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Acylation of proteins of the archaebacteria *Halobacterium cutirubrum* and *Methanobacterium thermoautotrophicum*

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Abstract

Although the membrane lipids of extremely halophilic archaebacteria are exclusively derived from diphytanylglycerol diether, which is non-acylated, small amounts of fatty acids have been detected in these organisms. These fatty acids are formed by the action of a fatty acid synthase (FAS), shown to be present in the extreme halophile *Halobacterium cutirubrum*, despite the fact that only a fraction of the activity of FAS remains at the high salt concentration (> 4 M) present in the cytoplasm. It has now been demonstrated that fatty acids do not occur in lipid-bound form but largely in the form of acylated proteins in the red membrane of *H. cutirubrum*. In contrast, the bacteriorhodopsin of the purple membrane of this extreme halophile does not appear to be acylated. The thermophilic methanogen, *Methanobacterium thermoautotrophicum* had a much higher fatty acid synthase activity than the extreme halophile, and the synthase activity of the methanogen was optimal under its normal (anaerobic) growth conditions. The methanogen also utilized the resulting fatty acids to acylate its membrane proteins. The major fatty acids in both organisms were palmitic and stearic acids with small amounts of myristic and 18:1 acids, and these were bound to protein through both ester and amide linkages.

Keywords: Fatty acid; Fatty acid synthetase; Acylated protein; Purple membrane; Red membrane; (Archaebacterium)

1. Introduction

Membrane lipids of archaebacteria ('Archaea') [1,2] contain chemically stable ether bonds [3–5] instead of ester bonds found in phospholipids and glycolipids of eubacteria and other organisms. The polar lipids of the extremely halophilic archaea, e.g., *Halobacterium cutirubrum*, consist exclusively of derivatives of the dialkylglycerol diether, 2,3-diphytanyl-*sn*-glycerol [3] whereas those of methanogenic archaea, e.g., *Methanobacterium thermoautotrophicum*, consist of derivatives of both diphytanylglycerol diether and its dimer, dibiphytanyldiglycerol tetraether [4–9]. These ether-linked lipids presumably have a survival value for archaebacteria which grow in harsh environments [4,5]. In addition to the ether-linked lipids, a small amount of fatty acid has been detected in *H. cutirubrum* [10], suggesting that a fatty acid synthase (FAS) system might be operative in this organism. Support for this hypothesis was obtained by the finding that whole cells of *H. cutirubrum* incorporated [1-^{14}C]acetate into fatty acids [11], and by the isolation from this organism of a cell-free enzyme system that catalyses the biosynthesis of fatty acids from acetyl-CoA and malonyl-CoA and is strongly inhibited by high salt concentration [12]. The existence of fatty acids in extreme halophiles, although in small amounts, raised the question as to their cellular function in these organisms.

Recent reports that fatty acids are covalently linked to many membrane proteins [13–15], including mammalian rhodopsin [16,17], suggest that fatty acid acylation of proteins may be a feature of membrane proteins that is common to all organisms including the archaea. These findings led us to investigate whether there are membrane proteins in *H. cutirubrum* that are acylated by the fatty acids known to be present in this organism. It was antici-

Abbreviations: FAME, fatty acid methyl ester; FAS, fatty acid synthase; PAGE, polyacrylamide gel electrophoresis; PG, phosphatidylglycerol (diether analogue); PGP-Me, phosphatidylglyceromethylphosphate (diether analogue); PGS, phosphatidylglycerosulfate (diether analogue); SDS, sodium dodecyl sulfate; S-TGD-1, sulfated triglycosyl diphytanylglycerol.

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pated that bacteriorhodopsin, by analogy with mammalian rhodopsin [16,17], might also be acylated, and our studies therefore included an examination of the purple membrane as well as the red membrane of *H. cutirubrum*.

