

A. (1985) *J. Biol. Chem.* 260, 4688-4693.  
 Yamashita, K., Tachibana, Y., Matsuda, Y., Katsunuma, N.,  
 Kochibe, N., & Kobata, A. (1988) *Biochemistry* 27,  
 5565-5573.

Yoshima, H., Takasaki, S., & Kobata, A. (1980) *J. Biol. Chem.* 255, 10793-10804.  
 Yoshima, H., Matsumoto, A., Mizuochi, T., Kawasaki, T., &  
 Kobata, A. (1981) *J. Biol. Chem.* 256, 8476-8484.

## Study of $Q_B^-$ Stabilization in Herbicide-Resistant Mutants from the Purple Bacterium *Rhodopseudomonas viridis*

Laura Baciou,<sup>†</sup> Irmgard Sinning,<sup>‡</sup> and Pierre Sebban<sup>\*‡</sup>

UPR 407, Bat 24 CNRS, Centres Réactionnels Photosynthétiques, Gif Sur Yvette 91198, France, and Max-Planck-Institut für Biophysik, Heinrich-Hoffmann Strasse 7, D-6000 Frankfurt 71, Germany

Received November 29, 1990; Revised Manuscript Received June 27, 1991

**ABSTRACT:** The pH dependences of the rate constants of  $P^+Q_B^-$  ( $k_{BP}$ ) and  $P^+Q_A^-$  ( $k_{AP}$ ) charge recombination decays have been studied by flash-induced absorbance change technique, in chromatophores of three herbicide-resistant mutants from *Rhodopseudomonas (Rps.) viridis*, and compared to the wild type.  $P$ ,  $Q_A$ , and  $Q_B$  are the primary electron donor and the primary and the secondary quinone acceptors, respectively. The triazine resistant mutants T1 (Arg L217 → His and Ser L223 → Ala), T3 (Phe L216 → Ser and Val M263 → Phe), and T4 (Tyr L222 → Phe), all mutated in the  $Q_B$  binding pocket of the reaction center, have previously been characterized (Sinning, I., Michel, H., Mathis, P., & Rutherford, A. W. (1989) *Biochemistry* 28, 5544-5553). The pH dependence curves of  $k_{BP}$  in T4 and the wild type are very close. This confirms that the sensitivity toward DCMU of T4 is mainly due to a structural rearrangement in the  $Q_B$  pocket rather than to a change in the charge distribution in this part of the protein. In T3, a 6-fold increase of  $k_{AP}$  is observed ( $k_{AP} = 4200 \pm 300 \text{ s}^{-1}$  at pH 8) compared to that of the wild type ( $k_{AP} = 720 \pm 50 \text{ s}^{-1}$  at pH 8). We propose that the Val M263 → Phe mutation induces a free energy decrease between  $P^+Q_A^-$  and  $P^+I^-$  ( $\Delta G^\circ_{IA}$ ) ( $I$  is the primary electron acceptor) of about 49 meV. The very different pH dependence of  $k_{AP}$  in T3 suggests a substantial change in the  $Q_A$  pocket. The 2.5 times increase of  $k_{AP}$  above pH 9.5 in the wild type is no longer detected in T3. Instead, a decrease of  $k_{AP}$  is observed above pH 9.5 ( $k_{AP} = 5100 \pm 300 \text{ s}^{-1}$  at pH 9.5 and  $k_{AP} = 3700 \pm 300 \text{ s}^{-1}$  at pH 11). Since in *Rps. viridis* the  $k_{AP}$  variations reflect the changes of  $\Delta G^\circ_{IA}$ , it seems that the protonatable groups(s) involved in the increase of  $k_{AP}$  in the wild type above pH 9.5 has shifted closer to  $I^-$  than to  $Q_A^-$  in T3. The pH dependence of  $k_{BP}$  in T3 is also very different from that of the wild type. The 6-fold increase observed in the wild type in the pH range 5.5-8 is no longer detected in T3. We suggest that the Phe L216 → Ser mutation has an overall effect of shifting to lower pH the pK of the group ( $pK \sim 6.5$ ) involved in the  $\Delta G^\circ_{BA}$  (free energy difference between  $P^+Q_B^-$  and  $P^+Q_A^-$ ) variations at low pH in the wild type. The temperature dependences of  $k_{AP}$ ,  $k_{BP}$ , and  $K_2$ , the  $Q_A^-Q_B^- \leftrightarrow Q_AQ_B^-$  equilibrium constant, have been determined in T3 and the wild type. At pH 8, the energy barrier between  $Q_A^-$  and  $Q_B^-$  is substantially increased in T3 ( $\Delta G^\circ_{BA} = -0.224 \pm 0.015 \text{ eV}$ ) compared to that of the wild type ( $\Delta G^\circ_{BA} = -0.131 \pm 0.015 \text{ eV}$ ). The relative contribution of enthalpic and entropic terms to  $\Delta G^\circ_{BA}$  is very different in T3 and the wild type. In T1, above pH 7,  $Q_B^-$  is destabilized compared to the wild type. Assuming that this effect is mainly due to the absence of the positive charge present on Arg L217, we suggest that the apparent pK of His L217 in T1 is  $8.3 \pm 0.2$ . The  $K_2$  values in T1, T4, and wild type have been compared with the previously measured relative binding affinities of  $Q_B$  ( $Q_{50}$ 's). The  $Q_B$  binding pocket of the wild type looks well designed for a simultaneous optimization of  $K_2$  and  $Q_{50}$ 's.

**T**he absorption of light energy by photosynthetic organisms results in the creation of a transmembrane charge-separated state of the reaction centers, in less than a nanosecond. The photosynthetic reaction center from *Rhodopseudomonas (Rps.) viridis* is composed of four polypeptides, the so-called H, M, and L subunits and a tightly bound cytochrome *c*. The three-dimensional structure of the reaction centers from *Rps. viridis* and *Rhodobacter (Rb.) sphaeroides* became known since their successful crystallization and X-ray structure

analysis (Allen et al., 1988; Arnoux et al., 1989; Chang et al., 1986; Deisenhofer et al., 1985). The primary charge separation occurs in these reaction centers between a dimer of bacteriochlorophyll, *P*, and a quinone,  $Q_A$ , bound to the M polypeptide. The electron present on  $Q_A$  is then transferred to a secondary quinone,  $Q_B$  (bound to the L polypeptide), which can be doubly reduced.  $Q_B^-$  is tightly bound to the reaction centers (Wraight, 1981), whereas  $Q_B^{2-}$  is loosely bound and is supposed to leave the reaction center in its quinol state,  $Q_BH_2$ , after two protons have been uptaken from the cytoplasm. In vivo, the redox potentials of the quinone molecules are very different compared to their values measured in different solvents (Gunner et al., 1986; Woodbury et al.,

\* To whom correspondence should be addressed.

<sup>†</sup> Centres Réactionnels Photosynthétiques.

<sup>‡</sup> Max-Planck-Institut für Biophysik.

