IDENTIFICATION OF A BACTERIO-OPSIN SPECIES WITH A N-TERMINALLY EXTENDED AMINO ACID SEQUENCE

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1. Introduction

Halobacterium halobium, when induced for purple membrane production, synthesizes the integral membrane protein bacterio-opsin at a much higher rate than other membrane proteins [1]. Bacterio-opsin is therefore an attractive model for studying the problem of synthesis of intrinsic membrane proteins [2].

Protein synthesis of bacterio-opsin and some other membrane proteins in vivo is selectively disturbed when Mg^{2+} are removed from the medium, whereas no effect on the synthesis of cytoplasmic proteins can be observed. Re-addition of Mg^{2+} to the cell suspension reconstitutes an almost normal membrane protein pattern. Instead of bacterio-opsin however, a protein species with a slightly higher apparent molecular weight appears, which is referred to as 27 000 M_r protein [3].

Here we show that the 27 000 M_r protein is a bacterio-opsin molecule which is elongated by an additional peptide at the N-terminus. The N-terminal amino acid sequence of the protein was determined up to position 5 by manual Edman radiosequencing. We found the sequence:

H_2N—Met—Leu— ? —Leu—Leu—(Leu)—... .

2. Materials and methods

2.1. *Strain and culture conditions*

Halobacterium halobium R_1M_1 [4] was used for all experiments and grown in a peptone medium [5] as in [3].

2.2. *Radioactive labelling of bacterio-opsin*

The labelling of bacterio-opsin with radioactive amino acids is described in [3].

2.3. *Radioactive labelling and isolation of 27 000 M_r protein*

Halobacteria were converted into spheroplasts as in [3], $MgCl_2$ was then added to the spheroplast suspension (2 ml) to 0.1 mol/l final conc. After addition of radioactive amino acids (15 μCi L-[U-^{14}C]-leucine, 350 mCi/mmol or 50 μCi L-amino acid mixture [U-^{14}C] or 20 μCi L-[^{35}S]methionine, 500 Ci/mmol) the cells were gently stirred at 37°C and illuminated with yellow light (slide projector with an OG 515 nm cut-off filter) for 60 min.

The cells were lyzed and the membrane fraction, containing 27 000 M_r protein, was isolated as described for bacterio-opsin [3]. The 27 000 M_r protein was purified by SDS–polyacrylamide gel electrophoresis on 12% slab gels [6], localized by autoradiography and recovered by elution. The eluted protein was lyophilized, redissolved in water and SDS removed by precipitation with KCl [7]. Glycine and other buffer substances were removed by gel filtration on Sephadex G-25.

2.4. *Peptide mapping*

Radioactive proteins mixed with unlabelled bacterio-opsin were dissolved in the smallest possible volume of 70% formic acid, containing 0.1% phenol. A 50-fold excess of BrCN (w/w), was added and the mixture flushed with nitrogen and kept in the dark at room temperature for 24 h [8]. The cleavage products were lyophilized, redissolved in 80% formic acid, containing 0.1% phenol, and the peptides layered onto a Biogel P 30 column (100–200 mesh; 4 × 1500 mm), and eluted with the formic acid/phenol solvent. Column fractions (0.4 ml) were lyophilized, redissolved in a small volume of 60% formic acid, and spotted onto silica gel thin-layer chromatography

plates (20 × 20 cm, without fluorescent indicator, Merck), and developed with 2-butanol/water/formic acid (70/21/9 by vol.). The peptides were visualized with Fluram [9], and radioactive fragments located by fluorography [10].

2.5. Edman sequence analysis

Radio-sequencing was performed by adding 1 mg myoglobin as protein carrier to $3-5 \times 10^5$ cpm of labelled 27 000 M_r protein. The samples were manually degraded as in [11]; the extraction and conversion procedures were done as in [12]. Radioactive PTH-amino acids were identified by fluorography of thin-layer chromatograms [13].

