Synthesis of α-Ketoglutarate by Reductive Carboxylation of Succinate in *Veillonella, Selenomonas,* and *Bacteroides* Species

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Evidence for reductive carboxylation of succinate to synthesize α-ketoglutarate was sought in anaerobic heterotrophs from the rumen and from other anaerobic habitats. Cultures were grown in media containing unlabeled energy substrates plus [14C]succinate, and synthesis of cellular glutamate with a much higher specific activity than that of cellular aspartate was taken as evidence for α-ketoglutarate synthase activity. Our results indicate α-ketoglutarate synthase functions in *Selenomonas ruminantium, Veillonella alcalescens, Bacteroides fragilis, Bacteroides vulgatus, Bacteroides uniformis, Bacteroides distasonis,* and *Bacteroides multaciudus.* Evidence for this carboxylation was not found in strains representative of 10 other species.

Most microbes apparently synthesize α-ketoglutarate (αKG) needed for biosynthesis by forward tricarboxylic acid reactions via isocitric dehydrogenase. Some anaerobes, however, accomplish this by reductive carboxylation of succinate. This αKG synthase reaction was first demonstrated as a physiologically significant reaction in green photosynthetic bacteria (11, 13):

\[
\text{succinyl-CoA} + \text{CO}_2 + \text{ferredoxin}_{\text{red}} \rightarrow \alpha\text{-KG} + \text{CoA} + \text{ferredoxin}_{\text{ox}}
\]

where CoA is coenzyme A. We reported (2) that an important rumen bacterium, *Bacteroides ruminicola,* carboxylated succinate to αKG, and that this was probably the major, if not the sole, reaction used for synthesis of αKG. Evidence for this included data from experiments with cell extracts, from isotopic competition experiments, and from the position of label in glutamic acid (Glu) synthesized from [1,4-14C]succinate. The specific activity (SA) of Glu in protein from *B. ruminicola* cells, grown in a medium containing unlabeled glucose as the main carbon and energy source plus 14C-labeled succinate, was nine times the SA of aspartic acid (Asp). Although the nature of the low potential electron carrier for the reductive carboxylation in *B. ruminicola* and the organisms studied here is not yet clear, for the sake of brevity we refer to this carboxylation as the αKG synthase reaction.

Milligan (23) estimated that 20 to 40% of the Glu synthesized by a mixed population of ruminal microbes was synthesized by reactions involving the reductive carboxylation of succinate (16, 27); however, until now, there has been only one report of αKG synthase activity in pure cultures of anaerobic chemoorganotrophic bacteria (2).

In the present study, a variety of anaerobic bacteria were tested for αKG synthase activity. The basic premise of the experiments is that after growth in media containing an unlabeled energy plus 14C-labeled succinate, forward reactions of the tricarboxylic acid cycle cannot explain the SA of cellular Glu being decidedly higher than that of Asp; a functional αKG synthase reaction is the most probable explanation.


MATERIALS AND METHODS

Organisms. *Bacteroides fragilis* ATCC 23745; *B. vulgatus* ATCC 8482; *B. uniformis* ATCC 8492; and *B. distasonis* ATCC 8503 were from the American Type Culture Collection. *B. hypermegas* 61/42 (now ATCC 25560) and 58/74 (now NCTC 10571); *B. vulgatus* Au 21/27, EBF 77/84, and EBF 77/47; *B. multaciudus* 402/44 and 374/14; and *Fusobacterium necrogenes* EBD 1/4a (now ATCC 25556) were isolated from cecal contents from chickens (5) and obtained from E. Barnes. *Veillonella alcalescens* 221 was obtained from H. R. Whiteley. *Selenomonas sputigena* was obtained from R. Smibert. The *Vibrio succinogenes* culture was the strain isolated by Wolin et al. (31) from the rumen. The other rumen bacteria tested were strains obtained from M. P. Bryant.
Culture methods. The anaerobic culture techniques used were those of Hungate (19). Most of the organisms were cultured in medium A, a defined medium similar to that described by Bryant and Robinson (9), except that casein hydrolysate was deleted and the following ingredients (milligrams per 100 ml) were added: glucose, 300; soluble starch, 300; methionine, 4.5; and hemin, 0.2. V. alcalescens was grown in a medium containing minerals, resazurin, vitamins, Na₂CO₃, and cysteine as in medium A; sodium lactate, 0.2% (wt/vol); and enzymatic casein hydrolysate (vitamin free, Nutritional Biochemicals Corp., Cleveland, Ohio), 0.05% (wt/vol). This concentration of casein hydrolysate was suboptimal for growth in that the absorbance at 600 nm (A₆₀₀) reached 0.46, whereas with twice that concentration of casein hydrolysate, A₆₀₀ of 0.70 was observed.

