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Role of palmitic acid on the isolation and properties of halorhodopsin

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Abstract

Purified halorhodopsin was isolated from *Halobacterium halobium* as previously described (Duschl, A. et al. (1988) J. Biol. Chem. 263, 17016–17022). Two purple bands were eluted from phenyl-Sepharose column, indicating the presence of differently retained halorhodopsin forms; the absorption spectra of the two halorhodopsin bands in the dark were not different. By gas chromatography/mass spectrometry we could identify palmitate (which is only a minor lipid component of archaeal cells) among lipids associated with purple fractions. Typically the palmitate content of the first eluted band was higher than that of the second, indicating a correlation between the palmitate content and the retention time; from one to two fatty acid molecules per halorhodopsin molecule were present depending on the fraction analysed. Very little or no palmitate was released from denaturated halorhodopsin. By adding palmitate to buffers used in the phenyl-Sepharose chromatography, only one sharp purple band was collected, corresponding to the less retained halorhodopsin fraction. Pentadecanoic fatty acid could also affect the halorhodopsin chromatography. Chromatography of halorhodopsin in the presence of β-mercaptoethanol showed only one band, corresponding to the more retained halorhodopsin form. The two halorhodopsin fractions had different photoreactivity; the less retained halorhodopsin fraction (at higher palmitate content) showed an higher rate of decay of the absorbance at 570 nm upon illumination. By following the decay of the absorbance at 570 nm upon addition of alkali in the dark, we found that the two halorhodopsin fractions had different pK_a values of deprotonation.

Keywords: Archaebacterium; Halophile; Halorhodopsin; Fatty acid

1. Introduction

Bacterial and animal rhodopsins share several structural and functional features: the polypeptide chain of both kinds of proteins traverses the membrane 7 times, the chromophore forms an aldimine bond with a lysine of the C-terminal intramembrane segment, similar intermediates have been described in the photocycle.

While it is known that bovine rhodopsin is fatty acid acylated [1,2], very little experimental work has been done to know whether bacterial rhodopsins are also acylated.

Although the presence of fatty acids in archaebacteria, as minor lipid components, has been demonstrated more than 20 years ago, only recently the functional role of fatty acids in these bacteria has been examined [3]. Palmitic, stearic and octadecenoic acids have been found in extreme halophiles and methanogenic bacteria; it has been demonstrated that these archaeal cells utilize fatty acids to acylate red membrane proteins of *Halobacterium cutirubrum*, while bacteriorhodopsin of the purple membrane did not appear to be acylated [4].

To our knowledge this is the only study in which the question of acylation of a bacterial rhodopsin has been examined.

There is now an increasing interest in the covalent modification of cell proteins by fatty acids because this feature is shared by a number of regulatory proteins, membrane receptors, signal transduction proteins and oncogen proteins [5] and appears to have a regulatory function on these proteins playing a key role in the control

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of cellular functions [6]. Furthermore the fact that the very little amount of fatty acids present in archaeal cells appears to be only utilized to acylate membrane proteins strongly suggests that this modification of polypeptide chain could be relevant for the protein function and therefore selected by evolution.

These considerations led us to examine the question of the acylation of bacterial rhodopsins, notwithstanding the previous negative result concerning bacteriorhodopsin.

Here we report data obtained investigating whether halorhodopsin, the Cl^- pump of halobacteria, is post translationally modified by the addition of fatty acids.

Two adjacent cysteine residues in the C-terminal cytoplasmic fragment of bovine rhodopsin are palmitoylated [2].

As regards halorhodopsin, Cys residues are absent in the C-terminal fragment, but there are serine and threonine residues which could be sites of acylation as well [7]. Furthermore the halorhodopsin sequences from various *Halobacterium halobium* strains show two well conserved intramembrane Cys residues [7,8] in D and E helices, whose function is still not well understood.

We isolated two different fractions of purified halorhodopsin and analysed them separately for the content of fatty acids by gas chromatography/mass spectrometry. Our data indicate that different amounts of palmitate are associated to the two different halorhodopsin bands at constant fatty acid/protein molar ratio.

The binding of palmitate to halorhodopsin, rather than being the consequence of random association between proteins and lipids during membrane solubilization, appeared to be highly specific, as indicated by a number of experiments showing that palmitate strongly influences chromatography, photoreactivity and deprotonation in the dark of halorhodopsin.

