# Halocyanin, an Archaebacterial Blue Copper Protein (Type I) from *Natronobacterium pharaonis*†

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ABSTRACT: A small blue copper protein with a molecular mass of about 15.5 kDa has been isolated from the haloalkaliphilic *Natronobacterium pharaonis*. This protein, which was named halocyanin, contains one $Cu^{2+}$. The circular dichroism spectrum in the region of the absorption of the peptide bond reveals mainly $\beta$-structural elements. The absorption spectrum in the visible range has three maxima with the main band at 600 nm. The circular dichroism spectrum in this region is characterized by four extrema with maxima at 413 and 590 nm and minima at 477 nm as well as 721 nm. The electron paramagnetic resonance spectrum displays a hyperfine structure which shows a close resemblance to those of plastocyanin. The cumulative spectral data agree well with those of other small blue copper proteins with axial symmetry of the Cu ligands. In analogy, one can deduce that halocyanin may adopt a type I copper binding site with two His, one Met, and one Cys as probable ligands. This conclusion is confirmed by the C-terminal sequence which contains, homologous to other known sequences of type I copper proteins, three of the four copper ligands. Halocyanin can only be removed from the membrane by mild treatment with detergents. This observation indicates that halocyanin is a peripheral membrane protein which serves as a mobile electron carrier. The presence of type I blue copper proteins in archaebacteria might lead to further insights into their phylogenetic origin.

The element copper is found in a variety of proteins whose functions involve e.g. electron-transfer reactions, oxygen transport, reduction and oxidation of metabolites, or denitrification (for a review see Lontie (1984)). These copper proteins were classified into three groups distinguished by their different spectroscopic properties.

Type I copper proteins are intensely blue due to a broad absorption maximum at about 600 nm which is attributed to a charge-transfer transition of a cysteine residue to the $Cu^{2+}$ center (Solomon et al., 1980). Their electron-paramagnetic resonance (EPR[1]) spectra have an unusually small hyperfine coupling constant, $A_{\parallel}$. These two spectroscopic properties are unique for type I Cu complexes and point to a special coordination of the ligands. Type I copper is found in small single-copper proteins but also in association with type II and type III centers in multicopper enzymes (reviewed by Fee (1975)). Type II copper is sometimes referred to as colorless copper because its visual absorption is much smaller than that of other copper chromophores. It is present in all blue multicopper oxidases like e.g. superoxide dismutase (reviewed by Adman (1991)). The EPR spectra are similar to those of simple copper(II) complexes. The third class type III copper is also found in multicopper oxidases and has an essential role in the reduction of oxygen. However, it is not detectable by EPR and its absorption band is centered around 330 nm. Examples for type III proteins are the multimeric hemocyanins which serve as oxygen-transport proteins (reviewed by Adman (1991)).

The best characterized copper-containing proteins are small proteins with one type I copper center. Their molecular masses range from 10 to about 20 kDa. According to the information available so far, the physiological functions involve electron-transfer reactions. Therefore Adman (1985) proposed to name these single-domain copper proteins cupredoxins in analogy to ferrodoxins. Blue copper proteins have been found only in eucaryotic cells and in eubacteria.

A well-known example of a small copper protein is plastocyanin, which is present in vascular plants as well as in eukaryotic green algae and cyanobacteria (Sykes, 1990). Plastocyanin serves as an electron carrier between the cytochrome $b_6/f$ complex to the oxidized primary donor of photosystem I. From non-photosynthetic tissues of higher plants several small blue copper proteins (e.g., stellacyanin and plantacyanin) have also been isolated, but their physiological role remains obscure.

Further sources of type I copper proteins are eubacteria. Sutherland and Wilkinson (1963) isolated a blue protein from *Bordetella*, which they named azurin. Azurins with closely related amino acid sequences have now been found in many bacterial strains. They seem to be involved in the bacterial redox systems where they transport electrons between cytochrome $c_{551}$ and cytochrome oxidase (Farver et al., 1982, and literature therein). For another example of a bacterial blue protein, rusticyanin from *Thiobacillus ferroxidans* (Cox & Boxer, 1978; Ronk et al., 1991), a role in the iron-oxidation system was postulated (Blake et al., 1991). Amicyanin is found to be the acceptor of methylamine dehydrogenase in *Pseudomonas*, a methylotrophic bacterium (Tobari & Harada, 1981; Chen et al., 1992). Another blue copper protein with known function, pseudoazurin, serves as an electron donor to nitrite reductase (Liu et al., 1986). A recently discovered blue copper protein from *Chloroflexus aurantiacus*, auracyanin, may be involved in photosynthetic electron transfer. It is capable of donating an electron to cytochrome $c_{554}$. Interestingly, auracyanins are peripheral membrane proteins (McManus et al., 1992).

The copper enviroments in all blue copper proteins (type I) seem to be similar. The secondary structures consist predominantly of $\beta$-barrels with differences only in the loop

† Dedicated to Benno Hess on the occasion of his 70th birthday.

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[1] Abbreviations: CD, circular dichroism, CPC, cucumber peeling cupredoxin; EPR, electron paramagnetic resonance; MeCN, methyl cyanide; SDS, sodium dodecyl sulfate.

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regions. X-ray crystallographic data revealed that the tertiary structure is quite well preserved (e.g. Guss and Freeman (1983); for a review see Adman (1991)) although the primary sequences are only homologous in regions which participate in copper binding. Most strikingly, a C-terminal sequence with the general feature Cys-Aax$_n$-His-Aax$_m$-Met has been found in all protein sequences determined so far. An exception from this general scheme is found in stellacyanin (Bergmann et al., 1977) and a cupredoxin from cucumber peelings (CPC) (Mann et al., 1992) where Met is exchanged by Gln.

