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Photoreaction Center of *Ectothiorhodospira* sp. Pigment, Heme, Quinone, and Polypeptide Composition†

Suzie Lefebvre, Rafael Picorel, Yves Cloutier, and Gabriel Gingras*

ABSTRACT: The photoreaction center of *Ectothiorhodospira* sp., a member of the family Chromatiaceae, was isolated from its photosynthetic membranes with a yield of approximately 25–35%. The preparation is free of antenna bacteriochlorophyll and associated proteins. Its pigment complement is 4 mol of bacteriochlorophyll, 2 mol of bacteriopheophytin, and 1 mol of spirilloxanthin. On this basis, its molar extinction coefficient was calculated. Whereas chromatophores contain both ubiquinone and menaquinone, the photoreaction center contains only menaquinone, which, therefore, probably is the primary electron acceptor. The protein is composed of three different subunits of apparent molecular weights of 39 100, 31 300, and 24 800. The sum of these weights is very close to the protein minimal molecular weight of the photoreaction center based on its molar extinction coefficient and amino acid content. This indicates a 1:1:1 molar stoichiometry. Four moles of heme *c* per mole of photoreaction center and cytochromes *c*-555 and *c*-552 are also present. Specific staining after polyacrylamide gel electrophoresis shows that all the heme is bound to the 39K polypeptide. A good amino acid composition homology is found between the L and M polypeptides from *Ectothiorhodospira* sp. and from *Rhodospirillum rubrum*. A membrane-bound cytochrome from *Chromatium vinosum* seems to be homologous to the heaviest subunit of the former two organisms.

Ectothiorhodospira is a bacteriochlorophyll *a* containing member of the family Chromatiaceae. These bacteria carry out anoxygenic photosynthesis in which sulfide is oxidized to sulfate via a series of intermediates among which is elemental sulfur. Unlike the other Chromatiaceae, *Ectothiorhodospira* does not accumulate sulfur granules in its cytoplasm. Its photosynthetic machinery is localized in cytoplasmic membranes piled up as lamellar stacks (Truper & Pfennig, 1978; Remsen, 1978).

† From the Département de Biochimie, Université de Montréal, Montréal, Québec, Canada H3C 3J7. *Received January 24, 1984.* Supported by a grant from the Natural Sciences and Engineering Research Council of Canada, by a bursary from the F.R.S.Q. (Québec) to S.L., and by a fellowship from the C.S.I.C. (Spain) to R.P.

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Our interest in the photoreaction center of *Ectothiorhodospira* sp. was spurred by the finding (Mar et al., 1983) that the charge recombination between its primary electron donor and acceptor is very nearly temperature independent, much like in the dehydrated photoreaction center from *Rhodopseudomonas sphaeroides* (Clayton, 1978). Moreover, unlike native preparations from *Rp. sphaeroides* and *Rhodospirillum rubrum*, this temperature dependency fits (Mar et al., 1983) the theoretical predictions of an activationless model proposed by Jortner (1980).

The photosynthetic apparatus of *Ectothiorhodospira* is similar in several respects to that of the better known *Chromatium vinosum*. Their light-harvesting antenna is composed of B-800–850 and of B-880 holochomes with similar absorption and CD spectra (Hayashi et al., Picorel et al., 1984), and with similar relative susceptibilities to chemical oxidation (Goedheer, 1960; Picorel et al., 1984). However, unlike *Chr. vinosum* (Thornber et al., 1978), the species of *Ectothiorhodospira* that we studied here shows no evidence of a B-800–820 holochrome.

The Bchl[1] *a* containing Chromatiaceae and Rhodospirillaceae have holochromes that differ by some of the criteria mentioned above. But their photosynthetic apparatus also differs with respect to photoreaction center composition. The better characterized photoreaction centers from Bchl *a* containing species such as *Rp. sphaeroides*, *Rs. rubrum*, and *Rp. capsulata* contain no cytochromes and typically are composed of three different polypeptides (Gingras, 1978; Feher & Okamura, 1978). Since the photoreaction center from *Ectothiorhodospira* has not been characterized before, we will draw on the known properties of preparations from *Chr. vinosum* as representative of this group.

In *Chr. vinosum* the primary electron acceptor is menaquinone (Feher & Okamura, 1976; Romijn & Amesz, 1977), ubiquinone possibly playing the role of secondary acceptor, whereas in Bchl *a* containing *Rhodospirillaceae*, ubiquinone acts as both primary and secondary acceptor (Feher & Okamura, 1978; Crofts & Wraight, 1983). The exact polypeptide composition of the photoreaction center from *Chr. vinosum* has not been established: some preparations were reported to contain five polypeptides with apparent molecular weights ranging from 20 000 to 45 000 (Halsey & Byers, 1975; Lin & Thornber, 1975) while another was reported to contain only three with apparent molecular weights between 20 000 and 30 000 (Feher & Okamura, 1978). Another peculiarity of the *Chromatium* photoreaction center preparations is that they contain cytochromes *c*-552 and *c*-555 (Halsey & Byers, 1975; Lin & Thornber, 1975; Romijn & Amesz, 1977). Both these cytochromes donate electrons to the primary electron donor (Parson, 1969; Parson & Case, 1970; Dutton, 1971), and *c*-552 does so even at liquid helium temperature (Devault & Chance, 1966). The latter observation indicates a close proximity of the *c*-552 heme to the Bchl of the primary electron donor. However, the nature of their association is unclear: should cytochrome be considered as part of the photoreaction center or is it a mere hydrophobic contaminant? This is one of the questions that we set out to answer for the photoreaction center of *Ectothiorhodospira* sp.

The present paper reports on the isolation and partial characterization of the photoreaction center from *Ectothiorhodospira* sp. It contains Bchl, Bpheo, and spirilloxanthin in a 4:2:1 mole ratio. Its only quinone component appears to be menaquinone, although ubiquinone is also present in the chromatophores. It also contains four heme groups per mole. Spectroscopic analysis indicates that two of these are associated with a high-potential *c*-555 cytochrome and two with a low-potential *c*-552 cytochrome. The photoreaction center is composed of three subunits in a 1:1:1 mole ratio, and one of them, a M_r 39 000 polypeptide, carries all the heme. We find a reasonably good amino acid composition homology between this subunit, the H subunit of *Rs. rubrum*, and the membrane-bound *c*-552–*c*-555 cytochrome purified from *Chr. vinosum* by Kennel & Kamen (1971). There is a high amino acid composition homology between the respective M and, especially, L subunits of the photoreaction centers from *Ectothiorhodospira* sp. and *Rs. rubrum*.

Materials and Methods

Organism and Growth Conditions. *Ectothiorhodospira* sp. a generous gift of Dr. Elaine Newman, Concordia University, Montreal, was cultured as in Bognar et al. (1982). The cells were grown at 37 °C in 13-L cylindrical bottles completely filled with culture medium and stoppered with rubber stoppers. Illumination was provided by three 150-W photoflood lamps placed at 40 cm from the center of the culture bottles. Two lamps were used for the first 16 h after inoculation, and the third lamp was turned on for the rest of the growth period. After approximately 52 h of culture, the cells were harvested by centrifugation for 20 min at 2500*g* (Sorvall HG-4L rotor). The cells were resuspended in 3 times their weight of distilled water and centrifuged for 10 min at 16300*g* (Sorvall rotor GSA). Washing was repeated until the supernatant was colorless.