2. Materials and methods

2.1. Strains and growth media

Halobacterium halobium strains overexpressing halorhodopsin were used. Data here reported have been obtained using a wild-type (WT) strain kindly offered by R. Needleman (unpublished data). Similar results were obtained by analysing halorhodopsin isolated from D2 strain [9], available thanks to J. Tittor. Routinely the growth medium, containing neutralized bacteriological peptone (L 34, from Oxoid), was prepared as described in [10], except that the antibiotics novobiocin or mevinolin (1 μg/ml) were added, respectively, to WT or D2 strains to select for the halorhodopsin superproducer cells.

Furthermore, when specifically indicated, we used a growth medium of chemically defined composition, not containing fatty acids, as described in [11].

2.2. Halorhodopsin isolation and purification

Halorhodopsin was isolated following the method of Duschl et al. [10].

In brief, bacterial cells grown on peptone medium (3 liters, $A_{500} > 1$), after washing, were dialysed overnight against water; the purple membranes were collected by centrifugation, washed and solubilized with cholate in the presence of 4 M NaCl (30 ml of membrane suspension plus 10 ml of 20% cholate). After stirring for 30 min, the supernatant was separated by centrifugation ($A_{570} = 0.5$). The extract (about 200 nmol of HR) was then applied over a phenyl-Sepharose (Pharmacia) column 1.5×5 cm (10 ml of gel) previously equilibrated with cholate buffer containing 4 M NaCl, 25 mM Tris-Cl (pH 7.2), 0.4% sodium cholate. The column was washed with 300 ml or 150 ml of cholate buffer and then eluted with octyl glucoside buffer containing 4 M NaCl, 25 mM Tris-Cl (pH 7.2), 0.5% octyl glucoside.

Chromatography was performed at room temperature, in the dark or under dim light; the collection of the purple fractions (2.5 ml) started after elution of about 70 ml of octyl glucoside buffer. After the collection of purple fractions, phenyl-Sepharose was regenerated following the instructions given by Pharmacia.

The absorbance at 570 nm and 280 nm of each fraction was measured spectrophotometrically; fractions collected after one chromatographic step had typically a 280/570 absorbance ratio not higher than 1.5 and were analysed for the content of associated fatty acids without further purification.

2.3. Chromatography of halorhodopsin in the presence of fatty acids

To test the effect of saturated fatty acids (16:0) or (15:0) on the chromatographic process, columns were pre-equilibrated and washed with cholate buffer containing 15 μM fatty acid before elution with octyl glucoside buffer containing 780 μM fatty acid.

2.4. Determination of palmitic acid in halorhodopsin fractions

The protocol followed to determine fatty acids bound to halorhodopsin is schematically reported in Fig. 1; analyses were performed on both denaturated and native protein.

Immediately after the collection halorhodopsin was dialysed two times for 1 h against 100-fold volume of distilled water. The blue-purple precipitated protein was then collected by centrifugation and, when indicated, exposed to organic solvents to delipidate it.

Samples were vigorously mixed for 2 min after the addition of isopropanol/n-hexane (1:2, v/v) (or MeOH/$CHCl_3$ (2:1, v/v)) and the lipid phase separated from the aqueous phase containing the denaturated

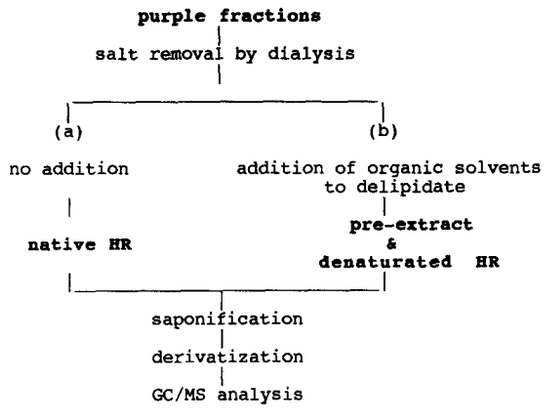

Fig. 1. Scheme of the protocol to determine fatty acids bound to native and denaturated halorhodopsin.

halorhodopsin. The extraction was repeated four times. All n-hexane (or $CHCl_3$) phases were combined (in the following named pre-extract) and dried under N_2.

Undenaturated (blue pellet after dialysis) or denaturated (white powder after lyophilization) halorhodopsin (sample size 30–100 nmoles retinal) and pre-extract were supplemented with 74 nmoles (200 ng/μl injected) of pentadecanoic fatty acid (15:0) as internal standard (IS) and then saponified with 1 M NaOH at 100°C for 2 h. Lipids released were extracted with MeOH/$CHCl_3$ four times; the $CHCl_3$ phases were dried under N_2 and subjected to methanolysis in 0.5 ml of methanolic 0.6 M HCl for 1 h at 100°C. Methyl derivatives were extracted with 1.5 ml n-hexane four times. The combined n-hexane phases were dried under N_2 and the residue solubilized in 100 μl n-hexane to be analysed by gas chromatography/mass spectrometry (GC/MS).

