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MOLAR EXTINCTION COEFFICIENTS AND OTHER PROPERTIES OF AN IMPROVED REACTION CENTER PREPARATION FROM *RHODOPSEUDOMONAS VIRIDIS*

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Summary

Reaction centers have been purified from chromatophores of *Rhodopseudomonas viridis* by treatment with lauryl dimethyl amine oxide followed by hydroxyapatite chromatography and precipitation with ammonium sulfate. The absorption spectrum at low temperature shows bands at 531 and 543 nm, assigned to two molecules of bacteriopheophytin *b*. The 600 nm band of bacteriochlorophyll *b* is resolved at low temperature into components at 601 and 606.5 nm. At room temperature the light-induced difference spectrum shows a negative band centered at 615 nm, where the absorption spectrum shows only a weak shoulder adjacent to the 600 nm band. The fluorescence spectrum shows a band at 1000 nm and no fluorescence corresponding to the 830 nm absorption band. Two molecules of cytochrome 558 and three of cytochrome 552 accompany each reaction center. The differential extinction coefficient (reduced minus oxidized) of cytochrome 558 at 558 nm was estimated as $20 \pm 2\ mM^{-1} \cdot cm^{-1}$ through a coupled reaction with equine cytochrome *c*. The extinction coefficient of reaction centers at 960 nm was determined to be $123 \pm 25\ mM^{-1} \cdot cm^{-1}$ by measuring the light-induced bleaching of *P*-960 and the coupled oxidation of cytochrome 558. The corresponding extinction coefficient at 830 nm is $300 \pm 65\ mM^{-1} \cdot cm^{-1}$. The absorbance ratio a_{280nm}/a_{830nm} in our preparations was 2.1, and there was 190 kg protein per mol of reaction centers. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed three major components of apparent molecular weights 31 000, 37 000 and 41 000.

Introduction

We have developed a relatively simple method for preparing photochemical reaction centers from *Rhodopseudomonas viridis*. These reaction centers are

Abbreviation: LDAO, lauryl dimethyl amine oxide.

more pure, in terms of chromophore : protein ratio than others that have been reported [1—3]. We shall present data on the composition of these reaction centers, and some optical parameters including absolute extinction coefficients.

Methods

Preparation of reaction centers. Rp. viridis NHTC 133 was grown in modified Hutner's medium, and purified chromatophores prepared from the cells, as described elsewhere [4,5]. The procedure for isolating reaction centers was closely similar to a method we have developed [6] for the purification of reaction centers from *Rp. gelatinosa*.

Purified chromatophores were suspended in 0.01 M Tris · HCl, pH 7.5 (hereafter called Tris buffer) to an absorbance of 50 (1 cm path) at the long wave absorption maximum near 1020 nm. 60 ml of these chromatophores were mixed with 12 ml of 30% lauryl dimethyl amine oxide (LDAO) and allowed to stand 5 min. The mixture was put onto a 100 ml hydroxyapatite column that had been flushed with 0.01 M potassium phosphate, pH 7.0 (hereafter called phosphate buffer) containing 0.1% LDAO. The column was eluted, first with 0.01 M phosphate buffer plus 0.1% LDAO until the eluate was nearly colorless (about 250 ml), and then with a linear gradient, 0.01—0.1 M phosphate buffer containing 0.1% LDAO, total volume 500 ml. The flow rate was kept below 4 ml/min. The reaction centers emerged at about 0.2 M phosphate, relatively free from antenna bacteriochlorophyll *b* and other pigments except cytochromes. The reaction center-rich part of the eluate was treated with 1.5 volume saturated ammonium sulfate and centrifuged at about 10 000 × *g* for 10 min. This step removed a contaminating pigment (probably oxidized bacteriochlorophyll *b*) absorbing near 690 nm, which remained soluble while the reaction centers formed a floating solid phase. The solid phase was dissolved in 0.01 M Tris buffer containing 0.1% LDAO and dialyzed overnight against the same Tris/LDAO solution. The dialyzed sample was then made 2% in LDAO and put onto a 50 ml hydroxyapatite column, eluted as before but with half the volumes and flow rate. The reaction center-rich fractions were again precipitated with ammonium sulfate, the solid phase redissolved and dialyzed as before. If desired, the final materials could be concentrated by membrane filtration ("Minicon Concentrator", Amicon Corp., Lexington, Mass. 02173). All steps of the reaction center purification were performed at about 4°C with minimal exposure to light.