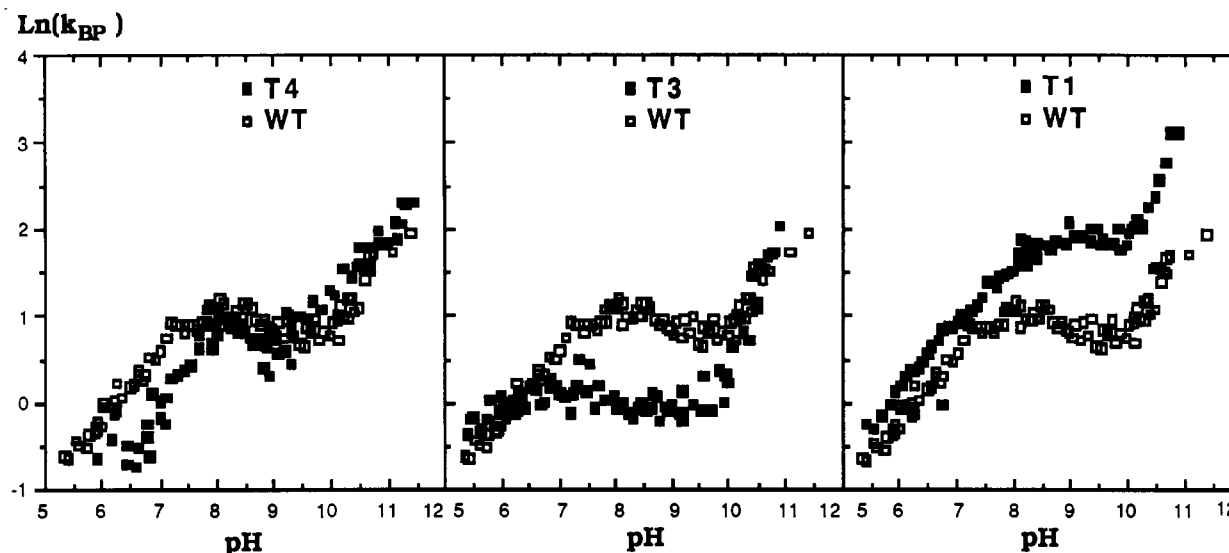


FIGURE 1: pH dependence of the rate constant,  $k_{BP}$ , of  $P^+Q_B^-$  charge recombination in chromatophores from the three mutants, T4, T3, and T1, compared to the wild type.  $T = 21^\circ\text{C}$ . For the buffers, see Materials and Methods.

1986). In *Rb. sphaeroides*, where  $Q_A$  and  $Q_B$  are both ubiquinone 10, the free energy difference between the  $Q_A^-$  and  $Q_B^-$  states ( $\Delta G^\circ_{AB}$ ) is about 70 meV. In *Rps. viridis*, where  $Q_A$  is a menaquinone 9 and  $Q_B$  is a ubiquinone 9,  $\Delta G^\circ_{AB}$  is about 120 meV. A convenient way of studying the degree of stabilization of these states is by measuring the charge recombination kinetics from either the  $P^+Q_B^-$  state or the  $P^+Q_A^-$  state in the presence of inhibitors of the  $Q_A^-$  to  $Q_B$  electron transfer. In chromatophores from *Rps. viridis*, at pH 8, the decay lifetimes from  $P^+Q_A^-$  and  $P^+Q_B^-$  are about 1.5 ms and 300 ms, respectively. At room temperature,  $P^+Q_A^-$  recombines, in *Rps. viridis*, via thermal reexcitation of the intermediate charge-separated state,  $P^+I^-$  (Shopes & Wraight, 1987). It was previously demonstrated that in *Rb. sphaeroides* the  $P^+Q_B^-$  decay proceeds, by more than 95%, via  $P^+Q_A^-$  (Kleinfeld et al., 1984). This is also the case in *Rps. viridis*. It was recently shown that the rate constants of  $P^+Q_A^-$  and  $P^+Q_B^-$  charge recombination display substantial pH dependence in the pH range 5–11, suggesting the presence of protonatable amino acid side chains that modulate the relative free energy levels (or redox potentials) of  $Q_A^-$  and  $Q_B^-$  (Baciou et al., 1990; Sebban & Wraight, 1989; Shopes & Wraight, 1987). The modulations of the redox potentials of the quinones are likely to be due to the differences in the close protein environments of  $Q_A$  and  $Q_B$ . Therefore, it is of interest to study the energetics of the quinone system in herbicide-resistant mutants that are modified in the  $Q_B$  binding site. From *Rps. viridis*, mutants have been selected on their ability to grow photosynthetically in the presence of the herbicide terbutryn [2-(methylthio)-4-(ethylamino)-6-(*tert*-butylamino)-1,3,5-triazine], and the mutations have previously been characterized (Sinning & Michel, 1987; Sinning et al., 1989a). In this work we have used three of these mutants: T1 (Arg L217  $\rightarrow$  His and Ser L223  $\rightarrow$  Ala), T3 (Phe L216  $\rightarrow$  Ser and Val M263  $\rightarrow$  Phe), and T4 (Tyr L222  $\rightarrow$  Phe). We have studied the influence of the amino acid changes on the free energy level of  $Q_B^-$  as a function of pH, and compared these data with the binding affinity of  $Q_B$  for its site, which has previously been measured (Sinning et al., 1989a).

#### MATERIALS AND METHODS

Cells of *Rps. viridis* wild type and mutants were grown as described (Sinning et al., 1989a). Chromatophores were isolated as reported by Michel (1982).

The room temperature flash-absorption spectroscopy apparatus was the same as that previously used (Baciou et al., 1990). In these experiments, the optical density of the chromatophores was about 1 at 1020 nm. The decays of  $P^+Q_A^-$  and  $P^+Q_B^-$  were monitored at 800 nm. For low-temperature measurements, the light-induced absorbance changes were detected at 1300 nm.

pH buffers used were MES [2-(*N*-morpholino)ethanesulfonic acid] (Sigma) between pH 5.5 and 6.5; Bis-Tris-propane [1,3-bis[tris(hydroxymethyl)methyl]amino]propane] (Sigma) between pH 6.3 and 9.5; CAPS [(cyclohexylamino)propanesulfonic acid] (Calbiochem), above pH 9.5.

For the mutants, the  $P^+Q_A^-$  charge recombination rate constants were measured in the presence of 8 mM *o*-phenanthroline. For the activation energy measurements, temperature was monitored by using a NiCr–Ni thermocouple with a precision of  $\pm 0.3^\circ\text{C}$ ; pH was readjusted at different temperatures.

#### RESULTS AND DISCUSSION

The pH titrations of the rate constant of  $P^+Q_B^-$  charge recombination ( $k_{BP}$ ) from T1, T3, T4, and WT chromatophores are shown in Figure 1. In WT chromatophores from *Rps. viridis*, the  $P^+Q_B^-$  decay is not exponential, but rather biphasic. We have previously interpreted this phenomenon as due to the existence of two conformations of the reaction centers, which exist in the dark and whose equilibrium time in the charge-separated state is in the time scale of the decay lifetime of  $P^+Q_B^-$ , i.e.,  $\approx 500$  ms, at pH 7 (Baciou et al., 1990). The above kinetic behavior of the  $P^+Q_B^-$  decay was also observed here in the T1 and T4 mutants, but nearly no heterogeneity was detected in T3. The pH dependence curves of  $k_{BP}$  for the two kinetic phases display similar shapes. In addition, for T1 and T4, the  $Q_A-Q_B \leftrightarrow Q_AQ_B^-$  equilibrium constant ( $K_2$ ) is nearly the same for the two phases independently of the pH (data not shown), as we have previously demonstrated for the WT. Therefore, for simplification, we have plotted here the overall rate constants, taken as the inverse of the average lifetime of the two kinetic phases associated with the two putative conformations of the reaction centers. This approximation does not change the conclusions of the present paper but rather simplifies them. Actually, we are interested in the present work by the effects of the mutations on the energy level of  $Q_B^-$  in the mutants compared to the wild

type. A detailed study of the modifications induced by the mutations on the distribution of the two states of the reaction centers is in progress and will be presented elsewhere.