A VG Trio-2000 instrument (VG BIOTECH, Altrincham, UK) was used for GC/MS analysis. A SPB-5 fused silica capillary column (30 m, 0.20 mm i.d., 0.20 mm film thickness, Supelco), connected to an 'on-column' injector through a retention gap, was used with the following temperature program: 80°C for 2.5 min, linear to 180°C at 20 C°/min, linear to 280°C at 15 C°/min, isothermal at 280°C for 15 min to assure elution of all sample components. Helium was used as carrier gas. The mass spectrometer was operated in electron impact (EI) mode (70 eV). 1 μl of sample was typically injected.

The error of chromatographic measurements was about 20%.

The retinal content of samples analysed by GC/MS was estimated from the absorbance at 570 nm using a molar extinction coefficient of 50000 $M^{-1}\,cm^{-1}$.

2.5. Determination of palmitic acid in halobacterial cells grown in synthetic medium

An aliquot (20 ml of culture, $A_{500} > 1$, total protein content about 1.5 mg) of HR superproducer cells, grown in synthetic medium not containing fatty acids, were harvested by centrifugation and washed twice with 4 M NaCl. The pellet of cells, after adding 10 μg of pentadecanoic fatty acid, was resuspended in 1 ml of 1 M NaOH using a syringe and incubated at 100°C for 2 h. Lipids released by alkali were extracted with methanol/chloroform (2:1, v/v) four times and chloroform phases were dried under N_2. Dried lipids were derivatized and analysed as before described.

Lipids of synthetic medium were also analysed (not shown). The palmitate background found in the synthetic medium (blank) was subtracted from lipid samples derived from halobacterial cells.

2.6. Photobleaching of halorhodopsin

Halorhodopsin samples were continuously exposed to a 250 watt tungsten Oriel lamp at 90°; an interference orange filter (590–610 nm) was inserted between excitation lamp and sample. The irradiance was measured with the help of a calibrated radiometer detector (Laser power meter, model 460-1A, EG and G, Electro-Optics Division). The absorbances at 570 and 410 nm were simultaneously followed by means of an optical multichannel analyzer EG and G Instruments equipped with a photodiode array detector (512 pixel) and a holographic grating by Thermo Javell Ash. Corp. (600 g/nm, blaze 450 nm).

2.7. Halorhodopsin titration by alkali

The pH of halorhodopsin fractions I and II was changed by adding aliquots of 1 M NaOH solution and measured by a Beckman pH meter. After each addition a spectrum was recorded to follow the changes in the absorbances at 570 and 410 nm. To calculate pK_a, corrections were made to account for the dilution of the sample determined by alkali addition.

2.8. Protein determination

Protein content was determined with the method of Bradford [12].

3. Results

3.1. Two distinct halorhodopsin bands on phenyl-Sepharose

Fig. 2 shows that, after washing a 10 ml gel column with 300 ml cholate buffer, halorhodopsin eluted with octyl glucoside buffer in two clearly distinct purple bands of different colour intensity: the first eluted band appeared to be much more diluted than the second. This splitting of halorhodopsin in two bands was not described in the original method [10].

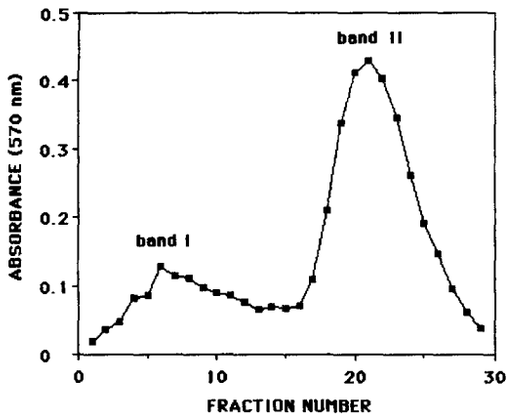

Fig. 2. Chromatography of halorhodopsin after washing with 300 ml of cholate buffer. The extract was loaded on 10 ml phenyl-Sepharose CL-4B column after washing with 300 ml 0.4% sodium cholate, 4 M NaCl, 25 mM Tris-Cl (pH 7.2) (cholate buffer); the purple protein was eluted with 0.5% octyl glucoside, 4 M NaCl, 25 mM Tris-Cl (pH 7.2) (octyl glucoside buffer) in two clearly distinct bands (band I and II). Elution in the dark at about 20°C.

