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EXCITED STATES OF PHOTOSYNTHETIC REACTION CENTERS AT LOW REDOX POTENTIALS

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SUMMARY

In preparations of photochemical reaction centers from *Rhodopseudomonas spheroides* R-26, lowering the redox potential so as to reduce the primary electron acceptor prevents the photochemical transfer of an electron from bacteriochlorophyll to the acceptor. Measuring absorbance changes under these conditions, we found that a 20-ns actinic flash converts the reaction center to a new state, P^F, which then decays with a half-time that is between 1 and 10 ns at 295 °K. At 25 °K, the decay half-time is approx. 20 ns. The quantum yield of state P^F appears to be near 1.0, both at 295 and at 15 °K. State P^F could be an intermediate in the photochemical electron-transfer reaction which occurs when the acceptor is in the oxidized form.

Following the decay of state P^F, we detected another state, P^R, with a decay half-time of 6 μs at 295 °K and 120 μs at 15 °K. The quantum yield of state P^R is approx. 0.1 at 295 °K, but rises to a value nearer 1.0 at 15 °K. The kinetics and quantum yields are consistent with the view that state P^R forms from P^F. State P^R seems likely to be a side-product, rather than an intermediate in the electron-transfer process.

The decay kinetics indicate that state P^F cannot be identical with the lowest excited singlet state of the reaction center. One of the two states, P^F or P^R, probably is the lowest excited triplet state of the reaction center, but it remains unclear which one.

INTRODUCTION

The primary photochemical reaction in bacterial photosynthesis is the transfer of an electron from bacteriochlorophyll to an acceptor whose identity remains controversial [1, 2]. In *Rhodopseudomonas spheroides*, the electron transfer occurs in a "reaction center" which is a pigment-protein complex containing four molecules of bacteriochlorophyll and two of bacteriopheophytin [3]. We shall use the symbol "P" to denote the ground electronic state of the set of pigment molecules that share in the donation of the electron, and "P^+" to denote the same set in its oxidized (radical cation) state. Following the excitation of the pigment complex to its lowest-lying singlet state (P*), the electron transfer occurs with high speed and with extraordinarily high yield (102±4 %) [4].

If one lowers the ambient redox potential of a reaction-center preparation so as to reduce the electron acceptor, the photochemical electron-transfer reaction cannot occur [1, 5]. P* must then decay to P by alternative paths. One mechanism for such a decay is fluorescence. The fluorescence yield at low redox potentials is three to five times that at higher potentials [6, 7], but this increase in fluorescence yield is surprisingly small. If the reduction of the electron acceptor changes only the rate constant for the reaction that leads to electron transfer, without altering the rate constants for fluorescence and other routes of decay, a drop in the yield of the photooxidation from 98 % to near zero would allow a 50-fold increase in fluorescence. This calculation assumes, however, that both fluorescence and the electron-transfer reaction occur directly from P*. If the electron-transfer reaction proceeds from a different state with lower energy, inhibition of the electron transfer might have only a minor effect on the amount of fluorescence that occurs from P*.

A triplet state of the reaction center could, in principle, play the role of intermediate between P* and electron transfer. Electron spin resonance (ESR) studies of reaction-center preparations have shown that flash excitation at low redox potentials results in the formation of a triplet state [8]. The quantum yield of the triplet has not been measured absolutely, because the ESR measurements require a cryogenic temperature (< 20 °K) which introduces experimental complications, but the yield of the triplet is the same as that for the photooxidation of P at this temperature [9].

An additional impetus for supposing that the photooxidation of the reaction center might proceed by way of a triplet state comes from model studies of chlorophylls and bacteriochlorophyll in solution. Flash excitation of chlorophyll *a* or *b* in organic solvents generates triplets in quantum yields of approx. 0.7 [10–12]. The triplet state of bacteriochlorophyll also forms readily although its quantum yield appears to be near 0.2 (ref. 13 and Connolly, J. S., personal communication). The bacteriochlorophyll triplet in pyridine lies at an energy of approx. 5500 cm^{-1} above the ground state. The chlorophylls and bacteriochlorophyll in their triplet states enter readily into electron-transfer reactions, reducing aromatic nitro compounds [13, 14], quinones [15], or $FeCl_3$ [16] at rates that can approach the limit of diffusion control.

This report describes a spectrophotometric study of short-lived states of the reaction center that appear upon flash excitation of reaction centers at low redox potentials. We have discovered two such states, which are kinetically and spectrally distinguishable. One of the two (P^F) has a high quantum yield and appears to be a precursor of the other (P^R). We conclude that P^F could be on the main photochemical pathway, but that P^R probably is not. The lifetime of P^F is too long for P^F to be identical with P*, but it is not clear whether P^F or P^R represents the triplet state of the reaction center.