The structural and partial sequence homologies between the small blue copper proteins are of interest with respect to evolutionary questions. Because an archaebacterial blue copper protein had not yet been described in the generally available literature, it was proposed that blue copper proteins (type I) originate from an ancestral purple photosynthetic *Chlorobium*-like bacterium (Rydén, 1984). More insight into the evolution of blue copper proteins could be gained if more sequence data from other species become available. This would be particularly valid if blue copper proteins (type I) were also present in the third kingdom of living cells, the archaebacteria. So far, only in *Halobacterium salinarium* a small blue copper protein has been described (Steiner, 1983). In the present work, the identification and purification of another archaebacterial blue copper protein is presented. Thus, the blue copper proteins (type I) are universally distributed among the living organisms suggestive of a very early evolutionary origin.

The blue copper protein described here was isolated from the haloalkaliphilic bacterium *Natronobacterium pharaonis*. Because of the habitat of this species (Soliman & Trüper, 1982), namely, the brines of the Wadi Natrun lakes with pH values around 11 and a salinity of 30%, it is proposed to name this archaebacterial blue copper protein halocyanin.

## MATERIALS AND METHODS

*Materials.* Triton X-100 and octyl glucoside were purchased from Boehringer (Mannheim, Germany). Sodium deoxycholate was obtained from Fluka (Neu-Ulm, Germany). DEAE-Sepharose CL-6B, Sephacryl S-100 HR, and Superose 12 prep grade were products of Pharmacia (Freiburg, Germany). Hydroxylapatite Bio-Gel HT was purchased from Bio-Rad (München, Germany). TPCK-treated trypsin was obtained from Serva, (Heidelberg, Germany) whereas the endoprotease Glu-C from *Staphylococcus aureus* was a product of Boeringer (Mannheim, Germany). All chemicals used were of analytical grade.

*Strain and Cell Culture. N. pharaonis* strain SP1(28) (kindly provided by W. Stoeckenius) was grown aerobically for 72 h to an optical density at 800 nm of 1.6 (ca. $4 \times 10^9$ cells/mL) as described by Lanyi et al. (1990) in peptone medium supplemented with 4 $\mu$M $CuSO_4$. The cells were harvested by centrifugation and washed three times with 4 M NaCl.

*Preparation of Cell Membranes.* Washed *N. pharaonis* cells from 20 L of cell culture were resuspended in 150 mL of 4 M NaCl containing 20 mg of DNase and lysed by dialysis against 8 L of $H_2O$ for 16 h at 8 °C. Membrane fragments were collected by centrifugation for 60 min at 360000*g* (lower centrifugal forces are also sufficient to pellet the membranes) and washed three times with 0.1 M NaCl. To remove remaining bacterioruberine, a sucrose gradient centrifugation as described by Steiner and Oesterhelt (1983) (omitting Tween) was carried out.

*Extraction.* Washed membranes were stirred for 30 min at room temperature in 0.5% (w/v) Triton X-100 in 50 mL of 0.1 M NaCl, 10 mM Tris, pH 8.0 (final protein concentration: ca. 17 mg/mL) and then centrifuged at 360000*g*. The greenish supernatant was collected, and the pellet was treated again with the detergent. The combined supernatant fractions were used for further purification of halocyanin. Throughout the isolation, the halocyanin content was determined by its absorption at 600 nm.

*Ion-Exchange Chromatography.* The supernatant liquid was loaded on an ion-exchange column (5.0 × 4.0 cm) of DEAE-Sepharose CL-6B equilibrated with the extraction buffer. Halocyanin adsorbed as a blue band at the top of the column. The column was washed thoroughly with three bed volumes of 0.2 M NaCl (10 mM Tris, 0.5% Triton X-100, pH 8.0) followed by two bed volumes of 0.3 M NaCl. Halocyanin was subsequently eluted (flow rate 40 mL/h) by increasing the ionic strength of the buffer to 0.4 M NaCl.

*Hydroxylapatite Chromatography.* To remove remaining cytochromes, the blue pool fraction of the ion-exchange chromatography was applied to a hydroxylapatite column (2.6 × 4.0 cm) which had been equilibrated with 20 mM $Na_2HPO_4$, 50 mM NaCl, and 0.25% sodium deoxycholate, pH 8.5. The column was washed with one bed volume of 20 mM $Na_2HPO_4$, 50 mM NaCl, and 0.25% sodium deoxycholate, pH 8.5. The blue material was eluted with 300 mL of a linear gradient from 20 to 200 mM $Na_2HPO_4$ in 50 mM NaCl and 0.25% sodium deoxycholate, pH 8.5, with a flow rate of 20 mL/h. During this chromatographic step, Triton X-100 was exchanged by sodium deoxycholate, which has convenient properties for spectroscopic measurements.

*Gel Filtration Chromatography.* For analytical measurements, the protein was further purified by gel filtration. The fractions from the hydroxylapatite column were concentrated in Centriprep CP10 and Centricon C10 to a protein concentration of 4–8 mg/mL, and 0.5 mL was subjected to FPLC gel filtration (Superose 12TM prep grade, HR16/50 column). The column was equilibrated and developed in 0.25% sodium deoxycholate and 10 mM $Na_2HPO_4$, pH 8.5, at 1.0 mL/min, and the fractions containing halocyanin were manually collected.

*Peptide Digestion.* For the tryptic digest, 1 mg of halocyanin was first pyridylethylated according to the procedure of Fujita et al. (1984). The modified protein was treated with trypsin (30 $\mu$g) in 0.1 M Tris and 10 mM $CaCl_2$, pH 8.5, for 14 h at room temperature. The digestion was stopped by lyophilisation.

