Uncovering the [2Fe2S] domain movement in cytochrome bc₁ and its implications for energy conversion

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In crystals of the key respiratory and photosynthetic electron transfer protein called ubihydroquinone:cytochrome (cyt) c oxidoreductase or cyt bc₁, the extrinsic [2Fe2S] cluster domain of its Fe-S subunit assumes several conformations, suggesting that it may move during catalysis. Herein, using Rhodobacter capsulatus mutants that have modifications in the hinge region of this subunit, we were able to reveal this motion kinetically. Thus, the bc₁ complex (and possibly the homologous bcf complex in chloroplasts) employs the [2Fe2S] cluster domain as a device to shuttle electrons from ubihydroquinone to cyt c₁ (or cyt f). We demonstrate that this domain movement is essential for cyt bc₁ function, because a mutant enzyme with a nonmoving Fe-S subunit has no catalytic activity, and one with a slower movement has lower activity. This motion is apparently designed with a natural frequency slow enough to assure productive Qo site charge separation but fast enough not to be rate limiting. These findings add the unprecedented function of intracomplex electron shuttling to large-scale domain motions in proteins and may well provide a target for cyt bc₁ antibiotics.

Rhodobacter capsulatus | photosynthetic and respiratory electron transfer | mitochondrial complex III | protein domain motion | Rieske Fe-S subunit

W hen different crystal structures reveal dramatically different protein conformations, large amplitude domain movements are often inferred. However, in only a few cases such as myosin (1), flagellar motor (2), and ATP synthase (3, 4) have such movements been visualized. The cytochrome (cyt) bc₁ (or its cyt bc₁ counterpart in chloroplasts) is a key component of respiratory and photosynthetic electron transfer chains (5, 6). Recent crystal structures of the mitochondrial cyt bc₁ have revealed that the extrinsic [2Fe2S] cluster domain of the Fe-S subunit occupies various locations within this enzyme complex (7–10). It has been observed in either a position proximal to the ubihydroquinone (OH₂) oxidation catalytic site (Qo position) from which it takes electrons or a position close to cyt c₁ subunit (c₁ position) to which it donates electrons (Fig. 1). Because of the large distances observed between the electron-donating and electron-accepting cofactors of the cyt bc₁ in the different structures, no one of these locations can support sufficiently rapid electron tunneling (11) to meet the observed turnover rates (12, 13) and the specific substrate–product interactions (14) that occur at the OH₂ oxidation site. Thus, an unprecedented intracomplex electron shuttle motion to transfer electrons during catalysis has been suggested (8). However, neither the presumably essential movement nor the electron transfer associated with it has been visualized before this work.

In light-activated energy transduction systems, such as the one provided by the photosynthetic bacterium R. capsulatus, a short flash of light (<10-μs duration) can activate the photochemical reaction center, thereby inducing oxidation of two equivalents of cyt c (c₁) and a mixture of c₂/c₃ that are presented to each cyt bc₁ in about 50 μs (12, 13). The cyt bc₁ is then primed to complete its catalytic cycle (Fig. 2 Left A and B). Extensive electron paramagnetic resonance (EPR) spectroscopy data establish that the equilibrium position of the reduced [2Fe2S] cluster domain is located at the Qo position (14). Both the amount and rate of flash-oxidized cyt c reduction depend on the arrival of electrons via the initially reduced [2Fe2S] cluster and, later, from the millisecond oxidation of OH₂ at the Qo site (12, 13). Fig. 2 Left C–H depicts how the electrons might get from the Qo site to cyt c₁ if brought about by the movement of the [2Fe2S] cluster domain.

