Cytochrome $b_6$ Arginine 214 of Synechococcus sp. PCC 7002, a Key Residue for Quinone-reductase Site Function and Turnover of the Cytochrome $b_f$ Complex*

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Quinone-reductase (Q) domains of cyanobacterial/chloroplast cytochrome $b_f$ and bacterial/mitochondrial $bc$ complexes differ markedly, and the cytochrome $b_f$ Q site mechanism remains largely enigmatic. To investigate the $b_f$ Q domain, we constructed the mutation R214H, which substitutes histidine for a conserved arginine in the cytochrome $b_6$ polypeptide of the cyanobacterium Synechococcus sp. SPCC 7002. At high light intensity, the R214H mutant grew ~2.5-fold more slowly than the wild type. Slower growth arose from correspondingly slower overall turnover of the $b_f$ complex. Specifically, as shown in single flash turnover experiments of cytochrome $b_6$ reduction and oxidation, the R214H mutation partially blocked electron transfer to the Q$_i$ site, mimicking the effect of the Q$_i$ site inhibitor R214H, which substitutes histidine for a conserved arginine in the cytochrome $b_6$-4-hydroxyquinoline-N-oxide. The kinetics of cytochrome $b_6$ oxidation were largely unaffected by hydroxyl-deutium exchange in the mutant but were slowed considerably in the wild type. This suggests that although protonation events influenced the kinetics of cytochrome $b_6$ oxidation at the Q$_i$ site in the wild type, electron flow limited this reaction in the R214H mutant. 

Redox titration of membranes revealed midpoint potentials ($E_{\text{m}}$) of the two b hemes similar to those in the wild type. Our data define cytochrome $b_6$ Arg$^{214}$ as a key residue for Q$_i$ site catalysis and turnover of the cytochrome $b_f$ complex. In the recent cytochrome $b_f$ structures, Arg$^{214}$ lies near the Q$_i$ pocket and the newly discovered c$_i$ or x heme. We propose a model for Q$_i$ site function and a role for Arg$^{214}$ in plastoquinone binding.

The cytochrome $b_f$ complex of oxygenic photosynthesis transfers electrons between the photosystem II and I reaction centers.

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1 The abbreviations used are: ISP, Rieske iron-sulfur protein; $b_{10}$, low potential cytochrome $b_6$ heme; $b_{10}$, high potential cytochrome $b_6$ heme; c$_i$ (x), heme c$_i$ (or x); $f$, cytochrome $f$ heme; Q$_o$, plastoquinone oxidation site; Q$_i$, plastoquinone reduction site; FCPP, cabonylcyanide-p(trifluoro-methoxy)phenyl-hydrazide; NQNO, 2-N-4-hydroxyquinolino-N-oxide; MOPS, 3-(N-morpholino)propanesulfonic acid; Sm, streptomycin; Sp, spectinomycin.
The locations of the redox centers are taken from the cytochrome complex, proposed (12), according to which the quinol and the oxidized (reviewed in Ref. 12). An interpretative model has been concerted manner in cytochrome molecule in two electrons can be injected consecutively onto the quinone space. This model has been confirmed experimentally for cytochrome bc complexes. The inhibitor NQNO (29), which seems to operate by a mechanism similar to that of antimycin, only partially blocks the Qi site quinone and cyclic flow from photosystem I. No functional evaluation of the cytochrome bf site mechanism has yet been obtained because mutations of this site have not been available from oxygenic, photosynthetic organisms. Prior to elucidation of the cytochrome bf crystal structures, we have initiated a molecular genetic study to probe the mechanism of quinone reduction in the cytochrome bf complex. Although loss-of-function mutations are not allowed in cyanobacterial bf complexes (reviewed in Ref. 5), the feasibility of generating stable mutations within this complex in cyanobacteria is now well established (31, 32). To dissect the catalytic mechanism of the Q site, we generated mutation R214H in the PetB, cytochrome b, polypeptide of Synechococcus sp. PCC 7002. This changes a conserved arginine in the cytochrome bf complex to a histidine that is the conserved, corresponding residue near the Q, pocket of cytochrome bc complexes. The reverse H217R mutation in the bc complex of Rhodobacter capsulatus increases the binding affinity of the Q, semiquinone, increases the amplitude of b heme reduction, and slows electron transfer through the complex (33). We report here that the PetB R214H mutation in Synechococcus decreases the overall growth and cytochrome bf turnover rates, slows electron transfer across the membrane between the two b hemes, and thus defines a key residue for quinone-reductase (Q, site) function in cytochrome bf complexes. These data are interpreted in light of the bc and bf structures, and a model is proposed for Q, site catalysis in the cytochrome bf complex.