**T4 (Tyr L222 → Phe).** The pH titration curve of  $k_{BP}$  in T4 chromatophores (Figure 1) is very close to that of the wild type except for the values in the pH range 6–8, where  $Q_B^-$  seems to be slightly more stabilized in T4. It is a rather small effect since the ratio of the  $k_{BP}$  values for the wild type versus T4 never exceeds 1.6. In addition the pH dependence of the rate constant of  $P^+Q_A^-$  charge recombination kinetics ( $k_{AP}$ ) was found to be nearly superimposable with that of the wild type (data not shown). This leads to unchanged  $K_2$  values [ $K_2 = k_{AP}/k_{BP} - 1$  (Wraight, 1981)] in T4 compared to the wild type, as a function of pH. We have also measured the limiting value of  $k_{AP}$  at 80 K,  $k_{TA}$ . The measurements were achieved in 66% glycerol. At low temperature the  $P^+Q_A^-$  charge recombination proceeds directly to the ground state via an activationless electron-transfer process (Shopes & Wraight, 1987).  $k_{TA}$  appears to be the same in the wild type and in T4,  $132 \pm 10 \text{ s}^{-1}$  and  $140 \pm 10 \text{ s}^{-1}$ , respectively. The data presented in Figure 1 show that the replacement of Tyr L222 [conserved in the L subunit of all purple bacteria and also in the green bacterium *Chloroflexus aurantiacus* (Shiozawa et al., 1989; Ovchinnikov et al., 1988)] by Phe has almost no effect either on  $k_{BP}$  or on  $K_2$ . However, it was previously shown that this mutation [which confers sensitivity to DCMU (diuron)] induces an appreciable change in the tertiary structure of this region by suppressing a possible hydrogen bond between Tyr L222 and the carbonyl group of Asp M43. The structural change appears to have a small effect on the overall arrangement of the charge distribution of the amino acid side chains in the vicinity of  $Q_B$ . We confirm that the sensitivity to DCMU in T4 (Sinning et al., 1989b) is probably due to the structural rearrangement of the  $Q_B$  pocket, which has been determined by X-ray structure analysis (Sinning et al., 1990a).

**T3 (Phe L216 → Ser; Val M263 → Phe).** The second mutation in T3 is located in the M subunit (Val M263 → Phe) and does not involve an ionizable group. However, at pH 8,  $k_{AP}$  is found about 6 times as high as in the wild type:  $k_{AP} = 4200 \pm 300 \text{ s}^{-1}$  (Figure 2). Since in *Rps. viridis*,  $P^+Q_A^-$  recombines at room temperature via a relaxed state of  $P^+I^-$  (Kleinfeld et al., 1985; Gopher et al., 1985; Gunner et al., 1986; Sebban, 1988; Shopes & Wraight, 1987; Woodbury et al., 1986),  $k_{AP}$  changes reflect variations of the free energy gap between  $P^+Q_A^-$  and  $P^+I^-$  ( $\Delta G^\circ_{IA}$ ). Thus, the 6-fold difference of  $k_{AP}$  between the wild type and T3 suggests that the Val M263 → Phe mutation substantially decreases  $\Delta G^\circ_{IA}$ . This is accounted for by the equations

$$k_{AP_{T3}} = k_d e^{-(\Delta G^\circ_{IA_{T3}}/kT)} + k_{TA_{T3}}$$

$$k_{AP_{WT}} = k_d e^{-(\Delta G^\circ_{IA_{WT}}/kT)} + k_{TA_{WT}}$$

then

$$\Delta G^\circ_{IA_{WT}} - \Delta G^\circ_{IA_{T3}} = kT \ln \left( \frac{k_{AP_{T3}} - k_{TA_{T3}}}{k_{AP_{WT}} - k_{TA_{WT}}} \right) \quad (1)$$

where  $k_d$  is the rate constant of the  $P^+I^-$  charge recombination decay. For the same reasons as previously pointed out (Shopes & Wraight, 1987), we took  $k_d = 2.10^{-7} \text{ s}^{-1}$ . These equations assume that  $k_d$  is the same for T3 and the wild type. This is likely since there is no mutation close to P or I. We have measured  $k_{TA}$  in T3 and in the wild type at 80 K in 66% glycerol:  $k_{TA} = 166 \pm 15 \text{ s}^{-1}$  and  $132 \pm 15 \text{ s}^{-1}$ , respectively. Taking the  $k_{AP}$  values measured at room temperature, at pH 8, leads to a  $\Delta G^\circ_{IA}$  decrease in T3 of  $49 \pm 5 \text{ meV}$ . In the

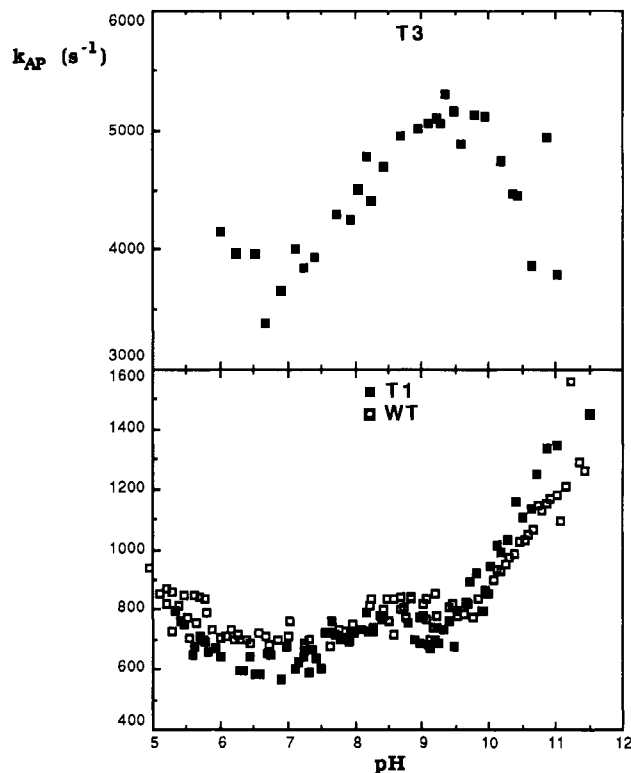


FIGURE 2: pH titration of the  $P^+Q_A^-$  rate constant,  $k_{AP}$ , of charge recombination in the chromatophores from T3, T1, and the wild type.  $T = 21^\circ \text{C}$ . For the buffers, see Materials and Methods.

wild-type structure, Val M263 is located close to  $Q_A$  (distance  $\sim 4.5 \text{ \AA}$ ). There seems to be enough space for the aromatic ring of Phe M263 in T3 to be parallel to  $Q_A$ , which would result in the sandwiching of  $Q_A$  between Phe M263 and Trp M250. That could be at the origin of the observed subsequent free energy change of  $Q_A^-$ . Rearrangements that would lead to a destabilization of  $Q_A^-$  cannot be ruled out, since until now we have no structural data on the T3 mutant to prove the assumptions based on the wild-type structure.

