# Purification of membrane-bound cytochromes and a photoactive P840 protein complex of the green sulfur bacterium *Chlorobium limicola* f. *thiosulfatophilum*

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A photoactive P840 protein complex and a fraction enriched in cytochrome *b* from chromatophores of *Chlorobium limicola* f. *thiosulfatophilum* are described. The former is identical in pigment composition to the 'core complex' of *Prostecochloris aestuarii* [(1983) Biochim. Biophys. Acta 725, 361–367]. It consists of 3 major polypeptides. The dominating one, of 65 kDa and carrying bacteriochlorophyll, is the P840 reaction center protein. It is probably very similar to the P700 reaction center protein of chloroplasts and cyanobacteria. A 24-kDa protein could be identified as an ascorbate-reducible cytochrome *c*-550.5. The third polypeptide of 32 kDa might be a Rieske-type FeS protein. The cytochrome *b* fraction consists of 2 polypeptides of 42 kDa, representing cytochrome *b*-562, and 24 kDa, representing ascorbate-reducible cytochrome *c*-550.5 again. Conditions can be varied to obtain cytochrome *b*-562 in pure form. A first characterization of the components is presented.

Chlorobium *chromatophore* *Electron transport* *Membrane protein* *Cytochrome* bc *complex* *Reaction center P840* *Bacterial photosynthesis*

## 1. INTRODUCTION

Compared to purple, photosynthetic bacteria relatively little is known about membrane-bound electron transport components of the green, photosynthetic bacteria (Chlorobiaceae [1,2]). EPR measurements suggest that their reaction center complex is similar to Photosystem I in chloroplasts, with FeS centers as electron acceptors [3,4], capable of pyridine nucleotide reduction without further energy requirement [5]. The presence of a quinone pool [6], of photooxidizable cytochrome *c* [7] and photoreducible cytochrome *b* [8,9], which is stimulated by antimycin A [19], together with the possible occurrence of a Rieske-type FeS center [3] indicate the existence of a quinol-cytochrome *c* oxidoreductase in the form of a cytochrome *bc* complex, as found for mitochondria, chloroplasts and other bacteria [11].

The pigment system of Chlorobiaceae can be separated into a large membrane-attached bacteriochlorophyll (BChl) *c* antenna, the chlorosome [12–14] and a photosystem–protein complex within the photosynthetic membrane containing BChl *a* [8,15–17]. The latter can be resolved into a water-soluble 42-kDa BChl *a*–protein [12,18], and a 'reaction center complex' [9,18,19], which in addition to BChl *a* contains carotenoids and varying amounts of cytochrome *b* and *c*. Recently, a still smaller core complex was obtained from the reaction center complex of *Prosthecochloris aestuarii*, with 3 polypeptides, of 60, 43 (dominating) and 32 kDa [20]. This preparation had lost photochemical activity.

We report here on the purification of membrane-bound cytochrome *b* and *c*, and of a photoactive P840 reaction center from *Chlorobium limicola* f. *thiosulfatophilum*.

