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Interaction Site for High-Potential Iron−Sulfur Protein on the Tetraheme Cytochrome Subunit Bound to the Photosynthetic Reaction Center of *Rubrivivax gelatinosus*†

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ABSTRACT: We have recently demonstrated, using site-directed mutagenesis, that soluble cytochromes interact with the *Rubrivivax gelatinosus* photosynthetic reaction center (RC) in the vicinity of the low-potential heme 1 (*c*-551, E_m = 70 mV) of the tetraheme cytochrome subunit, the fourth heme from the special pair of bacteriochlorophyll [Osyczka, A., et al. (1998) *Biochemistry 37*, 11732−11744]. Although the mutations generated in that study did not show clear effects on the electron transfer from high-potential iron−sulfur protein (HiPIP), which is the major physiological electron donor to the RC in this bacterium, we report here that other site-directed mutations near the solvent-exposed edge of the same low-potential heme 1, V67K (valine-67 substituted by lysine) and E79K/E85K/E93K (glutamates-79, -85, and -93, all replaced by lysines), considerably inhibit the electron transfer from HiPIP to the RC. Thus, it is concluded that HiPIP, like soluble cytochromes, binds to the RC in the vicinity of the exposed part of the low-potential heme 1 of the cytochrome subunit, although some differences in the configurations of the HiPIP−RC and cytochrome *c*−RC transient complexes may be postulated.

The photosynthetic reaction center complex (RC)[1] of purple bacteria participates in the primary steps of light-induced cyclic electron transfer, by catalyzing a transmembrane charge separation between the photooxidized special pair of bacteriochlorophyll and a system of quinone electron acceptors. In most species, the oxidized special pair is rapidly rereduced by the hemes of the RC-bound tetraheme cytochrome subunit (*1*). The subsequent reduction of the cytochrome subunit occurs with the participation of periplasmic soluble electron carriers, which shuttle electrons from the cytochrome bc_1 complex to the RC. Depending on the species, these mobile mediators are cytochromes (cytochrome c_2, cytochrome c_8) and/or high-potential iron−sulfur protein (HiPIP) (*2*, *3*).

The formation of a functional transient complex between soluble electron carriers and the RC is achieved through the specific recognition of the binding domains located on the surface of two interacting proteins. The three-dimensional structure of the *Blastochloris* [formerly called *Rhodopseudomonas* (*4*)] *viridis* RC revealed that all four hemes of the RC-bound cytochrome subunit are located close enough to the surface to accept electrons from mobile electron donors (*5*, *6*). We recently identified the region of the surface of the

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[1] Abbreviations: RC, reaction center; HiPIP, high-potential iron−sulfur protein; E_m, redox midpoint potential; DAD, 2,3,5,6-tetramethylphenylenediamine; PCR, polymerase chain reaction.

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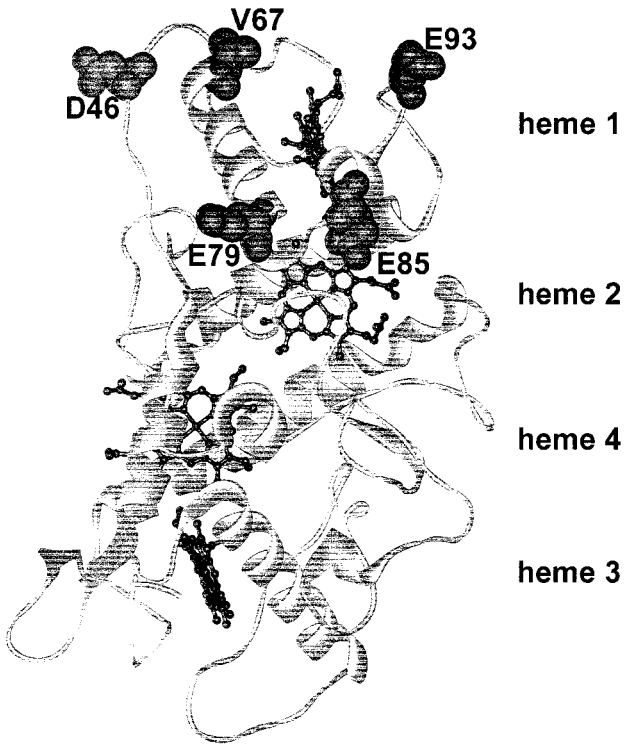

