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PROPERTIES OF REACTION CENTERS OF *RHODOPSEUDOMONAS SPHAEROIDES* IN DRIED GELATIN FILMS

LINEAR DICHROISM AND LOW TEMPERATURE SPECTRA

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Summary

Methods of preparing dried gelatin films containing purified reaction centers of *Rhodopseudomonas sphaeroides* are described. The spectral properties of reaction centers in solution are essentially maintained in dried gelatin films. These films are uniform and have excellent optical properties, showing little particulate scattering at temperatures down to about 4K. Film contraction on cooling to 90K is less than 1% in linear dimension. Linear dichroism spectra are reported for films at room and low temperature. Reaction centers show a moderate amount of linear dichroism in unstretched gelatin films; the magnitude of the linear dichroism becomes much greater when the films are stretched. In stretched films, linear dichroic ratios ($A_{\parallel}/A_{\perp}$; absorbance measured with electric vector parallel and perpendicular to stretching direction) between 1.7 and 2.2 were obtained for the 860 nm absorption band of the bacteriochlorophyll component that undergoes primary photooxidation. The relative polarizations of light-induced absorption changes of reaction centers in stretched films are similar to those reported by Vermeglio and Clayton ((1976) Biochim. Biophys. Acta 449, 500—515) and support their hypothesis that absorbance decreases, maximal near 860 and 810 nm, and an increase near 790 nm are associated with the respective disappearance and appearance of discrete bands characteristic of the reduced and oxidized bacteriochlorophyll dimer. This interpretation is also supported by the polarization of the absolute absorption spectrum near 810 and 860 nm. An absorption band near 540 nm, ascribed to the Q_x transitions of two molecules of bacteriopheophytin in the reaction center, is split at low temperatures into two bands having similar polarizations. This splitting is probably not due to exciton coupling of the two molecules, since excition theory predicts different polarizations.

Abbreviation: LDAO, lauryl dimethyl amine oxide.

Introduction

The study of linear dichroism is a valuable tool in pursuit of structure vs. function relationships in molecular biology. Linear dichroism studies of reaction centers of *Rhodopseudomonas sphaeroides* should be particularly informative since these complexes consist of a small known number of pigment molecules bound to a protein moiety (four bacteriochlorophyll and two bacteriopheophytin molecules per reaction center) with sensible correlations between optical absorption bands and transition moments of the chromophores [1,2]. In addition, much is known about the primary photochemistry and electron transfer reactions of these complexes [3]. Maximum information concerning the orientation of these pigment molecules requires methods for preparing specimens of oriented reaction centers which are uniform, of a practical size, and devoid of major optical imperfections.

Previously, Penna et al. [4] were able to orient purified reaction centers of *Rp. sphaeroides* on a glass slide by gently brushing a micellar solution until dry. Unfortunately, the dichroic ratios of the absorption bands were extremely small and the sense of orientation (e.g. uniaxial or planar) and the angular relationships of the transition dipoles could not be determined. Vermeglio and Clayton [5] were able to orient reaction centers by drying chromatophores of *Rp. sphaeroides* in which antenna pigment was selectively bleached by oxidation with K_2IrCl_6. High dichroic ratios were observed and the orientation of different transition dipoles were related approximately to the chromatophore membrane plane. Their experiments concerning polarized light-induced absorbance changes also helped to elucidate the spectral properties of the bacteriochlorophyll "special pair" that acts as photochemical electron donor. Their measurements below 600 nm were marginal because the dried chromatophore films showed strong scattering of light.

In the present study, we were able to prepare specimens containing highly oriented reaction centers of *Rp. sphaeroides* by drying mixtures of detergent-solubilized reaction centers and gelatin so as to form dried films. Orientation of reaction centers in the film plane was induced by stretching the films *. For measurements of linear dichroism with unstretched gelatin films, the axis of the measuring beam made an angle of 60° with a line normal to the plane of the film. The electric vector was either parallel to the film or made an angle of 60° with the plane of the film. With stretched films the axis of the measuring beam was normal to the film and the electric vector was either perpendicular or parallel to the direction of stretching. Our use of gelatin films to orient reaction centers of photosynthetic bacteria was prompted by a publication of Wright et al. [6] in which micellar vertebrate rhodopsin was found to be oriented in unstretched gelatin films.

