Different Mechanisms of the Binding of Soluble Electron Donors to the Photosynthetic Reaction Center of *Rubrivivax gelatinosus* and *Blastochloris viridis*

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The tetraheme cytochrome subunits of the photosynthetic reaction centers (RCs) in two species of purple bacteria, *Rubrivivax gelatinosus* and *Blastochloris (Rhodopseudomonas) viridis*, were compared in terms of their capabilities to bind different electron-donor proteins. The wild-type RCs from both species and mutated forms of *R. gelatinosus* RCs (with amino acid substitutions introduced to the binding domain for electron-donor proteins) were tested for their reactivity with soluble cytochromes and high potential iron-sulfur protein. Cytochromes from both species were good electron donors to the *B. viridis* RC and the *R. gelatinosus* RC. The reactivity in the *R. gelatinosus* RC showed a clear dependence on the polarity of the charges introduced to the binding domain, indicating the importance of the electrostatic interactions. In contrast, high potential iron-sulfur protein, presumed to operate according to the hydrophobic mechanism of binding, reacted significantly only with the *R. gelatinosus* RC. Evolutionary substitution of amino acids in a region of the binding domain on the cytochrome subunit surface probably caused the change in the principal mode of protein-protein interactions in the electron-transfer chains.

In purple bacterial photosynthesis, periplasmic, soluble electron carrier proteins mediate the electron transfer from the cytochrome bc1 complex to the reaction center (RC) complex. Two types of protein are known to perform this function, cytochromes (c,c) and/or high potential iron-sulfur proteins (HiPPIs). In some species (*e.g.* *Blastochloris (Rhodopseudomonas) viridis*, *Rhodospirillum molischianum*, *Rhodospirillum rubrum*) only one type of electron carrier is present. In other species (*e.g.* *Rubrivivax gelatinosus*, *Rhodocyclus tenuis*, and *Rhodobacter fermentans*) both cytochrome c and HiPIP operate as the electron mediators (1–7).

The RC of *R. gelatinosus* contains a tetraheme cytochrome subunit that is capable of accepting electrons from both HiPIP and cytochromes (2, 8). Using site-directed mutagenesis, we found that the binding domain for these two types of soluble electron carriers is formed by the vicinity of the surface-exposed edge of the low potential heme 1 of the subunit, the most distant heme from the special pair of bacteriochlorophylls (9–11). However, the mechanisms of the cytochrome c-RC interaction and the HiPIP-RC interaction appear to be different, even though the binding regions overlap. In the case of cytochrome c, the electrostatic interactions between the oppositely charged residues of the binding domains of cytochrome c (lysines) and the RC (aspartates and glutamates) are of primary importance (9), whereas the binding of HiPIP seems to be controlled mainly by the hydrophobic coupling of the encounter surfaces of the two proteins (10, 11). These two proposed models of interaction are consistent with the structural properties of the binding region. The surface around the exposed edge of heme 1 is formed not only by hydrophobic residues near the heme crevice but also by acidic residues that cluster the heme from a further distance. Clearly, both hydrophobic and electrostatic components contribute to the binding domain, thus explaining its ability to anchor both HiPIP and cytochrome c.

Even though an acidic cluster in the region around the heme 1 of the cytochrome subunit appears to be conserved in several species of purple bacteria (12), the relative contribution of the electrostatic and hydrophobic components of the putative binding domains seems to vary in different species. As a result, each domain has its own structural characteristics that contribute to the selectivity observed in some electron donor-RC interactions (13). For example, the RC of *B. viridis* was shown to accept electrons efficiently from some types of soluble cytochromes c but not from HiPPIs (13).

To obtain further insights into the mechanisms and evolution of the RC-soluble electron donor interactions, here we compare the tetraheme cytochrome subunits from *R. gelatinosus* (the wild-type and mutants) and *B. viridis* RCs in terms of their ability to bind and exchange electrons with different electron carrier proteins. The results provide an interesting example of evolutionary changes in the mechanism of protein-protein interaction caused by the changes in physicochemical characteristics of surface amino acids.

