Site-Directed Mutagenesis of Arginine-114 and Tryptophan-129 in the Cytochrome *b* Subunit of the bc_1 Complex of *Rhodobacter sphaeroides*: Two Highly Conserved Residues Predicted To Be near the Cytoplasmic Surface of Putative Transmembrane Helices B and C†

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ABSTRACT: The cytochrome *b* subunit of the ubiquinol:cytochrome *c* oxidoreductase (the bc_1 complex) contains two heme prosthetic groups, cytochrome b_L and cytochrome b_H. In addition, this subunit also provides major elements of the quinol oxidation site (Q_o) and a separate quinone reductase site (Q_i), which are thought to be located on opposite sides of the membrane. Site-directed mutagenesis has been used to explore the role(s) of specific amino acid residues in this subunit from the photosynthetic bacterium *Rhodobacter sphaeroides*. Previous work identified five residues, Gly48 (Gly33), Ala52 (Gly37), His217 (His202), Lys251 (Lys228), and Asp252 (Asp229), as being either at or near the quinone reductase site (the residue numbers in parentheses designate the equivalent positions in the yeast mitochondrial enzyme). These residues are predicted to be near the cytoplasmic boundaries of transmembrane helices: helix A (G48, A52), helix D (H217), or helix E (K251, D252). In the current work, the importance of two additional highly conserved residues, which are also predicted to be near the cytoplasmic boundaries of transmembrane helices, is explored by site-directed mutagenesis. R114 (helix B) has been substituted with K, Q, and A, and W129 (helix C) has been changed to A and F. The results suggest that a positively charged residue at position 114 is important. The R114K mutation causes only subtle effects, which appear to be localized to cytochrome b_H and the quinone reductase site. In contrast, R114Q is not assembled, and R114A, although partially assembled, is nonfunctional and appears to have a very low amount of cytochrome *b* associated with the complex. Both mutants at position 129 (W129A and W129F) are able to support the photosynthetic growth of the organism, but show abnormal characteristics. The defects associated with the W129A mutation appear to be primarily associated with the quinone reductase site and cytochrome b_H, whereas the W129F mutation appears to result in more global defects that also perturb the cytochrome b_L locus. The results are consistent with the placement of residues R114 and W129 near the cytoplasmic side of the membrane, but suggest that these residues are important for the assembly and overall stability of the complex.

The ubiquinol:cytochrome *c* oxidoreductases, also known as the bc_1 complexes,[1] catalyze electron transfer between ubiquinol and cytochrome *c* in mitochondria and bacteria and (as the b_6f complex) between plastoquinol and plastocyanin in chloroplasts [reviewed in Crofts (1985), Hauska et al. (1988), and Trumpower (1990)]. All bc_1 and b_6f complexes contain a cytochrome *b* subunit that contributes major structural elements to both a quinol oxidase site (Q_o or Q_z site) and a quinone reductase site (Q_i or Q_c site). The two sites are proposed to be on opposite sides of the membrane, with quinol oxidation occurring on the periplasmic side and quinone reduction occurring on the cytoplasmic side (Crofts et al., 1983; Mitchell, 1976).

The cytochrome *b* subunit of the bc_1 complexes has been remarkably conserved during evolution. A two-dimensional model of the cytochrome *b* subunit is shown in Figure 1, in which eight transmembrane helical spans are indicated (Crofts et al., 1987). The presence of these transmembrane spans and their approximate boundaries are consistent with a large body of experimental evidence, and the arguments in favor of this model have been reviewed recently (Crofts et al., 1992; Degli Esposti et al., 1993; Yun et al., 1991b). A comparison of cytochrome *b* sequence data from over 930 species reveals a set of about 17 totally conserved residues and an additional number of highly conserved residues (Degli Esposti et al., 1993; M. Degli Esposti, personal communication). It seems likely that these conserved residues are important for the structure and/or function of the enzyme or for the assembly process.

