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The effect of very high magnetic fields on the reaction dynamics in bacterial reaction centers: implications for the reaction mechanism

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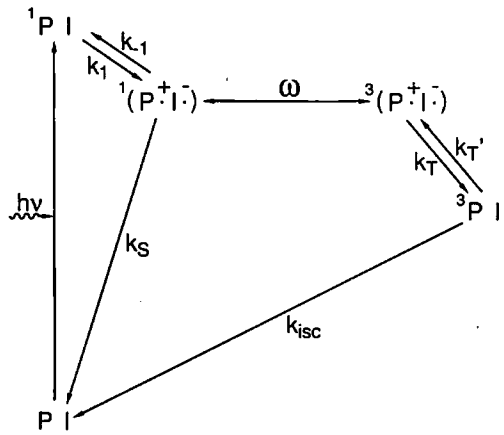
Magnetic field effects have proven to be a powerful tool for elucidating the mechanism of the initial charge separation and recombination steps in bacterial photosynthesis. Since the first data were obtained at high magnetic field strengths, there have been difficulties reconciling the quantitative values obtained for spin-dependent radical-pair decay rate constants with other data within the framework of the conventional reaction mechanism. These difficulties are further highlighted by the recent extensions of the measurements of the triplet quantum yield and decay kinetics and delayed fluorescence to very high magnetic fields. In this paper we analyze the conventional reaction scheme and several others which have been proposed in light of all of the available data. Of the four schemes discussed in detail, three, including the one in most general use, are inadequate in explaining the experimental results. The fourth scheme, postulating that the formation of the initial charge-separated state proceeds via formation of the radical-pair state in an unrelaxed form that undergoes nuclear coordinate relaxation on the nanosecond time-scale (Woodbury, N.W. and Parson, W.W. (1984) Biochim. Biophys. Acta 767, 345–361), is consistent with all data available to date. A quantitative analysis of this scheme provides some information on the nature of this early charge-separated state.

Introduction

The initial electron transfer reaction in bacterial photosynthesis occurs between the photoexcited singlet state of the electron donor (denotes ^1P) and the electron acceptor (denoted I) to form the radical-pair state, P^+I^-. Extensive spectroscopic studies [1–3] and analyses of the X-ray structures of reaction centers (RCs) from the photosynthetic bacteria *Rhodopseudomonas viridis* [4] and *Rhodobacter sphaeroides* [5–7] have identified the electron donor as a bacteriochlorophyll dimer, often called the special pair. The precise identity of the initial electron acceptor is less certain, but it is believed to be a bacteriopheophytin monomer [8]. When further electron transfer is blocked by prior reduction or removal of the quinone secondary acceptor Q_A, the radical pair decays by charge recombination to either re-form ^1PI or the ground state, PI, or form ^3PI, the excited triplet state of the donor, depending upon the electronic spin state of the radical pair (see Scheme I). An applied magnetic field can affect the evolution of this electronic spin state and thus affect the competition between spin-dependent decay pathways.

The ability to alter the reaction dynamics by applying an external magnetic field has proven to be a powerful technique for exploring these charge transfer processes. Both the theory and experimental results



Scheme I. The reaction scheme most widely used to model the effect of external magnetic fields on the reaction dynamics of *Rb. sphaeroides* reaction centers when Q_A is either removed or pre-reduced.

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have been reviewed by several investigators [9–11]. We and others have used Scheme I to analyze these magnetic field effects quantitatively. There have been indications, however, that Scheme I is inadequate in explaining certain observations quantitatively. As discussed below, recent detailed measurements of the quantum yield of ^{3}PI ($\Phi_{^3PI}$), the ^{3}PI decay rate (k_{obs}), and the time evolution of the delayed fluorescence at very high field indicate that the predictions based on Scheme I are not fulfilled. These observations highlight the need to revise the present theoretical and mechanistic models. In the following, we consider several alternative reaction schemes in order to obtain a consistent interpretation of these magnetic field data and the results of the other experiments. These schemes have been considered previously by us and others; a critical review of the literature, however, indicates a number of oversimplifications or errors in previous analyses. Further, our analysis is now guided by the RC X-ray crystal structure [4–7] which helps both to eliminate physically unreasonable reaction schemes and to suggest likely participants in more reasonable schemes. The schemes considered here are by necessity only a subset of all possibilities. Our purpose is to provide a critical analysis of these schemes in order to gain insight into what types of reaction scheme are conceivable and the criteria which would have to be met for new schemes, and to stimulate new experimental work.