The endoproteinase Glu-C proteolysis was carried out with carboxymethylated halocyanin (Mattar, 1993). A 1-mg sample of modified halocyanin was incubated with 20 $\mu$g of endoproteinase Glu-C in 25 mM phosphate buffer, pH 7.8, for 14 h at room temperature. The reaction was stopped by lyophilization.

*Isolation of Peptides for Sequence Analysis.* The peptides of the tryptic and Glu-C digest were separated by high-performance liquid chromatography (HPLC) on a reversed-phase column ($\mu$-Bondapak C-18, Waters, Eschborn, Germany) using a linear gradient (0.1% TFA in water/0.1% TFA in MeCN) (for further details see S. Mattar et al., in preparation).

The purified peptides were sequenced by automated Edman degradation with an Applied Biosystems 470A sequencer using standard procedures.

*Analytical Procedures.* Gel electrophoresis was performed essentially using the procedure of Laemmli (1970) in a 2-mm flat gel in a linear gradient from 10 to 17.5% acrylamide.

Table I: Purification Scheme of Halocyanin from *N. pharaonis*[a]

| purification step | protein (mg) | halocyanin content (μmol) | specific content (nmol/mg) | purification factor | yield (%) |
|---|---|---|---|---|---|
| membrane fraction | 850 | 3.7[b] | 4.3 | 1 | 100 |
| Triton extract | 295 | 3.3[b] | 11.2 | 2.6 | 90 |
| DEAE-Sepharose fraction | 76 | 2.7 | 35.5 | 8.3 | 73 |
| hydroxylapatite fraction | 42 | 2.1 | 50 | 11.6 | 57 |
| gelfiltration eluate | 30 | 1.9 | 63 | 14.7 | 51 |

[a] The purification was carried out by starting from 20 L of cell culture. The protein content was determined by amino acid analysis, and the halocyanin concentration was measured spectroscopically at 600 nm. [b] An approximate content is based on an estimation.

The native molecular mass of halocyanin was determined by gel filtration on a Sephacryl S-100 HR column (2.6 × 91 cm) equilibrated with 0.25% sodium deoxycholate and 10 mM $Na_2HPO_4$, pH 8.5, with a constant flow of 10.2 mL/h. For calibration, horse heart cytochrome *c* (12 500 $M_r$), chymotrypsinogen (25 000 $M_r$), carboanhydrase (29 000 $M_r$), ovalbumin (45 000 $M_r$), and bovine serum alumin (64 000 $M_r$) were used. The molecular mass was also determined by electrospray mass spectrometry as described elsewhere (S. Mattar et al., in preparation).

Amino acid analysis was performed on a Biotronik automatic analyzer (Model 7000) after total hydrolysis of the samples in 6 N HCl with 0.1% phenol at 112 °C for 24, 48, or 72 h. For the determiniation of cysteine and methionine, protein samples were first oxidized according to Hirs (1967). Tryptophan was measured by the method of Yokote et al. (1986).

The thermal unfolding curve was determined by measuring the absorption change at 600 nm at different temperatures using a temperature-controlled sample holder. The temperature was measured directly in the cuvette.

The midpoint potential was determined by electrochemical redox titration according to Moss et al. (1991).

*Spectroscopic Measurements.* Absorption spectra were taken on a Perkin-Elmer Lambda 9 double-beam spectrophotometer.

The circular dichroism (CD) spectra were recorded on a Jobin-Yvon M III dichrograph. The raw data were averaged and stored on a Nicolet 1074 (Nicolet, Madison, WI) transient recorder and transformed to ellipticity ($\theta$) using a home-made program. The CD spectrum in the UV region was kindly provided by F. X. Schmidt (Bayreuth, Germany).

Electron paramagnetic resonance (EPR) measurements were made on a Varian E-9 spectrometer at 113 K. Other conditions of EPR spectroscopy: microwave power, 50 mW; microwave frequency, 9.13 GHz; modulation amplitude, 1.6 G; modulation frequency, 100 kHz. The data were averaged and stored on a Nicolet 1070.

The copper content was determined on a Hitachi Z-8000 absorption spectrometer by calibrating the peak height with a Cu standard.

## RESULTS

*Purification.* The purification scheme is shown in Table I. In the first step, the membrane preparation was treated with Triton X-100. The detergent extraction yielded a fraction with halocyanin as the main protein component (20%) as proven by SDS–PAGE (data not shown). The subsequent chromatography on DEAE-Sepharose removed large amounts of contaminating proteins, particularly most of the cytochromes as well as yellow flavoproteins. From this step on, the halocyanin content could be determined spectroscopically.

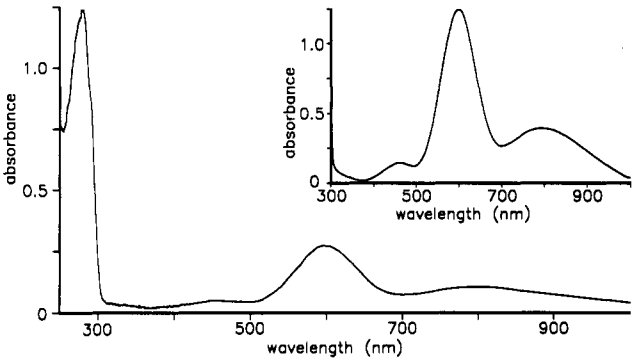


FIGURE 1: Absorption spectrum of halocyanin in the visible and ultraviolet regions at room temperature.