FIGURE 1: Ribbon diagram of the modeled structure of *Rvi. gelatinosus* RC-bound cytochrome subunit. The mutated residues in the vicinity of the solvent-exposed edge of low-potential heme 1 are shown as a space-filling model. The heme arrangement (represented as a ball-and-stick model) is oriented vertically, with the bottom closest to the membrane (heme 3 closest to the special pair of bacteriochlorophyll). The coordinates were taken from the 3-D model built for *Rvi. gelatinosus* on the basis of the crystallographic structure of *Blc. viridis* RC as described in (*7*).

subunit involved in the electrostatically controlled binding of soluble cytochromes by introducing a series of site-directed mutational charge substitutions to the *Rubrivivax gelatinosus* RC (*7*). In this species, the cytochrome subunit exhibits a high degree of homology with *Blc. viridis* cytochrome (*8, 9*), which rationalized the structural predictions based on the 3-D structure of the *Blc. viridis* RC (see model in Figure 1). The introduced mutations have shown that the interaction between bound and soluble cytochromes occurs in the region near the solvent-exposed edge of the low-potential heme 1 [*c*-551, $E_m = 70$ mV (*10*)], the most distant heme from the special pair [in *Blc. viridis* it corresponds to heme *c*-554, $E_m = -60$ mV (*11*)]. The site-specificity of the electrostatic effects observed for individual single mutations indicated that the acidic residues forming a cluster around the heme 1 participated in the binding, and that this process was primarily controlled by E93 and E79, and facilitated by the contribution of E85 and D46 (for the positions of these residues, see Figure 1). The double mutants E93K/E85K and E79K/E85K, which showed additive inhibitory effects, provided additional evidence for the proposed site of interaction. Using the same set of mutants, we also observed that the reactivity of HiPIP, which is the main physiological electron donor to the *Rvi. gelatinosus* RC (*12*), was not influenced by any of the mutated positions to the extent that would allow us to identify its binding site (*7*). This was interpreted as an indication that the binding of this protein might occur in a different region of the subunit, or that even if it did occur in the region around heme 1, it would be controlled by a different set of amino acids or by factors other than electrostatics.

In this study, we further examined the region around the solvent-exposed edge of heme 1 of the subunit with the aim of verifying its possible involvement in the binding of HiPIP. We found that two, newly generated mutations, a triple mutation E79K/E85K/E93K (glutamate-79, -85, and -93, all replaced by lysines) and a single charge substitution V67K (valine-67 to lysine) (Figure 1), considerably affect the electron transfer from HiPIP to the RC, indicating that the vicinity of heme 1 participates in the formation of the HiPIP−RC transient complex.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Rvi. gelatinosus strains with the mutated RC-bound tetraheme cytochrome subunit were generated and analyzed as described in (*7*). Site-specific mutagenesis was performed on the basis of the Kunkel method, as implemented in the Mutan-K mutagenesis kit (Takara Shuzo Co., Ltd.) using the pGI7-1 plasmid (*7*) as the template DNA. The single mutation V67K was introduced into the *puf*C gene using the oligonucleotide 5′-CGTGAACTCCGCTTTCGACAGATGG-3′ (mismatch position is underlined). The template with the triple-point mutation E79K/E85K/E93K was constructed by the addition of a single mutation E79K to the pGI7-1 plasmid containing E93K and E85K using the oligonucleotides described in (*7*). Mutations and the sequence integrity of the entire *puf*C gene were further confirmed by DNA sequence analysis. The mutated pGI7-1 plasmids were incorporated into the genomic DNA of the ΔC deletion background (*7*), and the presence of desired mutations was confirmed by DNA sequence analysis of the *puf*C gene amplified by PCR from the genomic DNA of mutated strains.

Membranes, Proteins, and Kinetic Measurements. Membrane fractions containing the wild-type and mutated RC complexes were prepared as described (*7*, *13*). *Rvi. gelatinosus* HiPIP was isolated and purified as described (*13*). Horse mitochondrial cytochrome *c* (type VI) was from Sigma.