No differences were observed in the halorhodopsin UV-visible absorption spectra of bands I and II; the absorption maximum in the visible region of both bands was at 570 nm and the 280/570 absorbance ratio was not higher than 1.4, a value indicating a good degree of halorhodopsin purity after only one chromatographic step.

The appearance of two halorhodopsin bands on phenyl-Sepharose column was also previously reported [13]; as the material from the two halorhodopsin fractions showed the same protein band on SDS-PAGE and the same absorption spectrum, the appearance of the two purple bands in the phenyl-Sepharose chromatography was considered an artifact [13].

3.2. GC / MS analyses

We analysed separately the two halorhodopsin bands for their fatty acid content.

In particular we looked for the presence of palmitate,

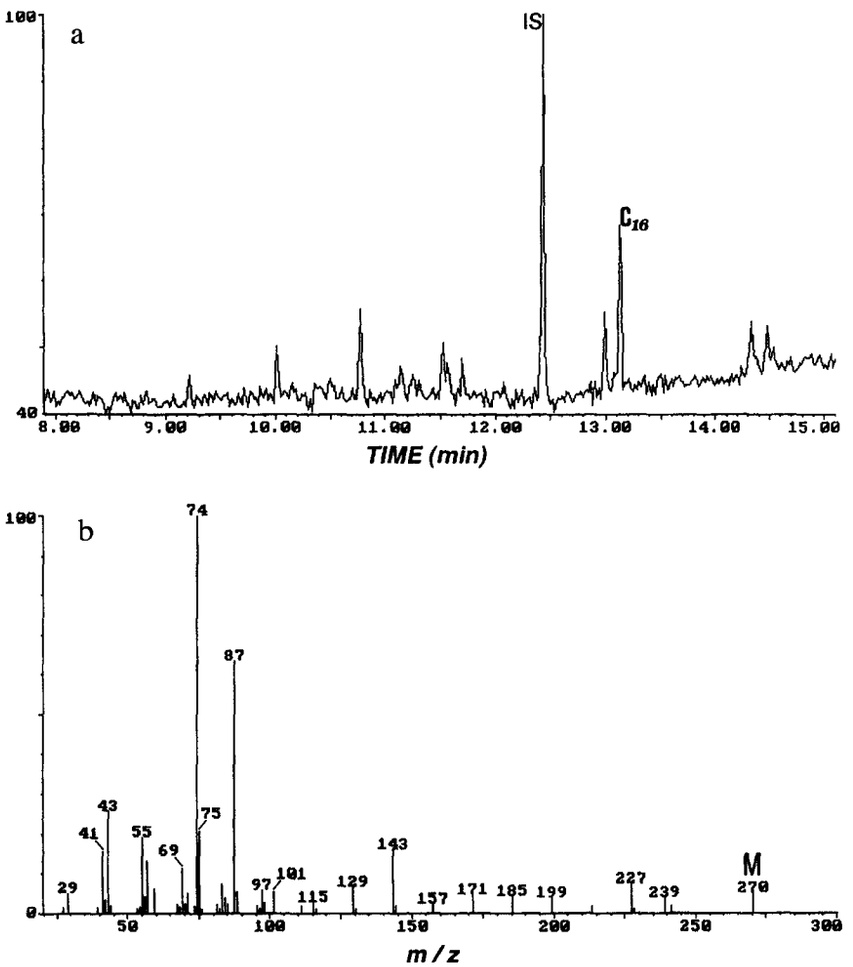

Fig. 3. (a) Total ion current gas chromatogram on SPB-5 capillary column of methyl ester derivatives of lipids released by alkali from undenaturated halorhodopsin (band I) and (b) electron impact mass spectrum of the peak with retention 13.1 min. IS, internal standard (15:0); C_{16}, palmitate methyl ester (16:0).

which was shown to be the major fatty acid acylated to bovine rhodopsin [14] and to red membrane proteins of extreme halophiles [4].

We found only very little or no palmitate in delipidated (i.e., denaturated) halorhodopsin samples (Table 1), while we could clearly identify and quantitate methyl palmitate (16:0) in the extract after methanolysis of lipids released by native (i.e., not denaturated) halorhodopsin.

Fig. 3a shows a typical GC-MS chromatogram relevant to the methylated extract (see Section 2) of native halorhodopsin (band I). Peak eluting at 13.1 min could be unequivocally identified as the methyl ester of hexadecanoic (palmitic) acid on the basis of a perfect match of retention time and electron impact (EI) mass spectrum (see Fig. 3b, $M = 270$) with those of an authentic standard.

From data in Table 1 it can be seen that the (16:0)/retinal molar ratio of first band was two-fold that of the second.