The 40% rumen fluid broth medium was similar to RGC medium (8) except that agar was deleted and soluble starch (0.4%) and Trypsinase (BBL Microbiology Systems, Cockeysville, Md.) (0.05%) were added. V. succinogenes was grown in the VSP and VSN media of Kafkowitz (20). Methanobacterium ruminantium was grown in the inoculum medium of Bryant (7). The gas mixture used for culture of M. ruminantium was CO₂-H₂ (1:1), while all other organisms were grown with a CO₂ gas phase.

Incorporation of radiochemicals. Incorporation of labeled substrates was usually measured in cultures (10 or 20 ml) grown from a 1 or 2% inoculum of a 16- to 24-h culture. All strains were grown in media containing [1,4-¹⁴C]succinic acid (Cal-Atomic, Los Angeles, Calif.). Some of the organisms were also grown in media containing [2,3-²⁴šC]succinic acid (New England Nuclear Corp., Boston, Mass.), d-[U-¹⁴C]glucose or [2-¹⁴C]propionic acid (both from Amersham Corp., Arlington Heights, Ill.).

Except when otherwise specified, [¹⁴C]succinate was added to the medium at a concentration of 0.05 mM (10 mCi/mmoll). Growth was measured as A₆₀₀ in test tubes (18 by 150 mm) with a Spectronic 20 colorimeter (Bausch & Lomb, Inc., Rochester, N. Y.). Radioactive materials were filter sterilized (0.45-µm filter; Millipore Corp., Bedford, Mass.) and were bubbled with CO₂ before they were added to culture media. Unlabeled materials added to culture media in competition experiments were usually filter sterilized, but materials known to be heat stable were autoclaved.

Results

αKG synthase in Selenomonas ruminantium. S. ruminantium cultures incorporated ¹⁴C during growth in the presence of [1,4-¹⁴C]succinate (Table 1). Radioactivity in the protein fraction of the cells was mainly in the glutamate family of amino acids, Glu, proline (Pro), and arginine (Arg). When unlabeled succinate was added to cultures of strain GA192 so that the SA of succinate was diluted from 10 to 0.5 or 0.125 µCi/µmol, the SA of Glu synthesized declined, but not in direct proportion to the change in SA of the succinate supplied (Table 2). At all concentrations of succinate, radioactivity in cellular amino acids was mainly in the Glu family of amino acids.

αKG synthase in Bacteroides. Most of the

<table>
<thead>
<tr>
<th>Table 1. Distribution of ¹⁴C after growth of S. ruminantium strains in [1,4-¹⁴C]succinatea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>GA192</td>
</tr>
<tr>
<td>HD-1</td>
</tr>
<tr>
<td>HD-4</td>
</tr>
<tr>
<td>D</td>
</tr>
</tbody>
</table>

*a Cultures (10 ml) were in medium A plus [1,4-¹⁴C]succinate (0.05 mM [10 mCi/mmol]). Measurements were made with the amino acid analyzer flow cell system.

*b Values in parentheses are the estimates (where data was available) of the SA (counts per minute per nanomole).
\[ 14^C \text{ in amino acids from the hydrolyzed protein fraction of } B.\ vulgarus \text{ 8482 and } B.\ fragilis \text{ 23745 cells grown in medium A plus } [1,4-14^C] \text{succinate was in Glu, Pro, and Arg (Fig. 1). The ratios of the SA of Glu/SA of Asp were 20.1 for } B.\ fragilis \text{ and 128 for } B.\ vulgarus. \] The latter value was the highest measured for any organism and is due to the barely detectable amount of \[ 14^C \] in Asp from the \[ B.\ vulgarus \] hydrolysate.