The pH dependence of  $k_{BP}$  in T3 is very different from that of the wild type (Figure 1). In the pH range 7–10, a substantial stabilization of  $Q_B^-$  is observed in T3 compared to the wild type. Since the  $Q_A^-$  as well as the  $Q_B^-$  free energy levels are much affected by the mutations present in T3, it seemed interesting to compare the energetics of these states between T3 and the wild type. The temperature dependence of the different kinetic parameters involved in these processes may be expressed as

$$k_{AP} = k_d e^{-\Delta G_{IA}^\circ/kT} + k_{TA}$$

$$k_{BP} = k_d e^{-\Delta G_{IB}^\circ/kT} + k_{TB}$$

which leads to

$$\ln \left( \frac{k_{AP} - k_{TA}}{k_{BP} - k_{TB}} \right) = -\frac{\Delta G^\circ_{BA}}{kT} = -\frac{\Delta H^\circ_{BA}}{kT} + \frac{\Delta S^\circ_{BA}}{k} \quad (2)$$

The first equation concerning  $k_{AP}$  is the same as for eq 1. The second one, where  $\Delta G^\circ_{IB}$  represents the standard free energy gap between  $P^+I^-$  and  $P^+Q_B^-$ , assumes that  $P^+Q_B^-$  is in thermal equilibrium with  $P^+I^-$ . This is probably correct for the following reasons. In *Rb. sphaeroides*,  $P^+Q_B^-$  was suggested to recombine via  $P^+Q_A^-$  by more than 95% (Kleinfeld et al., 1984). This is likely to be also the case in *Rps. viridis*. Since the rate constant of electron transfer from  $Q_A^-$  to  $Q_B$  [ $\tau \approx 30 \text{ \mu s}$  at pH 8 in the chromatophores (Carithers &

Table I: Activation Parameters,<sup>a</sup> at pH 8, for the P<sup>+</sup>Q<sub>A</sub><sup>-</sup> and P<sup>+</sup>Q<sub>B</sub><sup>-</sup> Charge Recombination Processes and for the Q<sub>A</sub><sup>-</sup>Q<sub>B</sub><sup>-</sup> ↔ Q<sub>A</sub>Q<sub>B</sub><sup>-</sup> Equilibrium Constant in Chromatophores from *Rps. viridis* Wild Type and T3

process	ΔH° (eV)	-TΔS° <sup>b</sup> (eV)	ΔG° (eV)
T3			
P <sup>+</sup> Q <sub>A</sub> <sup>-</sup>	0.078 ±0.010	0.132 ±0.012	ΔG° <sub>IA</sub> = 0.21 ±0.01
P <sup>+</sup> Q <sub>B</sub> <sup>-</sup>	0.56 ±0.010	-0.126 ±0.012	ΔG° <sub>IB</sub> = 0.434 ±0.01
Q <sub>A</sub> <sup>-</sup> Q <sub>B</sub> <sup>-</sup> ↔ Q <sub>A</sub> Q <sub>B</sub> <sup>-</sup>	-0.476 ±0.012	0.252 ±0.015	ΔG° <sub>BA</sub> = -0.224 ±0.015
WT			
P <sup>+</sup> Q <sub>A</sub> <sup>-</sup>	0.202 ±0.010	0.057 ±0.012	ΔG° <sub>IA</sub> = 0.259 ±0.015
P <sup>+</sup> Q <sub>B</sub> <sup>-</sup>	0.222 ±0.010	0.167 ±0.012	ΔG° <sub>IB</sub> = 0.388 ±0.015
Q <sub>A</sub> <sup>-</sup> Q <sub>B</sub> <sup>-</sup> ↔ Q <sub>A</sub> Q <sub>B</sub> <sup>-</sup>	-0.076 ±0.012	-0.056 ±0.015	ΔG° <sub>BA</sub> = -0.131 ±0.015

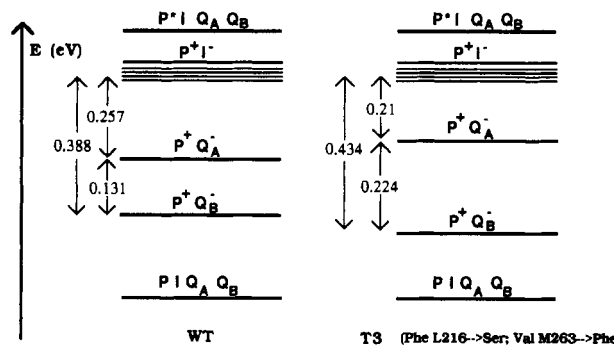
<sup>a</sup>These parameters are derived from the Arrhenius plots derived from eqs 1 and 2.  $k_{TA}$  was taken as 132 and 166 s<sup>-1</sup> for the WT and the T3 mutant, respectively.  $k_{TB}$  was taken as 0.1 s<sup>-1</sup>. <sup>b</sup>T = 298 K.

Parson, 1975);  $\tau \approx 25 \mu\text{s}$  (P. Mathis, I. Sinning, and H. Michel, manuscript in preparation)] is much higher than the deactivation rate from either P<sup>+</sup>Q<sub>A</sub><sup>-</sup> or P<sup>+</sup>Q<sub>B</sub><sup>-</sup>, the equilibrium between these states is established. Since P<sup>+</sup>Q<sub>A</sub><sup>-</sup> is also in equilibrium with P<sup>+</sup>I<sup>-</sup>, P<sup>+</sup>Q<sub>B</sub><sup>-</sup> and P<sup>+</sup>I<sup>-</sup> are in equilibrium too.  $k_{TB}$  is the rate constant of P<sup>+</sup>Q<sub>B</sub><sup>-</sup> charge recombination via the "direct route", i.e., directly to the ground state. In *Rb. sphaeroides*,  $k_{TB}$  was estimated to be less than 0.1 s<sup>-1</sup> (Kleinfeld et al., 1984). That value is likely to be affected by the mutations occurring in the Q<sub>B</sub> pocket, especially if the polarity is changed. However, we have assumed here a  $k_{TB}$  value of 0.1 s<sup>-1</sup>. An error in this parameter would affect the relative contributions of the enthalpic and entropic terms to the ΔG° values derived from Arrhenius plots but would affect only slightly the absolute ΔG° values. ΔG°<sub>BA</sub> represents the standard free energy gap between the P<sup>+</sup>Q<sub>A</sub><sup>-</sup> and P<sup>+</sup>Q<sub>B</sub><sup>-</sup> states. The  $(k_{AP} - k_{TA})/(k_{BP} - k_{TB})$  term reflects the equilibrium between Q<sub>A</sub><sup>-</sup> and Q<sub>B</sub><sup>-</sup>, corrected for the direct recombination pathways from P<sup>+</sup>Q<sub>A</sub><sup>-</sup> and P<sup>+</sup>Q<sub>B</sub><sup>-</sup>.

