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Palmitic acid is associated with halorhodopsin as a free fatty acid Radiolabeling of halorhodopsin with ^{3}H-palmitic acid and chemical analysis of the reaction products of purified halorhodopsin with thiols and $NaBH_4$

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Abstract

Halorhodopsin, isolated from *Halobacterium salinarium* cells incubated with tritiated palmitic acid, co-elutes with labeled palmitate in phenylsepharose CL-4B chromatography. Halorhodopsin-bound ^{3}H-palmitate is not readily displaced by prolonged exposure to a large excess of detergents and by re-chromatography of radiolabeled halorhodopsin on phenylsepharose. On other hand, the association of labeled palmitate with purified halorhodopsin is not resistant to denaturation induced either by isopropanol/hexane or by SDS gel electrophoresis. We have tested the hypothesis that tightly associated palmitate is bound to halorhodopsin through a thioester bond, which is unstable in denaturing conditions. Using GC/MS, we have analysed the reaction products of native halorhodopsin with specific thioester reagents, thiols and $NaBH_4$, which are inactive on free fatty acids. The results of this analytical approach indicate that there is no thioester bond between halorhodopsin and palmitic acid and that palmitic acid is associated with halorhodopsin as a free fatty acid. © 1998 Elsevier Science B.V.

Keywords: Halorhodopsin; Palmitic acid; Free fatty acid; (Archaea)

1. Introduction

Fatty acids are only minor lipid components of Archaea, representing less than 1% of the total cell lipids. Recent studies have shown that in Archaeal cells fatty acids are only used to acylate membrane proteins [1].

In previous work [2] we have shown that *Halobacterium salinarium* cells are able to synthesize palmitate, and that this fatty acid is associated with purified halorhodopsin. The quantitative analysis of palmitate associated with native halorhodopsin indicated that from one to two fatty acid molecules per protein molecule were present. Furthermore, we reported that palmitate strongly affects photoreactivity and deprotonation in the dark of the Archaeal chloride pump.

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Here we present further evidence of the association of palmitate with halorhodopsin and we clarify the nature of the interaction between this protein and fatty acids.

This report demonstrates that purified halorhodopsin, isolated from halobacterial cells incubated with tritiated palmitate, appears to be labeled with radioactive palmitate.

To determine if this association represents acylation we have used a chemical approach in which we examine the effects of specific reagents of thioesters on this association.

Because of previous data indicating that fatty acids are removed from halorhodopsin after denaturation, for the present investigation we have selected a couple of specific thioester reagents that are unable to react with free fatty acids and that, therefore, could be used on native halorhodopsin, namely thiols and $NaBH_4$.

In the past, these two reagents have been largely used to characterize the chemistry of bacteriorhodopsin [3] and bovine rhodopsin [4].

Hydroxylamine, another typical reagent of thioesters, was not suitable because it easily reacts with free fatty acids.

Chemical analyses of the reaction products of native halorhodopsin with thiols and $NaBH_4$ were performed by means of GC/MS.

2. Materials and methods

2.1. Materials

An engineered *H. salinarium* strain overexpressing halorhodopsin, kindly offered by Richard Needleman, was used in this study. These cells have an insertion in the gene encoding bacteriorhodopsin and therefore produce no bacteriorhodopsin. The growth medium, containing neutralized peptone (L 34, Oxoid) and novobiocin (from Serva, at final 1 μg ml^{-1}), was prepared as previously described [5].

[9,10-^{3}H]Palmitic acid (50 Ci $mmol^{-1}$) was from Amersham. DNase and *n*-octyl-β-glucopyranoside (octylglucoside) were from Sigma, sodium cholate from Serva and phenylsepharose CL-4B from Pharmacia. Bis-(trimethylsilyl)trifluoroacetamide (BSFTA) was from Supelco.

2.2. Halorhodopsin isolation and radiolabeling

A concentrated suspension of halobacterial cells in 4 M NaCl (10 ml) was incubated with ^{3}H-palmitic acid (200 μCi) in ethanol (1% final) at 37°C for 1 h with shaking.