Preparation of the Chromatophores. The bacteria were ground with 1.5–2 times their weight of levigated alumina (Fisher Scientific Co.), with a mortar and pestle. After approximately 40 min of grinding, the bacteria were resuspended in distilled water and centrifuged at 2500*g* (Sorvall GSA rotor) to remove most of the alumina and cell debris. The supernatant was centrifuged at 16300*g* (Sorvall SS-34 rotor) for 20 min to remove the rest of the alumina and cell debris, and the supernatant of this second centrifugation was centrifuged again at 100000*g* for 1.5 h (Beckman 45-Ti rotor). The pellet was homogenized in distilled water and recentrifuged at 10000*g* for 1.5 h; the new pellet was resuspended in distilled water to a final A_{855} of 75.

Preparation of Ectothiorhodospira sp. Photoreaction Center. Unless otherwise noted, all steps were carried out at 4 °C, in darkness if possible, or at least in subdued light. To the chromatophore suspension was added an equal volume of 100 mM phosphate buffer (pH 7.5) containing ca. 0.9% (w/v) lauryldimethylamine *N*-oxide (LDAO). This detergent concentration is only given as guidance and should be adjusted by preliminary tests before any preparative batch. This mixture was incubated for 1 h at 40 °C in darkness, with occasional stirring. Solubilization was stopped by dilution with a sufficient volume of cold 50 mM phosphate (pH 7.5) to bring the final detergent concentration to 0.1%. The solution was next centrifuged for 2 h at 151000*g* (Beckman 45-Ti rotor). The supernatant, containing the photochemical activity, was carefully pipetted out by suction. Purification was by ammonium sulfate fractionation and by DEAE-cellulose chromatography. Precipitation was carried out in darkness at 4 °C by first slowly adding 226 g/L (final concentration) finely powdered ammonium sulfate under constant stirring. After standing 30 min, the solution was centrifuged 20 min at 16300*g* (Sorvall rotor GSA). Ammonium sulfate was added

[1] Abbreviations: Bchl, bacteriochlorophyll; DEAE, diethylaminoethyl; LDAO, lauryldimethylamine *N*-oxide: SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Bpheo, bacteriopheophytin.

to the supernatant to a final concentration of 305 g/L, the solution was centrifuged as above, and the precipitate was collected by filtration on Whatman No. 1 filter paper in a Buchner funnel. The precipitate was resuspended in 50 mM Tris-HCl (pH 8.0) and dialyzed for about 16 h against the same buffer containing 0.1% LDAO. The dispersion was centrifuged for 20 min at 16300*g* and filtered as above to remove any particulate material that might clog up the DEAE-Sephacel column used in the subsequent step. The sample, typically 65 mL with $A_{879} = 0.7$, was next applied to a two-thirds filled 1.6 × 30 cm DEAE-Sephacel column equilibrated with 10 mM Tris-HCl (pH 8.0)–0.1% (w/v) LDAO. After adsorption, the sample was washed with 50 mL of the same solution and then with approximately 150 mL of the same solution containing 120 mM NaCl until the eluate was colorless. Elution was with the same buffer containing 175 mM NaCl. The eluate was dialyzed, rechromatographed as above, and dialyzed against 50 mM Tris-HCl (pH 8.0)–0.1% LDAO. The first colored column fractions were found to contain impurities and were discarded. In some preparations a third chromatography was performed by substituting 0.1% Triton X-100 for LDAO.

Analytical Methods. (*1*) *Pigment Analysis.* Bchl and carotenoids were quantitatively extracted from lyophilized photoreaction center with acetone–methanol and assayed spectroscopically according to van der Rest & Gingras (1974). The following extinction coefficients in acetone–methanol were used: for Bchl, $E_{475} = 1.9$ mM^{-1} cm^{-1}, $E_{747} = 38.3$ mM^{-1} cm^{-1}, and $E_{771} = 65.3$ mM^{-1} cm^{-1}; for Bpheo, $E_{475} = 3.0$ mM^{-1} cm^{-1}, $E_{747} = 45.1$ mM^{-1} cm^{-1}, and $E_{771} = 12$ mM^{-1} cm^{-1}; for spirilloxanthin, $E_{475} = 94$ mM^{-1} cm^{-1}.

To determine the nature of the carotenoids present in the photoreaction center, the extracts were transferred to ethyl ether and analyzed by thin-layer chromatography on silica gel plates. Two solvent systems were used for development: (1) petroleum ether (boiling range 40–60 °C)–acetone (9/1 v/v) and (2) benzene–chloroform (1/1 v/v). The spots were eluted with dry ethyl ether and analyzed by absorption spectroscopy (van der Rest & Gingras, 1974).

(*2*) *Quinone Analysis.* The quinones were extracted from the photoreaction center and chromatographed on thin-layer silica gel plates, essentially as described by Vadeboncoeur et al. (1979). One milliliter of photoreaction center ($A_{879} = 3$) was pipetted into 10 mL of acetone–methanol (1/1 v/v) at −78 °C. After 15 min of slow agitation at 22 °C followed by centrifugation at 5000*g* for 10 min, the supernatant was extracted with successive volumes of 10, 8, and 8 mL of petroleum ether (boiling range 60–110 °C). The pooled petroleum ether extracts were then mixed with 1 mL of spectroscopic grade ethanol, and the solution was evaporated under a gentle stream of nitrogen gas to a final volume of about 0.1 mL. The samples were spotted on 20 × 20 cm silica gel thin-layer plates (Macherey-Nagel silica gel G, 0.25 mm thickness) along with synthetic samples of ubiquinone Q_6 and 3-phytylmenadione (vitamin K_1) (Sigma Chemicals). Before use, the plates were activated by heating at 90 °C for 1 h, then washed with 100% ethanol, activated again by heating for 1 h at 90 °C, and cooled about 30 min before use. The plates were developed in darkness with a mixture of petroleum ether and acetone (9/1 v/v). Detection of the quinone spots was with either methylene blue or Nile blue according to Barr & Crane (1971).

(*3*) *Heme Content.* Heme was assayed by the pyridine hemochromogen method described by Bartsch (1978). To detect hemes *a* and *b*, we first extracted the photoreaction center preparation with acetone–HCl, centrifuged the extract on a tabletop centrifuge, and evaporated the supernatant under a stream of nitrogen gas. The residue was dissolved in alkaline pyridine, and the reduced *minus* oxidized spectrum was recorded between 450 and 650 nm. Reduction was with sodium dithionite. Heme *c* was assayed by directly adding alkaline pyridine to the photoreaction center preparation and taking the oxidized *minus* reduced difference spectrum as above. A $\Delta E_{550} = 20$ mM^{-1} cm^{-1} was used for quantitative determinations.

(*4*) *Amino Acid Analysis.* The following operations were carried out in the same tube. An amount (approximately 130 μg of protein) of purified photoreaction center of known A_{880} or of isolated subunits (approximately 70 μg) was lyophilized. A total of 0.5 mL of 6 N HCl (constant boiling: Pierce Chemical Co.) was added along with a known amount of nor-L-leucine as a standard. The tubes were then sealed under vacuum and kept at 110 °C for 48 h. The hydrolysates were diluted with deionized water, filtered on Millipore No. 1300 filters, and dried under a gentle stream of nitrogen gas. The hydrolysates were then dissolved in 0.2 M citrate buffer to an equivalent of 1 mg/mL protein. The amino acid analyses were performed with a Durrum Model D500 amino acid analyzer. Samples of photoreaction center were hydrolyzed for 24, 48, 72, and 96 h in order to check for amino acid loss as a function of time. Since no obvious difference was observed, uncorrected analyses of 48-h hydrolysates are reported.

