Electrostatic interaction between redox cofactors in photosynthetic reaction centers

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Running title: Electrostatic interactions between redox centers
Abstract

Intramolecular electron transfer within proteins is an essential process in bioenergetics. Redox cofactors are embedded in proteins and this matrix strongly influences their redox potential. Several cofactors are usually found in these complexes and they are structurally organized in a chain with distances between electron donor and acceptor short enough to allow rapid electron tunneling. Amongst the different interactions that contribute to the determination of the redox potential of these cofactors, electrostatic interactions are important but restive to direct experimental characterization. The influence of interaction between cofactors is evidenced here experimentally, by means of redox titrations and time-resolved spectroscopy, in a chimeric bacterial reaction center (Maki et al. (2003) J. Biol. Chem., 278(6):3921-3928) composed of the core subunits of Rubrivivax gelatinosus and the tetraheme cytochrome of Blastochloris viridis. The absorption spectra and orientations of the various cofactors of this chimeric reaction center are similar to those found in their respective native protein, indicating that their local environment is conserved. However, the redox potentials of both the primary electron donor and its closest heme are changed: the former is downshifted in the chimeric reaction center when compared to the wild type, conversely the latter is upshifted. We propose a model in which these reciprocal shifts in the midpoint potentials of two electron transfer partners are explained by an electrostatic interaction between them.
Introduction

Proteins exert a fine electrochemical tuning of the redox potential of the cofactors they bind in order to perform the various electron transfer reactions that are involved in biological processes. As a famous example, the redox potentials ($E_m$) of $c$-type cytochromes are tuned by more than 500 mV (see (1) and references therein). The physicochemical basis of such wide range modulations have been rationalized by various authors who all agree that the protein medium has the unique property of providing a dielectric environment in which the redox cofactors are embedded with an intricate charge or dipole distribution (1-3). From a general standpoint, the free energy difference between the oxidized and reduced forms of any redox cofactor in a protein is the sum of several terms. Amongst these are $\Delta G_{\text{conf}}$ and $\Delta G_{\text{el}}$. $\Delta G_{\text{conf}}$ accounts for any conformational change that the change in the redox state of the cofactor may induced (including proton or ion binding or release). $\Delta G_{\text{el}}$ results from the electrostatic potential at the cofactors resulting from the individual charged groups and the permanent dipoles within the protein. Estimating the respective values of these different terms is a difficult task, yet their sum is readily accessible experimentally since it can be obtained by comparing the absolute values of the $E_m$ in solution and in the protein. The latter may be obtained by two different methods. The most commonly used one is equilibrium redox titration. The other relies on the determination, by a functional analysis, of the free energy change associated with an electron transfer reaction between two cofactors. Such a change in free energy is generally considered as equal to the difference in $E_m$’s between the electron donor and acceptor. Thus, provided one of these two redox potentials is known, the other one is readily inferred. However, these two methods sometimes yield different results and this has led to the distinction between ‘equilibrium redox potential’ and ‘operating redox potential’. These differences arise because the two methods do not probe the same state of the redox
cofactors. Two types of phenomena may account for these differences. One comes from the distinct time-domain involved in equilibrium redox titration and functional analysis. Whereas redox titrations require thermodynamic equilibrium between the sample and the solution poised at a given potential, the functional analysis allows one to probe transient states whose free energy may differ significantly from that of the equilibrated states. Indeed, in response to the change in the redox state of a given cofactor, the protein environment may undergo energetic relaxation (such as proton transfer, conformational changes) which may be slower than the lifetime of the transient oxidized (or reduced) cofactor. Armstrong and colleagues have nicely illustrated this point with the ‘fast-scan electrovoltammetric’ technique (4). Other examples are found in photosynthetic reaction centers (RC) (see e.g. (5,6)). An alternative but non-exclusive explanation of the different $E_m$'s yielded by redox titration and functional analysis relies on the fact that most of the membrane proteins involved in electron transfer reactions bind several cofactors which are usually located at less than 15 Å one from the other. Such short distances may result in significant electrostatic interactions between the different cofactors. Thus, for a given cofactor, $\Delta G_{el}$ includes the electrostatic contributions of the nearby electron carriers. Such a contribution has been nicely illustrated in the case of the tetraheme cytochrome of *Blastochloris* (formerly *Rhodopseudomonas viridis*) (7,8). It is noteworthy however that throughout a redox titration, all the cofactors undergo an identical charge change in terms of sign (*i.e.* all are either reduced or oxidized), whereas in an electron transfer chain, two nearby cofactors involved in an electron transfer reaction will undergo charge changes of opposite sign (one will be oxidized at the expense of the other). Consequently, if the electrostatic interaction between the two is significant, the difference between their equilibrium $E_m$'s will be greater than the free energy change associated with the electron transfer between them. In this paper we will illustrate the importance of such electrostatic interactions in electron transfer chains. Although electron transfer chains
embedded in a single protein are found in many biological pathways, the photosynthetic chains are ideally suited for such studies. Indeed not only do they allow redox titration of the various redox cofactors but also the kinetics of electron transfer reaction may be characterized with an unequalled time-resolution. In particular, the bacterial photosynthetic RC of *Blc. viridis* and its associated tetraheme cytochrome have been intensively studied. Its three dimensional structure has been solved (9) and the spectroscopic or redox properties of the various cofactors are known (see (10) for a review). Further, the different electron transfer steps have been characterized (11). In membrane fragments as well as purified RC of *Blc. viridis* the reduction of the oxidized primary electron donor P\(^+\) by the closest heme, c\(_{559}\), is multiphasic (12). Interestingly, this feature has been interpreted along the lines of either of the two phenomena which has just been described: a conformational heterogeneity yielding a distribution of substates (11) or a low equilibrium constant of this electron transfer reaction (13). To reconcile this latter hypothesis with the equilibrium constant of \(\sim 100\) expected from the difference in midpoint potentials of the P\(^+\)/P and c\(_{559}\)\(^+/\)/c\(_{559}\), Baymann and Rappaport proposed that an electrostatic interaction between P and its closest heme raises the redox potential of the P\(^+\)/P couple (13). As discussed below, the present results support this hypothesis.