MATERIALS AND METHODS

Cyano bacterial Cultures—Stock cultures of Synechococcus sp. PCC 7002 were grown under cool white fluorescent lamps (20–30 μmol photons m⁻² s⁻¹) in medium A as described previously (31). R214H mutant cultures were supplemented with streptomycin (Sm) and spectinomycin (Sp) at 50 μg/ml. For growth rate determinations, mid-log phase cells were inoculated into medium A (bubbled with 1% CO₂ in air) and incubated at 39 °C and 250 μmol photons m⁻² s⁻¹ light intensity. Cell density was determined by direct turbidity measurements on 18-mm-diameter culture tubes in a Spectronic 20D spectrophotometer (Milton Roy, Rochester, NY). Cultures for assays were grown at 200–250 μmol photons m⁻² s⁻¹ light intensity and harvested at mid-log phase. Chlorophyll concentrations were determined as described in Ref. 34. Synechococcus strain BstU1 (31) is wild type for petB but carries the same Sm/Sp resistance cassette upstream of petB as in the R214H mutant. The BstU1 strain was used as a control in some experiments. Its salient properties are indistinguishable from the wild type.

Site-directed Mutagenesis of the Synechococcus petB Gene—Escherichia coli strains were grown in LB medium supplemented with streptomycin (Sm) and spectinomycin (Sp) as needed with 150 μg/ml of ampicillin, 15 μg/ml tetracycline, or 25 μg/ml each of Sm and Sp as described previously (31). Standard molecular genetic manipulations were performed as in (35). Plasmid pA6.2 carrying the Synechococcus petBD genes was used for site-directed mutagenesis of petB as described in Ref. 31. The petB R214H mutation was created by means of the mutagenic oligonucleotide 5'-CCACTTCCTCATGATTC-3' (loccalized in the R214H mutant), and the underlying indicates the newly created Hinfl restriction site tag. The R214H mutation in plasmid pA6.2 was verified by plasmid isolation from E. coli transformants, PCR amplification from the primers TK-1 (5'-GACAGGAGCACTGTTCCCGTC-3') and TK-2 (5'-GACATCCACCGGAGACGCAG-3') (locations shown in Fig. 2), and restriction tests for the Hinfl tag.

Plasmid pA6.2-R214H carrying the petB R214H mutation was introduced into Synechococcus by transformation (31). Because this plasmid cannot replicate in Synechococcus, selection for Sm/Sp resistance forces integration into the genome of Synechococcus and flanking petBD sequences as illustrated in Fig. 2. Integration of the petB-R214H mutation and segregation and loss of the wild type (petB) allele were confirmed by: (i) PCR amplification of petB from Synechococcus transformants and restriction tests for the Hinfl tag as described above for E. coli, (ii) allele-specific PCR detection of the petB-R214H allele according to Ref. 31 as shown in Fig. 2B, and (iii) DNA sequencing of petB PCR products from the TL-F and TK-2 primers. Forward primers for allele-specific PCR were 5'-CCACTTCCTCATGATCGCG-3' (for detection of wild type petB) or 5'-CCACTTCCTCATGATCA-3' (for detection of...
Cytochrome bf Quinone-reductase Site