Cells were then disrupted by dialysis overnight in the cold room. Membranes (containing about 2 mg of halorhodopsin) were collected and washed twice by centrifugation; membrane solubilization was obtained by incubation at RT for 1 h with 5% cholate buffer containing 4 M NaCl, buffered with 25 mM Tris/HCl pH 7.2. The extract, obtained after spinning the solubilized membranes at $100{,}000 \times g$ for 1 h in an ultracentrifuge, was incubated for 1 h at RT with phenylsepharose CL-4B, pre-equilibrated in cholate buffer, containing 4 M NaCl, 25 mM Tris/HCl pH 7.2, 0.4% sodium cholate. At the end of the incubation, the gel appeared purple and no halorhodopsin was left in the buffer. The binding capacity of the phenylsepharose gel in our experimental conditions was found to be 5 mg of halorhodopsin per 1 ml of settled gel. In a typical experiment about 150 μl of gel was used. The purple gel was then poured in an insulin syringe and washed with 14 ml cholate buffer; the washing was interrupted after checking that no significative radioactivity was present in the washing fractions. The column was then eluted with octylglucoside buffer (4 M NaCl, 25 mM Tris/HCl pH 7.2, 0.5% octylglucoside); about 20 fractions, 300 μl each, were collected from a column packed with about 150 μl of gel.

The absorbance at 570 and 280 nm of each fraction was measured and its radioactivity was determined by counting 50 μl aliquots of each fraction.

2.3. Binding of ^{3}H-palmitate to phenylsepharose CL-4B in the absence of halorhodopsin

In a parallel experiment, the ability of phenylsepharose CL-4B to bind ^{3}H-palmitate in the absence of halorhodopsin was tested. ^{3}H-palmitate (in 5% cholate buffer and at the same specific activity as that of the extract) was incubated with phenylsepharose under the same experimental conditions used for the sample. The amount of ^{3}H-palmitate bound to the gel in the absence of halorhodopsin was subtracted from the sample.

2.4. SDS–PAGE of radiolabeled halorhodopsin

^{3}H-palmitic acid labeled halorhodopsin (50 μg) was analysed by SDS–PAGE. A 15% acrylamide gel was run at 10°C and the protein visualized by staining with Coomassie Blue. The broad halorhodopsin band was excised and incubated overnight at 60°C with hydrogen peroxide (1 ml at 20%); the radioactivity of resulting solution was measured in a liquid scintillation counter.

2.5. Treatment with free thiols

Pentanethiol (20 μl) was added to halorhodopsin (200 nmol suspended in 1 ml water) under nitrogen flow; the sample was incubated overnight at 37°C under shaking. After this time the sample appeared to be completely bleached. An aliquot of halorhodopsin not treated with thiols was run in parallel as a control. The reaction was performed at room light. Lipids associated to the control and bleached samples were extracted by addition of isopropanol/hexane (1:2, v/v). After addition of organic solvents, the sample was vigorously shaken for 2 min on a vortex mixer and, after phase separation, the organic phase was saved. The extraction was repeated for two more times and hexane phases combined. Hexane was removed under nitrogen, the dried residue solubilized in ethylether (100 μl) and analysed by GC/MS.

2.6. Synthesis of pentanethiolpalmitate

To a stirred solution of pentanethiol (0.42 g, 4 mmol) and pyridine (0.24 g, 3 mmol) in anhydrous benzene (5 ml) palmitoyl chloride was added dropwise (0.56 g, 2 mmol) at room temperature. The reaction mixture was continuously stirred overnight. After filtration and evaporation of the solvent at reduced pressure, a yellow oil was obtained. The final product was characterized by GC/MS spectrometry. In the following, we report m/e values and relative heights (in brackets) of mass spectrum peaks (70 eV): 271 (12), 239 (63), 123 (7), 109 (13), 99 (7), 98 (9), 97 (14), 85 (28), 83 (22), 71 (49), 69 (28), 57 (78), 55 (71), 43 (100), 41 (68).