MATERIALS AND METHODS

Reaction centers were isolated from the *Rps. spheroides* carotenoidless mutant R26 with the use of lauryldimethylamine oxide, as described by Clayton and Wang [17]. Concentrated solutions of reaction centers were dialyzed against 0.05 % Triton X-100, 50 mM Tris · Cl, pH 7.5, for 2 days to free them of the lauryldimethylamine oxide. For study, they were diluted with the same Triton/Tris solution, or with mixtures of this solution and glycerol (1 : 3, v/v). Bacteriochlorophyll was obtained from

wild type *Rps. spheroides* by extraction of lyophilized cells with 2 % methanol in petroleum ether. It was purified by chromatography on sugar, essentially as described by Strain and Svec [18].

Absorbance changes following flash excitation were measured using an apparatus which has been described previously [19], with the following modifications. For many of the experiments, the actinic flash source was a liquid dye laser, which was pumped axially by a Q-switched ruby laser. One window of the dye laser cavity had a dielectric coating with > 90 % reflectivity between 780 and 900 nm and > 90 % transmittance at 694 nm. The 694 nm ruby pulse entered the cavity through this window. The second (output) window had approx. 90 % reflectivity at 694 nm, and a reflectivity of about 2 % between 730 and 900 nm. A Corning 2600 filter removed residual ruby light from the dye laser beam. To measure the laser wavelength, we directed a portion of the beam from a ground-glass scatterer through a monochromator to an RCA C31034 photomultiplier. With $2 \cdot 10^{-5}$ M 3,3'-diethylthiatricarbocyanine iodide in dimethylsulfoxide as the dye, the emission maximum was 834 nm. With 3,3'-diethylthiadicarbocyanine iodide, it was 720 nm. The width of the flash at half-maximum amplitude was usually about 20 ns. Flash energies were measured with a ballistic thermopile.

For the fastest measurements, the measuring light source was a Xe flash lamp (United States Scientific Instruments, CP-2n), which discharged 13 J (26 μF, 1 kV) through a series inductance. The width of the flash at half-maximum amplitude was 20 μs. The measuring beam passed through a monochromator, the sample, a second monochromator and/or auxilliary filters, to an RCA C70114C photomultiplier. The photomultiplier anode current proceeded to a two-stage preamplifier. The first stage (Optical Electronics Inc., Model 9816) provided a current-to-voltage transimpedance of 1 KΩ. The second stage (National Semiconductor LH0063CK) was a current driver, allowing the use of 50 Ω terminated cable. The preamplifier signal continued to the 7A19 vertical amplifier of a Tektronix 7903 oscilloscope. The overall risetime of the detection circuitry was approximately 5 ns.

The measurements in the μs time range employed a continuous tungsten measuring light, and a preamplifier with narrower bandwidth but higher gain. For some of these measurements, the preamplifier signal was digitized and stored in a Biomation model 610 transient recorder, and then transferred to a home-built digital signal averager. When signal averaging was used, several minutes elapsed between individual measurements.

For the experiments at room temperature, the redox potential of the sample was controlled and measured with Pt and calomel electrodes in an anaerobic cuvette, as described elsewhere [20]. Two different cuvettes were used in the low-temperature experiments. Neither of them provided a measurement of the redox potential, but a sufficiently low potential was achieved by the addition of excess solid $Na_2S_2O_4$ shortly before the sample was cooled. One low-temperature cuvette was a sealed chamber with a 1-mm light path in an acrylic block. The acrylic block resided in a copper chamber which was screwed to the cold-tip of an Air Products Corp. cryogenic refrigerator. A thermocouple at the cold-tip provided a measure of the temperature. The temperature of the sample itself was somewhat above the temperature that the thermocouple sensed, because of radiation from the walls of the refrigerator shroud and imperfect heat sinking to the cold-tip. We attempted to minimize this error by

enclosing the sample chamber and the cold-tip in a copper heat-shield which was held at liquid N_2 temperature by an earlier stage of the refrigerator. The second low-temperature cuvette, which was used for measurements down to 77 °K, has been described previously [21]. In this cuvette, the thermocouple was immersed in the sample itself.

RESULTS

1. *General observations and difference spectra*

Fig. 1 shows records of the absorbance changes that occur when reaction-center preparations are illuminated with short flashes. For Trace A, the redox potential was poised at +250 mV, and the absorbance increase at 430 nm that persists after the flash is due to the photooxidation of the reaction center ($P \rightarrow P^+$). Figs 2A and 2B show the difference spectrum of the absorbance changes that accompany this reaction.

For Trace B in Fig. 1, the redox potential was poised at −165 mV. This reduces the primary electron acceptor, which has an apparent midpoint potential of −50 mV [5, 22]. At the time of the flash, there is an absorbance increase at 420 nm which is followed rapidly by a relaxation, returning the absorbance almost to the resting level. We shall call the state of the system that forms and decays most rapidly under these conditions "P^F", and the one that remains after the fast relaxation "P^R".

