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Enzymes involved in anaerobic degradation of acetone by a denitrifying bacterium

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Abstract

The pathway of anaerobic acetone degradation by the denitrifying bacterial strain BunN was studied by enzyme measurements in extracts of anaerobic acetone-grown cells. An ADP- and $MgCl_2$-dependent decarboxylation of acetoacetate was detected which could not be found in cell-free extracts of acetate-grown cells. It is concluded that free acetoacetate is formed by ATP-dependent carboxylation of acetone. Acetoacetate was converted into its coenzyme A ester by succinyl-CoA: acetoacetate CoA transferase, and cleaved by a thiolase into acetyl-CoA. The acetyl residue was completely oxidized in the citric acid cycle. The ADP-dependent decarboxylation of acetoacetate was inhibited by EDTA, but not by avidin. High myokinase activities led to equilibrium amounts of ATP, ADP, and AMP in the reaction mixtures, and prevented determination of the decarboxylase reaction stoichiometry, therefore.

Abbreviations: ADP – adenosine diphosphate, AMP – adenosine monophosphate, ATP – adenosine triphosphate, BSA – bovine serum albumine, MOPS – 3-(N-morpholino)propanesulfonic acid, PIPES – piperazine-N,N′-bis- (2-ethanesulfonic acid), PHB – poly-β-hydroxybutyrate, Tris – Tris- (hydroxymethyl-) aminomethane

Introduction

Acetone is used as a growth substrate by aerobic and anaerobic bacteria. From aerobic enrichment cultures with propane or acetone as substrate, Gram-positive bacteria were isolated which attack acetone by oxygenase-dependent oxidation to acetol (Lukins & Foster 1963; Taylor et al. 1980). Oxidation forms methylglyoxal and pyruvate which is finally oxidized to carbon dioxide and water. All enzymes of this pathway, except for acetone monooxygenase, were demonstrated (Taylor et al. 1980). In the absence of molecular oxygen, acetone is degraded after initial condensation with carbon dioxide to a C_4-compound, probably free acetoacetate. Evidence was provided first by studies with the phototrophic bacterium *Rhodocyclus gelatinosus* (*Rhodopseudomonas gelatinosa;* Siegel 1950) and later confirmed by ^{14}C-labelling experiments for a fermenting bacterium in a methanogenic coculture (Platen & Schink 1987), and for denitrifying bacteria (Bonnet-Smits et al. 1988; Platen & Schink 1989). As an alternative reaction of oxygen-independent acetone activation, hydration to 1,2-propanediol was suggested (Rudney 1954) but never proven.

In the present paper the enzymes involved in anaerobic acetone degradation by the denitrifying strain BunN are described.

Materials and methods

Growth conditions and preparation of cell-free extracts

All experiments were carried out with the denitrifying bacterial strain BunN (Platen & Schink 1989). Cells were cultivated at 28° C under anoxic conditions in 1 l cultures in a phosphate-buffered mineral medium containing 10 mM acetone and 20 mM sodium nitrate (Platen & Schink 1989).

1 l cultures at the late logarithmic growth phase were centrifuged for 20 min at 6000 × *g*. Cells were resuspended and washed twice in 100 ml PIPES-NaOH buffer (50 mM, pH 6.9). After resuspension in 10 ml of the same buffer cells were disrupted by a French press (110 Mpa), and cell debris was removed by centrifugation (1 h; 48000 × *g*). This supernatant (cell-free extract) was used for enzyme determinations.

Enzyme assays

Photometric measurements were carried out with a model 100-40 photometer (Hitachi, Tokyo, Japan) at 20° C in 1 ml-cuvettes with 1 cm light path. All reaction mixtures were prepared under aerobic conditions and contained 10 to 100 μg extract protein. Control experiments under anaerobic conditions did not yield any higher enzyme activities. E.C. numbers were taken from International Union of Biochemistry (1984).