Materials and Methods

Preparation of reaction centers. Carotenoidless mutant strain R-26 of *Rp. sphaeroides* was grown according to the method of Clayton [7]. Reaction

* We are highly indebted to C.S. Schenck and W.W. Parson of the University of Washington for suggesting stretching as a way to improve the orientation, based on their experience with stretched polyvinyl alcohol films containing reaction centers.

centers were then isolated as described by Clayton and Wang [8]. Reaction centers were stored in a solution consisting of 0.3% (v/v) LDAO (lauryl dimethyl amine oxide; Onyx Chemical Co., Jersey City, N.J.) and 0.01 M Tris · HCl, pH 7.5, and were kept at −20°C until use.

Preparation of gelatin films. A 10% (w/v) solution of calf skin gelatin (Eastman), isoelectric point = 4.8, was softened for about 15 min at 45°C. A mixture was then made at room temperature, with a total volume of 2.0 ml, consisting of 3.0% (w/v) gelatin, 0.15% (v/v) LDAO, 0.005—0.01 M Tris · HCl, pH 7.5, and 2.7—27 μM reaction centers (based on $\epsilon_{802nm} = 2.88 \cdot 10^5\ M^{-1} \cdot cm^{-1}$ [1]). This mixture was pipetted onto the surface of a rectangular glass microscope slide (2.5 × 7.5 cm), the edges of which were coated with paraffin. The available surface area of the slide was approx. 17 cm^2. The solution was drawn with the tip of a pipette to uniformly cover the slide within the paraffin barrier. A glass funnel, inverted over the slide, provided a space which could be purged with a stream of dry nitrogen to hasten drying of the film. Ambient temperature was near 21°C. Within 15 min of deposition a fairly rigid gel was formed. After about 4 h, depending on rate of nitrogen flow, the dried film separated itself from the slide surface. In a dry nitrogen environment the film was somewhat brittle, so before handling it was allowed to hydrate for several minutes in room air with a relative humidity of 40—60%. In the course of drying, the film thickness decreased about 30-fold and was 0.03—0.05 mm in the completely dried film. Reference films were prepared in an identical way, excluding reaction centers from the original mixture. Films were normally stored in sealed bottles at −20°C until use and were stable for weeks under these conditions.

In the course of developing this recipe for dried gelatin films, we investigated the influence of pH and of the detergent/gelatin ratio of the original mixture on the optical properties of the films. The pH of the gelatin/LDAO/Tris · HCl reaction center mixture was approx. 5.0 and was determined primarily by the gelatin. (As supplied by Eastman, calf skin gelatin in 10% aqueous solution has a pH of 4.8.) On addition of reaction centers to the gelatin/LDAO/Tris · HCl solution, the reaction centers precipitated. However, the reaction centers became soluble again while the film was drying, and the final film was quite transparent with no detectable scattering. The pH of the starting mixture could be adjusted with HCl to as low as 3.5 and clear films were still produced. However, decomposition of the reaction centers was observed at pH values below 4.5. If the pH of the gelatin/LDAO/Tris · HCl solution was adjusted to values greater than 5.5—6.0, the reaction centers remained soluble when added. However, reaction centers then precipitated on drying and the final film was opaque. Opaque films were also produced if the detergent/gelatin ratio of the original mixture was increased more than 3-fold. The detergent/gelatin ratio could be decreased to a residual level set by the LDAO concentration of the reaction center preparation and clear films were still produced. Thicker dried films can be made if more gelatin is added to the starting mixture, but we have found no distinct advantage in using thicker films. We observed that the degree of orientation of reaction centers in stretched or unstretched films as measured by the dichroic ratios of different absorption bands was essentially independent of the pH and detergent/gelatin ratio as long as the film was optically clear. If

reaction centers were precipitated in the dried film, then little or no orientation was observed.