Site-directed mutagenesis studies have been directed toward elucidating the functional or structural roles of specific residues. For example, invariant histidines H111 (helix B) and H212 (helix D) have been identified as the axial ligands of cytochrome b_H. Nonfunctional, but assembled, variants of the bc_1 complex that lack cytochrome b_H have been generated by placing other amino acids in either of these positions (Yun et al., 1991a). Other highly conserved residues are not so critical for function; S102, located near one of the axial ligands of cytochrome b_L in helix B, has been changed to alanine without any measurable effect other than a small perturbation of the spectrum of cytochrome b_L (Yun et al., 1992). Overall,

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[1] Abbreviations: bc_1 complex, ubiquinol:cytochrome c_2 oxidoreductase; BChl, bacteriochlorophyll; cyt, cytochrome; cyt b_H, higher potential cytochrome *b* of the bc_1 complex (with an $E_{m,7}$ of 50 mV in wild-type strains); cyt b_L, low-potential cytochrome *b* of the bc_1 complex (with an $E_{m,7}$ of −90 mV in wild-type strains); Q_i site, the quinone reduction site (also known as Q_n or Q_c site); Q_o site, the quinol oxidation site (also known as Q_p or Q_z site); RC, reaction center.

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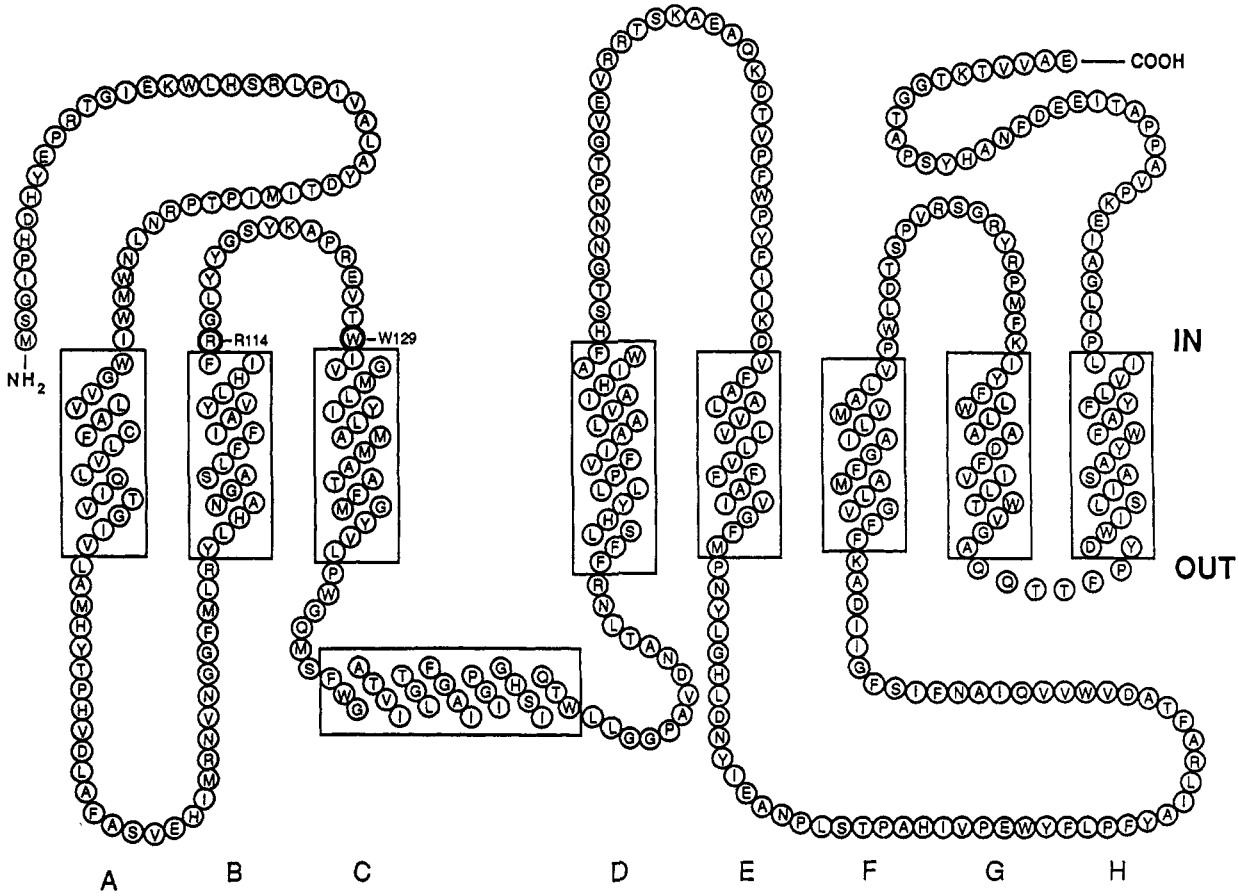