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## 2. MATERIALS AND METHODS

*C. limicola* f. *thiosulfatophilum*, strain Tassajara (Deutsche Sammlung von Mikroorganismen, D-3400 Göttingen) was grown in 10-l bottles as in [21]. Cells were harvested 3 days after inoculation and were stored as a concentrated suspension in 50 mM phosphate (pH 6.8) at −20°C until use. All further steps were carried out below 5°C. Thawed cells from a 20 l culture (about 30 g wet wt) were washed by resuspension and centrifugation, and were finally suspended in 150 ml phosphate buffer plus 1 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was homogenized by sonication and chromatophore membranes were sedimented at 300000 × *g* as in [10]. The chromatophores were suspended in 100 ml of 20 mM Tris–HCl (pH 7.8), 100 mM KCl, 1 mM EDTA and centrifuged again. Then they were extracted in two steps, first at low, subsequently at high salt: For the first extraction the washed chromatophores were suspended in 40 ml Tris–HCl (pH 7.8), followed by 1:1 dilution with 60 mM β-D-octylglucoside (Sigma), 1% cholate, 300 mM KCl, 0.5 mM PMSF, 20 mM Tris–HCl (pH 7.8). After 15 min stirring on ice and 1 h centrifugation at 300000 × *g*, a yellow-green supernatant was obtained, which contained most of a dithionite-reducible cytochrome *c*-552.5 [9], representing a heme–protein of 32 kDa on SDS-polyacrylamide gel electrophoresis (SDS–PAGE), but contained only a small amount of cytochrome *b*. The pellet, corresponding to 325 *A* at 670 nm in 80% methanol, was further extracted in 100 ml of 30 mM octylglucoside, 0.5% cholate, 0.4 M ammonium sulfate, 0.5 mM PMSF and 20 mM Tris–HCl (pH 7.8), for 30 min on ice before centrifugation was repeated as above. The blue-green supernatant contained ascorbate-reducible cytochrome *c*-550.5 and dithionite-reducible cytochrome *b*-562 [9] in a ratio of about 1:1, each about 3 μM (assuming $\epsilon = 20\ mM^{-1} \cdot cm^{-1}$ at the α-band), plus residual cytochrome *c*-552.5. Only about 4% of total BChl was solubilized by the second, and even less by the first extraction, mainly as BChl *a*. The *Chlorobium* vesicles with BChl *c* [12–14] were not solubilized, and could be sedimented. To the second extract Triton X-100 was added to 0.25%, which allowed better fractionation in the subsequent step. Ammonium sulfate was then added solid to 42% saturation (note that the extract already contained 10%). The precipitate was resuspended in about 4 ml of 20 mM Tris–HCl (pH 7.8), 0.2% Triton X-100 and was dialyzed for 1 h against 30 mM phosphate (pH 6.8), 0.1% Triton X-100. The sample was then loaded onto a hydroxyapatite column (inner diameter = 2 cm, *h* = 3 cm). Elution with 50 mM phosphate buffer, 0.1% Triton X-100 yielded a fraction containing the reaction center P840 together with cytochromes *c*-550.5 and *b*-562 (ratio 1:2), leaving most of a 42-kDa BChl *a*–protein [18,20] on the column, together with many other polypeptides. After precipitation with 40% saturated ammonium sulfate and resuspension in a small volume of 50 mM phosphate/Triton buffer, the sample was applied on a sucrose density gradient, 10–30% sucrose in 50 mM phosphate (pH 6.8), 0.1% Triton X-100, and centrifuged for 22 h at 60000 rpm in a Beckman SW 61 rotor. Four pigmented zones could be discerned after the run – a green one on the top, at about 10% sucrose, containing no polypeptides, a brownish one at about 16%, another green one at about 24% and a blue-green one at about 27% sucrose, containing the residual BChl *a*–polypeptide of 42 kDa.

Other methods are given in the figure legends.

## 3. RESULTS AND DISCUSSION

Seven heme-carrying polypeptides could be detected in homogenates of *Chlorobium* cells by SDS–PAGE [22], corresponding to molecular masses of 37, 32, 24, 20, 16, 14 and 10 kDa. No *c*-type cytochromes of 45–60 kDa [23,24] could be seen. The 14- and 10-kDa species are water soluble, since they were largely lost to the supernatants during preparation of washed chromatophores. The latter represents cytochrome *c*-555 [23,24]. A dominating, chromatophore-bound *c*-type cytochrome, reducible by dithionite only, could be largely extracted by octylglucoside/cholate at moderate ionic strength. It had an α-band at 552.5 nm and corresponded to a 32-kDa polypeptide. The rest of the cytochromes was extracted at high salt. Subsequently ascorbate-reducible cytochrome *c*-550.5, corresponding to the 24-kDa heme-protein, and cytochrome *b*-562 were precipitated by 42% ammonium sulfate, together with

BChl *a*, residual cytochrome *c*-552.5 and a 37-kDa heme–protein remaining in the supernatant.

