The cytochrome subunit structure in the photosynthetic reaction center of *Chromatium minutissimum*

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Received 31 October 1997; revised version received 17 December 1997

Abstract Gel-electrophoretic assay revealed that the photosynthetic reaction center (RC) of *Chromatium minutissimum*, in contrast to the well-known RC *Rhodopseudomonas viridis*, consists of five rather than four subunits with molecular masses of 37, 34, 25, 19, and 17 kDa. The 37- and 19-kDa subunits are stained with tetramethylbenzidine for the cytochrome *c* hemes. Absorption spectra show that the concentration of reduced cytochromes in the *C. minutissimum* RC poised at redox potential of −150 mV (fully reduced pool of hemes) is about three times more than in the *C. minutissimum* RC poised at redox potential of +260 mV (only high-potential hemes are reduced). The results of redox titration of absorption changes at the cytochrome *c* α-band are most appropriately approximated by a six-component theoretical curve with the midpoint potentials of $E_{m1} = 390$ mV, $E_{m2} = 320$ mV, $E_{m3} = 210$ mV, $E_{m4} = 100$ mV, $E_{m5} = 20$ mV, and $E_{m6} = -50$ mV. Possible functions of the cytochromes with the midpoint potentials 210 and 100 mV, which have not been found in purple bacteria before, are discussed.

Key words: Photosynthetic bacterium; Reaction center; Cytochrome; *Chromatium minutissimum*

1. Introduction

The three-dimensional structure of the bacterial photosynthetic reaction center (RC) has been solved by X-ray diffraction with high atomic resolution [1,2]. The RC of the purple bacteria *Rhodopseudomonas viridis* consists of three membrane subunits (L, M, and H or, according to a new classification, A, B, and C) and a peripheral tetraheme cytochrome subunit. The structure of three membrane subunits was shown to be remarkably similar in two bacterial species studied by X-ray diffraction (*R. viridis* and *Rhodobacter spaeroides*) [1,2]. However, indirect data demonstrate a species-specific variability of the RC tetraheme cytochrome subunit. Direct data on the variability of the cytochrome subunit structure are not available, because only one of the species studied by X-ray diffraction (*R. viridis*) contains this subunit. The variability in the midpoint redox potentials of the cytochrome hemes [3], the temperature dependence of the rate constant of cytochrome photooxidation [3,4], and the strength of binding of the cytochrome subunit to RC [5] in different species of purple bacteria can be regarded as indirect evidence of the species specificity of the subunit structure.

The structure and functions of the cytochrome subunit of bacterial RC appear to be an ideal model for studying the mechanisms of electron transfer in biological systems. Indeed, the mechanism of electron tunneling in biological systems has been proposed for the first time by Chance and DeVault more than 30 yr ago to describe the temperature dependence of this reaction in the *Chromatium vinosum* bacteria [6]. The discovery of a unique two-phase character of the dependence greatly influenced the development of electron-transfer theories over the last three decades, giving rise to a large number of theoretical treatments, which are still being continued [7]. However, the structure of the cytochrome subunit in *Chromatium* remains obscure. A method of preparation of the isolated RC of *C. minutissimum* containing photoactive cytochromes *c* has been described in our previous work [8]. We also studied the temperature dependence of the rate constant of oxidation of cytochrome *c* and the molecular dynamics of the preparations obtained [9]. The goal of the present work was to elucidate the structure of the cytochrome subunit in the isolated preparations of RC *C. minutissimum*.

2. Materials and methods

Culture of *C. minutissimum* was grown, chromatophores were isolated, and RC were prepared and purified as described in [8]. Absorption spectra were recorded on a Hitachi-557 spectrophotometer (Japan). Kinetics of light-induced reactions of electron transfer was measured with an automated single-beam spectrophotometer with laser photoactivation [10]. Redox titration and spectral measurements of the RC preparations poised at various redox potentials were performed as described earlier [10,11]. The methods of gel electrophoresis, staining the heme-containing fractions, and densitometry were described in Ref. [8]. Experimental data were processed on a Herald Electronics personal computer (Great Britain) using standard software. Kinetic measurements on the automated single-beam laser spectrophotometer were run using the same personal computer and locally developed software.