Analyses. Methods of protein estimation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and measurement of optical absorption and difference spectra at room temperature and 30 K are described in a companion paper dealing with *Rp. gelatinosa* [6].

We attempted to determine pigment composition of the reaction centers by extraction with acetone/methanol followed by acidification to convert bacteriochlorophyll *b* to bacteriopheophytin *b*, but failed. Upon acidification, the absorption bands of bacteriochlorophyll *b* vanished but the bands characteristic of bacteriopheophytin *b* did not appear.

The high- and low-potential cytochromes, cytochrome 558 and cytochrome 552, were assayed by the difference spectra induced by reduction with sodium

ascorbate and sodium dithionite, respectively, compared with material either untreated or oxidized with potassium ferricyanide. Although the low-potential cytochrome associated with reaction centers from *Rp. viridis* has been assigned an α peak at 553 nm ("cytochrome 553") in the literature [1—3], we found the peak to be at 552 nm. We assumed a differential extinction coefficient (reduced minus oxidized) of 20 $mM^{-1} \cdot cm^{-1}$ for each cytochrome at the peak of its α band, and showed this value to be correct for cytochrome 558 by measuring the reduction of cytochrome 558 coupled to the oxidation of equine cytochrome *c* (see later).

Molar extinction coefficients of reaction centers were determined by comparing light-induced absorbance changes at 960 nm (photochemical oxidation of bacteriochlorophyll *b*, "*P*-960") with those at 558 nm. The latter showed the oxidation of cytochrome 558, presumably coupled to the rereduction of *P*-960. The molar extinction coefficients then enabled us to estimate the concentration of reaction centers in our samples.

Fluorescence spectra were measured with a fluorometer described elsewhere [7]. Light-induced absorbance changes were measured kinetically with a split-beam absorption spectrometer, using either a tungsten-iodine lamp or a Q-switched ruby laser for excitation [8].

Results and Discussion

Absorption and emission spectra

Absorption spectra of reaction centers from *Rp. viridis*, prepared as described here and suspended in 0.01 M Tris buffer with 0.1% LDAO, are shown in Fig. 1. The ordinate shows the absolute extinction coefficient as determined in this study (see later). The inset shows part of the absorption spectrum at 30 K; the peaks at 531 and 543 nm are probably due to the Q_x transitions of two molecules of bacteriopheophytin *b* in the reaction center. Note that the band of bacteriochlorophyll *b* near 600 nm is resolved at low temperature into a peak at 606.5 nm and a shoulder at 601 nm. In the near infrared the absorption spectrum at 30 K was similar to that reported by Trosper et al. [3], with principal maxima at 833 and 1000 nm, lesser ones at 790 and 808 nm, and a poorly resolved shoulder near 850 nm.

The light-induced difference spectrum at room temperature showed a negative band centered at 615 nm, where the absorption spectrum (Fig. 1) shows a weak shoulder *. In the near infrared the light-induced difference spectrum at room temperature showed positive maxima at 807 and 823 nm and negative ones at 834 and 965 nm.

Room temperature fluorescence spectra, with excitation at 600 nm, showed a maximum at 1000 nm well resolved from fluorescence components below 800 nm. There was no indication of fluorescence corresponding to the 830 nm absorption band, in samples with or without added dithionite.

* We could not obtain reliable light-induced differences spectra showing oxidation of bacteriochlorophyll *b* at low temperature, using the Cary spectrophotometer with cross-illumination, probably because of the rapidity of the back-reaction of the primary photoproducts.

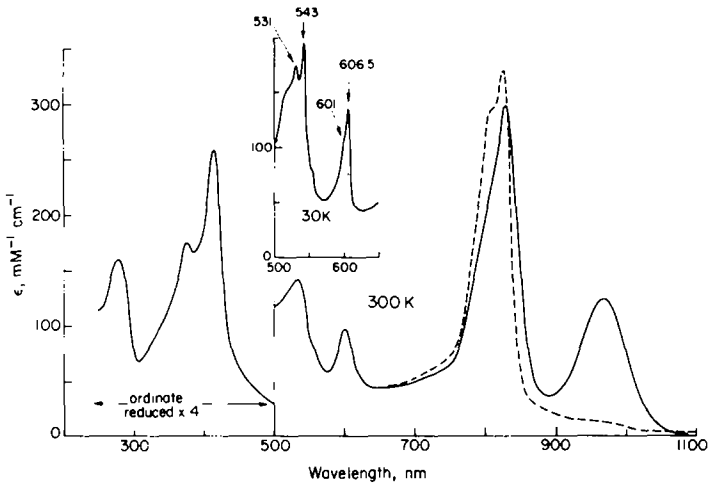

Fig. 1. Absorption spectra of reaction centers from *Rp. viridis*. Solid curves, sample in weak monochromatic light during measurement. Dashed curve, sample in strong white light (No. 2 IR Mode of the Cary 14R) during measurement. The ordinate is correct for wavelengths greater than 500 nm; it has been reduced 4-fold in the spectrum below 500 nm.

