL-[Me-^{14}C]carnitine chloride from The Radiochemical Centre, Amersham, Buchs, England. γ-[Me^{14}C]butyrobetaine was synthesized as described earlier (9). Other chemicals were commercially available of analytical grade.

γ-Butyrobetaine hydroxylase was purified from human kidney (10). The enzyme which had a specific activity of 1.5-2.7 μkat x g protein^{-1}, was contaminated to a minor degree by other proteins as judged by polyacrylamide gel electrophoresis. The purification of γ-butyrobetaine hydroxylase to homogeneity from Pseudomonas sp AK 1 has been described (2).

Incubating conditions and product analysis

The 2-oxoglutarate-degrading activity was measured as $^{14}CO_2$ evolved from 2-oxo[1-^{14}C]glutarate (11). The composition of the incubation mixture for the human enzyme was: enzyme (5-100 μg of protein), γ-butyrobetaine (1.4 mM) or carnitine (1.4 mM), 2-oxo[1-^{14}C]glutarate (1.4 mM, 0.14-0.42 mCi/l), $FeSO_4$ (0.6 mM), sodium ascorbate (14 mM), catalase (1.4 g/l) and potassium phosphate buffer at pH 6.5 (14 mM). The total volume was 0.35 ml and the incubations were carried out at 37°C for 10 min. When the pseudomonad enzyme activity was measured the enzyme (0.2-1.2 μg of protein) was incubated with the same incubation mixture as the human enzyme but the pH was changed to 7.0. The incubations were stopped by addition of 0.3 ml of trichloroacetic acid (0.6 M).

When the stoichiometry between CO_2 and carnitine was determined, CO_2 was measured as $^{14}CO_2$ evolved from 2-oxo [1-^{14}C]glutarate and carnitine was either measured as [Me-^{14}C]carnitine formed from γ-[Me^{14}C]butyrobetaine (11) or determined by a modification (12) of the enzymatic method of Cederblad and Lindstedt (13). In this case the supernatants were neutralized with a potassium hydroxide solution (1 M). The standard solutions of carnitine also contained the cofactors used in the incubations to compensate for an inhibition of carnitine acetyl transferase.

When the stoichiometry between CO_2 and succinate was determined 2-oxo[U-^{14}C]glutarate was used. The reaction was stopped by trichloroacetic acid and $^{14}CO_2$ was collected for 1 h at 37°C. After centrifugation of the incubation mixtures, the supernatants were neutralized with potassium hydroxide solution (1 M). The neutralized samples were put onto columns (0.5 ml) of AG 1 X8 (formate form). The columns were washed with water and succinate was eluted with formic acid (2 M).

Possible products (9) obtained after incubation with DL-[Me-^{14}C]carnitine (1.4 mM, 1.4-3 mCi/l) were searched for by chromatography on a column of AG 50 W X8 (1 x 45 cm, 35 ml) in hydrochloric acid (1 M) and by thin layer chromatography in solvent system I and V described by Lindstedt & Lindstedt (14). The plates were scanned for radioactivity.

Table I

Requirements for the complete and partial reaction catalyzed by γ-butyrobetaine hydroxylase from human kidney. The enzyme concentration was 0.15 g/l.

Compound omitted	CO_2 formed, nmol×min^{-1}	
	Complete reaction (γ-butyrobetaine)	Partial reaction (D-carnitine)
None	7.7	3.1
Substrate	0.05	0.05
Fe^{2+}	< 0.01	< 0.01
Ascorbate	2.0	1.6
Catalase	3.0	1.7

RESULTS

The decarboxylation of 2-oxoglutarate was linear with concentration of the human enzyme both in the presence of γ-butyrobetaine and of D-carnitine (Fig. 1A). With γ-butyrobetaine, 2-oxoglutarate decarboxylation was linear with time for at least 30 min, whereas the CO_2 formation with D-carnitine was linear for only 15 min (Fig. 1B). The cofactor requirements of the complete and of the partial reactions were similar (Table I).

In Table II the degree of 2-oxoglutarate decarboxylation in the presence of equimolar concentrations of D-, L- or DL-carnitine is compared with the degree of decarboxylation in the presence of γ-butyrobetaine for both the bacterial and the human enzyme. There was a marked difference in uncoupling between the human enzyme and the pseudomonad enzyme, as was also apparent in the stoichiometry between γ-butyrobetaine hydroxylation and 2-oxoglutarate decarboxylation. With the bacterial enzyme the stoichiometry between CO_2 production and carnitine formation was close to one when carnitine was determined with two different

Table II

Effect of substrate analogues on the decarboxylation of 2-oxoglutarate catalyzed by γ-butyrobetaine hydroxylase from human kidney and from Pseudomonas sp. AK1.