Fig. 2 Right also shows the effects of two powerful natural antibiotics, myxothiazol and stigmatellin, that are commonly used in the laboratory to inhibit the Qo site reaction in quite different ways. When myxothiazol is present, no OH₂ oxidation occurs, simply because myxothiazol displaces OH₂. In this case, cyt c reduction kinetics suggest that the initially reduced [2Fe2S] cluster can still move from the Qo site to the cyt c₁ position and contribute to cyt c reduction, which is due only partially to their similar redox potentials. However, this electron transfer from the [2Fe2S] cluster to cyt c₁ heme, which is now independent of the Qo site catalysis, has never been observed (Fig. 2 Right Top C and D). Thus, if the [2Fe2S] domain moves as proposed, it must occur on a time scale so as to be hidden within the 50-μs envelope required to activate the entire system. When stigmatellin is added, the [2Fe2S] cluster domain is trapped in the Qo position, as evidenced by EPR (15), crystallographic (8), and reconstitution (16) data. Under these conditions, no electron transfer takes place from the [2Fe2S] cluster to cyt c₁ heme, leaving the cyt c component fully oxidized (Fig. 2 Right Middle C and D). A critical test of the model would be to devise ways to impede the movement sufficient enough to bring it out of the 50-μs envelope but not to create a situation as encountered with stigmatellin, where it is stopped altogether. Only then will the reduction of the flash-oxidized cyt c visibly depend on the rate of this movement and be strictly controlled by it in the presence of myxothiazol, as shown in Fig. 2 Right Bottom C and D.

Comparison of various crystallographic data (7–10) indicates that movement of the [2Fe2S] cluster domain would require conformational changes of the linker region encompassing amino acid residues 67–73 in the bovine sequence (corresponding to 43–49 in R. capsulatus numbering) and connecting its fixed hydrophobic anchor and extrinsic carboxyl-terminal portions (Fig. 1). We considered that this putative hinge region might therefore be vulnerable to mutations that could obstruct this mobility. Herein, using R. capsulatus mutants with alanine

Abbreviations: cyt, cytochrome; Q, ubiquinone; OH₂, ubihydroquinone; EPR, electron paramagnetic resonance.

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residue insertions, we were able to reveal this motion kinetically and demonstrate that it is essential for the function of the cyt bc1.

Materials and Methods

Bacterial Strains and Growth Conditions. Escherichia coli or R. capsulatus strains were grown as described in ref. 17 in Luria–Bertani broth or mineral-peptone-yeast-extract enriched medium, respectively, and in the presence of appropriate antibiotics. Respiratory or photosynthetic growth of R. capsulatus strains was at 35°C in the dark under semiaerobic conditions or in anaerobiosis under continuous light, respectively. MT-RBC1 is a bc1− strain in which the chromosomal copy of the petABC operon has been deleted and replaced by a gene cartridge conferring resistance to spectinomycin (17). The strain pMTS1/MT-RBC1 corresponds to MT-RBC1 complemented in trans with the plasmid pMTS1, which provides resistance to kanamycin and contains a wild-type copy of petABC.

Molecular Genetic Techniques. Engineering of mutations located in the linker region of the Fe-S subunit was facilitated by the creation of a unique MluI restriction site in pMTS1 via a silent mutation at its codon Ala-40, yielding plasmid pMTS1-MluI (E.D., M.V.-V., and F.D., unpublished work). The alanine insertion mutations were created by PCR with an upstream common primer (5′-GTCCTGGGCTCTGAA-3′) and the following downstream specific primers: +1Ala, 5′-ATGAACGCGTCGGCAGTCAAGGCCGCGATGCGC-ATCGATCTTCG-3′; +2Ala, 5′-ATGAACGCGTCGGCAGTCAAGGCCGCGATGCGC-ATCGATCTTCG-3′; +3Ala, 5′-ATGAACGCGTCGGCAGTCAAGGCCGCGATGCGC-ATCGATCTTCG-3′; and +3Ala, 5′-ATGAACGCGTCGGCAGTCAAGGCCGCGATGCGC-ATCGATCTTCG-3′. The MluI–ApaLI fragment containing the mutation thus generated was then exchanged with its wild-type counterpart in pMTS1-MluI, and the newly constructed plasmids were introduced into MT-RBC1 via triparental crosses (17). In all cases, the presence of the desired mutation and absence of any additional mutation on the insert thus exchanged was confirmed by DNA sequencing.