Strains of some of the other species of \[ Bacteroides \] also synthesized Glu that had a much higher SA than did Asp during growth in medium A containing \[ [1,4-14^C] \text{succinate} \] (Table 3). Thus, \[ \alpha K\text{G synthase probably functions in } B.\ fragilis, B.\ distasonis, B.\ uniformis, B.\ vulgarus, B.\ multiacidus, \] and, as previously shown (2), in \[ B.\ ruminicola. \] The patterns of \[ 14^C \] distribution in amino acids of protein hydrolysates from \[ B.\ distasonis \] ATCC 8503, \[ B.\ uniformis \] ATCC 8492, and \[ B.\ vulgarus \] EBF 77-84 and Au 21/27 were similar to those for \[ B.\ vulgarus \] and \[ B.\ fragilis \] (Fig. 2), except that less \[ 14^C \] was found in Arg.

With \[ B.\ amylphilus \] 70 and \[ B.\ succinogenes \] S85 hydrolysates, however, more \[ 14^C \] was recovered in Asp than in Glu. SA of these amino acids was not measured, but the proportions of Asp and Glu were similar in most of these protein hydrolysates and, thus, relative proportions of \[ 14^C \] in Asp and Glu were not greatly different from relative SA measurements (Table 3). The SA of Glu from \[ B.\ hypermegas \] was only slightly higher than that of Asp. We do not consider this as evidence either for or against the function of \[ \alpha K\text{G synthase in } B.\ amylphilus, B.\ succinogenes, \] or \[ B.\ hypermegas. \]

After growth of \[ B.\ ruminicola \] 23 in medium containing \[ [1,4-14^C] \text{succinate}, \] about 12% of the \[ 14^C \] was recovered as carbonate in the culture medium (2). The labeling pattern in protein hydrolysates from cells of strain 23 grown in either \[ [1,4-14^C] \text{succinate or } [14^C] \text{CO}_3 \] is presented (Fig. 2). In this experiment, the ratio of the SA of Glu/SA of Asp from cellular protein was 9.01 for cells grown in \[ [14^C] \text{succinate and 1.37 for cells grown in } [14^C] \text{CO}_3 \]. This indicates that the production of Glu with a much higher SA than that of Asp by cells grown in \[ [14^C] \text{succinate was not due to utilization of } [14^C] \text{CO}_3 \text{ produced from the carboxyl-labeled succinate}. \] The results in Fig. 2 also support previous findings (2) obtained when amino acids from \[ B.\ ruminicola \] 23 cells grown in \[ [1,4-14^C] \text{succinate were separated by paper chromatography.} \]

Addition of unlabeled succinate to a medium containing \[ d-[U-14^C] \text{glucose led to marked reduction of relative amounts of } 14^C \text{ recovered in the Glu family of amino acids, but had little effect on } 14^C \text{ incorporation into Asp by } B.\ ruminicola \text{ 23 (2). Similar findings were obtained here with } B.\ fragilis \text{ 23745 grown in a medium that contained } d-[U-14^C] \text{glucose (8.3 mM, 0.12 mCi/mmol). In this experiment, however, Glu} \] and Pro were not adequately resolved by the

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**Table 2. Incorporation of \[ 14^C \text{ from } [1,4-14^C] \text{succinate by } S.\ ruminantium \text{ GA192}**

<table>
<thead>
<tr>
<th>Culture no.</th>
<th>SA of succinate ((\mu\text{Ci}/\mu\text{mol}))</th>
<th>Succinate concn (mM)</th>
<th>% of culture [ 14^C \text{ in:}</th>
<th>SA (cpm/nmol)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CO(_2) Cells Protein</td>
<td>Glu Arg Asp Lysine Ileu</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>0.05</td>
<td>22.2 18.2 12.1</td>
<td>531 28 20 23</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>1</td>
<td>8.8   4.3  3.0</td>
<td>138 233 11 11 21</td>
</tr>
<tr>
<td>3</td>
<td>0.125</td>
<td>4</td>
<td>6.4   4.1  3.5</td>
<td>60 94 5.6 6.7 11</td>
</tr>
</tbody>
</table>

*Other amino acids with appreciable radioactivity were Pro and threonine, but SA measurements for these were not obtained. Measurements were made as given in Table 1.