Cytochrome content

All of the cytochrome 552 and most of the cytochrome 558 was in its oxidized state in reaction centers as shown in Fig. 1. The small amount of reduced cytochrome 558 could be oxidized by illuminating or by adding potassium ferricyanide. Addition of sodium ascorbate reduced all of the cytochrome 558 and a little of the cytochrome 552, as indicated by the appearance of a peak centered at 557 nm in the absorption spectrum. All of this reduced cytochrome could then be oxidized by continuous illumination. A single laser flash caused the reversible oxidation of about half of the cytochrome 558 in reaction centers with ascorbate; the spectrum of the flash-induced change showed a negative peak centered at 558 nm. The cytochrome 552 could be reduced by adding sodium dithionite. Table I gives quantitative data on the cytochrome content of reaction centers as revealed by the difference spectra induced by adding ferricyanide, ascorbate or dithionite. We have made the following assumptions: The differential extinction coefficient (reduced minus oxidized) is $20 \pm 2\ mM^{-1} \cdot cm^{-1}$ for each cytochrome at its α peak (this was verified for cytochrome 558; see later). The extinction coefficient of reaction centers at 830 nm is $300 \pm 65\ mM^{-1} \cdot cm^{-1}$ (see later). Ferricyanide oxidizes all of the cytochrome, and relatively little of the cytochrome 552. In our calculations we made the approximation that ascorbate reduced none of the cytochrome 552; consequently we may have overestimated the content of cytochrome 558 and underestimated that of cytochrome 552 by as much as 15%. Aside from this reservation, and taking other experimental uncertainties into account, there were about two molecules of cytochrome 558 and three of cytochrome 552 associated with each reaction center. These values agree with those published by Trosper et al. [3]. Pucheu et al. [2] reported quite different ratios; about 1 cytochrome 558: 0.3 cytochrome 553. Pucheu et al. [2] found that most of the cytochrome 558 in

TABLE I

CYTOCHROME CONTENT OF REACTION CENTERS FROM *RHODOPSEUDOMONAS VIRIDIS*

Reaction center concentration was based on an extinction coefficient of 300 $mM^{-1} \cdot cm^{-1}$ at 830 nm; the differential extinction coefficient (reduced minus oxidized) for each cytochrome at its α peak was taken to be 20 $mM^{-1} \cdot cm^{-1}$ (see the text). We assumed that ferricyanide oxidized all of the cytochrome, ascorbate reduced all the cytochrome 558 and none of the cytochrome 552, and dithionite reduced all of the cytochrome. This assumption is discussed in the text. The confidence limits reflect probable degrees of uncertainty in the extinction coefficients; spectrophotometric accuracy was better than ±0.001 absorbance. Reaction center sample in 0.01 M Tris buffer, with or without solid reagents (about 1 mM).

	Preparation No. 1	Preparation No. 2	Mean
a_{830nm}	0.33	0.285	
Reaction center concentration (μM)	1.1	0.95	
Δa_{558nm} (untreated versus ferricyanide)	0.008	0.003	
Concentration of reduced cytochrome 558 (μM)	0.4	0.15	
Δa_{558nm} (ascorbate versus ferricyanide)	0.046	0.034	
Total cytochrome 558 concentration (μM)	2.3	1.7	
Δa_{552nm} (dithionite versus ascorbate)	0.065	0.054	
Total cytochrome 552 concentration (μM)	3.25	2.7	
Cytochrome 558 per reaction center	2.1	1.85	2.0 ± 0.4
Cytochrome 552 per reaction center	2.95	2.85	2.9 ± 0.7

their reaction center preparations was in its reduced state without the addition of reducing agents.