Method of stretching films. A film with dimensions of 2.0 × 2.0 cm was cut from a previously prepared dried film and two opposite edges firmly clamped. The film was then suspended in a chamber in which the air was saturated with water vapor at 25—30°C. After 30—60 min, the film became soft and pliable. The film was then removed to the room environment (21°C; relative humidity of air, 40—60%), each of the clamps grasped by opposite hands, and the film immediately stretched 2.5—3 times its original length. While tension was still applied to the film, a stream of room air (blown by a fan) was directed onto the surface of the film. In 1—2 min, the film once again became rigid and the clamps were removed. The stretched film could then be handled and stored in the same way as an unstretched film without detectable loss of induced orientation of reaction centers. The length of the hydration period and the temperature of the water vapor-saturated environment must be reproduced fairly closely for successful stretching. Otherwise, the film is either too brittle or too soft and is easily broken in the stretching attempt.

Measurement of absorption and linear dichroism. Techniques of data acquisition, data manipulation, and photoexcitation of samples are described by Vermeglio and Clayton [5]. In measureing the polarization absorption spectra we used a modified arrangement of optical components which, in sequence, was: beam depolarizer, sample, Glan-Thompson crystal polarizer, phototube. Films were taped to a brass plate in which a window had been cut; the plate could be mounted in the sample space at any desired angle relative to the measuring beam. For measurements at low temperature [9] the film was sandwiched between two glass plates which were then mounted in the sample holder of the cryostat.

Results

Unpolarized absorption

Absorption spectra of an unstretched gelatin film containing reaction centers, at 300 and 87 K, are shown in Fig. 1. The measuring beam was unpolarized and its axis was normal to the plane of the film. At 300 K, these films have excellent optical properties with no obvious scattering; they resemble Kodak-Wratten optical filters. The absorbance is uniform over various parts of the film to within a few percent, and the absorption spectra of reaction centers in solution are retained in the films with little change. The only substantial difference is that the band near 860 nm in solution is shifted in the films to 850 nm, as it is in solution at high concentrations of LDAO or low pH.

On lowering the temperature to about 90 K, the gelatin-reaction center films retain their excellent optical properties and show changes in their absorption spectra similar to the changes seen in liquid samples upon cooling; principally a bathochromic shift of the long wave band and a splitting of the band near 535 nm (Q_x transition of bacteriopheophytin) into two bands (see Fig. 1). Care must be taken to cool the film to about 200 K before evacuating the sample chamber of the cryostat; otherwise dehydration of the film may cause it to become brittle and to craze on further cooling. By marking the glass plates between which the film was sandwiched, we established that the contraction of

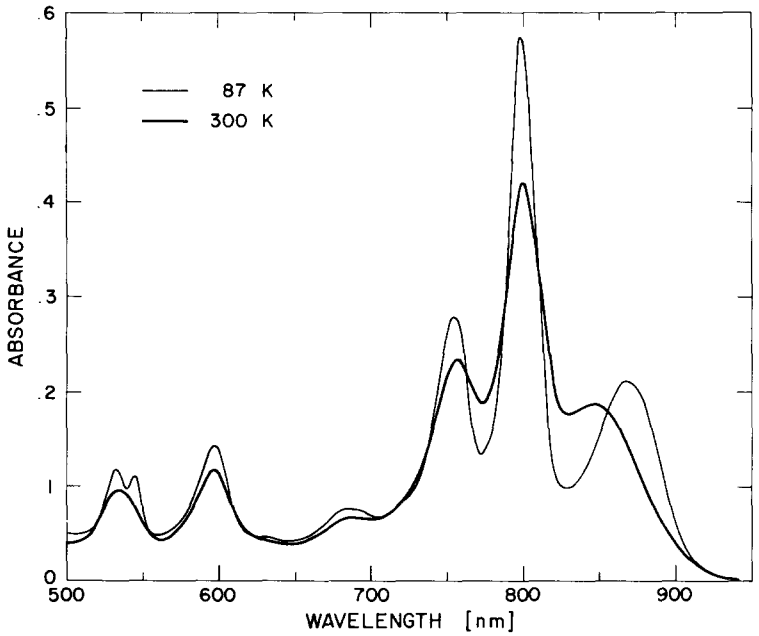

Fig. 1. Absorption spectra of reaction centers from *Rp. sphaeroides* in a dried unstretched gelatin film. The measuring beam was unpolarized and was incident normal to the film surface.

the film on cooling was no greater (within 1% in linear dimension) than that of the glass. Gelatin films were also immersed directly in liquid helium. Even with this extreme treatment, the films remained intact and optically clear.