Fig.1, lane 1 shows that the eluate from the hydroxyapatite column contains 4 major polypeptides of molecular masses 65, 42, 32 and 24 kDa. Only the latter is heme-positive on SDS–PAGE, and therefore represents cytochrome *c*-550.5. Lanes 2 and 3 in fig.1 demonstrate that of the 4 polypeptides in the hydroxyapatite eluate, the two at 65 and 32 kDa are found in the green band at 24% sucrose while a polypeptide of 42 kDa, different from the BChl *a*–protein, is present in the brownish zone. Heme-positive cytochrome *c*-550.5 is found in both zones, in discrete concentration peaks. The spectra of the brownish zone in fig.2 show that only a small amount of BChl *c* (or bacteriopheophytin [25]) and about 0.25 cytochrome *c*-550.5 per cytochrome *b*-562 are present (assuming $\epsilon = 20\ mM^{-1} \cdot cm^{-1}$ at the $\alpha$-peaks). Redox titrations, performed at pH 6.2 as in [26], gave a midpoint potential of +220 mV ($n = 1$) for cytochrome *c*-550.5. This value is similar to that reported for cytochrome *c* photooxidation in *Chlorobium* chromatophores [7], but also lower values for photooxidizable cytochrome *c* have been reported [15,27]. For cytochrome *b*-562 more than one component was found ($n < 1$), with an average midpoint potential of +8 mV. A similar heterogeneity is known for cytochromes *b* in all quinol-cytochrome *c* oxidoreductases tested so far [11].

The spectra of the green band at 27% sucrose in fig.3 suggest that this fraction resembles what has been called the core complex of the photosystem with respect to pigment composition [20]. In addition the presence of cytochrome *c*-550.5 is documented (about 1 per 20 BChl *a* [9]). Noteworthily, our preparation contains little of the 42-kDa polypeptide compared to the core complex described in [20]. Furthermore, during electrophoresis at 4°C in the presence of lithium dodecyl sulfate (LiDS)