**EXPERIMENTAL PROCEDURES**

Genetic procedures for generation of *R. gelatinosus* strains with the mutated RC-bound tetraheme cytochrome subunit were described in Refs. 9–11. Membrane fractions containing the wild-type and mutated RC complexes of *R. gelatinosus* were prepared as described (9, 14), and the same procedure was used to obtain the membrane fractions from *B. viridis*. *R. gelatinosus* HiPIP and cytochrome c8 were isolated and...
purified as described (14). Cytochrome c<sub>2</sub> from *B. viridis* was purified according to established procedures (15).

Xenon flash-induced absorbance changes accompanying electron transfer were recorded as described in Refs. 9–11. Experiments were performed aerobically in 5 or 10-mm-path length cuvettes with 2 mM Tris-HCl (pH 8) containing 20 μM DAD and 0.1 mM sodium ascorbate. The concentration of membranes was adjusted to 2.0 μM DAD and 0.1 mM sodium ascorbate.

The model of *R. gelatinosus* cytochrome c<sub>R</sub> was built on the basis of the coordinates of the crystal structure of *Pseudomonas aeruginosa* cytochrome c<sub>cyt</sub> (16) using the amino acid sequence alignment described in Ref. 17. The modeling procedures were as described in Refs. 9 and 11.

RESULTS

Fig. 1 shows kinetic traces for the reaction of *B. viridis* cytochrome c<sub>2</sub> with the wild-type and mutated tetraheme cytochrome subunits of *R. gelatinosus* RC. In comparison to the wild-type RCs, which reacted with cytochrome c<sub>2</sub> with the second-order rate constant of 3.1 × 10<sup>-2</sup> M<sup>−1</sup>s<sup>−1</sup> (Fig. 1a), all mutants shown in this figure considerably accelerated the reaction rate. The single mutants V67E and R72E reacted with the second-order rate constant of 9.6 × 10<sup>−2</sup> M<sup>−1</sup>s<sup>−1</sup> (Fig. 1b) and 1.2 × 10<sup>−1</sup> M<sup>−1</sup>s<sup>−1</sup> (Fig. 1c), respectively. The double mutant V67E/R72E reacted with the rate of 2.6 × 10<sup>−1</sup> M<sup>−1</sup>s<sup>−1</sup> (Fig. 1d). Apparently, both V67E and R72E accelerated the reaction rate to a similar degree, and an additive effect was observed for V67E/R72E.

*B. viridis* cytochrome c<sub>2</sub> is a unique cytochrome that reacts faster with the mutants V67E, R72E, and V67E/R72E than with the wild-type RCs. Similar acceleratory effects were observed in the case of horse mitochondrial cytochrome c<sub>8</sub> (11) and yeast mitochondrial cytochrome c (data not shown). Moreover, all these cytochromes showed the highest reactivity in the reaction with V67E/R72E. The reactivity of *B. viridis* cytochrome c<sub>2</sub> with V67E/R72E remained, however, lower than its reactivity with its physiological electron acceptor, the *B. viridis* RC. As shown in Fig. 2a, the reaction of *B. viridis* cytochrome c<sub>2</sub> with the *B. viridis* RC occurred with the second-order rate constant of 1.0 × 10<sup>−1</sup> M<sup>−1</sup>s<sup>−1</sup>. For comparison, horse cytochrome c reacted with *B. viridis* RC slower than *B. viridis* cytochrome c<sub>2</sub> (second-order rate constant of 4.0 × 10<sup>−2</sup> M<sup>−1</sup>s<sup>−1</sup>, Fig. 2b) but similarly to its reactivity with the wild-type *R. gelatinosus* RC (9). On the other hand, the acceleration of the reaction rate caused by mutations V67E, R72E, and V67E/R72E was larger in the case of horse cytochrome c (one and two orders of magnitude for the single and double mutants, respectively) (11) than in the case of *B. viridis* cytochrome c<sub>2</sub> (up to one order of magnitude for the double mutant) (Fig. 1).

The concentration of membranes was adjusted to 88 μM soluble electron donors: *B. viridis* cytochrome (cyt) c<sub>2</sub> (a), horse mitochondrial cytochrome c (b), *R. gelatinosus* cytochrome c<sub>R</sub> (c), and *R. gelatinosus* HiPIP (d). All traces are plotted on the same time resolution scale. Reaction conditions were as in Fig. 1.