Difference spectra for the absorbance changes signalling formation of states P^F and P^R are given in Figs 2A, 2B, and 2D. The spectra for the two states are similar, but they differ significantly from each other, particularly around 420 and 540 nm, and both of them differ from the $P \rightarrow P^+$ spectrum. In the near infrared, the difference spectrum for formation of state P^R resembles that for formation of P^+ in its bleaching of the major absorption band at 870 nm. It differs, however, in showing only a slight bleaching in the 800-nm absorption band, in place of a blue shift. We could not determine the spectrum of state P^F in the infrared, because of fluorescence artifacts. The formation of either state P^F or state P^R causes a bleaching in the bacteriochlorophyll absorption band at 380 nm. The band at 600 nm also bleaches, but new, broad absorption bands develop in this region of the spectrum, causing the net absorbance

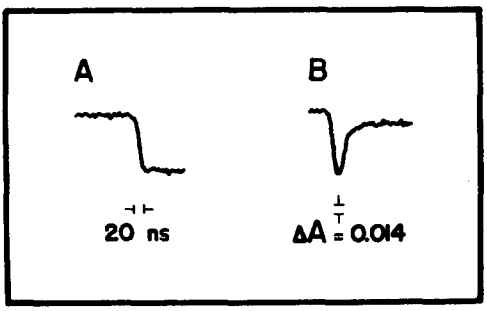


Fig. 1. Flash-induced absorbance changes in reaction centers. The abrupt downward deflections indicate absorbance increases caused by the 834-nm dye laser flashes, which were of saturating intensity. The traces are reproduced from photographs of the oscilloscope screen. (A) Reaction-center concentration, 5.84 μM; indigodisulfonate, 50 μM; Triton X-100, 0.05 %; Tris · Cl, pH 7.7, 50 mM; E = approx. +250 mV; λ = 430 nm; light path, 1 cm. (B) Same sample, but E = −165 mV; λ = 420 nm.

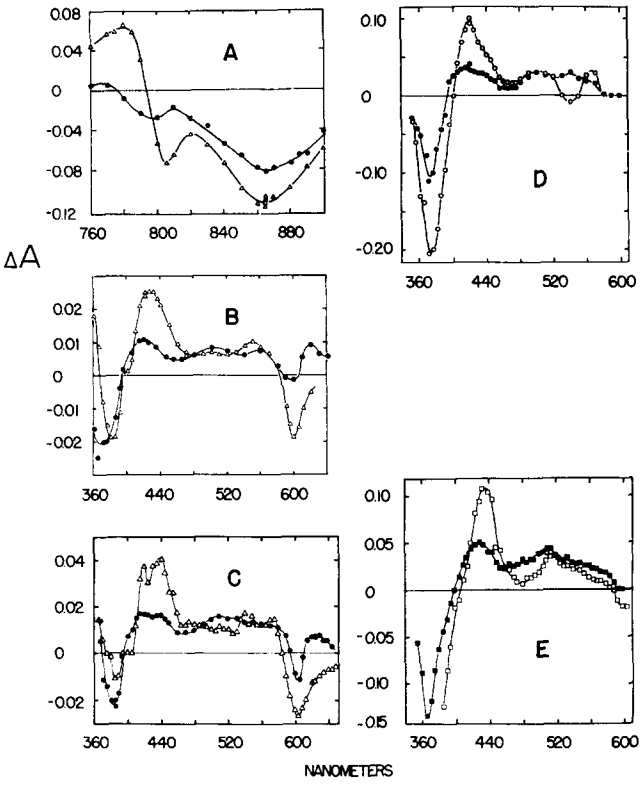


Fig. 2. (A) △—△, difference spectrum of near-infrared absorbance changes accompanying photooxidation of P to P^+ at room temperature. E = approx. +250 mV. Individual measurements were similar to those of Fig. 1A, except that the preamplifier signals were digitized and stored in a Biomation model 802 transient recorder, operating with a 2-ms full sweep. The absorbance changes have been normalized to $\Delta A_{865\ nm} = -0.112$, which is the expected ΔA for complete photooxidation of 1 μM P [3]; ●—●, similar spectrum for conversion of P to P^R. Conditions similar to those of Fig. 1B, except that 50 μM anthraquinone sulfonate also was present; E = −250 ± 30 mV. Preamplifier signals were captured in a Biomation model 610, operating with a 20-μs sweep, and then transferred to a computer of average transients. An average of ten measurements was collected. To avoid artifacts due to fluorescence and the laser flash itself, the measurements were made with comparatively weak (13 % saturating) ruby laser flashes (694 nm). The absorbance changes that would result from saturating flashes then were calculated from a comparison of the effects of saturating and attenuated flashes, measured at 420 nm, where artifacts were negligible. The data also are normalized to 1-μM reaction centers from an actual concentration of 0.4 μM. (B) Difference spectra of visible absorbance changes for $P \rightarrow P^+$ (△—△) and $P \rightarrow P^R$ (●—●) at room temperature. Conditions and measurement techniques as in corresponding experiments of A. All data are normalized to 1-μM reaction centers and saturating flashes. (C) Difference spectra of absorbance changes for $P \rightarrow P^+$ at 77 °K (△—△) and $P \rightarrow P^R$ at approx. 15 °K (●—●). Reaction centers, 14.5 and 19.4 μM, respectively. The data are not normalized to 1 μM. They show the results of measurements made with saturating 834-nm dye laser flashes, as recorded on the oscilloscope without signal averaging. For the P^R spectrum, E was lowered by the addition of excess $Na_2S_2O_4$ before cooling. The buffer contained 67 % glycerol; 33 % 50 mM Tris · Cl, pH 7.6; 0.017 % Triton X-100. (D) Difference spectra of absorbance changes for $P \rightarrow P^F$ (○—○) and $P \rightarrow P^R$ (●—●) at room temperature. Conditions and measurement techniques essentially as in Fig. 1B. 5.4 μM reaction centers (data not normalized); E = −200±25 mV. (E) Difference spectra of absorbance changes accompanying conversion of bacteriochlorophyll to its lowest-lying triplet state in pyridine (□—□) and in ethanol pyridine (7 : 2, v/v) (■—■). Bacteriochlorophyll, 18 μM; flash, 720-nm dye laser. The flashes were not saturating.

