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PIGMENT CONTENT AND MOLAR EXTINCTION COEFFICIENTS OF PHOTOCHEMICAL REACTION CENTERS FROM *RHODOPSEUDOMONAS SPHEROIDES*

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SUMMARY

Reaction center particles isolated from carotenoidless mutant *Rhodopseudomonas spheroides* were studied with the aim of determining the pigment composition and the molar extinction coefficients.

Two independent sets of measurements using a variety of methods show that a sample with $A_{800\ nm}=1.00$ contains $20.8\pm0.8\ \mu M$ tetrapyrrole and that the ratio of bacteriochlorophyll to bacteriopheophytin is 2:1.

Measurements were made of the absorption changes attending the oxidation of cytochrome *c* coupled to reduction of the photooxidized primary electron donor in reaction centers, using laser flash excitation. The ratio of the absorption change at 865 nm (due to the bleaching of P870) to that at 550 nm (oxidation of cytochrome) was found to be 5.77.

These results, combined with other data, yield a pigment composition of 4 bacteriochlorophyll and 2 bacteriopheophytin molecules in a reaction center. Based on this choice, extinction coefficients are determined for the 802- and 865-nm bands: $\varepsilon_{802\ nm}=288\ (\pm14)\ mM^{-1}\cdot cm^{-1}$ and $\varepsilon_{865\ nm}=128\ (\pm6)\ mM^{-1}\cdot cm^{-1}$. For reversible bleaching of the 865-nm band, $\Delta\varepsilon^{red-ox}_{865nm}=112\ (\pm6)\ mM^{-1}\cdot cm^{-1}$ (referred to the molarity of reaction centers). Earlier reported values of photochemical quantum efficiency are recomputed, and the revised values are shown to be compatible with those obtained from measurements of fluorescence transients.

INTRODUCTION

Photosynthetic reaction center preparations have become a valuable tool in studying the photochemistry and associated electron transport of bacterial photosynthesis[1–15]. When computing stoichiometries and quantum efficiencies in these

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studies, we have had to rely on earlier values of extinction coefficients determined with relatively crude preparations[16]. Recent refinements in the purification of reaction center particles from *Rhodopseudomonas spheroides*[10,17] have allowed an improved evaluation of the molecular composition and optical parameters of these particles. New data on these matters will be reported here.

Absorption spectra of reaction center particles[10,12,17] show maxima near 537 and 757 nm identified with bacteriopheophytin and maxima near 600, 803, and 865 nm ascribed to bacteriochlorophyll. Absorption (A) by a sample of reaction center particles can be correlated with the amounts of bacteriochlorophyll and bacteriopheophytin extractable from that sample. If we can decide how many molecules of these pigments are in one reaction center, we can translate these data into extinction coefficients based on the molarity of reaction centers. For this we must define a reaction center, as either a functional or a morphological entity, not necessarily identical with a reaction center particle. Functionally a reaction center can be defined as that constellation of molecules capable of effecting the photochemical translocation of one electron with the use of one quantum. Morphologically a reaction center is the smallest particle capable of performing the characteristic photochemistry. Recent analyses by Feher *et al.*[18] suggest that a morphologically defined reaction center is a distinct smaller part of the reaction center particle as prepared by Clayton and Wang[17] or Feher[10].

MATERIALS AND METHODS

*Section I**

Reaction center particles were isolated from carotenoidless mutant *Rhodopseudomonas spheroides* strain 2.4.1./R-26 as described by Feher[10]. They were kept cold and in the dark until ready for use. Bacteriochlorophyll was extracted from *Rhodospirillum rubrum* by standard methods[20]. The ratio of the absorption in the red band in ether to that in acetone (1.37) and of the absorption in the blue band to that in the red band in both ether (0.78) and acetone (0.96) agreed very well with the data of Sauer *et al.*[21], and their extinction coefficients were used in calculating total moles of pigment. Bacteriochlorophyll was quantitatively converted to bacteriopheophytin by adding 1/100 vol. of 1 M HCl to an acetone solution. The bacteriopheophytin was crystallized from acetone–water. Again the spectra agreed quantitatively with published values[21]. The conversion of bacteriochlorophyll to bacteriopheophytin in ether required stronger acid: 2% (v/v) of methanesulfonic acid. All extractions of reaction center particles and manipulations of the pigments were carried out in a darkroom under dim green light. All solvents were freshly distilled, as was the aqueous HCl. The distilled water was twice re-distilled in quartz, and glassware was carefully cleaned.