Scheme I

The initial photochemistry in Q_A-depleted reaction centers from the bacterium *Rb. sphaeroides* R-26 mutant has generally been modeled with Scheme I. The radical pair is initially formed from ^{1}PI in a singlet spin configuration, denoted $^1(P^+I^-)$, with rate k_1. $^1(P^+I^-)$ can decay to PI (rate k_S), reform ^{1}PI (rate k_{-1}), or undergo coherent electron spin evolution to form the triplet spin configuration of the radical pair $^3(P^+I^-)$. $^3(P^+I^-)$ either decays by charge recombination to generate ^{3}PI (rate k_T), or it evolves back to $^1(P^+I^-)$. ^{3}PI can decay by intersystem crossing (rate k_{isc}) or re-form $^3(P^+I^-)$ (rate $k_{T'}$). At zero magnetic field, the nuclear hyperfine interaction causes interconversion of the singlet radical-pair state and all three triplet radical-pair states. When the magnetic field B is increased from 0 to about 1 kG, the rate of interconversion decreases because of the loss of the near degeneracy of the singlet and two of the triplet radical-pair states, causing a decrease in $\Phi_{^3PI}$. As the field is increased beyond 1 kG, the interconversion rate increases as the *g*-factor difference between P^+ and I^- starts to become the dominant mechanism for interconversion, resulting in an increase in $\Phi_{^3PI}$ above the zero-field value. As the applied magnetic field is increased further, Scheme I predicts that the resulting equilibration between the $^1(P^+I^-)$ and $^3(P^+I^-)$ states will cause a saturation of $\Phi_{^3PI}$ at the 'infinite field' limit:

$$\Phi_{^3PI}^{\infty} = \frac{k_T}{k_S + k_T} \tag{1}$$

Because the radical-pair lifetime decreases as $\Phi_{^3PI}$ increases, it was concluded that $k_T > k_S$ [13]. As a result, Eqn. 1 predicts that $\Phi_{^3PI}^{\infty} > 0.5$. The value of k_T obtained from the delayed fluorescence measurements described in the accompanying paper is $k_T = (4.0 \pm 0.3) \cdot 10^8\ s^{-1}$ [14], consistent with the range of values obtained indirectly from analysis of reaction yield detected magnetic resonance (RYDMR) data [15–17]. The value of k_S obtained by analyzing the dependence of the radical-pair lifetime as a function of $\Phi_{^3PI}$ is $k_S = (4.6 \pm 0.1) \cdot 10^7 s^{-1}$ (see below). Using these values, Eqn. 1 predicts that $\Phi_{^3PI}^{\infty} \simeq 0.9$ at room temperature. This prediction conflicts with our earlier conclusion that $\Phi_{^3PI}^{\infty}$ is approx. 0.5, based on measurements of the quantum yield of ^{3}PI measured from 1 to 12 kG extrapolated to the infinite field limit [18]. We recently extended these measurements up to 135 kG and determined that the effect of the magnetic field on the quantum yield of ^{3}PI does saturate [19]. Using previously obtained values for $\Phi_{^3PI}(B = 0) = 0.32 \pm 0.04$ [13], we obtained a value of $\Phi_{^3PI}$ at saturation of $\Phi_{^3PI}^{\infty} = 0.52 \pm 0.07$. This indicates that Eqn. 1 is not followed, and reaction Scheme I is at best incomplete.

As mentioned above, there have been other indications that Scheme I is inadequate. For instance, discrepancies between theory and experiment have been noted in the relationship between the magnetic field dependence of k_{RP}, the decay rate of the radical-pair state measured by transient absorption spectroscopy, and of $\Phi_{^3PI}$. Using an expression for k_{RP} generalized to account for the possibility of a non-exponential decay of the radical-pair state:

$$k_{RP} = \left[\int_0^{\infty} [P^+I^-(t)]\, dt\right]^{-1} \tag{2}$$

where $[P^+I^-(t)]$ is the concentration of the radical-pair state at time t normalized to the amount of sample excited, Haberkorn and Michel-Beyerle derived an expression for k_{RP} as a function of $\Phi_{^3PI}$ [20]:

$$k_{RP} = \left[\tau^0 + \Gamma \frac{\Phi_{^3PI}(B)}{\Phi_{^3PI}(B=0)}\right]^{-1} \tag{3}$$

The relationship between k_{RP} and $\Phi_{^3PI}$ can be expressed in the form of Eqn. 3 for all of the schemes analyzed in this paper, where τ^0 and Γ can be expressed as a function of the rate constants of the various reaction pathways. For Scheme I, $\tau^0 = 1/k_S$ and $\Gamma = \Phi_{^3PI}(B = 0)\ [(1/k_T) - (1/k_S)]$. Measurements of k_{RP} and $\Phi_{^3PI}$ [13] can be fit to Eqn. 3 with $\tau^0 = (2.18$

$\pm 0.06) \cdot 10^{-8}$ s (giving $k_S = (4.6 \pm 0.1) \cdot 10^7$ s^{-1}) and $\Gamma = (-9.6 \pm 0.6) \cdot 10^{-9}$ s. These values, when combined with the value of $\Phi_{^3PI}(B = 0) = 0.32 \pm 0.04$, are incompatible with a positive value of k_T. Likewise, Schenck and co-workers observed a larger change in k_{RP} for Q_A^--containing RCs upon application of a low magnetic field than is compatible with Eqn. 3 for any non-negative value of k_T [12].