Substrate	Human enzyme[a]		Pseudomonad enzyme[b]	
	CO_2 formed nmol x min^{-1}	Relative activity %	CO_2 formed nmol x min^{-1}	Relative activity %
γ-Butyrobetaine	5.6	100	5.6	100
D-Carnitine	2.0	36	0.53	9.5
L-Carnitine	0.51	9.1	< 0.02	< 0.36
DL-Carnitine	1.0	18	0.26	4.6
None	0.04	0.71	0.02	0.36

[a] Enzyme concentration, 0.13 g/l; [b] Enzyme concentration, 0.0013 g/l

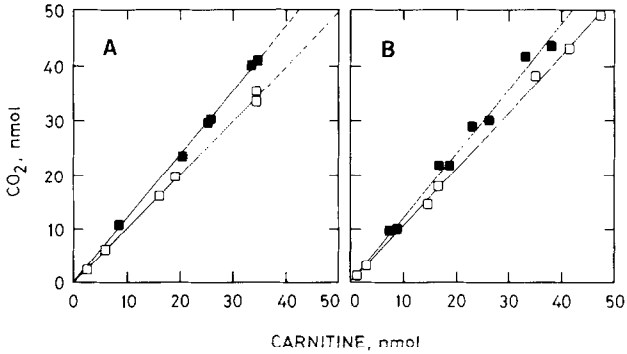


Figure 2. Relationship between the formation of carnitine from γ-butyrobetaine and the production of CO_2 from 2-oxoglutarate at different concentrations of the human (■) and the pseudomonad (□) enzymes. (**A**) The incubations were carried out with γ-[Me-^{14}C]butyrobetaine (0.6 mM, 1.8 mCi/l) and 2-oxo[1-^{14}C]glutarate (0.6 mM, 0.14 mCi/l) (**B**) The incubations were carried out with γ-butyrobetaine (1.4 mM) and 2-oxo[1-^{14}C]glutarate (0.6 mM, 0.28 mCi/l). Carnitine was determined by an enzymatic method (13).

methods, whereas with the human enzyme there was about 20% overproduction of carbon dioxide both when the amount of product formed was dependent on enzyme concentration (Fig. 2) and on substrate concentration. There was a 1:1 stoichiometry between CO_2 and succinate formation in the D-carnitine-dependent reaction with the human enzyme (Fig. 3).

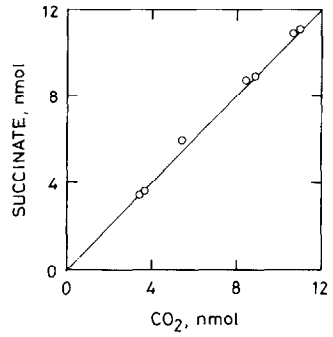
With the human enzyme, the activities for D-carnitine- and γ-butyrobetaine-dependent 2-oxoglutarate decarboxylation were equally sensitive to heat inactivation (Fig. 4). When the enzyme was heated at 50°C, the half-lives of decrease of the two activities were about 12 min.

The substrate-dependent velocity of 2-oxoglutarate decarboxylation with the human enzyme in the presence of γ-butyrobetaine and D-carnitine were very similar (Fig. 5). K_m for 2-oxoglutarate was found to be 0.25 mM and 0.20 mM and for γ-butyrobetaine and D-carnitine 0.14 mM.

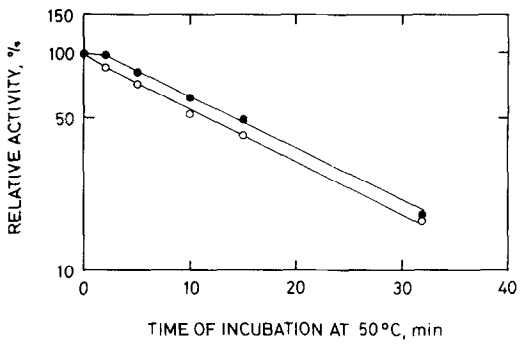
To detect possible product formation from carnitine, we incubated DL-[Me^{14}C]carnitine with both enzymes. In an experiment in which about 10% conversion of carnitine would have been expected if there was a 1:1 stoichiometry between 2-oxoglutarate decarboxylation and hydroxylation of γ-butyrobetaine, ion-exchange chromatography resulted in only one radioactive peak which coincided with carnitine. The reaction products were further analyzed both in an acid and in an alkaline TLC system, but still only carnitine was found. A product formation at the level of about 1% would have been detected in these experiments.