change at 600 nm to be very small. The photooxidation causes a pronounced absorbance decrease at this wavelength.

The spectra of both states P^F and P^R generally resemble spectra of the triplet state of bacteriochlorophyll in organic solvents, which include major new absorption bands near 420 and 510 nm (Fig. 2E and refs 13 and 23). The relative strengths of the 420 and 510 nm absorbance changes depend strongly on the solvent. The difference spectrum for the formation of state P^R is similar to that for the bacteriochlorophyll triplet in ethanol/pyridine (7 : 2, v/v); that of state P^F bears more resemblance to the triplet difference spectrum in pure pyridine. The influence of the solvent prevents the spectra from affording a decisive identification of either P^F or P^R with the triplet state of the reaction center. It is worth noting, however, that the absorbance decrease at 540 nm in the difference spectrum for P^F is not typical of the triplet bacteriochlorophyll spectra and may indicate the involvement of the bacteriopheophytin of the reaction center.

Both states P^F and P^R form upon flash excitation at very low temperatures, as well as at room temperature. Fig. 2C shows the difference spectrum for the P^R state at approx. 15 °K, along with a P^+ spectrum at 77 °K for comparison. The P^R spectrum at low temperature is similar to that at room temperature, but some of the bands are sharper, the 380-nm band is shifted to the red, and the 420-nm band is split.

The absorption spectrum for state P^F in Fig. 2D must contain a contribution from state P^R, with a magnitude that depends on how rapidly state P^R forms after the flash. Our working hypothesis (see Discussion) is that state P^R forms as state P^F decays. If this is correct, spectra measured at the earliest times probably give a fair picture of the true spectrum of state P^F. It is possible, however, that state P^R forms immediately during the flash, and that state P^F decays only to the ground state. In this case, the true spectrum of state P^F would be the difference between a spectrum measured at the earliest times and one measured at a later time.

Both the P^F and the P^R states can be observed at redox potentials as low as −450 mV, and the magnitudes of the absorbance changes that are associated with their formation and decay are independent of the redox potential between −100 and −450 mV. There was no indication of absorbance changes due to the P^F or P^R states when reaction-center preparations were exposed to flashes at redox potentials that were high enough to oxidize P to P^+ in the dark ($\geqslant$ 550 mV).

2. *Quantum yields*

Fig. 3B shows the extent of the absorbance changes due to the formation of state P^R, as a function of the strength of the actinic flash. For comparison, Fig. 3A shows a similar plot of the $P \rightarrow P^+$ absorbance changes, as measured at a higher redox potential. Using the known [3] extinction coefficients for the P and P^+ states, the initial slope of the latter plot represents a quantum yield near 1.0.

With saturating flashes, the maximal absorbance change at 425 nm due to the formation of state P^R is about 9 % of the maximal change at 865 nm due to the formation of P^+ (compare Figs 3A and 3B). If a saturating flash places every reaction center in state P^R, this comparison would imply that the differential extinction coefficient for the formation of state P^R is approx. 10 $mM^{-1} \cdot cm^{-1}$ at 425 nm. This assumption and the initial slope of the plot in Fig. 3B indicate a quantum yield of 0.15. The assumption that saturation places every reaction center in state P^R can be seriously in