*Not determined.
TABLE 3. Radioactivity in glutamate and aspartate from protein hydrolysates of Bacteroides spp. grown in [1,4-14C]succinate

<table>
<thead>
<tr>
<th>Organism</th>
<th>Relative 14C of Glu/Asp</th>
<th>SA of Glu/Asp</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. vulgatus 77/47</td>
<td>7.4</td>
<td>8.0</td>
</tr>
<tr>
<td>B. distasonis ATCC 8603</td>
<td>8.83</td>
<td>9.6</td>
</tr>
<tr>
<td>B. uniformis ATCC 8492</td>
<td>18.6</td>
<td>19.2</td>
</tr>
<tr>
<td>B. multiacidus 402/44</td>
<td>9.2</td>
<td>7.6</td>
</tr>
<tr>
<td>B. multiacidus 374/14</td>
<td>4.4</td>
<td>3.9</td>
</tr>
<tr>
<td>B. hypermegas NCTC 10570</td>
<td>1.07</td>
<td>1.1</td>
</tr>
<tr>
<td>B. hypermegas ATCC 25660</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>B. amylophilus 70</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>B. succinogenes S85</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>B. ruminicola GA33</td>
<td>17.3</td>
<td></td>
</tr>
</tbody>
</table>

* Analysis conducted by Dave Peterson, University of Minnesota, Minneapolis.

Based upon measurements of 14C from amino acids separated by two-dimensional paper chromatography (2). All other measurements made as indicated in Table 1.

Fig. 2. Distribution of 14C in amino acids of hydrolyzed protein from B. ruminicola 23 grown in medium containing [1,4-14C]succinate (A) or 14CO3 (B). (C) Measurement of ninhydrin-reactive substances of the protein hydrolysate from cells grown in 14CO3.

amino acid analyzer. The ratios of 14C recovered in Glu + Pro/Asp were 1.29, 0.98, and 0.61 for cultures with unlabeled succinate added at 0, 10^-3, and 10^-2 M, respectively. These results indicate that the added succinate diluted the SA of [14C]succinate produced from labeled glucose and led to a dilution of the 14C incorporated into aKG by B. fragilis.

aKG synthase in V. alcalescens. When V. alcalescens cells were grown in the presence of [1,4-14C]succinate, 19% of the 14C in the culture supernatant was recovered as carbonate. An appreciable portion of the 14C in cellular protein was present in amino acids of the Asp family (Fig. 3), and the ratio of the SA of Glu/Asp was 2.23. This was not considered to be strong evidence for the function of aKG synthase. Evidence for this activity was obtained, however, when V. alcalescens was grown in [2,3-14C]succinate. Only 0.13% of the 14C in the culture supernatant was present as CO2. The quantity of 14C in Asp was barely detectable, and the relative proportions of 14C in Glu, Pro, and Ileu (Fig. 3) were 1.0, 0.21, and 0.51, respectively.

Because V. alcalescens produces propionate from succinate, it seemed pertinent to compare the labeling pattern in amino acids from cells grown in [14C]propionate with that obtained with [14C]succinate. When cells were grown in a medium containing [2-14C]propionate (0.083 mM, 12 mCi/mmol), the only amino acids with enough 14C to be detected were Glu, Pro, and isoleucine (Ileu). The relative proportions of 14C in these, as determined by the flow cell amino acid analyzer system, were 1.0, 0.19, and 1.26 for Glu, Pro, and Ileu, respectively (data not shown).