Section II

Cells of *Rps. spheroides* 2.4.1./R-26 were grown, and chromatophores were prepared, as described in ref. 17. Whole cells and chromatophores were always used as suspensions in de-ionized water containing 0.01 M Tris·HCl, pH 7.5. Reaction

* D.C.M. contributed the results in Section I, S.C.S. and R.K.C. the results in Section II, and W.W.P. and R.K.C. those in Section III.

center particles were isolated according to the method of Clayton and Wang[17] and were used as chilled, dark-adapted suspensions in de-ionized water containing 0.01 M Tris·HCl, pH 7.5 *plus* ≐ 0.3% (v/v) of the detergent lauryl dimethyl amine oxide*.

Lauryl dimethyl amine oxide (Onyx Chemical Company, Jersey City, N.J.) was incubated with catalase (≐10 μg per ml of 30% lauryl dimethyl amine oxide) for about 1 h at room temperature to remove H_2O_2 (≐0.02%). Triton X-100 (Rohm and Haas) was treated with $SnCl_2 \cdot 2H_2O$ to reduce organic peroxides[22]. Ubiquinone-30 (Sigma Chemical Company) was dissolved in ethanol and stored in a freezer.

All absorption spectra and light-induced absorption changes were measured with a Cary 14R spectrophotometer. The monochromator was calibrated against the α peak (at 550 nm) of reduced equine cytochrome *c*, and the readings of optical density were calibrated with the use of a standard $CuSO_4$ solution. The solution itself had been calibrated by direct measurements of transmittance. A sample in the Cary spectrophotometer could be cross-illuminated through a port in the sample chamber.

In all extractions of reaction center particles, the pellets of sedimented, denatured protein were re-extracted once: additional extractions yielded no detectable pigment. Bacteriochlorophyll was quantitatively converted to bacteriopheophytin by the addition of 1/60 volume of 5 M HCl. For the determination of the extinction coefficient of bacteriopheophytin in a mixture of 7 vol. of acetone and 2 vol. of methanol (acetone–methanol (7:2)), aliquots of acidified extracts of chromatophores, having known spectra, were evaporated to dryness and the bacteriopheophytin redissolved in dry acetone–methanol (7:2), chloroform, and ether. The spectrum of bacteriopheophytin was unchanged by the addition of water to 5% by volume, and the extinction coefficient obtained with dry solvents was applied to the (wet) extracts of reaction center particles.

Section III

Preparations of reaction center particles were made both with lauryl dimethyl amine oxide[17] and with Triton X-100[1].

Sigma Type III cytochrome *c* was reduced with $Na_2S_2O_4$ under N_2, chromatographed anaerobically on Sephadex G-25, and stored under N_2 at 0 °C.

Absorption changes due to photooxidation of the primary electron donor** and cytochrome were measured after saturating flashes from a Q-switched ruby laser[23]. On each measurement, the spectrophotometer signal was digitized and stored in the memory of a Biomation Model 802 transient recorder. The results of four measurements then were averaged in a digital computer. The individual measurements were made 20 min apart, with the sample in darkness between measurements. A shutter introduced the measuring light beam 1–2 ms before the recording of each measurement began. When necessary, the baseline signal without an actinic flash or flash

* The concentration of lauryl dimethyl amine oxide is not known accurately, since knowledge of its physical properties and of its binding to reaction center particles is incomplete; there is no simple assay for the concentration of this detergent in a suspension of reaction center particles. Thus it is not accurately known how much lauryl dimethyl amine oxide is carried along, closely associated with the reaction center particles during manipulations of the concentration of detergent; see Results, Section I.

** By primary electron donor, P, we mean that portion of the reaction center, not necessarily a single molecule, which is oxidized in the primary photochemical separation of charge.

artifacts that occurred in the absence of the measuring light also were recorded and were subtracted from the full signal in the computer.

RESULTS

Section I

A volume* of exhaustively dialysed reaction center particles was centrifuged and the pellet extracted with 6 ml, then 1 ml, of acetone. The spectrum of an aliquot of the weighed combined extracts was determined. The mixture of bacteriochlorophyll and bacteriopheophytin was quantitatively converted to the latter with HCl, and the spectrum re-determined. Several variations on the basic theme were carried out, with no significant difference in the results: the pellet of reaction center particles was lyophillized before extraction, the acetone was buffered with Tris, and the acetone extract was highly diluted into ether to determine the spectra and convert to bacteriopheophytin.

The extraction was repeated with smaller samples, using 17 different solvents. Many of these solvents caused difficulties, either failing to extract the pigments completely or producing complex bacteriochlorophyll spectra (probably arising from mixtures of different solvation isomers of bacteriochlorophyll, as in dimethylsulfoxide). Two useful solvents were dichloromethane and pyridine; these gave the same ratio of bacteriochlorophyll to bacteriopheophytin (2:1) as was found with acetone.

