SOLUBILIZATION AND SEPARATION OF TWO *b*-TYPE CYTOCHROMES FROM A CAROTENOID MUTANT OF *HALOBACTERIUM HALOBIUM*

Christina HALLBERG and Herrick BALTSCHEFFSKY
Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

Received 5 January 1981; revised version received 2 February 1981

1. Introduction

The respiratory system in the membranes of *Halobacterium halobium* contains a predominant complex of *b*-type cytochromes, a low level of a *c*-type cytochrome and two CO-reactive hemoproteins identified as cytochromes *o* and a_1 [1]. We have attempted to purify and characterize some of these cytochromes. This is of special interest, since halobacteria appear to have some unique biochemical properties [2].

We report here the solubilization and separation of two membrane-bound *b*-type cytochromes from a carotenoid mutant of *Halobacterium halobium*. The difference absorption spectra of these cytochromes differ in their α-, β- and γ-maxima. The solubilization was performed with Triton X-100 and separation was achieved on a column with hydroxyapatite. Two *b*-type cytochromes were previously identified in *H. halobium* membranes [1], but were not solubilized from the membranes nor identified separately.

Here, a preparation of *b*-type cytochromes was obtained, which was ~6-times more purified (protoheme/mg protein) than the purest fraction in [3].

Wild-type strains of *H. halobium* contain red carotenoids, which interfere with the measurements of the cytochrome spectra. Thus, a mutant strain of *H. halobium* (913-7) lacking carotenoid absorption in the visible region was used here.

2. Materials and methods

2.1. *Growth and harvest of bacteria*

Mutant 913-7 of *Halobacterium halobium* was a gift from Professor Janos Lanyi (Max Planck Institut für Biochemie, Munich). The cells were grown aerobically (on a rotary shaker at 100 rev./min) in 3 liter Erlenmeyer flasks (1.2 liter in each flask) for 70 h at 35°C. The medium contained per liter: NaCl 250 g; $MgSO_4 \cdot 7\ H_2O$ 20 g; Na_3-citrate 3 g; KCl 2g; $CaCl_2 \cdot 2\ H_2O$ 0.2 g; and Oxoid Bacteriological Peptone L37 10 g (pH was adjusted to 7.4 before sterilization) [4]. The cells were harvested by centrifugation at 7000 × *g* for 45 min at 4°C and washed once in basal salt [4].

2.2. *Membrane preparation*

The method used for preparation of membranes was suggested in [5]. The cells were broken by freezing them in liquid nitrogen and thawing. Subsequently the broken cells were homogenized in 3 M NaCl, deoxyribonuclease was added and the suspension was stirred for 60 min at room temperature. After centrifugation at 30 000 × *g* for 60 min the membranes were washed once with 3 M NaCl and centrifuged again at 30 000 × *g* for 30 min. The pellet was suspended in 10 mM MOPS buffer (pH 7.2) and centrifuged at 114 000 × *g* for 2 h. The pellet 'membrane preparation' was supended in 10 mM MOPS buffer (pH 7.2). The centrifugation steps were performed at 4°C.

2.3. *Solubilization*

The membrane preparation was treated at room temperature with Triton X-100, so that a protein/detergent ratio of ~1:1.5 was obtained. This was then centrifuged at 105 000 × *g* for 2 h. The supernatant contained the solubilized cytochromes.

2.4. *Hydroxyapatite column chromatography*

Hydroxyapatite was kindly supplied by Professor G. von Jagow's laboratory (Munich). A column was

Abbreviations: MOPS, morpholinopropane sulfonic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylene diamine dihydrochloride

packed with hydroxyapatite (diam. 2.2 cm, length 1.2 cm) and the gel was equilibrated with 10 mM MOPS buffer (pH 7.2) containing 0.1% Triton X-100. About 10 mg protein/ml bed vol. was applied upon the column. The flow rate was 18 ml/h. The proteins were eluted stepwise from the column by using sodium phosphate buffers described in section 3. All steps were performed at room temperature.