From an experimental standpoint *Blc. viridis* has the drawback of being unable to grow heterotrophically making the mutagenesis approach uncertain, despite few successful attempts. Recently, Maki *et al.* (14) succeeded in transferring the membrane bound cytochrome of *Blc. viridis* to another bacterium, *Rvi. gelatinosus*, in which mutagenesis strategy may be planned (15,16).

**Fig 1** In this paper, we further investigated this chimeric RC (here after named VC-F) and show that most of the various cofactors at the donor side of this RC show absorption spectra and relative orientations similar to that found in the respective native RC’s, indicating that the backbone
structure of the protein as well as the interactions between the hemes and the side chains are conserved. These findings make the chimeric RC suited for the direct characterization of electrostatic interactions between cofactors by the mean of equilibrium redox titration. Indeed, in this RC, two cofactors (the primary electron donor, P, and the closest heme $c_{559}$) are expected to have redox potentials differing by less than 50 mV (see Figure 1). Such a case allows the direct observation of a putative electrostatic interaction since it should manifest itself by a deviation to a one-electron Nernst curve. This contrasts with the case where the two interacting redox centers have strongly different midpoint potential (as in the *Bcl. viridis* case e.g.) for they are then expected to titrate in well separated redox potentials range yielding titration curves that follow the classical Nernst equation. Accordingly, we found that the redox titration of the primary donor (P/P$^+$) of the chimeric RC displayed a deviation to a one electron Nernst curve. The redox potential of the primary donor was downshifted by 50 mV in the chimera with respect to the WT *Rvi. gelatinosus* strain. Interestingly the redox potential of the closest heme (the $c_{559}$ heme) underwent a converse upshift of similar amplitude. These data evidence that electrostatic interactions between cofactors in proteins modulate their redox potentials.

**Experimental procedures**

Interspecific replacement of the gene coding for the RC-bound cytochrome subunit in *Rvi. gelatinosus*, used to produce VC-F mutant, is described in (14). The site directed substitution, by a two-step PCR method, of Arg204 for a Leu in the cytochrome subunit of the VC-F strain is described in (17).
Cells of the VC-F mutant of *Rvi. gelatinosus* were grown for 24 h, in the light and in anaerobic conditions in Hutner medium with 50 μg.mL⁻¹ kanamycin and 20 μg.mL⁻¹ ampicilin. For membrane preparations, cells were harvested by centrifugation at 4000 g for 10 min, resuspended in 20 mM Tris-HCl (pH 7), and disrupted by French Press at 50 MPa. The remaining intact cells were separated from the membrane supernatant by centrifugation at 10000 g for 10 min. Then, the membrane fragments were collected after centrifugation at 250000 g for 90 min and resuspended in 20 mM Tris-HCl, 100 mM KCl (pH 7) for equilibrium redox titrations.

**EPR spectroscopy**: Membrane fragments were resuspended in 20 mM MOPS, pH 7, and oxidized by addition of 2 mM potassium ferricyanide. The membranes were then washed free from ferricyanide by renewed pelleting and resuspension in 20 mM MOPS, pH 7. EPR spectra were taken at 15 K using a Bruker ER 300 X-band spectrometer equipped with an Oxford Instruments helium cryostat and temperature-control system. The instrument settings were: microwave power, 6.7 mW; microwave frequency, 9.43 GHz; modulation amplitude, 2.5 mT. Angular dependence of EPR signals was investigated on oriented membrane multilayer obtained by drying the membrane fragments onto Mylar sheets (18).

Redox titrations were performed as in (13,19) in an electrochemical cell (100 μm optical path length), with three electrodes: a platinum electrode, a gold grid (InterNet Inc.) modified by PATS (2-pyridinecarboxaldehyde thiosemicarbazone, Sigma) to avoid irreversible adsorption of the proteins onto the gold grid, and an Ag/AgCl reference electrode in 3M KCl. The redox mediators were used at 20 μM each: 1,4-benzoquinone (Eₘ = +280 mV), 1,1-dimethyl ferrocene (Eₘ = +340 mV), ferrocene (Eₘ = +420 mV), monocarboxylic acid ferrocene (Eₘ = +530 mV).