(*5*) *Polyacrylamide Gel Electrophoresis.* Analytical gel electrophoresis in the presence of sodium dodecyl sulfate was carried out according to Laemmli (1970) in a Pharmacia GE-2/4LS apparatus. The slab gels (80 × 140 × 2.7 mm) were comprised of a 2.5 cm long stacking gel [3% acrylamide, 0.8% bis(acrylamide)] and of an 11.5 cm long separating gel [12% acrylamide, 0.32% bis(acrylamide)]. The buffers and SDS concentrations were as described by Laemmli (1970). Before electrophoresis, the protein samples (1 mg of protein/mL) were incubated at 30 °C for 2 h in 62.5 mM Tris-HCl (pH 6.8) containing 7% sucrose and 2% SDS. Unless otherwise mentioned, no 2-mercaptoethanol was present in this denaturing solution. The molecular weight markers (Pharmacia Fine Chemicals) were lactalbumin (M_r 14000), soybean trypsin inhibitor (M_r 20000), carbonic anhydrase (M_r 30000), and ovalbumin (M_r 43000). The gels were stained for proteins with Coomassie G250 Brilliant Blue according to Lopez & Siekewitz (1973). Staining for heme was carried out as described by Thomas et al. (1976) by employing 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide.

(*6*) *Preparative Gel Electrophoresis.* The protein samples were denatured as above in the absence of 2-mercaptoethanol. Under those conditions, heme remains bound to its apocytochrome protein and, being colored, serves as a position marker on the gel. Electrophoresis was carried out in the same conditions as above except that the gels were formed in cylindrical tubes of 200 × 6 mm. Approximately 200 μg of photoreaction center protein was applied on each gel. By use of the cytochrome band as a position marker, the three protein bands were cut out, and the gel was crushed in 50 mM Tris-HCl (pH 8.0) and eluted by diffusion in the cold. The samples were then dialyzed (4 °C) for 24 h in 4 L of the same buffer, assayed for protein content, and hydrolyzed. Throughout this work, the method of Lowry et al. (1951) was used for routine protein assay using bovine serum albumin as the standard.

Spectroscopic Methods. Absorption spectra were recorded at room temperature with a Cary 14R spectrophotometer in 1-cm path-length cuvettes. The actinic cross-illumination setup

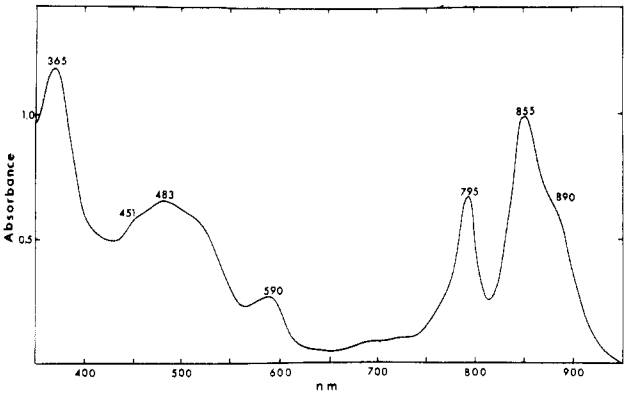

FIGURE 1: Absorption spectrum of a chromatophore suspension from the photosynthetic bacterium *Ectothiorhodospira* sp. The chromatophores were in distilled water.

Table I: Main Characteristics of the Photoreaction Center Preparation in the Course of the Various Extraction and Purification Steps

fraction	A_{280}/A_{799}	$\Delta A_{879}/A_{879}$	fraction of photochemical activity recovered
chromatophores		0.008	1
151 000*g* supernatant	4.6	0.4	0.85
ammonium sulfate precipitate	2.8	0.45	0.75
after one chromatographic step	1.9	0.8	0.50
after two chromatographic steps	1.7	0.94	0.25–0.35

used to measure photochemical activity of the preparations was described before (Vadeboncoeur et al., 1979). Electron paramagnetic resonance spectra were measured in a Varian E-line spectrometer operating at a microwave frequency of 9.4 GHz in an E-231 cavity. Modulation frequency of the magnetic field was 100 MHz. The spectra were recorded at room temperature in flat quartz cells.

Results

Figure 1 shows the absorption spectrum of a suspension of chromatophores from *Ectothiorhodospira* sp. The near-infrared region of this spectrum shows absorption bands at 795, 855, and 890 nm. The first two bands are attributable, for the major part, to a B-800–850 holochrome and the second to a B-880 holochrome.

The photoreaction center was successfully extracted and purified from such chromatophores with LDAO as detergent; in order to obtain reasonable yields of practically antenna-free crude photoreaction center, the solubilization step had to be carried out at fairly high temperatures. Temperatures below 40 °C tend to favor extraction of the B-880 holochrome along with the photoreaction center. Under the conditions described here, approximately 85% of the light-induced reversible bleaching present in the chromatophores was recovered in the 151000*g* supernatant following solubilization with LDAO. The yield dropped approximately to 75% after ammonium sulfate fractionation, to 50% after one chromatography on DEAE-Sephacel, and to 25–35% after a second chromatographic step (Table I). Ammonium sulfate fractionation and chromatography bring about a considerable decrease in the A_{280}/A_{799} ratio which, in the purest fractions, is nevertheless still high compared to other photoreaction center preparations: about 1.7 in the present preparation compared to 1.25 in the preparations from *Rs. rubrum* and *Rp. sphaeroides* (Gingras, 1978). Similarly, the ratio of A_{799}/A_{879} is 2.85 in this preparation compared to 2.3 for the A_{800}/A_{870} in *Rs. rubrum* and *Rp. sphaeroides* photoreaction centers (Gingras, 1978). The purified preparations were found to be more stable in Tris-HCl (pH 8.0) buffer than in phosphate buffer where the primary electron donor is easily autooxidized. In Tris-HCl, the preparation could be kept for several weeks at 4 °C without any appreciable loss in activity.

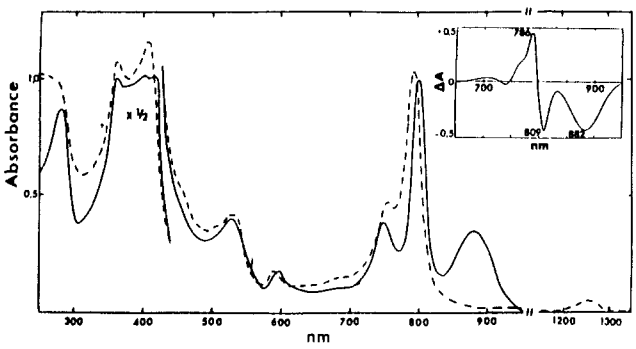

FIGURE 2: Absorption spectrum of photoreaction center from *Ectothiorhodospira* sp. in 50 mM Tris-HCl (pH 8.0)–0.1% LDAO. (—) Spectrum of the reduced preparation recorded with weak monochromatic light; (---) spectrum of the same preparation recorded with an intense nonmonochromatic beam of light. (Inset) reduced *minus* oxidized near-infrared difference spectrum. Oxidation of the primary electron donor was with saturating actinic light provided by side illumination. The A_{879} of the preparation was 0.5.