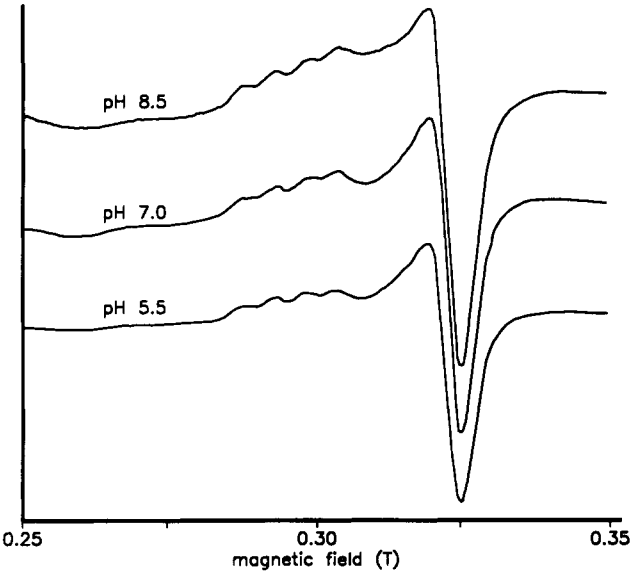


FIGURE 2: Electron paramagnetic resonance spectra of halocyanin recorded at 113 K at the pH values indicated. The microwave power was 50 mW at a frequency of 9.13 GHz. The modulation amplitude was set at 1.6 G with a modulation frequency of 100 kHz.

The subsequent chromatography on hydroxylapatite separated halocyanin from remaining cytochromes. From an estimated content of 3.7 μmol/20 L of cell culture, 30 mg of pure halocyanin could be isolated, which amounts to a 51% yield (Table I). Under the assumptions that almost no loss has occurred during the extraction step and that the yield of the first chromatographic step is about 80%, the halocyanin content in 1 L of cell culture can be approximated to be 2.9 mg. With a cell number of $4 \times 10^9$ cells/mL, it can be estimated that about 25 000 halocyanin molecules are contained in each cell.

*Classification.* The absorption and EPR spectra were used for a preliminary classification of the isolated blue protein. The absorption spectrum in Figure 1 is typical for blue copper proteins with a maximum at 600 nm and two secondary maxima at 790 and 460 nm. The main peak at 280 nm corresponds mainly to the absorption of tyrosine and tryptophan residues. The ratio of $A_{280}$:$A_{600}$ is about 4.8 whereas $A_{600}$:$A_{460} \cong 0.1$. The absorption coefficient at 600 nm was determined to be 4190 $M^{-1}$ $cm^{-1}$ on the basis of a molecular mass of halocyanin of 15.5 kDa.

The EPR spectra of oxidized halocyanin at different pH values are shown in Figure 2. The spectra are not pH dependent in the measured range from pH 4 to pH 8.5. The absorption band has the typical appearance of the unpaired electrons of $Cu^{2+}$ with $g_\perp = 2.024$ and $g_\parallel = 2.209$. The spectrum has considerable similarities to that of plastocyanin

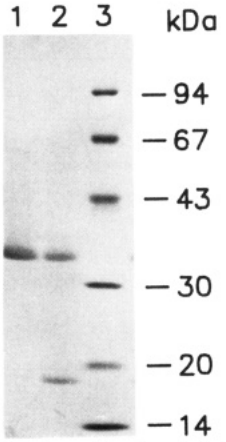


FIGURE 3: SDS gel of halocyanin: lane 1, halocyanin preincubated at 95 °C for 10 min; lane 2, halocyanin without the heating step; lane 3, molecular mass markers.

Table II: Amino Acid Composition of Halocyanin

| amino acid | no. of residues | amino acid | no. of residues |
|---|---|---|---|
| Asx | 27 | Leu | 3 |
| Thr | 12 | Tyr | 3 |
| Ser | 4 | Phe | 8 |
| Glx | 19 | His | 3 |
| Pro | 6 | Lys | 0 |
| Gly | 20 | Arg | 5 |
| Ala | 12 | Cys[a] | 2 |
| Val | 15 | Met | 2 |
| Ile | 3 | Trp[b] | 3 |

total of 146 residues; mass = 15.54 kDa[c]

[a] Determined after performic acid oxidation. [b] Determined in the presence of thioglycolic acid after TFA/HCl hydrolysis. [c] The molecular mass of 15.54 kDa was taken from preliminary electrospray mass spectrometric data (S. B. H. Kent, personal communication).

(Peisach & Blumberg, 1966; Aikazyan & Nalbandyan, 1979), with a hyperfine coupling only along the symmetrical axis, and it is distinct from that of e.g. stellacyanin (Peisach et al., 1967). Reduction of $Cu^{2+}$ to $Cu^{+}$ caused the disappearance of the EPR signal as well as of the blue color.

*Biochemical Characterization.* Halocyanin has an unusual migration behavior in gel electrophoresis under denaturating conditions. SDS-PAGE of the purified protein resulted in a single band with an apparent molecular mass of 35 kDa when the protein was heated 10 min at 95 °C prior to electrophoresis (Figure 3, lane 1). A sample, which was not preincubated at higher temperatures, showed an additional band with an apparent molecular mass of 18 kDa (lane 2). The appearance of the band with the higher apparent mass occurred simultaneously with an absorbance decrease at 600 nm, which indicated the loss of the chromophore. The midpoint ($T_m$) of this transition is 67 °C. Disulfide bonds are not involved in this process because it is not influenced by sulfides like e.g. dithiothreitol.