2.7. Reaction of $NaBH_4$ with palmitoylCoA, methylpalmitate and palmitic acid

$NaBH_4$ (10 mg) was added to palmitoylCoA or methylpalmitate or palmitic acid (30 μmol), solubilized in 1 ml water plus 300 μl tetrahydrofuran, and left for 15 min at 38°C.

The reaction of reductant with thioester was quenched by slowly adding 1 N HCl 700 μl, to destroy excess $NaBH_4$. The resulting mixture was extracted by $CHCl_3$ (1.5 ml for three times). The combined chloroform phases were dried under nitrogen and resolubilised in fresh chloroform (1 ml). One milliliter of water was added to the chloroform solution and it was vigorously shaken to remove salts from the chloroform phase. Finally, dried lipids were solubilised in pyridine (100 μl).

2.8. Reaction of $NaBH_4$ with halorhodopsin

A quantity of 10 mg $NaBH_4$ were added to 200 nmol of halorhodopsin resuspended in water (1% final), and reduction was carried out at room light as described above.

2.9. Derivatization of alcohols with BSFTA

BSFTA (100 μl) was added to the lipids extracted from reduced halorhodopsin and solubilized in pyridine; the derivatization was carried out at 100°C for 30 min. The silanised products were analysed by GC/MS.

2.10. GC / MS apparatus

GC/MS analyses were performed with a Hewlett-Packard instrument equipped with a mass selective detector MSD5970 and a SE-30 capillary column (30 m $\times$ 0.25 mm i.d.).

3. Results

3.1. Labeling of halorhodopsin with ^{3}H palmitic acid

Halorhodopsin superproducer *H. salinarium* cells were incubated with ^{3}H-palmitic acid.

The membrane extract obtained from these cells was incubated with phenylsepharose CL-4B gel. The

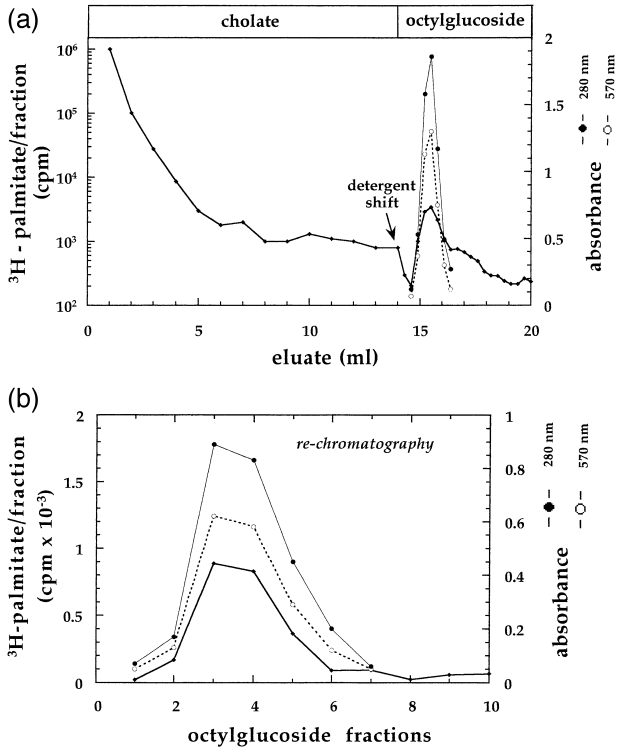

Fig. 1. Phenylsepharose CL-4B chromatography of halorhodopsin extracted from *H. salinarium* cells that have been incubated with ^{3}H-palmitic acid. (a) Co-elution of halorhodopsin and ^{3}H-palmitic acid. Radioactivity profile during washing and elution of phenylsepharose column is shown together with absorbances: absorbance at 570 nm (○) and 280 nm (●), radioactivity (♦). Details as in Section 2. (b) Rechromatography of radiolabeled halorhodopsin. Part of material collected from the chromatography in Fig. 1a (1.6 mg of HR, at specific activity 320 cpm/50 μl) was diluted 10-fold in 5% cholate buffer, incubated with gel and after shifting to octylglucoside halorhodopsin was recollected. Each fraction 300 μl. Absorbance at 570 (○) and 280 nm (●), radioactivity (♦).

resulting purple gel was washed in a syringe with 100 bed column volumes of cholate buffer, before shifting to octylglucoside buffer for halorhodopsin elution.