Quantitative analysis of the palmitate associated to the two different halorhodopsin fractions revealed that the molar ratio (16:0)/retinal was in the range 1.1 to 1.6 for band I and 0.6 to 0.8 for band II.

Furthermore, although we have not identified or quantified them, from Fig. 3a it is clear that other fatty acids besides palmitic are present in the chromatogram. This suggests that, if we had taken in account the other fatty acids, very likely the molar ratio of total fatty acids/retinal for band I and II would have been more close to 2 for band I and to 1 for band II.

Data of Table 1 clearly demonstrate that palmitate is present in the purple fractions from phenyl-Sepharose column (native halorhodopsin) and that is lost during the denaturation process.

Finally in order to exclude the possibility that palmitate associated to halorhodopsin is the result of a contamination due to the presence of fatty acids in the peptone present in the growth medium, we prepared a culture of halobacterial cells in a synthetic medium of chemically defined composition not containing fatty acids (see Section 2) and we determined the total palmitate content of these cells. The amount of fatty acid in the whole cells was determined by means of an internal standard (as in the case of the analyses of purple fractions) and related to the total protein cellular content, after corrections for palmitate in the blank. We found that halobacterial cells grown in the absence of fatty acids contain about 6 μg of palmitate per mg of total cellular proteins and therefore, in agreement with previous results of Kates [4], we conclude that halobacterial cells are able to synthetize fatty acids and that palmitate associated to halorhodopsin is not of exogenous origin.

Table 1
Palmitic acid content (expressed as palmitate methyl ester/retinal molar ratio) of native and/or denaturated halorhodopsin and of pre-extract relative to different purple fractions

Expt.	Band	Palmitic acid content		
		native	denat.	pre-extract
(a)	I	1.6		
	II	0.8		
(b)	I	1.1		
	II	0.6	traces	0.45
(c)	mixed		traces	0.69
(d)	mixed [a]		n.d.	0.63
(e)	mixed [a]		n.d.	0.44

Blanks correspond to samples not analysed. Pre-extract is defined in Section 2 and Fig. 1.
[a] Delipidated with MeOH/$CHCl_3$.

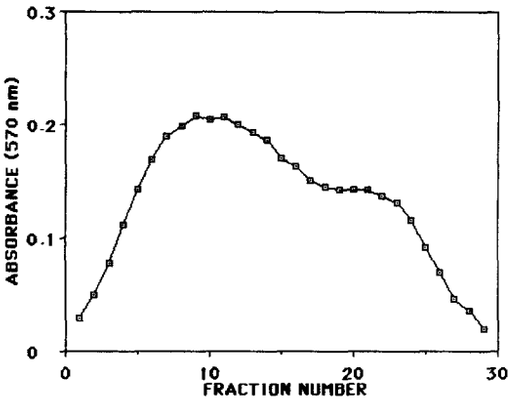

Fig. 4. Chromatography of halorhodopsin after washing with 150 ml of cholate buffer. Experimental details as in Fig. 2.

3.3. Role of palmitic acid on the chromatography of halorhodopsin

Fig. 4 shows the chromatography of halorhodopsin after washing the column with only half volume of cholate buffer used in the experiment illustrated in Fig. 2, i.e., 150 ml. A broad purple fraction, clearly resulting from the overlap of more than one band, was collected; the relative intensity of these bands appeared to be reversed compared to that obtained in the experiment shown in Fig. 2, indicating that by reducing the volume of washing buffer the major part of halorhodopsin loaded on the column tends to be less retained.

This was confirmed in further experiments in which halorhodopsin was eluted immediately after loading without washing with cholate buffer; in this last case the first band was much more coloured than the second one (not shown).

Interestingly the chromatography yield of experiments reported in Figs. 2 and 4 was the same (about 90%), indicating that the two different halorhodopsin forms could be linked one to the other through an equilibrium reaction, shifted to one direction or to the opposite depending on the experimental conditions.

Considering that these two halorhodopsin bands differ for the palmitate content, we made the hypothesis that the two distinct chromatographic bands could originate from

the hydrolysis of covalently bound palmitate, through a very reactive thioester bond (unstable in denaturating conditions, as suggested by data in Table 1). Therefore we tested the effect of adding palmitate and free thiols to the chromatography buffers.

Fig. 5 shows the chromatography of halorhodopsin in the presence of exogenous palmitic acid (profile a) and in the presence of a typical reagent of thioester bond, the β-mercaptoethanol (profile b). Interestingly we found that by adding exogenous palmitic acid to both cholate and octyl glucoside buffers halorhodopsin eluted as a single intense purple band, approximately corresponding to the first eluted band in Fig. 2. Instead the chromatographic profile of halorhodopsin in the presence of β-mercaptoethanol showed also one band, corresponding quite well to the second band in Fig. 2. Again the yield of halorhodopsin in the presence of exogenous palmitic acid was not different from that obtained in the experimental conditions of Figs. 2 and 4; while in the presence of thiols the yield was somewhat lower, about 75%.