The question also arose whether fatty acids (protein-bound or otherwise) are present in other archaea, such as methanogens, and whether these archaea also contain an active fatty acid synthase. The existence of fatty acids in methanogens, particularly *Methanobacterium thermoautotrophicum* has already been documented [6], but the presence of acylated proteins or of an active fatty acid synthase has not yet been established. We have therefore investigated these points in the thermophilic methanogen, *Methanobacterium thermoautotrophicum* by the same approach used for *H. cutirubrum* [11,12].

We report here the finding of covalently-bound fatty acids in the red membrane but not in the purple membrane of the extreme halophile, *H. cutirubrum*, and provide evidence for the existence of an active fatty acid synthase in the methanogen *M. thermoautotrophicum*, and for the formation of fatty acids covalently-bound to proteins in this synthase system.

2. Materials and methods

Fatty acid methyl ester (FAME) standards were from NuChek (Elysian, MN); SP2330 GLC liquid phase from Supelco (Bellefonte, PA) and [1(3)-^{14}C]malonyl-CoA from New England Nuclear (Wilmington, DE). Acyl carrier protein from *Escherichia coli* was a product of Sigma (St. Louis, MO).

2.1. Culture of organisms

Growth of *H. cutirubrum* cells for preparation of purple and red membranes was carried out in standard 4 M NaCl medium for extreme halophiles, as described elsewhere [18]. Cells were harvested by centrifugation and washed with 4 M NaCl.

Cultures of *M. thermoautotrophicum* (ATCC 29183) cell lines started from single colonies were transferred to the liquid medium described previously [19], and cultured at 60°C in the anaerobic hood. Growing cells were then serially transferred and used to inoculate a 75-litre fermenter (New Brunswick Scientific, Edison, NJ, USA). After 36–48 h, cells were harvested in a continuous-flow high-speed centrifuge (New Brunswick Scientific) under a flow of N_2. Cells were stored in sealed flasks at 0–2°C under CO_2/H_2 (1:4, v/v).

2.2. Isolation of purple and red membranes of H. cutirubrum

The purple and red membranes were isolated from osmotically disrupted cells of *H. cutirubrum* by centrifugation on a discontinuous sucrose gradient following the method of Oesterhelt and Stoeckenius [20], as modified by Kushwaha et al. [18]. In some experiments, where indicated, the red membrane was re-purified by a second passage through the sucrose density gradient.

The purity of the purple and red membranes was determined by absorbance spectroscopy and SDS-PAGE, as described previously [18]. Purified purple membrane showed the absorption spectrum typical of bacteriohodopsin with maxima at 565 and 275 nm (absorbance ratio of 1:2, respectively), and exhibited a single band with an apparent molecular mass of 19500 (M_r = 26000) on SDS-PAGE. These results indicate a high degree of purity of the purple membrane [18,20,21]. The purified red membrane had carotenoid absorbance maxima at 535, 500 and 470 nm (characteristic of bacterioruberines [18]), and showed 6 major protein bands on SDS-PAGE, as described previously [18]. In the experiments reported here, the apparent molecular masses of the red membrane proteins on SDS-PAGE ranged from: band 1, 10 kDa; band 2, 14 kDa; band 3, 16 kDa; band 4, 21 kDa; band 5, 36 kDa; and band 6, 62 kDa with an average molecular mass of 26.5 kDa. The membrane preparations were also characterized by their lipid composition [18,21]. Both the red and purple membranes contained phosphatidylglycerolmethylphosphate (PGP-Me) (see Ref. [22]) and phosphatidylglycerol (PG). In addition to these phospholipids, the purple membrane contained retinal and two sulfated lipid components, the glycolipid sulfate (sulfated triglycosyldiphytanylglycerol, S-TGD-1) and phosphatidylglycerosulfate (PGS), which were absent in the red membrane, as reported previously [18]. The red membrane used in these studies contained no trace of the two unidentified glycolipids seen earlier [18].