Polarized absorption and light-induced absorbance changes in stretched films

Absorption spectra of reaction centers in stretched gelatin films at 300K are shown in Fig. 2. The measuring beam was normal to the plane of the film; the two spectra are for the electric vector parallel ($A_{\parallel}$) and perpendicular ($A_{\perp}$) to the direction of stretching. The difference, $A_{\parallel} - A_{\perp}$, is shown as the linear dichroism spectrum in Fig. 3 for spectra measured at 300 K (corresponding to Fig. 2) and at 90 K. Dichroic ratios at the extrema seen in Fig. 3 are listed in Table I. The magnitudes of these ratios differed from film to film, with the value near 850 nm (300 K) ranging from about 1.7 to 2.2 for films stretched 2.5—3-fold, but the shapes of the spectra were constant.

Light-induced absorbance changes with unpolarized measuring light were similar to those seen in solution; the main features corresponded to the photo-oxidation of bacteriochlorophyll in the reaction centers. The maximum bleaching of the long wave band with saturating actinic light was about 70% of that seen in solution (compare Straley et al. [1]), suggesting that about 30% of the reaction centers were photochemically inactive in the films.

For measurements of light-induced changes with polarized light the film was set with its plane 45° from the axes of both the unpolarized actinic beam and the polarized measuring beam, with the measuring beam normal to the axis of stretching in the film. The electric vector of the measuring beam was then either parallel ($\Delta A_{\parallel}$) or perpendicular ($\Delta A_{\perp}$) to the stretch axis, but in the

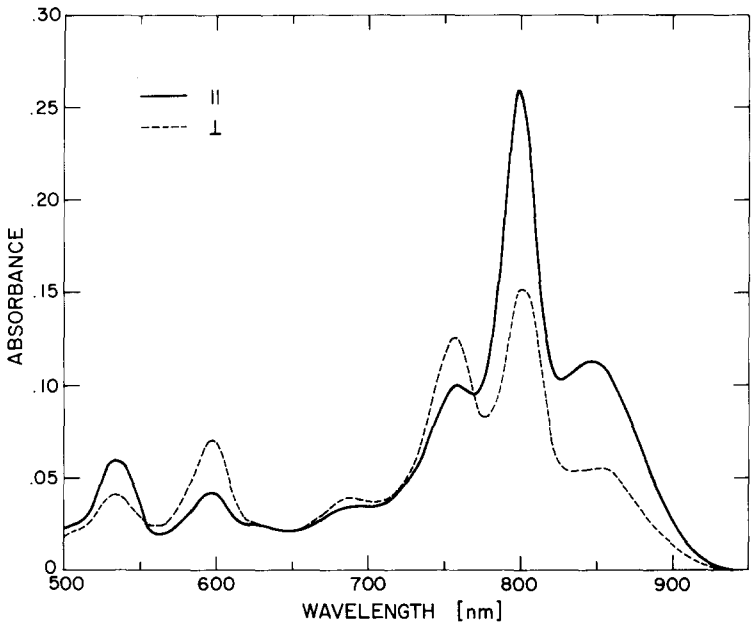

Fig. 2. Polarized absorption of reaction centers from *Rp. sphaeroides* in a dried stretched gelatin film. Absorption was measured with the electric vector of the measuring beam parallel (∥) and perpendicular (⊥) to the stretching direction. The measuring beam was incident normal to the film plane.

latter case the electric vector made an angle of 45° with the plane of the film. The spectra of light-induced changes measured in this way at 300 K are shown in Fig. 4; dichroic ratios * ($\Delta A_{\parallel}/\Delta A_{\perp}$) at extrema in the linear dichroism spectrum ($\Delta A_{\parallel} - \Delta A_{\perp}$ versus wavelength) are listed in Table II.