Biochemical and Biophysical Techniques. Chromatophore membrane preparation, protein determination, and 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzohydroquinone: cyt c reductase assays were performed as described (17). SDS/PAGE was performed by using an acrylamide concentration of 15% (wt/vol), and gels were stained with Coomassie blue. Immunoblot analyses were performed as described (17), with monoclonal or polyclonal antibodies specific for R. capsulatus cyt bc1, which was purified as described (18). Proteolysis experiments with thermolysin were done at room temperature in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl, 0.01% dodecyl maltoside, and 20 μM stigmatellin, when specified (M.V.-V., E.D., C. R. Moomaw, C. A. Slaughter, and F.D., unpublished work). Aliquots were analyzed by immunoblotting with polyclonal antibodies against the Fe-S protein of R. capsulatus.

Light-induced, single-turnover, time-resolved kinetics were performed as described (19) by using chromatophore membranes and a single wavelength spectrophotometer (Biomedical Instrumentation Group, University of Pennsylvania) in the presence of 2.5 μM valinomycin, N-ethyl-dibenzoylamine ethyl sulfate, N-methyl-dibenzoylamine methyl sulfate, 2,3,5,6-tetramethyl-1,4-phenylenediamine, and 2-hydroxy-1,4-naphthoquinone. Transient cyt c reduction kinetics initiated by a short
saturating flash (8 μs) from a xenon lamp were followed at 550–540 nm, and cyt b reduction was followed in the presence of antimycin at 560–570 nm. The concentrations of antimycin, myxothiazol, and stigmatellin used were 5, 5, and 1 μM, respectively, and the ambient potential was poised at 100, 200, or 400 mV as indicated.

Oxidative titrations of the Fe-S subunit [2Fe2S] cluster in chromatophore membranes were conducted potentiometrically according to Dutton (20) in the presence of 100 μM tetrachlorohydroquinone, 2,3,5,6-tetramethyl-1,4-phenylenediamine, 1,2-naphthoquinone-4-sulfonate, 1,2-naphthoquinone, N-ethyl-dibenzopyrazine ethyl sulfate, and N-methyl-dibenzopyrazine methyl sulfate, and in the presence of 100 μM stigmatellin or 300 μM myxothiazol when indicated. EPR spectroscopy of these samples was performed as described (21) by using a Bruker (Billerica, MA) ESP-300E, equipped with an Oxford Instruments (Oxon, England) ESR-9 helium cryostat, under the following conditions: sample temperature, 20 K; microwave power, 2 mW; modulation amplitude, 20.243 G; modulation frequency, 100 kHz; microwave frequency, 9.45 GHz.

Chemicals. All chemicals were as described (22).

Results and Discussion

The Alanine Insertion Mutants and Their Initial Characterization. In an attempt to interfere with the function of the Fe-S subunit hinge region (corresponding to residues 43–49 in R. capsulatus), mutants with insertions of one (+1Ala), two (+2Ala), or three (+3Ala) alanine residues were engineered between position 46 and 47 (corresponding to residues 70 and 71 in bovine number-
ing; Fig. 1). These mutants properly assembled their cyt bc1, as shown by immunoblot analyses of the subunits and spectroscopic quantification of their cofactors (b- and c-type hemes, [2Fe2S] cluster). The [2Fe2S] cluster–Qo site interactions of the mutants were normal, and their [2Fe2S] cluster domains were located in the Qo position when reduced, as indicated by the position and lines shapes of their EPR g signals (14) in the absence and presence of stigmatellin (data not shown). However, the +2Ala and +3Ala mutants were unable to grow, and the +1Ala mutant grew poorly under photosynthetic growth conditions. This finding indicated a cyt bc1 defect, because in phototrophic bacteria like R. capsulatus, this enzyme is required for cyclic electron transport. In agreement with these results, the cyt bc1 of the +1Ala mutant was poorly functional, and the cyt bc1 of the +2Ala or +3Ala mutants was nonfunctional as revealed by their single turnover activities (Table 1). Surprisingly, the steady-state activity of the +1Ala mutant was abnormally high in detergent-dispersed membranes.

