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ORIGINAL PAPER

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# Mechanisms of acetate formation and acetate activation in halophilic archaea

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**Abstract** The halophilic archaea *Halococcus (Hc.) saccharolyticus*, *Haloferax (Hf.) volcanii*, and *Halorubrum (Hr.) saccharovorum* were found to generate acetate during growth on glucose and to utilize acetate as a growth substrate. The mechanisms of acetate formation from acetyl-CoA and of acetate activation to acetyl-CoA were studied. *Hc. saccharolyticus*, exponentially growing on complex medium with glucose, formed acetate and contained ADP-forming acetyl-CoA synthetase (ADP-ACS) rather than acetate kinase and phosphate acetyltransferase or AMP-forming acetyl-CoA synthetase. In the stationary phase, the excreted acetate was completely consumed, and cells contained AMP-forming acetyl-CoA synthetase (AMP-ACS) and a significantly reduced ADP-ACS activity. *Hc. saccharolyticus*, grown on acetate as carbon and energy source, contained only AMP-ACS rather than ADP-ACS or acetate kinase. Cell suspensions of *Hc. saccharolyticus* metabolized acetate only when they contained AMP-ACS activity, i.e., when they were obtained after growth on acetate or from the stationary phase after growth on glucose. Suspensions of exponential glucose-grown cells, containing only ADP-ACS but not AMP-ACS, did not consume acetate. Similar results were obtained for the phylogenetic distantly related halophilic archaea *Hf. volcanii* and *Hf. saccharovorum*. We conclude that, in halophilic archaea, the formation of acetate from acetyl-CoA is catalyzed by ADP-ACS, whereas the activation of acetate to acetyl-CoA is mediated by an inducible AMP-ACS.

**Keywords** Halophilic archaea · Acetate formation · Acetate activation · ADP-forming acetyl-CoA synthetase · AMP-forming acetyl-CoA synthetase · Acetate kinase · Phosphate acetyltransferase

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**Abbreviations.** *Hc. Halococcus · Hf. Haloferax · Hr. Halorubrum · Hb. Halobacterium*

## Introduction

Acetate is an important end product of fermentation processes of many anaerobic and facultative prokaryotes. The mechanisms of acetate formation from acetyl-CoA, a central intermediate of metabolism, have recently been shown to be different in the domains of Bacteria and Archaea (for literature see Schäfer et al. 1993; Schönheit and Schäfer 1995): (1) In all eubacteria analyzed, acetyl-CoA is converted to acetate by the "classical" mechanism involving two enzymes, phosphate acetyltransferase (acetyl-CoA+$P_i \rightleftarrows$acetyl-phosphate+CoA), PTA (EC 2.3.1.8), and acetate kinase (acetyl-phosphate+ADP$\rightleftarrows$acetate+ATP), AK (EC 2.7.2.1). ATP is formed in the acetate kinase reaction by the mechanism of substrate-level phosphorylation (Thauer et al. 1977). (2) In all acetate-forming archaea studied so far, including anaerobic hyperthermophiles and one species of aerobic mesophilic halophiles (*Halobacterium saccharovorum*), the conversion of acetyl-CoA to acetate and the formation of ATP from ADP and phosphate is catalyzed by only one enzyme, an acetyl-CoA synthetase (ADP-forming) (acetyl-CoA+ADP+$P_i \rightleftarrows$acetate+ATP+CoA). This enzyme represents a novel mechanism of ATP synthesis by the mechanism of substrate-level phosphorylation. ADP-forming acetyl-CoA synthetase is also present in the eukaryotic protists *Entamoeba histolytica* and *Giardia lamblia*, and homologous proteins have been found in the genomes of several bacteria (see in Musfeldt et al. 1999; Sánchez et al. 2000). The metabolic function of these putative synthetases in bacteria remains to be shown.

Acetate is also a substrate of catabolism and anabolism in several aerobic and anaerobic prokaryotes. Two main mechanisms have been described for the activation of acetate to acetyl-CoA, which is the first step prior to its utilization in metabolism: Acetate is either activated by a single enzyme, an AMP-forming acetyl-CoA synthetase

(E.C.6.2.1.1) (acetate+CoA+ATP→acetyl-CoA+AMP+$PP_i$) or by the acetate kinase/phosphate acetyltransferase couple operating in the reverse direction as described above. In a few anaerobic bacteria, acetate is activated by means of a succinyl-CoA:acetate CoA transferase (see Thauer et al. 1989).

The mechanism of acetate activation in the domain of Archaea has been studied in detail so far only in the strictly anaerobic methanogens, particularly in acetoclastic species growing at the expense of acetate conversion to $CH_4$ and $CO_2$. *Methanothrix* species utilize an AMP-forming acetyl-CoA synthetase, whereas *Methanosarcina* species activate acetate by acetate kinase and phosphate acetyltransferase (Jetten et al. 1992; Ferry 1997). Both mechanisms of acetate activation have also been demonstrated in $H_2$-utilizing methanogens, which require acetate for cell carbon synthesis (see in Oberlies et al. 1980; Schäfer et al. 1993; Shieh and Whitmann 1987).

The mechanism of acetate activation in aerobic archaea, which include extreme halophiles, has not been clearly identified. Various halophilic archaea have been described to grow on acetate as energy and carbon source (Torreblanca et al. 1985; Oren et al. 1988; Kauri et al. 1990; Kevbrina and Plakunov 1992; Oren and Gurevich 1995). Furthermore, the mechanism of acetate formation from acetyl-CoA has been analyzed only for one halophilic archaeon, *Hb. saccharovorum* (Schäfer et al. 1993). This organism has been shown to generate acetate via an ADP-forming acetyl-CoA synthetase (ADP-ACS). Since this enzyme catalyzed in vitro a reversible reaction (acetyl-CoA+ADP+$P_i$⇄acetate+ATP+CoA), it is tempting to speculate that it is also involved in the activation of acetate to acetyl-CoA in vivo – in analogy to the reversible bifunctional acetate kinase/phosphate acetyltransferase system in bacteria.