2.3. Separation of protein and lipid components of membranes

The protein and lipid components of the membrane preparations were separated by a modified Bligh and Dyer procedure [23], as follows. To 2 ml of a suspension of the membranes (containing 12–16 mg protein) was added 7.5 ml of chloroform/methanol (1:2, v/v); the contents were thoroughly mixed and allowed to stand at 4°C for 1 h. The protein pellet was collected by centrifugation and washed twice with 7.5 ml of chloroform/methanol (1:2, v/v). The lipid components were isolated from the combined chloroform/methanol extracts by formation of a biphasic system (chloroform and methanol/water (10:9, v/v)),as described elsewhere [23]. A known quantity (120 nmol) of an internal standard, methyl heneicosanoate (21:0 FAME), was then added to the protein pellet and to the total lipid extract. A solvent blank containing 2 ml water (plus 120 nmol of 21:0 FAME) was also prepared and carried through the entire extraction procedure.

2.4. Determination of fatty acid composition of protein and lipid components of membranes

The protein and lipid components were hydrolysed separately, as indicated in the legends of the tables, either by: (a) 0.6 M methanolic-HCl for 2 h at 75–80°C for ester/thioester-bound acyl groups; (b) 2 M methanolic-HCl for 20 h at 75–80°C for amide-bound acyl groups; or (c) 6 M aqueous HCl in a sealed glass tube in vacuo for 20 h at 125°C for amide-bound or more strongly bound acyl groups. After acid hydrolysis, the reaction mixtures were adjusted to pH 10 by the addition of 8 M NaOH, and were heated again for 1–2 h at 80°C. The unsaponifiable fraction was removed by extraction with petroleum ether, and after acidification with 6 M aqueous HCl, free fatty acids were extracted with the same solvent and converted to methyl esters by heating in 0.6 M methanolic-HCl at 80°C for 1–2 h [23]. The FAMEs were analyzed by GLC on a column of 10% SP2330 at 180°C and quantitated using the previously added 21:0 FAME as internal standard [23]. Values were corrected for the solvent blank. Note that all reactions, except for (c), were carried out in screw-cap (Teflon-lined) tubes (20 ml) heated in a block heater [23]. Methanolic-HCl was prepared by adding an appropriate amount of concentrated (12 M) HCl to methanol to achieve the desired concentration.

2.5. Preparation of fatty acid synthases (FAS) and ACPs

FAS from *E. coli* and *H. cutirubrum* were prepared as described previously [12], but modified as follows: Cells of *E. coli* (10 g wet weight) were suspended in 10 ml of 0.1 M phosphate buffer (pH 7.4) containing 1 mM 2-mercaptoethanol and cells of *H. cutirubrum* (10 g wet wt.) were suspended in the same buffer containing 4 M NaCl. All subsequent operations were performed as described previously [12], except that the final dialysis of the *H. cutirubrum* synthase was against 0.1 M phosphate buffer (pH 7.4) containing 1 mM 2-mercaptoethanol and 4 M NaCl.

The FAS from *M. thermoautotrophicum* was prepared by the same method as for *H. cutirubrum* except that all operations were done in an anaerobic hood under an atmosphere of hydrogen.

Acyl carrier protein from both *H. cutirubrum* and *M. thermoautotrophicum* was prepared under aerobic conditions by the isopropanol extraction method of Rock and Cronan [24], modified as follows: Cells of *M. thermoautotrophicum* (10 g wet weight) were suspended in 10 ml of 0.1 M phosphate buffer (pH 7.4) containing 1 mM 2-mercaptoethanol; cells of *H. cutirubrum* (10 g wet wt.) were suspended in the same buffer plus 2-mercaptoethanol, containing 4 M NaCl. The cell suspensions were sonicated under aerobic conditions as described previously [12], centrifuged at $40\,000 \times g$ for 1 h and the supernatant solutions were heated at 100°C for 30 min. Denatured protein was removed by centrifugation and the heat-stable supernatant solutions were cooled to 4°C adjusted to pH 3.0 and stirred at 4°C for 30 min. The ACP precipitates were collected by centrifugation, redissolved in 0.1 M phosphate buffer (pH 7.4) plus 1 mM 2-mercaptoethanol and dialysed overnight against the same buffer. The dialysates were then diluted with an equal volume of 2-propanol and the mixtures were stirred at 4°C for 1 h. Denatured protein was removed by centrifugation and the supernatant solutions containing ACP were flash-evaporated at room temperature to remove 2-propanol, dialysed against 1 mM 2-mercaptoethanol and stored at −20°C.