DISCUSSION

In our previous studies, we showed that the binding of soluble cytochromes to the RC-bound tetraheme cytochrome subunit in *R. gelatinosus* is primarily controlled by Glu<sup>79</sup> and Glu<sup>83</sup> and facilitated by the contribution of Glu<sup>85</sup> and Asp<sup>46</sup>, all of which form an acidic cluster surrounding the exposed part of the low potential heme 1 of the subunit (c<sub>8</sub>-type, midpoint redox potential (E<sub>1/2</sub>) = 70 mV) (9). The importance of the negative charge exposed in the binding region was indicated by the inhibitory effects of the mutations that replaced aspartates or...
glutamates with lysines or histidines. In these assays two types of cytochromes, horse mitochondrial cytochrome c and B. viridis cytochrome c2, displayed the same kinetic behavior, which suggested that they had the same binding mechanism (9). These findings and a high sequence homology between the R. gelatinosus and B. viridis subunits (18) supported the earlier proposal that in B. viridis the binding domain for cytochrome c2 may be formed by the homologous region around the heme 1 of the subunit (c, E_m = −60 mV) (19).

Considering the distribution of charged amino acids, the putative binding domain of B. viridis, consisting of Glu79, Glu85, Glu67, and Glu48 shows a high structural analogy to that of R. gelatinosus. With the exception of the region near the position 67 (note that in R. gelatinosus Glu67 is changed to Val67), similarities can also be seen in the delocalized electrostatic potentials of the encounter surfaces (Fig. 4, a and b). Furthermore, the mutational introductions of an additional negative charge to the R. gelatinosus binding domain (generation of the domains with the area of negative potential comparable or even larger than that in B. viridis; see Fig. 4) resulted in a significant acceleration of the reaction with horse cytochrome c (the effect of the mutations V67E, R72E, and V67E/R72E more efficiently than with the wild-type R. gelatinosus RC (Fig. 1). These results demonstrate that B. viridis cytochrome c2 displays in its reactivity with the RC a clear dependence on the polarity of charge introduced to the binding domain. This provides further evidence for the electrostatic mechanism of the binding and supports the idea that the docking site exists near heme 1 in the B. viridis tetraheme subunit (19, 20).

The electrostatic model of the binding assumes the involvement of attractive interactions between the lysines of cytochrome c and glutamates/aspartates of the RC. As shown in Fig. 5, the distribution of lysines encircling the heme crevice of B. viridis cytochrome c2 and horse cytochrome c is highly similar. This explains the similarities in their kinetic behavior in the reactions with the wild-type and mutated RCs.

In contrast to B. viridis cytochrome c2 and horse cytochrome c, R. gelatinosus cytochrome c2 displays a less uniform distribution of lysines on its encounter surface (Fig. 5), and this is also observed for other c2-type cytochromes (21, 22). The lysines accumulate only above and below the heme crevice (as viewed in Fig. 5), at a somewhat larger distance from the heme. This specific surface charge distribution is apparently optimized for the coupling with the R. gelatinosus RC (Fig. 3a), and introducing an additional negative charge to the RC counterpart (V67E) does not improve the interaction (Fig. 3b). On the other hand, an additional positive charge (V67K) produces a clear inhibitory effect (Fig. 3c) consistent with the effects of the mutations replacing glutamates/aspartates with lysines/histidines (9). This confirms that, as in the case of other soluble cytochromes, electrostatics are important in the binding of cytochrome c to the RC.

In describing the RC-cytochrome c interactions, it should be emphasized that factors other than electrostatics also influence the binding. This can be seen by comparing the binding domains of R. gelatinosus and B. viridis RCs in their reactions with the same soluble cytochromes. Although the mutants V67E, R72E, and V67E/R72E display similar or even higher negative charges of the binding domain in comparison with that of the putative B. viridis domain (Fig. 4), their reactivity with B. viridis cytochrome c2 (Fig. 1, b–d) still remains lower than the level of physiological interaction (Fig. 2a). Although
the B. viridis binding domain has an additional glutamate near heme 1 (Glu), horse cytochrome c reacts similarly with both B. viridis and R. gelatinosus RCs, whereas introducing glutamate at position 67 to the R. gelatinosus domain accelerates the reaction (11). On the other hand, R. gelatinosus cytochrome c, which has a less positive charge on its encounter surface (Fig. 5), reacts more efficiently with R. gelatinosus RC than with B. viridis RC (Fig. 3, a and d). Taken together, not only the net charge of the binding domains but also their structural compatibility appear to be important in the RC-cytochrome c interactions.

