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Isolation and characterization of cytochrome *b*-562 from cytochrome *b*-c_1 complex of the photosynthetic bacterium *Rhodopseudomonas sphaeroides* R-26

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A *b*-type cytochrome (cytochrome *b*-562) was highly purified from the isolated cytochrome *b*-c_1 complex of *Rhodopseudomonas sphaeroides* R-26 by treatment with sodium *N*-lauroyl sarcosinate (sarkosyl) and sodium thiocyanate, followed by gel filtration. The purified cytochrome *b*-562 was electrophoretically homogeneous, and its minimal M_r was estimated to be 16000 by SDS-polyacrylamide gel electrophoresis. It showed absorption maxima at 278, 415 and 534 nm in the oxidized form, and 428, 530 and 562 nm in the reduced form. Redox titration of this cytochrome was biphasic, giving two midpoint potentials, 55 and −100 mV, at pH 7.0. This indicates that the cytochrome is intact.

Cytochrome b-*562* *Cytochrome* b-c_1 *complex* Rhodopseudomonas sphaeroides

1. INTRODUCTION

A cytochrome *b*-c_1 complex has been isolated from a photosynthetic bacterium *Rhodopseudomonas sphaeroides* [1–3]. This complex contains two *b*-type cytochromes, which correspond to cytochrome *b*-566 (*b*-90) and cytochrome *b*-561 (*b*-50) of the intact chromatophore membrane, and one cytochrome c_1, one iron-sulfur center, ubiquinones as essential redox components. They are analogous to those of complex III of mitochondria [4].

For years, kinetic studies have led to several working hypotheses for the behavior of these components in the cyclic electron transfer system of *R. sphaeroides* [5–7]. One of the disputable points is how the second electrogenic step across the membrane, which is registered by carotenoid bandshift phase III, occurs through the cytochrome *b*-c_1 complex. It was explained by a Q-cycle mechanism [7] that phase III reflects the movement of electrons from a quinol oxidase site (Q_z-site) to a quinone reductase site (Q_c-site) through cytochrome *b*-566 and cytochrome *b*-561 of the cytochrome *b*-c_1 complex. Studies of the effects of antimycin and myxothiazol on the carotenoid bandshift change suggested that the electron passing from cytochrome *b*-566 to cytochrome *b*-561 is about 35–50% of the distance across the whole chromatophore membrane [8]. However, there is no biochemical information about the topological arrangement of the two *b*-type cytochromes in the bacterial membrane.

Here, we have isolated from cytochrome *b*-c_1 complex of *R. sphaeroides* R-26 a *b*-type cytochrome, *b*-562, which has two electrochemically distinct redox centers. Its M_r was estimated to be 16000, which is much smaller than that obtained by others [9,10].

Abbreviations: sarkosyl, sodium *N*-lauroyl sarcosinate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride

2. MATERIALS AND METHODS

R. sphaeroides strain R-26 was kindly supplied by Dr W.W. Parson, Dept. of Biochemistry,

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University of Washington. Cells were cultured anaerobically in synthetic medium as described in [11]. The cytochrome *b*-c_1 complex from *R. sphaeroides* R-26 was prepared by the procedures in [1] except that 1 mM PMSF was added to the cell suspension before the cell passage through a French pressure cell to avoid protein digestion by proteases. The purified cytochrome *b*-c_1 complex in 50 mM Tris-HCl, pH 7.5, containing 0.2% Tween 20 and 20% glycerol (v/v) was stored at −80°C until use. The complex catalyzed cytochrome *c* reduction with duroquinol as an electron donor and the rate was typically 3.8–4.5 μmol cytochrome *c* · nmol cytochrome $c_1^{-1} \cdot h^{-1}$. Also, it had about equal amounts of two *b*-type cytochrome components with midpoint potentials of 60 mV and −70 mV at pH 7.0, values which are compatible with those reported in [1].

SDS-PAGE was carried out as described in [12]. Redox titration was as in [13]. Protoheme was determined by the formation of alkaline pyridine hemochrome according to [14]. Absorption spectra were measured with a Shimadzu UV-240 spectrophotometer and a Hitachi 356 spectrophotometer.

