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$^{14}CO_2$ exchange with acetoacetate catalyzed by dialyzed cell-free extracts of the bacterial strain BunN grown with acetone and nitrate

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The nitrate-reducing bacterial strain BunN is able to grow with acetone and nitrate under anoxic conditions. Dialyzed crude cell-free extracts of acetone-plus-nitrate-grown cells of strain BunN catalyzed the exchange of $^{14}CO_2$ into acetoacetate in an ADP-dependent reaction. The rates of exchange catalyzed by extracts of acetate-grown or 3-hydroxybutyrate-grown cells were only 13% of that catalyzed by extracts of acetone-grown cells. The activity was enzymic since it was destroyed by boiling and was proportional to the amount of added extract. The optimal acetoacetate concentration was 100 mM and the apparent K_m was 11.1 mM. The pH optimum was 6.5, the exchange was not dependent on the addition of biotin, and the activity was not inhibited by avidin. The exchange activity was not stimulated (less than two fold) by a variety of metal ions or by a range of possible cofactors. Under optimal conditions (100 mM acetoacetate, 5 mM ADP, 10 mM $NaHCO_3$, pH 6.5, under N_2), the exchange activity was 2.7 nmol · min^{-1} · mg $protein^{-1}$; 2% of the *in vivo* carboxylation activity of acetone-plus-nitrate-grown cultures. It is suggested that the exchange reaction is a partial reaction catalyzed by the enzyme (or enzyme complex) that carboxylates acetone, and that the methods developed in this study provide a means with which to investigate this reaction further.

Keywords. Carboxylation; acetone; CO_2 exchange.

The degradation of acetone under anoxic conditions appears to involve an initial carboxylation step. This has been suggested by labelling studies with nitrate-reducing bacteria (Bonnet-Smits et al., 1988; Platen and Schink, 1989), sulfate-reducing bacteria (Platen et al., 1990), and fermenting bacteria (Platen and Schink, 1987). The growth rate of the acetone-oxidizing, nitrate-reducing bacterial strain BunN shows Monod-type kinetics in dependence on the CO_2/HCO_3^- concentration in the growth medium (Platen and Schink, 1989). Most of the enzymes involved in the catabolism of acetone by this nitrate-reducing bacterium have been determined (Platen and Schink, 1990), but the carboxylation step in this organism and in other acetone-degrading bacteria has not been measured *in vitro*.

The carboxylation of acetone to acetoacetate is an endergonic reaction ($\Delta G^{\circ\prime}$ calculated according to Thauer et al., 1977):

$$CH_3COCH_3 + HCO_3^- \rightarrow CH_3COCH_2COO^- + H_2O$$

$$(\Delta G^{\circ\prime} = +17.1 \text{ kJ} \cdot \text{mol acetone}^{-1}).$$

This reaction suggests a reversal of the acetoacetate decarboxylase reaction catalyzed by an enzyme from *Clostridium acetobutylicum* (Hamilton and Westheimer, 1959; Westheimer, 1969). Our knowledge of this enzyme, however, does not suggest that it is a membrane protein or reversible ion pump (Walsh, 1979; Gerischer and Dürre, 1990). Several microorganisms are able to couple the decarboxylation of an organic acid (by an ion-pumping decarboxylase) to the generation of a transmembrane ion gradient, which can then be harnessed to generate ATP. This is often in the form of a sodium-ion gradient (Dimroth, 1987), and, *in vitro*, such gradients can drive carboxylation reactions in reconstituted proteoliposomes (Dimroth and Hilpert, 1984). Although a carboxylation reaction driven by an electrochemical gradient appears feasible, it is not known to occur in whole organisms. Energetically unfavourable reactions can be driven by such gradient as occurs, for example, in the transport of compounds across the cytoplasmic membrane (Harold, 1986; Maloy, 1990) or during reversed electron transport to drive endergonic redox reactions (Klingenberg and Schollmeyer, 1960; Aleem, 1966; Paulsen et al., 1986).

The direct utilization of ATP to drive a reaction requiring an energy input of +17.1 kJ · mol^{-1} is feasible, and could involve the formation of a free (i.e. not enzyme bound) phosphorylated intermediate, such as the phospho*enol*acetone postulated by Platen and Schink (1990), or of an enzyme-bound intermediate, analogous to the different mechanisms by which pyruvate is carboxylated (Walsh, 1979). The observation of ADP stimulation of acetoacetate decarboxylation (although at very low rates) by crude cell-free extracts of the nitrate-reducing, acetone-carboxylating bacterium BunN (Platen and Schink, 1990) could support such a hypothesis. A phosphorylated intermediate, phenylphosphate, has recently been identified in the carboxylation of phenol by a nitrate-reducing bacterium (Lack and Fuchs, 1992, 1994). There is no information as to what sort of mechanism is involved in the carboxylation of acetone.