In this report, the mechanism of acetate formation and acetate activation was studied in halophilic archaea during growth on glucose and on acetate, respectively. The phylogenetic distantly related species *Halococcus saccharolyticus* (Montero et al. 1989), *Haloferax volcanii* (Mullakhanbhai and Larsen 1975; Torreblanca et al. 1985), and *Halorubrum saccharovorum* (Tomlinson and Hochstein 1976; McGenity and Grant 1995) (for taxonomy see Kamekura 1998; Grant et al. 1998) were analyzed. The data indicated that, in these halophilic archaea, acetate formation from acetyl-CoA is catalyzed by ADP-forming acetyl-CoA synthetase, whereas the activation of acetate is catalyzed by an inducible AMP-forming acetyl-CoA synthetase.

## Materials and methods

### Growth of halophilic archaea on glucose and acetate

*Halococcus (Hc.) saccharolyticus* (DSM 5350), *Halorubrum (Hr.) saccharovorum* (DSM 1137) and *Haloferax (Hf.) volcanii* (DSM 3757) were from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany). All halophilic archaea were grown aerobically at 37 °C according to Tomlinson and Hochstein (1973) and Schäfer and Schönheit (1993) with modifications. The medium for the growth on glucose contained (per liter): 250 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, 19.5 g MES, 2 g KCl, 1 g Na-glutamate, 3 g Na-citrate, 2.5 g yeast extract, 5.0 g casamino acids, 10 ml vitamin solution according to Staley (1968), and 10 ml trace element solution containing (per liter) 1.5 g EDTA, 0.01 g $Na_2MoO_4 \cdot 2H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g CoCl, 0.1 g $ZnSO_4$, 0.01 g $CuSO_4 \cdot 5H_2O$. *Hc. saccharolyticus* and *Hf. volcanii* were grown with 25 mM glucose, *Hr. saccharovorum* with 50 mM glucose as carbon and energy source. The pH was adjusted to 7.35 with 10 N NaOH. The medium for the growth on acetate was the same as for the growth on glucose with the following modifications: The medium (1 l) contained 6 g Tris/HCl, 0.5 g yeast extract, 40 mM Na-acetate as carbon and energy source and no casamino acids. The medium for the growth of *Hf. volcanii* on acetate contained 125 g NaCl per liter. The acetate-containing growth medium (1 l) of *Hf. volcanii* and *Hr. saccharovorum* was supplemented with 0.5 g $NH_4Cl$, 0.1 g $CaCl_2$, and 0.05 g $K_2HPO_4$. The pH was adjusted to 7.35 with 10 N NaOH. Growth experiments were performed in 500-ml flasks filled with 100 ml medium. The cultures were shaken at 200 rpm on a gyratory shaker. Growth was followed by measuring the optical density at 578 nm ($\Delta OD_{578}$). A $\Delta OD_{578}$ of 1 corresponded to a protein content of 0.5–0.6 mg/ml for all halophiles tested. Glucose and acetate were determined enzymatically.

### Cell suspension experiments with *Halococcus saccharolyticus*

*Halococcus saccharolyticus* was grown in 2-l flasks filled with 400 ml medium containing either glucose or acetate. Glucose-grown cells, both from the exponential and the stationary growth phase, and acetate-grown cells from the exponential growth phase were harvested by centrifugation at 5,000×*g* and 4 °C for 20 min. Cells were washed once in a buffer containing (per liter) 19.5 g MES, 2 g KCl, and 250 g NaCl, pH adjusted to 7.35 and finally resuspended in the same buffer at a protein concentration of 12–18 mg/ml. The suspension experiments were carried out at 37 °C in 100-ml flasks filled with 10 ml of cell suspension supplemented with 40 mM acetate. The suspensions were shaken at 200 rpm on a gyratory shaker. At the times indicated, samples were taken from the suspensions, the cells were removed by centrifugation, and acetate consumption was determined.

### Preparation of cell extracts

For the preparations of cell extracts cells, of each of the three extreme halophilic archaea were harvested at various growth phases as indicated by centrifugation at 5,000×*g* (*Hc. saccharolyticus*) or 8,000×*g* (*Hr. saccharovorum* and *Hf. volcanii*) and 4 °C for 20 min, washed once in 100 mM Tris/HCl (pH 7.5) containing 200 g NaCl per liter, and finally resuspended in the same buffer. Cells were stored at –20 °C. Cells were disrupted by sonication in the same buffer used for washing the cells. Cell debris were removed by centrifugation at 13,400×*g* for 15 min. The supernatant (=cell extract) was stored on ice or at 4 °C for several hours. Protein was determined by the Biuret method according to Bode et al. (1968) using bovine serum albumin as a standard.

### Gel filtration of cell extracts

Cell extracts were dialyzed at 4 °C in buffer containing 50 mM Tris/HCl (pH 7.5), 2 M NaCl, 5 mM $MgCl_2$, and 10% glycerol. The dialyzed extract was centrifuged for 30 min at 13,400×*g* and the supernatant was filtered through a 0.2-μm pore size syringe filter (Sartorius, Göttingen, Germany). After filtration, the extracts were applied to a Superdex TM200 16/60 column equilibrated with the same buffer as used for dialysis. The proteins were eluted from the column at a flow rate of 1 ml/min. Fractions (1 ml) were collected and analyzed for acetyl-CoA synthetase activity. For the

gel filtration of cell extracts, a fast protein liquid chromatography system (FPLC) from Pharmacia Biotech (Freiburg, Germany) was used. The procedure was carried out at 17 °C under oxic conditions. The molecular masses of the native enzymes were calculated using the following standard proteins (Biorad): thyroglubin (670 kDa), IgG (158 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin $B_{12}$ (1.35 kDa).