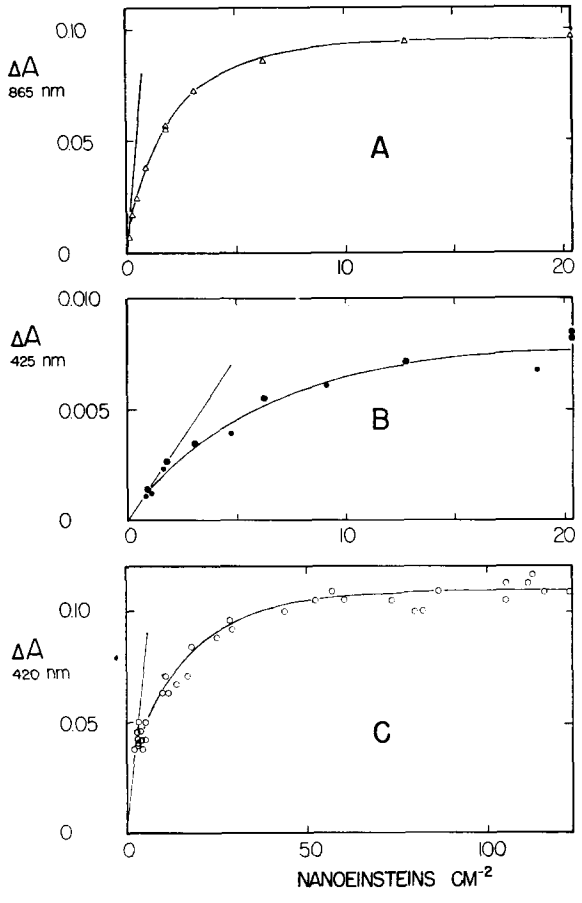


Fig. 3. Quantum yields of states P^+, P^R, and P^F. (A) Absorbance changes due to $P \rightarrow P^+$, measured at 865 nm. Reaction centers, 0.87 μM; Tris · Cl, pH 7.5, 10 mM; Triton X-100, 0.05 %; anthraquinone sulfonate, 50 μM; indigodisulfonate, 50 μM; E = $+180\pm5$ mV; flash λ, 694 nm. The abscissa gives the absorbed irradiance, as calculated from the incident irradiance and the absorbance of the sample at the laser wavelength. The absorbance was measured with weak, continuous light. This may not give an accurate measure of the amount of light absorbed from the strongest flashes, because of depletion of the ground state population. The straight line shows the initial slope of the curve expected for a quantum yield of 1.0. (B) Absorbance changes due to $P \rightarrow P^R$, measured at 425 nm. Conditions as in A, except E = -240 ± 40 mV. The different sized symbols indicate two separate experiments. (C) absorbance changes due to $P \rightarrow P^F$, measured at 420 nm. Reaction centers, 5.84 μM; Tris · Cl, pH 7.7, 50 mM; Triton X-100, 0.05 %; indigodisulfonate, 50 μM; E = -180 ± 12 mV; flash λ, 834 nm.

error if state P^R is formed from another state (e.g. P^F) whose lifetime is significant with respect to the width of the flash. An alternative assumption is that the promotion of every reaction center to state P^R would cause the same degree of bleaching in the 865-nm absorption band as does the complete conversion to P^+. On this assumption, saturating flashes place about 70 % of the units in state P^R (Fig. 2A). The differential extinction coefficient at 425 nm then is 14 $mM^{-1} \cdot cm^{-1}$, and the quantum yield with weak flashes is 0.10.

The quantum yield of P^F is even more difficult to determine accurately, because of the rapid turnover of the state during and after the flash. Fig. 3C shows the results of an attempt to measure the yield in an experiment similar to that of Fig. 3B. On the assumption that saturating flashes convert all of the reaction centers to this state, the apparent differential extinction coefficient at 425 nm is 19 $mM^{-1} \cdot cm^{-1}$. The coefficient is an instrumental or "apparent" number because state P^F turns over at a rate which is comparable to the instrumental response speed. The number can be used to compute the quantum efficiency nevertheless, as long as the absorbance change is proportional to the fraction of the reaction centers that has been converted to state P^F. Using 19 $mM^{-1} \cdot cm^{-1}$, the initial slope of the data in Fig. 3C gives a quantum yield of 0.8.

Fig. 4A shows the results of another experiment in which we attempted to compare the quantum yield of state P^F with that of state P^R. At room temperature, the