Propan-2-ol: NAD^+ oxidoreductase (no E.C. #), propan-2-ol: $NADP^+$ oxidoreductase (E.C. 1.1.1.80), 3-hydroxybutanoate: NAD^+ oxidoreductase (E.C. 1.1.1.30), 3-hydroxybutanoate: $NADP^+$ oxidoreductase (no E.C. –ER), 3-hydroxyacyl-CoA: NAD^+ oxidoreductase (E.C. 1.1.1.35), 3-hydroxyacyl-CoA: $NADP^+$ oxidoreductase (E.C. 1.1.1.36), and malate: NAD^+ oxidoreductase (E.C. 1.1.1.37) were determined in 100 mM potassium phosphate buffer (pH 7.0) or MOPS-KOH buffer (100 mM, pH 7.2) containing 300 μM NADH or NADPH and cell-free extract). The reaction was started in every case by addition of 30 μl substrate from stock solutions (300 mM acetone, 300 mM Li-acetoacetate, 10 mM acetoacetyl CoA, or 200 mM oxaloacetic acid). NAD (P) H decrease was monitored at 365 nm wavelength ($\varepsilon = 3.4\,mM^{-1}\,cm^{-1}$ and $3.5\,mM^{-1}\,cm^{-1}$, respectively; Bergmeyer 1983).

Succinyl-CoA: acetoacetate CoA transferase (E.C. 2.8.3.6) and thiolase (E.C. 2.3.1.9) were measured in 100 mM Tris-HCl buffer (pH 8.1 yo 8.3) containing 10 mM $MgCl_2$, 100 mM acetoacetyl-CoA, and cell-free extract. The reaction was started by addition of 10 μl sodium succinate solution (1 M) or Coenzyme A solution (10 mM), and decrease of the acetoacetyl-CoA-Mg^{2+} complex was followed at 303 nm wavelength ($\varepsilon = 14\,mM^{-1}\,cm^{-1}$; Stern 1956).

ATP'ase (E.C. 3.6.1.3) and myokinase (E.C. 2.7.4.3) were measured in 100 mM MOPS-KOH buffer (pH 7.2) containing 10 mM $MgCl_2$, 2.5 mM phosphoenolpyruvate, 300 μM NADH, 10 units of pyruvate kinase, 28 units of lactate dehydrogenase, and cell-free extract. After monitoring background oxidation of NADH, ATP was added to a final concentration of 4.5 mM. Further decrease of NADH was due to ATP'ase activity. Addition of AMP to 5 mM final concentration allowed to measure also myokinase activity.

The assay for malate synthase (E.C. 4.1.3.2) was carried out in 100 mM Tris-HCl buffer (pH 8.3) containing 5 mM $MgCl_2$, 100 μM acetyl-CoA, and cell-free extract. 10 μl of 100 mM sodium glyoxylate was added, and decrease of acetyl-CoA was measured at 233 nm wavelength ($\varepsilon = 4.44\,mM^{-1}\,cm^{-1}$; Dixon & Kornberg 1959).

Isocitrate lyase (E.C. 4.1.3.1) was detected in 100 mM potassium phosphate buffer (pH 7.0) in the presence of 5 mM $MgCl_2$, 3 mM phenylhydrazine, and 20 and 100 μg extract protein. The reaction was started by addition of D,L-isocitrate to a final concentration of 5 mM, and the absorption increase at 324 nm wavelength was monitored ($\varepsilon = 17\,mM^{-1}\,cm^{-1}$; Dixon & Kornberg 1959).

For detection of carbonic anhydrase (E.C. 4.2.1.1) a standard method was used (Boehringer 1983). The reaction mixture was stirred by a magnetic stirrer (300 rpm), the change of pH was measured wird a pH electrode (Ingold, Steinbach, FRG).

Acetoacetate decarboxylation was assayed in

25 ml serum bottles which contained 450 µl of non-diluted cell-free extract (protein content: 3 to 4 mg · ml^{-1}) supplemented with various reagents from stock solutions (ADP, $MgCl_2$ e.g.; details see under 'Results') to a final volume of 500 µl, and closed with butyl rubber stoppers. The gas phase was nitrogen at 1.2 to 1.5 bar pressure. The reaction was started by addition of 10 µl Li-acetoacetate solution (1 M) or 20 µl Na-acetoacetate solution (0.5 M). The reaction mixture was shaken (130 rpm) in a water bath at 28° C, and gas samples of 300 µl volume were taken at intervals of 30 to 90 minutes for carbon dioxide determination. For calibration, 10 or 20 µl of 100 mM $NaHCO_3$ was added to 500 µl buffer in a 25 ml serum bottle.