*Copper Content.* For the determination of the copper content, the sample was first dialyzed against 10 mM EDTA. Atomic absorption spectrophotometry resulted in 1.24 ± 0.11 atoms of copper per protein molecule (mass = 15.5 kDa). Other metals like Fe, Mn, and Zn were only detected in substoichiometric amounts (<0.03 M/halocyanin molecule).

*Amino Acid Composition and Partial Sequence.* The amino acid composition of halocyanin is shown in Table II. Noticeable is the high content of Asx and Glx, which could explain the low p*I* of about 4.5 determined by free flow electrophoresis. Interestingly, Lys could not be detected. Also Trp existed

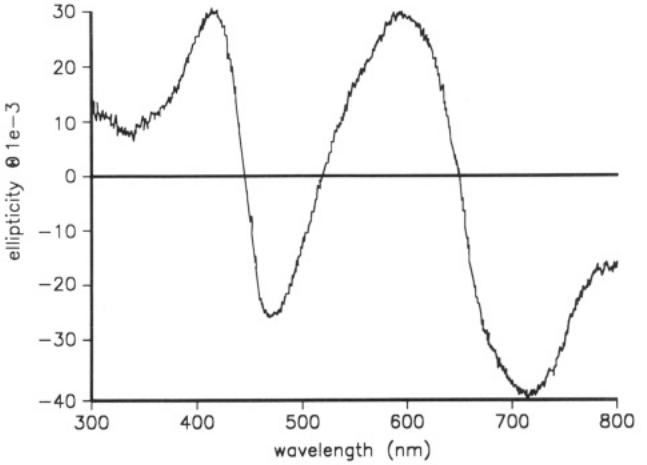


FIGURE 4: Visible CD spectrum of 0.6 mM halocyanin in 50 mM NaCl/20 mM $Na_2HPO_4$/1% octyl glucoside, pH 7.0, with a 0.1 cm path length cell; average of four scans.

only in a few copies. As possible ligands for copper, three His, two Cys, and two Met could be identified.

An amino-terminal sequence could not be obtained, indicating that the N-terminus might be blocked. For further analysis of the protein, a tryptic digestion was carried out and the proteolysate was separated by reversed-phase HPLC. The peptides were then sequenced by automated Edman degradation. One of the isolated peptides (T6) comprised the carboxy-terminus. The sequence of this peptide is Ala-Gln-Gly-Met-Tyr-Gly-Ala-Val-Ile-Val-Glu. After the 11th step of sequencing, the concentration of the major component dropped to the background level. For an internal tryptic peptide, one would expect a Lys or Arg residue and not Glu as the terminal amino acid, so that T6 can be assumed to constitute the carboxy-terminal peptide. Further sequence analysis of peptides derived from a digest with the endoprotease Glu-C revealed a peptide (G10) which overlapped with T6. It has the sequence Gly-Val-Ala-Leu-Tyr-Val-Cys-Thr-Pro-His-Arg-Ala-Gln-Gly-Met-Tyr-Gly-Ala-Val-Ile... (Mattar, 1993). From this combined sequence information, the C-terminus thus contains three (Cys, His, and Met) of the four copper ligands (see Table IV).

*CD Spectroscopy.* The CD spectrum between 300 and 800 nm (Figure 4) is characterized by four extrema at 413 and 590 nm (maxima) as well as 477 and 721 nm (minima). The CD spectrum of halocyanin is quite similar to those of other small blue copper proteins, with differences mainly in the positions of the extrema (e.g.: Plastocyanin, Gewirth and Solomon (1988); auracyanin, Trost et al. (1988)).

Figure 5 shows the CD spectrum of halocyanin between 185 and 245 nm in the range of the n–$\pi^*$ and $\pi$–$\pi^*$ transitions of peptide bonds. A comparison with CD spectra of reference proteins (Brahms & Brahms, 1980; Johnson, 1990) allows an assignment to the secondary structure of the protein. The spectrum with a maximum at 196 nm and a single minimum at 220 nm points to mainly $\beta$-structural features. The zero transition at 209 nm and the low ellipticities also indicate that halocyanin has almost no or less than 5% $\alpha$-helical parts.

## DISCUSSION

*N. pharaonis* is a haloalkaliphilic archaebacterium that grows optimally at pH 8.5–9.5 and 4 M NaCl. It was first isolated from extreme alkaline soda lakes in Africa (Imhoff et al., 1979; Tindall et al., 1980; Soliman & Trüper, 1982). The cells are rod-shaped, Gram-negative, and strictly aerobic.

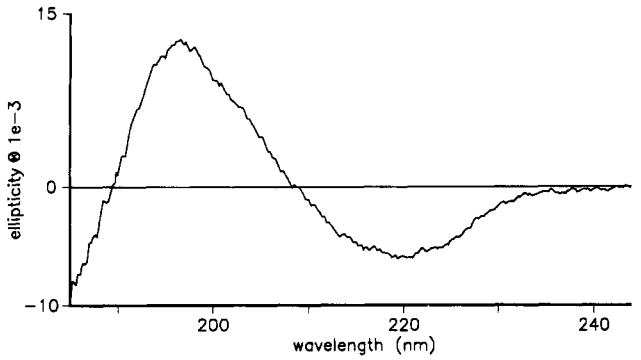


FIGURE 5: Ultraviolet CD spectrum of 6 $\mu$M halocyanin after dialysis against $H_2O$ with a 0.01 cm path length cell; average of 16 scans.

The haloalkaliphiles share many characteristics in common with the "classical" archaebacterial halophiles, including the quinone content (Collins et al., 1981) and the bacterioruberin synthesis (Tindall et al., 1980). On the other hand, they constitute a distinct phenotypic group because they have an obligate requirement for high pH and a very low $Mg^{2+}$ tolerance (Tindall et al., 1980; Soliman & Trüper, 1982). They seem to be only distantly related to the *Halobacteria* and were classified into the new genera *Natronococcus* and *Natronobacterium* (Tindall et al., 1984).