### Determination of enzyme activities

All enzyme assays were performed under oxic conditions at 37 °C in cuvettes filled with 1 ml assay mixture, unless mentioned otherwise. The auxiliary enzymes were generally added shortly before the start of the reaction, and it was ensured that these enzymes were not rate-limiting. One unit (1 U) of enzyme activity is defined as 1 μmol substrate consumed or product formed per minute.

#### *Acetyl-CoA synthetase (ADP-forming or AMP-forming)*

The formation of acetyl-CoA from acetate, ATP, and HSCoA was assayed according to Aceti and Ferry (1988) by monitoring the $Fe^{3+}$-acetyl-hydroxamate complex formation from acetyl-CoA and hydroxylamine at 540 nm. The assay mixture contained 100 mM Tris/HCl (pH 9.0), 1.5 M KCl, 10 mM $MgCl_2$, 400 mM potassium acetate, 700 mM hydroxylamine hydrochloride, 10 mM ATP, 1.5 mM HSCoA, and extract. This assay was used to determine the specific activity and apparent $K_m$ values of the acetyl-CoA synthetase (AMP-forming) in acetate-grown cells of *Hc. saccharolyticus* and the ACS activity in the fractions of the gel filtration.

#### *Acetyl-CoA synthetase (ADP-forming) (E.C.6.2.1.13)*

Acetyl-CoA synthetase was measured as HSCoA and acetate-dependent ADP formation from ATP by coupling the reaction with the oxidation of NADH at 365 nm via pyruvate kinase and lactate dehydrogenase (Schäfer and Schönheit 1991). The assay mixture contained 100 mM Tris/HCl (pH 9.0), 1.5 M KCl, 10 mM $MgCl_2$, 10 mM sodium acetate, 2 mM ATP, 1 mM HSCoA, 2.5 mM phosphoenolpyruvate, 0.3 mM NADH, 15 U lactate dehydrogenase, 4 U pyruvate kinase, and extract. This assay was used to determine the specific activities, the apparent $K_m$ values, and the specificity of the enzyme for ADP in the active fractions of the gel filtration.

#### *Acetyl-CoA synthetase (AMP-forming) (E.C.6.2.1.1.)*

Acetyl-CoA synthetase (AMP-forming) was measured as HSCoA and acetate-dependent AMP formation from ATP by coupling the reaction with the oxidation of NADH at 365 nm via pyruvate kinase, lactate dehydrogenase, and adenylate kinase according to Oberlies et al. (1980). The assay mixture contained 100 mM Tris/HCl (pH 9.0), 1.5 M KCl, 10 mM $MgCl_2$, 10 mM sodium acetate, 2 mM ATP, 1 mM HSCoA, 2.5 mM phosphoenolpyruvate, 0.3 mM NADH, 15 U lactate dehydrogenase, 4 U pyruvate kinase, 3.6 U myokinase, and extract. This assay was used to determine the specificity of the enzyme for AMP in the active fractions of the gel filtration.

#### *Acetate kinase (E.C.2.7.2.1.)*

Acetate kinase was measured using two different assay systems. (1) The acetate-dependent ADP formation from ATP was measured by coupling the reaction with the oxidation of NADH at 365 nm via pyruvate kinase and lactate dehydrogenase (Schäfer and Schönheit 1991). The assay mixture contained 100 mM Tris/HCl (pH 9.0), 1.5 M KCl, 10 mM $MgCl_2$, 10 mM sodium acetate, 2 mM ATP, 2.5 mM phosphoenolpyruvate, 0.3 mM NADH, 15 U lactate dehydrogenase, 4 U pyruvate kinase, and extract. (2) The formation of acetyl-phosphate from acetate and ATP was assayed according to Aceti and Ferry (1988) by monitoring the $Fe^{3+}$-acetyl-hydroxamate complex formation from acetyl-phosphate and hydroxylamine at 540 nm. The assay mixture contained 100 mM Tris/HCl (pH 9.0), 1.5 M KCl, 10 mM $MgCl_2$, 400 mM potassium acetate, 700 mM hydroxylamine hydrochloride, 10 mM ATP, and extract.

#### *Phosphotransacetylase (E.C.2.3.1.8.)*

Phosphotransacetylase was monitored according to Srere et al. (1963) as $P_i$-dependent HSCoA release from acetyl-CoA with Ellman's thiol reagent, 5′5-dithiobis (2-nitrobenzoic acid) (DTNB), by measuring the formation of thiophenolate anion at 412 nm. The assay mixture contained 100 mM Tris/HCl (pH 9.0), 1.5 M KCl, 5 mM $MgCl_2$, 0.1 mM DTNB, 0.1 mM acetyl-CoA, 5 mM $KH_2PO_4$, and extract.

#### *Isocitrate lyase (E.C.4.1.3.1.)*

Isocitrate lyase (isocitrate→glyoxylate+succinate) was assayed according to Serrano et al. (1998) by following the isocitrate- and phenylhydrazine-dependent formation of glyoxylate phenylhydrazone at 324 nm. The assay mixture contained 70 mM HEPES buffer (pH 7.0), 1.9 M KCl, 5 mM $MgCl_2$, 4 mM phenylhydrazine, 4 mM D,L-isocitrate, and extract.

#### *Malate synthase (E.C.4.1.3.2.)*

Malate synthase (acetyl-CoA+glyoxylate→malate+HSCoA) was monitored according to Serrano et al. (1998) as glyoxylate-dependent HSCoA release from acetyl-CoA with Ellman's thiol reagent, DTNB, by measuring the formation of thiophenolate anion at 412 nm. The assay mixture contained 20 mM Tris/HCl (pH 8.0), 3 M KCl, 5 mM $MgCl_2$, 0.1 mg/ml DTNB, 2 mM EDTA, 0.2 mM acetyl-CoA, 0.5 mM glyoxylate, and extract.