These discrepancies involve measurements of the absolute value of $\Phi_{^3PI}$, a difficult measurement [21]. While there is some disagreement on the exact value, it is unlikely that $\Phi_{^3PI}(B = 0)$ is large enough to reconcile the apparent discrepancies noted above. Furthermore, Eqns. 1 and 3 can be combined to express the *relative quantum* yield of ^{3}PI in terms of the observed radical-pair decay rates:

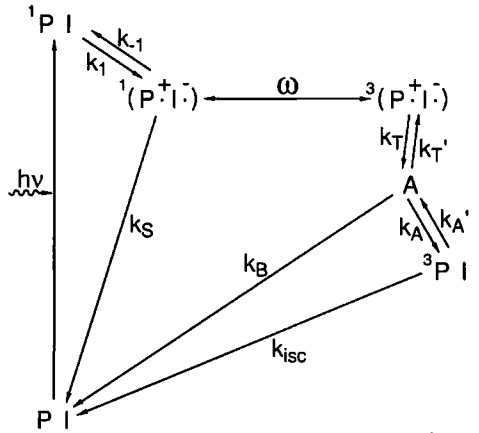
$$\frac{\Phi_{^3PI}^{\infty}}{\Phi_{^3PI}(B = 0)} = \frac{k_{RP}(B = 0)(k_T - k_S)}{(k_S + k_T)(k_{RP}(B = 0) - k_S)} \tag{4}$$

The relative quantum yield can be measured much more accurately, and fewer assumptions are required in the analysis of the data. Using $k_{RP}(B = 0) = (7.7 \pm 0.6) \cdot 10^7$ s^{-1} [13], $k_s = (4.6 \pm 0.1) \cdot 10^7$ s^{-1} and $k_T = (4.0 \pm 0.3) \cdot 10^8$ s^{-1} [14], Eqn. 4 yields $\Phi_{^3PI}^{\infty}/\Phi_{^3PI}(B = 0) = 2.0 \pm 0.2$, as compared with the observed value of $\Phi_{^3PI}(B = 135$ kG$)/\Phi_{^3PI}(B = 0) = 1.61 \pm 0.03$ [19].

We stress that Eqn. 1, 3 and 4 are not specific to any of the details of the singlet-triplet spin evolution, but are direct results of Scheme I; as a result, the conclusions based on these equations are not dependent upon the values of magnetic parameters or the exchange interaction, or the validity of any of the simplifications or assumptions made in the analysis of the singlet-triplet mixing process. Since the minimum number of states in Scheme I appears inadequate to explain the data, we procede to elaborate upon Scheme I by systematically adding a single additional state at various points and evaluating the consequences.

Scheme II

One modification to the reaction scheme that has been suggested is a branching point, State A, in the $^3(P^+I^-)$ decay path, as shown in Scheme II [12,13,21]. It was postulated that spin interconversion from state A to the singlet ground state might occur by spin-orbit interactions [13]. Assuming the identifications of P with the special pair and I with the bacteriopheophytin in the X-ray crystal structure are correct, spin-orbit interactions between P^+ and I^- would likely be too weak to provide a decay path to the ground state which is fast compared with k_T. Another possibility is that state A involves charge separation between the two bacteriochlorophylls comprising P. The parallel orientation of the two bacteriochlorophylls is, however, inconsistent



Scheme II. Postulating a braching point, state A, in the $^3(P^+I^-)$ decay path, as proposed by Schenck and Chidsey with their respective co-workers [12,13].

with the need for rotation of the transferred electron's orbital during spin conversion. A further possibility is that the decay of the triplet radical pair involves an electron transfer from I^- to the monomer bacteriochlorophyll located between the special pair and the bacteriopheophytin; spin–orbit coupling may be possible here, given the orientation of the chromophores.

Defining γ as the branching ratio:

$$\gamma = \frac{k_A}{k_A + k_B} \tag{5}$$

where k_A and k_B are as shown in Scheme II, we obtain:

$$\Phi_{^3PI}^{\infty} = \gamma \frac{\eta k_T}{k_S + \eta k_T} \tag{6}$$

where

$$\eta = \frac{k_A + k_B}{k_A + k_B + k_{T'}} \tag{7}$$

Since no intermediate state has been observed spectroscopically [3], the concentration of A must be small relative to the concentration of P^+I^-. Making this assumption and solving for k_{RP} as a function of $\Phi_{^3PI}$ yields:

$$\tau^0 = 1/k_S \tag{8}$$

and

$$\Gamma = \Phi_{^3PI}(B = 0)\left(\frac{1}{\eta\gamma k_T} - \frac{1}{\gamma k_S}\right) \tag{9}$$

All of the discrepancies discussed above are now resolvable, with $k_S = 1/\tau^0$ and $k_{T'}$ and k_B given by:

$$k_{T'} = \frac{(k_T - k_S)\Phi_{^3PI}(B = 0) + (k_T + k_S)k_S\Gamma\Phi_{^3PI}^{\infty}}{2k_S\Phi_{^3PI}^{\infty}\Phi_{^3PI}(B = 0)} k_A \tag{10}$$

$$k_B = \frac{k_A}{2\Phi_{^3PI}^{\infty}} - \frac{k_S k_A \Gamma}{2\Phi_{^3PI}(B = 0)} - k_A \tag{11}$$