Determination of optical extinction coefficients

When reaction centers in 0.01 M Tris buffer with 0.1% LDAO were subjected to a saturating laser flash, the 960 nm absorption band became bleached and then recovered with half-time about 150 ms. This is shown in Fig. 2, trace a. Upon addition of 4 mM sodium ascorbate, this change (photo-oxidation of bacteriochlorophyll *b*) could not be seen within the resolving time, about 10 ms, of the instrument (trace b). Instead, a maximal light-induced oxidation of cytochrome 558 was evident (trace c). We assume that the oxidation of cytochrome 558 was coupled to the rapid re-reduction of oxidized bacteriochlorophyll *b*, with each reaction center capable of transferring a single electron from cytochrome 558 to oxidized bacteriochlorophyll *b* after a saturating flash. It is on this "one-electron" basis that we define the reaction center as a functional entity. The extinction coefficient of reaction centers at 960 nm could then be computed from the relative sizes of the absorbance changes at 960 nm (trace a) and 558 nm (trace b), taking the differential extinction coefficient for cytochrome 558 to be 20 $mM^{-1} \cdot cm^{-1}$ and noting that the oxidation of bacteriochlorophyll *b* in reaction centers causes a complete bleaching of the long wave band *. For this reason the absolute extinction coefficient ϵ_{960} of reaction centers in their dark resting state is equivalent to the differential (light-dark) coefficient $\Delta\epsilon_{960}$.

* In the example shown in Fig. 1 the long wave band was not entirely bleached by strong light (dashed curve), but in other measurements with ubiquinone-30 added to the reaction centers, it was.

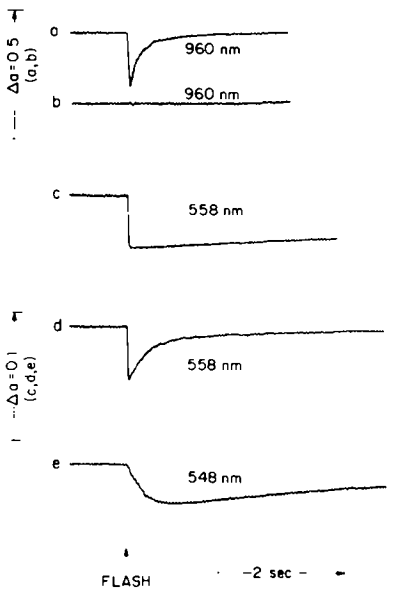

Fig. 2. Laser flash-induced absorbance changes in reaction centers from *Rp. viridis*. Reaction centers, $a_{830nm} = 0.57$, in 0.01 M Tris buffer with 0.1% lauryl dimethyl amine oxide and 20 μM ubiquinone-30. Traces b—e, 4 mM sodium ascorbate added. Traces d and e, 20 μM equine cytochrome *c* also added.

Two corrections were applied in this computation. First, in the reaction center sample without ascorbate, 9% of the cytochrome 558 was in its reduced state. We assume that 9% of the reaction centers, containing reduced cytochrome 558, could not show flash-induced bleaching of *P*-960 within the time resolution of the spectrometer. Second, the ratio of absorbance at 960 and 830 nm was 0.385 in the sample used for these measurements prior to the addition of ascorbate, whereas this ratio rose to 0.41 after ascorbate was added. We assume that in only 0.385/0.41 or 94% of the reaction centers, the *P*-960 was in its reduced state prior to the actinic flash. Combining these factors, we expect that the observed change at 960 nm (trace a) was less than the maximum change that could have occurred with all reaction centers participating, by a factor of (0.91)(0.94) = 0.86. We therefore multiplied the changes observed at 960 nm by 1/0.86 or 1.17 to give corrected values.

The spectrum of the flash-induced change with ascorbate showed a peak at 558 nm and no change at 548 nm; from this we infer that any flash-induced oxidation of cytochrome 552, present in small amounts in its reduced form, was negligible.

In addition to comparing amplitudes of flash-induced changes as in traces a and c of Fig. 2, we compared initial slopes of the same changes induced by continuous light of wavelength 830 nm. In these measurements we used actinic intensities of 0.3 and 0.6 mW/cm^2 and averaged the results. The data and results of these experiments, with both flashing and continuous light, are shown in Table II. The extinction coefficient of reaction centers at 960 nm was computed by

$$\epsilon_{960} = \Delta\epsilon_{558}(\Delta a_{960nm}/\Delta a_{558nm}) \times 1.17$$

TABLE II

COMPARISON OF LIGHT-INDUCED ABSORBANCE CHANGES AT 960 nm (PHOTOOXIDATION OF BACTERIOCHLOROPHYLL *b*) AND 558 nm (COUPLED OXIDATION OF CYTOCHROME 558) IN REACTION CENTERS FROM *Rp. VIRIDIS*

The ratios of the magnitudes of these absorbance changes are used to compute the extinction coefficients of the reaction centers. Reaction centers (a_{830nm} = 0.57) in 0.01 M Tris buffer with 0.1% lauryl dimethyl amine oxide and 20 μM ubiquinone-30, with or without 4 mM sodium ascorbate. Continuous actinic illumination was at 830 nm; 0.3 mW/cm^2 (A) and 0.6 mW/cm^2 (B).