We have attempted, unsuccessfully to date, to measure an *in vitro* carboxylation of acetone using various assay systems. We therefore attempted to measure a partial reaction, the exchange of radioactively labelled CO_2 with the carboxyl group of the

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Enzymes. Acetoacetate decarboxylase (EC 4.1.1.4); adenosinetriphosphatase (EC 3.6.1.3); 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30); succinyl-CoA:acetoacetate CoA-transferase (EC 2.8.3.6)

proposed carboxylation product, acetoacetate, in an attempt to characterize the carboxylation activity. In this study we report the development of an assay method for the partial reaction and the preliminary characterization of the exchange activity.

MATERIALS AND METHODS

Organism and culture conditions. Strain BunN (Platen and Schink, 1989) was maintained in liquid culture after isolation. Stock cultures were grown in 100 ml volumes in 125-ml glass bottles. A bicarbonate-buffered medium containing vitamins and trace elements (Dörner and Schink, 1990) was modified by the omission of sulfide and the addition of 2 mM Na_2SO_4, 10 mM acetone, and 20 mM $NaNO_3$. Larger culture volumes were grown under a headspace of N_2 plus CO_2 (80:20, by vol.) in 500-ml or 1000-ml glass bottles sealed with rubber stoppers. Some cultures were grown with 10 mM acetate or 10 mM DL-3-hydroxybutyrate instead of acetone. All cultures were incubated in the dark at 30°C.

Preparation of cell extracts. Cells were harvested by centrifugation at 3000 *g* for 30 min under strictly anoxic conditions (Janssen and Schink, 1993), and washed and resuspended in buffer A (anoxic 100 mM Mops, 10 mM $MgCl_2$, 2.5 mM dithioerythritol, pH adjusted to 7.0 with NaOH). Cell extracts were prepared by disruption in a French press (Janssen and Schink, 1993).

Servapor dialysis tubing (16-mm diameter, 10–14-kDa cut-off; Serva) was boiled in 2% (mass/vol.) $NaHCO_3$ plus 1 mM EDTA (pH 8.0 adjusted with NaOH) for 10 min, washed in distilled water, boiled again for 10 min in 1 mM EDTA (pH 8.0 adjusted with NaOH), allowed to cool slowly, washed thoroughly with distilled water, and stored at 4°C in buffer A under N_2. Cell extracts were dialyzed in 3–5-ml aliquots against 1 l buffer A for 6 h under N_2 in the dark with slow stirring. All handling was carried out in an anoxic chamber under N_2/H_2 (95:5, by vol.).

Centricon 10 microconcentrators (10-kDa cut-off, Amicon) were used in sealed metal centrifuge containers under N_2/H_2 (95:5, by vol.), and centrifuged at 5000 *g* for 6 h at 4°C. The retentate was resuspended in buffer A. Membrane and cytoplasmic fractions were prepared by centrifugation at 200000 *g* for 30 min at 4°C. The pellet was resuspended in a volume of buffer A equivalent to the original extract volume.

Measurement of exchange activity. The standard assay was carried out in 1 ml buffer A in 2-ml vials sealed with rubber stoppers. The reaction mixture contained (unless noted otherwise) 30 mM $NaHCO_3$, 5 mM lithium acetoacetate, 5 mM ADP, 20 mM KH_2PO_4, 250 kBq or 500 kBq $Na_2{}^{14}CO_3$, and approximately 2–10 mg dialyzed extract protein. Modifications and other additions are noted in the text as appropriate. The gas phase normally consisted of N_2 plus CO_2 (80:20, by vol.). The reactions were incubated at 30°C. Samples of 200 µl were taken at intervals using Unimetric syringes (Macherey-Nagel, Düren, Germany), and added to 25 µl 70% (mass/vol.) perchloric acid. The preparations were allowed to stand for 30 min, centrifuged in a benchtop microcentrifuge at 5000 *g*, gassed with CO_2 at 10 ml · min^{-1} for 30 min, and centrifuged again using the same conditions. The radioactively labelled compounds were separated by reverse-phase and ion-exchange HPLC (Brune and Schink, 1990; Friedrich and Schink, 1993). Radioactivity was monitored on-line (Gorny and Schink, 1994), using [1-^{14}C]benzoate as an external standard. CoA-esters were detected at 206 nm, and organic acids were detected either at 206 nm or using a refractive index detector. After establishing that acetoacetate was the only labelled product, 50-µl samples (acidified and gassed as above) were added to 4 ml Pico-Aqua LSC-cocktail (Canberra-Packard) and the radioactivity was measured in a PW 4700 double-channel liquid scintillation counter (Phillips).

Other methods. A solution of titanium(III) chelated by nitrilotriacetic acid was prepared as described by Moench and Zeikus (1983). Protein was quantified by the micro-assay of Bradford (1976) using bovine serum albumin as standard.