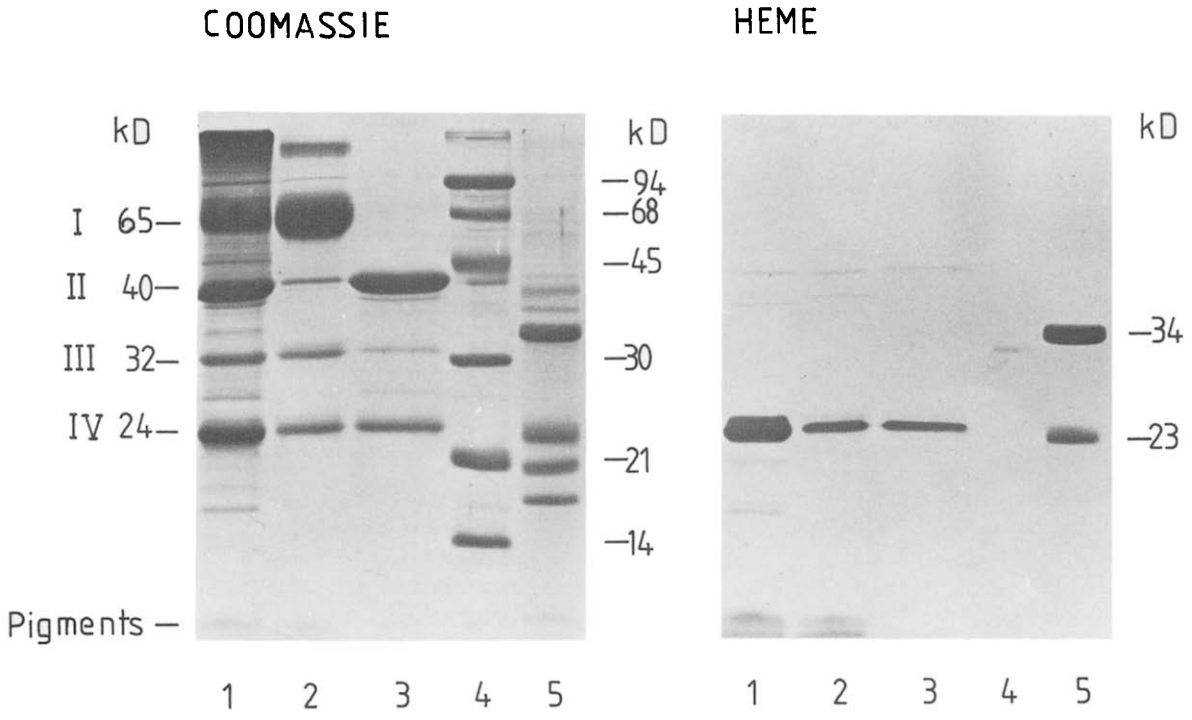


Fig.1. SDS–PAGE of fractions containing P840 and cytochromes. Electrophoresis on 15% polyacrylamide gel was performed as in [35]. The gel was stained for heme (right panel) as in [22], followed by staining with Coomassie Blue (left panel). The following samples were applied: (1) eluate from hydroxyapatite, 800 pmol cytochrome *b*-562; (2) green band at 24% sucrose, 150 pmol cytochrome *c*-550.5; (3) brown band at 16% sucrose, 630 pmol cytochrome *b*-562; (4) protein standard; (5) cytochrome $b_6f$ complex from chloroplasts as heme standard [33], 200 pmol cytochrome *f*.

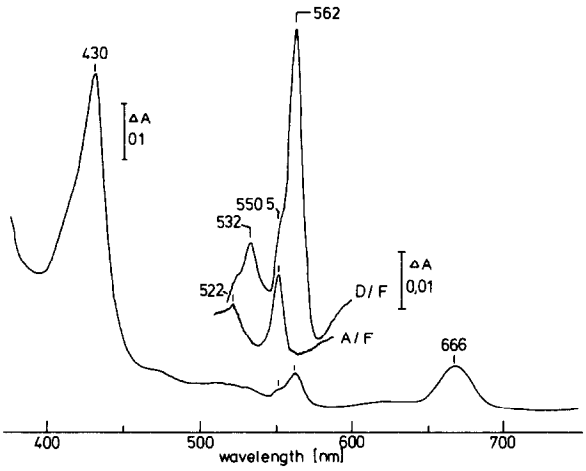


Fig.2. Spectra of the brown band on the density gradient. Spectra were taken in an Aminco DW2 spectrophotometer. The absolute spectrum was recorded in the presence of dithionite. The redox difference spectra (A/F, ascorbate – ferricyanide; D/F, dithionite – ferricyanide) were recorded at 10× amplification.

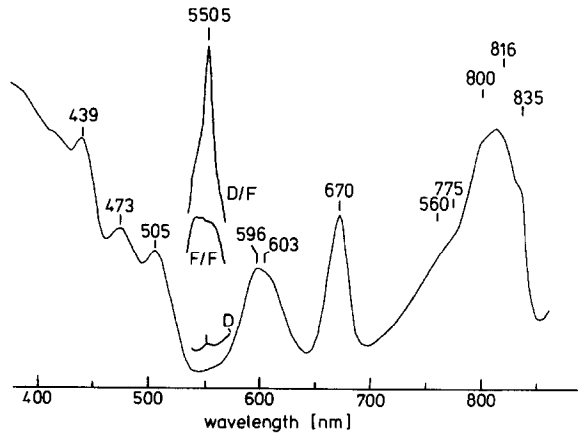


Fig.3. Spectra of the P840 fraction on the density gradient. The absolute spectra were recorded without any addition. The corresponding spectrum with dithionite (D) is only shown in the $\alpha$-band region for cytochromes *b* and *c*. The upper two insets show difference spectra at 20× amplification. D and F, dithionite and ferricyanide, respectively. The dotted spectrum in the long wavelength region was taken above liquid nitrogen.

[28], the major polypeptide of 65 kDa carries BChl *a*. We therefore conclude that this polypeptide is the apoprotein of the P840 reaction center, which thus would resemble the P700–protein of Photosystem I in chloroplasts and cyanobacteria also with regard to its size [29]. The band at still higher molecular mass (fig.1, lane 1) might be an aggregate, since it disappeared on LiDS–PAGE at lower temperature. The nature of the 32-kDa component is not yet clear, but it might be the Rieske-type FeS protein, since a cross-reaction with an antiserum against the Rieske FeS protein from chloroplasts [30] was found by immunoblotting [31].