Deproteinization of cell-free extracts

0.5 ml of cell-free extract was mixed with 1 ml $HClO_4$. The precipitate was removed by centrifugation (1 min, 13000 × *g*). 1 ml of the supernatant was neutralized by addition of 735 µl of 1 M $KHCO_3$ solution, the precipitate was removed as before and the supernatant used for determination of phosphate, acetoacetate, or acetone.

Chemical analyses

Protein was determined by the microbiuret method (Zamenhoff 1957). Phosphate was determined in deproteinized cell-free extracts according to Herbert et al. (1971). Carbon dioxide was determined by gas chromatography (Vega 6000 gas chromatograph; Carlo Erba, Milano, Italy) with a HWD 430 thermal conductivity detector (block temperature 170° C; filament 270° C). Carrier gas was helium (40 ml · min^{-1}). The columns (2 m × 2 mm) were packed with 60/80 Carbosieve S II (Supelco Inc., Bellefonte, USA) and heated at 180° C. Sample volume was 300 µl, the detection limit was 1 to 2 nmol CO_2 per sample.

Acetone and acetoacetate were determined enzymatically. 850 µl deproteinized cell-free extract (or a diluted quantum with up to 200 nmol of acetone or acetoacetate) was supplemented with 100 µl 1 M potassium phosphate buffer (pH 7.0) and 50 µl 6 mM NADH. The initial absorption was read at 365 nm wavelength and the reaction was started by addition of either 0.2 units 3-hydroxybutyrate dehydrogenase (Sigma, Deisenhofen, FRG) or 0.2 units isopropanol dehydrogenase (partially purified from crude extracts of strain BunN; Platen 1989). After 50 min of incubation at 22° C the reaction was complete. The absorption change was correlated to acetone or acetoacetate content. The system was calibrated with known amounts of acetoacetate or acetone.

Preparation of acetoacetate salts

Lithium acetoacetate was prepared by the method of Hall (1962). Purity of the preparation was 96% as proven by absorption spectra (Hall 1962) and enzymatic assays using commercially available Li-acetoacetate (Serva, Heidelberg, FRG) as reference substance. In this preparation, the lithium content was reduced by approximately 80% by the following procedure: To 750 µl of 1 M Li-acetoacetate solution, 50 µl 10 N NaOH and 500 µl 0.5 M Na_3PO_4 were added and the precipitate (Li_3PO_4) was removed by centrifugation (1 min, 13000 × *g*). The supernatant was neutralized with 40 to 50 µl 10 M HCl and contained 520 mM acetoacetate (determined by the enzymatic method) and 50 mM phosphate.

Results

Metabolic and anaplerotic enzymes

Enzymes were determined in cell-free extracts of acetone-or 3-hydroxybutyrate-grown cells (Table 1). Isopropanol, 3-hydroxybutyrate, and 3-hydroxybutyryl-CoA were preferentially oxidized with NAD^+ as coenzyme. 3-hydroxybutyrate and 3-hydroxybutyryl-CoA were also oxidized with $NADP^+$, but only at a rate of 5 to 10% of the NAD^+-dependent reaction. $NADP^+$ dependent propan-2-ol oxidoreductase activity was negligible. Propan-2-ol-oxidoreductase activity was always found in cell-

free extracts of acetone- and 3-hydroxybutyrate-grown cells (about 10–20% of activity of acetone-grown cells), but never in acetate-grown cells. Acetoacetate could be converted to its coenzyme A ester by succinyl-CoA: acetoacetate CoA transferase. The enzyme was detectable in cell-free extracts of acetone-, 3-hydroxybutyrate-, and acetate-grown cells. Carbonic anhydrase was not detected; ATP'ase and myokinase were always present at high activities.

The anaplerotic enzymes malate synthase and isocitrate lyase were always present in cell-free extracts of acetone-, 3-hydroxybutyrate-, and acetate-grown cells at activities of approximately one to five percent of that of the catabolic enzymes.

Decarboxylation of acetoacetate

Decarboxylation of acetoacetate was detectable in non-diluted cell-free extracts (1 to 3 mg protein · ml^{-1}) of acetone-grown cells. ADP and Mg^{2+} were needed to drive the reaction (Fig. 1A). Quantification of acetone (enzymatic method) after decarboxylation experiments confirmed that carbon dioxide was released from acetoacetate: a total amount of 1.60 and 1.52 μmol acetone was found in two independent experiments in which 2.00 and 1.85 μmol carbon dioxide were formed, respectively (recovery: 80 to 82%; part of acetone evaporated into the gas phase).