3. Results and discussion

Rod-shaped halobacteria can be converted into spheres by complexing Mg^{2+} in the medium with EDTA. These spheroplasts lose the ability to produce bacterio-opsin; however, re-addition of Mg^{2+} leads to the synthesis of a membrane protein with a slightly higher apparent molecular weight (27 000 M_r) than authentic bacterio-opsin (26 000 M_r) (fig.1).
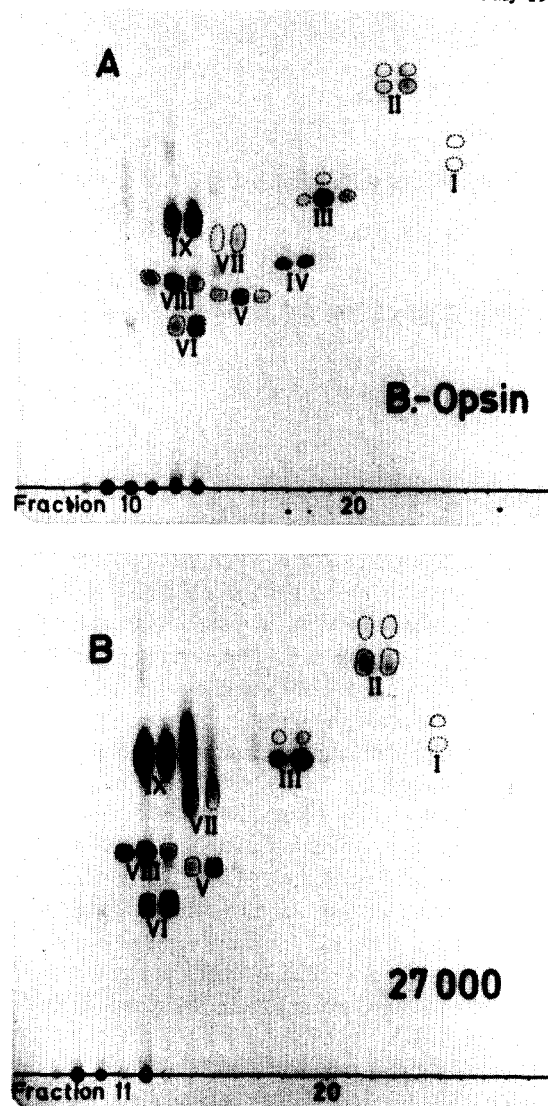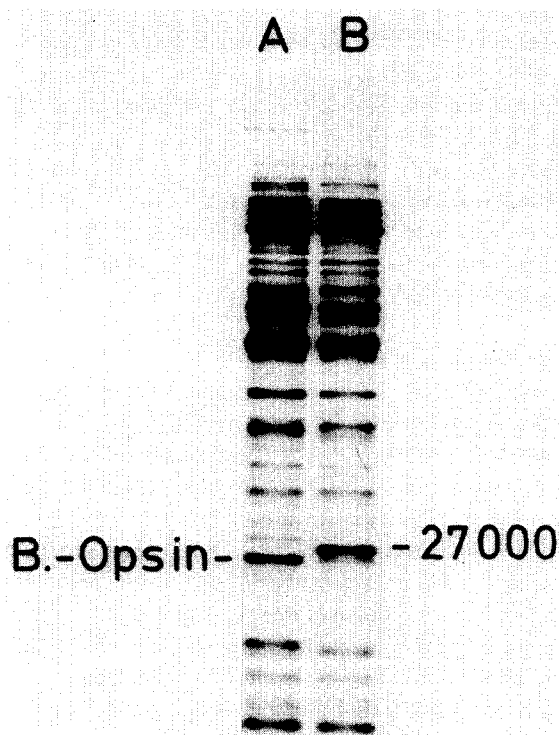
Fig.1. Fluorograms of SDS—polyacrylamide gels after electrophoresis of proteins synthesized by halobacteria before and after conversion to spheroplasts. Pulse labelling (20 min, 20 μCi [³⁵S]methionine) and the preparation of spheroplasts were performed as in section 2. (A) Protein pattern synthesized by rod-shaped halobacteria; (B) protein pattern synthesized by spheroplasts in the presence of 100 mM Mg^{2+}.