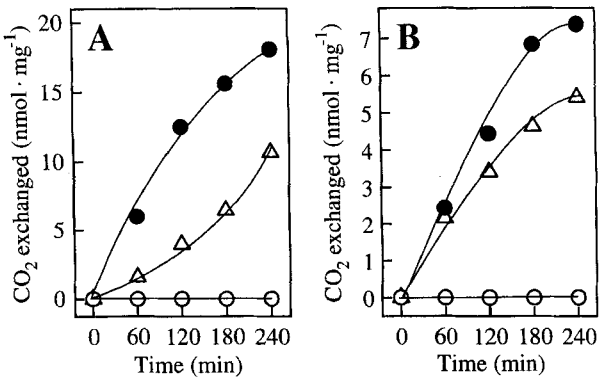

Fig. 1. CO_2 exchange catalyzed by dialyzed and fractionated cell-free extracts of acetone-plus-nitrate-grown cells of strain BunN. (A) The reaction mixtures containing dialyzed cell-free extracts (2.4–3.0 mg protein · ml^{-1}; standard assay conditions were used except as stated) were supplemented with (●) 5 mM ADP and 20 mM KH_2PO_4, (△) 5 mM ATP, or (○) one of 100 µM (+)-biotin, 100 µM thiamine pyrophosphate, 100 µM pyridoxal 5-phosphate, or no addition. ADP and KH_2PO_4 were added only where specifically stated. (B) Crude (undialyzed) extract was separated into a low-molecular-mass fraction (less than 10 kDa) and protein fraction (greater than 10 kDa). The reactions mixtures (standard assay conditions were used except as stated) contained (△) protein fraction plus low-molecular-mass fraction (2.1 mg protein · ml^{-1}) and no ADP and KH_2PO_4, (●) protein fraction (2.1 mg protein · ml^{-1}), 5 mM ADP and 20 mM KH_2PO_4, (○) protein fraction (2.1 m g protein · ml^{-1}) or low-molecular-mass fraction (equivalent to 2.2 mg protein · ml^{-1}), both without ADP and KH_2PO_4.

RESULTS

Identification of exchange activity. Crude cell-free extracts of acetone-plus-nitrate-grown cells of strain BunN catalyzed the incorporation of $^{14}CO_2$ into acid-stable products in the presence of 5 mM added acetoacetate. Rates of approximately 30–60 pmol · min^{-1} · mg $protein^{-1}$ were measured (data not shown). Dialysis of the crude extract prior to acetoacetate addition resulted in abolishment of this activity. The addition of ADP plus KH_2PO_4 to dialyzed cell extracts restored the ability to incorporate radiolabel into acid-stable products in the presence of acetoacetate, at rates of up to 100 pmol · min^{-1} · mg $protein^{-1}$ (Fig. 1A). There was virtually no incorporation of radiolabel in the absence of added acetoacetate. The specific exchange rates varied with different batches of dialyzed extract. The addition of ATP also restored the exchange activity, but with rather different kinetics (Fig. 1A). The addition of (+)-biotin, thiamine pyrophosphate, or pyridoxal 5-phosphate (each tested individually at 100 µM) did not restore label-incorporation activity. When crude cell-free extracts were separated using a microconcentrator into a greater than 10-kDa fraction, and less than 10-kDa fraction, neither fraction alone was able to catalyze the incorporation of $^{14}CO_2$ into acid-stable products (Fig. 1B). The combination of the two fractions in the presence of acetoacetate did restore radiolabel fixation activity. The greater than 10-kDa fraction was also able to catalyze radiolabel fixation if ADP plus KH_2PO_4 was added to assays containing acetoacetate.

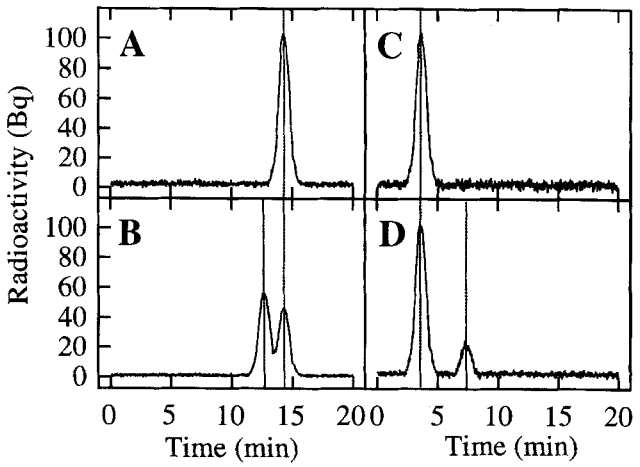

Fig. 2. Radiochromatograms of protein-free reactions mixtures eluted by ion-exchange and reverse-phase HPLC after $^{14}CO_2$ exchange catalyzed by dialyzed cell-free extracts of acetone-plus-nitrate-grown cells of strain BunN. Radiochromatograms obtained by ion-exchange HPLC after 4 h incubation with (A) 5 mM acetoacetate, and (B) 5 mM acetoacetate plus 2 mM NADH. Standard assay conditions were used except as stated, with 3.9 mg protein · ml^{-1}. 3-Hydroxybutyrate eluted at 12.8 min, acetoacetate at 14.4 min. Radiochromatographs obtained by reverse-phase HPLC after a 4-h incubation with (C) 5 mM acetoacetate, and (D) 5 mM acetoacetate to which 1 mM succinyl-CoA was added 45 min before termination of the incubation. Standard assay conditions were used (except as stated) with 5.83 mg protein · ml^{-1}. Acetoacetate eluted at 3.6 min and acetoacetyl-CoA eluted at 7.4 min.