Non-specific decarboxylation of acetoacetate was tested with BSA in control experiments. Up to 4 mg BSA · ml^{-1} did not cause significant spontaneous decarboxylation whereas 20 mg BSA · ml^{-1} resulted in high carbon dioxide formation (Table 2). The specific decarboxylation activity caused by BSA was in the range of 0.4 nmol · min^{-1} · mg^{-1} protein.

Decarboxylation of acetoacetate (20 mM) by cell-free extracts of acetone-grown cells in the presence of ADP (10 mM) and $MgCl_2$ (10 mM) occurred at rates of 4 to 8 nmol · min^{-1} · mg^{-1} protein, ADP- and $MgCl_2$-free controls gave decarboxylation rates below 2 nmol · min^{-1} · mg^{-1} protein. This activity was also measured in cell-free extracts of acetate-grown cells. In these preparations, the decarboxylation rate was not affected by addition of ADP and $MgCl_2$ (Fig. 1b).

Heating for 20 min at 80° C destroyed decarboxy-

Table 1. Enzymes detected in cell-free extract of strain BunN after growth with acetone (act) or 3-hydroxybutyrate (3-hb). The specific acetone consumption rate of cells growing at 28° C in the exponential growth phase was 150 (± 20) nmol · min^{-1} · mg^{-1} protein (calculated from μ- and Y_s-values published for this strain; Platen & Schink 1989).

#	Enzyme	Specific activity*	
		(act)	(3-hb)
1	Propan-2-ol: NAD^+ oxidoreductase	395	57
2	Propan-2-ol: $NADP^+$ oxidoreductase	< 3	< 3
3	3-hydroxybutanoate: NAD^+ oxidoreductase	210	361
4	3-hydroxybutanoate: $NADP^+$ oxidoreductase	< 3	< 1
5	3-hydroxyacyl: NAD^+ oxidoreductase	362	398
6	3-hydroxyacyl: $NADP^+$ oxidoreductase	29	39
7	Thiolase	317	147
8	Succinyl-CoA: acetoacetate CoA transferase	162	277
9	Malate: NAD^+ oxidoreductase	2602	2011
10	ATP'ase	28	nd
11	Myokinase	1110	nd
12	Malate synthase	10	10
13	Isocitrat lyase	3	2
14	Carbonic anhydrase	0	nd

* nmol · min^{-1} · [mg protein]$^{-1}$ at 20° C.
nd: not determined.

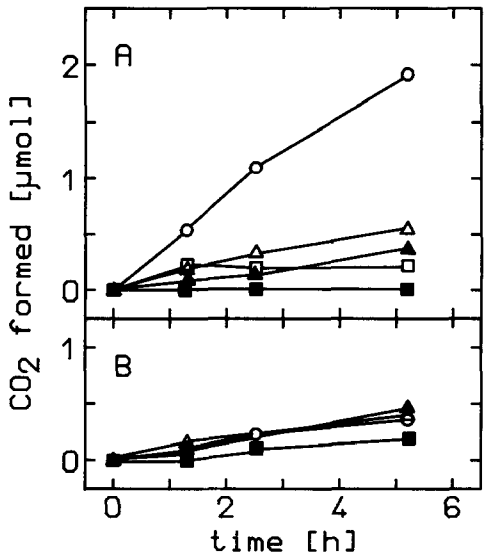

Fig. 1. Carbon dioxide formation from acetoacetate by cell-free extracts of strain BunN in PIPES-NaOH buffer (50 mM, pH 6.9). All reaction mixtures (500 µl) contained 0.67 mg extract protein and 2 mM inorganic phosphate. The reaction was started by addition of acetoacetate (sodium salt). [□] no acetoacetate, 10 mM ADP, 10 mM $MgCl_2$; [■] 20 mM acetoacetate; [△] 20 mM acetoacetate, 10 mM ADP; [▲] 20 mM acetoacetate, 10 mM $MgCl_2$; [○] 20 mM acetoacetate, 10 mM ADP, 10 mM $MgCl_2$. A. Cell-free extract of cells grown with acetone. Specific malate dehydrogenase activity was 1.83 µmol · min^{-1} · [mg protein]$^{-1}$. B. Cell-free extract of cells grown with acetate. Malate dehydrogenase activity was 1.72 µmol · min^{-1} · [mg protein]$^{-1}$.

lating activity completely. The reaction rate was correlated with the amount of protein in the reaction mixture (Table 2). Membrane fractions of acetone- grown cells (48000 × *g* sediment) decarboxylated acetoacetate at a rate of 1.33 nmol · min^{-1} · mg^{-1} protein. This activity was not affected by ADP or $MgCl_2$. Replacing ADP for ATP also led to an increase in the carbon dioxide formation rate (Fig. 2); in some experiments, the same decarboxylation rate was obtained as with ADP in the reaction mixture (data not shown). AMP did not enhance carbon dioxide formation (Fig. 2).