The results of these solvent extractions of reaction center particles suggest that the pigments are buried in a strongly hydrophobic core of the protein, surrounded by a more polar shell containing the detergent lauryl dimethyl amine oxide. The relatively low permeability by "poor" solvents (such as hexane, methylcyclohexane, benzene, *o*-xylene, diethyl ether, diisopropyl ether, and formamide) and the retention of some photoactivity by the reaction center particles after extraction with these solvents are evidence suggesting the presence of a polar shell in reaction center particles. This shell apparently is disrupted by "good" solvents (such as acetone, dichloromethane, and pyridine), and the protein is denatured. "Powerful" solvents (such as dimethylsulfoxide, dimethylformamide, and *N*-methylformamide) so scramble the protein structure that it is insoluble even in sodium dodecyl sulfate.

A complication in the spectral analyses was caused by the detergent lauryl dimethyl amine oxide, strongly bound to the reaction center particle but extractable with "good" organic solvents. This detergent forms tight complexes with bacteriochlorophyll in most solvents. In acetone the resulting spectral changes were minor in the near ultraviolet and far red bands of bacteriochlorophyll (peak $\Delta\varepsilon$ +1.5% and −4.2%, respectively), but can cause larger errors in estimating the ratio of bacteriochlorophyll to bacteriopheophytin. The sharpening of the 576-nm band in acetone on complexing is large: the full bandwidth at half-maximum decreases from 38 to 33 nm; the increase in peak absorption is 11.5%. Titration of bacteriochlorophyll with lauryl dimethyl amine oxide showed that it is approximately saturated with this detergent on extraction from reaction center particles. Only a lower limit

* The volume was chosen so as to give a value of 10–20 for the product of the volume (ml) times the absorption by the reaction center particles at the peak near 803 nm ($A_{800\,nm}$). This allowed work with a constant number of reaction center particles.

of several detergent molecules per bacteriochlorophyll, *i.e.* >10 per reaction center particle, could thus be set. These results were confirmed upon chromatography of the pigment on sugar. The pigments ran faster on the column in the presence than in the absence of lauryl dimethyl amine oxide, and the separation of bacteriochlorophyll and bacteriopheophytin was hindered. These effects could be duplicated by adding this detergent to bacteriochlorophyll. Repeated chromatography did separate the complex, but differential losses were too great for this procedure to be of use for analysis. We therefore based our analyses on the spectral properties of bacteriochlorophyll and bacteriopheophytin in the presence of the saturating amounts of lauryl dimethyl amine oxide bound to the reaction center particle.

The spectral analysis yielded two pieces of information: the concentration of total pigment in a sample of reaction center particles having $A_{800\ nm}=1.00$ and the ratio of bacteriochlorophyll to bacteriopheophytin. The ratio was obtained from the following equation:

$$\frac{\text{Bacteriochlorophyll}}{\text{Bacteriopheophytin}}=\frac{A_2\varepsilon_1{}^{\mathrm{p}}-A_1\varepsilon_2{}^{\mathrm{p}}}{A_1\varepsilon_2{}^{\mathrm{c}}-A_2\varepsilon_1{}^{\mathrm{c}}}$$

where A_1 and A_2 are the absorption at two wavelengths, $\varepsilon_1{}^{\mathrm{p}}$ is the extinction coefficient for bacteriopheophytin at wavelength 1, $\varepsilon_1{}^{\mathrm{c}}$ the same for bacteriochlorophyll, *etc.* The wavelengths 1 = 522 nm and 2 = 576 nm were chosen to maximize the differential extinction coefficients and thus minimize the error. For these wavelengths, the normalized extinction coefficients in the presence of lauryl dimethyl amine oxide are: $\varepsilon_1{}^{\mathrm{p}}=1$, $\varepsilon_2{}^{\mathrm{p}}=0.078$, $\varepsilon_2{}^{\mathrm{c}}=0.855$, and $\varepsilon_1{}^{\mathrm{c}}=0.128$. Both the far red and visible bands gave the same ratio, but the near ultraviolet band did not, indicating that some ultraviolet-absorbing material other than bacteriochlorophyll and bacteriopheophytin was also extracted. It is known[11] that ubiquinone is extracted from the reaction center particles.