### Source of material

Enzymes, coenzymes, and phosphoenolpyruvate were from Boehringer (Mannheim, Germany). MES and Tris were from Biomol (Hamburg, Germany), yeast extract from Gibco BRL (Eggenstein, Germany), and casamino acids from Difco (Stuttgart, Germany). All other chemicals were reagent grade and obtained from E. Merck (Darmstadt, Germany), Sigma (Munich, Germany), or Roth (Karlsruhe, Germany). The Superdex 200 16/60 column was prep grade and obtained from Pharmacia Biotech (Freiburg, Germany).

## Results

### Growth of halophilic archaea on glucose: formation and consumption of acetate

*Halococcus saccharolyticus* and *Haloferax volcanii* were grown on complex media (0.25% yeast extract, 0.5% casamino acids) containing 25 mM glucose. Both organisms grew exponentially, with a doubling time ($t_d$) of about 10–12 h, up to optical densities ($\Delta OD_{578}$) of about 6–7 (3–4 mg protein/ml). During exponential growth, glucose was completely consumed and – in parallel – acetate was excreted into the medium up to concentrations of 10 mM. In the stationary phase, the excreted acetate was almost completely consumed (Fig. 1). In the absence of

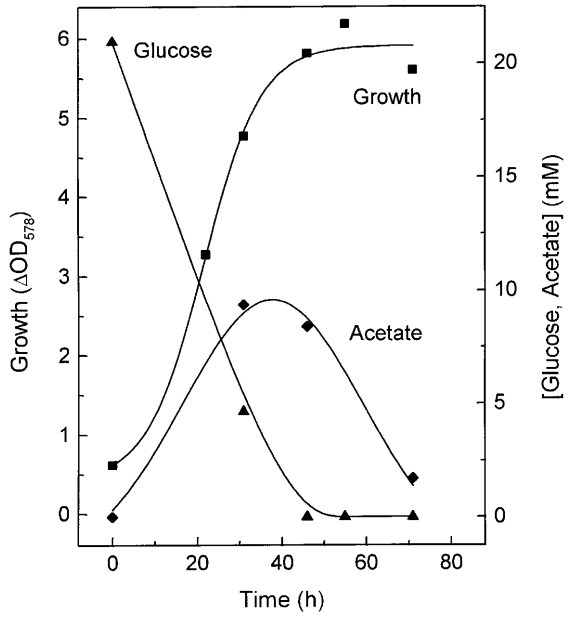


**Fig. 1** Growth of *Halococcus saccharolyticus* on glucose in the presence of 0.25% yeast extract and 0.5% casamino acids. Cultures were grown at 37 °C in 500-ml flasks filled with 100 ml medium containing 25 mM glucose. Growth (■) and the concentrations of glucose (▲) and acetate (◆) were followed over time

sugars, the organisms grew on complex constituents of the medium up to densities of $\Delta OD_{578}$ 3–4; under these conditions acetate was not formed in significant amounts. Similar results were obtained with *Hr. saccharovorum*: During growth on 45 mM glucose, the cells excreted up to 40 mM acetate in the exponential growth phase (doubling time 13 h). In the stationary phase, 20 mM of acetate were consumed within 20 h. The findings indicate that halophilic archaea excrete and reutilize acetate when grown on glucose as carbon and energy source.

## Mechanism of acetate formation: ADP-forming acetyl-CoA synthetase

Glucose-grown cells of *Hc. saccharolyticus* and *Hf. volcanii*, obtained from the exponential growth phase in which acetate was formed (Fig. 1), contained ADP-forming acetyl-CoA synthetase (ADP-ACS) (acetyl-CoA+ADP+$P_i \rightleftarrows$acetate+ATP+CoA). The specific enzyme activities (37 °C), measured as acetate- and CoA-dependent ADP formation from ATP in the pyruvate kinase/lactate dehydrogenase (PK/LDH) assay, were about 0.04 U/mg (*Hc. saccharolyticus*) and 0.1 U/mg, (*Hf. volcanii*), respectively. Extracts of both organisms contained ATPase activity, measured as ADP formation from ATP in the PK/LDH assay at specific activities of 0.02 U/mg and 0.01 U/mg. Acetate kinase and phosphate acetyltransferase could not be detected in either organism. The ADP-ACS activity of *Hc. saccharolyticus* requires $Mg^{2+}$ (30 mM) for maximal catalytic activity. Long-term stability (>48 h) requires high salt concentrations, i.e., 1–2 M of KCl or NaCl. Enzyme activity showed a pH optimum at pH 8, and a temperature optimum at 43 °C. The apparent $K_m$ values for acetate, CoA, and ATP were 5.3 mM (Fig. 4), 0.41 mM, and 1 mM. ADP-ACS activity in *Hr. saccharovorum* forming 40 mM acetate during exponential growth on glucose was about 0.2 U/mg (see also Schäfer et al. 1993). The findings indicate that acetate formation from acetyl-CoA in phylogenetically distantly related halophilic archaea is catalyzed by ADP-ACS.

## Growth of halophilic archaea on acetate

*Hc. saccharolyticus*, *Hf. volcanii* and *Hr. saccharovorum* were grown on media containing acetate and 0.05% yeast extract. A growth curve of *Hc. saccharolyticus* is shown in Fig. 2. The cells grew exponentially with a doubling time of 25 h up to cell densities of $\Delta OD_{578}$=1.6 (0.3 mg protein/ml). In the absence of acetate cells grew on 0.05% yeast extract up to densities of 0.3 ($\Delta OD_{578}$). Growth of *Hf. volcanii* and *Hr. saccharovorum* on acetate (40 mM) and 0.05% yeast extract required ammonium ions. In the presence of 0.5 g $NH_4Cl$ per liter the cells showed similar growth kinetics as *Hc. saccharolyticus*; they grew with doubling times of 10 h up to optical densities $\Delta OD_{578}$ of about 1.7, control assays without acetate reached $\Delta OD_{578}$ of about 0,4.