For T3 and the wild type, at pH 8, the room temperature dependences of  $k_{AP} - k_{TA}$ ,  $k_{BP} - k_{TB}$ , and  $(k_{AP} - k_{TA})/(k_{BP} - k_{TB})$  were found linear in Arrhenius plots. The activation parameters resulting from these plots are presented in Table I. ΔG°<sub>IA</sub> is equal to 0.259 ± 0.015 eV in the wild type and 0.21 ± 0.01 eV in T3. As calculated on the basis of eq 1, the Val M263 → Phe mutation decreases ΔG°<sub>IA</sub> by about 49 meV. On the other hand, the Phe L216 → Ser results in a stabilization of Q<sub>B</sub><sup>-</sup> of about 46 meV in T3 compared to the wild type, at pH 8. ΔG°<sub>IB</sub> increases from 0.388 ± 0.015 eV in the wild type to 0.434 ± 0.01 eV in T3. This energy change probably originates from the disappearance of the ring of Phe. As a consequence, ΔG°<sub>BA</sub> is substantially increased in T3. As shown in Table I, ΔG°<sub>BA</sub> is equal to -0.131 ± 0.015 eV in the wild type and -0.224 ± 0.015 eV in T3. It must be pointed out that ΔH°<sub>BA</sub> and -TΔS°<sub>BA</sub> are very different in T3 and the wild type (for the wild type, ΔH°<sub>BA</sub> = -0.076 ± 0.012 eV and -TΔS°<sub>BA</sub> = -0.056 ± 0.015 eV; for T3, ΔH°<sub>BA</sub> = -0.476 ± 0.012 eV and -TΔS°<sub>BA</sub> = 0.252 ± 0.015 eV). That could be due to the changes in the protein environments of Q<sub>A</sub> and Q<sub>B</sub> between the wild type and T3. Scheme I summarizes these results.

Kleinfeld et al. (1984) have shown that in *Rb. sphaeroides*, where ΔG°<sub>BA</sub> is about -67 meV, the charge recombination pathway from P<sup>+</sup>Q<sub>B</sub><sup>-</sup> proceeds via P<sup>+</sup>Q<sub>A</sub><sup>-</sup> to more than 95%.

Scheme I: Comparison between the Energetics of the Charge-Separated States in T3 and the Wild Type, at pH 8<sup>a</sup>



<sup>a</sup>P<sup>+</sup>I<sup>-</sup> includes substates that were suggested by Sebban and Barbet (1984) and by Woodbury and Parson (1984) to be related to structural fluctuations of the reaction center proteins. P<sup>+</sup>Q<sub>A</sub><sup>-</sup> is supposed to recombine via the same sublevel of P<sup>+</sup>I<sup>-</sup> in T3 and the wild type.

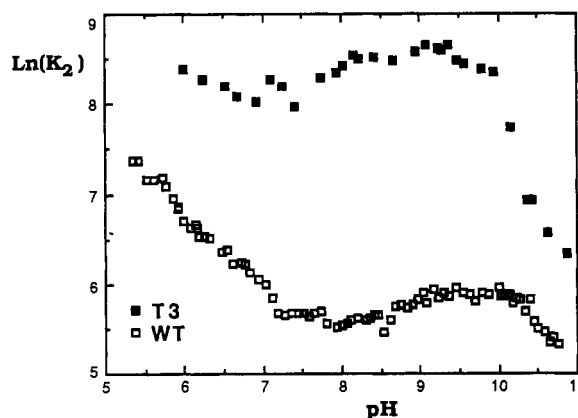


FIGURE 3: pH dependence of  $K_2$  the Q<sub>A</sub><sup>-</sup>Q<sub>B</sub><sup>-</sup> ↔ Q<sub>A</sub>Q<sub>B</sub><sup>-</sup> equilibrium constant, in chromatophores from T3 and the wild type. T = 21 °C. For the buffers, see Materials and Methods.

In *Rps. viridis* wild type, where ΔG°<sub>BA</sub> is about -0.13 eV, this is also the case. It is noteworthy that in T3, where ΔG°<sub>BA</sub> is increased to -0.224 eV, this phenomenon remains unchanged. That could arise from a very small coupling between Q<sub>B</sub><sup>-</sup> and P<sup>+</sup> that makes unlikely the direct back-electron transfer from Q<sub>B</sub><sup>-</sup> to P<sup>+</sup>.

As shown in Figure 2,  $k_{AP}$  for T3 displays a weak pH dependence in the pH range 5–12. The overall pH titration is very different from that of the wild type. A small  $k_{AP}$  decrease (about 30%) is observed in the pH range 9.5–11 whereas in the wild type,  $k_{AP}$  increases more than 2 times between pH 8 and 11 (Figure 2). It has previously been suggested that in *Rps. viridis*, some protonable groups located between Q<sub>A</sub> and I modulate the free energy difference between P<sup>+</sup>Q<sub>A</sub><sup>-</sup> and P<sup>+</sup>I<sup>-</sup> (Shopes & Wraight, 1987; Sebban & Wraight, 1989). Thus, in the wild type, the  $k_{AP}$  increase at high pH can be understood in terms of the presence of a protonatable group closer to Q<sub>A</sub><sup>-</sup> than to I<sup>-</sup>. It seems likely that this group(s) has been displaced closer to I<sup>-</sup> than to Q<sub>A</sub><sup>-</sup> in T3.

Marked differences are also observed between the pH dependences of  $k_{BP}$  in T3 and the wild type in the pH range 5.5–10.3 (Figure 1). The 6-fold increase of this rate constant in the wild type, in the pH range 5.5–8, is no longer detected in T3. Consequently, in the pH range 5.5–8, in T3,  $K_2$  displays a much flatter pH dependence (Figure 3). The big increase of ΔG°<sub>BA</sub> in T3 could affect the recombination pathway from P<sup>+</sup>Q<sub>B</sub><sup>-</sup> so that the direct route would become the main phenomenon. However, as mentioned above, the temperature dependence of  $k_{BP}$  in T3 seems to rule out this hypothesis.

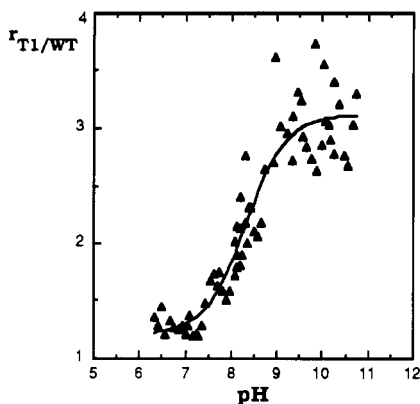
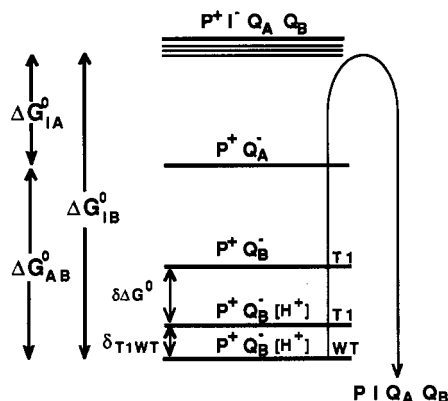


FIGURE 4: pH dependence of  $r_{T1/WT}$ , the ratio between  $k_{BP}$  measured in T1 and the wild type. The line is the result of the fitting according to eq 3. A  $pK$  value of  $8.3 \pm 0.2$  is obtained for His L217 in the T1 mutant.