Spectroscopic Measurements—Turnover of the cytochrome bf complex was monitored by measuring the rate of reduction of cytochrome $f$ following a saturating 40-ms light pulse as described previously (31, 34). Time-resolved absorbance changes were measured in the $a$-band region ($530-570$ nm) of the cytochromes. Because the spectra of cytochromes $f$ and $c_6$ are largely superposed, and transfer between them is rapid, no effort was made to monitor these carriers separately (31). To improve the signal to noise ratio, the measurements were averaged with an interval between flashes of $7-10$ s. The cells were suspended at $1-5$ μM chlorophyll in reaction medium containing $5$ mM Hepes, pH 7.5, $10$ mM KCl buffer containing the redox mediators anthraquinone $2$-sulfonate ($-225$ mV), anthraquinone $1,5$-disulfonate ($-170$ mV), $2$-hydroxy-1,4-naphthoquinone ($-145$ mV), anthraquinone ($-100$ mV), $2,5$-dihydroxy-$2$-benzoquinone ($-60$ mV), menadione ($0$ mV), and $1,4$-naphthoquinone ($+60$ mV), each at a concentration of $100$ μM. Cytochrome $b_6$ absorbance spectra in the $540-580$ nm range were recorded at $-25$ mV intervals from samples poised at electrical potentials of $-200$ mV to $+35$ mV. The cytochrome $b_6$ peak absorbance values at $564$ nm were plotted as a function of potential. The midpoint potentials of the $b_6$ hemes were calculated by fitting these data to a sum of two Nernst equations (each $n = 1$) as in previous analysis of wild type Synechococcus (38). Alternatively, a global fit analysis was performed as was in Ref. 38, where the entire spectra were deconvoluted and fitted to a sum of Nernst curves. Both procedures yielded essentially identical results.

RESULTS

Construction of the PetB R214H Mutant and Characterization of Growth—As stated, both photosynthetic and respiratory electron transport in cyanobacteria require the cytochrome $b$ complex, and therefore essential subunits cannot be inactivated (reviewed by Ref. 5). Accordingly, the PetB R214H mutation was constructed by allele replacement (31). This method leaves a copy of the antibiotic resistance cassette upstream of the targeted gene in the Synechococcus genome as illustrated in Fig. 2A. The Sm/Sp cassette does not detectably alter the growth or electron transfer properties of control cells (31). Synechococcus R214H mutant segregants were confirmed as shown in Fig. 2B and detailed under “Materials and Methods.”

Synechococcus mutant cells carrying the R214H mutation in the presumptive quinone-reductase site were initially characterized for growth properties and possible sensitivity to the cytochrome $b\_Q$ site inhibitor antimycin A. At $39^\circ C$, $250$ μmol photons m$^{-2}$ s$^{-1}$ and $1%$ CO$_2$, the R214H mutant grew $2.5$-fold more slowly than the wild type. Fig. 3 shows a typical experiment. Specific growth rates and doubling times under these conditions were $\sim 0.053$ h$^{-1}$ (doubling time, $\sim 13$ h) and $0.19$ h$^{-1}$ (doubling time, $\sim 3.5$ h) for the mutant and control.
cells, respectively. Under slower growth conditions (32 °C, 80 μmol photons m⁻² s⁻¹, and atmospheric CO₂), mutant and control cells grew at similar rates. Mutant and control cultures were insensitive to antimycin (30 μM) under all conditions (not shown). These data indicate that the R214H mutation did not alter the antimycin insensitivity of the cytochrome bf Q₁ site but did suggest a partial blockage of electron flow to this site resulting in slower growth under conditions of high electron flux.

**Turnover of Cytochrome bf Complexes in Wild Type and R214H Mutant Cells—Plastoquinol oxidation in the cytochrome bf complex operates via a branched pathway, where one electron is transferred to the high potential chain and the second electron is transferred to the low potential chain (Fig. 1). The overall rate of electron flow through the complex can therefore be evaluated by measuring the rate of cytochrome f/c₆₆ rereduction (the spectra of the two redox partners are largely superposed) after a long flash (31). The rationale for this measurement is that light induces the oxidation of the redox cofactors (the P700 reaction center of photosystem I, cytochromes f and c₆, and the Rieske ISP 2Fe-2S cluster) that are functionally located downstream of the rate-limiting, plastoquinol oxidation step. When the light is turned off, plastoquinol oxidation drives the reduction of the ISP cluster, cytochromes f and c₆, and P700. This process is proportional to the rate of plastoquinol oxidation but is also indicative of the overall turnover of the bf complex. If the Q cycle is continuously engaged during cytochrome bf turnover, as proposed (39), then the cytochrome f/c₆₆ rereduction rate also depends on the Q₁ site reduction of plastoquinone. The complex has to perform several turnovers to generate all of the reducing equivalents required to reduce the pool of electron carriers in the high potential chain (31, 38).