In parallel, as control, the same amount of phenylsepharose gel was incubated with cholate buffer containing ^{3}H-palmitate at the same specific activity as the extract (i.e., in the absence of halorhodopsin) and used as blank column; the radioactivity collected from the blank column was never higher than 10% of sample radioactivity.

In Fig. 1a, the radioactivity of sample fractions collected during cholate washing together with that of the octylglucoside fractions is reported on a semilogarithmic scale. It can be seen that washing with cholate removes all non-specific radioactivity bound to phenylsepharose CL-4B and that after shifting to octylglucoside buffer a peak of radioactivity is observed.

The radioactivity in octylglucoside fractions coeluted with the 570 nm absorbance of the chromophore and the 280 nm absorbance of the protein portion of halorhodopsin.

Similar results were obtained after repeating the experiment with halorhodopsin from *Natronobacterium pharaonis*.

3.2. Characteristics of ^{3}H-palmitic acid labeled halorhodopsin

^{3}H-palmitic acid labeled halorhodopsin was concentrated on an Amicon membrane (Microcon cut-off 10,000) and two volumes of fresh octylglucoside buffer were added to the protein; the sample was further concentrated and finally the volume was readjusted to the starting value. At the end of the ultrafiltration process, 85% of starting protein was recovered together with 70% of starting radioactivity; taking in account the small loss of protein, no more than 20% of radiolabeled palmitate was displaced from the halorhodopsin recovered after this experiment (see data in Table 1).

Another interesting piece of information on the resistance of halorhodopsin radiolabeling to long and

Table 1
Radioactivity associated with halorhodopsin before and after detergent exchange and after treatment with organic solvents

Sample	Radioactivity (%)
Halorhodopsin control	100
After detergent exchange[a]	70
Hexane phase after addition of isopropanol/hexane[b]	95

[a]A 300 μl aliquot of ^{3}H-labeled halorhodopsin in octylglucoside buffer was concentrated on an Amicon membrane (Microcon cut-off 10,000). One volume of octylglucoside buffer was added to the protein on filter and the protein was again concentrated; after repeating this step once, the volume was readjusted to 300 μl with octylglucoside buffer. Protein yield: 85%.

[b]Isopropanol/hexane 1:2 was added to another 300 μl HR aliquot (three times); after phase separation hexane phases were collected, dried under nitrogen and resuspended in 300 μl hexane.

harsh detergent exposure, was obtained from the re-chromatography of halorhodopsin.

Radiolabeled halorhodopsin fractions were pooled and diluted 10 fold with 5% cholate buffer. In the presence of such a large excess of cholate, we assume that the protein molecules are surrounded by cholate instead of octylglucoside. Moreover, after binding to phenylsepharose, halorhodopsin was washed on the gel with about 100 volumes of bed column of cholate buffer and finally halorhodopsin elution was obtained with octylglucoside buffer.

The re-chromatographated halorhodopsin fractions were found to be still associated with significative amounts of labeled palmitate (see Fig. 1b).

The yield of the second phenylsepharose chromatography was quite low, as only 60% of loaded protein was recovered and the ratio ^{3}H-palmitic acid/halorhodopsin after re-chromatography was found to be reduced to a half.

The results reported in Table 1 and in Fig. 1b indicate that the binding of ^{3}H-palmitate to halorhodopsin is pretty strong, as it is not easily removed by prolonged treatments with detergents.

On other hand, most of radioactivity found in association with purified halorhodopsin did not resist to treatment with organic solvents used to delipidate proteins, such as $CHCl_3/CH_3OH$ or hexane/isopropanol. Data in Table 1 confirm previous observations obtained by analysing the GC/MS fatty acid content of native and delipidated halorhodopsin [2].

Finally, an aliquot of labeled halorhodopsin was subjected to SDS polyacrylamide gel electrophoresis; the halorhodopsin band was excised and the radioactivity in the gel slice measured. Almost all the radioactivity originally present in the purple fractions was lost during SDS–PAGE (not shown).