ACP from *H. cutirubrum* was assayed with the *E. coli* fatty acid synthase as described previously [12]. ACP from *M. thermoautotrophicum* was assayed by the same procedure [12] but using the synthase from *M. thermoautotrophicum*.

The ACP from *H. cutirubrum* showed a single band on SDS-PAGE with apparent molecular mass, after reduction with 2-mercaptoethanol or dithiothreitol, of 27 ± 0.5 kDa, which was essentially unchanged (28 kDa) after alkylation with iodoacetamide to reduce dimers to monomers. For comparison, the *E. coli* ACP had an apparent molecular mass of 19.4 ± 0.8 kDa after reduction with 2-mercaptoethanol and alkylation with iodoacetamide (M_r of *E. coli* ACP: calc. for the monomer, 8847 Da [25]). Thus, under the conditions used here for SDS-PAGE, the *E. coli* ACP appears to run as a dimer, whereas the *H. cutirubrum* ACP appears to run as a trimer. The ACP from *M. thermoautotrophicum* showed one major and two minor bands on SDS-PAGE with apparent molecular masses of 37 kDa, 34.9 kDa and 32 kDa, respectively, after reduction with either 2-mercaptoethanol or dithiothreitol. The major band may represent a tetramer.

2.6. Assay of fatty acid synthases

Fatty acid synthases were assayed by measuring the incorporation of ^{14}C from [1(3)^{14}C]malonyl-CoA into fatty acids [12], except that at the end of the incubation, the reaction products were separated into 'ester/thioester-bound' and 'amide-bound' ^{14}C-labelled fatty acids as follows. The reaction products were hydrolysed by alkali as described [12]; the unsaponifiable fraction was removed by extraction with petroleum ether, and after acidification with concentrated HCl, 'ester/thioester-bound' ^{14}C-labelled fatty acids were isolated by a second petroleum ether extraction. To isolate the 'amide-bound' fatty acids, the reaction mixture was then diluted with an equal volume of 4 M methanolic HCl and hydrolysed at 80°C for 20 h. The reaction mixture was neutralized with NaOH and salt was removed by centrifugation. The clear supernatant was removed, diluted with an equal amount of 0.6 M NaOH and heated at 80°C for 1 h. After removal of the unsaponifiable fraction by extraction with petroleum ether,

the reaction mixture was acidified, and ^{14}C-labelled fatty acids which had been amide-bound were isolated by petroleum ether extraction. 'Ester-bound' and 'amide-bound' ^{14}C-labelled fatty acids were counted in a Beckman scintillation counter; counts were corrected for background, quenching and counting efficiency.

^{14}C-labelled products were further identified as free fatty acids by TLC on silica gel H plates in petroleum ether/diethyl ether (80:20, v/v), or in petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v), followed by autoradiography and staining with Rhodamine 6G [23]. Further analysis of these ^{14}C-labelled fatty acids by radio-GC or by argentation-TLC was not performed because of insufficient material.

2.7. Analytical methods

Protein was determined according to the method of Lowry et al. [26] and SDS-PAGE by the method of Weber and Osborn [27] on 12% polyacrylamide gels.

3. Results

3.1. Quantitative determination of the amounts of fatty acids in protein and lipid components of membranes

The purple and red membranes were separated into protein and lipid components, and both components were hydrolysed with 0.6 M methanolic, 2 M methanolic and 6 M aqueous HCl, as described in Methods and materials, to release the 'ester-bound' and 'amide-bound' fatty acids. Hydrolysis in 0.6 M methanolic HCl at 75°C for 2 h was sufficient to release all of the fatty acids from the lipid components, but for the protein components 2 M methanolic HCl at 75°C was required; hydrolysis with 6 M HCl did not result in any further release of fatty acids. Therefore, in subsequent experiments, the lipid and protein components were hydrolysed only with 0.6 M and 2 M methanolic HCl, respectively.