We employed this technique as a first approach to gain information on the turnover efficiency of the cytochrome complex in the R214H mutant cells. Fig. 4A presents the absorbance changes from 540 to 570 nm measured in intact wild type and mutant cells following a 40-ms flash. Here reduction and oxidation processes are shown as increases or decreases in absorbance, respectively. The spectra show that the absorbance change following illumination is made up of contributions from cytochrome f (α-band maximum 556 nm in *Synechococcus* sp. PCC 7002, Ref. 38) and cytochrome c₆ (α-band maximum 553 nm in *Synechococcus*, Ref. 38) in both strains.

Fig. 4B presents a typical experiment where the kinetics of electron flow in cytochrome f/c₆ were estimated in both the wild type and mutants strains. In the absence of inhibitors, the half-times of cytochrome f/c₆ reduction were 18 ± 1 ms in the wild type, in substantial agreement with previous estimates (31), and 46 ± 2 ms in the R214H mutant, indicating a significant decrease in cytochrome bf turnover efficiency. These values did not change upon the addition of antimycin A (data not shown) indicating, as in the growth experiments, that the simple replacement of cytochrome b₆ Arg₂¹⁴ with histidine did not confer specific antimycin binding and inhibitor sensitivity.

**Single Turnover Flash-induced Cytochrome b₆ Redox Changes—**Having demonstrated that the overall turnover of the cytochrome bf complex was slowed in the R214H mutant, we tried to gain more direct information on the specific reaction step affected. To this end, we studied flash-induced, single-turnover absorption changes associated with redox events in the b₆ hemes. These reactions are indicative of electron transfer processes that occur both at the Q₁ (cytochrome b₆ reduction) and Q₉ (cytochrome b₆ oxidation) sites. The experiments were performed with intact cells, as described previously (38). The results of such measurements are presented in Fig. 5.

Fig. 5 (A and B) shows flash-induced absorbance changes recorded in the 550–580-nm region, in the presence of FCCP (solid squares) or FCCP and NQNO (open squares). Data(16,39),(979,989) from both the wild type (Fig. 5A) and R214H mutant (Fig. 5B) revealed spectra with absorbance maxima at ~563 nm, typical of cytochromes b₆ (38). The difference between absorbance at 563 nm and a base line drawn between 545 and 573 nm was subsequently monitored to track the b₆ heme reduction and oxidation kinetics shown in Fig. 5, C and D. In wild type cells (Fig. 5C), illumination resulted in the appearance of a small signal increase (reflecting cytochrome b₆ reduction), followed by a large signal decrease. The latter is attributable to oxidation of the cytochrome b₆ heme at the Q₁ site. Indeed, no redox signal changes are expected during electron transfer between the b₆ and b₅ hemes, because their spectra in *vivo* are almost identical (Refs. 11, 38, and 40 and references therein). Upon the addition of the Q₁ site inhibitor, NQNO (29), the cytochrome b₆ oxidation kinetics were slowed considerably, whereas the reduction process remained largely unaffected (compare the initial positive slopes). Consequently, the amplitude of the reduction phase increased at the expense of the oxidation signal, and the rate of electron injection into the low potential (cytochrome b₅ and b₆) chain could be correctly estimated.

From these single-turnover data, we estimated a b₆ for cytochrome b₆ reduction in the 2-ms range in agreement with a previous estimation based on the deconvolution of time-resolved spectra (38). Note that because of the bifurcated reaction mechanism at the Q₁ site, electron transfers to the high (cytochrome f/c₆) and low (b₅ heme) potential branches are expected to occur at similar rates as confirmed here (not shown) and demonstrated previously for *Synechococcus* 7002 (38). The almost 1 order of magnitude difference between our single-flash estimation of Q₉ site turnover based on b₆ heme reduction and that obtained from cytochrome f/c₆ reduction after long flash...
FIG. 5. Light-induced redox changes in the 555-580-nm spectral region measured upon single turnover flash illumination (A and B) and kinetics of cytochrome b₆ redox changes (C and D). Cells were collected during exponential growth and resuspended in Hepes 20 mm pH 7.2, with the addition of 20% (w/v) Ficoll to prevent sedimentation. The cells were illuminated with red flashes at a frequency of 0.15 Hz. 3,5-di-tert-butyl-4′-chlorophenyl)-1,1-dithiylene and hydroxyamine were added at concentrations of 10 mM and 1 mM, respectively, to block photosystem II activity. The ionophore FCCP (1 μM) was added to remove the electrochemical proton gradient and its effects of the rate of plastoquinone reduction at the Q₁ site. Data shown are from cells in the absence (solid) and presence (open) of the Q₁ site inhibitor NQNO at 4 μM. A (wild type, WT) and B (R214H mutant). The spectra were recorded 40 ms after the actinic flash illumination and normalized at their extremes to correct for the contribution of cytochrome f/c₆ absorption. By this time, however, the cytochromes f/c₆ were largely reduced, and their contribution to the overall absorption did not exceed 10%. C (wild type) and D (R214H mutant) kinetics. Flash intensity was 20% of the saturating value to prevent multiple turnovers of the electron transfer chain. ΔI/I is the difference (ΔI) in light transmission between the sample and reference cuvettes, normalized to light transmission (I) by the reference cuvette.