The absorption spectrum of photoreaction center from *Ectothiorhodospira* sp. (Figure 2) has characteristic near-infrared bands at 879, 799 and 750 nm due to the Q_y transitions of Bchl and Bpheo and a band at 600 nm due to the Q_x transition of Bchl. Other bands are observed at 525 nm (due to carotenoids and Bpheo) and at 417, 403, and 365 nm. The three latter bands are attributed to the Soret bands of heme (417 nm; see below), of Bpheo, and of Bchl. Figure 2 (dotted line) also shows the absorption spectrum of the preparation recorded with a bright nonmonochromatic source of illumination (IR 2 mode). Under these conditions, the primary electron donor is oxidized, and this gives rise to classic spectral modifications such as a bleaching of the 879-nm band accompanied by a blue shift of the 799-nm band and the appearance of new bands in the 1250-nm region. Some of these spectral changes are better seen on the oxidized *minus* reduced spectrum (Figure 2, inset). It displays large negative bands at 809 and 882 nm and a positive band at 786 nm with a shoulder at 760 nm.

The EPR spectrum of the oxidized primary electron donor measured at room temperature (Figure 3) has a *g* value of 2.0025 as determined by calibration of the instrument with a photoreaction center preparation from *Rs. rubrum* whose primary acceptor *g* value has been determined by Loach & Walsh (1969). The peak to peak line width of the signal is 11.2 G, considerably wider than that of the *R. rubrum* primary electron donor.

Pigment Composition and Extinction Coefficient. The pigments were quantitatively extracted from the lyophilized photoreaction center with acetone–methanol (7/2 v/v) and assayed spectrophotometrically according to van der Rest & Gingras (1974). We quote the average of 16 extraction experiments on eight different preparations; standard deviation was ±10%. For preparations with $A_{879} \times$ volume (mL) = 1.52, we found the acetone–methanol extracts to contain 5.04 mol of Bchl and 2.67 mol of Bpheo; thus, the Bchl *a*/Bpheo *a* mole ratio is 1.9. When the reasonable assumption is made that

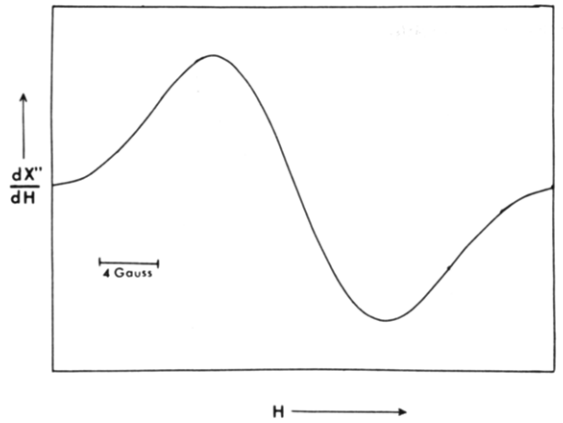

FIGURE 3: Electron paramagnetic resonance spectrum of the oxidized primary electron donor of the photoreaction center from *Ectothiorhodospira* sp. The preparation was in 50 mM Tris-HCl (pH 8.0). Oxidation of the primary donor was with saturating white light. The spectrum was recorded at room temperature at 9.4 GHZ. Microwave power was 5 mW, and the modulation amplitude of the magnetic field was 4 G.

this photoreaction center, like those from *Rs. rubrum* and *Rp. sphaeroides*, contains 4 mol of Bchl and 2 mol of Bpheo per mol of photoreaction center, it is possible to calculate a molar extinction coefficient. We arrive at values of $E_{799} = 339$ mM^{-1} cm^{-1} and $E_{879} = 121$ mM^{-1} cm^{-1}. The reduced *minus* oxidized E_{882} is 114 mM^{-1} cm^{-1}.

The carotenoid composition was similarly established by a quantitative acetone–methanol extraction of the lyophilized material, transfer to dry ethyl ether, and thin-layer plate chromatography on silica gel. Although we did not systematically investigate the matter, the chromatophores contain at least five different carotenoids, and spirilloxanthin represents only about 35% of their total amount. With photoreaction center extracts, each one of the two solvent systems that we used resolved three carotenoid bands. The most abundant was, by far, spirilloxanthin which represented 90% of the carotenoids present in the extract. It was identified by cochromatography with spirilloxanthin extracted from *Rs. rubrum* and by its absorption spectrum with characteristic bands in benzene at 479, 510, and 549 nm. Another carotenoid, present as 4% of the carotenoids in the extract, was identified as lycopene by cochromatography with lycopene from tomatoes and by absorption spectroscopy. The third carotenoid representing 6% of the carotenoids could not be positively identified but is thought to be rhodopin. Quantitative assays of the carotenoids in the total acetone–methanol extract using the extinction coefficients cited under Materials and Methods give 1.1 mol of carotenoid per 4 mol of Bchl and 2 mol of Bpheo (average of 16 extractions on eight different preparations).

Quinone Content. We qualitatively analyzed the quinone content of chromatophores and of purified photoreaction center from *Ectothiorhodospira* sp. by acetone–methanol extraction followed with thin-layer plate chromatography. The plates were sprayed either with reduced methylene blue or with reduced Nile blue. Methylene blue readily revealed the spots corresponding to ubiquinone but was a poor stain for menaquinone. Reduced Nile blue, on the other hand, was oxidized by both types of quinone. Figure 4 is a photograph of a plate stained with Nile blue. The samples were in the following order: synthetic ubiquinone Q_6 and 3-phytylmenadione (lane 1), an extract from the chromatophores of *Ectothiorhodospira* sp. (lane 2), an extract from the photoreaction center of *Ectothiorhodospira* sp. (lane 3), and an extract from the photoreaction center of *Rs. rubrum* (lane 4).

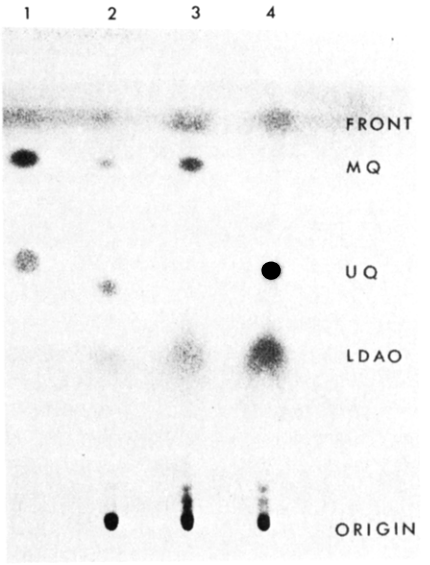

FIGURE 4: Thin-layer plate chromatograms of an organic solvent extract of the photoreaction center from *Ectothiorhodospira* sp. The spots were revealed with a Nile blue spray. The chromatograms were developed as described under Materials and Methods. (Lane 1) Authentic samples of ubiquinone Q_6 and 3-phytylmenadione; (lane 2) extract from 1 mL of *Ectothiorhodospira* sp. chromatophores (A_{855} = 19); (lane 3) extract of 25 nmol of photoreaction center from *Ectothiorhodospira* sp.; (lane 4) extract from 25 nmol of photoreaction center of *Rs. rubrum*.