The amount of fatty acid in the protein and lipid components of the purple and red membrane of *H. cutirubrum* was determined by means of an internal standard, and related to the protein content of the corresponding membrane, after correction for fatty acids in the blank. Although some fatty acid could be detected in both the protein and lipid components of the purple membrane, the actual amount of fatty acid was very low and negligible relative to the amount of protein in the purple membrane (molar ratio fatty acid/protein, 0.026 and 0.067 for protein and lipid components, respectively) (Table 1). These results indicate that at most about 3 in 100 molecules of the purple membrane protein are acylated with one molecule of fatty acid and that the fatty acid content of this membrane, both protein-bound and lipid-bound, is too low to account for the amount of fatty acid known to be present in whole cells of *H. cutirubrum* [10–12].

The red membrane, on the other hand, did contain significant amounts of fatty acids which were bound to the protein but not to the lipid components (Table 1). In the red membrane prepared as described in Materials and methods (Table 1, preparation 1) 88% of the total fatty acid was protein-bound; after further purification (Table 1, preparation 2), 96% of the fatty acid was found to be protein-bound. The fatty acid/protein molar ratios in preparations 1 and 2 of red membrane were 0.87 and 1.3, respectively, based on an average protein molecular mass of 26.5 kDa. However, since the red membrane contains at least six protein components, the actual mol of fatty acid per mol protein for each of the six protein components cannot be reliably calculated from this data. The data do show, however, that the red membrane contains significant levels of protein-bound fatty acid, but essentially little or no lipid-bound fatty acid (Table 1).

3.2. Fatty acid composition of purple and red membranes of H. cutirubrum

The major protein-bound fatty acids in the red membrane were palmitic and stearic acids, with small amounts of myristic and 18:1 acids and traces of lauric and pentadecanoic acids (Table 2). No significant differences were observed in composition of the protein-bound fatty acids of the red and purple membranes, with the possible excep-

Table 1
Relative amounts of fatty acid in protein and lipid components of membranes of *H. cutirubrum*

Membrane	Membrane protein		Fatty acid in protein			Fatty acid in lipid		
	μg	nmol	μg	nmol	molar ratio FA/protein	μg	nmol	molar ratio FA/protein
Purple	12	462 [b]	3	12 [d]	0.026	8	31 [d]	0.067
Red								
Prepn. 1	25	943 [c]	210	820 [d]	0.87	28	109 [d]	0.116
Prepn. 2 [a]	15	566 [c]	192	750 [d]	1.33	8	31 [d]	0.055

[a] Preparation 2 was re-purified by a second sucrose density gradient centrifugation.
[b] Based on molecular mass of 26 kDa for bacteriorhodopsin [20].
[c] Based on average molecular mass of 26.5 kDa.
[d] Based on average molecular weight of 256 or 262 for lipid- or protein-bound fatty acids, respectively, estimated from data in Table 2.

Table 2
Fatty acid composition of protein and lipid components of purple and red membranes *H. cutirubrum* [a]

Chain	Purple membrane		Red membrane	
	protein-bound	lipid-bound	protein-bound	lipid-bound
12:0	4.0± 0.5	7.2±1.2	5.8±0.4	5.3±0.5
14:0	14.2± 1.5	12.5±1.0	9.9±0.7	13.0±0.3
15:0	4.3± 0.4	4.5±0.2	6.2±0.4	3.7±0.3
16:0	42.5±10.0	46.5±7.6	39.6±4.2	48.4±5.4
18:0	26.3± 5.0	17.5±0.5	26.3±3.1	14.8±0.7
18:1	11.5± 1.1	11.5±0.6	12.2±0.5	14.8±0.6

[a] Protein components were hydrolyzed with 2 M methanolic HCl for 20 h at 75°C and lipid components with 0.6 M methanolic HCl for 2 h at the same temperature. Data are corrected for the solvent blank and are given as mol% (means ± S.D., $n = 3$ independent experiments).

tion of a somewhat higher content of protein-bound myristate in the purple membrane (Table 2).