To have information on the specificity of palmitate effect on the halorhodopsin chromatography, we compared the effect of palmitate with that of another saturated fatty acid (15:0).

Fig. 6 illustrates the chromatography of halorhodopsin in the absence of fatty acid (profile c) or in the presence of palmitic (a) and pentadecanoic (b) acid, at 30°C. By adding a pentadecanoic fatty acid (15:0) to the chromatography buffers instead of the hexadecanoic fatty acid (16:0), still a splitting of halorhodopsin in two bands was visible at the beginning of the chromatographic run, even if after collection the first eluting band was too diluted to be detected. The rate of migration of halorhodopsin in the presence of pentadecanoic fatty acid was slower than in the presence of the hexadecanoic fatty acid. It can be seen that the first band was scarcely visible even in the profile of the control and furthermore, by comparing the fraction numbers in chromatograms of Figs. 2 and 4 with those in Fig. 5, that by increasing the temperature halorhodopsin was more retained on the column, even in the presence of exogenous palmitic acid.

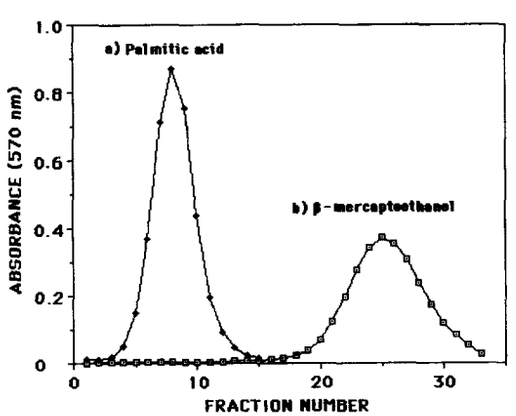

Fig. 5. Chromatography of halorhodopsin in the presence of exogenous palmitic acid (a) and of free thiols (b). Column (a) was prewashed with 150 ml cholate buffer containing 15 μM palmitic acid and eluted with octyl glucoside buffer containing 780 μM palmitic acid. In (b) cholate and octyl glucoside buffers contained 4 mM β-mercaptoethanol instead of palmitic acid. The same amount of extract was loaded on the two different columns. Elution in the dark at 16°C. Other experimental details as in Fig. 2.

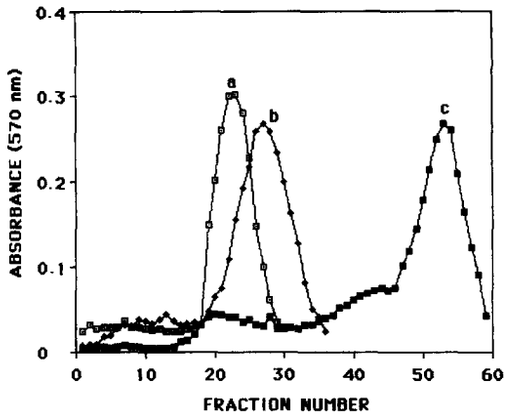

Fig. 6. Effect of two different fatty acids on the chromatography of halorhodopsin. The figure illustrates the chromatography of halorhodopsin at 30°C in three different experimental conditions; in all cases the same amount of extract was loaded (about 200 nmol). In the absence of fatty acids, the column (c) was washed with 300 ml cholate buffer before eluting with octyl glucoside buffer as in the experiment of Fig. 2. In the presence of fatty acids, the columns (a) and (b) were washed with 150 ml cholate buffer before eluting with octyl glucoside buffer; the palmitic (a) or pentadecanoic (b) fatty acids were present in the chromatography buffers at the concentration reported in Fig. 5.

3.4. Properties of halorhodopsin band I and II

In order to establish if the two halorhodopsin fractions containing different amount of palmitate are functionally similar, we compared the rate of decay of the absorbance at 570 nm (HR_{570} decay), corresponding to a simultaneously rise in the absorbance at 410 nm (rise of HR_{410}, not shown), upon illumination. Fig. 7 shows the time-course of HR_{570} decay for both fractions of halorhodopsin, continuously illuminated with orange light in the range of linear light dependence of the reaction; it can be seen that the rate of HR_{570} decay was much higher for the halorhodopsin band I, having an higher molar ratio palmitate/retinal. From data in Fig. 7 we calculated the initial rates of HR_{570} decay finding $9.7 \cdot 10^{-10}$ Ms^{-1} and $1.77 \cdot 10^{-10}$ Ms^{-1} for I and II band, respectively; saturation of HR_{570} decay rates was reached only after about several minutes illumination (not shown).