3. Results and discussion

The cytochrome composition of the *C. minutissimum* RC preparations was assayed spectrophotometrically from the differential (reduced minus oxidized) absorption spectra of the RC preparations poised at various redox potentials of medium (E_h) (Fig. 1). The cytochromes *c* chemically reduced with sodium ascorbate (3 mM, $E_h = 260$ mV) were characterized by the α-band absorption maximum at 555 nm (Fig. 1, curve 1), whereas the cytochromes *c* chemically reduced with sodium dithionite (3 mM, $E_h = -150$ mV) were characterized by the α-band absorption maximum at 551.5 nm and a shoulder at 555 nm (Fig. 1, curve 3). The shoulder in the spectrum shown in Fig. 1, curve 3, is likely to be associated with the cytochrome hemes reduced by ascorbate. The spectrum recorded at an intermediate level of redox potential ($E_h = 80$ mV) was characterized by an intermediate position of the spectral maximum (Fig. 1, curve 2).

It is well known that in chromatophores and RC prepara-

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PII S0014-5793(98)00013-1

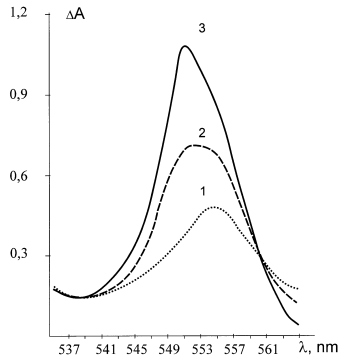


Fig. 1. Differential spectra (reduced minus oxidized) of the *C. minutissimum* RC preparations poised at different redox potentials of medium. The chemically oxidized RC preparation poised at E_h = 480 mV was a reference sample. Experimental sample was poised at: (1) E_h = 260 mV; (2) E_h = 80 mV; (3) E_h = −150 mV, pH 7.0. Redox mediators: 30 μM 2,3,5,6-tetramethyl-1,4-phenylenediamine (3,6-diaminodurene), 100 μM benzoquinone, 50 μM phenazine methosulfate, 100 μM menadione, 100 μM hexamineruthenium, 100 μM 4-dimethylamine-1,4-naphthoquinone, and 100 μM 2-methoxy-1,4-naphthoquinone. Sodium ascorbate (1,2) or sodium dithionite (2,3) were used as reducing agents.

tions of other species of photosynthetic bacteria, ascorbate is able to reduce only so-called high-potential hemes *c*, whereas dithionite is able to reduce the whole pool of RC hemes. Thus, it can be suggested from the spectral maximum amplitudes shown in Fig. 1 that the total number of hemes *c* in the *C. minutissimum* RC preparations tested is three times higher than the number of high-potential hemes *c* (provided that the extinction coefficients of all hemes are comparable). It is also known that the number of high-potential hemes in all the bacterial species which contain the multiheme cytochrome subunit, is two [1,3,5,11,12]. Thus, it can be suggested that the total number of hemes *c* in the RC *C. minutissimum* preparations studied in this work differs from the total number of hemes in RC *R. viridis*, which has been identified by X-ray diffraction [1].