	Continuous actinic light: Initial slope, da/dt, arbitrary units		Corrected * $(da/dt)_{960}$	Corrected $\frac{(da/dt)_{960}}{(da/dt)_{558}}$	ϵ_{960} (mM^{-1} · cm^{-1}) (assuming ϵ_{558} = 20)	ϵ_{830} = $\epsilon_{960}/0.41$
	960 nm, without ascorbate	558 nm, with ascorbate				
A	6.5	1.56				
	5.6	1.46				
	5.7	1.16				
	6.4	1.38				
		1.08				
		1.46				
		1.44				
Mean						
	6.05	1.36	7.1	5.2		
B	12.4	2.6				
	11.9	2.55				
	11.9	2.55				
	12.5	2.15				
		2.5				
		2.5				
		2.45				
Mean						
	12.2	2.47	14.3	5.8		
	Mean of A and B			5.5	110 ± 25	270 ± 40
	Laser flashes: Δa_{960nm} without ascorbate	Δa_{558nm} with ascorbate	Corrected * Δa_{960nm}	Corrected $\frac{\Delta a_{960nm}}{\Delta a_{558nm}}$	ϵ_{960} (mM^{-1} · cm^{-1})	ϵ_{830}, (mM^{-1} · cm^{-1})
	0.21	0.037				
	0.24	0.034				
	0.20	0.035				
	0.18	0.038				
Mean						
	0.21	0.036	0.245	6.8	136 ± 20	330 ± 50
	Mean of "continuous" and "flash" experiments				123 ± 25	300 ± 65

* Multiplied by 1.17; see the text.

the 1.17 being the aforementioned correction factor and $\Delta\epsilon_{558}$ taken as 20 mM^{-1} · cm^{-1}. Giving equal weight to the measurements with flashes and with continuous light, we found

ϵ_{960} = 123 ± 25 mM^{-1} · cm^{-1}

and

$\epsilon_{830} = \epsilon_{960}/0.41 = 300 \pm 65\ \mathrm{mM^{-1} \cdot cm^{-1}}$

Our confidence limits are based conservatively on the average deviations in the measurements.

When the reaction center sample of Fig. 2, with 4 mM sodium ascorbate, was treated with 20 μM equine cytochrome *c*, the photochemically oxidized cytochrome 558 mediated in turn the oxidation of the cytochrome *c*. This is illustrated by traces d and e. In trace d (in contrast to trace c) we see that the oxidized cytochrome 558 returned rapidly to its reduced form after a flash. Concomitantly the cytochrome *c* became oxidized, as indicated by the change at 548 nm shown in trace e. The oxidized cytochrome *c* was then re-reduced slowly, presumably by the ascorbate. The absorbance change for oxidation of cytochrome *c* was maximal at 550 nm, but at 548 nm it was not contaminated by changes due to cytochrome 558. Assuming perfect coupling between the re-reduction of cytochrome 558 and the oxidation of cytochrome *c*, we could estimate the differential extinction coefficient of cytochrome 558 by comparing the absorbance changes and taking $\Delta\epsilon$(cytochrome *c*, 550 nm, reduced minus oxidized) equal to 19.6 $\mathrm{mM^{-1} \cdot cm^{-1}}$ [9]. We confined our comparisons to the first 0.4 s after a flash, to minimize errors due to the slow re-reduction of oxidized cytochrome *c*.

Although the initial flash-induced absorbance change at 558 nm was probably due solely to oxidation of cytochrome 558, it was evident that the oxidation of cytochrome *c* made a contribution at this wavelength at later times: The trace (d) did not return to its original baseline over a period of 5 s. The early part of the decay curve at 558 nm after a flash was therefore a composite of the decay of oxidized cytochrome 558 and the growing-in of oxidized cytochrome *c*, the latter a small but not negligible contribution. We proceeded as follows: At 0.4 s after a flash, the decay at 558 nm was 68% complete with reference to the post-flash baseline. We took this to represent a 68% completion of the reaction between oxidized cytochrome 558 and reduced cytochrome *c*. The change at 548 nm, at 0.4 s after a flash, was then 68% of the maximum change that would have been expressed if the oxidized cytochrome *c* has not begun to react slowly with ascorbate. Accordingly we divided the 548 nm change at 0.4 s by 0.68, and compared that result with the initial change at 558 nm. We shall illustrate this with the example shown in Fig. 2:

$\Delta A_{548\,\mathrm{nm}}$ (0.4 s after flash) = 0.027.