While offering an explanation for the observed yield of ^{3}PI, this scheme conflicts with the magnetic field effect on the observed decay rate of ^{3}PI, k_{obs}. For Scheme I, k_{obs} is given by [22]:

$$k_{obs} = k_{isc} + \tfrac{1}{3}k_S\Phi_{^3PI}\, e^{-\Delta G^0\beta} \tag{12}$$

where ΔG^0 is the free-energy difference between the ^{3}P and $^3(P^+I^-)$ states, and $\beta = 1/kT$, where k is the Boltzmann constant, and T is the absolute temperature. Including state A leads to a modification of Eqn. 12 by introducing a new decay path for ^{3}P via state A:

$$k_{obs} = k_{isc} + (1-\gamma)k_{A'} + \frac{\gamma}{3}\frac{k_{A'}k_{T'}}{k_A k_T}k_S\Phi_{^3PI} \tag{13}$$

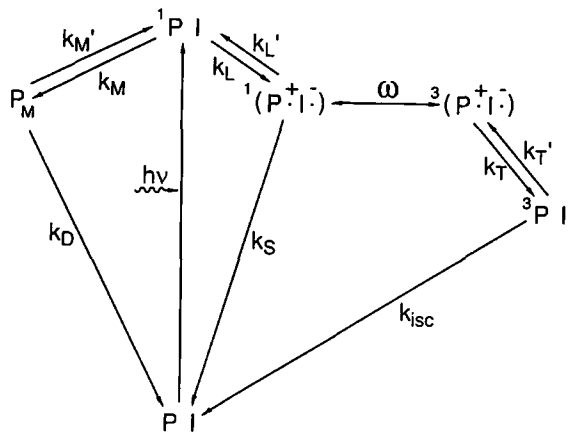
Substituting $\Phi^{\infty}_{^3PI}/\Phi_{^3PI}(B=0) = 1.61 \pm 0.03$, $\Phi_{^3PI}(B=0) = 0.32 \pm 0.04$, $\tau^0 = (2.18 \pm 0.06)\cdot 10^{-8}$ s, $\Gamma = (-9.6 \pm 0.6)\cdot 10^{-9}$ s, and $k_T = (4.0 \pm 0.3)\cdot 10^8$ s^{-1} into Eqn. 13 with the expressions for $k_{T'}$ and k_B given by Eqns. 10 and 11 results in the conclusion that less than 3% of the ^{3}PI decay should proceed by the magnetic-field-dependent decay route at room temperature, in contrast to the large magnetic-field-dependent component observed at high field [19].

Scheme III

The X-ray crystal structure of both *R. viridis* and *Rb. sphaeroides* RCs reveals two potential electron transfer chains, both beginning with special pair P [4–7]. The chromophores in these chains are related by a local total two-fold axis, and it is not obvious at the level of the chromophore structure that one electron transfer pathway is preferred over the other. A considerable body of spectroscopic and kinetic data suggests that electron flow proceeds almost exclusively down one chain under ordinary conditions [3,23]. Michel-Beyerle and co-workers have recently considered the possibility that the initial charge separation can occur to either of the two chromophore branches, forming either P^+I^- or P_M, where P_M is the state where the unpaired electrons are on P and on the alternative electron transfer chain, as shown in Scheme III [23,24]. Indirect evidence for the existence of this route is provided by the experiments of Tiede and co-workers [25,26], who showed that it is possible to accumulate RCs with the bacteriopheophytin on the alternative (M-side) electron-transfer chain reduced. The question then arises as to whether this additional path can reconcile the discrepancies noted above.

The stochastic Louiville equation used to describe the equation of motion of the system [21,27] can be modified to include the additional state to yield:

$$\frac{d}{dt}[P_M] = k_M[^1PI] - (k_{M'} + k_D)[P_M] \tag{14}$$



Scheme III. The reaction scheme proposed by Michel-Beyerle and co-workers [23,24]. In this scheme, the initial charge separation can occur to the bacteriopheophytin on either of the two chromophore branches.

$$\frac{d}{dt}[^1PI] = -(k_M + k_L)[^1PI] + k_{M'}[P_M] + k_{L'}\mathrm{Tr}\{P^S\rho\} \tag{15}$$

$$\frac{d}{dt}\rho = \frac{-i}{h}\{\mathcal{H},\rho\}_- - \frac{k_{L'}+k_S}{2}\{P^S,\rho\}_+ - \frac{k_T}{2}\{P^T,\rho\}_+ + \frac{k_L[^1PI]}{\mathrm{Tr}[P^S]}P^S \tag{16}$$

where $[^1PI]$ and $[P_M]$ are the concentrations of ^{1}PI and P_M normalized to the amount of sample excited, respectively, ρ is the density matrix representing P^+I^-, P^S and P^T are the singlet and triplet spin projection operators, respectively, $\mathcal{H}$ is the electron spin Hamiltonian, $\{\}_-$ and $\{\}_+$ are the commutators and anti-commutators, respectively, and Tr indicates trace. At room temperature, the Q_x absorbance bands of the two bacteriopheophytins largely overlap, so transient absorption which is used to monitor the radical pair decay kinetics is not very sensitive to the difference between P_M and P^+I^-. We can, therefore, represent k_{RP} by:

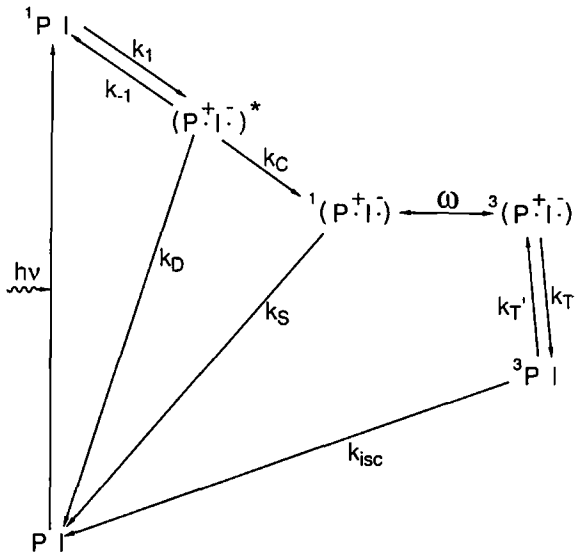
$$k_{RP} = \left[\int_0^{\infty}\{[P_M(t)] + \mathrm{Tr}\{\rho\}\}\,dt\right]^{-1} \tag{17}$$

There is a range of values for the rate constants which predict values of $\Phi_{^3PI}(B=0)$, $\Phi^{\infty}_{^3PI}/\Phi_{^3PI}(B=0)$, τ^0, Γ, and k_T within experimental error of the observed values. All of these possible solutions have appreciable decay (quantum yield > 0.4) of ^{1}PI through formation of P_M and decay through k_D. Assuming various rate constants are not changed when Q_A is present, and that electron transfer to Q_A can occur only from I^-, this sets a maximum yield for the formation of $P^+IQ_A^-$ of 0.6, in conflict with the observation of near unity quantum yield for the state [28]. Modifying Scheme III to include singlet-triplet mixing in P_M would require more decay through k_D to match the values of the various observed quantities, increasing this discrepancy.

Scheme IV

Another alternative reaction mechanism analyzed originally by Haberkorn and co-workers [29] involves an intermediate state between ^1PI and $^1(P^+I^-)$. This was suggested to reconcile the rapid rate of electron transfer from ^1P to I with the small exchange interaction between the unpaired electrons in P^+ and I^-. This proposal is no longer favored by one of the authors [30]; however, we believe it provides an important conceptual framework for the following discussion, and it forms the basis for recent contributions by Marcus [31,32]. In a different form, such a modified scheme was also proposed by Woodbury and Parson [33] to explain the complex pattern of delayed fluorescence from RCs. They neglected, however, to include explicity the effects of spin dynamics in their analysis. In Haberkorn's model, the electron transfers to form a radical pair in what was called a 'close configuration', state C, and then subsequently transfers to a 'distant configuration', state D, where there is negligible exchange interaction and singlet-triplet mixing takes place. Back electron transfer to either PI or ^3PI occurs through re-formation of C. It was suggested by Haberkorn and co-workers that the close state may involve an unpaired electron residing on a monomeric bacteriochlorophyll, now known to reside between P and the bacteriopheophytin as revealed by the X-ray crystal structure. We have shown, however, that electron transfer to this monomeric bacteriochlorophyll does not compete with fluorescence from ^1P by measuring the electric-field-induced fluorescence anisotropy [34]. In order to resolve the discrepancies reflected in Eqns. 1, 3 and 4, state C has to have appreciable concentration during the lifetime of the radical-pair state. In addition, the quantum yield of state D must be significantly less than unity. An intermediate state in which the bridging bacteriochlorophyll anion is present in appreciable concentration has not been detected optically, despite much effort [3,35]. Also, if Q_A reduction in Q_A-containing RCs proceeded only from D, the quantum yield of D formation sets an upper limit for the quantum yield of $P^+IQ_A^-$, in conflict with the observation of near unity quantum yield for this product [28].

A solution to these concerns is to elaborate upon the model of Woodbury and Parson [33], postulating that the intermediate state represents a form of P^+I^- in which the nuclei of P^+, I^- and the surrounding protein solvent are in an unrelaxed state. This state, denoted $^1(P^+I^-)^*$, then evolves on a nanosecond time-scale to a relaxed form of $^1(P^+I^-)$ [(P^+I^-) will be used in the following discussion to denote the relaxed form of the radical-pair state]. By invoking such a mechanism, the unrelaxed form $^1(P^+I^-)^*$ would be optically indistinguishable from the relaxed form, preventing its observation as a distinct intermediate in transient absorption measurements. In addition, Q_A reduction could proceed directly from $^1(P^+I^-)^*$, allowing for formation of $P^+IQ_A^-$ with unity quantum yield. This proposed model is shown as Scheme IV. Note that this hypothesis is not inconsistent with the existence of other intemediate states en route to formation of $^1(P^+I^-)^*$.