To test this suggestion and to elucidate the midpoint redox potentials of individual hemes, we carried out a redox titration of the absorption changes in the α-band maximum of the absorption spectra of cytochromes *c* in the RC preparations isolated from *C. minutissimum*. The results of titration are shown in Fig. 2A. The structural model of the reaction center of *R. viridis* [1] suggests that the titration curve of the cytochrome subunit should contain four components. The curve of that type has been successfully used for simulating experimental results of redox titration of the cytochromes *c* in *R. viridis* [14–18]. However, in the case of *C. minutissimum*, we found that the four-component curve does not provide a satisfactory fit of the titration results. The curves of higher order (five-, six-, or seven-component) are a better bet, the theoretical curve plotted from the Nernst equation for six single-electron transitions with equal amplitudes being most appropriate. It should also be noted that the spectra of the *C. minutissimum* reaction centers poised at different redox potentials (Fig. 1) also suggest that the cytochrome subunit of the *C. minutissimum* RC contains six hemes. According to the semilogarithmic anamorphosis of the titration curve (Fig. 2B), the midpoint potentials of six hemes are: E_{m1} = 390 mV, E_{m2} = 320 mV, E_{m3} = 210 mV, E_{m4} = 100 mV, E_{m5} = 20 mV, and E_{m6} = −50 mV.

Thus, in contrast to the tetraheme model of cytochrome subunit structure, which gained a wide recognition because of the X-ray diffraction study of the *R. viridis* RC preparations [1], the results obtained in this work suggest that the *C. minutissimum* RC preparations contain six rather than four hemes *c*. It should also be noted that the literature contains controversial evidence on the stoichiometry of the cytochrome *c* hemes bound to the *Chromatium* RC. According to various authors, the stoichiometric ratio ranges from 1:4 [19] to 1:9 [20]. Spectral and potentiometric characteristics of hemes *c* associated with the *C. minutissimum* RC studied in this work are summarized in Table 1. According to the commonly accepted nomenclature, the RC-bound cytochrome hemes with the midpoint potential (E_m) of about 300 mV, are called high-potential, whereas the hemes with E_m of about 0 mV are called low-potential [3]. Thus, it can be suggested that the *C. minutissimum* RC cytochrome *c* hemes with E_{m1} = 390 mV and E_{m2} = 320 mV belong to the group of high-potential cytochromes, and the cytochrome *c* hemes with E_{m5} = 20 mV and E_{m6} = −50 mV belong to the group of low-potential cytochromes. By analogy, the RC-bound cytochrome *c* hemes with E_{m3} = 210 mV and E_{m4} = 100 mV, which have been discovered for the first time in this work, can be called mid-potential cytochromes.

Polypeptide composition and molecular weight of components of RC-cytochrome complex were assayed by electrophoresis in polyacrylamide gel (PAGE). Native gel electrophoresis of the RC preparations which were not treated with either SDS or β-mercaptoethanol, revealed only one electrophoretic band (Fig. 3A). This suggests that the *C. minutissimum* RC preparation obtained is a homogeneous single protein that does not contain low-molecular components. The gel electrophoresis of the RC preparations treated with SDS and β-mercaptoethanol revealed five subunits with apparent molecular mass of 37, 34, 26, 19, and 17 kDa. Specific staining for hemes *c* with tetramethylbenzidine revealed that only the bands with molecular mass of 37 and 19 kDa were stained. The polypeptides with molecular weight of 34, 26, and 17 kDa are likely to be the components of the membrane subunit triad (L, M, and

Table 1
Spectral and potentiometric characteristics of hemes *c* in the preparations of photosynthetic reaction centers of *C. minutissimum*

No. of heme	Midpoint redox potential (mV)	Maximum of α-band of absorption spectrum (nm)
1	390	556
2	320	555
3	210	554.5
4	100	553
5	20	551.5
6	−50	551

Note: Midpoint redox potentials were measured at pH 7.0.

H), whereas the polypeptides with molecular weight of 37 and 19 kDa are cytochrome subunits. By the molecular weight, the 37-kDa subunit is close to the tetraheme cytochrome subunit of the *R. viridis* RC (40 kDa) [1]. In principle, the 19-kDa polypeptide containing cytochrome *c* could be a product of dissociation of the 37-kDa cytochrome subunit. However, in no other bacterial species studied, the PAGE method employed in our experiments caused a dissociation of the cytochrome subunit into smaller fragments [21,22].