Scheme II: Differences in the Energetics of the Protonation States of  $Q_B^-$  between T1 and the Wild Type<sup>a</sup>



<sup>a</sup>See Scheme I for the meaning of the sublevels of  $P^+I^-$ .  $P^+Q_A^-$  is supposed to recombine via the same sublevel of  $P^+I^-$  in T1 and in the wild type.

Preliminary data on the mutant MAV3 from *Rps. viridis* (Ewald et al., 1990), which only has the Phe L216 → Ser mutation, seems to also rule out that possibility.

The pH dependence of  $k_{BP}$  at low pH in *Rps. viridis* wild type suggests the presence of a group with a  $pK$  of  $\sim 6.5$  (Figure 1). In *Rb. sphaeroides* His L190 was suggested as a putative primary proton donor to  $Q_B^-$  (Feher et al., 1990). This residue could be a candidate for the above protonatable group. In T3, there is almost no pH dependence of  $k_{BP}$  between pH 5.5 and 8. Therefore, it seems likely that the Phe L216 → Ser mutation in the  $Q_B$  site has notably shifted the  $pK$  of the group involved in the apparent protonation of  $Q_B^-$  in the wild type to lower pH (Figure 1). The instability of T3 at low pH did not allow us to titrate  $k_{BP}$  below pH 5.5.

**T1 (Arg L217 → His; Ser L223 → Ala).** The main effect observed in the T1 mutant is the destabilization of the  $Q_B^-$  state compared to the wild type as shown by the  $k_{BP}$  pH dependence (Figure 1). Whereas this effect is small at low pH, it becomes significant above pH 7.5. Since the pH titrations of  $k_{AP}$  for T1 and the wild type are superimposable (Figure 2), the pH titration of the ratio of  $k_{BP}$  ( $r_{T1/WT}$ ) between T1 and the wild type reflects the relative variations of  $K_2$  between both strains. The pH dependence of  $r_{T1/WT}$  is shown in Figure 4. Starting from about 1.2 at pH 7,  $r_{T1/WT}$  reaches a value of 3 at pH 9.5 and stays on a plateau. This variation can be interpreted in terms of deprotonation of His L217. Since above pH 9.5  $r_{T1/WT}$  remains constant, it can be assumed that the  $r_{T1/WT}$  values measured above pH 9.5 are

due to the electrostatic effect of the absence of Arg L217 in T1 (we consider that  $pK$  of Arg L217  $> 11$ ). The replacement of Ser L223 by Ala can probably be neglected in terms of its electrostatic effect because at pH 7 where His L217 is still protonated in T1 the pH titration of  $r_{T1/WT}$  starts from a value close to 1. Scheme II summarizes the pathways of  $P^+Q_B^-$  charge recombination in the wild type and T1.

According to Scheme II,  $k_{BP}$  for the wild type and T1 may be written for T1 as

$$k_{BPT1} = k_{AP_{T1}} e^{-(\Delta G^\circ_{BA} - \delta_{T1WT})/kT} F(pH)$$

with

$$F(pH) = \frac{e^{\delta \Delta G^\circ/kT} + 10^{(pK_{217} - pH)}}{1 + 10^{(pK_{217} - pH)}}$$

Replacing  $k_{AP}$  in the above equation by its value given in eq 1 gives the equation

$$k_{BPT1} = (k_d e^{-\Delta G^\circ_{IA}/kT} + k_{T_{B_{T1}}}) e^{-(\Delta G^\circ_{BA} - \delta_{T1WT})/kT} F(pH)$$

Finally,

$$k_{BPT1} = (k_d e^{-(\Delta G^\circ_{IB} - \delta_{T1WT})/kT} + k_{T_{B_{T1}}} e^{-(\Delta G^\circ_{BA} - \delta_{T1WT})/kT}) F(pH)$$

With the same approach,  $k_{BP}$  for the WT is given as

$$k_{BP_{WT}} = k_d e^{-\Delta G^\circ_{IB}/kT} + k_{T_{B_{WT}}} e^{-\Delta G^\circ_{BA}/kT}$$

Thus

$$r_{T1/WT} = \frac{k_d e^{-(\Delta G^\circ_{IB} - \delta_{T1WT})/kT} + k_{T_{B_{T1}}} e^{-(\Delta G^\circ_{BA} - \delta_{T1WT})/kT}}{k_d e^{-\Delta G^\circ_{IB}/kT} + k_{T_{B_{WT}}} e^{-\Delta G^\circ_{BA}/kT}} F(pH) \quad (3)$$

$pK_{217}$  represents the apparent  $pK$  of His L217 in T1.  $\delta_{T1WT}$  is the free energy difference between the protonated states of T1 and the wild type.  $\delta_{T1WT}$  is very small according to the observed  $r_{T1/WT}$  value below pH 7, where both T1 and the wild type are protonated.  $\delta \Delta G^\circ$  is the free energy difference between the protonated and deprotonated states (in terms of apparent His deprotonation) of T1. The pH dependence of  $\Delta G^\circ_{IB}$  is due to the presence of protonatable amino acids other than His L217. We have considered that  $\Delta G^\circ_{IB}$  is the same for T1 and the wild type. That is certainly correct since the  $r_{T1/WT}$  value is constant and close to 1 at low pH and constant above pH 9.5. Consistent with this, only minor changes have been detected by X-ray crystallography in the three-dimensional structure of T1 compared to that of the wild type (Sinning et al., 1990b).  $k_{T_{B_{T1}}}$  was determined at 80 K in 66% glycerol:  $160 \pm 10 \text{ s}^{-1}$ . As above,  $k_{T_{B_{WT}}}$  was taken as  $132 \text{ s}^{-1}$ ; at pH 8,  $\Delta G^\circ_{AB} = -\Delta G^\circ_{BA} = 0.131 \text{ eV}$ ,  $\Delta G^\circ_{IB} = 0.388 \text{ eV}$ ;  $k_d = 2.10^{-7} \text{ s}^{-1}$ .  $k_{T_b}$ , which was previously taken equal to  $0.1 \text{ s}^{-1}$ , is negligible in the above equations so we did not include it. The line drawn in Figure 4 was obtained from a fitting procedure according to eq 3. The resulting parameters are  $\delta_{T1WT} = 3 \pm 1 \text{ meV}$  and  $pK_{217} = 8.3 \pm 0.2$ . The  $\delta_{T1WT}$  value agrees with similar energy levels for the  $P^+Q_B^-$  states in T1 and the wild type below the  $pK$  of His L217. The  $pK$  of His L217 in T1 is different from the usual  $pK$  value of His ( $\approx 6.5$ ) in proteins given in the literature. Ionized amino acids in the environment of L217 could be responsible for the high  $pK$  value. In *Rb. sphaeroides*, a  $pK$  of 9.5 was suggested for the glutamic acid L212, well above its expected  $pK$  value (Paddock et al., 1989).  $\delta \Delta G^\circ$  is found equal to  $0.028 \pm 0.003 \text{ eV}$ . The side chain of Arg L217 is at about 11 Å from the center of  $Q_B$ . If we assume that the free energy change due to the