The +2Ala Mutant Contains a Nonmoving Fe-S Subunit. Further insights toward the effects of the alanine insertion mutations were gained by analyzing their cyt c reduction kinetics in the presence of the Qo site inhibitors stigmatellin and myxothiazol. In the case of the +2Ala (Fig. 3B) and +3Ala (data not shown) mutants, cyt c reduction kinetics without inhibitor or with myxothiazol or stigmatellin were almost identical to those obtained with the native cyt bc1 (EPR spectra not shown) and a higher Eₚ value of the [2Fe2S] cluster (ΔEₚ = +100 mV; Table 1). These indicate a stronger interaction of the [2Fe2S] cluster domain with the Qo site, of the kind observed in the presence of stigmatellin. Furthermore, the +2Ala mutant Fe-S subunit showed resistance to thermolysin-mediated, conformation-sensitive proteolysis (ref. 24 and M.V.-V., E.D., C. R. Moomaw, C. A. Slaughter, and F.D., unpublished work) that matched that observed with the native cyt bc1 in the presence of stigmatellin (Fig. 4). This resistance contrasts with the uninhibited native cyt bc1, which is readily proteolysed by thermolysin to release an 18-kDa Fe-S subunit fragment lacking its first 46 amino acid residues. Thus, the conformation of the +2Ala mutant either with or without stigmatellin resembles that of the native Fe-S subunit in the presence of stigmatellin.

Movement of the Fe-S Subunit Extrinsic Domain Is Required for cyt bc1 Turnover but Not for QH2 Oxidation at the Qo Site. Light-induced, single-turnover kinetics experiments monitoring cyt b reduction in
the presence of antimycin and performed at 400 mV (a redox potential at which the [2Fe2S] is initially oxidized) revealed that transient cyt b reduction kinetics observed with the +2Ala mutant were similar to those seen with a wild-type strain (QH₂ to cyt b about 50 s⁻¹; data not shown). Therefore, the absence of the Fe-S subunit extrinsic domain movement does not prevent QH₂ oxidation, even though it abolishes the turnover of the cyt bc₁ by impeding the electron shuttling to cyt c₁. This finding further highlights that the concerted electron transfer step is located between the cyt b and the Fe-S subunit of the cyt bc₁.

**Visualizing Electron Transfer from the [2Fe2S] Cluster to cyt c₁ Heme with the +1Ala Mutant.** Remarkably, in the case of the +1Ala mutant, the cyt c reduction kinetics were now slow enough to lie between the wild type (too fast) and the +2Ala mutant (too slow), and hence they were measurable; in the presence and absence of myxothiazol, their half-times were 3 and 10 ms, respectively (Fig. 3C). Moreover, cyt c reduction kinetics could be rendered even more obvious by performing the experiments at pH 9.0. Under these conditions, the Eₘ of the [2Fe2S] cluster (but not that of cyt c₁ heme) decreases, thus shifting the redox equilibrium between these two cofactors to favor the reduction of cyt c₁ (Fig. 3C, pH 9). Again, diminished cleavage by thermolysin of the +1Ala mutant cyt bc₁ (Fig. 4), increased Q content trapped at the Q₀ site of the purified complex (EPR spectra not shown), and increased Eₘ of the [2Fe2S] cluster (ΔEₘ = +60 mV; Table 1) indicated a more favored Q₀ position for the [2Fe2S] cluster domain.

It could be argued that the slower cyt c reduction kinetics observed in the alanine insertion mutants are due to the increased Eₘ values of their [2Fe2S] clusters thermodynamically displacing the redox equilibrium between the [2Fe2S] cluster and cyt c₁ heme. However, other mutants with increased Eₘ values, such as the Fe-S subunit A46T mutant with Eₘ = 386 mV (Table 1), are still functional (21). Further, in the case of the +2Ala mutant, one would expect that in the uninhibited state, the cyt bc₁ should still function despite this uphill reaction (25). Moreover, in the presence of myxothiazol, the Eₘ differences vanish (E.D., and F.D., unpublished work). Therefore, such thermodynamic equilibrium cannot alone account for the impeded electron transfer observed in the alanine insertion mutants.