3.3. Reaction of halorhodopsin with thioester reagents

Taken all together, data presented in Section 3.2 seem to indicate that the binding of palmitate to halorhodopsin is non-covalent.

However, considering that some covalent bonds are unstable in denaturing conditions—as is the case with the aldimine bond of retinal to lysine of G helix in halorhodopsin itself—it cannot be excluded that palmitate can bind halorhodopsin through a very reactive covalent bond which is unstable in denaturing conditions (e.g., a thioester bond).

In this section, we describe experiments done to test the hypothesis that there is a thioester bond between palmitate and halorhodopsin which has two cysteines.

Fatty acid acylation of membrane protein is routinely investigated by gas chromatography of derivatised fatty acids, which are released from delipidated protein after alkali or hydroxylamine treatment.

The previous data [2] and the data in Table 1 indicate that fatty acids associated to halorhodopsin are removed by denaturation. Here, we have used thioester reagents that are inactive on free fatty acids and are therefore suitable for such an analysis of native halorhodopsin.

Thioesters are known to be able to transfer the fatty acyl group to free thiols (transesterification). Therefore, if a thioester bond was present in halorhodopsin between protein cysteine residues and fatty acids, the products of reaction between halorhodopsin and thiols should be the thioester formed between free thiol and palmitate together with the deacylated protein.

The finding of such newly formed thioester among the reaction products of purified halorhodopsin with free thiols would be evidence for the presence of thioester in halorhodopsin.

In the present study, we have synthesized the expected product of the reaction of halorhodopsin with pentanethiol and used GC/MS analysis to detect it.

Although β-mercaptoethanol is the typical reagent for studying oxidized SH residues of proteins, in this study we have selected pentanethiol because it does not contain other functional groups potentially reactive in the course of thioester synthesis.

The characteristics of pentanethiolpalmitate (obtained from the reaction of palmitoyl chloride with thiol) were checked by means of IR, NMR and GC/MS spectroscopy (peaks and relative heights of pentanethiolpalmitate mass spectrum are reported in Section 2.6).

Furthermore in preliminary experiments, the effect of pentanethiol on chloride transport was tested in cell envelope vesicles, by following light induced alkalinization of the extravesicular medium. We found that pentanethiol has the ability to inhibit the pho-

toactivated Cl^- transport mediated by halorhodopsin to a similar extent as observed with β-mercaptoethanol.

The results of GC/MS analysis of the lipid extract obtained from pentanethiol treated halorhodopsin indicated that a thioester bond is not present in significant amounts in halorhodopsin (not shown).

$NaBH_4$ is a quite specific reagent used to determine the chemical nature of fatty acid–protein linkages, as thioester, but not oxyester, are rapidly cleaved; the fatty acid involved in the linkage is reduced to the corresponding long chain alcohol [6].

We have verified that in our experimental conditions: (a) palmitic acid is not reduced to the corresponding long chain alcohol by the reductant and (b) palmitoyl-S-CoA, but not methylpalmitate, is easily reduced, in agreement with a previous observation [7].

Fig. 2 reports the GC/MS of silanised lipid extract obtained after borohydride reduction of halorhodopsin. In chromatogram (Fig. 2A) a very small peak, having the retention time of silanised cetyl alcohol, is present (marked with **a**). The mass spectrum of this component (in Fig. 2B) was found to