Our P840 preparation was photochemically active. Either photobleaching of P840 (605–540 nm [9]), or photooxidation of cytochrome *c*-550.5 (550.5–540 nm, suppressible by dithionite) could be observed, as will be described in detail elsewhere. From the extent of the former a BChl *a*:P840 ratio of 20 could be calculated [9], which is lower than previously reported for reaction center P840 preparations [9,19]. All of the cytochrome *c*-550.5 present was photooxidizable. Also excess cytochrome *c*-550.5, added in the form of the brown band in the sucrose gradient, could be fully photooxidized. Addition of soluble cytochrome *c*-555 [23,24] had no effect on extent and kinetics. It is possible that in *Chlorobium* the membrane-bound cytochrome *c*-550.5 functions as the immediate electron donor for the reaction center chlorophyll. On the other hand, the kinetics have not been resolved, and slow photooxidation of cytochrome $c_1$, in the absence of mediating cytochrome $c_2$, has been found for *Rhodospirillum rubrum* [32] and *Rhodopseudomonas sphaeroides* (B.A. Melandri, personal communication). However, also the reaction centers of other purple photosynthetic bacteria, i.e., *Chromatium* and *Rhodopseudomonas viridis*, contain bound cytochrome *c*, while others do not [33].

In contrast to reports for *Chlorobium* chromatophores [8–10], we have not yet been able to observe photoreduction of cytochrome *b*-562, either with the eluate of the hydroxyapatite column, or in a system recombining the cytochrome *b* and P840 fraction of the sucrose gradient. Also oxidant-induced reduction [11] of cytochrome *b*-562 could not be detected.

The distribution of cytochrome *c*-550.5 between these two fractions varied complementarily in dif-

ferent preparations (0.5–1.5 per P840 were found). This distribution was influenced if samples were frozen prior to sucrose density centrifugation, or if dithionite was added.

Pure cytochrome *b*-562 could be obtained if 0.25% soybean lecithin was included in the Triton X-100/phosphate buffer for resuspending the ammonium sulfate precipitate of the hydroxyapatite eluate, and in the sucrose gradient. In this case the brown band was found at 14% sucrose, contained only one polypeptide of 42 kDa and gave a pure cytochrome *b*-562 spectrum, with a midpoint potential heterogeneity similar to the preparation above. The P840 fraction banded at 28% sucrose, together with the residual 42-kDa BChl *a*–protein. It contained all of the cytochrome *c*-550.5. These observations argue against a tight cytochrome *bc* complex in the *Chlorobium* chromatophore membrane.

We were unable to measure menadiol-cytochrome *c* oxidoreductase activity, using mammalian cytochrome *c* or *Chlorobium* cytochrome *c*-555 [23,24] as electron acceptors – either with the final cytochrome *bc* fraction, or with chromatophores and samples of the intermediary purification steps. This is caused by a very high uncatalyzed rate with menadiol, even at low pH [33]. Also ubi- or plastoquinol was ineffective. In view of the direct photooxidation of cytochrome *c*-550.5 it is possible, however, that reduction of a soluble cytochrome *c* by menadiol does not occur in *Chlorobium*.

In conclusion it remains uncertain whether green sulfur bacteria oxidize quinol in an energy-conserving mechanism, catalyzed by a cytochrome *bc* complex, similar to mitochondria, chloroplasts or other bacteria [11]. The obvious similarities of cytochrome *c*-550.5 and cytochrome *b* -562 to the cytochromes in the $bc_1$ complexes of mitochondria and the purple bacterium *Rps. sphaeroides* [11,34] are still suggestive, however.