Polarized absorption of unstretched films

When the film was positioned so that the polarized measuring beam was incident normal to the film surface, no difference in absorption was observed as the polarizing crystal was turned through 90°, indicating no orientation of reaction centers within the plane of the film. However, if the film was rotated around some axis which lay within the plane of the film but was normal to the direction of the measuring beam, then a difference in absorption was observed for light polarized parallel to the axis, $A_{\parallel}(\theta)$, and light polarized perpendicular to it, $A_{\perp}(\theta)$. The angle of rotation of the film about this axis is denoted θ and is equal to the angle between a line drawn normal to the film surface and the direction of the measuring beam. $A_{\parallel}(0 \leqslant \theta \leqslant 90°)$ is then a measure of the transition dipole moment vectors projected onto the plane of the film. At angles greater than 0°, $A_{\perp}(\theta)$ is a measure of both in-plane and out-of-plane projections [11,12]. The linear dichroism spectrum of reaction centers in unstretched gelatin films, $A_{\parallel}(60) - A_{\perp}(60)$, is qualitatively similar to the linear dichroism

* The dichroic ratio of the 850 nm band determined from polarized absorption spectra of the same film with reaction centers in the reduced state (dark) was 1.73. The difference between this value and the one obtained from the light-dark difference spectra (1.56) may arise for several reasons. One reason is that cross-illumination, even with unpolarized light, constitutes a photoselection experiment [10].

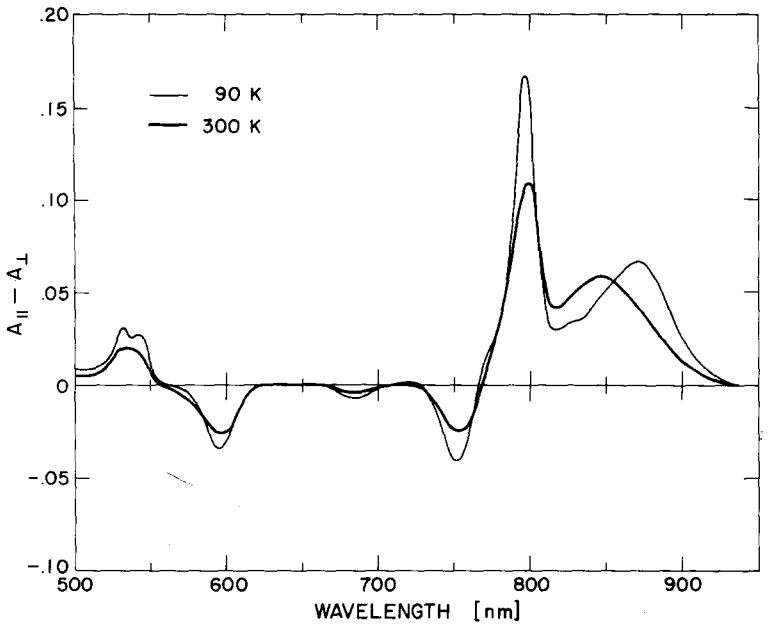

Fig. 3. Linear dichroism spectra derived from the spectra of Fig. 2 and from similar spectra at low temperature.

spectrum in stretched gelatin films (Fig. 3), but the magnitudes of the dichroic ratios of the different bands are substantially less (e.g. $A_{\parallel}(60)/A_{\perp}(60)$ 850 nm band, = 1.14).

Determination of refractive index

The refractive index (n) of an unstretched gelatin film containing reaction

TABLE I

DICHROIC RATIOS FOR THE POLARIZED SPECTRA OF REACTION CENTERS IN STRETCHED GELATIN FILMS

The ratios are listed at the extrema shown in the linear dichroism spectra of Fig. 3.

Temperature (K)	Wavelength (nm)	Dichroic ratio ($A_{\parallel}/A_{\perp}$)
300	535	1.46
	597	0.59
	685	0.87
	754	0.79
	799	1.72
	848	2.08
90	532	1.62
	543	1.63
	595	0.61
	686	0.90
	752	0.74
	798	1.86
	873	2.14