Xenon-flash-induced absorbance changes accompanying electron transfer were recorded using a single-beam spectrophotometer assembled in our laboratory (*14*). Experiments were performed aerobically in 10-mm path length cuvettes with 2 mM MOPS−NaOH (pH 7) (for the reaction with HiPIP) or 2 mM Tris-HCl (pH 8) (for the reaction with cytochrome *c*) containing 20 μM DAD and 0.1 mM sodium ascorbate. The concentration of membranes was adjusted to $A_{850} = 1.0$. The reduction of bound cytochrome was followed by monitoring at wavelengths of 555 or 556 nm for the reaction with HiPIP or cytochrome *c*, respectively.

RESULTS AND DISCUSSION

Both E79K/E85K/E93K and V67K, mutations which are located near the solvent-exposed edge of heme 1 (Figure 1), significantly affected the reaction of the cytochrome subunit not only with soluble cytochrome *c* but also with the main physiological electron donor, HiPIP. The effects of these mutations on the reactivity of HiPIP are shown in Figure 2. The wild-type cytochrome was efficiently reduced by HiPIP with a second-order rate constant of 2.8×10^8 M^{-1} s^{-1}

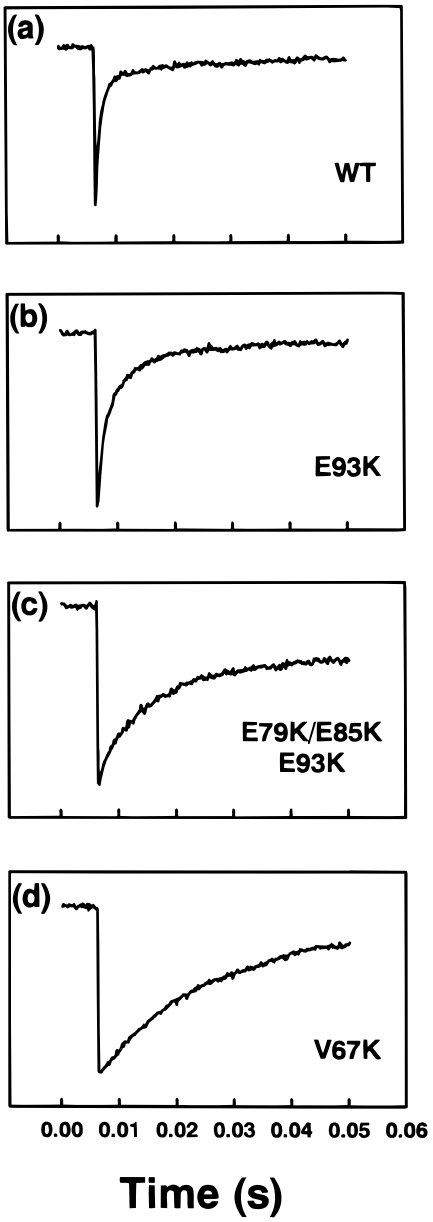

FIGURE 2: Single-flash-induced oxidation and reduction of RC-bound hemes measured at 555 nm for the reaction of wild-type (a) and mutated (b−d) tetraheme cytochrome subunits of *Rvi. gelatinosus* RC with 3 μM *Rvi. gelatinosus* HiPIP. All traces are plotted on the same time resolution scale. The height of each panel corresponds to ΔABS of 2.0×10^{-3}. Buffer solution contained 2 mM MOPS−NaOH (pH 7), 20 μM DAD, and 0.1 mM sodium ascorbate.