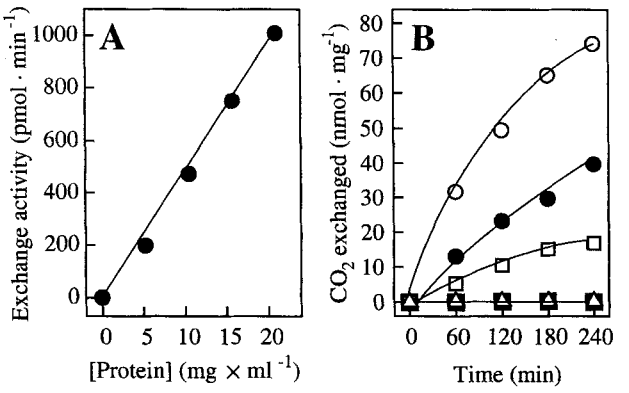

Fig. 3. CO_2 exchange catalyzed by different fractions of dialyzed cell-free extracts of acetone-plus-nitrate-grown cells of strain BunN. (A) Linear response of the initial rate of the exchange activity with increasing protein concentrations in the assay. (B) Kinetics of the exchange catalyzed by (○) cytoplasmic (6.4 mg protein · ml^{-1}) and (□) membrane (1.7 mg protein · ml^{-1}) fractions, and by (●) complete (10.2 mg protein · ml^{-1}) and (■) boiled (10.2 mg protein · ml^{-1}) preparations, and by (△) bovine serum albumin (10.0 mg protein · ml^{-1}). The cytoplasmic and membrane fractions were prepared from a dialyzed extract (10.2 mg protein · ml^{-1}) by centrifugation, and the membrane fraction was resuspended in the equivalent volume of buffer A (protein recoveries in the two fractions were 75%). Standard assay conditions were used, and all assays were made using the same preparation of dialyzed extract.

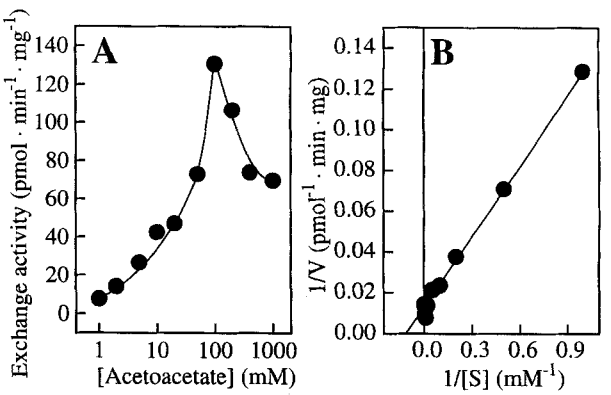

Fig. 4. The effect of acetoacetate concentration on the CO_2 exchange catalyzed by dialyzed cell-free extracts of acetone-plus-nitrate-grown cells of strain BunN. (A) The effect of acetoacetate concentration on the initial rate of the exchange reaction (standard assay conditions, 3.2–4.0 mg protein · ml^{-1}). (B) Double reciprocal plot used to calculate the apparent K_m (11.1 mM).

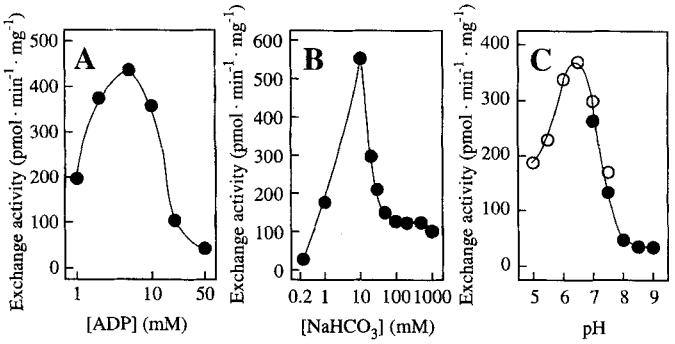

Fig. 5. Characterization of the CO_2 exchange catalyzed by dialyzed cell-free extracts of acetone-plus-nitrate-grown cells of strain BunN. (A) The effect of ADP concentration on the initial rate of the exchange activity (standard assay conditions, 3.1 mg protein · ml^{-1}). (B) The dependence of the initial rate of the exchange activity on the concentration of $NaHCO_3$ in the assay (4.6 mg protein · ml^{-1}, standard assay conditions were used, except that only N_2 was added to the headspace). (C) Effect of pH of the assay mixture on the initial rate of the exchange activity. Standard assay conditions were used (2.6 mg protein · ml^{-1}), except that (○) 50 mM Mes plus 50 mM Mops, or (●) 50 mM Mops plus 50 mM Tricine were used instead of Mops in the assay buffer (pH adjusted with NaOH).