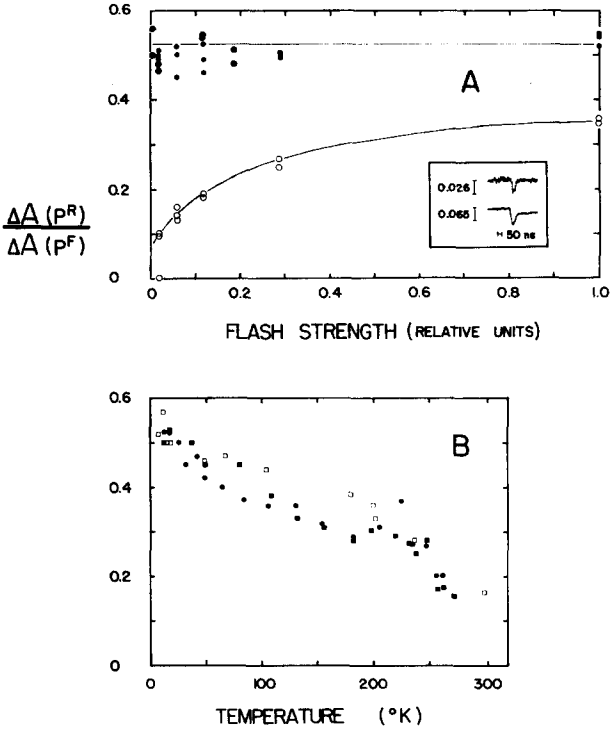


Fig. 4. (A) Effect of flash strength on the relative magnitudes of the absorbance changes due to $P \rightarrow P^R$ and $P \rightarrow P^F$. The ordinate gives the ratio of the two absorbance changes as measured at 425 nm; the abscissa, the irradiance in relative units. Reaction centers, 29.2 μM. Conditions as in Fig. 2C. *E* lowered with excess $Na_2S_2O_4$. ○—○, room temperature; ●—● and ●—● (2 different experiments), 15±5 °K. The inset shows typical measurements at room temperature: The reaction-center concentration was 5.4 μM; the incident irradiance was 5.1 neinsteins $\cdot$ cm^{-2} for the upper trace, and 273 neinsteins $\cdot$ cm^{-2} for the lower. (B) Effect of temperature on the relative magnitudes of the absorbance changes due to $P \rightarrow P^R$ and $P \rightarrow P^F$, as measured at 425 nm with comparatively weak flashes (approx. 40 % saturating for P^R at room temperature). Conditions as in Fig. 2C. Measurements were made as the temperature was being lowered (■) and raised again (○) with one sample, and as it was being lowered with another (□).

ratio of the absorbance changes due to the formation of the two states depends critically on the strength of the flash. Absorbance changes due to state P^F are still relatively large with flashes that are so weak that state P^R is barely detectable. This means that the quantum yield of state P^F is substantially greater than that of state P^R. If we assume that a saturating flash places every reaction center in state P^F, and (at a later time) 70 % of the reaction centers in state P^R, then the difference of 2.8-fold in the absorbance changes with strong flashes (Fig. 4A) implies that the apparent differential extinction coefficient at 425 nm for the formation of state P^F is about twice that for the formation of state P^R. (The apparent extinction coefficient for P^F in this experiment is not necessarily the same as that in the experiment of Fig. 3C, because of small differences between the flash lengths and the instrumental response speeds.) Using this information on the extinction coefficients, the 10 to 20-fold difference in the size of the absorbance changes that follow weak flashes (Fig. 4A) indicates that the quantum yield of state P^F is 5 to 10 times that of state P^R. These calculations obviously are not very exact. It seems clear, however, that the quantum yield of P^F is comparatively high, and that it could be close to 1.0.

At very low temperatures, the ratio of the absorbance changes due to the formation of states P^F and P^R becomes independent of the flash strength (Figs 4A and B). This evidently results from an increase in the quantum yield of state P^R, rather than from a decrease in that of state P^F. Figs 4B and 5A show the temperature dependence of the amplitudes of the two absorbance changes, for comparatively weak flashes. The absorbance changes due to state P^F are essentially independent of temperature; those due to P^R increase steadily with decreasing temperature. These results

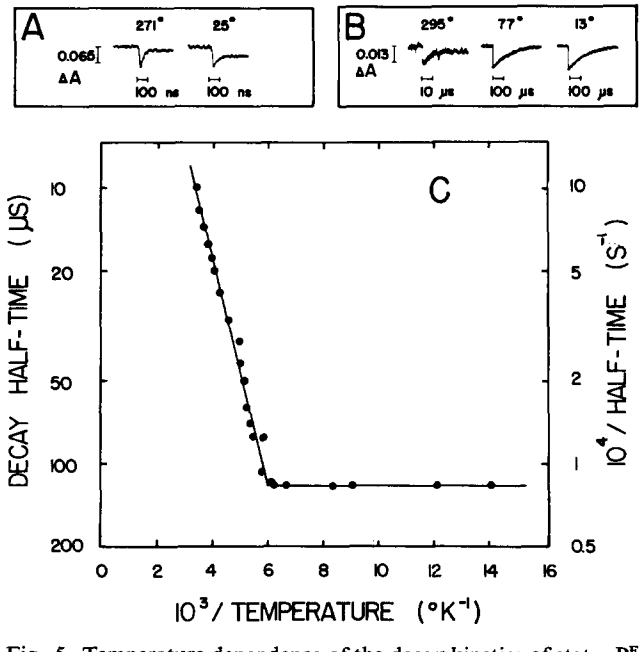


Fig. 5. Temperature dependence of the decay kinetics of states P^F and P^R. Conditions as in Fig. 2C. (A) Decay kinetics of state P^F at 271 and 25 °K. (B) Decay kinetics of state P^R at 295, 77 and 13 °K. (C) Arrhenius plot of decay kinetics of state P^R between 265 and 77 °K.

suggest that the quantum yields of both states are near 1.0 at very low temperatures. If this is correct, the two states cannot arise independently, and state P^F must be a precursor of P^R. The increase in the quantum yield of P^R at low temperature accompanies a decrease in the rate of decay of state P^F; this effect is discussed further below.