Addition of EDTA to the reaction mixture decreased the carbon dioxide formation rate to a value similar to the control without ADP and $MgCl_2$ (Table 3). Avidin (0.25 to 1.0 mg; corresponding to 3 to 12 units) did not affect acetoacetate decarboxylation. Use of potassium phosphate buffer (50 mM, pH 7.0) instead of PIPES-NaOH

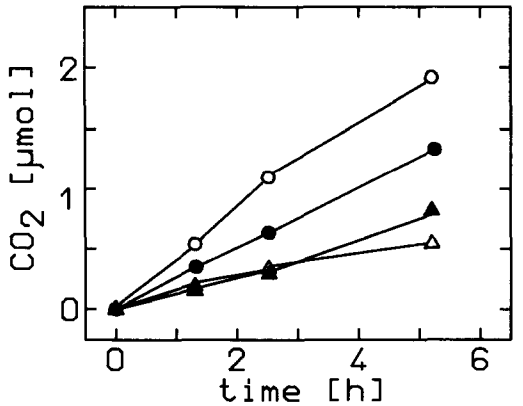

Fig. 2. Effect of ATP [●], ADP [○], and AMP [▲] (10 mM each) on decarboxylation of acetoacetate by cell-free extracts of strain BunN (grown with acetone). The reaction mixture contained PIPES-NaOH buffer (50 mM; pH 6.9), 20 mM Na-acetoacetate, 10 mM $MgCl_2$, 2 mM sodium phosphate, and 0.67 mg extract protein. Specific activities of ATP'ase and myokinase were 28 nmol ATP hydrolyzed · min^{-1} · [mg protein]$^{-1}$, and 1.11 µmol ATP formed from ADP · min^{-1} · [mg protein]$^{-1}$. [△] control without adenine nucleotide.

buffer (50 mM, pH 6.9) for preparation of cell-free extracts caused a decrease of activity from 3.95 nmol · min^{-1} to 1.09 nmol · min^{-1} (28% residual activity in one experiment). In another experiment, the decarboxylation rate was lowered by 40% from 4.59 nmol · min^{-1} to 2.85 nmol · min^{-1} by addition of sodium phosphate buffer (pH 7.0) to a final concentration of 50 mM to the PIPES-NaOH buffered reaction mixture. At this concentration, precipitates were observed, especially after addition of $MgCl_2$. Addition of phosphate to final concentrations of 2, 7, or 22 mM did not alter the reaction rate. The endogenous phosphate concentration in cell-free extracts prepared with PIPES-NaOH buffer was 130 to 200 µM.

Coenzyme A (2 mM), acetyl-CoA (2 mM), dithioerythritol (2 mM), glutathion (1 mM), $MnCl_2$ (2 mM) or pyruvate (10 mM) did not affect decarboxylation of acetoacetate. The same was true for monovalent cations such as Na^+ (up to 50 mM), K^+ (25 mM) or Li^+ (20 mM). 20 mM NH_4Cl caused 50% inhibition of enzyme activity.

Table 2. Effect of protein concentration on acetoacetate decarboxylation. All reaction mixtures (500 µl) contained 50 mM PIPES-NaOH buffer (pH 6.9) and 20 mM acetoacetate. A. Non-specific decarboxylation by BSA. B. ADP/$MgCl_2$-dependent decarboxylation by cell-free extract of strain BunN. Reaction mixtures contained 10 mM ADP, 10 mM $MgCl_2$, and 2 mM phosphate. Only the end point values of carbon dioxide formed are given; carbon dioxide formation was linear with time in all cases as proven by analyzing gas samples at intervals of approximately 90 minutes.