2.5. *Assays*

Cytochromes were identified by spectrophotometric measurements. Reduced minus oxidized difference absorption spectra were performed at room temperature with a Shimadzu UV-210 A double-beam spectrophotometer using a slit of 0.5 nm and 10 mm light-path cuvettes. Reduced samples were obtained by adding solid dithionite. Untreated samples were considered fully oxidized, as addition of ferricyanide did not increase the oxidation state further. Protein was determined by the Lowry method [6]. Protoheme contents (A_{556}–A_{575}) were estimated from reduced pyridine hemochromogen difference spectra using $\epsilon = 30\ mM^{-1} \cdot cm^{-1}$ [1].

3. Results and discussion

Solubilization of the *b*-type cytochromes from the bacterial membranes were carried out with the non-ionic detergent Triton X-100. Different concentrations of the detergent were tested in the absence and presence of NaCl. The results show that the best condition for solubilization was achieved in the absence of NaCl at 0.2% (v/v) detergent and 1.25 mg protein/ml (fig.1). In these experiments the best protein/detergent ratio was found to be ~1:1.6. About 60% of the *b*-type cytochromes were solubilized under these conditions. For higher protein concentrations also higher detergent concentrations were used so that a protein/detergent ratio of ~1:1.5 always was obtained.

The solubilized material was applied upon a hydroxyapatite column, prepared as in section 2. Hydroxyapatite chromatography in Triton X-100 has also been used for purification of the bc_1-complex from beef heart mitochondria [7,8]. The *b*-type cytochromes were eluted with a stepwise gradient consisting of sodium phosphate buffers (pH 7.2) containing 0.1% Triton X-100. Elution buffers with 10 mM, 50 mM, 70 mM, 80 mM, 90 mM, 100 mM and

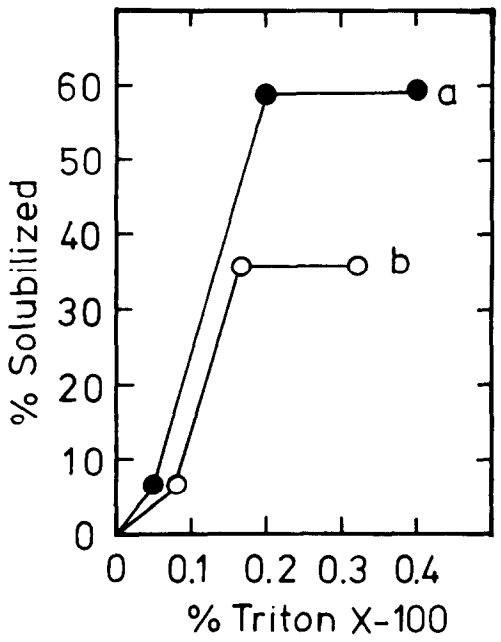

Fig.1. Solubilization of *b*-type cytochromes from the bacterial membranes with different concentrations of Triton X-100. The percentage of *b*-type cytochromes solubilized, was calculated from the amount of *b*-type cytochromes found in the supernatant after centrifugation at 105 000 × *g* for 2 h, compared to the amount in the suspension before centrifugation. *b*-Type cytochromes were quantitated from difference absorption spectra at 560 nm. Solubilization with Triton X-100 in: (a) 10 mM MOPS buffer (pH 7.2) at 1.25 mg protein/ml; (b) 10 mM MOPS buffer (pH 7.2) with 0.4 M NaCl at 1.25 mg protein/ml.