$\Delta A_{548\,\mathrm{nm}}$ corresponding to complete reaction of cytochrome *c* with cytochrome 558 = 0.027/0.68 = 0.040.

Initial $\Delta a_{558\,\mathrm{nm}}$ (oxidation of cytochrome 558) = 0.041.

$$\frac{\Delta A_{558\,\mathrm{nm}}\ (\text{cytochrome 558 oxidation})}{\text{adjusted } \Delta A_{548\,\mathrm{nm}}\ (\text{equivalent cytochrome } c \text{ oxidation})} = 0.041/0.040 = 1.02.$$

The average of seven such measurements gave this ratio to be 1.05 ± 0.1. Taking $\Delta\epsilon$(cytochrome *c*, reduced minus oxidized) to be 19.6 $\mathrm{mM^{-1} \cdot cm^{-1}}$ at 550 nm

[9], the value at 548 nm is 18.9 $mM^{-1} \cdot cm^{-1}$. Then for cytochrome 558, $\Delta\epsilon$(reduced minus oxidized, 558 nm) = 18.9(1.05 ± 0.1) = 20 ± 2 $mM^{-1} \cdot cm^{-1}$.

Molecular weight and protein components

For the reaction center sample shown in Fig. 1, the ratio of absorbance at 280 nm to that at 830 nm was 2.1; the lowest value reported previously has been 2.5 [2]. We found 190 kg protein per mol of reaction centers for this sample, the concentration of reaction centers being based on our newly determined extinction coefficient.

A polyacrylamide gel electrophoretogram for the same material is shown in Fig. 3; trace 1 shows two major components a and c and a shoulder b. Extraction of the sample with acetone prior to digestion for electrophoresis did not change these results. In trace 2 the sample contained reaction centers from *Rp. sphaeroides*, giving the polypeptides H, M and L as markers. Taking the molecular weights of H, M and L to be 27 500, 22 500 and 20 000 [6], the apparent molecular weights of components a, b and c are 41 000, 37 000 and 31 000, respectively. Pucheu et al. [2] reported bands of apparent molecular weights 45 000, 37 000, 29 000 and 23 000. We do not know whether any of the bands a, b and c should be identified with cytochrome 558 or cytochrome 552. If either of these cytochromes has a molecular weight as low as 12 000, it would comprise no more than about 20% of the total protein, and need not appear as a striking feature in the electrophoretogram. We note also, from the rise of the trace near the origin, that some stainable material remained there (these gels were stained with Coomassie Brilliant Blue). At any rate it is clear that the polypeptide composition of reaction centers from *Rp. viridis* is distinct from the familiar pattern, seen in reaction centers from *Rp. sphaeroides*, *Rhodospirillum rubrum* and *Chromatium vinosum*, of three polypeptides in the approximate range 20 000—30 000 [10]. It is also different from the pattern of two major components at 33 000 and 25 000 daltons found in reaction

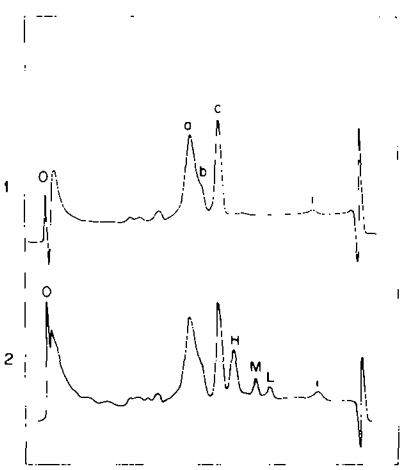

Fig. 3. Polyacrylamide gel electrophoretograms showing the polypeptide components of reaction centers from *Rp. viridis*. The origin of the gel is labeled o and the end (ion front) is labeled i. Trace 1, no additions. Trace 2, reaction centers from *Rp. sphaeroides*, giving components H, M and L, added as marker.

centers from *Rp. gelatinosa* [6]. Thus the various species described as *Rhodopseudomonas* show considerable diversity in the polypeptide composition of their reaction centers.

Acknowledgements

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