Protein content in 500 µl mg	Incubation time min	CO_2 formed µmol	Net protein dependent CO_2 formation µmol	Specific CO_2 formation rate nmol · min^{-1} mg^{-1}
A. Experiments with BSA				
0	337	0.49	–	–
0.5	337	0.64	0.15	0.90
1.0	337	0.64	0.15	0.45
2.0	337	0.80	0.31	0.46
10.0	337	1.85	1.36	0.40
B. Experiments with cell-free extract				
0	368	0.28	–	–
0.49	368	0.97	0.69	3.84
0.98	368	1.90	1.62	4.49
1.46	368	2.93	2.65	4.93

Attempts to measure acetone carboxylation in cell-free extracts

Undiluted cell-free extract (3 ml; 1 to 3 mg protein · ml^{-1}) in 50 mM PIPES-NaOH buffer (pH 6.9) was incubated at 28° C with 30 mM acetone, 30 mM $KHCO_3$, 30 mM ATP, and 15 mM $MgCl_2$. The reaction was started by transfer of the test tubes from ice to a 28° C water bath. 0.5 ml samples were taken periodically and analysed for acetoacetate content after protein precipitation. During 7 hours of incubation, no ATP- and carbon dioxide- dependent acetoacetate formation was detected. The endogenous acetoacetate concentration in cell-free extracts was in the range of 40 to 50 nmol per mg extract protein. No exchange of $^{14}CO_2$ with acetoacetate was observed in cell-free extracts, neither in the presence nor in the absence of ADP and $MgCl_2$ (details not shown).

Discussion

Since anaerobic degradation of acetone depends on the presence of carbon dioxide (Siegel 1950; Platen & Schink 1987; Bonnet-Smits et al. 1988; Platen & Schink 1989) it was concluded that acetone is introduced into the intermediary metabolism by carboxylation to acetoacetate. In the present paper we present results indicating that ace-

Table 3. Effect of EDTA and $MgCl_2$ on acetoacetate decarboxylation by cell-free extracts of strain BunN. The reaction mixture (500 µl) was PIPES-NaOH-buffered (50 mM; pH 6.9) and contained in all cases 0.67 mg protein, 20 mM acetoacetate, 10 mM ADP, and 2 mM inorganic phosphate.

Compound added			Specific CO_2 formation rate nmol · min^{-1} · mg^{-1}	% Activity of 'complete'
10 mM $MgCl_2$	20 mM EDTA			
+	–	('complete')	9.18	100
–	–		1.91	21
+	+		2.78	30
–	+		2.10	23

tone is carboxylated in an ATP-dependent reaction by cell-free extracts of the denitrifying bacterium strain BunN.

Free acetoacetate is activated by CoA transfer from succinyl-CoA, the specific activity of succinyl-CoA: acetoacetate CoA transferase is sufficient to explain a central role of free acetoacetate in acetone metabolism. However, this enzyme may also play an important role in mobilization of PHB (strain BunN produces PHB up to 30% of cell dry mass; Platen & Schink 1989) as shown for other bacteria (Senior & Dawes 1973). Acetoacetyl-CoA is cleaved by a thiolase to acetyl-CoA. The acetyl residue is completely oxidized to carbon dioxide through the citric acid cycle. Growth with acetone via acetoacetate requires anaplerotic enzymes to maintain operation of the citric acid cycle; isocitrate lyase and malate synthase were detected. The proposed pathway of acetone metabolism by strain BunN is given in Fig. 3.

A photometric test for the formation of free acetoacetate from acetone and carbon dioxide by cell-free extracts could not be developed. Direct photometric detection of acetoacetate is not sensitive enough ($\varepsilon 270 = 26.7\,M^{-1}\,cm^{-1}$; calculated from Fridovich 1972). Coupling of acetoacetate formation with NADH oxidation using commercially available 3-hydroxybutyrate dehydrogenase was not applicable either because of high propan-2-ol: NAD^+ oxidoreductase activity in cell-free extracts. All attempts to detect acetone-dependent $^{14}CO_2$-fixation in cell-free extract as described for the fungus *Agaricus bisporus* (Rast & Bachofen 1967) failed so far.