200 mM sodium phosphate were used sequentially. About 90% of the protein was eluted with a gradient of 10–90 mM sodium phosphate (fig.2). The first *b*-type cytochrome was eluted from the column with the same concentrations of sodium phosphate, but the other *b*-type cytochrome came out later with 100–200 mM sodium phosphate (fig.2). The two cytochromes have different absorption maxima in the difference spectra. Fig.3 shows representative spectra of the two different *b*-type cytochromes, one spectrum (a) from fraction 6 and another spectrum (b) from fraction 10 after hydroxyapatite column (see fig.2). The *b*-type cytochrome eluted first has maxima at 562 (α), 531 (β) and 432 (γ) nm and the second one has maxima at 559 (α), 527 (β) and 427 (γ) nm (fig.3). These maxima can be compared to the maxima for *b*-type cytochromes observed in *H. halobium* membranes [1]. A *b*-type cytochrome with absorp-

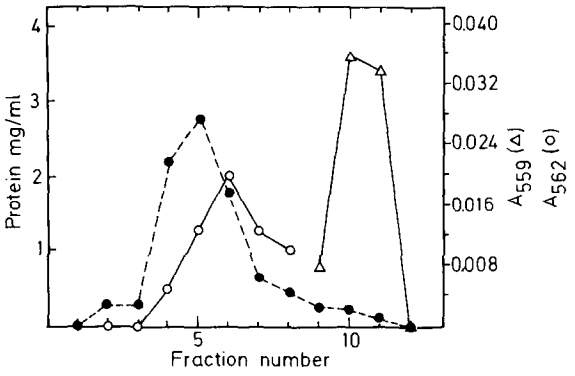

Fig.2. Elution profile of a Triton X-100 extract of membranes after hydroxyapatite chromatography. Solubilized material (10 ml) with 5 mg protein/ml was applied upon the column. Fractions of 5 ml were collected. The different fractions were eluted with a stepwise sodium phosphate gradient. Fraction numbers 4, 5, 6, 7, 8, 9, 10 and 11 were eluted, respectively, with 10, 50, 70, 80, 90, 100, 200 and 200 mM sodium phosphate buffers (pH 7.2) containing 0.1% Triton X-100. The cytochromes were measured with difference absorption spectra. One *b*-type cytochrome with α-peak at 562 nm was eluted with 10–90 mM sodium phosphate and another *b*-type cytochrome with α-peak at 559 nm was eluted with 200 mM sodium phosphate. (•) Protein; (○) *b*-type cytochrome with A_{max} at 562 nm in dithionite-reduced minus oxidized difference spectra; (△) *b*-type cytochrome with A_{max} at 559 nm in dithionite-reduced minus oxidized difference spectra.

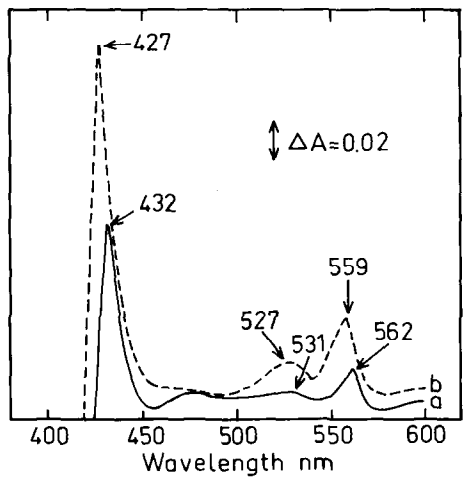

Fig.3. Dithionite-reduced minus oxidized difference absorption spectra of the two different *b*-type cytochromes after separation on the hydroxyapatite column. Spectrum of: (a) fraction 6 (1.8 mg protein/ml) which contains a *b*-type cytochrome with α-peak at 562 nm; (b) fraction 10 (0.25 mg protein/ml) which contains a *b*-type cytochrome with α-peak at 559 nm.