As a member of the order halobacteriales, *Natronobacteria* are representatives of the archaebacteria, one of the three kingdoms of living cells. Some of the biochemical properties of archaebacteria are unique; others are shared with either eubacteria or eukaryotes (Fox et al., 1980; Woese et al., 1990). Due to their unusual and unexpected habitats, including extremes of temperature, ionic strength, and proton concentration, they are interesting objects for biological studies focused e.g. on bioenergetics at high pH values or on structural and functional stability of biological matter at extreme temperatures. Furthermore, the archaebacteria contribute to the discussion about the nature of a universal common ancestor ("progenote"). The comparison of archaebacterial enzymes with those of procaryotes and eucaryotes may help to resolve the question at what time and at which step the divergence between the single kingdoms took place (Bayley & Morton, 1978). In this communication, the isolation and biochemical classification of a blue copper protein from *N. pharaonis* is described, a class of proteins which has not yet been isolated and purified in archaebacteria.

The amount of knowledge about blue copper proteins is a result of the relative ease with which the blue copper proteins (type I) can be assayed and isolated. Halocyanin was first discovered because of its blue color. Its isolation is easily performed with a few chromatographic steps (Table I).

The biochemical data for halocyanin are congruent with typical blue copper proteins (type I) (Table III) with an intense absorption band at 600 nm and an EPR spectrum having a close resemblance to that of plastocyanin with the typical hyperfine coupling (Peisach & Blumberg, 1966; Aikazyan & Nalbandyan, 1979). The EPR data suggest that the copper atoms are of the type I class with an axial character. A correlation between the rhombicity of the EPR spectrum and the ratio of the optical densities at 460 and 600 nm has been proposed by Lu et al. (1993). Halocyanin has a ratio of $A_{460}$: $A_{600} \cong 0.1$, which is close to the value of plastocyanin (0.06) or azurin (0.11) (Lu et al., 1993). These data indicate that halocyanin should be grouped with plastocyanin into the same diverse subgroup of the small type I blue copper proteins.

Table III: Biochemical Data for Selected Small Blue Copper Proteins[a]

| protein | source | $M_r$ | p*I* | $E^\circ$ (mV) | $\lambda_{max}$ (nm) ($\epsilon$) |
|---|---|---|---|---|---|
| halocyanin | *Natronobacterium pharaonis* | 15 500 | 4.5 | 223 | 600 (4190) |
| plastocyanin | plants/algae | 10 500 | 4.2 | 375 | 597 (4500) |
| azurin | Bacteria | 14 000 | 5.4 | 305 | 625 (5200) |
| stellacyanin | *Rhus vernicifera* | 20 000 | 9.9 | 184 | 608 (4080) |
| amicyanin | *Thiobacillus versutus* | 11 000 | 4.7 | 260 | 596 (3900) |
| rusticyanin | *Thiobacillus ferrooxidans* | 16 000 | 9.1 | 680 | 597 (2240) |
| auracyanin | *Chloroflexus aurantiacus* | 12 800 | 4.0 | 240 | 596 (2900) |

[a] Data taken from Sykes (1990) and Trost et al. (1988) (auracyanin).

The circular dichroism spectrum in the region where peptide bonds absorb provides an estimate of secondary structural elements. As is found in other blue copper proteins, halocyanin has a high degree of $\beta$-structure. The midpoint potential of 223 mV (at pH 7; data not shown) falls within the range of those found for small copper proteins. As could be expected, it proved to be higher than that of the $Cu^{2+}/Cu^{+}$ couple with 154 mV and to fall into the range of values for several other small blue copper proteins (Table III). While most of them clustered around a value of 300 mV, the lowest potential was obtained for stellacyanin with 184 mV and the highest for rusticyanin with 680 mV.

The similarity to other type I blue copper proteins is also evident in the analysis of the CD and absorption spectra in the visible range. The assignments of the observed bands have been discussed by several authors (e.g. Solomon et al. (1980) and Trost et al. (1988). A careful analysis of the CD, MCD, and visible absorption spectra of plastocyanin led Gewirth and Solomon (1988) to the conclusion that the band at 600 nm is due to a cysteinate S $p\pi \rightarrow$ Cu $d_{x^2-y^2}$ transition. Other transitions from His $\pi_1$ and Met thioether were assigned to bands in the region 425–465 nm. In a recent publication, Han et al. (1993) concluded from resonance Raman spectra of azurin that the 600- and 460-nm bands have substantial (Cys)S $\rightarrow$ Cu(II) charge-transfer character. Taking these data into account, and in analogy to other small blue copper proteins, it seems that the copper site in halocyanin consists of imidazole nitrogens, thiolate sulfur, and thioether sulfur. With the exception of stellacyanin and CPC, all blue copper proteins (type I) so far analyzed are liganded to two His, one Met, and one Cys. The amino acid composition of halocyanin indicates that there are three histidine, two cysteine, and two methionine residues per protein molecule and thus per copper atom.