The optical spectroscopic measurements were performed on two different laboratory built absorption spectrophotometers: a xenon flash-lamp microsecond time-resolution one (20)
used for the titration of the cytochromes in the $\alpha$-band; and a Nd:YAG pulsed laser nanosecond time-resolution one (21) used for the kinetic experiments and the flash-induced titration of the primary electron donor.

**Results**

*Redox characteristics of the $P^+/P$ couple in the WT and chimeric RCs*

Fig. 2 The primary electron donor $P$ was titrated in membrane fragments purified from *Rvi. gelatinosus* and *Blc. viridis* WT strains and the chimeric VC-F strain (Figure 2, left panel) by measuring the flash induced absorption changes at 605 nm 50 ns after the actinic flash. Both the oxidative or reducing waves yielded similar results indicating that thermodynamic equilibrium was reached during the titration (not shown). The titration curve in *Blc. viridis* could be satisfyingly fitted with a Nernst curve with $E_m = +500$ mV. The $P^+/P$ couple in *Rvi. gelatinosus* titrates with an $E_m$ of $\sim +400$ mV, consistent with previous findings (22), but, interestingly, the titration curve showed a slight deviation to a one-electron Nernst equation (dotted line). In the VC-F chimeric RC the results were significantly different from that in *Rvi. gelatinosus* WT: i) the deviation to the one-electron Nernst equation (dotted line) was more pronounced, ii) the overall midpoint potential was lower by about 50 mV.

Fig. 3 Several non-exclusive hypotheses may account for such a difference. The first one relies on a strong heterogeneity amongst the RC’s. As a possible reason for such an heterogeneity, one may consider that the tetraheme subunit is lost in a fraction of RC’s during the preparation of the membrane fragments. To test this hypothesis we measured (Figure 3) the amount of long-lived $P^+$ under conditions where only the $c_{556}$ and $c_{559}$ hemes were reduced in the dark (Figure 3, squares) and under conditions where the high and low potential hemes were reduced in the dark (Figure 3, triangles). Whereas in the former case about 50% of $P^+$ was still present 200
μs after the flash, this fraction of long lived P+ was only 3 % when the low potential hemes were reduced. Under these latter conditions the free energy change associated with P+ reduction is expected to be large owing to the low midpoint potentials of the electron donor (see figure 1). Thus, after this equilibrium is reached (i.e. in the hundreds of μs time range, see figure 1), the amount of P+ remaining should be too low to be detectable. Conversely, in the eventual RC’s devoid of the tetraheme subunit, P+ is expected to decay via charge recombination i.e. in the tens of ms time range (23). Thus the amount of P+ still detectable at 200 μs after the actinic flash can be taken as an indication of the amount of RC’s with no tetraheme subunit. Moreover, the absorption changes measured 200 μs after the flash were indicative of the flash-induced oxidation of a low-potential heme (not shown). We take these results as an evidence that most of the RC’s (97%) have a bound and functional cytochrome subunit.

Another possible explanation of the different titration curves in the VC-F and WT RC’s is a structural modification of the protein matrix around the BChl dimer resulting from the chimeric association with the Blc. viridis cytochrome subunit. We consider this hypothesis as unlikely as well since the absorption changes associated with the formation of the P+ state in the VC-F mutant were similar to that observed with the WT Rvi. gelatinosus RC (Figure 4A). Further, EPR measurements performed on VC-F oriented membrane multilayer dried on Mylar sheets showed an angle dependence of the g_2 signal of the hemes identical to that obtained for Blc. viridis. Nitschke and Rutherford (24) assigned the EPR g_2 signals of each of the four hemes in the Bcl. viridis cytochrome on the basis of their respective E_m. They ascribed the g-value of 3.09 to the highest potential c_{559} heme, and g-values comprised between 3.29 and 3.32 to the other hemes. The global binding of the Bcl. viridis cytochrome onto the RC of Rvi. gelatinosus was investigated in the same way. Oriented multilayers of VC-F membrane fragments exhibited peaks at g-values of 3.1 and 3.3, with orientation
dependent intensities (Figure 4B). Polar plots of EPR signal amplitudes allowed one to orient the \( g_z \) of 3.1 along the 0° axis, whereas the maximum of the \( g_z = 3.3 \) line was found at 45° (Figure 4C). The \( g_z \) vector of a heme being perpendicular to the porphyrin ring, we concluded that the highest potential heme \( c_{559} \) was almost perpendicular (90°) to the membrane plane, as for \textit{Bcl. viridis} (24) (compare Figure 4C to Figure 7 in (24)). Moreover, the absorption spectra of the four hemes bound to the tetraheme subunit are conserved in the chimeric RC with respect to the WT \textit{Bcl. viridis} RC (see Figure 5A for a comparison of absorption changes associated with the oxidation of the \( c_{556} \) and \( c_{559} \) hemes in both strains). Furthermore, the \( E_m \)'s of the three lower potential hemes embedded in the cytochrome subunit are remarkably conserved when compared to those found in \textit{Bcl. viridis} (14). Based on the sensitivity of both the spectroscopic and redox properties of a redox cofactor to any significant alteration of its protein environment, we take these results as a strong indication in favor of a conserved structure.