tion maxima at 561 (α), 530 (β) and 430 (γ) nm was observed in ascorbate–TMPD reduced minus oxidized membranes. A difference spectrum obtained with dithionite-reduced membranes recorded against ascorbate–TMPD-treated membranes showed another *b*-type cytochrome with maxima at 564 (α), 535 (β) and 434 (γ) nm [1]. We have also made similar difference spectra of the two separated *b*-type cytochromes. The *b*-type cytochrome with absorption maximum at 559 nm (α-band) was partially reduced by ascorbate–TMPD, but the other *b*-type cytochrome with absorption maximum at 562 nm (α-band) was only reduced by dithionite. It was suggested [1] that cytochrome *b* with the α-peak at 561 nm is involved in the 'ascorbate and ascorbate–TMPD oxidase systems' in the membranes of *H. halobium*. In this system cytochrome *b* (α-peak at 561 nm) can reduce a *c*-type cytochrome [1]. Our cytochrome *b* with the α-peak at 559 nm appears to correspond to this cytochrome *b*, being partly reduced by ascorbate–TMPD. The question of whether there exists a cytochrome bc_1 complex (complex III) in the cell membrane of *H. halobium* can not be answered at present, but we have spectral indications in the 550 nm region of a *c*-type cytochrome in the same fraction, after hydroxyapatite column chromatography, as the *b*-type cytochrome with the α-peak at 559 nm.

Different *b*-type cytochromes have also been found in *Halobacterium cutirubrum*. In [9] two *b*-type cytochromes with absorption maxima at 559 nm and 563 nm were found in the membranes of this bacterium. This can be compared to the two *b*-type cytochromes with maxima at 559 nm and 562 nm, which we have isolated from *H. halobium*. A scheme was proposed for the electron transfer system in *H. cutirubrum*, where 'cytochrome *b* (563)' and 'cytochrome *b* (559)' are placed in two alternative pathways [9]. It would be premature to attempt to distinguish between parallel and serial schemes for our two separated *b*-type cytochromes. Also, more information is needed for detailed consideration of the possibility that they are related to the *b*-type cytochromes of blue–green algae and chloroplasts, for example cytochrome b_{559} and cytochrome b_6 (b_{563}), a relationship which ferredoxin sequence data [10] may have made credible.

Cytochrome *b*, measured as protoheme, has been purified to a higher degree in this study than in [3]. Fractions 10 and 11 after hydroxyapatite chroma-

Table 1
Enrichment and yield of cytochrome *b*, measured as protoheme, before and after hydroxyapatite column chromatography

Fraction	Protoheme/protein (nmol/mg)	Purification	Yield (%)
Supernatant after Triton X-100 extraction and centrifugation at 105 000 × *g*	0.8	1	100
Fractions eluted with 200 mM sodium phosphate from the hydroxyapatite colum	12	15	50

tography (see fig.2) contain after pooling 12 nmol protoheme/mg protein, table 1, compared to 2 nmol protoheme/mg protein in [3].

Acknowledgements

We wish to thank Professor J. Lanyi for valuable advice and discussions and Professor D. Oesterhelt for providing laboratory facilities. We want to express our great gratitude to Professor G. van Jagow and to Dr H. Schägger for very valuable help and suggestions. This work was supported by grant no. K2292-106 from the Swedish Natural Science Research Council to H. B. and by a travel grant from Wallenbergs-stiftelsens Jubileumfond to C. H.

References

[1] Cheah, K. S. (1970) Biochim. Biophys. Acta 205, 148–160.
[2] Bayley, S. T. (1979) Trends Biochem. Sci. 4, 223–225.
[3] Hallberg, C. and Baltscheffsky, H. (1979) Acta Chem. Scand. B33, 600–601.
[4] Oesterhelt, D. and Stoeckenius, W. (1974) Methods Enzymol. 31, 667–678.
[5] Lanyi, J. K. (1980) personal communication.
[6] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[7] Riccio, P. Schägger, H., Engel, W. D. and Von Jagow, G. (1977) Biochim. Biophys. Acta 459, 250–262.
[8] Von Jagow, G., Schägger, H., Riccio, P., Klingenberg, M. and Kolb, H. J. (1977) Biochim. Biophys. Acta 462, 549–558.
[9] Lanyi, J. K. (1968) Arch. Biochem. Biophys. 128, 716–724.
[10] Hase, T., Wakabayashi, S., Matsubara, H., Kerscher, L., Oesterhelt, D., Rao, K. K. and Hall, D. O. (1977) FEBS Lett. 77, 308–310.