Chromatophores of *Ectothiorhodospira* sp. clearly contain both ubiquinone and menaquinone, but only menaquinone could be detected in its photoreaction center. Since, in our hands, the lower limit of detection of this method was approximately 5 nmol, and since the extracts that we spotted on the plates were from 25 nmol of photoreaction center, we estimate the latter to contain at most 1/5 mol of ubiquinone/mol. The photoreaction center from *Rs. rubrum*, presented here as a control, contains only ubiquinone, very likely Q_{10}. LDAO, the detergent employed for solubilizing the photoreaction center, is also stained by Nile blue.

Heme Content. To determine whether this photoreaction center preparation contains any cytochromes, we used the alkaline pyridine hemochromogen method. Since even in the purest of our preparations heme was obviously present according to this test, we attempted to determine whether it was heme *a*, *b*, or *c*. Hemes *a* and *b* not being covalently bound to their apocytochrome moiety can be extracted by acid acetone and assayed by formation of their pyridine hemochromogens. This test gave negative results: no heme was ever detected in the acid acetone extract even though it was always present in the residue. Added to the fact that the pyridine hemochromogen of the residue had a reduced *minus* oxidized difference spectrum (not shown) with a peak at 550 nm, we conclude that the preparation contains only heme *c*. Quantitative determination of heme in known amounts of photoreaction center allowed us to establish a mole ratio of 4 hemes/4 mol of Bchl, i.e., per mole of photoreaction center. To make sure that this stoichiometry was truly characteristic of our preparation, we followed it through the various steps of the purification procedure. The mole ratio was 9.6 in the ammonium sulfate precipitate, 4.8 after one chromatography on DEAE-Sephacel, and 4.2 after two such chromatographic steps. No significant change was observed after three or more chromatographic steps.

Spectroscopic Analysis of the Cytochromes. Figure 5 shows the difference spectra recorded between the nontreated and reduced photoreaction center. Reduction with either ascorbate

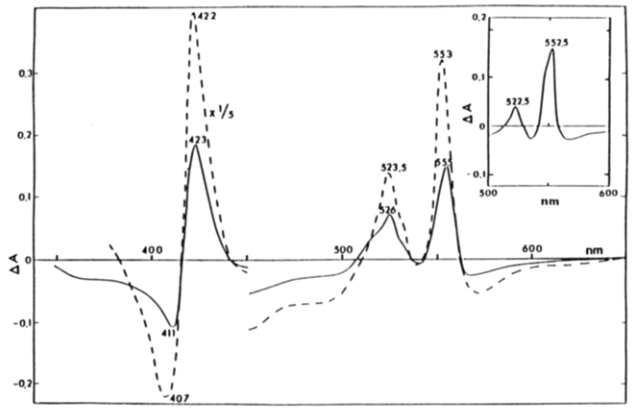

FIGURE 5: Reduced *minus* oxidized difference spectra of photoreaction center from *Ectothiorhodospira* sp. The sample and reference cuvettes contained 5 μM photoreaction center in 50 mM Tris-HCl (pH 8.0)–0.1% Triton X-100. The sample cuvette also contained either sodium ascorbate (—) or sodium dithionite (---). (Inset) Difference between the spectrum of the photoreaction center from *Ectothiorhodospira* sp. reduced with sodium dithionite *minus* the spectrum of the preparation reduced with sodium ascorbate.

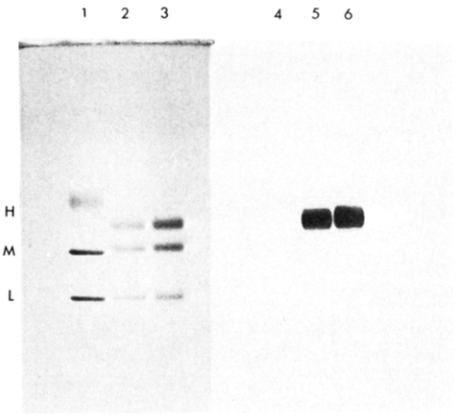

FIGURE 6: Electrophoretogram of the photoreaction center isolated from *Ectothiorhodospira* sp. Polyacrylamide gel electrophoresis was run in the presence of SDS. After electrophoresis, the gel slab was cut in two identical halves; the first (lanes 1–3) was stained for proteins with Coomassie blue, and the second (lanes 4–6) was stained for heme with 3,3′,5,5′-tetramethylbenzidine. (Lanes 1 and 4) Denaturation with 2% SDS and 4% 2-mercaptoethanol. (Lanes 2 and 5) Denaturation with 2% SDS but without 2-mercaptoethanol. In lanes 3 and 6 denaturation was with 2% SDS and 4 M urea but without 2-mercaptoethanol.

or dithionite is not followed by any absorbance changes in the near-infrared, indicating that the primary electron donor is completely reduced in their absence. Addition of ascorbate reveals peaks at 555, 526, and 422 nm and a through at 411 nm. Reduction with dithionite shows peaks at 553, 523.5, and 422 nm and a through at 407 nm. These clearly correspond to the reduced *minus* oxidized spectra of two different cytochromes. The spectrum of the inset is the difference between the spectra of Figure 5; it shows peak at 552.5 and 522.5 nm. From these spectra and assuming a molar ratio of *c*-555/*c*-552/P-879 = 1/1/1 and that all the high-potential cytochrome *c*-555 (reduction is slow) and none of the low-potential cytochrome *c*-552 are reduced by ascorbate, we can calculate corresponding extinction coefficients. We find reduced *minus* oxidized extinction coefficient of 14.9 mM^{-1} cm^{-1} at 555 nm for *c*-555 and 16.4 mM^{-1} cm^{-1} at 552.5 nm for *c*-552. We made no attempt to determine the redox potentials of these cytochromes. Presumably, each cytochrome contains two of the four hemes present per mol of photoreaction center.

SDS–Polyacrylamide Gel Electrophoresis. Figure 6 shows an electrophoretogram of purified photoreaction center from *Ectothiorhodospira* sp. In this experiment SDS–polyacrylamide gel electrophoresis was performed in duplicate on a single gel slab. The gel was cut in half, one of which was stained for proteins with Coomassie blue while the other identical half was stained for heme with 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide according to Thomas et al. (1976). Staining with Coomassie blue reveals the presence of three protein bands which we call H, M, and L in the order of decreasing apparent molecular weight. When the proteins are denatured either in the presence of 2% SDS (lanes 2 and 5) or in the presence of 2% SDS and 4 M urea (lanes 3 and 6), these polypeptides have apparent molecular weights of 36 300, 31 300, and 24 800. 3,3′,5,5′-Tetramethylbenzidine stains only band H which, therefore, must contain the heme. Interestingly, when denaturation is carried out with 2% SDS and 4% 2-mercaptoethanol (lanes 1 and 4), heme is no longer detected on the gel (lane 4) and the apparent molecular weight of polypeptide H increases from 36 300 to 39 100. For reasons explained under Discussion, we will use the higher apparent moleular weight as a better approximation of the true molecular weight.

Amino Acid Composition. We determined the amino acid composition of the photoreaction center from *Ectothiorhodospira* sp. with two aims in mind: first, to establish the molar stoichiometry of the three subunits of the complex and, second, to assess any possible compositional homology of the photoreaction center from *Ectothiorhodospira* sp. with the better known preparations from *Rs. rubrum* and *Rp. sphaeroides*. The photoreaction center was subjected to 24-, 48-, 72-, and 96-h acid hydrolysis. Since no clear decrease of the amino acid content was observed, no correction was applied to the raw data. The analyses were performed on known molar quantities of photoreaction center as determined by the absorbance at 879 nm by using an A_{879} of 121 mM^{-1} cm^{-1}. Summation of the molar contents of all the amino acid leads to a minimal molecular weight value of 92 700. Considering that the contents in Trp were not determined, this agrees well with the value of 95 200 that is obtained by summing in a 1:1:1 fashion the apparent molecular weights of the three polypeptides.