Cultures in which evidence for aKG synthase was not found. When V. succinogenes was grown in the fumarate medium (VSM) plus [1,4-14C]succinate, less than 0.1% of the 14C was

Fig. 3. Distribution of 14C in amino acids of hydrolyzed protein from V. alcalescens grown in media containing either [2,3-14C]succinate (A) or [1,4-14C]succinate (B). (C) Measurement of ninhydrin-reactive substances of the protein hydrolysate from cells grown in [1,4-14C]succinate.
incorporated into the cells. This low incorporation may have been due to extensive dilution of the SA of succinate by that produced from fumarate in the medium. With cells at mid-log phase of growth in medium VSN, which contained nitrate rather than fumarate as the terminal electron acceptor, 44% of the $^{14}$C from [2,3-$^{14}$C]succinate (0.056 mM, 13 mCi/mmol) was incorporated into cells during 3 h of growth from $A_{600}$ of 0.4 to 0.8. Most of the amino acids in the protein fraction of the cells were labeled (Fig. 4). Radioactivity, however, was not concentrated in the Glu family of amino acids. The SAs of glutamate and arginine relative to that of aspartate were 1.32 and 0.64, respectively.

We did not find evidence for $\alpha$KG synthase activity in a number of other bacteria that failed to incorporate appreciable (<0.5%) $^{14}$C when grown in media containing [$^{14}$C]succinate. These include: Ruminococcus flavefaciens C94, F. necrogenes ATCC 25556, M. ruminantium M1, Streptococcus bovis FD10, Butyrivibrio fibrisolvens D1, Selenomonas R-10 and R-11, Selenomonas spuitigina, anaerobic spirochete B35 from the rumen, and Succinivibrio dextrinosolvens 24.

**DISCUSSION**

In certain autotrophs (13, 17) and B. ruminicola (2), synthesis of $\alpha$KG by reductive carboxylation of succinate ($\alpha$KG synthase) appears to be an important reaction. The results presented here indicate that reductive carboxylation of succinate also occurs in various other anaerobic heterotrophs. Among those with this capacity are strains representative of species that are predominant in the rumen and in the gastrointestinal tract of humans and other animals.

Some of these species are also important clinically.

Our evidence for $\alpha$KG synthase activity is based on the premise that when cells are grown in medium containing unlabeled glucose (or lactate), significantly greater incorporation of labeled carbon from succinate into Glu as compared to Asp can best be explained by the action of $\alpha$KG synthase. This is in contrast to what would be expected if $^{14}$C from succinate entered Glu by forward reactions of the tricarboxylic acid cycle and carbon flowed through oxalate, the Asp precursor, to $\alpha$KG.

In a previous study, evidence for $\alpha$KG synthase in B. ruminicola, based on greater incorporation of $^{14}$C from succinate into Glu than into Asp, was supported by (i) data on the position of $^{14}$C in glutamate synthesized from [1,4-$^{14}$C]succinate, (ii) isotopic competition experiments, (iii) studies of glutamate synthesis from succinate by cell extracts, and (iv) the failure to demonstrate either citrate synthase or isocitrate dehydrogenase activities (2). It was thus suggested that the succinate carboxylation mechanism is the main, and perhaps only, pathway for synthesis of $\alpha$KG in B. ruminicola. Because the present study involved a number of organisms, it was not practical to obtain such confirmatory evidence for each species.

Ferredoxin supplies the reducing power for synthesis of $\alpha$KG from succinyl CoA and CO$_2$ in extracts from photosynthetic bacteria (12, 13). The electron carrier for this carboxylation in Methanobacterium thermooautotrophicum appears to be factor 420 (17, 32). Ferredoxin is present in V. alcalescens and is involved in degradation of $\alpha$KG to produce CO$_2$, H$_2$, and propionate (21) as well as in the exchange reac-