The lipid-bound fatty acids of both the purple and red membranes had essentially the same composition which did not differ significantly from that of the protein-bound fatty acids except for a somewhat lower stearic acid content in the lipid-bound fatty acids (Table 2).

3.3. Products of fatty acid synthases

The reaction products of the fatty acid synthase preparation from *H. cutirubrum* were hydrolysed with alkali, then by strong acid to release 'ester/thioester-bound' and 'amide-bound' fatty acids, respectively. As expected from previous studies [12], ester- or thioester-bound fatty acids were released from the reaction products by alkali (Table 3). An almost equal portion of fatty acid was released only after further strong acid hydrolysis under conditions required to hydrolyse amide-bonds (Table 3). Amide- or ester-bound fatty acids showed identical mobilities on TLC corresponding to authentic fatty acids or FAME, and neither contained spots corresponding to unsaponifiable material such as diphytanyl glycerol ether.

Similar results were obtained with the fatty acid synthase of *M. thermoautotrophicum* (Table 3). The reaction products of the synthase of this methanogen could also be separated into ester- and amide-bound fatty acid. The rate of synthesis of both ester- and amide-bound acids was higher when the reaction was carried out in an atmosphere of nitrogen at 60°C (comparable to normal culture conditions for the methanogen) than at 37°C or in oxygen at either temperature. The synthase from *M. thermoautotrophicum*, under all reaction conditions, was dependent on the addition of ACP but did not require reduced NADPH or acetyl-CoA; in fact NADPH appeared to be inhibitory. In contrast, the *H. cutirubrum* synthase was independent of the addition of ACP or NADPH (Table 3). It is noteworthy that the specific activity of the methanogen synthase is about ten times that of the *H. cutirubrum* synthase under the same conditions.

The relative amounts of the ester- and amide-bound products with the methanogen synthase ranged from 58–61% and 39–48%, respectively, depending on the incubation system used, with the exception of the system containing O_2 at 37°C in the absence of ACP. In the latter system the amount of amide-bound acids (56%) exceeded that of ester (0- and S-) bound acids (44%), whereas in the other systems the amount of ester-bound acids was much greater than the amide-bound acids.

Table 3
Specific activities and products of fatty acid synthases[a]

System	*H. cutirubrum*		*M. thermoautrophicum*	
	ester-bound fatty acid	amide-bound fatty acid	ester-bound fatty acid	amide-bound fatty acid
O_2, 37°C				
Complete	124 (46%)	144 (54%)	1340 (61%)	870 (39%)
Complete minus ACP	123 (47%)	137 (53%)	496 (44%)	638 (56%)
Complete minus NADPH	130 (47%)	145 (53%)	–	–
O_2, 60°C				
Complete	–	–	1460 (58%)	1060 (42%)
Complete minus ACP	–	–	510 (58%)	370 (42%)
N_2, 37°C				
Complete	–	–	1699 (58%)	1573 (48%)
Complete minus ACP	–	–	690 (61%)	440 (39%)
N_2, 60°C				
Complete	–	–	1924 (60%)	1257 (40%)
Complete minus ACP	–	–	690 (61%)	440 (39%)
Complete minus NADPH	–	–	3207 (60%)	2095 (40%)
Complete minus acetyl-CoA	–	–	1890 (61%)	1230 (39%)

[a] Data are corrected for the zero-time control and are given as pmol per 30 min per mg enzyme protein. The dash indicates 'not determined'. Numbers in parentheses are the mol% of total fatty acid. FA synthase activities were asayed as described in Ref. [12].

4. Discussion

The data presented here indicate that the archaebacteria *H. cutirubrum* and *M. thermoautotrophicum* contain fatty acids that are covalently linked to protein. This was shown by the separation of red and purple membranes of *H. cutirubrum* into protein and lipid components and the demonstration that fatty acid is localized largely in the protein components of the red membrane with little or no fatty acid in the purple membrane protein (Table 1). Although the nature of the linkage was not studied in detail in these studies, the fact that strong acid hydrolysis was required to release the protein-bound fatty acids suggests that the fatty acids are largely amide-linked to the protein (Tables 1 and 2). The presence of O- and/or S-linked fatty acids in the red and purple membranes is unlikely because no fatty acids were released by mild acid hydrolysis.