Table II gives the mole percent amino acid composition and the mole number of amino acid residues for the photoreaction center and its subunits. The mole number of amino acid residues for the photoreaction center and its H, M, and L subunits is based on respective molecular weights of 95 200, 39 100, 31 300, and 24 800. This is justified since the minimal molecular weight of the whole photoreaction center is experimentally indistinguishable from the sum of the apparent molecular weight of the subunits. Table II also compares, for each amino acid, the total number of amino acid residues found in the three subunits with that of the photoreaction center as a whole. Assuming a 1:1:1 molar stoichiometry for the subunits, the correspondence is very good. This is especially evident when considering that Met and Cys were not determined in the individual subunits and that more Tyr was undoubtedly destroyed during hydrolysis (Blackburn, 1978) of the subunits because it was carried out on samples of 70 μg of protein compared to 135 μg for the photoreaction center samples.

Discussion

We have isolated the photoreaction center from *Ectothiorhodospira* sp., a member of the Chromatiaceae. Our preparation is devoid of antenna holochrome, as indicated by a 94% photoinduced bleaching of its absorbance at 882 nm. This bleaching is totally reversible on switching on and off the

Table II: Amino Acid Composition of the Photoreaction Center from *Ectothiorhodospira* sp.[a]

amino acid	subunit H		subunit M		subunit L		sum of residues in subunits H, M, and L	photoreaction center	
	mol %	no. of residues	mol %	no. of residues	mol %	no. of residues		mol %	no. of residues
Asp	11.5	43	8.4	25	7.3	17	85	8.9	80
Thr	7.2	27	5.2	15	4.6	11	53	6.0	54
Ser	4.7	17	4.4	13	5.3	12	42	4.5	40
Glu	10.5	39	8.0	23	7.4	17	79	7.8	69
Pro	7.7	29	6.2	18	5.7	13	60	6.5	58
Gly	12.1	45	13.6	40	14.4	33	118	10.8	97
Ala	10.1	37	10.8	32	10.8	25	94	10.2	91
Cys	ND[b]		ND		ND			1.1	9
Val	6.5	24	6.4	19	6.6	15	58	6.3	56
Met	ND		ND		ND			3.1	26
Ile	4.6	17	6.4	19	6.5	15	51	5.7	50
Leu	8.6	32	11.7	34	12.3	28	94	11.1	99
Tyr	1.4	5	2.6	8	2.2	5	18	3.0	26
Phe	4.4	16	7.1	21	8.3	19	56	6.4	57
His	2.7	10	2.3	7	2.6	6	23	2.3	21
Lys	2.5	10	1.7	5	2.5	6	21	1.8	16
Arg	5.2	19	4.8	14	2.9	7	40	4.6	41
Trp	ND		ND		ND			ND	

[a] Average of two analyses from three different 48-h hydrolysates. Uncorrected values are reported. The number of residues was calculated by using average residue molecular weights of 105.4, 107.3, 107.6, and 106.8 and molecular weights of 39 100, 31 300, 24 800, and 95 200 for polypeptides H, M, and L and for the photoreaction center, respectively. [b] ND, not determined.

actinic light. Its absorption spectrum has some distinctive features. For instance, its two lowest energy bands have peaks at 879 and at 799 nm, compared to 868 and 803 nm in the photoreaction center from *Rs. rubrum* S1, and its A_{799}/A_{879} is 2.9 compared to $A_{803}/A_{868} = 2.3$ in *Rs. rubrum*. Another characteristic of the absorption spectrum is a relatively high absorbance at 417 nm which is largely due to the Soret band of heme *c*.

Like in other Rhodospirillales, oxidation of the primary electron donor causes bleaching of the 879-nm band accompanied by a blue shift of the 799-nm band and by the appearance of new bands in the 1250-nm region. The oxidized primary donor also gives rise to an EPR spectrum with a characteristic *g* value of 2.0025 and a line width of 11.2 G. Such a line width appears to be uncommonly high for a Bchl *a* containing photoreaction center [see Norris & Katz (1978)].

Pigment analysis shows that this photoreaction center contains Bchl, Bpheo, and a carotenoid, 90% of which is spirilloxanthin, in a 4/2/1 molar ratio. This indicates that, like photoreaction centers from *Rs. rubrum* and *Rp. sphaeroides* (van der Rest & Gingras, 1974; Straley et al., 1973; Cogdell et al., 1975), its pigment complement is also made up of 4 mol of Bchl, 2 mol of Bpheo, and 1 mol of a specific carotenoid. On these grounds, we determined a molar extinction coefficient that allowed us to express the other quantitative determinations on a molar basis.

The presence of 1 mol of carotenoid in the photoreaction center of *Ectothiorhodospira* sp. was expected since it appears to be of general occurrence in wild-type photoreaction centers (van der Rest & Gingras, 1974). This molecule has been shown to prevent the formation of singlet oxygen by triplet–triplet energy transfer from state P^R of the primary electron donor to oxygen (Cogdell et al., 1975; Boucher et al., 1977). That it is spirilloxanthin is no surprise since it is on the biosynthetic pathway of carotenoids in the Chromaticeae (Schmidt, 1978) and since it is a very efficient photoprotector (Boucher et al., 1977). Furthermore, spirilloxanthin was also reported in a photoreaction center preparation from *Chr. vinosum* (Romijn & Amesz, 1977).

While ubiquinone is clearly present in control extracts of the *Rs. rubrum* photoreaction center and of *Ectothiorhodospira* sp. chromatophores, we were unable to detect it in extracts of the photoreaction center from the latter organism. We conclude that ubiquinone is not the primary electron acceptor in this photoreaction center. Menaquinone which is present in extracts from both *Ectothiorhodospira* chromatophores and photoreaction center is a more likely candidate. This would be comparable to the situation reported for *Chr. vinosum* where menaquinone appears to be the primary acceptor (Fehrer & Okamura, 1976; Romijn & Amesz, 1977). In the present work, we made no attempt at a more precise identification of this quinone. However, that it is a naphthoquinone rather than a benzoquinone is indicated by its chromatographic behavior: despite its inability to resolve the quinones according to substituents, the chromatographic system that we used separates the benzoquinones and the naphthoquinones in two classes. Moreover, the low redox potential of this quinone, indicated by its inefficient oxidation of leucomethylene blue, is typical of naphthoquinone (Barr & Crane, 1971).

Another characteristic of the photoreaction center from *Ectothiorhodospira* sp. is that it contains 4 mol of heme *c* in the form of a low-potential and of a high-potential cytochrome, *c*-552 and *c*-555. Each cytochrome is probably made up of a pair of heme molecules. These cytochromes appear to be similar to those described for *Chr. vinosum* (Kennel & Kamen, 1971) in which two multiheme cytochromes *c*-552 ($E_{m,7} = 8$ mV) and *c*-555 ($E_{m,7} = 325$ mV) can donate electrons to the primary donor of the photoreaction center (Parson, 1969; Parson & Case, 1970; Dutton, 1971).