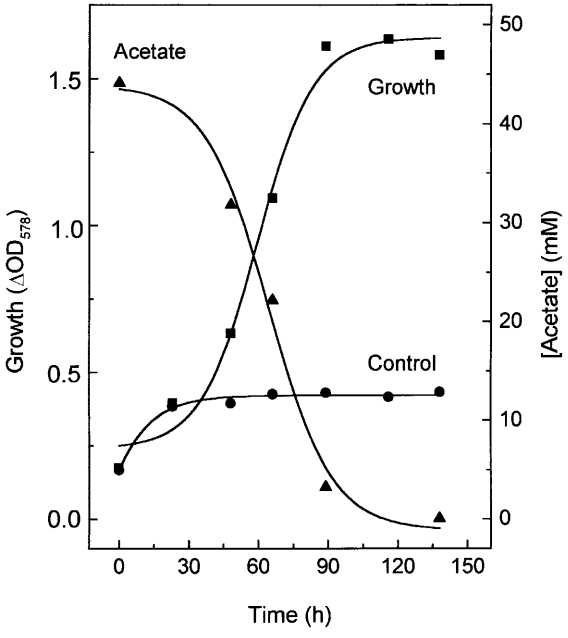


**Fig. 2** Growth of *Hc. saccharolyticus* on acetate in the presence of 0.05% yeast extract. Cultures were grown at 37 °C in 500-ml flasks filled with 100 ml medium containing 40 mM acetate. Growth (■) and the concentration of acetate (▲) were followed over time. As a control the organism was grown on the same medium without acetate (●)

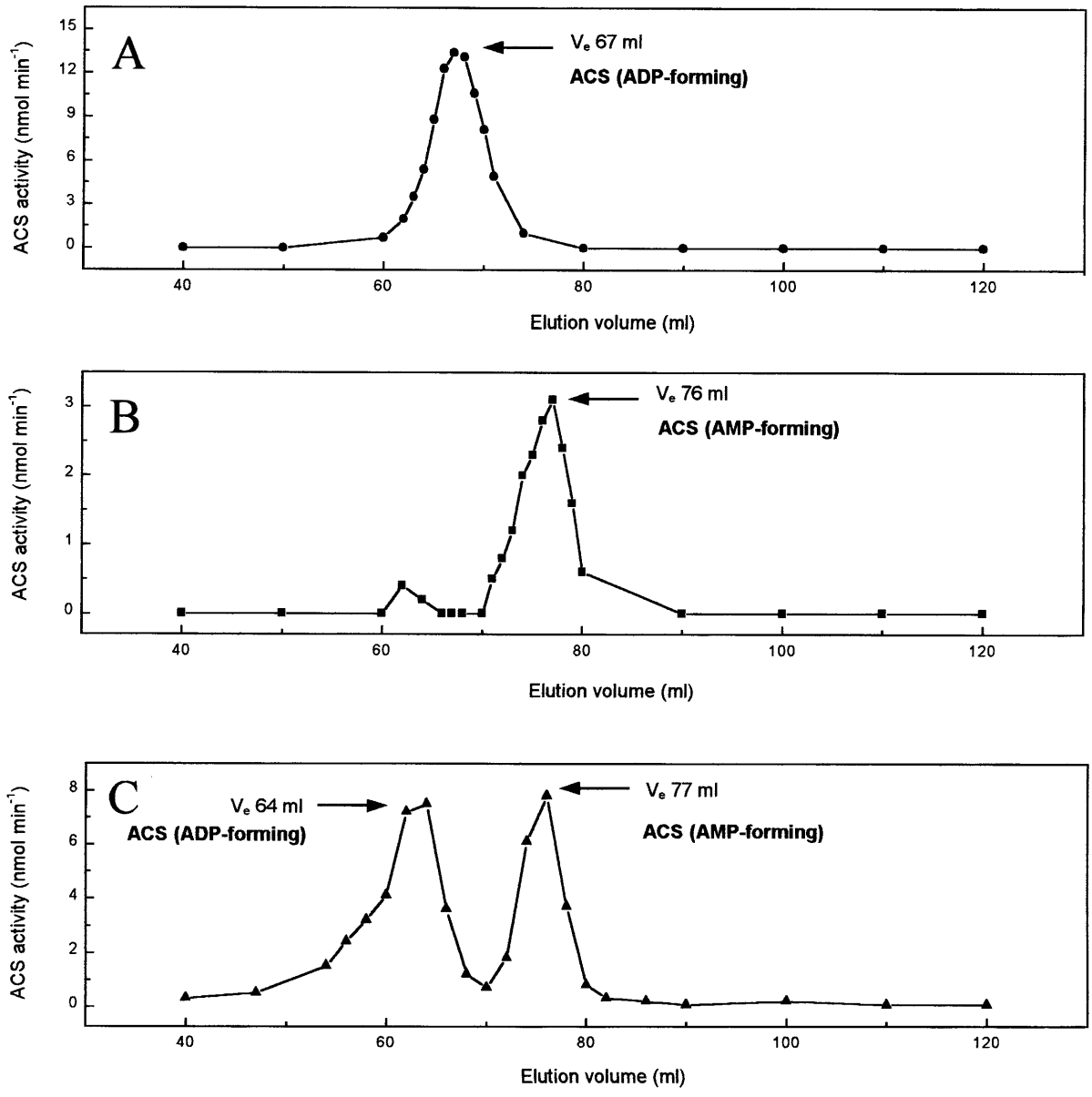


**Fig. 3A–C** Separation of acetyl-CoA synthetase activities in cell extracts of *Hc. saccharolyticus* via gel filtration on a Superdex TM200 HiLoad column. **A** Cell extracts of glucose-grown cells harvested in the exponential phase; **B** cell extracts of acetate-grown cells; **C** cell extracts of glucose-grown cells harvested in the stationary phase. The cell extracts were applied to a Superdex 200 HiLoad 16/60 column equilibrated with 100 mM Tris buffer (pH 7.5) containing 2 M NaCl, 5 mM $MgCl_2$, and 10% glycerol. The proteins were eluted with the same buffer at a flow rate of 1 ml/min. The fraction size was 1 ml. The ACS activity was determined in the hydroxamate assay according to Aceti and Ferri (1988)

## Mechanism of acetate activation: AMP-forming acetyl-CoA synthetase

*Hc. saccharolyticus, Hf. volcanii* and *Hr. saccharovorum*, forming acetate from glucose, contained ADP-ACS, which catalyzes in vitro the reversible conversion of acetyl-CoA to acetate (acetyl-CoA+ADP+$P_i \rightleftarrows$acetate+ATP+CoA). It was tested whether in these organisms ADP-ACS is also involved in the activation of actetate in vivo. Therefore, extracts of acetate-grown cells were analyzed for ADP-ACS, AMP-forming acetyl-CoA synthetase (AMP-ACS), and acetate kinase/phosphate acetyltransferase.

