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The fatty acid synthetase complex of *Haloanaerobium praevalens* is not inhibited by salt

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Abstract: Cell-free enzyme preparations of the obligately anaerobic halophilic eubacterium *Haloanaerobium praevalens* synthesize fatty acids from malonyl-CoA. The reaction is stimulated by NaCl and KCl at a concentration of 1 M, and only slightly inhibited by salt concentrations as high as 3 M. Thus, the fatty acid synthetase of *H. praevalens* is expected to be fully active at the high intracellular salt concentrations present, and it is the first fatty acid synthetase reported to be active in the presence of high salt concentrations.

Key words: Fatty acid synthetase; *Haloanaerobium praevalens*; Halophilic; Anaerobic

Introduction

Halophilic and halotolerant microorganisms have developed two fundamentally different strategies to cope with the high salt concentrations in their environment. The extremely halophilic archaebacteria (family Halobacteriaceae) maintain high concentrations of salt (mainly KCl) inside the cells. This mode of adaptation to life in hypersaline environments implies that all intracellular enzyme systems must be active in the presence of high salt concentrations. The aerobic halophilic and halotolerant eubacteria do not possess such specialized salt-tolerant or salt-requiring enzymes, and their intracellular salt concentration is low [1]. To maintain an osmotic balance between the cell cytoplasm and the surrounding medium, different organic solutes (glycine betaine, ectoine, and others) are synthesized and/or accumulated in high concentrations.

Fatty acid synthetase, the enzyme complex responsible for the synthesis of straight-chain fatty acids, is notably salt-sensitive in all cases examined. This is true both for non-halophilic bacteria such as *Escherichia coli* [2], moderately halophilic aerobic eubacteria [3], and even extremely halophilic archaebacteria of the genus *Halobac-*

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terium [2]. Sodium chloride and potassium chloride were reported to be equally inhibitory [2]. This salt-sensitivity is not expected to cause serious problems to the organisms, as the first two groups mentioned maintain low intracellular salt concentrations, while *Halobacterium* synthesizes only minor amounts of straight-chain fatty acids, the membrane lipids being of the archaebacterial isoprenoid-based type [1]. *Halobacterium* species do synthesize minor amounts of eubacterial-type fatty acids, which are covalently bound to membrane proteins (M. Kates, University of Ottawa, personal communication).

Halophilic obligatory anaerobic bacteria of the family Haloanaerobiaceae [4,5], though belonging to the eubacterial kingdom, share with the aerobic halophilic archaebacteria the property of high internal salt concentrations. Rather than accumulating organic solutes, they contain molar concentrations of KCl and NaCl, as was shown for *Haloanaerobium praevalens* [6], *Halobacteroides halobius* [6], and *Halobacteroides acetoethylicus* [7]. Accordingly, the intracellular enzymes have to be active in the presence of high salt concentrations, and this was indeed shown in the case of a number of enzymes of *Halobacteroides acetoethylicus* [7]. Being eubacteria, the members of the Haloanaerobiaceae contain straight-chain fatty acids in their lipids [8–10]. This implies that they should possess a salt-tolerant fatty acid synthetase complex, different from the fatty acid synthetases of non-halophilic bacteria, halotolerant aerobic eubacteria and halophilic archaebacteria. We therefore investigated the effect of salt on the fatty acid synthetase activity in the obligately anaerobic halophilic eubacterium *Haloanaerobium praevalens*, an organism abundant in the bottom sediments of the Great Salt Lake, Utah [8].

Materials and Methods

Strains and growth conditions

Haloanaerobium praevalens (DSM 2228) [8] was grown at 35°C in 120-ml stoppered bottles with 80 ml of medium containing (g l^{-1}): NaCl, 130; KCl, 1.0; $MgSO_4 \cdot 7H_2O$, 8.8; Bacto-tryptone (Difco), 10.0; Bacto-yeast extract (Difco), 10.0; cysteine · HCl, 0.5, and glucose, 2.5, pH 7.0, under a gas phase of nitrogen. Anaerobic medium was prepared by boiling under nitrogen. The glucose was added to the autoclaved medium from a separately sterilized anaerobic solution. *Halomonas elongata* (ATCC 33173) [11] was grown in 1-l Erlenmeyer flasks with shaking at 35°C in 500-ml portions of medium containing (g l^{-1}): NaCl, 100; $MgCl_2 \cdot 6H_2O$, 10.0; KCl, 1.0; Na_2SO_4, 0.5; yeast extract, 5.0; tryptone, 5.0, pH 7.0. *Halobacterium halobium* R1 was grown with shaking at 35°C in medium containing (g l^{-1}): NaCl, 250; KCl, 5.0; $MgCl_2 \cdot 6H_2O$, 5.0; NH_4Cl, 5.0; yeast extract, 5.0; pH 7.0. *Escherichia coli* K12 was grown with shaking at 35°C in nutrient broth (Difco).

Fatty acid synthetase assay

Cells from 400–1000 ml culture were collected by centrifugation, suspended in 5 ml of 50 mM Tris · HCl buffer (pH 7.0) containing 1 mM EDTA and 2.5 mM 2-mercaptoethanol. Cells were disrupted by sonication in the cold (6 periods of 30 s with intermittent cooling). The resulting suspensions were centrifuged (10 min, 12000 × *g* in the cold), and the supernatant fraction was used in the enzymatic assay. The protein content of the preparations was determined with the Lowry procedure.