Scheme IV. An elaboration of the scheme proposed by Woodbury and Parson [33], where the initially formed radical-pair state $^1(P^+I^-)^*$ relaxes on a nanosecond time-scale to a relaxed form $^1(P^+I^-)$ that undergoes singlet-triplet mixing.

For the purpose of this analysis, singlet-triplet mixing is assumed to occur only in P^+I^-. While this is a reasonable first approximation, it would be interesting to consider alternative schemes where this condition is relaxed. In addition, re-formation of $^1(P^+I^-)^*$ from $^1(P^+I^-)$ is not included. The value of the rate constant for formation of $^1(P^+I^-)$, k_C, and the difference in free energy between this state and $^1(P^+I^-)^*$, both estimated below, make this a reasonable assumption.

Defining k_{RP} as:

$$k_{RP} = \left[\int_0^\infty [(P^+I^-)^*] + [(P^+I^-)]\,dt\right]^{-1} \tag{18}$$

$\Phi_{PI}^{3\infty}$, τ^0, and Γ are given by:

$$\Phi_{PI}^{3\infty} = \frac{k_C k_T}{(k_C + k_D)(k_S + k_T)} \tag{19}$$

$$\tau^0 = \frac{k_C + k_S}{k_S(k_C + k_D)} \tag{20}$$

and

$$\Gamma = \Phi_{^3PI}(B = 0)\left[\frac{1}{k_T} - \frac{1}{k_S}\right] \tag{21}$$

Solutions of these equations are straightforward.

$$k_S = \frac{k_T \Phi_{^3PI}(B=0)}{\Phi_{^3PI}(B=0) - k_T \Gamma} \tag{22}$$

$$k_C = \frac{\Phi^{\infty}_{^3PI} k_S (k_S + k_T)}{k_S k_T \tau^0 - \Phi_{^3PI}(k_S + k_T)} \tag{23}$$

$$k_D = \frac{k_C + k_T}{k_S \tau^0} - k_C \tag{24}$$

Using the previously discussed values for $\Phi^{\infty}_{^3PI}/\Phi_{^3PI}(B=0)$, $\Phi_{^3PI}(B=0)$, τ^0, k_T, and Γ, the various rate constants are equal to: $k_S = (3.1 \pm 0.4) \cdot 10^7\ s^{-1}$, $k_C = (1.5 \pm 0.6) \cdot 10^8\ s^{-1}$, and $k_D = (1.2 \pm 0.4) \cdot 10^8\ s^{-1}$.

With these values of the decay rate constants, $^1(P^+I^-)^*$ is predicted to decay in about 3–7 ns. This is consistent with this state's being responsible for the short-lived, magnetic-field-independent components seen in the decay of the delayed fluorescence [14,33]. In addition, formation of $^1(P^+I^-)$ on the time-scale of the decay of $^1(P^+I^-)^*$ would obscure any quantum-beat phenomena in the delayed fluorescence from oriented RCs at very high magnetic field because different RCs in the sample would begin singlet-triplet mixing at different times. This is consistent with the observations detailed in the accompanying paper [14].

The initial amplitude of the delayed fluorescence was used by Woodbury and Parson to calculate a value for the standard free-energy difference between ^{1}PI and the intially formed radical-pair state of 1370 cm^{-1} at room temperature [33]. This value, however, when combined with the value of $k_1 = 3 \cdot 10^{11}\ s^{-1}$ [35–37] predicts a value for k_{-1} of approx. $3 \cdot 10^8\ s^{-1}$, too large to be consistent with the observed RYDMR linewidth [15–17] given the value of k_T obtained with the delayed fluorescence measurements [14]. We used the magnetic field dependence of k_{obs} to determine the free-energy difference between $^3(P^+I^-)$ and ^{3}PI which, when combined with the known energies of the ^{1}PI and ^{3}PI states [38], yielded a value for the standard free-energy difference between ^{1}PI and the radical-pair state of 2120 cm^{-1} [19]. Scheme IV can be used to resolve the discrepancy between these measurements: Woodbury and Parson's experiment, performed in the sub-nanosecond time regime, yields the free-energy difference between ^{1}PI and state $^1(P^+I^-)^*$, while our measurement, performed on the microsecond time-scale, provides the free-energy difference between ^{1}PI and state P^+I^-. Because singlet-triplet mixing is postulated to occur only in P^+I^-, a large value of k_{-1} would not contribute to the observed RYDMR linewidth. Since our calculation of $\Delta G°$ from the ^{3}PI decay rate data uses the value of k_S [19], the slight modification in the value of k_S that results from the use of Scheme IV ($3.1 \cdot 10^7\ s^{-1}$ vs. $4.6 \cdot 10^7\ s^{-1}$) would increase the calculated $^1PI - P^+I^-$ free-energy difference slightly to 2170 cm^{-1}. This indicates that the free-energy difference between $^1(P^+I^-)^*$ and $^1(P^+I^-)$ at room temperature is approx. 800 cm^{-1}, justifying the exclusion of re-formation of $^1(P^+I^-)^*$ from $^1(P^+I^-)$ in the reaction scheme. This free-energy difference is of the same order of magnitude as, though somewhat larger than, that postulated by Woodbury and Parson on the basis of their observed delayed fluorescence data [33]. There are, however, significant differences between their model and Scheme IV, most importantly their neglect of electron spin evolution.