The comparison of the binding domains of soluble cytochromes shown in Fig. 5 accentuates the following tendency: the ratio of charged to hydrophobic residues decreases from horse cytochrome c to B. viridis cytochrome c and further to R. gelatinosus cytochrome c. This may have its consequence in the binding mechanisms. In particular, the hydrophobic interactions may be expected to play a significant role in the binding of R. gelatinosus cytochrome c to the RC (note that the mutations of charged residues in the RC binding domain had a less pronounced effect on the reactivity of cytochrome c than on the reactivity of other cytochromes (9)). If this is the case, the cytochrome c binding mechanisms may represent an evolutionary intermediate between the electrostatic mode of binding characteristic for some cytochromes and the hydrophobic mode characteristic for HiPIP (11).

We previously observed that the high efficiency of HiPIP in the reaction with R. gelatinosus RC is related to the presence of a large hydrophobic domain near the heme 1 of the subunit (mainly the region around Val) (11). It is therefore possible that the charged residues in the homologous region of the B. viridis binding domain, Glu and Glu, (Fig. 4) despair the binding of HiPIP and are responsible for its poor reactivity with the RC (Fig. 2d). On the other hand, these residues do not disturb the binding of cytochrome c, which may be related to the greater involvement of electrostatic interactions in the binding. It is remarkable that the effects of the mutations in the R. gelatinosus binding domain (V67E and V67K) on the reactivity of cytochrome c and HiPIP show a similar phenomenon; replacing Val by glutamate does not change the reactivity of cytochrome c (Fig. 3), whereas any charged residue at this position (glutamate or lysine) strongly inhibits the reaction with HiPIP (11). In this context, the disparity between the high efficiency of cytochrome c and poor reactivity of HiPIP in their reactions with the B. viridis RC (Fig. 2, c and d, respectively) may be interpreted as another reflection of two different binding mechanisms.

It should be noted that recently determined crystal structures of the RC and HiPIP from Thermochromatium tepidum provide additional structural information strengthening the notion that the molecular recognition between HiPIP and the RC differs from the electrostatic binding of cytochromes c to the RC (23). The T. tepidum cytochrome subunit and HiPIP both possess clear hydrophobic domains on their putative encounter surfaces, and therefore the docking of HiPIP to the RC is expected to occur mainly through hydrophobic interactions (23).

We have recently succeeded in introducing the gene of cytochrome subunit of B. viridis into R. gelatinosus cells and exchanging the native cytochrome subunit with that of B. viridis. In the transformant, the photosynthetic growth rate was about three times slower than that in the wild type. This, in agreement with the results of the present study, probably reflects that HiPIP, the major electron donor in the R. gelatinosus cells, is not a good electron donor to the B. viridis cytochrome subunit.

The comparison of the binding domains of tetraheme cytochrome subunit presented in this study extends our knowledge of general principles that govern protein-protein interactions in electron transfer systems. It emphasizes that these processes are fairly complex and are not always mediated by favorable electrostatic interactions (processes involving some cytochromes, mitochondrial cytochrome c and cytochrome c). Alternative mechanisms based on hydrophobic interactions (like in the case of HiPIP) or a combination of electrostatic and hydrophobic interactions (cytochrome c) may actually exist in corresponding proteins of different species. There is growing evidence that, relevant to our study, points toward these types of interactions (23–28). These variations of the mode of protein-protein recognition may be important for the selectivity of the interaction in the electron-transfer and other systems. First, they ensure the interactions in chains where different mobile electron carrier proteins interchangeably react with the same membranous complexes (for example HiPIP-cytochrome c, plastocyanin-cytochrome c). Second, they secure the stability and robustness of the systems while retaining their plasticity with respect to the evolutionary changes. Third, they provide the systems with potential sites of regulation.

Highly specific effects of mutations at position 67 in the R. gelatinosus binding domain indicate that the alterations in the properties of individual side chains (in this case from hydrophobic to charged) can promote the change in the binding mechanisms and the selectivity toward a soluble protein partner. As these effects correlate with the selectivity of binding observed in the B. viridis subunit (in which position 67 is naturally occupied by a charged residue), it can be suggested that evolutionary substitutions of amino acids in the region of the binding domain represent one possible pathway leading toward more effective protein-protein interactions.

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