The same ratio of bacteriochlorophyll to bacteriopheophytin was also obtained by data at a single wavelength before and after conversion of bacteriochlorophyll to bacteriopheophytin. The following equation was used:

$$\frac{\text{Bacteriochlorophyll}}{\text{Bacteriopheophytin}}=\frac{A'-A}{A-\dfrac{\varepsilon^{\mathrm{c}}}{\varepsilon^{\mathrm{p}}}A'}$$

where A is the absorption of the mixture of bacteriochlorophyll and bacteriopheophytin at a given wavelength and A' is the absorption after the addition of HCl. For 768 nm, $\varepsilon^{\mathrm{c}}/\varepsilon^{\mathrm{p}}=4.17$ and for 522 nm, $\varepsilon^{\mathrm{c}}/\varepsilon^{\mathrm{p}}=0.128$.

The total pigment was most easily obtained following total conversion to bacteriopheophytin. It was also obtained from the spectra of the original extract, with the use of the ratio of bacteriochlorophyll to bacteriopheophytin.

Table I shows a representative series of data. The weighted results of 5 separate analyses (>10 ratio determinations) were: bacteriochlorophyll:bacteriopheophytin $=1.97\pm0.10$; $20.8\pm0.8\ \mu$M pigment in a sample having $A_{800\ nm}=1.00$.

TABLE I

A REPRESENTATIVE SET OF DATA ON PIGMENT EXTRACTION OF REACTION CENTER PARTICLES FROM *RHODOPSEUDOMONAS SPHEROIDES*

See the text for an explanation of the calculations.

$A_{800\,nm}$ × *volume of sample (ml)*	*Acetone extract*	*Absorption*			
		745 nm	*768 nm*	*522 nm*	*576 nm*
15.2	(1) 5.15 ml reaction center particles				
	(2) 250 μl (1) + 500 μl acetone	—	0.925	0.198	0.283
	(3) (2) + 10 μl 1 M HCl	0.892	0.295	0.480	0.036
$\frac{\text{Bacteriochlorophyll}}{\text{Bacteriopheophytin}}$	*via* 2 wavelengths (522 and 576 nm):	2.00			
$\frac{\text{Bacteriochlorophyll}}{\text{Bacteriopheophytin}}$	*via* acid:	2.06 (using $A_{768\,nm}$) 2.04 (using $A_{522\,nm}$)			

Total nanomoles of pigment= 300; therefore, 19.8 μM pigment in a sample having $A_{800\,nm}$= 1.00.

Absolute extinction coefficients ($M^{-1}\cdot cm^{-1}$):

for bacteriopheophytin,	$\varepsilon_{745\,nm}= 4.75\cdot10^4$	$\varepsilon_{522\,nm}= 2.50\cdot10^4$
for bacteriochlorophyll,	$\varepsilon_{768\,nm}= 6.90\cdot10^4$	$\varepsilon_{576\,nm}= 1.76\cdot10^4$

Section II

The number of moles of pigment (bacteriochlorophyll *plus* bacteriopheophytin) in a preparation of reaction center particles having a known spectrum was determined by measuring the amounts of bacteriopheophytin after extraction of the reaction center pigments with acetone–methanol (7:2, v/v), followed by acidification to convert bacteriochlorophyll to bacteriopheophytin. The measurements were based on the absorption at 525 nm.

The $\varepsilon_{525\,nm}$ for bacteriopheophytin in acetone–methanol (7:2, v/v)was found to be 2.34 ($\pm$0.03)$\cdot10^4$ $M^{-1}\cdot cm^{-1}$ by comparing the absorption at this peak for bacteriopheophytin in acetone–methanol (7:2, v/v) to those due to equal amounts of bacteriopheophytin in solvents for which the extinction coefficients have been reported in the literature[24–26].

Two control experiments were carried out to check for effects of lauryl dimethyl amine oxide on the spectra of reaction center pigments in solution. The first was a concentration series with this detergent in which chromatophores from *Rps. spheroides* 2.4.1./R-26 were extracted according to the procedure used for the measurement of the total pigment in reaction center particles. The second was a similar concentration series using suspensions of reaction center particles containing 0.05% Triton X-100 (and probably also a small, unknown amount of lauryl dimethyl amine oxide). Lauryl dimethyl amine oxide at a concentration as high as 0.6% in the suspension of chromatophores prior to extraction had no apparent effect on the spectrum of bacteriopheophytin in the extracts of chromatophores, and there was no apparent effect of this detergent at any of the concentrations (up to 1% in the suspension

before extraction) on the spectrum of bacteriopheophytin in extracts of reaction center particles. Any effects of the detergent on the visible band of bacteriochlorophyll (see *Section I*) did not affect this analysis based on the absorption peak of bacteriopheophytin.