The R214H mutant cells displayed rather different kinetics as shown in Fig. 5D. Here, the traces recorded in the absence and presence of NQNO were largely superimposed. In the mutant, the rate of cytochrome b₆ reduction (which represents the oxidation of the plastoquinol pool at the Q₁ site) was substantially unaffected, but the oxidation of the b₆ hemes was greatly slowed (t₁/₂ = ~0 ms relative to ~8 ms in the wild type). In sharp contrast to the wild type, the addition of the Q₁ site inhibitor did not further slow heme oxidation in the R214H mutant. This clearly suggests that the turnover of the Q₁ site was specifically altered by the mutation, in agreement with our expectations.

Isotopic Effect on Cytochrome b₆ Oxidation Kinetics—In green algae, the substitution of D₂O for H₂O influences the kinetics of cytochrome b₆ electron transfer reactions. In particular, this effect mostly concerns the reactions that follow plastoquinol oxidation at the Q₁ site (41) and plastoquinone reduction (or cytochrome b₆ oxidation) at the Q₁ site (37). The interpretation for this isotopic effect is that deprotonation and protonation of the quinones kinetically limits the reactions occurring in both the Q₁ and Q₉ sites.

To investigate whether the slow-down of cytochrome b₆ oxidation in the R214H mutant resulted from a decreased efficiency of electron transfer or a modification of the protonation process, we measured b₆ heme kinetics in cyanobacteria following H₂O-D₂O exchange according to procedures developed for C. reinhardtii (see “Materials and Methods”). With the wild type, we obtained results closely resembling those previously reported for C. reinhardtii (37). Both the reduction and oxidation rates of the cytochrome b₆ hemes decreased substantially in D₂O-enriched medium (compare the solid and open squares in Fig. 6A), consistent with the direct involvement of proton transfer events in these reactions. The isotopic effect on cytochrome b₆ oxidation was largely decreased by pretreatment with the Q₁ site inhibitor NQNO, suggesting that under these conditions, processes other than protonation of the Q₁ plastoquinone (for example, its binding to the Q₁ site or its reduction by the b₆ heme) limited the turnover of the Q₁ site.

In the R214H mutant, only a very small isotopic effect was observed on the cytochrome b₆ oxidation kinetics, independent of the presence of NQNO (Fig. 6B). This indicates that electron transfer reactions limited quinone reduction in the mutant and further supports the conclusion that the R214H mutation mimics the effect of NQNO.

Evaluation of the Cytochrome b₆ Redox Potentials in the R214H Mutant—Our data suggested that the kinetic consequences of the R214H mutation could be attributed either to a change in the redox potential of the Q₁ hemes (hemes b₆H and/or heme c₁ (x)) or to a decreased affinity of plastoquinone for its Q₁-binding site. To determine whether the redox potential of the b₆ heme was modified in the mutant, we performed an equilibrium redox titration of membranes extracted from the R214H mutant. In the −200 to +35 mV range, two components were clearly identified that had equivalent absorption spectra characteristic of cytochromes b₆ (Fig. 7). Their redox potentials were indistinguishable within experimental error from those previously determined for the wild type (38). We ruled out, therefore, that a modification of the b₆ heme redox potential was responsible for the kinetic effects observed in the R214H mutant.

In the spectral region analyzed, no evidence was found for a third redox component. This indicates that the c₁ (x) heme of the Q₁ domain was silent in the α band region, consistent with its expected high spin nature (9, 10). Because of strong chlorophyll absorbance, we were unable to perform redox titrations of membranes in the Soret region and therefore could not assess the redox properties of the c₁ (x) heme.