What is $^1(P^+I^-)^*$?

Although the analysis presented above can resolve many issues, it opens the obvious question: what is the nature of $^1(P^+I^-)^*$? It is intrinsically difficult to define precisely the relaxed state of the radical-pair state. Because P^+ and I^- are believed to be charged, very large local electric fields are generated which may cause a perturbation in the structure of the solvent around the ions. The nature of P^+, I^-, and their interactions with each other and with the protein solvent could all change with time. It is possible, as suggested by Woodbury and Parson [33], that there is not a distinct unrelaxed state, but rather a set of nuclear coordinate motions that occur independently at different rates. The quantitative analysis contained in this paper cannot provide a description of the intermediate states; it can, however, furnish information about what characteristics these states must have to be consistent with the experimental observations. For instance, in order to explain the discrepancy in ΔG^0 between ^{1}P and P^+I^- as calculated using the magnetic field dependence of k_{obs} [19] and as calculated using the initial amplitude of the delayed fluorescence relative to the prompt fluorescence signal [33], there has to be an energy difference between the relaxed and unrelaxed radical-pair states of approx. 800 cm^{-1} at room temperature, unless additional relaxation occurs in the ^{3}PI state. The time-scale of relaxation has to be on the order of nanoseconds, with significant charge recombination to the ground state occurring during this time. The qualitative results do not depend upon the absence of singlet-triplet mixing in $(P^+I^-)^*$. In order to explain the lack of beats in the delayed fluorescence time evolution, the singlet-triplet mixing has to be at least substantially different in the unrelaxed and relaxed forms of the radical-pair state. There are two reasons to suspect that such mixing may be absent. First of all, the small energy difference between ^{1}P and $(P^+I^-)^*$ combined with the large value of k_1 indicates that k_{-1} might be quite large, introducing a dephasing process that will interrupt the singlet-triplet spin evolution in $^1(P^+I^-)^*$. In addition, the exchange interaction in the unrelaxed radical-pair state might be quite large relative to exchange interaction in the relaxed radical-pair state. The exchange interaction is a difficult quan-

tity to calculate, and it is precisely the very small value of J (on the order of $1 \cdot 10^{-3}\,cm^{-1}$) that led Haberkorn et al. [20] and later Marcus [31,32] to argue in favor of an intermediate on route to the formation of P^+I^-. J is extremely sensitive to small changes in distance and to the nature of the bonding and non-bonding interactions between P^+ and I^-. If J were larger in the state $^1(P^+I^-)^*$ than in $^1(P^+I^-)$, singlet-triplet mixing might be effectively impeded [§].

The lifetime of the initial radical-pair state in ubiquinone-containing RCs before subsequent electron transfer from I^- to Q_A is only about 200 ps [40,41], short relative to the rate of the nanosecond relaxation from $^1(P^+I^-)^*$ to $^1(P^+I^-)$. Consequently, Q_A reduction would be expected to occur primarily from $(P^+I^-)^*$. If there is, however, a series of molecular rearrangements that occur in a range of time-scales, some coordinate motion may occur during the short radical-pair lifetime. There is evidence of further relaxation occurring after the formation of $P^+IQ_A^-$. The detection-wavelength dependence of the rate of the absorbance changes that occur on the sub-nanosecond time-scale in Q_A-containing RCs may be due to such motion [42,43]. The recombination kinetics of $P^+IQ_A^-$ are observed to be different if the sample is frozen in the dark or under illumination [44], possibly due to conformational motion either being frozen out or frozen in. The substitution of certain other quinones into the Q_A binding site apparently leads to a slowing of the rate of quinone reduction into the nanosecond time-scale [45]; consequently, nuclear coordinate relaxation in the radical pair state might compete with this forward electron transfer and the energetics of the $P^+I^-Q_A \rightarrow P^+IQ_A^-$ reaction would depend on time. Activated back electron transfer $P^+IQ_A^- \rightarrow PIQ$, which occurs for high potential quinones such as anthraquinone, proceeds through a state whose energy agrees well with the value we obtained from the analysis of the ^3PI decay rate, i.e., the state $^1(P^+I^-)$ [46,47]. This suggests either that memory of the process involved in the relaxation of $^1(P^+I^-)^*$ to $^1(P^+I^-)$ is preserved on the time-scale of this back electron transfer (approx. 1 ms) or that this or similar relaxation occurs in the $P^+IQ_A^-$ state [¶]. The latter possibility suggests that the relaxation process involves changes in or around P^+. If so, this indicates that the nature of nuclear coordinate relaxation following Q_A reduction may be directly related to the relaxation that occurs in Q_A-depleted RCs.