Nine separate extractions were performed, using three preparations of reaction center particles. Representative data are shown in Table II. The result agrees with that of *Section I*: 21 ± 1 nmoles of bacteriopheophytin are extracted from 1-ml sample of reaction center particles having $A_{800\ nm} = 1.0$. If the ratio of bacteriochlorophyll to bacteriopheophytin is 2:1 (see *Section I*; also see below), then two thirds of these 21 nmoles, or 14 nmoles, is due to bacteriopheophytin made from bacteriochlorophyll originally present in the sample.

TABLE II

A REPRESENTATIVE SET OF DATA FOR THE MEASUREMENT OF REACTION CENTER PIGMENTS AS THE AMOUNT OF BACTERIOPHEOPHYTIN IN ACIDIFIED ACETONE–METHANOL (7:2, v/v)

The $\varepsilon_{525\ nm}$ used for bacteriopheophytin in acetone–methanol (7:2, v/v) was $2.34\ (\pm 0.03) \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ (see text). Prior to extraction, reaction center particles were suspended in 0.01 M Tris·HCl, pH 7.5, and containing 0.3% lauryl dimethyl amine oxide.

$A_{800\ nm}$ of reaction center particles	*Raw data*: $A_{525\ nm}$ in acidified extract (includes reextraction)*	*Average*	*nmoles bacteriopheophytin in 1.0 ml of sample having $A_{800\ nm} = 1.0$*
16.3	0.381 0.383 0.372 0.390	0.38 ± 0.01	20 ± 1

* This is the $A_{525\ nm}$ of 4.07 ml containing the pigment from 0.20 ml of the suspension of reaction center particles.

The ratio of bacteriochlorophyll to bacteriopheophytin in the reaction center was determined by measuring the absorption due to bacteriopheophytin at its peak in the visible region in an extract of reaction center particles before and after acidification to convert bacteriochlorophyll to bacteriopheophytin. The solvent used for the extractions was methanol–ether (6:4, v/v). Before acidification, some of the absorption at 527 nm (the location of the peak due to bacteriopheophytin in methanol–ether (6:4, v/v)) was due to the tail of the bacteriochlorophyll band (peaking at about 606 nm). This contribution was estimated by fitting the experimentally measured spectrum (by means of a "least-squares" procedure) with spectra of bacteriochlorophyll and bacteriopheophytin. The latter spectra were obtained from a methanolic extract of cells of *Rps. spheroides* 2.4.1./R-26 which was diluted into methanol–ether (6:4, v/v). A small correction was applied to the spectrum of bacteriochlorophyll to discount the spectral contribution of bacteriopheophytin extracted along with bacteriochlorophyll from the cells.

Two types of controls were done to check for any effect of lauryl dimethyl amine oxide in this experiment. The first was to apply the same procedure used with reaction center particles to a suspension of chromatophores from *Rps. spheroides* 2.4.1./R-26 which contained 0.3% lauryl dimethyl amine oxide (or the appropriate volume of water). There was no significant effect from the 0.3% detergent on the peak height of the visible band of bacteriopheophytin in the suspension of chromatophores. However, in extracts containing detergent, the peaks of the visible bands of bacteriochlorophyll and bacteriopheophytin were blue-shifted by 1 nm relative to their positions in extracts without detergent. The second type of control was a concentration series using reaction center particles in the presence of 0.05% Triton X-100 (and probably also a small basal amount of lauryl dimethyl amine oxide). When the concentration of lauryl dimethyl amine oxide was 0.3%, small spectral changes were evident, and the calculated ratio of bacteriochlorophyll to bacteriopheophytin decreased by $\doteq 6\%$ (relative to the values obtained with 0 and 0.1% lauryl dimethyl amine oxide). Therefore, this control and the one with chromatophores indicate that the presence of $\geqslant 0.3\%$ lauryl dimethyl amine oxide in the suspensions of reaction center particles probably introduced error in the determinations of the ratio of bacteriochlorophyll to bacteriopheophytin but that the error was not serious at 0.3% lauryl dimethyl amine oxide.

Four determinations of the ratio of bacteriochlorophyll to bacteriopheophytin yielded the value 2.0. Two additional trials were carried out, in which the sample was acidified immediately after the solvent was added (instead of several minutes later, after a spectrum had been recorded of the nonacidified extract). This would minimize the exposure of bacteriochlorophyll to detergent, and it did result in a somewhat higher value of the ratio (2.1 for both trials). The simplest conclusion from these results is that the ratio of bacteriochlorophyll to bacteriopheophytin in the reaction center is 2 bacteriochlorophyll:1 bacteriopheophytin, a result which agrees with that of Section I.