DISCUSSION

Functional Phenotype of the R214H Mutant—We have shown here that replacement of the conserved Arg₂¹¹¹ with histidine in cytochrome b₆ dramatically impairs Q₁ site function, turnover of the cytochrome b₆ complex, and the growth of Synechococcus
cells. As mentioned, the cytochrome b₅ Qᵢ site and transmembrane electron transfer between the b₅ hemes have been enigmatic and difficult to study. Principally (i) there is no highly efficient inhibitor such as antimycin A to block electron transfer at the cytochrome b₅ Qᵢ site (16), (ii) no stable Qᵢ-semiquinone and accompanying EPR signal have been detected (16), and (iii) the spectral properties of the b₅ and b₆ hemes are virtually identical within intact cells (38, 40, 42). All of these factors have made it exceedingly challenging to track individual redox changes within the low potential chain. As a consequence, there has been much controversy about the role of the Qᵢ site for cytochrome b₅ turnover and whether the Q cycle mechanism of electron transfer (described above) operates requisitely or facultatively (see Refs. 5, 11, 43, and 44 for reviews). Several interesting alternative mechanisms for electron and proton transfer have been proposed that might operate in b₅ complexes under particular environmental conditions. These include “semiquinone” (36), “proton pump” (45–47), and “bypass” (43) models that involve transfer of semiquinones or protons across the membrane or the transfer of two electrons from plastoquinol to two Rieske ISP clusters in a cytochrome b₅ dimer on the luminal side of the membrane. Most of these mechanisms still require the operation of a quinone-reductase (Qₐ) site, in some form, on the electronegative side of the membrane.

To help elucidate Qᵢ site function in the cytochrome b₅ complex, we attempted to make the cytochrome b₅ complex of the cyanobacterium Synechococcus 7002 more like the bc complex. Because this project was initiated before the recent cyanobacterial (9) and algal (10) cytochrome b₅ structures, the rationale employed to design the mutation was based on homology to the cytochrome b protein of R. capsulatus (33). Cytochrome b₆, Arg²¹⁴ of Synechococcus corresponds to the conserved cytochrome b His²¹⁷ of R. capsulatus (and to cytochrome b His²⁰² of yeast shown in Fig. 8, right panel). The H₂¹⁷R mutation of R. capsulatus greatly increased (by ~10-fold) the binding affinity of semiquinone for the Qᵢ site and thereby slowed Qᵢ site turnover, increased the reduction level of heme b₆H, and slowed overall turnover of the bc complex.

Our corresponding R2¹⁴H mutation in Synechococcus did not generate an antimycin-sensitive cytochrome b₅ complex, nor did it alter the spectra of the b₅ hemes, but it did markedly alter Qᵢ site catalysis and the overall turnover of the cytochrome b₅ complex. In the cytochrome b₅ structures (9, 10), Arg²¹⁴ (equivalent to Arg²⁰⁷ in both Mastigocladus and Chlamydomonas) lies within or close to the Qᵢ pocket and the newly discovered c₁ (x) heme (Fig. 8, left and middle panels). Therefore, the removal of this positively charged residue might not only alter the affinity of the Qᵢ site for plastoquinone but also the midpoint potentials of the redox cofactors within its vicinity. Our redox titration of the b hemes from the R2¹⁴H mutant revealed midpoint potentials similar, within experimental error, to those from wild type Synechococcus. This seemed surprising in light of the relatively close proximity of Arg²⁰⁷ (Synechococcus Arg²¹⁴) to the b₅ heme and the likely electronic coupling of the b₅ and c₁ (or x) hemes via a heme b₅H and a water molecule in both the Mastigocladus and Chlamydomonas structures (9, 10). If the Qᵢ site structure is more like that in Fig. 8 (left panel), then the interaction of the c₁ (x) heme with Arg²⁰⁷ (Synechococcus Arg²¹⁴) should modulate the positive charge of the arginine, and there might be little direct influence on heme b₅H. Similarly, if Arg²⁰⁷ (Synechococcus Arg²¹⁴) has a direct role in binding plastoquinone, again as suggested in Fig. 8 (left panel), the effect of the R2¹⁴H mutation on heme b₅H might be minimized. For these reasons, we believe that our data favor a direct role for Arg²⁰⁷ (Synechococcus Arg²¹⁴) in plastoquinone binding at the Qᵢ site, although we cannot exclude other possibilities. We were unable to directly assess the midpoint potential of the c₁ (x) heme in the mutant because of the absence of either a distinguishing optical signal, as discussed above, or a well defined EPR signal.