The only successful assay for an acetone-carboxylating activity was to measure the reaction in the reverse direction, by CO_2 formation from acetoacetate. However, this reaction is carried out also non-specifically by proteins: blood plasma protein (60 to 80 mg protein · ml^{-1}; Lehninger 1983) converted under physiological conditions in 1 h about 40% of endogenous acetoacetate to acetone (Rossi 1938; Williamson et al. 1962). The protein content in our acetoacetate decarboxylation assays did not exceed 3 mg · ml^{-1}. Control experiments with BSA and with boiled cell-free extract showed that non-

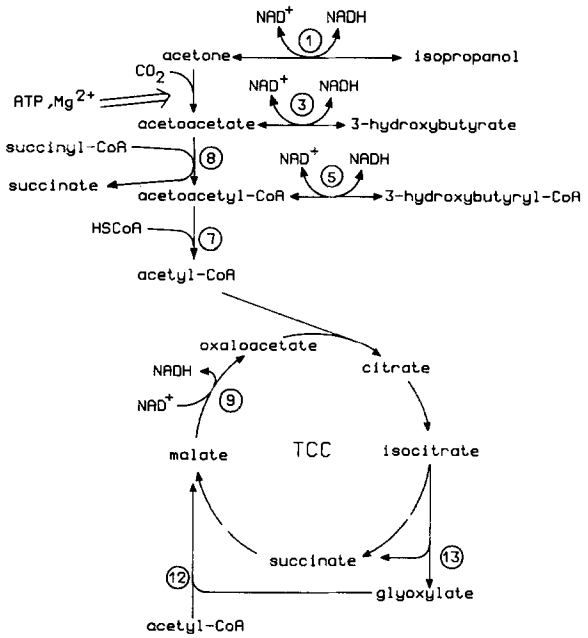

Fig. 3. Pathway of acetone degradation by the denitrifying strain BunN. The numbers at the arrows refer to the enzymes listed in Tab. 1. The stoichiometry of ATP consumption in the carboxylation of acetone is not yet known. TCC: tricarbonic acid cycle.

specific decarboxylation is low at this protein concentration.

Our results show that acetoacetate is specifically decarboxylated by cell-free extracts of acetone-grown cells from strain BunN in the presence of ADP and $MgCl_2$. This activity is not present in cell-free extracts of acetate-grown cells which indicates that the enzyme(s) is/are inducible. However, the specific activity of the decarboxylation reaction is only 2 to 5% of those of other metabolic enzymes, or of the specific acetone consumption rate of intact cells.

2 mM ADP and 3 mM $MgCl_2$ were sufficient for a maximum acetoacetate decarboxylation rate; it could not be enhanced by 10-fold higher concentrations of these compounds. No conclusion is possible on the reaction stoichiometry, therefore; 2 mM ADP caused a decrease in acetoacetate concentration of 4 to 6 mM. Obviously the high myokinase activity maintained an equilibrium of ATP, ADP, and AMP in the reaction mixture. ADP could not be replaced by AMP, but by ATP. This can be explained by ATP conversion to ADP by the

ATP'ase enzyme present at sufficient activity. Our results indicate that the following reaction was detected:

$$H_3C\text{-}CO\text{-}CH_2\text{-}COO^- + H_+ + ADP + P_i \rightarrow H_3C\text{-}CO\text{-}CH_3 + CO_{2(aq)} + ATP + H_2O$$
$$\Delta G^{\circ\prime} = +18.9\,kJ \cdot mol^{-1}$$

($\Delta G^{\circ\prime}$-values calculated after Thauer et al. 1977)

This reaction is endergonic under standard conditions and was possibly driven in our test system by diffusion of carbon dioxide to the gas phase, and by ATP hydrolysis to ADP and P_i. Therefore, the stoichiometry of the observed reaction remains unclear at present.

Carboxylation of acetone to acetoacetate can be compared with carboxylation of pyruvate to oxaloacetate. Two different pathways are possible for the latter reaction. Either pyruvate is carboxylated by a biotin-containing enzyme (pyruvate carboxylase: Scrutton & Young 1972); such a reaction was found for acetone-carboxylation by the fungus *Agaricus bisporus* (Rast & Bachofen 1967), but seems to be unlikely for acetone carboxylation by strain BunN because avidin did not inhibit the reaction. Or, pyruvate is carboxylated after activation to phosphoenolpyruvate (Cooper & Kornberg 1974) in a biotin-independent reaction (Utter & Kolenbrander 1972). A similar reaction sequence via 'phosphoenolacetone' should be considered for strain BunN. Further investigations require purification of the enzyme(s) and separation from ATP'ase, myokinase, propan-2-ol and 3-hydroxybutanoate dehydrogenase.

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