In the presence of 0.05% azide, a well known catalyst of the conversion of HR_{570} to HR_{410} [15], the initial rates were two orders of magnitude higher and the total absorbance change was about the same for the two bands, i.e., the two bands tend to reach the same photosteady state.

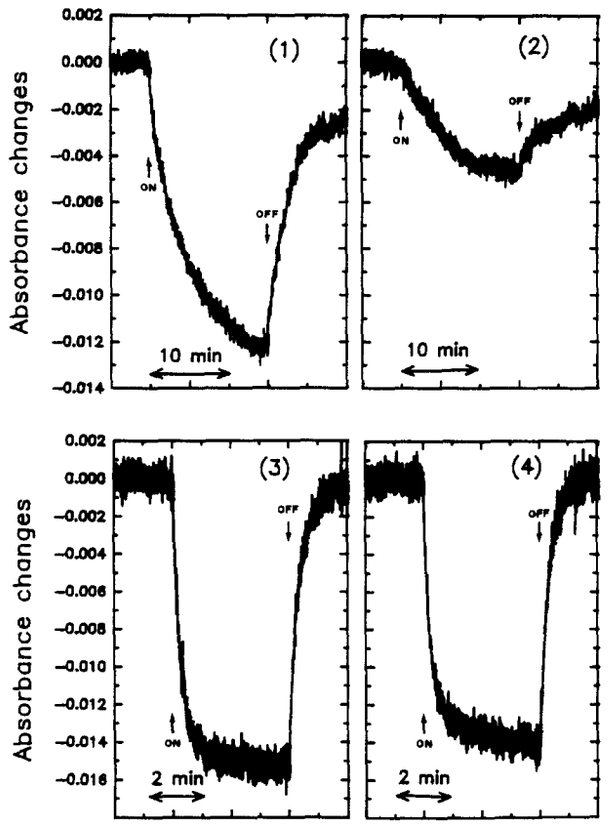

Fig. 7. Comparison of the decay of the absorbance at 570 nm of halorhodopsin band I and II under sustained illumination with orange light, in the absence (1,2) or in the presence of 0.05% NaN_3 (3,4). Irradiance was 1.9 $pEs^{-1}cm^{-2}$. The two halorhodopsin bands were at concentration about 4 μM in buffer of composition: 4 M NaCl, 0.5% octyl glucoside, 25 mM Tris-Cl (pH 7.2). Behaviour of band I without and with azide is shown in curves 1 and 3, respectively; while curves 2 and 4 refer to band II.

Furthermore as it is known that the formation of HR_{410} is accompanied by reversible deprotonation [13], we studied the interconversion of HR_{570} to HR_{410} induced by alkali in the dark. Fig. 8 illustrates data obtained by titrating halorhodopsin band I and II.

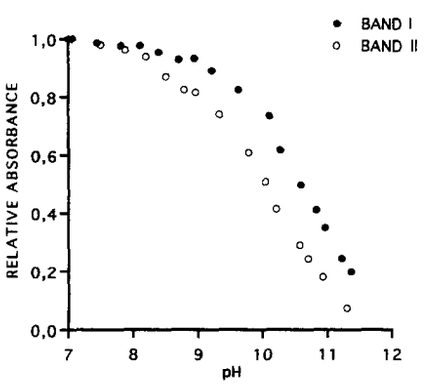

Fig. 8. Deprotonation in the dark of halorhodopsin band I and II induced by alkali. Each point was obtained from the spectra recorded at different pH values; the relative absorbance at 570 nm is plotted versus pH. A representative experiment over a number of three for each band is reported.

The fact that the two curves are shifted of about 0.4 pH unit indicates that halorhodopsin band I and II have different pK_a of the Schiff base.

As the response of halorhodopsin to titration by alkali has been previously explained in terms of deprotonation of the Schiff base [16], results illustrated in Fig. 8 could indicate that the environment of the Schiff base in the two different halorhodopsin forms is different.

4. Discussion

Results described in this paper show that the chromatographic behaviour of halorhodopsin is strongly affected by the addition of exogenous saturated fatty acids.

In the absence of exogenous fatty acids, by eluting halorhodopsin with octyl glucoside buffer, two purple bands were obtained. The relative intensity of these bands appeared to be dependent on the amount of cholate buffer used to wash the phenyl-Sepharose column.

In the presence of exogenous palmitic acid halorhodopsin migrated on phenyl-Sepharose as a single sharp blue-purple band, corresponding to band I.