Thanks to its unique position in the structure, Arg²⁰⁷ (Synechococcus Arg²¹⁴) may have additional roles in Qᵢ site catalysis. Besides plastoquinone binding and stabilization of a negative charge on heme c₁ (x) (see below), it may participate in proton transfer to the Qᵢ quinone, either by providing a direct path for protons or indirectly by participating in a network of residues involved in protonation. Such protonation networks are well established in the Qᵢ pocket of the bacterial reaction center (48, 49). In the cytochrome b₅ structures, Arg²⁰⁷ (Synechococcus Arg²¹⁴) may be hydrogen-bonded to plastoquinone via a water molecule. As detailed by Kurisu et al. (9) and Stroebe! et al. (10), heme c₁ (x), and the Qᵢ pocket are relatively accessible to the stromal, aqueous phase being bounded mostly by the N- and C-terminal loops of cytochrome b₅ and the N-terminal extension of subunit IV. Thus, Arg²¹⁴ could be a link in a short water chain such as that in cytochrome bc Qᵢ (50–52) or the reaction center Qᵢ sites (49, 53).

Our data showed a considerable kinetic isotope effect on both the reduction and oxidation kinetics of the b₅ hemes in wild type Synechococcus cells (Fig. 6). This implies that steps in both plastoquinol deprotonation at the Qᵢ site and plastoquinone protonation at the Qᵢ site affect the overall reactions at these sites as shown previously for chloroplasts (37, 54). The R2¹⁴H mutant showed a small isotope effect on b₅ heme reduction but virtually none on b₅ heme oxidation, indicating that an electron transfer step has been slowed at least as much as any of the protonation steps. These data do not preclude a role for Arg²¹⁴.
in Q₁ plastoquinone protonation in the native cytochrome b₆ complex.

Based on our findings, the main role of cytochrome b₆ Arg²¹⁴ (Arg²⁰⁷ in the cytochrome b₆ structure) seems to be in modulating the binding affinity of the Q₁ site for its plastoquinone substrate. In this sense, the R214H mutation of Synechococcus, which appears to weaken quinone binding, is indeed complementary to H217R of R. capsulatus where arginine at position 217 binds quinone more strongly than the native histidine.

**Structural Features of Cytochrome b₆ Arg²¹⁴**—Based on their characterization of the mutant phenotype, Gray et al. (33) proposed that His²¹⁷ binds quinone at the cytochrome b₆ Q₁ site in R. capsulatus via a hydrogen bond to one of the quinone carbonyls analogous to the interaction of a histidine with QB in reaction centers (58). To date, the distance between this histidine and quinone is too great for hydrogen bonding, favor the latter. These structural differences may reflect dynamic, functional aspects of cytochrome b₆ Q₁ domains as proposed in recent models of ubiquinone reductase (51, 52, 57). However, it remains controversial whether His²¹⁷ (or the equivalent histidine in other b₆ complexes) directly participates in binding forms of the Q₁-quinone or does so via a linking water molecule. Some cytochrome b₆ structures (18, 57) and EPR investigations (52, 57) support the former, whereas others (51, 56), where the distance between this histidine and quinone is too great for hydrogen bonding, favor the latter. These structural differences may reflect dynamic, functional aspects of cytochrome b₆ Q₁ domains as proposed in recent models of ubiquinone reductase and protonation (51, 52, 57). Fig. 8 (right panel) illustrates the juxtaposition of His²⁰² (equivalent to R. capsulatus His²¹⁷) and ubiquinone via hydrogen-bonds to a linking water molecule in the Q₁ pocket of the yeast cytochrome b₆ complex (56).