For the determination of $\Delta\varepsilon^{\text{red}-\text{ox}}_{865\,\text{nm}}$, a measurement was made of the fraction of the 865 nm absorption that is lost when reaction center particles are subjected to illumination intense enough to cause maximal bleaching. The reaction center particles, $A_{800\ \text{nm}}=0.72$, were in the presence of 30 μM ubiquinone-30 to retard the dark re-reduction of the photooxidized primary donor[12]. The sample was illuminated with saturating white light, causing 88% of the absorption at 865 nm to be reversibly bleached. Controls showed that the trace of ethanol added with the ubiquinone-30 in the experimental sample had no apparent effect. Tests with added reductant showed that a negligible fraction of the reaction center particles initially contained oxidized primary electron donor.

It is now possible to list optical parameters for reaction centers, using a range of assumptions for the number of bacteriochlorophyll molecules in a reaction center. Because of the strong electronic interactions between the pigment molecules, it is not meaningful to assign specific molecules to each band in the complex reaction center spectrum. However, one can still assign molar extinction coefficients to the reaction center. This has been done in Table III*. The relative height of the 865-nm

* The values of extinction coefficients quoted in ref. 19 are too large by 10% because of an arithmetical error. They should read $\varepsilon_{800\,\text{nm}}=2.9\cdot10^5\ \text{M}^{-1}\cdot\text{cm}^{-1}$ and $\varepsilon_{865\,\text{nm}}=1.2\cdot10^5\ \text{M}^{-1}\cdot\text{cm}^{-1}$ for 6 pigment molecules per reaction center.

TABLE III

OPTICAL PARAMETERS FOR REACTION CENTERS OF *RHODOPSEUDOMONAS SPHEROIDES*

*Assumed number of bacterio-chlorophylls per reaction center**	$A_{800\ nm}$ *per ml of sample containing 1 nmole of reaction centers***	$\varepsilon_{802\ nm}$ $(mM^{-1}\cdot cm^{-1})$	$\varepsilon_{865\ nm}$*** $(mM^{-1}\cdot cm^{-1})$	$\Delta\varepsilon^{red-ox}_{865\ nm}$§ $(mM^{-1}\cdot cm^{-1})$
2	0.144 ± 0.007	144 ± 7	63.9 ± 3.2	56.3 ± 2.8
4	0.288 ± 0.014	288 ± 14	128 ± 6	112 ± 6
6	0.432 ± 0.022	432 ± 22	192 ± 10	169 ± 8

* Assumptions of odd numbers of bacteriochlorophyll molecules per reaction center are ruled out by the finding of a value of 2 for the ratio of bacteriochlorophyll to bacteriopheophytin (see Sections I and II).

** Computed from the step: a 1-ml sample of reaction center particles having $A_{800\ nm} = 1.00$ contains $13.9/x$ nmoles reaction centers, where x is the number of bacteriochlorophyll molecules per reaction center, and 13.9 is the number of nmoles of bacteriochlorophyll contained in a 1-ml sample having $A_{800\ nm} = 1.00$. This latter number (13.9) is 2/3 of the 20.8 nmoles of tetrapyrrole contained in a 1-ml sample having $A_{800\ nm} = 1.00$ (see Sections I and II).

*** $\varepsilon_{865\ nm}$ is computed by the relation: $\varepsilon_{865\ nm} = \varepsilon_{802\ nm}/2.25$. The use of the value 2.25 for the ratio $A_{802\ nm}/A_{865\ nm}$ is explained in the text.

§ Computed with the assumption that 88% of the absorption at 865 nm is reversibly bleached in the primary photoact (see text).

band in reaction center particles apparently varies slightly in different preparations; a representative value of 2.25 has been used in Table III for the computation of $\varepsilon_{865\ nm}$ from $\varepsilon_{802\ nm}$.

Section III

A differential extinction coefficient for the absorption change reflecting photochemistry in reaction center particles can be determined by coupling the photochemistry to a well-characterized chemical process, the oxidation of mammalian cytochrome *c*. A molar differential extinction coefficient at 865 nm, presumably for the single-electron transfer in reaction center particles, can be computed from the known coefficient for oxidation of the cytochrome. This determines the molar extinction coefficient of the 865-nm band, for reaction centers defined functionally in terms of single-electron transfer. In turn this specifies the number of bacteriochlorophyll molecules per reaction center, when coupled with data on the extraction of pigments from reaction center particles.

Fig. 1A shows the oxidation of horse heart cytochrome *c* by reaction center particles, following a short actinic flash. Immediately after the flash, the absorption at 550 nm increased, presumably due to the formation of photooxidized primary electron donor, P^+, which is associated with broad absorption in the visible spectral region[27]. Cytochrome oxidation followed, giving a larger absorption decrease with a half-time of about 2 ms. In many experiments, a small, variable part of the cytochrome oxidation occurred with a half-time of about 20 μs, in agreement with the results of Ke *et al.*[7], but most of the reaction was much slower. The kinetics did

not appear to vary with pH between 7.5 and 8.5, nor did increasing the concentration of lauryl dimethyl amine oxide to 1.5% or replacing that detergent by 0.05% Triton X-100 affect them markedly.