The exchange activities catalyzed by dialyzed extracts (4.3 mg protein · ml^{-1}) were lower with 10 mM acetate (18% of control activity), 100 µM acetyl-CoA (8%), 10 mM 3-hydroxybutyrate (10%), 100 µM 3-hydroxybutyryl-CoA (2%) or 100 µM acetoacetyl-CoA (7%) as substrates, relative to 10 mM acetoacetate (100%). The radiolabel incorporated into acid-stable products by dialyzed cell extracts of acetone-plus-nitrate-grown BunN in the presence of acetoacetate eluted in a peak with the same retention time as acetoacetate (Fig. 2A and C). When NADH was included in the assay, an additional peak eluting with 3-hydroxybutyrate was detected (Fig. 2B). Similar radiochromatograms were obtained when undialyzed crude cell extracts were analyzed (data not shown). The addition of succinyl-CoA to the assay with dialyzed cell extract resulted in the formation of a radiolabelled peak representing a component that eluted with acetoacetyl-CoA (Fig. 2D). Acetoacetate was also measured by monitoring the ultraviolet absorbance at 206 nm using reversed-phase HPLC; the addition of a known quantity of acetoacetate to a sample which was then treated with perchloric acid and CO_2 (see Materials and Methods section) resulted in a 103% recovery of the added acetoacetate, showing it to be stable in the assay and subsequent preparation procedures.

Dialyzed cell extracts of strain BunN grown with acetate plus nitrate or with 3-hydroxybutyrate plus nitrate exhibited a much lower exchange activity into acetoacetate than did extracts prepared from acetone-plus-nitrate-grown cells. In assays using dialyzed extract containing 7.9–8.7 mg protein · ml^{-1}, prepared

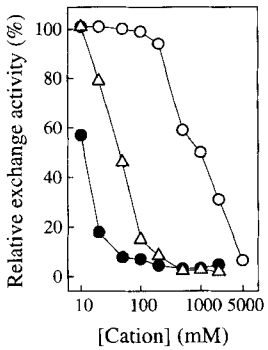

Fig. 6. Effect of different cations on exchange activity. The dependence of CO_2 exchange catalyzed by dialyzed cell-free extracts (2.3–5.2 mg protein · ml^{-1}) of acetone-plus-nitrate-grown cells of strain BunN on concentration of (○) NaCl, (△) KCl, and (●) NH_4Cl in assay (standard assay conditions were used). The results are presented relative to initial rates of the specific exchange activities in untreated controls using the same preparation of dialyzed extract.

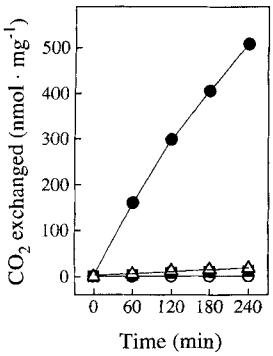

Fig. 7. CO_2 exchange catalyzed by dialyzed cell extracts under optimal conditions. Shown is the exchange catalyzed by (■) crude cell-free extracts (9.2 mg protein · ml^{-1}) under standard conditions, (△) by dialyzed cell-free extracts (9.0 mg protein · ml^{-1}) under standard conditions, and by dialyzed cell-free extracts (2.5 mg protein · ml^{-1}) using a modified assay in the presence (●) and absence (○) of acetoacetate. The modified assay was carried out in 50 mM Mes/50 mM Mops (pH 6.5 adjusted with NaOH) containing 10 mM $MgCl_2$, 2.5 mM dithioerythritol, 10 mM $NaHCO_3$, 5 mM ADP, 20 mM KH_2PO_4, 100 mM acetoacetate and 250 kBq $Na_2{}^{14}CO_3$ under N_2 (100%).

from cells grown on the appropriate substrates, the rates of exchange into acetoacetate were 3.8, 3.5, and 29 pmol · min^{-1} · mg $protein^{-1}$ for acetate-, 3-hydroxybutyrate- and acetone-grown cells, respectively.

Characterization of exchange activity. The exchange activity was proportional to the amount of cell-extract protein added to the exchange assay (Fig. 3A), and was destroyed by boiling (Fig. 3B). Bovine serum albumin did not catalyze the reaction. A significant exchange was catalyzed by the membrane/particulate fraction of a dialyzed extract (Fig. 3B). The cytoplasmic fraction of a dialyzed cell extract of acetone-plus-nitrate-grown cells contained most of the exchange activity; the specific activity was higher in the absence of membranes.