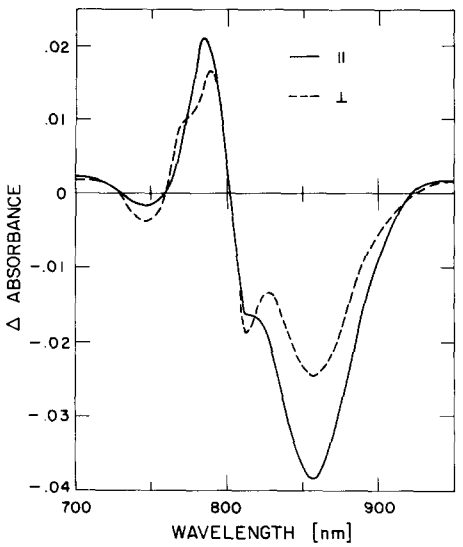

Fig. 4. Light minus dark difference spectra of reaction centers from *Rp. sphaeroides* in a dried stretched gelatin film. Difference spectra were measured with the electric vector of the measuring beam parallel (∥) and perpendicular (⊥) to the stretching direction. The film was positioned so that the measuring beam was incident normal to the stretching direction but at 45° to a line drawn perpendicular to the film plane. The intensity of the exciting light resulted in 70% saturation of the photochemistry in the steady state. The sample temperature was 300 K. $A_{\parallel}/A_{\perp}$ (850 nm, dark spectra) = 1.73.

centers was determined by use of Snell's law, $n = \sin\theta/\sin\phi$, where θ and ϕ are the angles of incidence and refraction for the measuring beam entering the film, respectively. The refracted angle ϕ was determined for several incident angles θ in the following way. $\cos\phi = L(0)/L(\theta)$, where $L(\theta)$ is the path length of the beam in the film. Since path length is proportional to the absorbance of a pigment uniformly distributed in the film, $\cos\phi = A_{\parallel}(0)/A_{\parallel}(\theta)$. The absorbance of an unstretched film containing reaction centers was measured at 800 nm for $\theta = 0°, 20°, 40°, 60°$, and 70°. (Since $A_{\parallel}(\theta)$ is a measure only of the in-plane projections of the transition dipoles contributing to the 800 nm band, $A_{\parallel}(\theta)$ depends only on path length.) The mean calculated value of refractive index was 1.43 ± 0.04 (95% confidence limits). From this value of the refractive index, Snell's law gives the angle of the measuring beam as it

TABLE II

DICHROIC RATIOS FOR THE POLARIZED SPECTRA OF LIGHT-INDUCED ABSORBANCE CHANGES

The ratios are listed at extrema in the difference between the two spectra shown in Fig. 4.

Wavelength (nm)	Dichroic ratio ($\Delta A_{\parallel}/\Delta A_{\perp}$)
746	0.45
≈764	≈0.5
784	1.46
812	0.86
858	1.56

traverses the gelatin film for any incident angle. In the case of the linear dichroism measurements on unstretched gelatin films, $\phi = 37.3° \pm 1.2°$ (95% confidence limits) when $\theta = 60°$.

Effect of hydration on the orientation of reaction centers

The linear dichroism spectra of both stretched and unstretched films containing reaction centers were determined for films mounted in air with normal room humidity (40—60%) and for films mounted in air saturated with water vapor. Hydration had no significant effect on the linear dichroism, $A_{\parallel}(60) - A_{\perp}(60)$, of an unstretched film. This shows that hydrated films, which were the starting material for stretched films, had the same orientation as dried films. Upon hydration of stretched films, the magnitude of the linear dichroism, $A_{\parallel} - A_{\perp}$, decreased; after 1 h, it was approx. 70% of the initial value. Redrying the film did not restore the original dichroism, indicating some permanent relaxation of in-plane asymmetry of the film.

Discussion

The linear dichroism spectra measured here are similar to those reported by Penna et al. [4] for reaction centers oriented by the brushing technique of Breton et al. [12] and to those obtained by Vermeglio and Clayton [5] using dried films of chromatophores with antenna pigments bleached. The dichroic ratios are similar to those observed by Vermeglio and Clayton [5] and about 100 times greater than those of Penna et al. [4]. In contrast to the report by Penna et al. [4], but in agreement with Vermeglio and Clayton [5], we observed no splitting of the long wave (850—870 nm) band in the linear dichroism spectrum. Our measurements indicate that this band corresponds to a single transition dipole, which could nevertheless be one member of a set of transitions arising from exciton coupling of two or more chromophores.