The analysis of the primary sequences and crystallographic X-ray studies of a variety of blue copper proteins (type I) (Adman, 1991) have conclusively demonstrated that three of the copper ligands are found in the C-terminal region of the proteins. There exists a general sequence motif Cys-$(Aax)_{2-4}$-His-$(Aax)_{2-4}$-Met- (Sykes, 1991) which can also be found in the C-terminal sequence of halocyanin: -***C***-T-P-***H***-R-A-Q-G-***M***-. The similarity to other proteins of this class is quite stringent. Especially, the copper ligands Cys, His, and Met are well preserved (Table IV). Thus, the biochemical properties and the C-terminal amino acid sequence integrate this protein clearly into the class of the small type I blue copper proteins. Therefore, it was named halocyanin, indicating its origin from the haloalkaliphilic *N. pharaonis*. Apparently, these proteins have evolved also in the third domain of life. That this is not a singular observation characteristic

Table IV: C-Terminal Amino Acid Sequence of Selected Small Blue Copper Proteins[a]

| | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Halocyanin (*Natronobact. pharaonis*) | A | L | Y | V | C | T | – | P | – | H | R | A | Q | G | M | Y | G | A | V | I | V | E | | | |
| Plastocyanin (*Populus nigra*) | Y | S | F | Y | C | S | – | P | – | H | Q | G | A | G | M | V | G | K | V | T | V | N | | | |
| Stellacyanin (*Rhus vernicifera*) | Y | – | Y | I | C | G | V | P | K | H | C | D | L | G | Q | K | V | H | I | N | V | T | | | |
| Plantacyanin (*Cucumis sativus*) | Y | – | F | I | C | N | F | P | G | H | C | Q | S | G | M | K | I | A | V | N | A | L | | | |
| Azurin (*Alcaligenes faecalis*) | Y | A | F | F | C | S | F | P | G | H | W | – | S | I | M | K | G | T | I | E | L | G | | | |
| Amicyanin (*Methylobact. extorquens*) | Y | D | Y | I | C | T | – | P | – | H | P | – | F | – | M | K | G | K | V | V | V | E | | | |
| Rusticyanin (*Thiobacillus ferrooxidans*) | Y | Y | Y | V | C | Q | I | P | G | H | A | A | T | G | M | F | G | K | I | V | V | K | | | |

[a] The frames indicate sequence similarities. The shaded areas mark the copper ligands. Data and alignment were taken from Ronk et al. (1991).

only for *N. pharaonis* is demonstrated by Steiner (1983) in his thesis, where he described a blue copper protein released from membrane preparations of *Halobacterium halobium*. The few available molecular data for this protein (EPR and absorption spectra, molecular mass, and copper content) indicate a close relationship to halocyanin.

The homology and similarity of the archaebacterial blue copper proteins to other blue copper proteins (type I) from eubacteria and eukaryotes raise the question of the phylogenetic origin of blue copper proteins. Rydén (1984, 1988) proposes an ancestral blue protein in a *Chlorobium*-like bacterium to be the common origin not only of the type I copper proteins but also of the multicopper oxidases. This ancestral tree has certainly to be revised once sequence data from archaebacterial blue copper proteins (type I) are available.

Interestingly, during the isolation procedure, halocyanin is fractionated with the membrane, indicating that it is not a cytoplasmic protein like most of the other known blue copper proteins (type I). It can only be released from the purified membrane by low amounts of a detergent like Triton. Congruent with these data is the observation that halocyanin cannot be recovered from a $C_{18}$ reversed-phase column. On the other hand, not only is the amino acid composition atypical for a hydrophobic protein, but also the high amount of charged amino acids (Table II) suggests it to be a soluble protein. Further evidence for unusual properties of the protein can be drawn from its SDS gel electrophoretic behavior. On heating of the sample prior to electrophoresis, the apparent molecular mass changes from 18 to 35 kDa, indicating a dimerization. From these observations, one can conclude that halocyanin is a peripheral membrane protein. However, the nature of a possible anchor remains unclear. It might be possible that a posttranslational modification of the protein has occurred similar to that observed in e.g. auracyanin, which is located at the periplasmic side of the membrane and was proven to be glycosylated (McManus et al., 1992). One might also speculate about a lipid anchor which could permit the attachment of halocyanin to the membrane.

The physiological role of halocyanin has not yet been clarified. Since this archaebacterial small blue copper protein was identified to be a peripheral membrane protein, it could be a mobile carrier in electron-transfer reactions. The attachment to the membrane not only confines halocyanin to a restricted part of the cell but also enhances the diffusional rate (by going from three- to two-dimensional diffusion), thereby increasing the chances of a collision with a reaction partner. From the midpoint potential of 223 mV, one might speculate that the reaction partner is an endoxidase replacing e.g. cytochrome *c* and adopting a role similar to that of plastocyanin or auracyanin. This might well be the case since in *N. pharaonis* several types of cytochromes, including cytochrome *c* and cytochrome *b*, have already been described (Scharf, 1992). Furthermore, an oxidase, cytochrome $ba_3$, was also identified which might function as the electron acceptor from halocyanin.