Thus, it can be suggested that in contrast to the RC of *R. viridis*, a thoroughly characterized protein, the RC protein of *C. minutissimum* consists of five polypeptide subunits, two of them containing heme groups. It can also be suggested that the mid-potential hemes with $E_{m3} = 210$ mV and $E_{m4} = 100$ mV, which are absent in *R. viridis* are associated with the 19-kDa subunit, which is also absent in *R. viridis*.

The question arises about the functional role of the mid-potential hemes in the electron transfer in RC. It is well known that both high-potential and low-potential cytochromes act as secondary electron donors to the photooxidized special pair of RC cytochromes and are intermediates

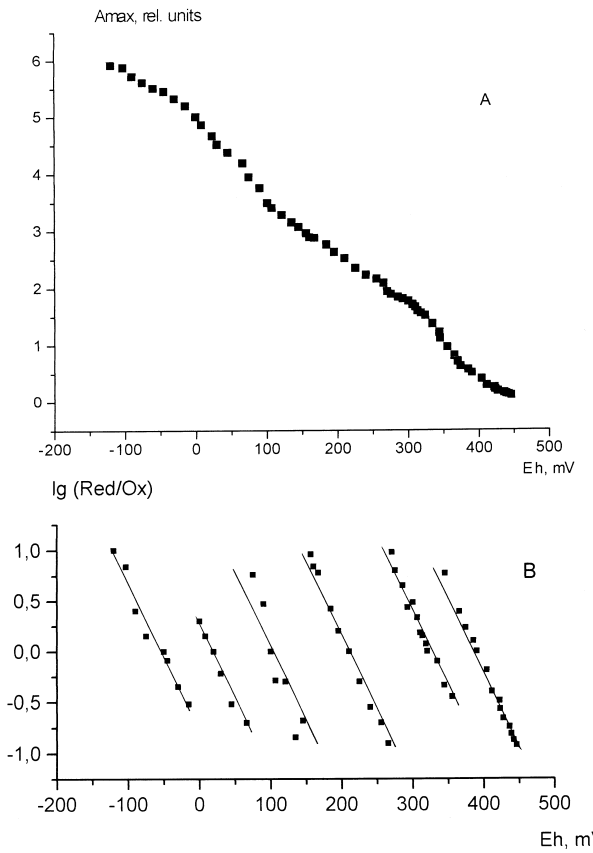


Fig. 2. A: Redox titration at the α-band maximum of the absorption spectra of cytochrome *c* in suspension of isolated RC *C. minutissimum*. The suspension medium contained: 75 mM sodium-phosphate buffer (pH 7.0), 0.1% Triton X-100, and 50 mM KCl. All measurements within the range of E_h from +100 to −150 mV were performed in an anaerobic cuvette. The redox mediators used are listed in Fig. 1. Titration agents were: potassium ferricyanide (oxidizing titration), sodium ascorbate, and sodium dithionite (reducing titration). The reference wavelength was 540 nm. B: Experimental points were approximated by the theoretical curve plotted from the Nernst equation for six single-electron transitions with equal amplitudes. The semilogarithmic anamorphosis of the titration curve is shown.

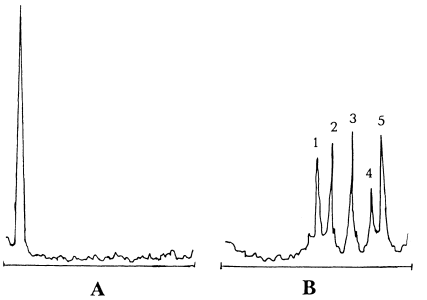


Fig. 3. Electrophoretic densitograms of isolated RC preparations of *C. minutissimum* untreated (A) and treated (B) with SDS (2%) and β-mercaptoethanol (1%). Bands no. B1 and B4 were stained with tetramethylbenzidine.