Our linear dichroism spectra and dichroic ratios show a minimum near 812 nm, at the long wave edge of the 800 nm absorption band, suggesting a transition near 812 nm, polarized differently from both the 800 nm band and the long wave band in reduced reaction centers. The presence of such a transition is indicated also by circular dichroism spectra [13,14] and by first derivative spectra at low temperature [5] for reaction centers in solution. The polarizations near 812 and 860 nm are seen also for the disappearance of bands (negative ΔA) in spectra of light-induced changes, as reported also by Vermeglio and Clayton [5]. These observations are consistent with the interpretation that the bands near 812 and 860 nm are properties of the "special pair" of bacteriochlorophyll molecules that serve as photochemical electron donor, showing exciton splitting as described by the molecular exciton model of Kasha et al. [15] for strong transition dipole coupling between two identical chromophores. This model predicts two transitions, polarized orthogonally, whose difference in energy depends on the specific geometry of the dimer.

The light-induced absorbance increase near 785 nm is polarized differently from the decrease near 812 nm, supporting the conclusion of Vermeglio and Clayton [5] that these extrema are not due to the shift of a single band. When the bacteriochlorophyll dimer is photo-oxidized, losing a single electron, the

exciton coupling is abolished and the absorption properties acquire some aspects of monomeric bacteriochlorophyll, giving rise to the band near 785 nm.

The light-induced changes centered at 746 and 764 nm are similarly polarized, consistent with the conclusion of Vermeglio and Clayton [5] that an absorption band of bacteriopheophytin in this region shifts to greater wavelengths. Finally, the band near 535 nm, assigned to the Q_x transition moments of two molecules of bacteriopheophytin in the reaction center, is split at low temperature into two components having virtually identical dichroic ratios. This observation is inconsistent with, but does not exclude rigorously, the interpretation of Penna et al. [4] and Sauer et al. [13] that the splitting is due to exciton coupling, since the molecular exciton model predicts different polarizations of the two transition moments. It is conceivable that different orientations gave similar dichroic ratios in our preparation.

We have not attempted to quantify the angular relationships between the transition dipoles of reaction centers of *Rp. sphaeroides*. To do this requires a description of the restraints which the gelatin medium imposes on the orientation of reaction centers as well as some information about the extent of orientation. These questions will be the subject of a subsequent publication. In stretched films, preliminary evidence suggests that reaction centers are oriented as if occupying the lattice points of a crystal with uniaxial symmetry.

Acknowledgements

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References

1 Straley, S.C., Parson, W.W., Mauzerall, D.C. and Clayton, R.K. (1973) Biochim. Biophys. Acta 305, 597–609
2 Norris, J.R., Uphaus, R.A., Crespi, H.L. and Katz, J.J. (1971) Proc. Natl. Acad. Sci. U.S. 68, 625–628
3 Clayton, R.K. (1977) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), Plenum Publ. Corp., New York, in the press
4 Penna, F.J., Reed, D.W. and Ke, B. (1974) in Proc. 3rd Int. Congr. Photosyn. Res., Rehovot (Avron, M., ed.), Vol. 1, pp. 421–425
5 Vermeglio, A. and Clayton, R.K. (1976) Biochim. Biophys. Acta 449, 500–515
6 Wright, W.E., Brown, P.K. and Wald, G. (1972) J. Gen. Physiol. 59, 201–212
7 Clayton, R.K. (1960) Biochim. Biophys. Acta 37, 503–512
8 Clayton, R.K. and Wang, R.T. (1971) Methods Enzymol. 23, 696–704
9 Clayton, R.K. (1977) Photosynthetic Organelles Special Issue of Plant and Cell Physiol. pp. 87–96
10 Albrecht, A.C. (1961) J. Mol. Spectrosc. 6, 84–108
11 Morita, S. and Miyazaki, T. (1971) Biochim. Biophys. Acta 245, 151–159
12 Breton, J., Michel-Villaz, M. and Paillotin, G. (1973) Biochim. Biophys. Acta 314, 42–56
13 Sauer, K., Dratz, E.A. and Coyne, L. (1968) Proc. Natl. Acad. Sci. U.S. 61, 17–24
14 Reed, D.W. and Ke, B. (1973) J. Biol. Chem. 248, 3041–3045
15 Kasha, M., Rawls, H.R. and El-Bayoumi, M.A. (1965) Pure Appl. Chem. 11, 371–392