As a third hypothesis which would account for the singular titration curve of the \( P^+/P \) couple in the VC-F strain, we would now like to consider the existence of a significant electrostatic interaction between the BChl dimer and its nearest redox-active neighbor: the \( c_{559} \) heme. Such an interaction between two close redox cofactors implies that the \( E_m \) of either one of the two cofactors depends on the redox state of its neighbor. In the present framework, one should thus consider the following scheme††:

\[
\begin{align*}
Pc & \xrightarrow{E_{pc}} P^+ c + e^- \\
Pc^+ & \xleftarrow{E_{pc}^-} P^+ c^+ + e^- \\
Pa & \xrightarrow{E_{pc}} Pc^+ + e^- \\
P^+ c & \xleftarrow{E_{pc}^-} P^+ c^+ + e^-
\end{align*}
\]
with
\[
\begin{align*}
\text{EP}(c^+) = E_{P(c)} + \Delta \Psi \\
\text{ERC}(P^-) = E_{c(P^-)} + \Delta \Psi
\end{align*}
\]

(5)

where \(E_{P(c)^+}\) and \(E_{P(c)}\) are the \(E_m\) of the \(P^+/P\) couple in the presence of the oxidized and reduced cytochrome, respectively, \(\Delta \Psi\) is the electrostatic interaction between both cofactors and \(E_{c(P^+)}\) and \(E_{c(P^-)}\) are the \(E_m\) of the cytochrome in presence of \(P^+\) and \(P\), respectively. The equation used to fit the titration curve is then derived from both following points:

1. The total amount of oxidized \(P^+\) or of reduced \(P\) can be written as :

\[
[P^+] = [P^+ c^+] + [P^+ c]
\]

(6)

\[
[P] = [P c^+] + [P c]
\]

(7)

2. Applying the Nernst equation at a given ambient potential \(E_h\) to the four electron transfer reactions just described yields:

\[
\frac{[P^+ c]}{[Pc]} = 10^{\frac{E_{A^+} - E_{P(c)} - \Delta \Psi}{60}}
\]

(8)

\[
\frac{[P^+ c^+]}{[Pc^+]} = 10^{\frac{E_{A^+} - E_{P(c)} - \Delta \Psi}{60}} = 10^\frac{E_{A^+} - E_{P(c)} - \Delta \Psi}{60}
\]

(9)

\[
\frac{[P c^+]}{[Pc]} = 10^{\frac{E_{A^-} - E_{c(P^-)}}{60}}
\]

(10)

\[
\frac{[P^+ c^+]}{[P^+ c]} = 10^{\frac{E_{A^-} - E_{c(P^-)}}{60}} = 10^\frac{E_{A^-} - E_{c(P^-)} - \Delta \Psi}{60}
\]

(11)

Incorporating equations 8-11 into equations 6 and 7, at a given \(E_h\), the fraction of \(P\) in the oxidized state, irrespective of the redox state of the cytochrome is:

\[
\frac{[P^+]}{[P^+]+[P]} = \frac{10^{\frac{E_{A^+} - E_{P(c)}}{60}} + 10^{\frac{2E_{A^-} - E_{c(P)} - E_{P(c)} - \Delta \Psi}{60}}}{1 + 10^{\frac{E_{A^-} - E_{P(c)}}{60}} + 10^{\frac{E_{A^-} - E_{c(P)} - E_{P(c)} - \Delta \Psi}{60}}}
\]

(12)
This equation allows one to distinguish several cases: i) each of the two extreme ones where $E_{P(c)} \ll E_{c(P)}$ or $E_{c(P)} \ll E_{P(c^+)}$ yields a redox titration which follows a one electron Nernst curve (with $E_m = E_{P(c)}$ and $E_m = E_{P(c^+) + \Delta \Psi}$, respectively). This reflects the fact that the redox changes of each cofactor occur in well-separated redox potential ranges, they thus do not interfere. A third, and experimentally more interesting, case arises when $E_{P(c)} \approx E_{c(P)}$ or $E_{c(P)} \approx E_{P(c^+)}$ since, according to the above equation, the titration curve should be markedly different from a classical Nernst curve. This latter case applies to the VC-F strain. The data presented in figure 2 could be satisfyingly fitted with equation 12 (Figure 2 solid lines), with $E_{c(P)}$, $E_{P(c)}$ and $\Delta \Psi$ as varying parameters. In the VC-F strain the best fit parameters were: $E_{c(P)} = +380$ mV, $E_{P(c)} = +350$ mV and $\Delta \Psi = +50$ mV. In the \textit{Rvi. gelatinosus} RC, the fit with equation 12 yielded: $E_{c(P)} = +300$ mV, $E_{P(c)} = +350$ mV and $\Delta \Psi = +50$ mV. In \textit{Blc. viridis}, because the $E_m$’s of the two interacting cofactors belong to more distinct redox potential regions, the deviation to a one electron Nernst curve was, as expected, less pronounced. The data could be satisfyingly fitted either with a one electron Nernst curve with $E_P = +500$ mV or according to equation 12 with $E_{c(P)} = +380$ mV, $E_{P(c)} = +450$ mV and $\Delta \Psi = +50$ mV. It is noteworthy that the values found for the $E_m$ of the $P^+/P$ couple as well as for $\Delta \Psi$ were similar in the chimeric and ‘parent’ RC from \textit{Rvi. gelatinosus}. Yet, one could argue that the value found here for the $E_m$ of the $P^+/P$ couple in \textit{Rvi. gelatinosus} WT is 50 mV smaller than the previously reported one of 400 mV. This is not unexpected since, in the WT, the $E_m$ of the closest heme to P is significantly lower than that of the $P^+/P$ couple so that the respective oxidation (or reduction) of the two cofactors occurs in distinct redox potential domains. Consequently, the most significant fraction of P should be oxidized in the presence of the oxidized cytochrome and the titration curve should yield, as a first approximation, a $E_m$ of $E_{P(c)} + \Delta \Psi = 350 + 50 = +400$ mV, in good agreement with previous reports (22).
To further test this interpretation, we have measured the equilibrium redox titration of the 
$P^+/P$ couple in a site-directed mutant of the VC-F strain in which the $E_m$ of the $c_{559}$ heme is 
expected to be decreased, thereby increasing the gap between the $E_m$’s of $P$ and its closest 
heme. According to the calculation of Gunner and Honig, the presence of a positively charged 
arginine residue (R264) in the vicinity of the $c_{559}$ heme of the $Blc. viridis$ cytochrome subunit 
contributes to raise the midpoint potential of this redox center (7). This was confirmed by 
Chen et al. who substituted, by site-directed mutagenesis, this residue for a lysine, and found 
a downshifted $E_m$ for the $c_{559}$ by about 110 mV (26). We have also targeted this Arg and 
substituted it for a Leu in the gene coding for the tetraheme subunit in the VC-F strain (17). 
Consistent with the previous findings from Chen et al., the $E_m$ of the $c_{559}$ heme in this mutant 
(R264L) was decreased to 130 mV (17) but the downshift was significantly larger, as 
extected from the less conservative mutation of the Arg residue into Leu rather than Lys. 