Table II: Comparison between the Apparent Binding Constant of Q<sub>B</sub> and the Apparent Equilibrium Constant between Q<sub>A</sub><sup>-</sup> and Q<sub>B</sub><sup>-</sup>, in the T1, T3, and T4 Mutants and the Wild Type from *Rps. viridis*

strain	apparent binding constant (μM) for ubiquinone 9 at pH 6 measured in RC's <sup>a</sup>	apparent equilibrium constant Q <sub>A</sub> <sup>-</sup> Q <sub>B</sub> ↔ Q <sub>A</sub> Q <sub>B</sub> <sup>-</sup> measured in chromatophores at pH 6 <sup>b</sup>	k <sub>BP</sub> (s <sup>-1</sup> )
WT	4.5	900 ± 50 <sup>c</sup>	0.95 ± 0.05
T1 Ser L223 → Ala Arg L217 → His	0.5	470 ± 50 <sup>c</sup>	1.30 ± 0.05
T4 Tyr L222 → Phe	100	1170 ± 50 <sup>c</sup>	0.6 ± 0.05
T3 Phe L216 → Ser Val M263 → Phe	80	4550 ± 200 <sup>d</sup>	0.9 ± 0.05

<sup>a</sup> From Sinning et al. (1989a). <sup>b</sup> From the present work. <sup>c</sup> The rate constant of P<sup>+</sup>Q<sub>A</sub><sup>-</sup> charge recombination was equal to 700 s<sup>-1</sup>. <sup>d</sup> The rate constant of P<sup>+</sup>Q<sub>A</sub><sup>-</sup> charge recombination was equal to 4100 s<sup>-1</sup>.

absence of Arg L217 in T1 is equivalent to the coulombic interaction energy between Arg and Q<sub>B</sub><sup>-</sup>, then

$$\delta\Delta G^\circ = \frac{1}{4\pi\epsilon_0} \frac{e^2}{d}$$

and then

$$\epsilon = \frac{1}{4\pi\epsilon_0} \frac{1}{\delta\Delta G^\circ} \frac{e^2}{d} \approx 47 \quad (4)$$

This is a quite high dielectric constant value if one considers its value in lipids (≈4) and in water (≈80). However, the polarity of the Q<sub>B</sub> pocket probably contributes to the increase in the dielectric constant. It is of interest to compare the above value to that derived from an Asp L213 → Asn mutant from *Rb. sphaeroides* (Takahashi & Wraight, 1990). Replacement of the negatively charged Asp by Asn results in a 60-meV decrease of the free energy level of P<sup>+</sup>Q<sub>B</sub><sup>-</sup>. Taking a distance of 6 Å between Asp L213 and the quinone and from  $\delta\Delta G^\circ = 60$  meV in eq 4 leads to an  $\epsilon$  value of 40, close to that calculated for T1.

Our evaluation of the  $\epsilon$  value between Arg L217 and Q<sub>B</sub><sup>-</sup> in *Rps. viridis* reaction centers is consistent with the estimation of the dielectric constant between interacting charges in proteins, as suggested by Warshel et al. (1984). These authors have suggested a Coulomb's type law with a distance-dependent dielectric constant of the form

$$\epsilon(r) = \{1 + 60[1 - e^{-0.1r}]\} (1 \pm 0.5)$$

where  $r$  is the distance between the two charges. Taking  $r = 11$  Å leads to a  $\epsilon$  value of  $41 \pm 20$  Å ( $r = 6$  Å leads to  $\epsilon = 28 \pm 14$ ), in good agreement with the above estimation.

It was already pointed out that a highly polar protein site could be of importance for the binding of herbicides in PSII and in purple bacteria (Shipman, 1981). Arg L217 was suggested to be a likely candidate for this site (Sinning et al., 1990b). On the basis of our data, Arg L217 (which is highly conserved in purple bacteria) could also be important in stabilizing Q<sub>B</sub><sup>-</sup> over a wide pH range.

**Concluding Remarks.** It is of interest to compare the values of the apparent binding constant for Q<sub>B</sub> measured by Sinning et al. (1989a), at pH 6, in reaction centers from the wild type and the mutants, with the  $K_2$  values measured here, on the basis of the P<sup>+</sup>Q<sub>A</sub><sup>-</sup> and P<sup>+</sup>Q<sub>B</sub><sup>-</sup> charge recombination rate constants (Table II). For the wild type, T1, and T4, it appears that the more Q<sub>B</sub> is bound to its site, the less Q<sub>B</sub><sup>-</sup> is stabilized. At pH 8, the differences observed for  $K_2$ , e.g.,  $K_{2T1} < K_{2WT} < K_{2T4}$ , are even more evident than at pH 6. This relationship between the  $K_2$  values may be understood in terms of the

relationship existing between the redox potential of Q<sub>B</sub>/Q<sub>B</sub><sup>-</sup> and the differential binding of Q<sub>B</sub> and Q<sub>B</sub><sup>-</sup>. The mutations present in T1, T3, and T4 seem to have affected the binding of Q<sub>B</sub> much more than that of Q<sub>B</sub><sup>-</sup>. That was previously pointed out by Wraight (1982) and Wraight and Shopes (1989). The three-dimensional structure of the Q<sub>B</sub> pocket and the interactions of single amino acids with Q<sub>B</sub> are important to ensure a sufficient decrease of the free energy of the P<sup>+</sup>Q<sub>B</sub><sup>-</sup> state compared to P<sup>+</sup>Q<sub>A</sub><sup>-</sup>. At the same time what is gained in free energy is apparently lost in the relative affinity of Q<sub>B</sub> for its site. From this point of view, the wild type is a compromise between these two requirements for optimal function of the quinone system in the reaction centers.

#### ACKNOWLEDGMENTS

Dr. P. Mathis is gratefully acknowledged for his collaboration in the low-temperature measurements of  $k_{Ta}$ . We thank Prof. H. Michel and Dr. A. L. Etienne for helpful discussions and careful reading of the manuscript. J. P. Roux and J. C. Barbet are acknowledged for their efficient work in the data acquisition and curve analysis program.