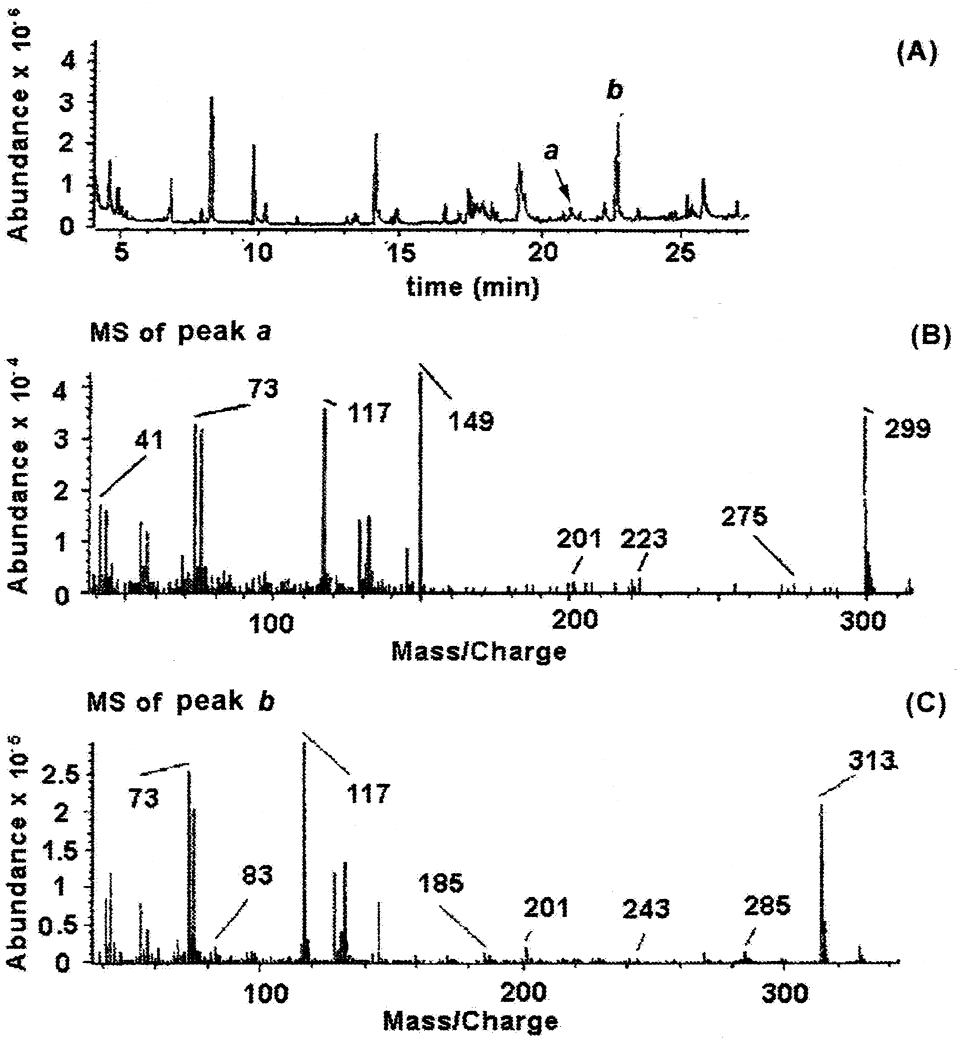

Fig. 2. GC/MS analysis of silyl derivatives of lipid extract obtained from $NaBH_4$ reduced halorhodopsin. (A) Chromatogram of silyl derivatives. Peak of silyl derivative of cetyl alcohol marked with (**a**), peak of unreduced silanised palmitic acid marked with (**b**). (B) Mass spectrum of peak (**a**). (C) Mass spectrum of peak (**b**).

be identical to that of the standard; in addition, a much bigger peak can be seen (marked with **b**). It has the retention time and the mass spectrum of silanised unreduced palmitic acid (Fig. 2C). These results indicate that most of the palmitate associated with purified halorhodopsin is present as free fatty acid.

4. Conclusions

In the first part of this work we have shown that tritiated palmitic acid and halorhodopsin co-elute in the course of phenylsepharose chromatography.

The association of tritiated palmitic acid with halorhodopsin was not easily removed by long treatments with detergents or by re-chromatography, but it did not resist to delipidation by means of organic solvents or by SDS–PAGE.

These results confirm previous observations [2] obtained with different techniques and seem to indicate that the association between halorhodopsin and palmitate is due to non-covalent binding.

This conclusion is supported by the analytical data of this paper which demonstrates that there is no thioester bond between halorhodopsin and palmitic acid, therefore excluding the possibility that the association of palmitate with halorhodopsin is due to protein palmitoylation.

Experiments are in progress to ascertain if the binding of palmitic acid to halorhodopsin as a free fatty acid has physiological significance.

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