Figure 2 (right panel) shows a comparison of the equilibrium redox titration curve of $P$ in the 
$Rvi. gelatinosus$, VC-F WT and R264L mutant strain (open circles, solid circles and triangles 
respectively). In line with the expectation that can be drawn from equation (12) the apparent 
midpoint potential of $P$ was upshifted in the R264L mutant with respect to the native VC-F 
RC and no significant deviation to a one electron Nernst curve was observed. This result is 
fully consistent with the present model according to which the deviation to a one electron 
Nernst that witnesses the electrostatic interaction between two close redox centers becomes 
less prominent when increasing the difference between the $E_m$ of the two centers. 

As a further support to our model, the $E_m$’s of the hemes that were found to interact 
electrostatically with $P$ when fitting the data with equation 12 ( +300 mV for $Rvi. gelatinosus$ 
WT and +380 mV for the VC-F strain) are consistent with the figures found in the literature 
for the closest hemes in the $Rvi. gelatinosus$ or $Blc. viridis$ cytochrome subunit, respectively 
(see Figure 1 for the data on the redox centers in the RC of these two species). We thus
propose that the peculiar redox titration curve observed for the $P^+/P$ couple reflects a significant electrostatic interaction between $P$ and the $c_{559}$ heme. One also expects the redox titration of the $c_{559}/c_{559}$ couple to be reciprocally affected by this interaction. To test this hypothesis we performed the dark equilibrium titrations of both $c_{559}$ and $c_{556}$ hemes in *Bleu. viridis* and VC-F RC’s.

**Redox characteristics of the exogenous cytochrome**

Absorbance changes were monitored in the $\alpha$-band region of the cytochromes (Figure 5). The spectra in panel A show the characteristic features of the oxidation spectra of the $c_{556}$ and $c_{559}$ hemes, respectively (11,25). They were found similar in the VC-F and *Bleu. viridis* cases. In both cases, the titration curves of the $c_{556}$ and $c_{559}$ hemes were derived from the spectra at different $E_h$: i) for the $c_{556}$ by taking the difference between 556 nm and 562 nm, which cancels the $c_{559}$ contribution (Figure 5B), ii) for the $c_{559}$ by taking the difference between 558 nm and 552 nm to cancel the contribution of $c_{556}$ (Figure 5C). The titration curve of the $c_{556}$ heme was satisfyingly fitted by a one electron Nernst equation and a $E_m$ of $+310$ mV in both the chimeric VC-F and WT *Bleu. viridis* case, in agreement with previous reports for the *Bleu. viridis* cytochrome subunit (11,25). But the $E_m$ of the $c_{559}$ heme was $430$ mV in the VC-F RC to be compared to the $+380$ mV found with *Bleu. viridis* RC (this latter value being similar to previous findings (13,25)). This 50 mV upshift matches nicely our expectations since, in the case of the VC-F strain, the $E_m$ of the $P^+/P$ couple was found lower than that of the $c_{559}$ heme. Thus, the majority of this heme should be oxidized (or reduced) in the presence of the oxidized form of $P$. Its apparent $E_m$ is thus expected to be: $E_{(P^+)\Delta \Psi} = 380 + 50 = +430$ mV, in agreement with the results shown in figure 2. We note however that the titration curve of $c_{559}$ does not display a similar deviation to a one electron Nernst curve as observed in the $P^+/P$
titration curve. This possibly arises from the difficulty to accurately cancel the contribution of the \(c_{556}\) heme in the titration curve of the \(c_{559}\) heme.