of cyclic electron transport, whereas low-potential hemes connect RC to a terminal electron donor (substrate of oxidation). We studied the kinetics of the dark reduction of P^+ under the redox conditions with only high-potential hemes ($E_h = 200$–300 bacteriochlorophylls (P^+) [1,3,4,9,11,12]; the high-potential mV), both high-potential and mid-potential hemes ($E_h = 50$–150 mV), or the three pairs of hemes (E_h from −20 to −60 mV) poised in a chemically reduced state (therefore, capable of donating an electron to P^+). It was found that the rate of the photoinduced electron transfer from high-potential hemes to P^+, measured at $E_h = 200$–300 mV, coincides with the reaction rate measured earlier in chromatophores and whole cells of this species of photosynthetic bacteria [9]. Similar coincidence was observed under redox conditions when the three pairs of cytochrome hemes were chemically reduced (E_h from −20 to −60 mV). The rate constants of the reaction were $k_{300} = 2.2 \cdot 10^5$ and $k_{-40} = 8.3 \cdot 10^5$/s, respectively (k_{300} and k_{-40} were measured at ambient redox potentials of +300 and −40 mV, respectively). Such a coincidence of the reaction kinetics in isolated RC preparations and in vivo supports the functional intactness of the RC preparations obtained. It should also be noted that regardless of the extent of chemical reduction of the mid-potential hemes, the reaction kinetics of the electron transfer to P^+ was invariable. Therefore, it can be concluded that the mid-potential hemes do not donate electrons to the photooxidized special pair. According to the value of midpoint redox potential, it seems reasonable to suggest that the mid-potential hemes are mediators of electron transfer along a thermodynamic gradient, i.e. from low-potential to high-potential hemes:

$$\mathbf{C_l} \dashrightarrow \mathbf{C_m} \dashrightarrow \mathbf{C_h} \dashrightarrow \mathbf{P890},$$

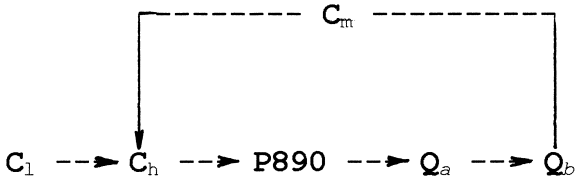
where $\mathbf{C_l}$ is the low-potential heme; $\mathbf{C_m}$ is the midpotential heme; $\mathbf{C_h}$ is the high-potential heme; **P890** is the special pair of RC bacteriochlorophyll molecules.

However, this suggestion is inconsistent with either the data on the structure of the tetraheme cytochrome subunit [1] or the kinetic findings on the reduction of high-potential hemes [3,11–13].

It was mentioned above that the high-potential cytochromes are intermediates of cyclic electron transport, which can bridge the acceptor side of the chain (the secondary quinone acceptor $\mathbf{Q_b}$ or cytochrome *bc*-complex) with its donor side.

In our opinion, the mechanism that mid-potential cyto-

chromes mediate the cyclic electron transport from the photoreduced quinone acceptors to high-potential hemes, is more realistic:



where $\mathbf{Q}_a$ is the primary quinone acceptor; $\mathbf{Q}_b$ is the secondary quinone acceptor. Other symbols as in Scheme 1.

However, additional studies are required to elucidate the functional role of the mid-potential hemes.

The interspecies difference in the RC structure is not very astonishing, particularly in case of the cytochrome component. Indeed, as mentioned above, the cytochromes connect the special pair of RC bacteriochlorophyll molecules with terminal electron donors. During the evolution, different species of photosynthetic bacteria were adapted to different growth substrates, and therefore to different terminal donors. This had a substantial impact on the electron transport chain structure. It is also well known that in addition to photosynthesis, cytochromes are involved in a variety of other processes in bacterial cells. These processes may also contribute to the species specificity observed.

Acknowledgements: This work was supported by the Russian Foundation for Basic Research (project no. 95-04-11857a).

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