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## REFERENCES

[1] Crofts, A.R. and Wraight, C.A. (1983) Biochim. Biophys. Acta 726, 149–185.
[2] Dutton, P.L. and Prince, R.C. (1978) in: The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R. eds) pp.525–576, Plenum, New York.
[3] Knaff, D.B. and Malkin, R. (1976) Biochim. Biophys. Acta 430, 244–252.
[4] Jennings, J.V. and Evans, M.C.W (1977) FEBS Lett. 75, 33–36.
[5] Evans, M.C.W and Buchanan, B.B. (1965) Proc. Natl. Acad. Sci. USA 53, 1420–1425.
[6] Collins, M. and Jones, D. (1981) Microbiol. Rev. 45, 16–54.
[7] Knaff, D.B., Buchanan, B.B. and Malkin, R. (1973) Biochim. Biophys. Acta 325, 94–101.
[8] Fowler, C.F. (1974) Biochim. Biophys. Acta 357, 327–331.
[9] Olson, J.M., Giddings, T.H. and Shaw, E.K. (1976) Biochim. Biophys. Acta 449, 197–208.
[10] Knaff, D.B. and Buchanan, B.B. (1975) Biochim. Biophys Acta 376, 549–560.
[11] Hauska, G., Hurt, E., Gabellini, N. and Lockau, W. (1983) Biochim. Biophys. Acta 726, 97–133.
[12] Olson, J.M. (1980) Biochim. Biophys. Acta 594, 33–51.
[13] Pierson, B.K. and Castenholz, R.W. (1978) in: The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R eds) pp.179–197, Plenum, New York.
[14] Staehelin, L.A., Golecki, J.R. and Drews, G. (1980) Biochim. Biophys. Acta 589, 30–45.
[15] Fowler, C.F., Nugent, N.A. and Fuller, R.C. (1971) Proc. Natl. Acad. Sci. USA 68, 2278–2282.
[16] Fowler, C.F., Gray, B.H., Nugent, N.A. and Fuller, R.C. (1974) Biochim. Biophys. Acta 292, 692–699.
[17] Olson, J.M., Philipson, K.D. and Sauer, K. (1973) Biochim. Biophys. Acta 292, 206–217.
[18] Olson, J.M. (1978) in: The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R. eds) pp.161–178, Plenum, New York.
[19] Swarthoff, T. and Amesz, J. (1979) Biochim. Biophys. Acta 548, 427–432.
[20] Vasmel, H., Swarthoff, T., Kramer, H.J.M. and Amesz, J. (1983) Biochim. Biophys. Acta 725, 361–367.
[21] Fuchs, G., Stupperich, E. and Jaenchen, R. (1980) Arch. Microbiol. 128, 56–63.
[22] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[23] Meyer, T.E., Bartsch, R.G., Cusanovich, M.A. and Methewson, J.H. (1968) Biochim Biophys. Acta 153, 854–861

[24] Steinmetz, M.A. and Fischer, U. (1981) Arch. Microbiol. 130, 31–37.
[25] Stanier, R.Y. and Smith, J.H.C. (1960) Biochim. Biophys. Acta 41, 478–484.
[26] Hurt, E. and Hauska, G. (1982) J. Bioenerg Biomembranes 15, 405–424.
[27] Prince, R.C. and Olson, J.M. (1976) Biochim. Biophys. Acta 423, 357–362.
[28] Delepelaire, P. and Chua, N.-H. (1979) Proc. Natl. Acad. Sci. USA 76, 111–115.
[29] Cogdell, R.J. (1983) Annu Rev. Plant Physiol. 34, 21–45.
[30] Hurt, E., Hauska, G. and Malkin, R. (1981) FEBS Lett. 134, 1–5.
[31] Thomas, P.E., Ryan, D. and Wayne, L. (1976) Anal. Biochem. 75, 168–176.
[32] Van der Wal, H.N. and Van Grondelle, P. (1983) Biochim. Biophys. Acta 725, 94–103.
[33] Hurt, E. and Hauska, G. (1981) Eur. J. Biochem. 117, 591–599.
[34] Gabellini, N., Bowyer, J R , Hurt, E., Melandri, A. and Hauska, G. (1982) Eur. J. Biochem. 126, 105–111.
[35] Laemmli, U.K. (1970) Nature 227, 680–685.