Exogenous pentadecanoic (15:0) fatty acid also increased the rate of migration, but not to the same extent of palmitic acid. This result together with the presence of other fatty acids besides palmitic in the pattern of Fig. 3 indicates that the effect of palmitic can be mimicked by other fatty acids.

We have analysed the purple fractions eluted from the phenyl-Sepharose column (in the absence of exogenous fatty acids) for the content of fatty acids.

To reveal the presence of covalently bound fatty acids we have applied the classical protocol of MeOH/HCl esterification of lipid products released by saponification from delipidated protein. Samples of native halorhodopsin were also analysed by saponification and esterification.

We have shown that palmitic acid was released only from native halorhodopsin. Only traces of palmitic acid or no fatty acids at all were obtained from delipidated (i.e., denaturated) halorhodopsin and at the same time palmitic acid was found in the organic solvents (pre-extract) used to delipidate.

The quantitative analysis of palmitate associated with the native halorhodopsin indicated that from one to two fatty acid molecules per protein molecule were present depending on the fraction analysed.

Band I (i.e., the less retained halorhodopsin form) had a palmitate content higher (molar ratio 2:1) than band II (1:1) (Table 1).

What is the nature of the association between palmitate and halorhodopsin?

The lack of resistance to organic solvents used to delipidate the protein may appear to indicate that most of

palmitate is associated to halorhodopsin in a non-covalent manner.

However, although the presence of covalently-bound palmitate could not be demonstrated unambiguously by the present methods, it cannot be excluded that a covalent bond may be unstable under denaturating conditions, as in the case of the halorhodopsin aldimine bond between retinal and Lys-216.

As we have shown that there is a correlation between the palmitate/retinal molar ratio and some important properties of halorhodopsin, the palmitate–halorhodopsin interaction does not seem to be random.

To explain how micromolar concentrations of non-covalently bound palmitate may significantly affect photobleaching rate (Fig. 7) and response to alkali in the dark (Fig. 8) of halorhodopsin, one needs to assume that palmitate must be located very close to the Schiff base in the hydrophobic channel of the protein.

Even if palmitate were only a contaminant (of intracellular origin) of halorhodopsin, the fact that it seems to interact with high affinity with the protein is of interest.

Again the lack of band I in the chromatogram obtained in the presence of free thiols may be considered an evidence in favour of the presence of a thioester bond between palmitate and halorhodopsin.

The ease with which the thioester can transfer the fatty acyl group to a free thiol, such as mercaptoethanol, together with the ability to bind spontaneously palmitate (i.e., in the absence of an acylating enzyme using palmitoyl-CoA as donor) were previously described in bovine rhodopsin studies [14].

In consideration of previous findings on bovine rhodopsin, it appears reasonable to suggest that acylated and deacylated halorhodopsin forms may be in equilibrium according to the equation

$$\text{HR-Cys-S-CO-R} + \text{H}_2\text{O} \rightleftharpoons \text{HR-Cys-SH} + \text{R-COOH}$$

On the basis of the above equilibrium reaction, we can predict that the free fatty acid will be moved away from halorhodopsin in the course of elution with detergents, continuously shifting the equilibrium to the right; on other hand the addition of fatty acid will shift the equilibrium to the left, reducing the trend of halorhodopsin to lose palmitate.

Furthermore denaturation by solvent extraction would also favour shifting the equilibrium to the right since the free fatty acid would be rapidly removed.

It is known that spontaneous hydrolysis of thioester is a slow process in neutral solution, unless the thiol formed is removed by specific reagents [17]. In the course of chromatography deacylated halorhodopsin would be separated from acylated halorhodopsin, because differently retained on column.

In a chemical characterization of Cys residues in halorhodopsin it was found that free thiol groups were only detectable in the denaturated and not in the native halorhodopsin [18]. These data can be also well explained by the presence of thioester bond in halorhodopsin; in fact if palmitate was covalently attached to halorhodopsin through Cys residues, these would be masked in the native state of the protein and therefore detectable only after the loss of palmitate determined by denaturation. Interestingly it was also previously reported that the light dependent chloride transport and the photochemical reactions appeared to be strongly affected by thiol specific reagents [19].

In conclusion, we suggest to interpret the effects of palmitic acid on halorhodopsin here described in terms of halorhodopsin acylation.

Experiments are in progress to ascertain whether the two Cys intramembrane residues, conserved in eight different sequenced halorhodopsins (Oesterhelt, D., personal communication) and located in the plane of retinal are sites of acylation and to shed light on the role of palmitate in the regulation of halorhodopsin function.

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