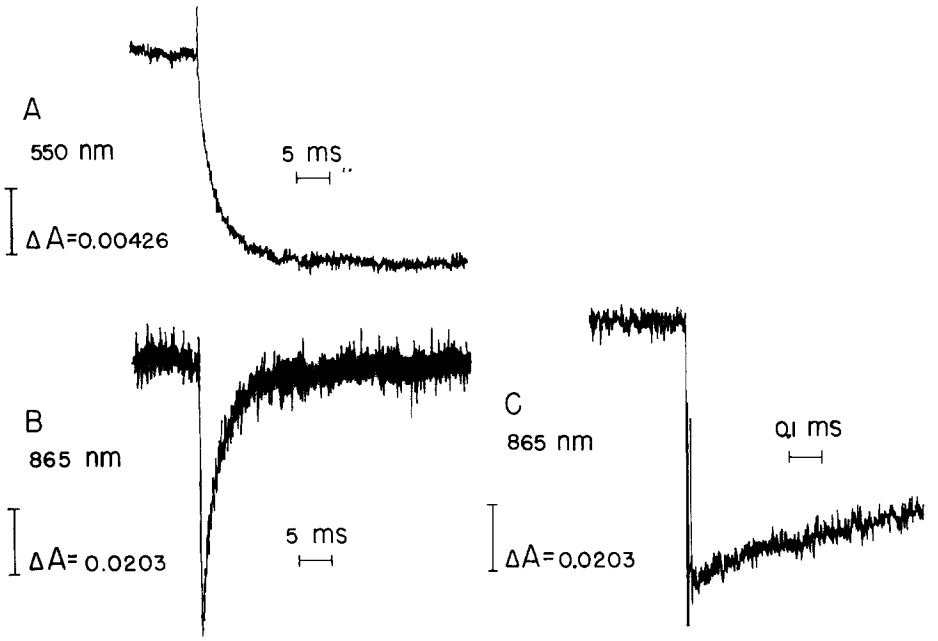

Fig. 1. Absorption changes on photooxidation of the primary electron donor P and cytochrome *c*. The sample contained reaction center particles (prepared with lauryl dimethyl amine oxide) giving an $A_{800\,nm}$ of 0.25, 0.5% lauryl dimethyl amine oxide, 11 μM cytochrome *c*, and 7 μM ubiquinone-30 in 3.1 ml of 0.01 M Tris·HCl, pH 7.5. The cuvette had a 1-cm light path and was rendered anaerobic by bubbling with N_2 through a rubber septum. In each trace, a downward deflection represents an absorption decrease. The same sample was used for all of the measurements in the figure. A. Cytochrome oxidation, measured at 550 nm, with a monochromator bandpass of 1.65 nm. B. P oxidation and reduction, measured at 865 nm, with a monochromator bandpass of 2.3 nm. A downward drift that occurred in the baseline without a flash has been subtracted from the full signal. C. P oxidation, as in B but on a shorter time scale. Artifacts that occurred at the time of the flash have been subtracted from the full signal.

Fig. 1C affords a measurement of the absorption change due to the initial photooxidation of P, and Fig. 1B shows that, within experimental error, all of the P^+ was reduced again as the cytochrome became oxidized. In the absence of cytochrome, P^+ reduction was negligible on this time scale. (This was also seen at 550 nm where, in the absence of cytochrome, the decay of the small positive absorption change required several seconds (not shown).) The rate of P^+ reduction in Fig. 1B is slightly slower than the rate of cytochrome oxidation in Fig. 1A. This probably resulted from the sequence of the measurements. In this experiment, the cytochrome measurements were made first, and the rate of the reaction decreased somewhat during the experiment. Experiments in which P was measured first gave results that were consistent with this interpretation.

The initial absorption decrease due to oxidation of P in Fig. 1C is 0.079; the

net absorption decrease due to cytochrome oxidation in Fig. 1A is 0.0138. The ratio is 5.72. Six similar experiments, four of which employed reaction center particles prepared with lauryl dimethyl amine oxide, and two of which used reaction center particles prepared with Triton X-100, gave an average of 5.77 ± 0.10 for this ratio, in complete agreement with the ratio of 5.8 that Ke *et al.*[7] reported for reaction centers prepared with Triton X-100.