Fig.2. Fluorography of BrCN-peptide maps. [¹⁴C]Leucine-labelled bacterio-opsin (A) and [¹⁴C]leucine labelled 27 000 M_r protein (B) were mixed with unlabelled bacterio-opsin as protein carrier and cleaved with BrCN. The peptides were separated by gel chromatography on Biogel P 30. Chromatographic fractions were spotted onto silica gel thin-layer and developed with 2-butanol/water/formic acid (70/21/9, v/v/v). The chromatograms were sprayed with Fluram. The peptides visible under UV, which derive from the carrier bacterio-opsin, were marked with dotted lines.

Table 1

Sequence analysis of water soluble BrCN-peptides of bacterio-opsin

Peptide	Sequence found	Correlated to the amino acid sequence	
I	Tyr–Leu–	Peptide	57– 60
II	Leu–Leu–Gly –Tyr –Gly –...	Peptide	61– 68
III	Gly –Leu–Gly –Thr –Leu–Tyr–Phe–...	Peptide	21– 32
IV	––––––––––––––––––––––	Peptide	1– 20
V	Gly –Val– X –Asp–Pro –Asp–Ala –...	Peptide	33– 56
VI	Val –Leu–Asp–Val– X –Ala–Lys–...	Peptide	210–248

Bacterio-opsin and 27 000 M_r protein, labelled with [^{14}C]leucine, were isolated by SDS–polyacryl-amide gel electrophoresis. The proteins were cleaved with cyanogen bromide and the peptides obtained were separated by chromatography on Biogel P30 followed by thin-layer chromatography on silica gel. Fig.2A shows the peptide pattern obtained from bac-terio-opsin. Using the known amino acid sequence data of bacterio-opsin [14,15], the water soluble pep-tides I, II, III, V, VI could be identified by manual Edman-degradation. Table 1 summarizes the results obtained. The other peptides, VII, VIII, and IX, were water insoluble and not sequenced. Peptide IV was identified as the blocked N-terminal cyanogen bromide
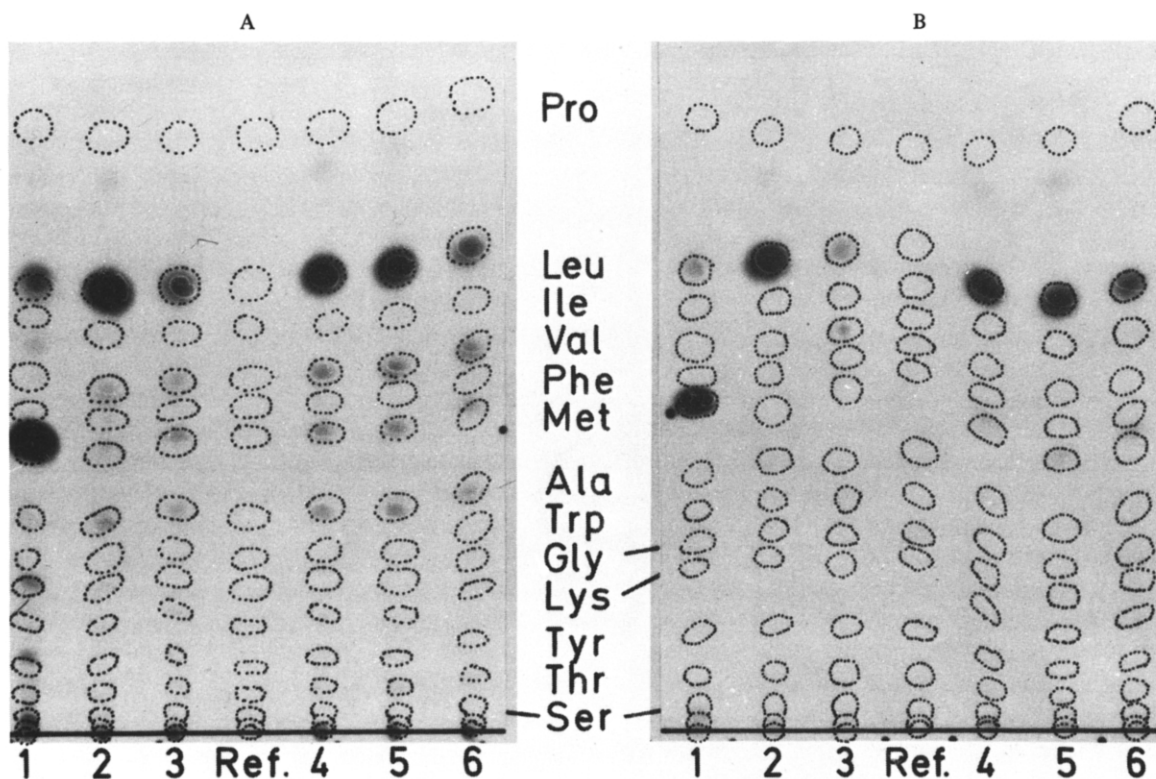