The exchange activity was maximal at 100 mM acetoacetate (Fig. 4A), with higher concentrations apparently causing an inhibition. An apparent K_m of 11.1 mM could be calculated from these data (Fig. 4B). Maximum activity was observed at an ADP concentration of 5 mM (Fig. 5A) and $NaHCO_3$ concentration of 10 mM (Fig. 5B). The optimal pH was 6.5 (Fig. 5C). The activity was very sensitive to NH_4Cl, less sensitive to KCl, but was not sensitive to NaCl (Fig. 6). Additions of 100 mM $(NH_4)_2SO_4$ or 100 mM LiCl resulted in activities of 9% and 78% relative to untreated controls, respectively. Treatment with air (gassing an extract with 3.1 mg protein · ml^{-1} with air at 10 ml · min^{-1} for 5 min, followed by gassing with N_2/CO_2 (80:20, by vol.) for 3 min) did not result in any significant activity loss (96% activity), compared to controls that had been gassed with N_2/CO_2 (80:20, by vol.) for 8 min (103% activity), or not gassed at all (nominally 100%).

The characterization of the exchange activity allowed us to develop an optimized assay, under N_2 (100%), in 50 mM Mes/50 mM Mops (pH 6.5 with NaOH) containing 10 mM $MgCl_2$, 2.5 mM dithioerythritol, 10 mM $NaHCO_3$, 5 mM ADP, 20 mM KH_2PO_4, 100 mM acetoacetate, 250 kBq $Na_2{}^{14}CO_3$, and 2.5 mg dialyzed extract protein. Under these conditions, an exchange activity of 2.7 nmol · min^{-1} · mg $protein^{-1}$ was obtained (Fig. 7).

Additions of various possible cofactors (all at 100 μM) and metal ions (all at 1 mM) had no dramatic effect on the obtained exchange activities. The additives tested (with the exchange activity relative to controls defined as 100%) were as follows: (+)-biotin (105%), thiamine pyrophosphate (88%), hydroxycobalamin (85%), pyridoxal 5-phosphate (114%), pyridoxal (146%), pyridoxamine (126%), pyridoxol (145%), $CoCl_2$ (128%), $FeCl_2$ (86%), $ZnCl_2$ (109%), $CaCl_2$ (95%), $MnCl_2$ (85%), $NiCl_2$ (90%) and $CuCl_2$ (115%). Similarly, no dramatic effect was observed with the following additions: 1.25 mM Ti^{3+} plus 5 mM nitrilotriacetate (74%), 10 mM acetone (119%), 100 μM succinyl-CoA (93%), 2% (by vol.) glycerol (103%), 1 mM EDTA (52%), 1 mM EGTA (103%), 1 mM NaCN (63%), or 80 kPa CO (110%). The addition of avidin at 50 μg · mg $protein^{-1}$ or 100 μg · mg $protein^{-1}$ resulted in activities of 100% and 99% relative to untreated controls, respectively. All of these assays contained 2.3–4.7 mg protein · ml^{-1}.

DISCUSSION

Crude cell-free extracts of acetone-plus-nitrate-grown strain BunN catalyzed an exchange of $^{14}CO_2$ into acid-stable products. This activity was increased by the addition of acetoacetate to the assay. Dialysis, or use of the protein fraction after separation by centrifugation of the low-molecular-mass components of the extract through a 10-kDa cut-off membrane, resulted in a complete loss of activity. The activity could be restored by addition of the low-molecular-mass fraction, or by addition of ADP or ATP. The low-molecular-mass fraction of crude extracts presumably contained ADP and ATP. The kinetics obtained after ATP addition suggested that the stimulation was due to ADP formation by the adenosinetriphosphatase present in cell extracts of this bacterium (Platen and Schink, 1990). The decarboxylation of acetoacetate by crude cell-free extracts of strain BunN is stimulated by ADP, but the rates are very low, typically 4–8 nmol · min^{-1} · mg $protein^{-1}$ (Platen and Schink, 1990). The rates of exchange of radiolabel between CO_2 and acetoacetate catalyzed by dialyzed cell extracts were low in comparison with the postulated minimum activity of a carboxylating enzyme of 144 nmol · min^{-1} · mg $protein^{-1}$ (calculated from the data of Platen and Schink, 1989). It is concluded that the exchange reaction is catalyzed by a protein in the cell extract and requires ADP for activity.