DISCUSSION

Both D- and L-carnitine are uncouplers of the reaction catalyzed by the human enzyme, whereas only D-carnitine is effective with the bacterial enzyme. There is also a marked difference in the degree of uncoupling between the human and the



(3)



(4)

Figure 3. Relationship between CO_2 and succinate formation from 2-oxo[U-^{14}C]glutarate (0.14 mM, 0.29 mCi/l) in the presence of D-carnitine. The concentration of human enzyme was 0.09-0.27 g/l.

Figure 4. The loss of γ-butyrobetaine-dependent (●) and D-carnitine-dependent (○) activities on denaturation with heat. The concentration of the human enzyme was 0.13 g/l. The initial enzyme activity was 7.1 nmol CO_2 per min with γ-butyrobetaine and 3.2 nmol CO_2 per min with D-carnitine.

bacterial enzyme but appropriate control experiments have been performed to rule out the possibility that the differences are due to a contaminating enzyme activity in the human enzyme. The degree of uncoupling is considerably higher than has been found with other 2-oxoglutarate-dependent enzymes, i.e. a maximum of 36% compared to 5-10% with prolyl hydroxylase (5) and thymine 7-hydroxylase (7,8).

Uncoupling occurs to a significant extent during normal catalysis with the human enzyme and possibly also with the bacterial enzyme, cf Fig. 2. This has not previously been demonstrated for this type of enzyme. The two enzymes both require Fe^{2+} and are stimulated by ascorbate and catalase, and the loosely coupled reaction observed for the human enzyme has no obvious explanation. When the enzyme is catalyzing the uncoupled reaction it is inactivated as judged from the time-progress curve. In the partial reaction the reaction cycle of the enzyme is not completed (8,15) and an oxidized enzyme is probably formed in spite of the high ascorbate concentration used in the assay.

The reaction catalyzed by γ-butyrobetaine hydroxylase is stereospecific (16) which means that further hydroxylation of the product, L-carnitine, cannot be expected. Hydroxylation of the product enantiomer was considered as a possibility in analogy with the hydroxylation of D-octopamin by dopamine β-hydroxylase (EC 1.14.17.1) (17). Sequential oxygenation of the same carbon also occurs in another 2-oxoglutarate-dependent dioxygenase reaction viz. the hydroxylation of thymine to 5-carboxyuracil in three consecutive hydroxylation reactions (18). The absence of any products from D-carnitine and the fact that also L-carnitine catalyzed the partial reaction rule out hydroxylation of D-carnitine as a cause of the observed decarboxylation of 2-oxoglutarate.

In healthy subjects D-carnitine is rapidly excreted by the kidneys, whereas in uremic patients D-carnitine is accumulated (19). Patients undergoing chronic

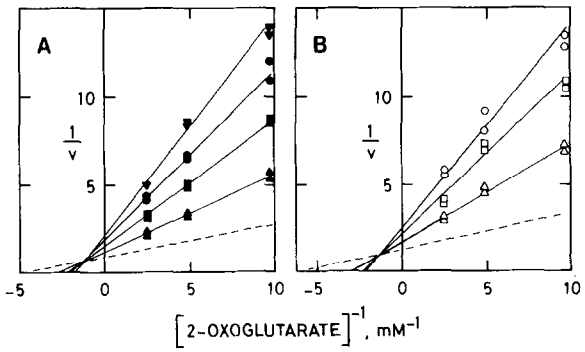


Figure 5. Initial velocity plots for human γ-butyrobetaine hydroxylase at variable concentrations of 2-oxoglutarate at different levels of γ-butyrobetaine (**A**) and of D-carnitine (**B**).
(**A**) The γ-butyrobetaine concentrations were: 0.075 mM (▼); 0.10 mM (●); 0.15 mM (■); 0.30 mM (▲). (**B**) The D-carnitine concentrations were: 0.10 mM (○); 0.15 mM (□); 0.30 mM (△). The dashed lines are the intercept replots i.e. the lines at infinite concentration of γ-butyrobetaine and D-carnitine, respectively. v is expressed in nmol $^{14}CO_2$ evolved per min.

dialysis become deficient in carnitine and when they are supplemented with DL-carnitine myastenia like symptoms have been reported in some patients. The symptoms have disappeared after administration of L-carnitine. If the adverse effects of D-carnitine can to some extent be explained by the interference in the reaction catalyzed by γ-butyrobetaine hydroxylase must await further investigation. At present we also abstain from speculations about any physiological role of the uncoupling effect of L-carnitine, e.g. in the regulation of carnitine biosynthesis. As a pratical aspect it should be noted that assays of enzymic hydroxylating activity based on decarboxylation of 2-oxoglutarate may give erroneous results, and a fixed relation between hydroxylation and decarboxylation cannot be taken for granted when assay conditions are changed. The use of tritium release assays must also be carefully considered, since the isotope effect observed (20), may be due to an increased uncoupling of hydroxylation due to the isotope substitution of γ-butyrobetaine.

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