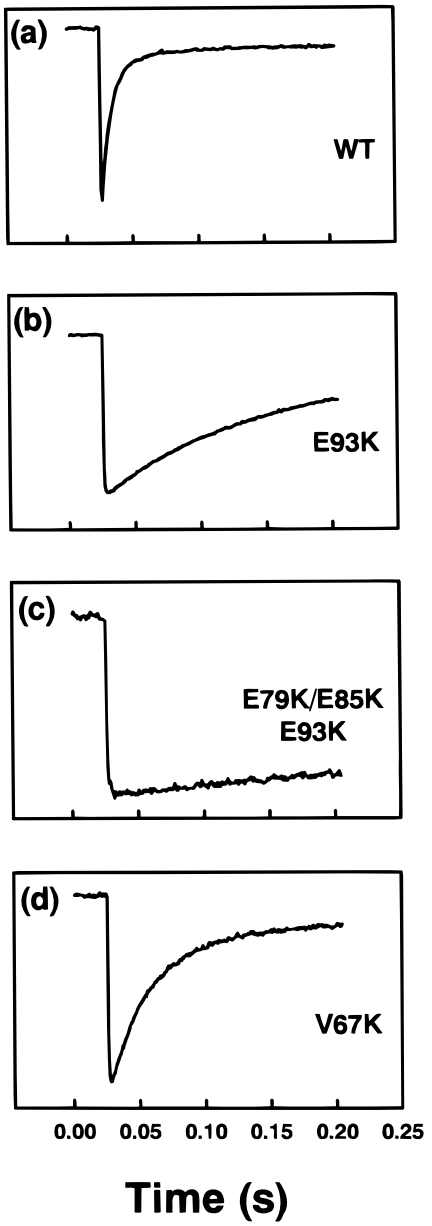

FIGURE 3: Single-flash-induced oxidation and reduction of RC-bound hemes measured at 556 nm for the reaction of wild-type (a) and mutated (b−d) tetraheme cytochrome subunits of *Rvi. gelatinosus* RC with 40 μM mitochondrial horse cytochrome *c*. All traces are plotted on the same time resolution scale. The height of each panel corresponds to ΔABS of 1.8×10^{-3}. Buffer solution contained 2 mM Tris-HCl (pH 8), 20 μM DAD, and 0.1 mM sodium ascorbate.

(Figure 2a). The triple mutant (E79K/E85K/E93K) reacted with a second-order rate constant of 5.6×10^7 M^{-1} s^{-1} (Figure 2c), whereas the single mutant V67K was reduced with a rate of 1.6×10^7 M^{-1} s^{-1} (Figure 2d). Apparently, the single substitution V67K (net change of one charge) resulted in much larger inhibition than the triple substitution E79K/E85K/E93K (net change of six charges). Single mutations at positions E79, E93, and E85, as representatively shown for the mutant E93K (Figure 2b), had no certain effects on the reaction with HiPIP, and the situation was the same in the case of double mutants E93K/E85K and E79K/E85K. Mutations in the regions where other hemes of the subunit are exposed did not show any effect either. These mutations included: E112K and D276H for the vicinity of the high-potential heme 2 (*c*-555, $E_m = 300$ mV), E197K for the high-potential heme 3 (*c*-555, $E_m = 320$ mV), and E314K for the low-potential heme 4 (*c*-551, $E_m = 130$ mV) (data not shown).

The effects of the single mutation V67K and the triple mutation E79K/E85K/E93K on the electron transfer from mitochondrial cytochrome *c* are shown in Figure 3. The reduction of bound cytochrome, which in the wild type occurs with the second-order rate constant of 4.5×10^6 M^{-1} s^{-1} (Figure 3a), was almost completely prevented in the triple mutant (Figure 3c). On the other hand, the mutant V67K reacting with the second-order rate of 1.0×10^6 M^{-1} s^{-1} (Figure 3d) inhibited the reaction to an extent comparable with the effect previously observed for mutation E85K (*7*).

Mutations at positions E79 and E93, as representatively shown for E93K (Figure 3b), have already been demonstrated to inhibit the reaction with horse cytochrome *c*, as expected for the change of two net charges (glutamate to lysine) in the interaction domain (*7*).

The results obtained with mutants V67K and E79K/E85K/E93K indicate that the vicinity of the heme 1 of the subunit forms the binding domain for both HiPIP and soluble cytochromes (Figure 1). In the case of HiPIP, it is demonstrated by the strong inhibition observed for mutant V67K and supported by the inhibitory effect of triple mutant E79K/E85K/E93K. In the case of mitochondrial cytochrome *c*, the effects of these mutations are fully consistent with the effects previously observed for single and double mutants of negatively charged amino acids in the vicinity of heme 1 (*7*) and provide additional evidence for the existence of the cytochrome *c* binding site in this region.