The ^{14}C-labelled product of the exchange reaction, in the presence of added acetoacetate, eluted with acetoacetate when analyzed by ion-exchange or by reverse-phase HPLC. The addition of NADH resulted in the formation of an additional labelled peak eluting with 3-hydroxybutyrate. This can be explained by the presence of 3-hydroxybutyrate dehydrogenase activity in the

dialyzed extract (Platen and Schink, 1990) reducing labelled acetoacetate to 3-hydroxybutyrate. The labelling of 3-hydroxybutyrate in undialyzed crude extracts is presumably due to the presence of NADH (or NADH generating activity) in the extracts. The addition of succinyl-CoA resulted in the formation of labelled acetoacetyl-CoA, since the dialyzed extract contained succinyl-CoA: acetoacetate CoA-transferase activity (Platen and Schink, 1990). The use of other organic acids in the assay, or their CoA-esters, resulted in much lower rates of exchange activity. It can thus be concluded that the incorporation of radiolabel into acid-stable products was due to an exchange of $^{14}CO_2$ with the carboxyl group of acetoacetate, although the position of the label was not verified.

The exchange activity was proportional to the amount of extract protein added, showed a pH optimum, was destroyed by boiling, and was not a non-specific exchange catalyzed by protein (e.g. bovine serum albumin). The exclusion of the latter possibility is particularly important, since a non-specific decarboxylation of acetoacetate is catalyzed by bovine serum albumin (Platen and Schink, 1990). The exchange reaction was catalyzed at much higher rates by dialyzed extracts prepared from acetone-plus-nitrate-grown cells of strain BunN than by dialyzed extracts from acetate-plus-nitrate or 3-hydroxybutyrate-plus-nitrate-grown cells. This shows that the activity was probably an enzyme specifically involved in acetone metabolism. The enzymes involved in acetone and 3-hydroxybutyrate metabolism (and associated poly-β-hydroxybutyrate formation) are the same (Platen and Schink, 1990), and the only difference that can be postulated is that an additional activity responsible for the carboxylation of acetone must be present in acetone-grown cells. We suggest that the exchange reaction measured in this study is a partial reaction carried out by the acetone carboxylating enzyme (complex). Under optimal conditions (100 mM acetoacetate, 10 mM $NaHCO_3$, 5 mM ADP, pH 6.5, under N_2) the exchange activity was 2.7 nmol $\cdot$ min^{-1} $\cdot$ mg protein^{-1}, which is 2% of the minimum *in vivo* activity (144 nmol $\cdot$ min^{-1} $\cdot$ mg protein^{-1}).

The specific activity could not be significantly increased (less than twofold) by the addition of various metal ions or cofactors. The specific activity of exchange was not higher in undialyzed extracts, showing that the low activity was not an effect due to loss during dialysis of a metal or cofactor other than ADP required for the reaction. The addition of avidin, an inhibitor of many biotin-containing carboxylases and decarboxylases, did not result in activity loss, nor did biotin addition result in an activity increase. This does not, however, exclude a biotin-dependent reaction, since biotin may be tightly bound or inaccessible to avidin. The activity was not sensitive to short (5 min) exposure to air.

The exchange activity was found in both the cytoplasmic and membrane fractions. Our experiments suggest that the exchange reaction is catalyzed by a component that does not have to be membrane associated to catalyze the exchange. It is, however, not possible to exclude an ion-driven carboxylation mechanism (Dimroth and Hilpert, 1984), since it is possible that the exchange is catalyzed by a component which is normally only loosely membrane associated. In such a carboxylation system, the ion pump/channel component driving the net carboxlyation reaction would be an integral membrane protein, while the exchange could be catalyzed by another component, easily removed in an active state from the cytoplasmic face of the membrane. The *in vivo* structure would be analogous to ion-pumping decarboxylases (Dimroth, 1987; Dimroth and Thomer, 1983; Buckel and Liedtke, 1986). The exchange reaction displayed a requirement for ADP. The data obtained thus suggested an ATP requirement for the net carboxylation of acetone to overcome the energetic restraints of the endergonic carboxylation reaction (+17.1 kJ $\cdot$ mol^{-1} under standard conditions). The exchange reaction is presumably catalyzed by an acetoacetyl-enzyme-ADP complex, possibly an intermediate of the carboxylation reaction. This model would support the cytoplasmic location of the enzyme, since an ATP-consuming mechanism has no need for any association with a transmembrane process. The assay described in this study provides a means to investigate the reaction further.

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REFERENCES

Aleem, M. I. H. (1966) Generation of reducing power in chemosynthesis. II. Energy-linked reduction of pyridine nucleotides in the chemoautotroph, *Nitrosomonas europaea, Biochim. Biophys. Acta 113,* 216–224.

Bonnet-Smits, E. M., Robertson, L. A., van Dijken, J. P., Senior, E. & Kuenen, J. G. (1988) Carbon dioxide fixation as the initial step in the metabolism of acetone by *Thiosphaera pantotropha, J. Gen. Microbiol. 134,* 2281–2289.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72,* 248–254.

Brune, A. & Schink, B. (1990) A complete citric acid cycle in assimilatory metabolism of *Pelobacter acidigallici*, a strictly anaerobic, fermenting bacterium, *Arch. Microbiol. 154,* 394–399.