3. RESULTS AND DISCUSSION

3.1. *Isolation and purification of cytochrome* b-*562 from the purified cytochrome* b-c_1 *complex*

The purified cytochrome *b*-c_1 complex was concentrated with a Diaflo XM-100A membrane filter (Amicon) and the concentration was adjusted to about 40 μM cytochrome *b*. Sarkosyl was added dropwise from a 10% solution with stirring at 0°C to give a concentration of 2% (v/v), after which sodium thiocyanate was added to the mixture at a final concentration of 15% (w/v). The mixture was incubated for 30 min at 0°C and applied to a Sephacryl S-200 column (4 × 95 cm) previously equilibrated with 50 mM Tris-HCl, pH 7.5, containing 1% sarkosyl and 1 M sodium thiocyanate, and then eluted with the same buffer. Cytochrome c_1 was eluted soon after the void volume followed by cytochrome *b*-562. The main fractions containing cytochrome *b*-562 were collected and concentrated with a Diaflo YM-30 membrane filter. The gel filtration was repeated under the same conditions until the ratio of absorbance at 562 nm in the reduced form to that at 280 nm became constant. Purified cytochrome *b*-562 was dissolved in 50 mM Tris-HCl, pH 7.5, containing 1% sarkosyl and 1 M sodium thiocyanate and stored at −80°C until use. About 15% of the cytochrome *b*-562 present in the cytochrome *b*-c_1 complex was recovered in the final purified preparation. It is remarkable that such simple procedures gave the highly purified cytochrome *b*-562 in a relatively high yield.

3.2. *Properties of purified cytochrome* b-*562*

The purified cytochrome *b*-562 preparation showed nearly a single band with an M_r of 16000 after Coomassie brilliant blue staining on SDS-PAGE (fig.1). The M_r corresponded to the smallest polypeptide of the complex obtained in our laboratory [1], and was much smaller than that from *R. sphaeroides* GA [9] and R-26 [10]. At present, we have no conclusive explanation for this discrepancy but it should be noted that the low value is neither due to protease digestion nor contamination by other low-M_r cytochrome. By Sephacryl S-200 gel filtration, the M_r of cytochrome *b*-562 was 62000, which showed this cytochrome is present in a multiple aggregation state. It was worth noting, however, that no M_r other than 62000 was observed in gel filtration.

The absorption spectra of the oxidized and reduced forms of purified cytochrome *b*-562 are shown in fig.2. It had peaks at 278, 415 and

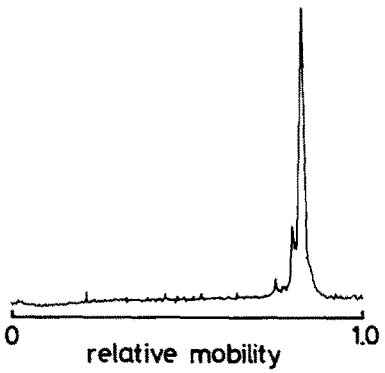

Fig.1. SDS-PAGE densitogram of cytochrome *b*-562 after protein staining. The concentration of polyacrylamide was changed linearly from 7.5 to 15%. The gel was scanned at 565 nm after staining the proteins with Coomassie brilliant blue.

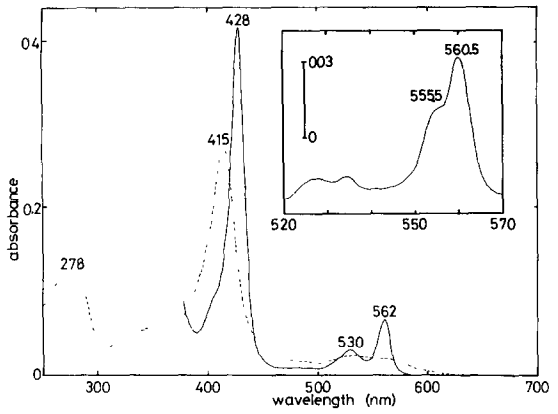

Fig.2. Absorption spectra of purified cytochrome *b*-562 at room temperature. Cytochrome *b*-562 was dissolved in 50 mM Tris-HCl, pH 7.5, containing 1% sarkosyl and 1 M sodium thiocyanate. Cytochrome concentration was 2.3 μM. (——) Absorption spectrum of dithionite-reduced form of the cytochrome; (– – –) absorption spectrum of the oxidized form. Inset: absorption spectrum of the reduced cytochrome at 77 K.