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## REFERENCES

Adman, E. T. (1985) in *Metalloproteins. Part 1: Metals Proteins with Redox Roles* (Harrison, P., Ed.) pp 1–42, Macmillan, London.
Adman, E. T. (1991) *Adv. Protein Chem.* 42, 145–197.
Aikazyan, V. T., & Nalbandyan, R. M. (1979) *FEBS Lett. 104*, 127–130.
Bayley, S. T., & Morton, R. A. (1978) *CRC Crit. Rev. Microbiol.*, 151–205.
Brahms, S., & Brahms, J. (1980) *J. Mol. Biol. 138*, 149–178.
Bergmann, C., Gangvik, E.-K., Nyman, P. O., & Strid, L. (1977) *Biochem. Biophys. Res. Commun. 77*, 1052–1059.
Blake, R. C., II, White, K. J., & Shute, E. A. (1991) *Biochemistry 30*, 9443–9449.
Chen, L., Durley, R., Poliks, B. J., Hamada, K., Chen, Z., Mathews, F. S., Davidson, V. L., Satow, Y., Huizinga, H., Vellieux, F. M. D., & Hol, W. G. J. (1992) *Biochemistry 31*, 4959–4964.
Collins, M. D., Ross, H. N. M., Tindall, B. J., & Grant, W. D. (1981) *J. Appl. Bacteriol. 50*, 559–565.
Cox, J. C., & Boxer, D. H. (1978) *Biochem. J. 174*, 497–502.
Farver, O., Blatt, Y., & Pecht, I. (1982) *Biochemistry 21*, 3556–3561.
Fee, J. A. (1975) *Struct. Bonding 23*, 1–60.
Fox, G. E., Stackebrandt, E., Hespell, R. B., Gibson, J., Maniloff, J., Dyer, T. A., Wolfe, R. S., Balch, W. E., Tanner, R. S., Magrum, L. J., Zablen, L. B., Blakemore, R., Gupta, R., Bonen, L., Lewis, B. J., Stahl, D. A., Luehrsen, K. R., Chen, K. N., & Woese, C. R. (1980) *Science 209*, 457–463.
Fujita, V. S., Black, S. D., Tarr, G. E., Koop, D. R., & Coon, M. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4260–4264.
Gewirth, A. A., & Solomon, E. I. (1988) *J. Am. Chem. Soc. 110*, 3811–3819.
Guss, J. M., & Freeman, H. C. (1983) *J. Mol. Biol. 169*, 521–563.
Han, J., Loehr, T. M., Lu, Y., Valentine, J. S., Averill, B. A., & Sanders-Loehr, J. (1993) *J. Am. Chem. Soc. 115*, 4256–4263.
Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 59–62.
Imhoff, J. F., Sahl, H. G., Soliman, G. S. H., & Trüper, H. G. (1979) *Geomicrobiol. J. 1*, 219–234.
Johnson, W. C. (1990) *Proteins: Struct. Funct. Genet. 7*, 205–214.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lanyi, J. K., Duschl, A., Hatfield, G. W., May, K., & Oesterhelt, D. (1990) *J. Biol. Chem. 265*, 1253–1260.
Liu, M.-Y., Liu, M.-C., Payne, W. J., & LeGall, J. (1986) *J. Bacteriol. 166*, 604–608.

Lontie, R., Ed. (1984) *Cooper Protins and Copper Enzymes*, Vol. 1, CRC Press, Boca Raton, FL.
Lu, Y., LaCroix, L. B., Lowery, M. D., Solomon, E. I., Bender, C. J., Peisach, J., Roe, J. A., Gralla, E. B., & Valentine, J. S. (1993) *J. Am. Chem. Soc. 115*, 5907–5918.
Mann, K., Schäfer, W., Thoenes, U., Messerschmidt, A., Mehrabian, Z., & Nalbandyan, R. (1992) *FEBS Lett. 314*, 220–223.
Mattar, S. (1993) Diplomarbeit, Ruhr-Universität Bochum.
McManus, J. D., Brune, D. C., Han, J., Sanders-Loehr, J. Meyer, T. E., Cusanovich, M. A., Tollin, G., & Blankenship, R. E. (1992) *J. Biol. Chem. 267*, 6531–6540.
Moss, D. A., Leonard, M., Bauscher, M., & Mäntele, W. (1991) *FEBS Lett. 283*, 33–36.
Peisach, J., & Blumberg, W. E. (1966) *Biochim. Biophys. Acta 126*, 269–273.
Peisach, J., Levine, W. G., & Blumberg, W. E. (1967) *J. Biol. Chem. 242*, 2847–2858.
Ronk, M., Shively, J. E., Shute, E. A., & Blake, R. C. (1991) *Biochemistry 30*, 9435–9442.
Rydén, L. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, R., Ed.) Vol. 1, pp 157–182, CRC Press, Boca Raton, FL.
Rydén, L. (1988) in *Oxidases and Related Redox Systems* (King, T. P., Manson, H., Eds.) pp 349–366, Liss, New York.
Scharf, B. (1992) Thesis, Ruhr-Universität Bochum.
Soliman, G. S. H., & Trüper H. G. (1982) *Zbl. Bakt. Hyg. I., Abt. Orig. C 3*, 318–329.
Solomon, E. I., Hare, J. W., Dooley, D. M., Dawson, J. H., Stephens, P. J., & Gray, H. B. (1980) *J. Am. Chem. Soc. 102*, 168–178.
Steiner, M. (1983) Thesis, Ludwig-Maximilian-Universität München.
Steiner, M., & Oesterhelt, D. (1983) EMBO *J. 2*, 1379–1385.
Sutherland, I. W., & Wilkinson, J. F. (1963) *J. Gen. Microbiol. 30*, 105–109.
Sykes, A. G. (1990) *Struct. Bonding 75*, 175–233.
Sykes, A. G. (1991) *Adv. Inorg. Chem. 36*, 377–408.
Tindall, B. J., Mills, A. A., & Grant, W. D. (1980) *J. Gen. Microbiol. 116*, 257–260.
Tindall, B. J., Ross, H. N. M., & Grant, W. D. (1984) *Syst. Appl. Microbiol. 5*, 41–57.
Tobari, J., & Harada, Y. (1981) *Biochem. Biophys. Res. Commun. 101*, 502–508.
Trost, J. T., McManus, J. D., Freeman, J. C., Ramakrishna, B. L., & Blankenship, R. E. (1988) *Biochemistry 27*, 7858–7863.
Woese, C. R., Kandler, O., & Wheelis, M. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4576–4579.
Yokote, Y., Murayama, A., & Akahane, K. (1986) *Anal. Biochme. 152*, 245–249.