In *Chr. vinosum* electron transfer from *c*-552 to primary donor takes place even at liquid helium temperature (DeVault & Chance, 1966), indicating a close proximity of heme to the Bchl primary electron donor. This hypothesis is consistent with the presence of these cytochromes in several photoreaction center preparations from this organism (Halsey & Byers, 1975; Lin & Thornber, 1975; Tiede et al., 1976; Romijn & Amesz, 1977). However, the degree of intimacy of this association has never been made clear: is cytochrome a hydrophobically bound contaminant or should it be considered an integral part of the photoreaction center? Some of the preparations that contained cytochrome were composed of five polypeptide bands (Halsey & Byers, 1975; Lin & Thornber, 1976), while another was composed of only three polypeptides, but its cytochrome

Table III: Number of Amino Acid Residues in the Polypeptide Subunits of the Photoreaction Centers from *Ectothiorhodospira* sp. and from *Rs. rubrum* Compared with Membrane-Bound Cytochrome from *Chr. vinosum*[a]

amino acid	photoreaction center		subunit H		subunit M		subunit L		membrane-bound cytochrome from *Chr. vinosum*
	Rs. rubrum	*Ectothio-rhodospira*	*Rs. rubrum*	*Ectothio-rhodospira*	*Rs. rubrum*	*Ectothio-rhodospira*	*Rs. rubrum*	*Ectothio-rhodospira*	
Asp	53	80	27	43	13	25	13	17	32
Thr	51	54	21	27	15	15	14	11	21
Ser	46	40	21	17	14	13	14	12	19
Glu	54	69	24	39	19	23	11	17	40
Pro	43	58	18	29	12	18	11	13	32
Gly	86	97	33	45	30	40	29	33	21
Ala	89	91	40	37	27	32	21	25	44
Cys	6	9							
Val	53	56	23	24	14	19	17	15	26
Met	18	26	5		7		5		6
Ile	54	50	19	17	16	19	17	15	8
Leu	83	99	26	32	28	34	26	28	20
Tyr	31	26	12	5	8	8	11	5	15
Phe	55	57	10	16	25	21	17	19	6
His	17	21	2	10	6	7	7	6	5
Lys	27	16	17	10	5	5	6	6	12
Arg	40	41	21	19	13	14	6	7	12
Trp	25		4		9		11		

[a] The values for the *Rs. rubrum* photoreaction center and subunits are drawn from Vandeboncoeur et al. (1979). The values for the membrane-bound cytochrome of *Chr. vinosum* are recalculated from Kennel & Kamen (1971), assuming 4 mol of heme/45 000 g of protein.

content was not reported (Feher & Okamura, 1978). Recently, a *c*-552 preparation was obtained from chromatophores of *Chr. vinosum* by the combined action of detergent and protease (Doi et al., 1983). It had a molecular weight of 20 000 and was free both of the photoreaction center and of *c*-555. But the use of protease precluded a satisfactory answer to the question of the type of association of *c*-552 with *c*-555 or with the photoreaction center protein. A similar problem exists in preparations from Bchl *b* containing bacteria such as *Rp. viridis* where the intermediary electron acceptor can be photoreduced and trapped at low redox potentials presumably through the two multiheme cytochromes. These preparations also contain three or four polypeptides, according to preparative procedures [reviewed in Gingras (1978); see Thornber et al. (1980) for a more recent treatment].

The present work brings strong evidence that, in *Ectothiorhodospira* sp., cytochrome is indeed an integral part of the photoreaction center. The simplest and most obvious interpretation of our electrophoresis and amino acid analysis data is that this photoreaction center contains only three different subunits in a 1/1/1 ratio. All the heme is bound to the heaviest subunit which, for convenience, we designate as H. Interestingly, the peroxidase activity of this subunit is lost after treatment with SDS and 2-mercaptoethanol (Figure 6). This may be due to the loss of iron from the heme as has been described for other *c* type cytochromes (Wood, 1980). We assume that the increase in the apparent molecular weight of polypeptide H that is observed (Figure 6) after this treatment is due to a more complete unfolding of the protein subsequent to removal of the iron.

The three subunit composition that we observe in this photoreaction center is in line with the situation in the great majority of preparations from Bchl *a* containing Rhodospirillaceae. These preparations, however, contain no cytochrome C and use ubiquinone as their primary electron acceptor. There are other similarities in the polypeptide compositions of the photoreaction centers from *Ectothiorhodospira* sp. and from Rhodospirillaceae such as *Rs. rubrum* or *Rp. sphaeroides*. First, their estimated molecular weights are very similar: M_r 39 100, 31 300, and 24 800 in *Ectothiorhodospira* sp. (Results) compared to M_r 36 100, 29 000, and 24 900 in *Rs. rubrum* (Vadeboncoeur et al., 1979). Second, a molar stoichiometry of 1:1:1 is the only one that will fit both the minimal molecular weight (92 700) and the sum of the apparent molecular weights of the polypeptide subunits (95 200) in the photoreaction center from *Ectothiorhodospira* sp. This stoichiometry has also been demonstrated for the photoreaction centers of *Rp. sphaeroides* (Steiner et al., 1974) and *Rs. rubrum* (Vadeboncoeur et al., 1979).

The similarities also extend to the finer level of the amino acid composition, itself a reflection of the primary structure of proteins. Table III compares the amino acid composition of the *Ectothiorhodospira* sp. photoreaction center and subunits with their homologues from *Rs. rubrum*. Such composition is nearly identical in *Rs. rubrum* and in *Rp. sphaeroides* (Steiner et al., 1974; Okamura et al., 1974; Vadeboncoeur et al., 1979). The comparison set up in Table III is not exact since Cys, Met, and Trp were determined in only some of the proteins considered. The two photoreaction centers have similar overall amino acid compositions. One of the most striking differences is that the photoreaction center of *Ectothiorhodospira* sp. contains a higher proportion of Asp and Glu with a smaller proportion of Lys; this suggests that this protein is more acidic. It also contains significantly more Pro, Gly, Met, Cys, His, and Leu residues.

A simple perusal of Table III shows a high composition homology between the L subunits of the two species. The homology between the M subunits is good but not as perfect, the main differences being in the contents of Asp, Pro, and Gly. Homology is poorest between the H subunits where large differences are found in the contents in Asp, Glu, Pro, Gly, Phe, Lys, and especially His. In this case, homology is not expected to be good, since the H polypeptide is larger and binds four molecules of heme *c* in the photoreaction center of *Ectothiorhodospira* sp. In that respect, and by analogy with soluble cytochrome *c*, the high His content in the H subunit from the latter preparation may perhaps be related to the role of this amino acid as a ligand of iron in heme *c*. By the same token, one might suggest that the excess Met and Cys found in the photoreaction center of *Ectothiorhodospira* sp. over that of *Rs. rubrum* may also be involved in the binding of heme to the H subunit.