The importance of these relaxations to the initial charge separation step is not apparent. One possibility is that such protein motion is important in the electron transfer from I^- to the quinone, and does not occur to a substantial degree during the 200 ps lifetime of the radical-pair state in ubiquinone-containing RCs. It is only when the radical-pair state lifetime is lengthened by quinone removal, pre-reduction, or substitution that there is sufficient time for these structural changes to occur in the initial radical-pair state. Another related possibility suggested by Miller [48] is that the RC minimizes the reorganization energy for the first electron transfer step by slowing the subsequent protein and chromophore rearrangement so that it occurs on a time-scale slow relative to the electron transfer. In this way, the electron transfer could occur to a quasi-equilibrium state, which does not relax on the time-scale of the lifetime of the radical-pair state, at least under physiological conditions when ubiquinone is present.

Recently, calculations of electron transfer pathways have been presented by Plato and co-workers [49] which use as their starting point detailed information on the unpaired electron spin density in P^+ and I^- obtained from ENDOR data. The implicit assumption in such analyses is that the radicals being studied by ENDOR provide information about the wavefunctions of P^+ and I^- which is relevant to very short time-scales, even though the measurements are made long after the radicals are formed chemically or photochemically. The present analysis indicates that the properties of the radical-pair state may be time-scale-dependent. Specifically, the properties of P^+I^- as observed on a nanosecond or longer time-scale may be significantly different from the properties of the radical-pair state that lives for 200 ps before decaying by electron transfer to ubiquinone, which in turn may be quite different from the radical-pair state that is formed by electron transfer from ^1P to I. Likewise, the quantitative description of magnetic field effects, especially at low fields, is only as reliable as the information on the magnetic properties of P^+ and I^- on the time-scale of the P^+I^- lifetime. This is one of the reasons why we have used information obtained at the highest possible fields, where the exact magnitude of the magnetic interactions in the radical-pair state are unimportant.

[§] Level crossing between the singlet radical-pair state and either the T_+ and T_- states (depending on the sign of the exchange interaction; see [9]) might occur at high field leading to a resonance in the high-field effects such as observed in simple biradicals [39]. No such resonance has been observed in any of our work; however, if the mixing interactions are not large enough, such effects might not be expected. In addition, if $^1(P^+I^-)^*$ does not exist as a distinct state, the time the RC spends in the level crossing region before further relaxation could be too short for significant singlet-triplet mixing.

[¶] We have recently observed that prolonged irradiation of Q_A-containing RCs at low temperature leads to a substantial change in the quantum yield of $P^+IQ_A^-$ formation, as well as changes in the fluorescence electric field effect (Lockhart et al., unpublished results). This photoconversion can be reversed by warming the sample to room temperature. This effect may be due to trapping of relaxed RCs.

Conclusion

In this paper we have considered a number of reaction schemes in order to reconcile the value of k_T obtained by the delayed fluorescence measurements described in the accompanying paper [14], the value of k_S derived from the measurement of the magnetic field dependence of the radical pair lifetime [20], the value of $\Phi_{^3PI}$ in the infinite field limit [19], and the decay rate of ^3PI [22]. The only scheme considered that successfully models these observations and explains the failure to observe other intermediate on the subnanosecond to nanosecond time-scale is Scheme IV, which postulates that P^+I^- is initially formed in an unrelaxed state, and then undergoes relaxation on the nanosecond time-scale. This scheme was also used by Woodbury and Parson to explain their observation of multi-exponential delayed fluorescence decay kinetics at low field [33], and provides an explanation for the absence of quantum beats in the delayed fluorescence decay at very high field [14]. In addition it helps to resolve the substantial difference in free energy for the initial electron transfer step measured by Woodbury and Parson [33] on the basis of their delayed fluorescence results, and our results based on the observed ^3PI decay rate [19]. This intermediate state could correspond with the state proposed by Haberkorn and co-workers [29], reconciling the high rate of the initial electron transfer step with the small exchange energy of the radical-pair state observed on the nanosecond time-scale.

We are not able at this time to identify the nuclear coordinate motions involved in the relaxation of $^1(P^+I^-)^*$ to $^1(P^+I^-)$. It is impossible to obtain unpaired spin densities for the radical intermediates on the nanosecond time-scale (the lifetime precludes spectral resolution better than hundreds of MHz); time-resolved resonance Raman spectroscopy, however, could provide more information, as could the calculations of the type recently initiated by Creighton and co-workers [50]. From the available information, it seems that a number of relaxation processes may be involved, occurring over a range of time-scales, most likely responding to the oxidation of the initial electron donor P. A quite sizable decrease in free energy on the order of 800 cm^{-1} is involved. It is not clear what purpose, if any, this rearrangement has for the functioning of the reaction center, or if it can only occur when the lifetime of the radical-pair state is increased by removal, pre-reduction, or substitution of the initial quinone acceptor. What this does indicate is that the properties of the radical-pair state may be time-dependent, and that information about this state measured on the nanosecond or longer time-scale may not be relevant for analyses of either the radical-pair state as it is formed by electron transfer from ^1P to I, or the radical-pair state as it exists during its 200 ps lifetime in quinone-containing RCs.

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