#### REFERENCES

- Allen, J. P., Feher, G., Yeates, T. O., Komiya, H., & Rees, D. C. (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85, 8487–8491.
- Arnoux, B., Ducruix, A., Reiss-Husson, F., Lutz, M., Norris, J., Schiffer, M., & Chang, C. H. (1989) *FEBS Lett.* 258 (1), 47–50.
- Baciu, L., Rivas, E., & Sebban, P. (1990) *Biochemistry* 29, 2966–2976.
- Carithers, R. P., & Parson, W. W. (1975) *Biochim. Biophys. Acta* 387, 194–211.
- Chang, C. H., Tiede, D., Tang, J., Smith, U., Norris, J., & Schiffer, M. (1986) *FEBS Lett.* 205, 82–86.
- Deisenhofer, J., Epp, O., Miki, K., Huber, R., & Michel, H. (1985) *Nature* 318, 618–624.
- Ewald, G., Wiessner, C., & Michel, H. (1990) *Z. Naturforsch.* 45C (5), 459–462.
- Feher, G., Mc Pherson, P. H., Paddock, M., Rongey, S. H., Schönfeld, M., & Okamura, M. Y. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. I, pp 39–46, Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Gopher, A., Blatt, Y., Schönfeld, M., & Okamura, M. Y. (1985) *Biophys. J.* 48, 311–320.
- Gunner, M. R., Robertson, D. E., & Dutton, P. L. (1986) *J. Phys. Chem.* 90 (16), 3183–3195.
- Kleinfeld, D., Okamura, M. Y., & Feher, G. (1984) *Biochim. Biophys. Acta* 766, 126–140.

- Kleinfeld, D., Okamura, M. Y., & Feher, G. (1985) *Biophys. J.* 48, 849-852.
- Michel, H. (1982) *J. Mol. Biol.* 158, 567-572.
- Ovchinnikov, Yu. A., Abdulaev, N. G., Zolotarev, A. S., Shmukler, B. E., Kargarov, A. A., Kutuzov, M. A., Telezhinskaya, I. N., & Levina, N. B. (1988) *FEBS Lett.* 231, 237-242.
- Paddock, M. L., Rongey, S. H., Feher, G., & Okamura, M. Y. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86, 6602-6606.
- Sebban, P. (1988) *FEBS Lett.* 233, 331-334.
- Sebban, P., & Barbet, J. C. (1984) *FEBS Lett.* 165 (1), 107-110.
- Sebban, P., & Wraight, C. A. (1989) *Biochim. Biophys. Acta* 974, 54-65.
- Sebban, P., Parot, P., Baciou, L., Mathis, P., & Verméglio, A. (1990) in *Reaction Centers of Photosynthetic Bacteria* (Michel-Beyerle, M. E., Ed.) Springer Series in Biophysics Vol. 6, pp 189-196.
- Shiozawa, J. A., Lottspeich, F., Oesterhelt, D., & Feick, R. (1989) *Eur. J. Biochem.* 180, 75-84.
- Shipman, L. L. (1981) *J. Theor. Biol.* 90, 123-148.
- Shopes, R. J., & Wraight, C. A. (1987) *Biochim. Biophys. Acta* 893, 409-425.
- Sinning, I., & Michel, H. (1987) *Z. Naturforsch.* 42C, 751-754.
- Sinning, I., Michel, H., Mathis, P., & Rutherford, A. W. (1989a) *Biochemistry* 28, 5544-5553.
- Sinning, I., Michel, H., Mathis, P., & Rutherford, A. W. (1989b) *FEBS Lett.* 256 (1), 192-194.
- Sinning, I., Koepke, J., & Michel, H. (1990a) in *Reaction Centers of Photosynthetic Bacteria* (Michel-Beyerle, M. E., Ed.) Springer Series in Biophysics Vol. 6, pp 199-208.
- Sinning, I., Koepke, J., Schiller, B., & Michel, H. (1990b) *Z. Naturforsch.* 45C, 455-458.
- Takahashi, E., & Wraight, C. A. (1990) *Biochim. Biophys. Acta* 1020, 107-111.
- Warshel, A., Russell, S. T., & Churg, A. K. (1984) *Proc. Natl. Acad. Sci. U.S.A.* 81, 4785-4789.
- Woodbury, N. W., & Parson, W. W. (1984) *Biochim. Biophys. Acta* 767, 345-361.
- Woodbury, N. W., Parson, W. W., Gunner, M. R., Prince, R. C., & Dutton, P. L. (1986) *Biochim. Biophys. Acta* 851, 16-22.
- Wraight, C. A. (1981) *Isr. J. Chem.* 21, 348-354.
- Wraight, C. A. (1982) in *Function of Quinones in Energy Conserving Systems* (Trumpower, B. L., Ed.) pp 181-197, Academic Press, New York.
- Wraight, C. A. (1989) in *Techniques and New Developments in Photosynthesis* (Barber, J., & Malkin, R., Eds.) pp 183-191, Plenum, New York.

## Structural Differences in Solution and Crystalline Forms of Met-Myoglobin<sup>†</sup>

K. Zhang,<sup>\*,†</sup> B. Chance,<sup>‡</sup> K. S. Reddy,<sup>‡</sup> I. Ayene,<sup>‡</sup> E. A. Stern,<sup>§</sup> and G. Bunker<sup>†</sup>

*Institute for Structural and Functional Studies, 3401 Market Street, Number 320 Philadelphia, Pennsylvania 19104, and  
Department of Physics, University of Washington, Seattle, Washington 98195*

*Received May 3, 1991; Revised Manuscript Received June 28, 1991*

**ABSTRACT:** For several decades X-ray diffraction studies have been the paragon of biological structure studies at atomic resolution. Diffraction provides three-dimensional structure information, which is essential to our fundamental understanding of protein function. However, since X-ray diffraction cannot be done to atomic resolution on proteins in their native solution or membrane-bound state, the possibility exists that the conformations of the protein in crystals are slightly different from the conformations in solution, and attempts to interpret details of the structure may be misleading and without physiological relevance. In this paper, we show that this concern is justified for a familiar protein, myoglobin. Performing X-ray absorption fine structure experiments on both solution and crystalline met-myoglobin (met-Mb), we find significant differences in the local environment of the iron between the two states. Specifically, the average iron-nearest neighbor atom distance in the crystalline form is 0.05 Å shorter than that in the solution form, and the iron-nearest neighbor bond is more rigid in the crystalline met-Mb. Possible artifactual explanations for the differences have been ruled out.

**I**t is generally believed that the function of a biological system directly depends on its three-dimensional structure. Most of our knowledge on protein structure at atomic resolution is obtained by X-ray diffraction on single crystal protein samples that are extracted from protein solution. Since the crystalline form of the protein is not the one performing physiological roles in vivo, the question of whether protein structures in solution and crystalline forms are really the same deserves a detailed

inquiry. More than 20 years ago Chance et al. (1966) reported that the reactivity of a suspension of horse ferrimyoglobin microcrystals with azide is much slower (about 21 times) than the reactivity of the soluble protein with azide. This decrease was attributed to a loss of flexibility in the crystalline structure that may limit the probability of the conformational change induced by ligation. However, direct comparison of the structure between solution and crystalline forms of met-myoglobin (met-Mb)<sup>1</sup> had not been made.

<sup>†</sup> This research was supported by NIH Grants HL-18708 and RR-01633.

<sup>\*</sup> To whom correspondence should be addressed.

<sup>‡</sup> Institute for Structural and Functional Studies.

<sup>§</sup> University of Washington.

<sup>1</sup> Abbreviations: met-Mb, OH<sub>2</sub> (Fe<sup>III</sup>) myoglobin; XAFS, X-ray absorption fine structure; 2-D NMR, two-dimensional nuclear magnetic resonance.