**Fig. 4.** Distribution of $^{14}$C in amino acids of hydrolyzed protein from V. succinogenes grown in VSN medium with [2,3-$^{14}$C]succinate (A). (B) Measurement of ninhydrin-reactive substances.
tion between CO₂ and α-keto acids (30). Ferredoxin was not detected in extracts from B. fragilis (18) or B. ruminicola (2). Clostridial ferredoxin, however, stimulated the carboxylation reaction by DEAE-cellulose-treated extracts from B. ruminicola (2). Ferredoxin also stimulated the αKG synthase activity demonstrated in crude extracts from the mixed ruminal population (16), but αKG synthase was not found when extracts were partially purified (15). A low-potential, iron-containing, electron-transfer protein isolated from extracts of mixed ruminal bacteria substituted for ferredoxin in the pyruvate synthase system (14). It may be that this or a similar substance is functional in the αKG synthase system of B. ruminicola and in certain other organisms studied here.

The incorporation of 14C into Asp when V. alcalescens was grown in 1,4-labeled succinate, but not when 2,3-labeled succinate was used (Fig. 3), suggests that 14CO₂ produced from the former was utilized to synthesize labeled oxalacetate (and thus aspartate), probably via carboxylation of phosphoenolpyruvate or pyruvate. This activity limits the sensitivity of our comparison of SA of Glu and Asp as an indicator of αKG synthase in organisms that degraded 1,4-labeled succinate to produce 14CO₂. In retrospect, thus, it would have been better to use succinate labeled in positions 2, 3 for all of this work.

Reductive carboxylation of propionate to synthesize α-ketobutyrate, an important intermediate in Ileu biosynthesis, was first demonstrated in cell-free preparations from Chromatium, Clostridium pasteurianum, and Desulfovibrio desulfuricans (10). Extracts prepared from mixed rumen bacteria also catalyze this α-ketobutyrate synthase reaction (15, 27). Some of the organisms in the present study (S. ruminantium and V. alcalescens) that are known to degrade succinate to propionate and CO₂ (25, 29) synthesized Ileu that had a higher SA than that of Asp (or threonine) during growth on [14C]succinate (Tables 1 and 2; Fig. 3). This suggests that α-ketobutyrate may have been synthesized from propionate rather than from Asp carbon via threonine. The experiment with V. alcalescens grown in [14C]propionate, in which more 14C was recovered in Ileu than in any other amino acid from cellular protein, further supports our hypothesis for the function of α-ketobutyrate synthase. Appearance of 14C in Glu and Pro after growth of V. alcalescens in [14C]propionate was also observed with Chromatium (10) and might be explained by reversal of reactions involved in αKG degradation to propionate via succinyl coenzyme A (21).

Propionate is a major end product of carbohydrate metabolism by B. hypermegas, and the extensive incorporation of 14C from succinate into Ileu suggests that propionate was produced from succinate and subsequently incorporated by α-ketobutyrate synthase. B. fragilis also produces propionate via succinate (22), but propionate is a minor rather than major end product of B. fragilis, and Ileu from protein of B. fragilis cells grown in [14C]succinate was not highly labeled. B. ruminicola produces propionate by the acrylate pathway (28), and, as might be expected, little 14C from succinate was incorporated into Ileu.

Results obtained with V. succinogenes (Fig. 4) illustrate an incorporation pattern with the SA of Asp similar to that of Glu. Thus, evidence was not obtained for the function of αKG synthase in this organism, but our data should not be interpreted as proof that αKG synthase does not function. V. succinogenes requires succinate for growth in media with formate as electron donor and nitrate as electron acceptor (24). The explanation of this requirement is still not known, but data given here indicate that carbon from succinate becomes widely distributed in various amino acids.

Blackburn and Hungate (6) estimated that about 25% of the labeled succinate added to mixed ruminal microbes was assimilated rather than degraded. They concluded from estimates of the turnover rate of succinate that this assimilation was quantitatively significant. Organisms belonging to the species B. ruminicola and S. ruminantium together comprise a significant portion of the bacteria in the rumen and could account for much of the ruminal Glu synthesis by the reductive carboxylation pathway (23). It seems probable that αKG synthase functions in a number of anaerobes other than those studied here, but whether or not it represents a major or sole pathway for synthesis of αKG in heterotrophs other than B. ruminicola remains to be shown.

LITERATURE CITED