Extracts of acetate-grown cells of *Hc. saccharolyticus* catalyzed the ATP- and CoA-dependent acetate conversion to acetyl-CoA which was quantified as acetyl-hydroxamate (Aceti and Ferry 1988). This enzyme activity (0.02–0.03 U/mg protein at 37 °C) is indicative of the presence of an acetyl-CoA synthetase. Acetate kinase activity was not found. The hydroxamate assay, however, does not discriminate between an ADP (+$P_i$)-forming or AMP (+$PP_i$)-forming acetyl-CoA synthetase.

The specificity of ACS in crude extracts could not be measured in the coupled assay systems (see Materials and methods section) due to the high background of ADP for-

mation by an active ATPase (0.1 U/mg). To identify the specificity of ACS (ADP- or AMP-forming), the crude extracts were fractionated by gel filtration on Superdex TM200 to separate the ACS activities from ATPase. As shown in Fig. 3B, ACS activity eluted from the column in one peak between 74 and 77 ml, corresponding to a protein of an apparent molecular mass between 50 and 60 kDa. The fractions catalyzed the acetate- and CoA-dependent AMP formation from ATP, measured in the coupled assay system (PK/LDH/adenylate kinase), indicating the presence of an AMP-forming acetyl-CoA synthetase. The specific activity of AMP-forming ACS in the peak fractions was between 0.06 and 0.09 U/mg, indicating a three- to four-fold enrichment of the enzyme compared to the crude extract. The ATPase background activity in these fractions (0.15–0.18 U/mg) was low enough to determine AMP-ACS activity correctly. The apparent $K_m$ values of AMP-ACS activity were determined to be 0.8 mM for acetate (Fig. 4), 0.9 mM for CoA, and 0.8 mM for ATP. An ADP-forming ACS activity could not be detected.

For comparison, extracts of cells of *Hc. saccharolyticus*, which formed acetate during exponential growth on glucose, were also fractionated on Superdex TM200 column. The acetyl-CoA synthetase activity eluted in one peak between 64 ml and 67 ml, corresponding to a protein with an apparent molecular mass of 150–170 kDa (Fig. 3A). The ACS was identified as ADP-forming synthetase measured in the PK/LDH assay. The enzyme activity in the peak fraction was 0.45 U/mg, indicating a ten-fold enrichment compared to crude extracts. An AMP-forming synthetase activity could not be detected.

Similar data were obtained for acetate-grown cells *Hf. volcanii* and *Hr. saccharovorum*. After fractionation of extracts of both halophiles by gel fitration, an AMP-forming ACS activity was found at elution volumes between 75 and 78 ml. In contrast to *Hc. saccharolyticus*, both *Hf. volcanii* and *Hr. saccharovorum*, contained in addition activities of ADP-forming ACS eluting between 64 and 67 ml from the Superdex TM200 column (not shown), indicating that ADP-ACS activity was not completely reduced (~0.02 U/mg). In comparison, glucose-grown cells (exponential growth phase) of *Hf. volcanii* and *Hr. saccharovorum*, forming acetate, contained only ADP-ACS activities. The data suggest that, during growth on acetate, halophilic archaea induced an AMP-forming ACS, which catalyzes the activation of acetate to acetyl-CoA.

Further indications for inducible AMP-forming ACS involved in acetate activation came from analysis of ACS activities in stationary phase cells of glucose-grown halophilic archaea. As shown in Fig. 1, *Hc. saccharolyticus* excreted acetate into the medium during exponential growth on glucose and consumed acetate in the stationary phase. Extracts of cells obtained from the stationary phase (at 70 h, Fig. 1) contained a lower ADP-ACS activity (0.03 U/mg measured in the PK/LDH assay) than those of cells of the exponential growth phase. Cell extracts of stationary phase cells were fractionated on Superdex TM200. As shown in Fig. 3C, ACS activity eluted in two peaks. In one peak, eluting at 65 ml–67 ml, ADP-ACS activity was detected. In the second peak, eluting between 75 and 77 ml, an AMP-ACS was found. The data suggest that an AMP-ACS is induced by *Hc. saccharolyticus* in the stationary phase, whereas the ADP-ACS decreased in