**Kinetic analysis of the electron transfer reactions**

From the redox titration we came to the conclusion that, under conditions where only the low potentials hemes are oxidized, the redox potentials of the \(P^+/P\), \(c_{559}^{+}/c_{559}\) and \(c_{556}^{+}/c_{556}\) are: +350 mV, +380 mV and +310 mV, respectively. Consequently, the reduction of \(P^+\) by the \(c_{559}\) heme is expected to be slightly uphill in energy and the oxidation of the \(c_{556}\) heme by \(P^+\) to be only weakly downhill. These two features may be addressed by studying the flash-induced absorption changes. Indeed, a single turnover flash given to a dark adapted sample results in the injection of a single oxidizing equivalent into the system and the time dependent distribution of this charge between the various redox centers is expected to depend on their respective midpoint potential.

Fig. 6 We first studied electron transfer reactions with membrane fragments poised at +200 mV (Figure 6A). Under such conditions both \(c_{556}\) and \(c_{559}\) hemes are reduced before the flash. Whereas in *Blc. viridis* the reduction of \(P^+\) occurs in the sub-\(\mu\)s time range (11,25), most of this reaction occurred in the chimeric RC with a half time of 14 \(\mu\)s. The \(c_{556}\) heme oxidation occurred concomitantly (t\(_{1/2}\) ~12 \(\mu\)s) whereas the transient oxidation of the \(c_{559}\) was hardly detectable.

To check the involvement of the \(c_{559}\) in the oxidation of the \(c_{556}\) heme by \(P^+\), we measured the kinetics of absorption changes poising the redox potential at +380 mV (Figure 6B). Under these conditions, owing to their respective \(E_{m}\)'s, a significant fraction of RC’s should be in the \(Pc_{559}c_{556}^+\) state (~32 %) before the actinic flash. In those RC’s the electron transfer step between \(P^+\) and the \(c_{559}\) heme should be clearly detectable. At 100 and 200 ns, the broad absorption increase reflects the photo-oxidation of P (see (13)). In the 500 ns to 5 \(\mu\)s time
range, a trough is observed with a minimum at 559 nm, suggesting the oxidation of the $c_{559}$ heme by the $P^+$. From 10 µs to 50 µs the trough shifts to a lower wavelength, consistent with inter-heme $c_{556}$-$c_{559}$ electron transfer. The kinetics monitored at various wavelengths was globally fitted with two exponentials. The half times of the fast and slow phases were found to be 700 ns and 11 µs, respectively.

One expectation that ensues from the redox titration is that the oxidation of the $c_{559}$ by $P^+$ should be energetically uphill. This prediction is supported by the present data. The fact that the $c_{559}$ oxidation step cannot be easily discriminated from the $c_{556}$ oxidation step suggests that the fraction of centers in the $P_{c_{559}}^+$ state in equilibrium with the $P^+c_{559}$ state is small. In the likely kinetic scheme describing the overall electron transfer reaction between $P^+$ and the $c_{556}$ heme, one can consider a two step process, where $k_1$, $k_1^-$, $k_2$ and $k_2^-$ are the forward and backward absolute rate constants of steps 1 and 2, respectively:

$$
(P^+, c_{559}, c_{556}) \xrightarrow{k_1, k_1^-}\ (P, c_{559}^+, c_{556}) \xleftarrow{k_2, k_2^-} (P, c_{559}, c_{556}^+)
$$

According to the $E_m$ of the three redox cofactors, the first step is energetically uphill and the second one is downhill. If, in addition, as in the case of *Blc. viridis*, $k_2<k_1$ (see figure 1), one expects the transiently formed $P_{c_{559}}^+c_{556}$ to be hardly detectable. Interestingly, decreasing the relative amplitude of the second step by e.g. increasing the redox poise in order to oxidize the $c_{556}$ heme should enhance the relative amplitude of the first step, facilitating its detection in good agreement with the present data.

The unusually slow oxidation of the $c_{556}$ heme found in the VC-F RC with respect to the parents RC also supports the occurrence of an uphill step in the overall electron transfer from the $c_{556}$ to $P^+$ *via* the $c_{559}$ heme. In *Blc. viridis* the apparent rate of the oxidation of the $c_{556}$ heme is $\sim 2.7 \times 10^5 s^{-1}$ (11,13), to be compared with the $6.3 \times 10^4 s^{-1}$ value found in the chimeric RC. As a first approximation, the apparent rate of oxidation of the $c_{556}$ heme is, according to the above scheme, $k_{app} = k_2/(1+k_1/k_1)$. Assuming that the absolute rate of electron transfer
between the two hemes \((k_2)\) is unchanged in the chimera relative to \textit{Blc. viridis} (an assumption which is supported by the above conclusion on the structural close similarities between both RC’s), and taking \(k_{-1}/k_1\) to \(10^{30/60} = 3.2\) (corresponding to the \(\Delta E_m\) between P and \(c_{559}\) of \(-30\) mV found here); one expects the apparent \(c_{556}\) oxidation rate to be \(k_{\text{app}} = 6.4 \times 10^4\) s\(^{-1}\), in agreement with the value of \(6.3 \times 10^4\) s\(^{-1}\) found here.