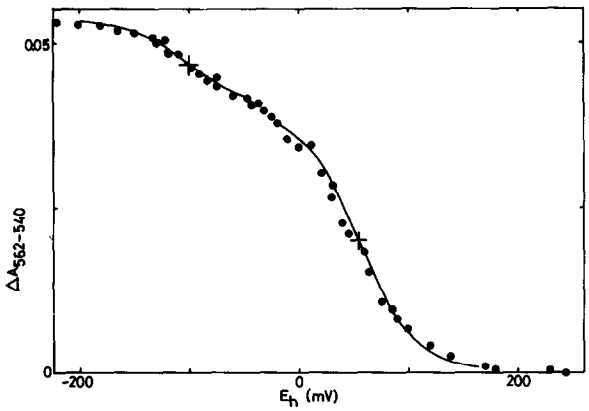

Fig.3. Redox titration of purified cytochrome *b*-562. Cytochrome *b*-562 was dissolved in 50 mM morpholino-propanesulfonic acid buffer, pH 7.0, containing 0.2% sarkosyl and 100 mM KCl. Oxidation-reduction levels of the cytochrome were monitored by measuring the difference in the absorbance at 562 and 540 nm. Redox mediators used were 50 μM ferrocyanide, and 20 μM each of 2,3,5,6-tetramethyl-*p*-phenylenediamine, *N*-methylphenazonium methosulfate, *N*-ethylphenazonium ethosulfate, and pyocyanine. Solid lines are the Nernst curves with $n = 1$. + indicates the midpoint potential of each component.

534 nm in the oxidized form and 562, 530 and 428 nm in the reduced form. The α-band of the reduced form was slightly asymmetric and the asymmetry became clearer at 77 K (fig.2, inset). Its α-band had an absorption maximum at 560.5 nm and a prominent shoulder at 555.5 nm. The millimolar absorption coefficient of cytochrome *b*-562 was 28.6 $mM^{-1} \cdot cm^{-1}$ at 562 nm.

Redox titration of the purified cytochrome *b*-562 showed two components with midpoint potentials of 55 mV and −100 mV (fig.3). The sarkosyl and sodium thiocyanate treatment slightly decreased the midpoint potential of each component from the original values in the cytochrome *b*-c_1 complex. But the clear biphasicity of the redox titration suggests that the cytochrome is nearly as native as in the cytochrome *b*-c_1 complex and intact membrane in spite of its low M_r. At any rate, it is highly probable that the 55 mV and −100 mV components correspond to cytochrome *b*-561 and cytochrome *b*-566 in the *b*-c_1 complex, respective-

Table 1

Amino acid composition (mol%) of purified cytochrome *b*-562

Amino acid	mol/100 mol
Lys	4.7
His	2.2
Arg	6.4
Asp	6.8
Glu	8.5
Thr	5.8
Ser	4.3
Pro	5.0
Gly	10.1
Ala	13.2
Cys	0.0
Met	2.1
Val	7.9
Ile	4.1
Leu	11.3
Tyr	2.5
Phe	3.3
Trp	1.8
Polarity (%)	38.7

Values were determined by the method of Spackman et al. [16]. Polarity was determined according to the method of Capaldi and Vanderkooi [17]

ly. This clear biphasicity is in contrast to the result obtained in [9]. The ratio of the amount of high-potential component to low-potential one was about 2:1. Probably, the low-potential component is more fragile than the high-potential one during the isolation procedure.

The amino acid composition of purified cytochrome *b*-562 is given in table 1. It shows a high content of nonpolar amino acids, especially valine and leucine. The low polarity of 38.7 (%) is characteristic of the integral membrane protein, which is higher than the calculated value (32.5%) for the cytochrome *b* from mitochondrial complex III [15] but lower than that obtained from the same bacterial species in other laboratory (40.8%) [10].

Further studies are being undertaken to explore the immunological relationship between cytochrome *b*-562 and other polypeptides of cytochrome b-c_1 complex of *R. sphaeroides*.

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