The addition of 1 % sodium dodecylsulfate to a preparation of reaction centers in 0.05 % Triton X-100 has an effect on the yield of state P^R similar to the effect of lowering the temperature. (Unlike treatment of reaction centers with sodium dodecylsulfate in the presence of lauryldimethylamine oxide [24], treatment in the presence of Triton X-100 does not alter the absolute absorption spectrum of the preparation in the near infrared.) The treatment with sodium dodecylsulfate also appears to slow the decay of state P^F slightly.

3. *Decay kinetics*

Both at room temperature and at cryogenic temperatures, the population of state P^F rises and falls approximately in parallel with the profile of the 20-ns actinic flash. This means that the decay time of the state must be short, relative to the width of the flash. We therefore have not been able to determine the decay kinetics accurately. We can, however, obtain an estimate as follows. In the experiment of Fig. 3C, the conversion of 50 % of the reaction centers into state P^F (at the peak of the flash) required the total absorption of about $6.5 \cdot 10^{-9}$ einsteins $\cdot$ cm^{-2}. If one approximates the flash envelope by an isosceles triangle with a base of 40 ns, the instantaneous intensity at the peak of the flash would be $3.2 \cdot 10^{-1}$ einsteins $\cdot$ $cm^{-2} \cdot s^{-1}$. Assuming that P^F is in a steady state at this point, and that the quantum yield for its formation is 1.0, P^F must be decaying at the rate of $3.2 \cdot 10^{-1}$ mol $\cdot$ $cm^{-2} \cdot s^{-1}$, or $3.2 \cdot 10^2$ $M \cdot s^{-1}$. Dividing by the instantaneous concentration of P^F ($2.9 \cdot 10^{-6}$ M) gives a decay rate constant of $1.1 \cdot 10^8$ s^{-1}, or a half-time of 6.3 ns.

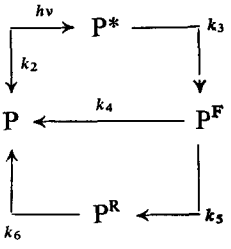
At low temperatures, the decay of state P^F appears to be slightly slower than it is at room temperature (Fig. 5A). Allowing for the length of the flash, the decay half-time may be approximately 20 ns at 25 °K.

State P^R lasts more than three orders of magnitude longer than state P^F does. At room temperature, the half-time of its decay is 6 μs (Figs 5B, 5C). At lower temperatures, the decay becomes still slower, displaying an activation energy of 200 cal $\cdot$ mol^{-1}, until the temperature reaches 160 °K. At this point, the decay rate becomes independent of temperature (at least down to 15 °K), maintaining a half-time of approx. 120 μs (Fig. 5C). This curious pattern is fully reversible. It is essentially the same for samples of reaction centers in frozen buffer solutions as it is in glasses containing 70 % glycerol.

DISCUSSION

Probably the simplest scheme which is consistent with our observations is the following (see the next page).

The rate constant k_2 for the direct decay of P* to P includes both radiative and non-radiative terms; we defer for the moment the question of whether P^+ forms from P*, from P^F, or from P^R. Measurements of the fluorescence yield at low redox potentials, when taken with a calculation of the natural radiative lifetime, indicate that the actual lifetime of P* is $2 \cdot 10^{-11}$ s [6, 7]. This means that P^F, which has a lifetime on



the order of 1 to 10 ns, cannot be P*. We propose that it is the rapid conversion of P* to P^F that accounts for the very short lifetime of P* and the very low yield of fluorescence.

P^F apparently decays by two routes, one of which gives rise to P^R. At room temperature, this path is unfavorable compared to the other, which we assume to be a direct decay back to P. Because the system can cycle between states P and P^F several times during the 20-ns flash, it will have repeated opportunities to give rise to P^R. The total yield of P^R therefore can continue to increase with flash strength, beyond strengths that are saturating for the formation of P^F. At lower temperatures, the conversion of P^F to P^R becomes more likely, perhaps because of a decrease in the rate of the direct decay path. At extremely low temperatures, virtually all of the decay proceeds by way of P^R, and the quantum yield of P^R approaches that of P^F.

The rate constant k_6 for the decay of P^R back to P also varies with temperature, following the complex pattern that is shown in Fig. 5C. One could interpret this pattern in terms of two mechanisms for the decay of P^R, one temperature-dependent and the other temperature-independent, or in terms of a single mechanism which depends critically on the frequency of a nuclear vibration [25]. The lifetimes of the triplet states of several different porphyrins have been shown to be independent of temperature, if quenching due to the formation of triplet excimers is prevented or discounted [26]. It is worth noting that near 160 °K, the temperature of the abrupt transition shown in Fig. 5C, there also occur changes in the temperature dependence of several different electron-transfer reactions in chromatophores. These include the reaction in which the reduced primary electron acceptor returns an electron to P^+ [27], and the reaction in which P^+ oxidizes a *c*-type cytochrome [25, 28].