Further evidence for protein-bound fatty acids was obtained from examination of the products of the FAS of *H. cutirubrum* and *M. thermoautotrophicum* (Table 3). The products of the synthases of both organisms could be separated into ester-bound (mild alkali-labile) and amide-bound (strong acid-labile) fatty acids (Table 3). The mild alkali-labile (ester-bound) fatty acids are presumably thioester-bound to ACP and the condensing enzyme, as in the *E. coli* FAS [28]. The strong acid-labile (amide-bound) fatty acids accounted for about 53–54% of the total fatty acid products of the *H. cutirubrum* synthase and for 39–56% of the products of the *M. thermoautotrophicum* synthase. The amide-bound fatty acids were assumed to be protein-bound since the small amount of fatty acid present in the extractable lipid fraction of *H. cutirubrum* membranes was only ester-bound, being released entirely on mild acid hydrolysis and no fatty acids were released on further strong acid hydrolysis necessary to break amide bonds (Tables 1 and 2). Also, no amide-bound fatty acids have been reported in lipids of either *H. cutirubrum* [3,4] or *M. thermoautotrophicum* [4–6].

The present findings raise questions concerning the type of FAS system present in archaebacteria. On the basis of the isolation procedure used for preparation of the archaeal FAS complexes and their similarity to the *E. coli* FAS (e.g., inhibition by high salt concentration; see Ref. [12] for halophile FAS), both the extreme halophile and the methanogen examined here appear to have the bacterial Type II FAS (Table 3, see Ref. [29]). The fatty acid pattern observed for the halophile (Table 2), although unusual in containing 18:1 but not 16:1 acids, more closely resembles that for bacteria than for higher organisms [29]. The major fatty acids of the methanogen, although not examined here, have been reported to be 18:0, 18:1, 17:0, 16:0, 15:0, 17:0-br and 15:0-br [6], clearly a bacterial pattern and similar to that for the halophile. The presence of the 18:1 acid rather than the 16:1 acid, at least for the methanogen, would be consistent with chain elongation at elevated temperatures of 16:1 to 18:1 by a β-ketoacyl-ACP synthetase II, as in *E. coli* [29]. However, it should be noted that the halophile and methanogen FAS differ from each other and from the *E. coli* FAS with respect to the requirement for ACP and/or NADPH (Table 3 and Ref. [12]). While ACP is clearly dissociable at high temperatures from the FAS of both the halophile and the methanogen, as for *E. coli*, the halophile ACP appears to be much more tightly bound than that of either the methanogen or *E. coli*, at optimal temperatures for FAS activity (Table 3 and Ref. [12]). On the other hand, the archaeal FAS systems appear to have adequate amounts of bound NADPH or other reducing cofactors and are independent of added NADPH (Table 3). These observations raise the question whether the archaeal FAS systems (including ACP) have evolved from bacterial FAS during adaptation to their 'harsh' environments. Further study of this point is required.

The present finding of protein-bound fatty acids in archaebacteria offers an explanation for the function of the small amounts of fatty acid previously found in *H. cutirubrum* [10–12], and in several methanogens including *M. thermoautotrophicum* [5,6]. Presumably acylation of some of the red membrane-bound proteins in *H. cutirubrum* serves to stabilize the membrane by increased protein–lipid interaction. In the purple membrane, the absence of long chain acylation of bacteriorhodopsin is consistent with the absence in this polypeptide of any cysteine residues [18,20], which are the acylated residues in mammalian rhodopsin [17]. The present data further shows that N-acylation of other amino acid residues in bacteriorhodopsin does not occur. The bacteriorhodopsin would appear to be sufficiently hydrophobic to interact adequately with lipid and form a stable purple membrane without acylation.

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