**Discussion**

Two main features arise from the combined study of the chimeric RC by either equilibrium redox titration or kinetic analysis: whereas the physico-chemical properties of the hemes embedded in the cytochrome subunit are remarkably conserved with respect to the parent species \textit{Blc. viridis}, the \(E_m\) of the P\(^+/P\) couple is down-shifted in the chimeric RC. Further, an electrostatic interaction of \(50\) mV raises the \(E_m\) of the P\(^+/P\) couple when the \(c_{559}\) is oxidized. Although the situation where this interaction can be directly observed, as in the present case, by the deviation to a one-electron Nernst curve is singular, we would like to argue that the existence of electrostatic interaction between nearby redox centers is likely to apply as well in other multi redox cofactors membrane proteins. Amongst these, the “parent” RC’s of the chimera should be first considered. Interestingly, the redox titration of the P\(^+/P\) couple in WT \textit{Rvi. gelatinosus} also displayed a significant deviation to a \(n=1\) Nernst curve. As discussed above, the deviation can be accounted for by an electrostatic interaction of \(50\) mV between P and the closest high-potential heme. Further it is noteworthy that the less pronounced deviation in the WT \textit{Rvi. gelatinosus} with respect to the chimeric RC is not an indication of a smaller electrostatic interaction. On the contrary, it can be rationalized by the combined effect of a larger difference in the \(E_m\) of P and its closest heme and a significant electrostatic interaction between these two redox centers. This point is illustrated by the disappearance of
the deviation to a one electron Nernst curve and a higher apparent $E_m$ for P in the R264L mutant of the VC-F RC where the $E_m$ of the closest heme to P has been downshifted. The $E_m$ of the $c_{559}$ heme being lower in the R264L mutant than in the native VC-F, the redox changes of P occur in a potential range where the closest heme is fully oxidized. As a consequence the electrostatic interaction between these two redox centers contribute to upshift the apparent $E_m$ of P when compared to the native VC-F RC. In *Blc. viridis*, the resolution of the three dimensional structure (9) and the determination of the order of the hemes in the cytochrome subunit (24,27,28) grounded several studies which emphasized the role of inter-cofactors electrostatic interaction. Based on the finding that the oxidation of the outermost solvent-exposed hemes was moderately electrogenic, Gao *et al.* estimated the interaction between $P^+$ and the $c_{559}$ heme in *Blc. viridis* to be 30 mV (29), in qualitative agreement with our finding. When their three dimensional structure is known, proteins are liable to electrostatic calculation. The Poisson-Boltzmann equation may be solved numerically and yield electrostatic interaction between cofactors (see e.g. (1,30,31)). Gunner and Hönig performed such calculations with the *Blc. viridis* RC and calculated the shift in $E_m$’s undergone by the four hemes relative to solution (7). Electrostatic interactions between the four hemes of the tetraheme subunit ranged from 5 to 77 mV (7). The $P^+/P$ couple was not treated in this study, likely because of the lack of reliable redox titration of this cofactor in solution owing to its peculiar dimeric character. Yet, based on a kinetic analysis of the reduction of $P^+$ by the $c_{559}$ in whole cells, Baymann and Rappaport concluded that the equilibrium constant of this reaction was significantly lower than predicted from the difference in $E_m$ of the two couples. The rationale for this apparent discrepancy was proposed to be an electrostatic interaction of 80 mV between these two cofactors (13).

As an example pertaining to respiratory chains the Succinate Quinol Reductase binds three iron sulfur clusters which are thought to act as the entry path for electron. Interestingly, as
determined by redox titration, the second cluster in the chain has a remarkably low redox potential (–250 mV) when compared to those of its two neighbors (–25 and –60 mV, (32)). If this $E_m$ corresponds to the operating potential of this cofactor, it would make its reduction highly endergonic (see (33) for a review). Although, an energetically uphill step in an overall exergonic chain is not, per se, to be excluded, it has been proposed that this highly reducing $E_m$ reflects an anticooperative electrostatic interaction between redox centers rather than the “true operating” midpoint potential of this singular cluster (34).

The suggestion reported here that the $E_m$ of a redox cofactor depends on the redox state of its neighboring redox cofactor raises the question of the dielectric properties of the protein medium. This question has been extensively studied and the emerging picture is that the screening of the electrostatic interaction between charges is best described by an effective distance-dependent dielectric constant. This phenomenological description was detailed by Warshel and coworkers (see e.g. (35)) and experimentally supported by various groups (see (36) for the *Rhodobacter sphaeroides* reaction center case and references therein). By studying the shift in $E_m$ of the $P^+/P$ couple in *Rhodobacter sphaeroides* reaction center induced by mutations of ionizable groups at selected sites, Johnson and Parson concluded that, although proteins are usually thought as low dielectric medium, electrostatic interactions resulting from a change in the charge distribution around P are efficiently screened with an average screening factor of about 40 (36). With an electrostatic interaction of 50 mV between P and its closest heme located at 20 Å (center to center) we get, by applying the Coulomb’s law, an average screening factor of 16 which is significantly larger than the value of 2-4 often used in electrostatic calculations but yet significantly lower than the value found by Johnson and Parson, suggesting that the screening of buried charges is less efficient in the present case than in their work. Among the various reasons which may concur to this smaller screening factor are: i) the fact that we are dealing here with electrostatic interaction between redox
cofactors and not between ionizable side chains and a redox cofactor, ii) the likely larger shielding from the bulk resulting from the binding of the cytochrome subunit to the RC in the present case at variance with the *Rhodobacter sphaeroides* RC and/or, iii) the preservation of the membrane environment in the present study. In line with this, Maki et al. measured the redox potential of the $c_{559}$ heme in RC from the VC-F strain either embedded in their native membrane or solubilized in detergent and found a lower value in this latter case than in the former (14).