Fig.3. N-terminal sequence of 27 000 M_r protein. 27 000 M_r protein labelled with [^{35}S]methionine and amino [^{14}C]acid mixture (A) and [^{35}S]methionine and [^{14}C]leucine (B) was manually degraded by the Edman procedure with myoglobin as protein carrier. PTH derivatives of each step were added to a mixture of unlabelled PTH-amino acids and chromatographed on silica gel thin-layer plates. PTH-amino acid standards were marked with dotted lines, the chromatographs were impregnated with 2-methylnaphtaline/ PPO (1000/4) and fluorographed [10]. Radioactive PTH-amino acids were identified by comparing the fluorograms with the PTH-amino acid standards.

fragment of bacterio-opsin, since:

1. It did not react with Fluram, which requires the presence of free amino groups.
2. Radioactively labelled lysine, tyrosine and valine could not be incorporated into peptide IV; the known sequence data show this peptide to lack these amino acids (as well as Ser, Phe, Asx). No other BrCN peptide lacks this combination of amino acids.
3. The amino acid analysis of purified peptide IV was in good agreement with the known composition of the N-terminal peptide.

Fig.2B shows the peptide pattern of 27 000 M_r protein. It exhibits the same peptides as bacterio-opsin, except peptide IV is missing and a new radioactive peptide appears near peptide IX.

In contrast to the bacterio-opsin polypeptide chain, in which the N-terminus is blocked by a pyroglutamyl residue [18], the 27 000 M_r protein released PTH-methionine in an Edman degradation experiment. To characterize the N-terminal amino acid sequence of 27 000 M_r, the protein was labelled with various radioactive amino acids, isolated in microquantities by SDS–polyacrylamide gel electrophoresis, and subjected to manual Edman degradation. Fig.3A shows the fluorography of PTH-amino acids of 27 000 M_r protein labelled with an amino [^{14}C] acid mixture supplemented with [^{35}S]methionine. Radioactive PTH-Met is detected in step 1, while radioactive PTH-Leu is found in step 2,4,5 and with low yield in step 6. This sequence is confirmed by Edman degradation of 27 000 M_r protein, labelled only with [^{35}S]Met and [^{14}C]Leu (fig.3B).

Due to the manual degradation method, much protein was lost during the extraction procedures. Therefore it was possible to analyze the sequence only up to position 5. At step 3, no radioactive amino acid was identified, but the analysis of 27 000 M_r protein labelled with the amino [^{14}C] acid mixture showed that [^{14}C]Asp, [^{14}C]Glu, [^{14}C]Gly and [^{14}C]Tyr were not or were very poorly incorporated. Furthermore, the amino[^{14}C]acid mixture did not contain [^{14}C]Asn, [^{14}C]Gln, [^{14}C]Cys and [^{14}C]Trp thus these amino acids would never be identified by this type of labelling experiment.

Our experiments show that the 27 000 M_r protein is a bacterio-opsin species with an additional (hydrophobic– N-terminal peptide. This indicates that at least one protein processing step is necessary during the biosynthesis and integration of this intrinsic membrane protein.

The proteolytic removal of an N-terminal peptide during membrane incorporation has been reported for several membrane proteins, for instance *E. coli* lipoprotein [17] and M 13 coat protein [18], but other membrane proteins are known; for example, staphylococcal α-toxin [19], which are incorporated into membranes without a proteolytic step.

Acknowledgements

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