There are clear differences among the three Q₁ site structures shown in Fig. 8. In the Chlamydomonas structure, Arg²⁰⁷ (Synechococcus Arg²¹⁴) lies close to the c₁ heme (Fig. 8, left panel). One of the arginine amino nitrogens is within easy hydrogen bonding distance of a c₁ heme propionate oxygen. The Q₁ site plastoquinone has not been unambiguously identified in the Chlamydomonas b₆ structure, but the quinone ring likely resides near the face of heme c₁ (10). This is represented by a red ring (viewed largely edge-on) in Fig. 8 (left panel). Because the quinone oxygens have not been assigned, likely hydrogen bonding interactions cannot be precisely deduced from the structure. However, depending on the orientation of these oxygens, the Arg²⁰⁷ (Synechococcus Arg²¹⁴) amino nitrogens could either be within direct, 3 Å, hydrogen bonding distance of the Q₁ plastoquinone (as illustrated in Fig. 8, left panel) or be linked via an intervening water molecule as in the His²⁰² ubiquinone linkage in the yeast Q₁ site (Fig. 8, right panel, and Ref. 50).

In the Mastigocladus structure, the Arg²⁰⁷ amines face away from heme x (c₁) and the assigned location of the Q₁ plastoquinone (Fig. 8, middle panel). In this position, direct hydrogen bonding to plastoquinone or heme x is not possible. The shortest distance from the arginine nitrogens to heme x propionates is ~8 Å, and the distance to the heme b₁ propionates is slightly greater. In this conformation, an interaction of Arg²⁰⁷ via an intervening water molecule or that of a different rotamer of the arginine with heme x might still be possible.

The observed structural differences of the cytochrome b₆ Q₁ domains may reflect: (i) evolutionary and functional differences between chloroplast and cyanobacterial cytochrome complexes; (ii) interesting conformational changes that occur during Q₁ site catalysis, as in the Q₁₄ sites of reaction centers (58), the quinol-oxidase (Qₐ) domains of b₆ complexes (12), and those postulated for b₆ Q₁ domains (51, 52, 57); or (iii) artifacts resulting from local differences in resolution and interpretation

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2 D. Picot, personal communication.
of electron densities. The phenotype observed here is more consistent with the description of the Q site obtained from Chlamydomonas. Nevertheless, our data cannot rule out other possible interpretations such as a very tight interaction of Arg214 with the c(i) heme. Further analysis will be needed to resolve this question.

**Electron Flow through Inhibited Q Sites in Cytochrome bf Complexes**—We have shown that the Synechococcus R214H mutation had an effect like that of NQNO, which slows bH heme oxidation and partially slows the turnover of the cytochrome bf complex (29). Surprisingly, the addition of NQNO to the R214H complexes—

**oxidation and partially slows the turnover of the cytochrome**

**b**

**heme**

**i**

of this type could imply that Arg214 provides a crucial binding site for NQNO and that the inhibitor binds poorly to the mutant complexes. Alternatively, electron transfer from heme bH to the Q, plastoquinone might already be largely (or perhaps completely) blocked either in the R214H mutant or by NQNO at 4 μM. The addition of NQNO to the mutant would then have no further impact. Turnover of the cytochrome complex under these conditions (~30% of that of the wild type) could only occur if there were a bypass or “electron leak” pathway that allows electrons to be diverted from Q, plastoquinone reduction.

The cytochrome bf structures reveal accessibility of the c(i) heme to the stromal compartment, raising the possibility of Q, site access to ferredoxin or ferredoxin NAD(P)H oxidoreductase in a cyclic electron pathway around photosystem I (9, 10, 59). This could imply that Arg214 provides a crucial binding site for NQNO and that the inhibitor binds poorly to the mutant complexes.

**The cytochrome bf complexes might operate in reverse depending on equilibrium midpoint potentials and sizes of electron pools as documented, for example, in the “reverse,” uphill electron transfer through the bc complex of Thiothrix ferrooxidans (60).**

**Conclusion**—Accumulating evidence indicates that under typical conditions in wild type organisms, electron transfer to the cytochrome bf Q site should proceed from heme bH to heme bH > heme c(i) > Q, plastoquinone. As mentioned, the cytochrome bf structures present compelling evidence for electronic coupling of hemes bH and c(i). It seems unlikely that the c(i) heme placement evolved for uniquely structural rather than functional reasons. We suggest that Synechococcus cytochrome bH Arg214 (Mastigocladus or Chlamydomonas Arg205) plays a central role in Q, plastoquinone binding. Moreover, data presented here demonstrate for the first time, that modification of the Q, site of the cytochrome bf complex dramatically alters overall photosynthetic efficiency. This finding supports previous indications that the quinone-reductase reaction is continuously engaged during steady state photosynthesis (39), thus modulating H+/H– stoichiometries and efficient photosynthetic CO2 assimilation.

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