Recent measurements of $\Delta\varepsilon^{red-ox}_{550\,nm}$ for equine cytochrome *c* range from 19.6 to 21.1 $mM^{-1} \cdot cm^{-1}$ (refs 28–31). With a value of 20.4 (± 0.7) $mM^{-1} \cdot cm^{-1}$, the ratio 5.77 gives $\Delta\varepsilon^{red-ox}_{865nm} = 118$ (± 4) $mM^{-1} \cdot cm^{-1}$, within experimental error of the $\Delta\varepsilon^{red-ox}_{865nm} = 112$ (± 6) $mM^{-1} \cdot cm^{-1}$ that is predicted on the assumption of 4 bacteriochlorophyll molecules per reaction center (see Table III). Other assumptions for the number of bacteriochlorophyll molecules per reaction center would require $\Delta\varepsilon$ values significantly different from the experimental value (*e.g.* 84.4 (± 4.2) and 141 (± 7) $mM^{-1} \cdot cm^{-1}$) for the assumptions of 3 or 5 bacteriochlorophyll molecules per reaction center, both ruled out, in addition, by the value of 2 for the ratio of bacteriochlorophyll to bacteriopheophytin.

DISCUSSION

Two lines of evidence favor a choice of 4 bacteriochlorophyll and 2 bacteriopheophytin molecules as the pigment composition of the reaction center. This choice in turn will determine which values for the optical parameters for reaction centers (listed in Table III) should be adopted.

We have presented two independent series of determinations of the ratio of bacteriochlorophyll to bacteriopheophytin in reaction center particles. Both series of measurements agree that the ratio is 2 bacteriochlorophyll:1 bacteriopheophytin*. This ratio, along with some spectral observations, allows a choice to be made of the minimum composition of pigments in reaction centers. At room temperature, the absorption spectrum of reaction center particles contains a band in the visible region, associated with bacteriopheophytin, which is markedly broad and flat (see refs 10 or 17, Fig. 1). At low temperatures, this band is resolved into two bands of similar magnitudes[13]. There is no reason to believe that this transition in bacteriopheophytin is degenerate[33]; it is more likely that the two bacteriopheophytin bands resolved at low temperature are due to the presence of a dimer of bacteriopheophytin in the reaction center. The foregoing evidence argues in favor of an even number of molecules of bacteriopheophytin per reaction center; the measured ratio of 2 bacteriochlorophyll:1 bacteriopheophytin therefore favors the choice of 4 bacteriochlorophyll and 2 bacteriopheophytin molecules as the minimum pigment composition of the reaction center.

Strong support for the choice of 4 bacteriochlorophyll and 2 bacteriopheophytin molecules as the composition of pigment in the reaction center comes from studies of the oxidation of cytochrome *c* by the photooxidized primary electron donor[5,34–36]. The comparison of the $\Delta A_{550\,nm}$ due to the oxidation of cytochrome *c* with the $\Delta A_{865\,nm}$ which recovers in the coupled reaction yields a value of $\Delta\varepsilon^{red-ox}_{865\,nm}$

* After completion of this work, the paper by Reed and Peters[32] appeared, giving results in agreement with ours.

which agrees well with the value that is predicted on the assumption of 4 bacteriochlorophyll molecules per reaction center.

Thus these considerations support the choice of 4 bacteriochlorophyll and 2 bacteriopheophytin molecules as the pigment complement of the reaction center. The resulting revision of $\Delta\varepsilon_{865nm}^{red-ox}$ from 100 $mM^{-1}\cdot cm^{-1}$ (ref. 13) to 112 $mM^{-1}\cdot cm^{-1}$ removes an apparent discrepancy between the values of the quantum efficiency of the primary photochemistry measured by the bleaching at 865 nm (*e.g.* refs 5, 13, 34 and 36) and by the rise in fluorescence from the 865-nm band in reaction center particles or from bulk bacteriochlorophyll in chromatophores[2,13,23]. The representative values for the quantum efficiency of 1.02 ± 12 (ref. 5), 0.9 ± 0.14 (ref. 13), and 1.0 ± 0.15 (ref. 13) become 0.81 ± 0.10, 0.79 ± 0.12, and 0.88 ± 0.13 when recomputed with the use of $\Delta\varepsilon_{865nm}^{red-ox}$ of 112 $mM^{-1}\cdot cm^{-1}$ instead of 93 or 100 $mM^{-1}\cdot cm^{-1}$ (refs 5 and 13, respectively). A representative value for the quantum efficiency measured from the rise in fluorescence is 0.7 (ref. 13); the values for the quantum efficiency measured in the two ways now agree within the limits of error in the measurements. These values are also compatible with values for the quantum efficiency, determined by measurements of electron spin resonance[5,37].

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