Obviously, the question of electrostatic interaction between cofactors should not be restricted to photosynthetic RC’s. In order to achieve electron transfer reactions with rates compatible with biological catalysis, the electron donor and acceptor are usually located within 15 Å apart (37), an edge-to-edge distance similar to the one between P and the closest high-potential heme, here. This principle is likely to apply to all the membrane bound proteins involved in bioenergetic processes.
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Footnotes

*Abbreviations: BChl bacteriochlorophyll, *Ble.* Blastochloris, E<sub>h</sub> ambient redox potential, E<sub>m</sub> midpoint redox potential, P primary electron donor, RC reaction center, r.u. relative units, *Rvi.* *Rubrivivax,* WT wild type.

†† the reduced and oxidized states of the cytochrome are noted c and c<sup>+</sup>, respectively for the sake of clarity. It does not mean that the absolute charge of the reduced cytochrome is null nor that it is equal to one for the oxidized state. Yet, the change in charge resulting from the oxidation is equal to one electron.
**Figure Legends**

**Figure 1:** schematic view of the bacterial reaction center of *Blc. viridis* and *Rvi. gelatinosus*. Midpoint redox potentials (\(E_m\)) are indicated for each species (see (10,13,25,38) for *Blc. viridis* and (10,22) for *Rvi. gelatinosus*). Electron transfer (E.T.) rates between cofactors (12,13), as well as orientation of the hemes relative to the membrane plane (determined by EPR spectroscopy (24)) are indicated for *Bcl. viridis*.

**Figure 2:** flash-induced equilibrium redox titration of the primary electron donor P
Flash-induced electrochemical redox titrations were performed at thermodynamic equilibrium by monitoring the light-induced absorbance changes at 605 nm (*i.e.* in the \(Q_x\) absorption band of bacteriochlorophylls), 50 ns after the saturating flash. The negative absorbance change, at this wavelength, is proportional to the amount of P that can be photo-oxidized and thus yields the amount of reduced P in the dark. Experimental data were fitted with a one electron Nernst curve (dashed lines) and with equation 12 (solid lines).
Left panel: *Blc. viridis* (closed squares) together with *Rvi. gelatinosus* WT and chimeric RC (open and closed circles respectively). Right panel: *Rvi. gelatinosus* WT (open circles), native chimeric RC (closed circles) and R264L mutant of the chimeric RC (triangles).

**Figure 3:** flash-induced kinetics of \(P^+\) reduction by the RC-bound cytochrome under moderately oxidizing potential (\(E_h \sim +200\) mV; ascorbate, squares) or low-potential conditions (\(E_h \sim 0\) mV; menadione+ dithionite, triangles). The redox potential of membrane fragments of the VC-F strain was poised by addition of ascorbate, menadione or sodium dithionite and monitored by a combined reference electrode.
**Figure 4:** A) difference absorption spectra of the primary electron donor P recorded in the Q_y band of the bacteriochlorophyll special pair: flash-induced oxidation of partially oxidized membranes of *Rvi. gelatinosus* (open squares) and VC-F (closed circles), 100 ms after the flash; and oxidized minus reduced purified VC-F RCs (solid line). B) EPR spectra recorded on oriented membrane multilayers of VC-F dried on mylar sheets. C) orientation dependence of the EPR signal amplitudes from panel C.

**Figure 5:** dark equilibrium redox titration of the high-potential hemes of VC-F and *Blc. viridis* RC. The absorbance changes were recorded in the α-band of the cytochromes. Panel A: difference absorption spectra of $c_{556}$ and $c_{559}$ oxidation with VC-F and *Blc. viridis* membrane fragments (symbols and lines, respectively). The $c_{556}$ and $c_{559}$ oxidation spectra were obtained by taking the difference between the spectra measured at +320 mV and +240 mV, and measured at +500 mV and + 400 mV, respectively. Panel B: titration curve of $c_{556}$ heme at 556 minus 562 nm with VCF (solid symbols) and *Blc. viridis* (open symbols) membrane fragments. Panel C: titration curve of $c_{559}$ heme at 558 minus 552 nm VCF (solid symbols) and *Blc. viridis* (open symbols) membrane fragments.

**Figure 6:** time-resolved spectra of the flash induced absorbance changes in the α-band of the cytochromes with VC-F membrane fragments. The redox potential was poised at +200 mV and +380 mV (left and right panel respectively). The experimental data (symbols) were fitted with a biexponential model and the computed values are represented as solid lines (see text for the amplitudes of the phases optimized by the global fit analysis).