Buckel, W. & Liedtke, H. (1986) The sodium pump glutaconyl-CoA decarboxylase from *Acidaminococcus fermentans*. Specific cleavage by *n*-alkanols, *Eur. J. Biochem. 156,* 251–257.

Dimroth, P. (1987) Sodium ion transport decarboxylases and other aspects of sodium ion cycling in bacteria, *Microbiol. Rev. 51,* 320–340.

Dimroth, P. & Hilpert, W. (1984) Carboxylation of pyruvate and acetyl coenzyme A by reversal of the Na^+ pumps oxaloacetate decarboxylase and methylmalonyl-CoA decarboxylase, *Biochemistry 23,* 5360–5366.

Dimroth, P. & Thomer, A. (1983) Subunit composition of oxaloacetate decarboxylase and characterization of the α-chain as carboxyltransferase, *Eur. J. Biochem. 137,* 107–112.

Dörner, C. & Schink, B. (1990) *Clostridium homopropionicum* sp.nov., a new strict anaerobe growing with 2-, 3-, or 4-hydroxybutyrate, *Arch. Microbiol. 154,* 342–348.

Friedrich, M. & Schink, B. (1993) Hydrogen formation from glycolate driven by reversed electron transport in membrane vesicles of a syntrophic glycolate-oxidizing bacterium, *Eur. J. Biochem. 217,* 233–240.

Gerischer, U. & Dürre, P. (1990) Cloning, sequencing, and molecular analysis of the acetoacetate decarboxylase gene region from *Clostridium acetobutylicum, J. Bacteriol. 172,* 6907–6918.

Gorny, N. & Schink, B. (1994) Hydroquinone degradation via reductive dehydroxylation of gentisyl-CoA by a strictly anaerobic fermenting bacterium, *Arch. Microbiol. 161,* 25–32.

Harold, F. M. (1986) *The vital force: a study of bioenergetics,* Freeman, New York.

Hamilton, G. A. & Westheimer, F. H. (1959) On the mechanism of the enzymatic decarboxylation of acetoacetate, *J. Am. Chem. Soc. 81,* 6332–6333.

Janssen, P. H. & Schink, B. (1993) Pathway of anaerobic poly-β-hydroxybutyrate degradation by *Ilyobacter delafieldii, Biodegradation 4,* 179–185.

Klingenberg, M. & Schollmeyer, P. (1960) Zur Reversibilität der oxydativen Phosphorylierung. Adenosintriphosphat-abhängige Atmungskontrolle und Reduktion von Diphosphopyridinnucleotid in Mitochondrien, *Biochem. Z. 333,* 335–350.

Lack, A. & Fuchs, G. (1992) Carboxylation of phenylphosphate by phenol carboxlyase, an enzyme system of anaerobic phenol metabolism, *J. Bacteriol. 174,* 3629–3636.

Lack, A. & Fuchs, G. (1994) Evidence that phenol phosphorylation to phenylphosphate is the first step in anaerobic phenol metabolism in a denitrifying *Pseudomonas* sp., *Arch. Microbiol. 161,* 132–139.

Maloy, S. R. (1990) Sodium-coupled cotransport, in *The bacteria, a treatise on structure and function: bacterial energetics* (Krulwich, T. A., ed.) vol. 12, pp. 203–224, Academic Press, San Diego.

Moench, T. T. & Zeikus, J. G. (1983) An improved preparation method for a titanium (III) media reductant, *J. Microbiol. Methods 1,* 199–202.

Paulsen, J., Kröger, A. & Thauer, R. K. (1986) ATP-driven succinate oxidation in the catabolism of *Desulfuromonas acetoxidans, Arch. Microbiol. 144,* 78–83.

Platen, H. & Schink, B. (1987) Methanogenic degradation of acetone by an enrichment culture, *Arch. Microbiol. 149,* 136–141.

Platen, H. & Schink, B. (1989) Anaerobic degradation of acetone and higher ketones via carboxylation by newly isolated denitrifying bacteria, *J. Gen. Microbiol. 135,* 883–891.

Platen, H. & Schink, B. (1990) Enzymes involved in anaerobic degradation of acetone by a denitrifying bacterium, *Biodegradation 1,* 243–251.

Platen, H., Temmes, A. & Schink, B. (1990) Anaerobic degradation of acetone by *Desulfococcus biacutus* spec. nov, *Arch. Microbiol. 154,* 355–361.

Thauer, R. K., Jungermann, K. & Decker, K. (1977) Energy conservation in chemotrophic anaerobic bacteria, *Bacteriol. Rev. 41,* 100–180.

Walsh, C. (1979) *Enzymatic reaction mechanisms,* Freeman, San Francisco.

Westheimer, F. H. (1969) Acetoacetate decarboxylase from *Clostridium acetobutylicum, Methods Enzymol. 14,* 231–241.