A second possibility could be an improper docking of the Fe-S subunit on cyt c₁ as a possible consequence of the hinge-region mutations. However, in the mitochondrial cyt bc₁ structure in which the Fe-S subunit extrinsic domain is in the c₁ position, one of the histidine ligands of the [2Fe2S] cluster (His-161 in bovine numbering) is located close enough to one of the propionates of cyt c₁ heme to form a hydrogen bond (8, 9). Electron-tunneling calculations indicate that electron transfer rates between these two cofactors should be extremely fast (∼1 μs; refs. 11 and 25). Thus, for the electron transfer between the [2Fe2S] cluster and cyt c₁ heme to become rate limiting to the level observed in this study (more than several milliseconds), the [2Fe2S] cluster should dock in the alanine insertion mutants at least 5–8 Å away from its native position, which is highly unlikely.

On the other hand, the higher Eₘ of the [2Fe2S] cluster, the higher Q content trapped in the Q₀ site on purification of the mutant cyt bc₁, and the increased resistance to thermolysin-mediated conformation-sensitive cleavage combine with the slow cyt c kinetics to indicate a strongly favored Q₀ position for the extrinsic domain of the Fe-S subunit. Therefore, the slow electron transfer rates observed in these mutants are at least partially due to a hindered movement from the Q₀ site to the cyt c₁ position caused by the alanine residue insertions. Further support for this conclusion is provided by the successful isolation of a faster-growing revertant of the +1Ala mutant with a second site suppressor mutation located at position 286 (262 in bovine numbering) of the e loop of *R. capsulatus* cyt b. This region represents the most conspicuous physical barrier that needs to be crossed during normal [2Fe2S] cluster domain movement (Fig. 1). Indeed, in this revertant, cyt c₁ reduction kinetics in the presence of myxothiazol have a fast unresolved phase (<50 μs) exactly like that seen in the wild type (data not shown).

Implications of the [2Fe2S] Cluster Domain Movement on Energy Conversion. The [2Fe2S] cluster domain motion on the 1- to 10-ms time scale in the +1Ala mutant may represent an engineering
boundary at slower times, beyond which the movement begins to interfere significantly with the rate of electron transfer through the cyt bc1 and the rate of physiological growth (Fig. 5). Based on the motion resolved in the +1Ala mutant, it is clear that the movement in the native cyt bc1 must, as proposed, be faster than the 50-μs resolution of light-induced kinetics and hence be very much faster than the 1,700-s\(^{-1}\) \(k_{\text{cat}}\) of the enzyme (12, 13). Thus, in the native enzyme, movement is required for catalytic activity but is neither rate-limiting nor essential for QH\(_2\) oxidation. Furthermore, our electron transfer simulations indicate that there may be another engineering boundary at faster times in the 1- to 10-μs range beyond which the cyt bc1 function may also become impaired (C.C.M., E.D., F.D., and P.L.D., unpublished work). These findings suggest that if the motion becomes too fast, then the system becomes vulnerable to short circuiting of electron transfer in the Q\(_{\text{b}}\) site that inevitably leads to a less efficient energy conversion in the Q cycle mechanism (26).

Large amplitude domain motions therefore represent an effective design to control the flux of electrons inside protein complexes with branched electron transfer pathways. However, this unusual control exposes the cyt bc1 to antibiotics designed to act, not as usual by displacing the substrate from the catalytic sites, but instead on the domain motion. This type of control might be achieved by impeding the helix-random coil conformational change in the [2Fe2S] hinge region, which, based on various structures (8–10) and mutants (24, 27), is required for the movement. The intraprotein electron shuttle motion of the Fe-S subunit demonstrated herein can now be explored by using “slow” mutants to analyze how this movement is controlled at the molecular level.

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