Kennel & Kamen (1971) isolated a membrane-bound cytochrome of the *c* type from *Chr. vinosum* chromatophores

by means of cholate solubilization. This cytochrome contained two moieties, a high-potential *c*-556 and a low-potential *c*-552.5. Electrophoresis on SDS–polyacrylamide gels resolved this cytochrome into a prominent band of apparent M_r 45 000 and two lesser bands of apparent M_r 29 000 and 23 000. The cytochrome contained 4–5 mol of heme/45 000 g of protein. In view of these observations, we might expect a high amino acid composition homology of this cytochrome with the H subunit of our preparation from *Ectothiorhodospira* sp. The amino acid composition reported by Kennel & Kamen (1971) as the number of residues per heme was recalculated by assuming their cytochrome contained four hemes per mole (Table III). Of all the subunits from *Rs. rubrum* and *Ectothiorhodospira* sp., composition homology is clearly best with the H subunit. For some residues, homology is better with the H subunit from *Rs. rubrum* (Asp, Thr, Gly, Tyr, Phe, and His) or with the H subunit from *Ectothiorhodospira* sp. (Glu, Pro, and Lys), and for some residues, homology is excellent with both (Ser, Ala, and Val). Compared to the H subunits of the other two species, the cytochrome from Chromatium appears to be poor in Ile, Leu, Phe, and Arg. Given the fact that these membrane proteins from *Ectothiorhodospira* sp. and—very likely also—from *Chr. vinosum* contain four hemes per mole, this kind of composition homology indicates that they probably are the same with superimposed interspecific variations.

According to amino acid composition and molecular weight, the M and the L polypeptides in *Rs. rubrum*, *Rp. sphaeroides*, and *Ectothiorhodospira* sp. probably have highly conserved structures. This is expected since they carry most of the reactants of the primary photoreaction (Feher & Okamura, 1978). Composition homology is not as unequivocal in the heme polypeptide that we have designated as "H". However, we feel that this attribution is probably correct since, at least in the above-mentioned species, this subunit has similar molecular weight and amino acid compositions and is present as one copy per mole of photoreaction center. This may offer a new perspective for the ill-understood structure–function of the colorless M_r 36 000 polypeptide of the Rhodospirillaceae. Our work also leads to the suggestion that the cytochromes that copurify with the photoreaction centers of other organisms such as *Chr. vinosum* and *Rp. viridis* may well be an integral part of these photoreaction centers. This can be tested only by a careful characterization of these photoreaction centers. Similarly, whether the 39 100 polypeptide of *Ectothiorhodospira* sp. is a true "H" subunit must await more information, particularly on the sequence of these proteins.

Acknowledgments

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Registry No. Bpheo, 17453-58-6; menaquinone, 1182-68-9; spirilloxanthin, 34255-08-8; cytochrome *c*-555, 9048-79-7; cytochrome *c*-552, 9048-78-6.

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Kinetic Properties of Na^+–H^+ Antiport in *Escherichia coli* Membrane Vesicles: Effects of Imposed Electrical Potential, Proton Gradient, and Internal pH†

Martine Bassilana, Evelyne Damiano, and Gérard Leblanc*

ABSTRACT: Modifications of the kinetic properties of the *Escherichia coli* (RA11) Na^+–H^+ antiport system by imposed pH gradients (ΔpH, interior alkaline) and membrane potential ($\Delta\psi$, interior negative) were studied by looking at the accelerating effects of ΔpH and $\Delta\psi$ on downhill Na^+ efflux from membrane vesicles incubated at different external pHs. First, variations of the Na^+ efflux rate (V_{Na}) as a function of imposed ΔpH appear to be strongly dependent on the external pH value. The individual V_{Na} vs. ΔpH relationships observed between pH 5.5 and pH 6.6 are all nonlinear and indicate the existence of a threshold ΔpH above which V_{Na} increases steeply as the ΔpH magnitude increases; threshold ΔpH values progressively decrease as the pH is raised from 5.5 to 6.6. In contrast, at or above neutrality, V_{Na} acceleration is linearly related to ΔpH amplitude. Strikingly, it is shown that the ΔpH-dependent variations in the Na^+ efflux rate measured in vesicles incubated at different external pHs can be accounted for by variations of internal pH; the observed relationship suggests that a high internal H^+ concentration inhibits the Na^+–H^+ antiport activity. This inhibition results from a drastic increase in the apparent K_m of the Na^+ efflux reaction as the internal H^+ concentration increases. On the other hand, imposed $\Delta\psi$ increases the Na^+ efflux rate linearly by a selective modification of the V_{max} value of the Na^+ efflux. Together, these data indicate that the internal H^+ concentration controls the Na^+–H^+ antiport activity and that the chemical and electrical proton gradients affect two different kinetic steps of the Na^+–H^+ exchange reaction.

It is now accepted that Na^+–H^+ exchange reactions catalyzed by the Na^+–H^+ antiporter present in bacterial membranes are involved in two important physiological functions: generation of a transmembrane Na^+ gradient (Harold & Altendorf, 1974; Lanyi, 1979) and regulation of the cytoplasmic pH (Padan et al., 1981). Operation of these cationic exchangers in the cytoplasmic membrane of bacteria was postulated by Mitchell (1968, 1970, 1973) within the frame of the chemiosmotic hypothesis, and their participation in the regulation of internal Na^+ concentration is thought to proceed in the following manner: during Na^+ extrusion, the antiporter obligatorily couples the Na^+ outflow to an inward movement of protons, the energy necessary for uphill Na^+ efflux being derived from the downhill H^+ influx along its favorable electrochemical potential gradient ($\Delta\bar{\mu}_{H^+}$)[1] created during activity of H^+-excreting devices such as the respiratory chain, H^+-ATPase, or the light-driven bacteriorhodopsin.

The existence of obligatory coupled Na^+ and H^+ movements (West & Mitchell, 1974; Zilberstein et al., 1979) as well as the dependence of the Na^+ extrusion process upon the generation of $\Delta\bar{\mu}_{H^+}$ (interior alkaline and electrically negative) has been convincingly demonstrated in many bacterial systems and derived right-side-out membrane vesicles [review in Lanyi (1979)]. Conversely, $\Delta\bar{\mu}_{H^+}$-dependent Na^+ accumulation by membrane vesicles with inverted orientation polarity has also been reported (Reenstra et al., 1980; Beck & Rosen, 1979).

Although there is general agreement that $\Delta\bar{\mu}_{H^+}$ drives the Na^+–H^+ antiport reaction, the precise mechanism underlying this activation is still unknown. In particular, the exact role of the electrical and chemical components of $\Delta\bar{\mu}_{H^+}$ remains to be elucidated. West & Mitchell (1974) first suggested that the Na^+–H^+ antiporter is solely influenced by ΔpH. Later on, Schuldiner & Fishkes (1978) proposed that $\Delta\psi$ drives the exchange reaction at alkaline external pH whereas ΔpH would specifically increase the antiporter activity at acidic pH. Finally, studies using everted membrane vesicles have shown that both ΔpH and $\Delta\psi$ can promote Na^+ accumulation in these preparations (Reenstra et al., 1980).

Recently, further insight into the mechanism of control of the antiport activity by $\Delta\psi$ and ΔpH was obtained by looking at the effects of these gradients on the kinetics of downhill Na^+ efflux catalyzed by the Na^+–H^+ exchanger in *Escherichia coli* membrane vesicles incubated at different external pHs (Bassilana et al., 1984). It was first observed that downhill Na^+ efflux is strongly enhanced in the presence of $\Delta\bar{\mu}_{H^+}$ (interior alkaline and negative). Furthermore, the results indicated that, at all external pHs tested between 5.5 and 7.5, both $\Delta\psi$ and ΔpH contribute to the acceleration of the downhill

† From the Laboratoire Jean Maetz du Département de Biologie du Commissariat à l'Energie Atomique, B.P. 68, 06230 Villefranche-sur-Mer, France. *Received February 2, 1984.*
* Address correspondence to this author. He is associated with CNRS (ER 247).

[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, electrochemical gradient of protons; ΔpH, proton gradient; $\Delta\psi$, electrical potential.

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