FIGURE 1: Two-dimensional model of the cytochrome *b* subunit of the bc_1 complex from *Rb. sphaeroides*. The positions where mutations have been introduced are indicated. In this model, the approximate borders of eight putative transmembrane spans are boxed, as is a putative amphipathic helix in the segment connecting transmembrane helices C and D. The periplasmic side of the membrane is labeled OUT.

the pattern that has emerged from mutagenesis studies is that when amino acid substitutions have significant functional effects, these generally result in perturbations that are localized. That is, the lesions appear to be primarily at the quinol oxidation site and its associated cytochrome b_L or at the quinone reductase site and its associated cytochrome b_H.

Identification of residue changes leading to resistance to inhibitors at the Q_i site (antimycin, diuron, HQNO) has provided important insights into the location of the site in the protein [see reviews by Colson (1993) and Degli Esposti et al. (1993)]. The distribution of mutations that perturb the quinol oxidase site or the quinone reductase site is consistent with the topology shown in the model in Figure 1 and with the assignments of the quinol oxidase site near the outside of the membrane (bacterial periplasm) and of the quinone reductase site on the inside of the membrane (bacterial cytoplasmic side) (Crofts et al., 1987; Colson, 1993; Gennis et al., 1993). In a more refined tertiary model, we used the pattern of mutations leading to resistance or functional modification to define the volumes of these catalytic sites (Crofts et al., 1992; Degli Esposti et al., 1993).

By targeting highly conserved residues predicted to be near the cytoplasmic side of the membrane, five different residues have been identified in the cytochrome *b* subunit from *Rhodobacter sphaeroides* as likely to be at or near the quinol reductase site. These are G48 and A52 in helix A, H217 in helix D, and K251 and D252 in helix E (Yun et al., 1992; Hacker et al., 1993). Of particular interest is that some strains with mutations in one or other of these positions (e.g., G48V, H217A, and D252A) are unable to rapidly reoxidize cytochrome b_H, and strains expressing these mutants are photosynthetically incompetent. The substitution of valine for G48 also results in spectroscopic perturbations to both cytochromes b_H and b_L, indicating changes to the structure beyond the immediate locale of the lesion.

The current work continues the exploration of the roles of highly conserved residues in the cytochrome *b* subunit by targeting R114 and W129 of helix B and helix C, respectively, which are both predicted to be at cytoplasmic boundaries (Figure 1). Although both of these residues are modeled to be at approximately the same level as G48, H217, and D252 with respect to the membrane bilayer, they are not predicted to impinge directly on the catalytic site in the tertiary model. W129 is invariant in aligned sequences of the cytochrome *b* subunit, whereas R114 is substituted by a lysine in some mitochondrial sequences, so that a positive charge is invariant at this position. The results from the site-directed mutagenesis are consistent with the conclusions from the phylogenetic comparisons, insofar as they show that R114K is tolerated whereas R114Q and R114A are both photosynthetically incompetent. R114Q is not assembled in the membrane and R114A appears to be poorly assembled. Both W129F and W129A can support photosynthetic growth, but both showed a modified phenotype, with the effects of the W129F mutations clearly extending to the cytochrome b_L locus. None of the assembled bc_1 complexes with mutations for R114 or W129 showed the dramatic inhibition of the reoxidation of cytochrome b_H observed previously with strains carrying mutations G48V, H217A, and D252A. While the previous data supported a model in which G48, H217, and D252 shared a common volume defining part of the quinone reductase site (Crofts et al., 1992; Hacker et al., 1993), our present results

provide no reason to include either R114 or W129 in this shared volume since the phenotypes of mutations at these positions are very different. However, the data do support the contention that both R114 and W129 are near the cytoplasmic boundary and are critical for the assembly and maintenance of the structure of the complex.