The identification of states P^F and P^R presents a dilemma. ESR measurements have shown that a triplet state of the reaction center is formed on flash excitation at low temperatures and low redox potentials, and the quantum yield of triplet appears to be high [9]. Optical absorbance changes must accompany this event. It is therefore disconcerting that at low temperatures, neither state P^F nor state P^R has the 6-μs decay half-time that Leigh and Dutton [29] have reported for the triplet ESR signal. Although the half-time of the decay of P^R is 6 μs at room temperature, we did not observe any component with a lifetime of this magnitude at low temperatures. At temperatures between 10 and 20 °K, the half-time for the decay of P^F is about 20 ns; that for P^R is 120 μs.

There are at least two possible solutions to this dilemma. First, the shorter lived state P^F could be the triplet state of the reaction center, if the 6μs decay time which Leigh and Dutton [29] observed was an instrumental response time, rather than the true decay of the triplet state. Leigh and Dutton [29] stated their response time as 0.5 μs, but they derived this number by examing the apparent rise-time of

the triplet signal itself (P. L. Dutton, personal communication). An independent measurement of their response speed is clearly desirable. It would seem unlikely, however, that a state with a lifetime as short as that of P^F would be detectable by ESR.

The second possibility is that P^R is the triplet state, and that the more rapid decay of the ESR signal reflects an approach to spin equilibrium among the three triplet sublevels. The initial intersystem crossing to the triplet favors the $M = 0$ sublevel strongly [29]. The ESR signal is exceptionally large because of this strong polarization, and its magnitude would decrease as the polarization decays. In principle, such a depolarization could be faster than the decay of the triplet population as a whole. To us, this interpretation seems somewhat more likely than the preceeding one. It is in better accord with the observation of Leigh and Dutton [29] that the decay rate increased if they raised the temperature above 10 °K. It also is in slightly better agreement with the spectra of Fig. 2, which favor the identification of P^R with the triplet. A half-time of 6 μs, however, would be unusually short for a spin depolarization at temperatures below 10 °K. Spin-lattice relaxation in the triplet of chlorophyll *b* in solution requires a time on the order of 100 μs at 77 °C, and it undoubtedly is even slower at lower temperatures [30]. On the other hand, the strong preference for population of the $M = 0$ level is itself quite unusual for anisotropic molecules. Further study of the ESR signal or of optically detected magnetic resonance will be necessary to clarify this puzzling situation.

If we exclude an identification of P^F with the $\pi\pi^*$ singlet state P*, because of its comparatively long lifetime, several additional possibilities remain open. These include $n\pi^*$, higher triplet, and P^- (anion) states. Charge-transfer states in which an electron has moved form one bacteriochlorophyll or bacteriopheophytin to another within P are also possible. Whatever the identity of P^F proves to be, the finding that state P^F accumulates in high quantum yield when photochemistry is blocked suggests that it may be an intermediate in the normal electron-transfer reaction. Similarly, the observation that the quatum yield of P^R is relatively low at room temperature supports the view that the electron transfer occurs directly from P^F, rather than proceeding by way of P^R. These conclusions must remain tentative, because it is possible that the reducing conditions that we have used to block photochemistry result in significant changes in one or more of the rate constants. In order to preserve the idea that photochemistry occurs directly from P*, however, one would have to postulate that the reduction of the primary electron acceptor causes an increase of at least 2 orders of magnitude in the rate constant for the formation of P^F (k_3). On the other hand, if photochemistry normally occurs through P^F, one is left to explain the 3-fold increase in fluorescence yield that does accompany the reduction of the acceptor. One possibility is that the enhanced fluorescence yield actually represents delayed fluorescence, resulting from the conversion of P^F back to P*. An examination of the temperature dependence of the fluorescence yield would test this possibility. An alternative is that the reduction of the electron-acceptor causes a 3-fold decrease in k_3. If k_3 is very large, a decrease of this relatively small magnitude could still leave the quantum yield of P^F close to 1. We are attemping to clarify the role of P^F in the photochemical reaction by seeking evidence for its formation under experimental conditions that do not require the reduction of the electron acceptor.

Illumination of chromatophores at low redox potentials causes the formation of states which differ significantly from states P^F and P^R in several respects [31–33].

These differences seem likely to result from the presence in chromatophores of antenna (light-harvesting) bacteriochlorophyll and carotenoids (T. Monger, R. Cogdell, and W. Parson, unpublished).

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Since the submission of this paper, picosecond kinetic studies under conditions that allow the photooxidation of P to occur have shown conclusively that P^F is an intermediate in the electron transfer reaction [34, 35].

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