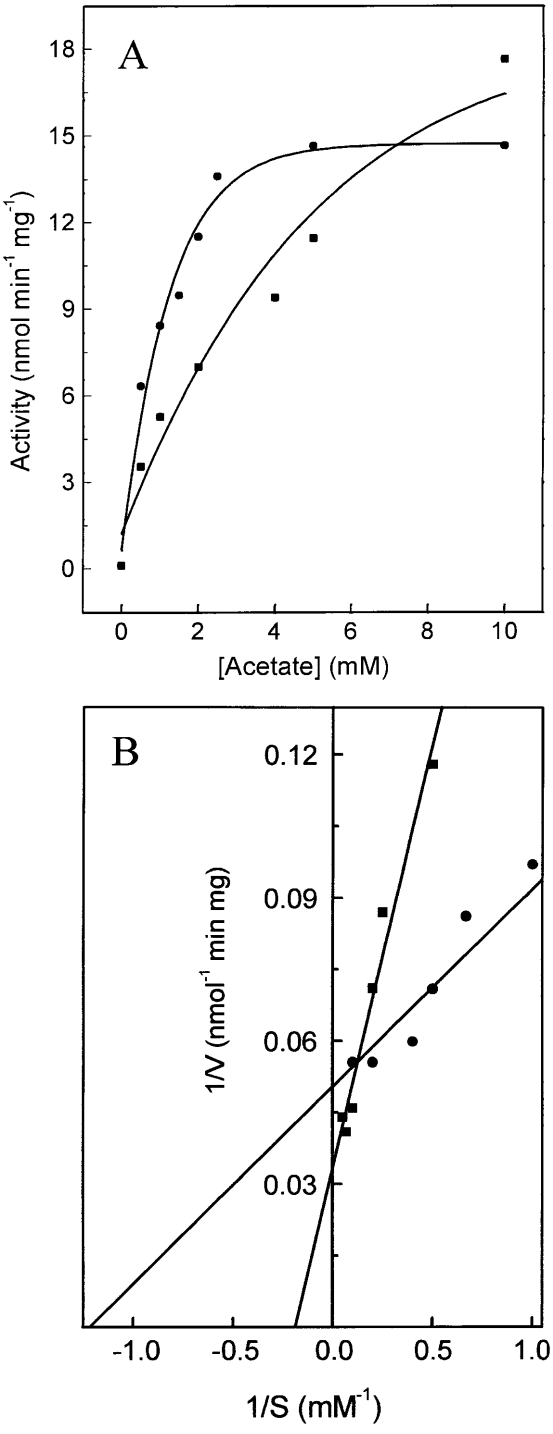


**Fig. 4A, B** Affinities for acetate of acetyl-CoA synthetase (ADP-forming) (■) and acetyl-CoA synthetase (AMP-forming) (●) in cell extracts of *Hc. saccharolyticus*. **A** Rate dependence on the concentration of acetate at 37 °C. **B** Double reciprocal plot of the rates vs the acetate concentration

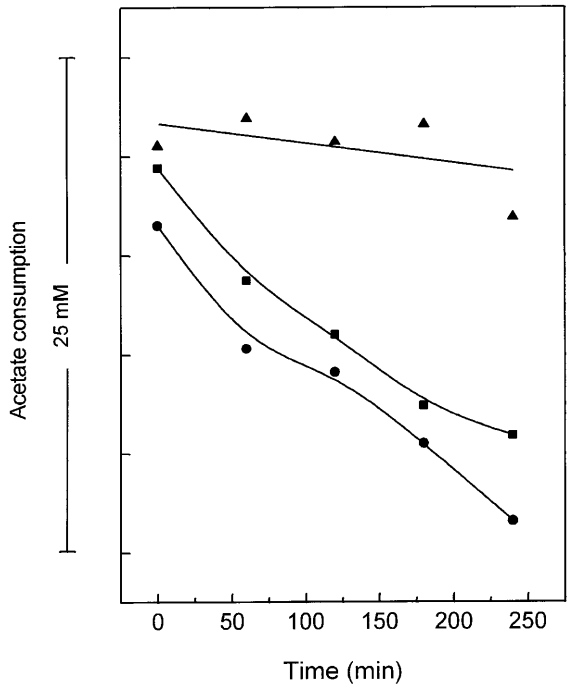


**Fig. 5** Acetate consumption in cell suspensions of *Hc. saccharolyticus*. Suspensions were prepared from glucose-grown cells harvested in the exponential phase (▲), glucose-grown cells harvested in the stationary phase (●) and acetate-grown cells (■). Ten ml of cell suspension containing 8–10 mg cell protein/ml and 40 mM acetate were incubated in 100-ml flasks. At the times indicated, the amount of acetate consumed was determined

this period. This finding supports the conclusion that an inducible AMP-ACS is involved in acetate activation.

### Acetate consumption by cell suspensions

Further evidence for the role of AMP-ACS in acetate activation was concluded from cell suspension studies. The consumption of acetate was followed in suspensions of *Hc. saccharolyticus* cells obtained either after growth on glucose, both in the exponential and stationary growth phase, and after growth on acetate. As shown in Fig. 5, acetate consumption was observed only in those cells in which AMP-ACS was present, i.e., in acetate-grown cells and in stationary phase cells after growth on glucose (see Fig. 3A–C). The rate of acetate consumption was about 4 nmol $min^{-1}$ (mg protein)$^{-1}$. Suspensions of exponential glucose-grown cells, containing only ADP-ACS activity but not AMP-ACS, did not consume acetate. These data support the previous finding that the activation of acetate is catalyzed by inducible AMP-ACS rather than by ADP-ACS.

### Other enzymes of acetate metabolism in halophilic archaea

Cell extracts of acetate-grown *Hc. saccharolyticus, Hf. volcanii*, and *Hr. saccharovorum* contained isocitrate lyase and malate synthase, the key enzymes of the glyoxylic acid pathway. The specific activities given in U/mg at 37 °C were 0.001, 0.035, 0.016 for isocitrate lyase and 0.004, 0.05, 0.048 for malate synthase. The presence of isocitrate lyase and malate synthase in the phylogenetically different strains indicates that the glyoxylic acid pathway is probably operative in the anabolism of all halophilic archaea growing on acetate. Isocitrate lyase and malate synthase of *Hf. volcanii* have recently been purified by Serrano et al. (1998).

## Discussion

In the present work, it was shown that in halophilic archaea the formation of acetate from acetyl-CoA was catalyzed by an ADP-forming acetyl-CoA synthetase (ADP-ACS) (acetyl-CoA+ADP+$P_i \rightleftarrows$acetate+ATP+CoA), whereas the activation of acetate to acetyl-CoA was mediated by an inducible AMP-forming acetyl-CoA synthetase (AMP-ACS) (acetate+CoA+ATP$\rightarrow$acetyl-CoA+AMP+$PP_i$).