A comparison between the kinetic behaviors of HiPIP and cytochrome *c* in the reaction with mutated RCs allows us to conclude that the cluster of acidic residues around heme 1 may play a less dominant role in the binding of HiPIP than it does in the binding of soluble cytochromes. The effects of individual mutations observed in the former reaction are much smaller than expected, if HiPIP was to form the Glu/Asp-Lys salt bridges upon the binding to the RC, as it is suggested and experimentally supported to occur during the binding of soluble cytochromes (*7*, *15*−*19*). The mutation of any acidic residue from the cluster (shown here for E93K) largely inhibits the reaction with cytochrome *c* (Figure 3b), while it has only a minor effect on the rate of electron transfer from HiPIP (Figure 2b). However, a significant inhibition of the reaction with HiPIP can be observed when the acidic cluster is entirely removed (Figure 2c), under which circumstances the binding of cytochrome *c* and its electron donation to the RC hardly occur (Figure 3c). Thus, it is possible that the cluster may facilitate the electrostatic recognition of HiPIP but not stabilize the final docking through the formation of ion pairs. On the other hand, a large inhibitory effect of V67K (Figure 2d) indicates that the surface in the vicinity of V67 (top left to heme 1 as viewed in Figure 1) is more dominantly involved in the recognition of HiPIP. Furthermore, the mutation V67K appeared to have a much more pronounced effect on the reactivity of HiPIP than it had on the reactivity of cytochrome *c* (V67K reacted over 1 order of magnitude more slowly with HiPIP, but only 4 times more slowly with cytochrome *c*). This observation, together with the difference in the extent of inhibition caused by the triple mutant E79K/E85K/E93K, suggests the possibility that the binding of HiPIP to the RC and the binding of cytochrome *c* to the RC are primarily controlled by different sets of amino acids. Since the region in the vicinity of V67 is occupied mainly by uncharged residues (see the model shown in Figure 7c, ref. *7*), it can be anticipated that the recognition of HiPIP occurs due to the complementation of delocalized electrostatic potentials of the binding domains of HiPIP and the RC, according to the mechanism proposed by Tiede et al. (*20*), and/or that factors other than electrostatics (distance and orientation, hydrophobic forces, van der Vaals interactions) contribute more significantly to the formation of the HiPIP−RC transient complex. To clarify the nature of the HiPIP−RC interaction, further experiments employing the RCs with mutations near the solvent-exposed edge of heme 1 are currently in progress.

It should be noted that the mutations shown in this study have the potential to induce some perturbations in the structural integrity of the cytochrome subunit. While we cannot exclude this possibility, the structural effects are expected to be minor with respect to the identification of the electron donor binding site, considering that all altered residues are located on the surface of the protein with the side chains facing the aqueous phase, and that all mutants exhibited no difference in the pattern of flash-induced oxidation of the bound cytochrome subunit.

In conclusion, we have shown that the vicinity of the low-potential heme 1 of the RC-bound tetraheme cytochrome subunit forms the binding site for both HiPIP and soluble cytochromes. Considering that HiPIP is the main physiological electron donor to the RC in *Rvi. gelatinosus* (*12*), this finding reinforces the idea that heme 1 accepts the electron from soluble periplasmic electron carriers and is actively involved in the cyclic photosynthetic electron-transfer pathway toward the photooxidized special pair of bacteriochlorophyll (*7*, *21*). HiPIP has been found to fulfill the role of cytochrome c_2 as an electron donor to the tetraheme cytochrome subunit in some other species belonging to the β-subclass [*Rhodocyclus tenuis* (*22*), *Rhodoferax fermentans* (*23*, *24*)], and is expected to act this way in many other species, particularly in those which lack soluble cytochrome c_2 in the periplasm (*2*, *3*, *25*). The observation that both types of periplasmic electron carriers, cytochrome *c* and HiPIP, interact with the *Rvi. gelatinosus* RC in the region near the exposed part of heme 1 of the cytochrome subunit suggests the possibility that this heme forms the binding site for soluble electron donors in all RC-bound tetraheme cytochromes of photosynthetic bacteria.

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