Fatty acid synthetase was assayed by measuring the incorporation of [2-^{14}C]malonyl-CoA into long-chain fatty acids. The complete system (0.5 ml) contained 1.96 nmol of [2-^{14}C]malonyl-CoA (Amersham, 51 mCi $mmol^{-1}$), 30 nmol of acetyl-CoA (Sigma), 120 nmol of NADPH (Sigma), 30 μmol of Tris · HCl buffer (pH 7.0), 5 μmol of 2-mercaptoethanol, and 150 or 250 μg acyl carrier protein (ACP) (from *E. coli*, purchased from Sigma). The reaction mixtures were adjusted to the desired salt concentration by the addition of concentrated solutions and/or solid NaCl or KCl. The reaction was started by the addition of cell protein (between 0.25 and 6.0 mg per system, as indicated). After 30 min incubation at 30°C, the reaction was stopped by the addition of 0.5 ml of 10 N NaOH, 50 μg of palmitic acid was added from a solution of 1 mg ml^{-1} in petroleum ether, and the mixtures were heated at 80°C for 10 min.

Free fatty acids were liberated by acidification with 0.7 ml of concentrated HCl, and extracted with three 2-ml portions of petroleum ether (b.p. 30–60°). The extracts were transferred to glass scintillation vials, dried under reduced pressure, 5 ml of Instagel (Packard) scintillation cocktail was added to each vial, and the radioactivity was counted in a scintillation counter. Activities were calculated as nmol malonyl-CoA incorporated per hour per mg protein, after correction for a zero-time control.

Results and Discussion

Cell-free enzyme preparations of the obligately anaerobic halophilic eubacterium *Haloanaerobium praevalens* synthesized fatty acids from malonyl-CoA in the presence of acetyl-CoA, NADPH and ACP. In the absence of salt, activities in the range of 0.16 to 0.20 nmol h^{-1} (mg protein)$^{-1}$ were measured. In the presence of 1 M NaCl or KCl the reaction rates were increased (13–20% and 5–60%, respectively). At higher salt concentrations, the reaction rates decreased somewhat, but even at salt concentrations as high as 3 M high rates were obtained (Fig. 1). The addition of *E. coli* ACP proved highly stimulatory: when ACP was omitted from the reaction mixture, the rate of fatty acid synthesis in the absence of salt was only 14% of that obtained in the presence of 150 μg ACP.

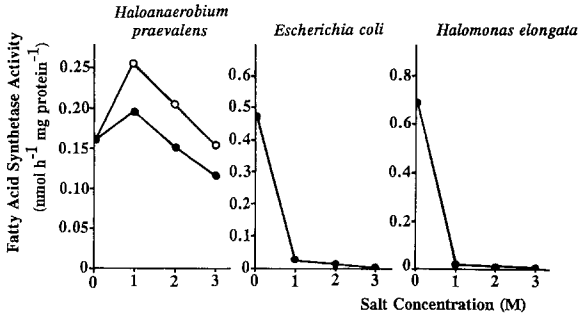

Fig. 1. Effect of sodium chloride (●) and potassium chloride (○) concentration on fatty acid synthetase activity in cell extracts of *Haloanaerobium praevalens* (left panel), as compared to *Escherichia coli* K12 (middle panel) and *Halomonas elongata* (right panel). Representative results are shown; the reaction mixtures contained 0.7, 0.27, and 0.87 mg protein, respectively, and additionally 150 μg acyl carrier protein from *E. coli*.

For comparison we determined the effect of NaCl on the fatty acid synthetase activity of cell extracts of *E. coli* and *H. elongata*. In both cases more than 90% inhibition was obtained already in the presence of 1 M NaCl (Fig. 1). *H. elongata*, being a halotolerant eubacterium, maintains an osmotic equilibrium with its surrounding medium by the accumulation of high concentrations of organic solutes: ectoine in minimal media, and glycine betaine in complex media [12]. Glycine betaine was found greatly superior to NaCl in supporting activity of fatty acid synthetase in extracts of *H. elongata*: in the presence of 3 M glycine betaine, activity was only 35% less than in a control experiment, as compared with 95.5% inhibition by 3 M NaCl. In *Vibrio* HX, another moderately halophilic eubacterium, glycine betaine in concentrations of 2–3 M was found to be required for optimal activity of fatty acid synthetase, and activities in its absence were extremely low [3]; we did not detect a similar phenomenon in *H. elongata*.

Fatty acid synthetase activities measured in *E. coli*, *H. elongata* and *H. praevalens* (between 0.16 and 0.86 nmol malonyl-CoA h^{-1} (mg protein)$^{-1}$) were seemingly low compared with the value of 4 nmol h^{-1} (mg protein)$^{-1}$ reported by Pugh et al. [2] in *E. coli*. However, in the latter work, the fatty acid synthetase content of the extract was enriched by ammonium sulfate fractionation.

Our attempts to demonstrate activity of fatty acid synthetase in extracts of *Halobacterium* R1 yielded very low values (around 0.0004 nmol malonyl-CoA h^{-1} (mg protein)$^{-1}$, near the lower limit of detection of our assay). NaCl at concentrations of 1 M and higher inhibited the activity, similar to what was reported for *Halobacterium cutirubrum*, where salt inhibits the malonyl-CoA : ACP transacylase step [2].

The results presented show that the general concept that fatty acid synthetases, both from halophilic and from non-halophilic microorgan-

isms, are strongly inhibited by salt, has to be modified. *Haloanaerobium praevalens* and other halophilic anaerobes of the family Haloanaerobiaceae combine a high internal salt concentration [6] with the need for synthesis of large amounts of straight-chain fatty acids [8]. Therefore it is not surprising that its fatty acid synthetase complex, similar to other enzyme systems [7] is active also in the range of salt concentrations occurring inside the cells. A thorough comparison of the molecular structure of the enzyme complex from anaerobic halophiles with that of other bacteria may yield information on the mechanism enabling anaerobic halophiles to synthesize fatty acids also in the presence of high salt concentrations.

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