### Formation of acetate by ADP-ACS

The mechanism of acetate formation from acetyl-CoA via ADP-ACS has been reported so far in anaerobic hyperthermophilic archaea and in one aerobic halophilic archaeon, *Hr. saccharovorum* (Schäfer et al. 1993). The presence of ADP-ACS in phylogenetically distantly related halophilic archaea (*Halococcous, Haloferax, Halorubrum*) support the idea that an ADP-forming acetyl-CoA synthetase is the general mechanism of acetate formation from acetyl-CoA in archaea. So far, ADP-ACS has been characterized only from the hyperthermophile *Pyrococcus furiosus* (Glasemacher et al. 1997; Mai and Adams 1996; Musfeldt et al. 1999). The native enzyme has an apparent molecular mass of 140 kDa. A similar apparent molecular mass (140–150 kDa) was concluded for ADP-ACS activity in halophilic archaea based on gel filtration experiments in this study.

### Activation of acetate by AMP -ACS

ADP-ACS in halophilic archaea catalyzed in vitro a reversible reaction (acetyl-CoA+ADP+$P_i \rightleftarrows$acetate+ATP+CoA), i.e., both the formation of acetate and the activation of acetate to acetyl-CoA. However, the results of this study indicate that, in vivo, the activation of acetate in halophilic archaea is catalyzed by an inducible AMP-forming ACS rather than by ADP-ACS operating in the reverse direction: (1) *Hc. saccharolyticus* contained only AMP-forming ACS rather than ADP-forming ACS when grown on acetate as carbon and energy source. (2) In the stationary phase during growth on glucose, in which acetate was consumed, an AMP-ACS activity was induced. (3) Acetate consumption by resting cell suspensions was observed only in those cells containing AMP-ACS, rather than by cells containing only ADP-ACS.

This is the first report of an AMP-ACS in aerobic archaea. A report on enzymes of acetate activation in the halophilic archaeon *Natronococcus occultus* did not give unambiguous results (Kevbrina and Plakunov 1992). So far, AMP-ACS has only been reported for the anaerobic methanogenic archaea. (Jetten et al. 1992; Shieh and Whitmann 1987). The enzyme from this *Methanothrix soehngenii* is a homodimer with an apparent molecular mass of 148 kDa (Jetten et al. 1989). About half the molecular mass (60–80 kDa) was calculated for AMP-ACS activity of halophilic archaea from gel filtration experiments.

An interesting result of this study is the metabolic switch from acetate formation to acetate consumption, observed during growth of halophilic archaea on glucose. An analogous situation is known for bacteria, e.g., *Escherichia coli* (Brown et al. 1977; Kumari et al. 1995, 2000) and *Bacillus subtilis* (see in Grundy et al. 1994). During growth of *E. coli* on media containing excess glucose or amino acids, the organism excreted acetate into the medium and consumed it in the stationary phase. The formation of acetate in this eubacterium is catalyzed by phosphate acetyltranferase (PTA) (acetyl-CoA+$P_i$⇄acetylphosphate+CoA) and acetate kinase (AK) (acetyl-phosphate+ADP⇄acetate+ATP), whereas for the activation of acetate an AMP-ACS is induced. Thus, *E. coli* and halophilic archaea have in common that they induce an AMP-ACS in the stationary phase during acetate consumption. However, both organisms differ in the – domain-specific – mechanisms of acetate formation from acetyl-CoA, which involve ADP-ACS in the archaeon *Hc. saccharolyticus* and PTA/AK in the bacterium *E. coli*. The induction of an AMP-forming ACS in *E coli* has been attributed to its higher affinity for acetate, as compared to that of acetate kinase, which enables the organism to scavenge for small concentrations of acetate (Brown et al. 1977; Kumari et al. 1995, 2000). Due to the lower affinity of the acetate kinase for acetate, the PTA/AK pathway is predominantly used in the energy-yielding formation of acetate in the catabolism (Brown et al. 1977; Thauer et al. 1977; Bock et al. 1999). An analogous situation was found in halophilic archaea. The AMP-forming ACS activity in extracts of *Hc. saccharolyticus* showed a six-fold higher affinity for acetate (apparent $K_m$ 0.8 mM) as compared to the ADP-ACS (apparent $K_m$ 5.3 mM) (Fig. 4), which is the archaeal counterpart of the bacterial PTA/AK system in the formation of acetate.

However, there are also examples that the PTA/AK pathway – in contrast to ADP-ACS in halophilic archaea – operates reversibly in vivo. It is, for example, the only mechanism for acetate activation, e.g., in the aerobic bacterium *Corynebacterium glutamicum* (Reinscheid et al. 1999) and in the anaerobic methanogenic archaeon *Methanosarcina* sp. (Ferry 1997). These organisms induce AK and PTA, rather than an AMP-ACS, during growth on acetate as energy and carbon source.

The molecular basis of the metabolic switch from acetate formation by ADP-ACS to acetate consumption by AMP-ACS during growth of halophilic archaea on glucose remains to be elucidated. ADP-ACS and AMP-ACS were not constitutive enzymes but were found to be regulated by growth substrates. Glucose-grown cells (exponential phase) contained only ADP-ACS, whereas in acetate-grown cells an AMP-ACS was induced. Furthermore, in the stationary phase during growth on glucose, the ADP-ACS activity decreased and the AMP-ACS activity increased. Regulation of enzyme activities might reflect repression or induction at the transcriptional level. It has been shown for *E. coli* that induction of AMP-ACS was the important step in the metabolic change of acetate formation to acetate activation during growth on glucose (Brown et al. 1977; Kumari et al. 1995, 2000). Purification of ADP-ACS and AMP-ACS from halophilic archaea, and cloning and functional expression of their encoding genes are in progress, which will allow a molecular analysis of the regulation of these acetyl-CoA synthetases in archaeal acetate metabolism.

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