MATERIALS AND METHODS

Materials. All restriction endonucleases and nucleic acid-modifying enzymes were obtained from New England Biolabs, Inc., or Bethesda Research Laboratories. Synthetic deoxyoligonucleotides for DNA sequencing and mutagenesis were synthesized at the Biotechnology Center of the University of Illinois on an Applied Biosystems Model 380A DNA synthesizer.

Bacteria and Plasmids. Escherichia coli strains were grown in L broth (Sambrook et al., 1989) at 37 °C. Plasmids and their derivatives were maintained in *E. coli* strains in the presence of ampicillin (75 mg/mL), kanamycin (50 mg/mL), or tetracycline (15 mg/mL). Growth medium used with strains of *Rb. sphaeroides* containing pRK415 derivatives was supplemented with 1 mg/mL tetracycline. *Rb. sphaeroides* strains were grown photosynthetically or aerobically in Sistrom's minimal medium A (Leuking et al., 1978) by vigorous shaking at 30 °C (Kiley & Kaplan, 1988; Yun et al., 1990). Photosynthetic growth was measured by growing cells anaerobically on Sistrom's plates. A Carolina Biological far-red 750 filter was used with photosynthetic *Rb. sphaeroides* cultures containing tetracycline to minimize photooxidation of the tetracycline. Dilutions of aerobically grown cultures were plated to give single colonies. Colonies for the photosynthetically competent mutants appeared on the plates after incubation for the same time as the wild-type (BC17C) control.

Preparation of Mutant Plasmids and Site-Directed Mutagenesis. The site-directed mutagenesis protocol for all mutants was described earlier (Hacker et al., 1993; Vandeyar et al., 1988; Yun et al., 1991a). All mutations were verified by DNA sequencing. The strain name, mutation at the protein level and at the nucleic acid level, and the oligonucleotide primers used for mutagenesis are listed here:

R114A Arg114Ala: CGC→
GCA (ATCTTCGCAGGCCTCTAC)

R114Q Arg114Gln: CGC→
CAA (ATCTTCCAAGGCCTCTAC)

R114K Arg114Lys: CGC→
AAG (ATCTTCAAGGGCCTCTAC)

W129A Trp129Ala: TGG→
GCG (GTCACCGCGATCGTGGGG)

W129F Trp129Phe: TGG→
TTC (GTCACCTTCATCGTGGGG)

Optical Redox Titrations. Chromatophores were prepared (Bowyer et al., 1979) and chemical redox titrations were performed as previously described (Dutton, 1978; Yun et al., 1991a). Unless otherwise indicated, the complement of mid-potential cytochrome *b* (referred to as $b_{50} + b_{150}$) was taken as that fraction titrating between E_h values of 200 and −20 mV, using the wavelength pair 561–569 nm. Data were taken from redox titrations in which the difference spectrum between 520 and 584 nm was measured at each value of E_h, using the spectrum at 200 mV as a reference. Antimycin-induced oxidation of cytochrome b_{150} in chromatophores was performed as described previously (Meinhardt & Crofts, 1984; Hacker et al., 1993).

Table 1: Summary of the Characteristics of Strains Containing Mutations

strain	mutation	cyt b_L $E_{m,7}$ (mV)	cyt b_{H150} $E_{m,7}$ (mV)	cyt b_{H50} $E_{m,7}$ (mV)	photosynthetic growth
BC17C		−90	150	50	+++
Gab*R114A*	R114A	−150	148	33	
Gab*R114Q*	R114Q				
Gab*R114K*	R114K	−94	147	12	+++
Gab*W129A*	W129A	−84	149	16	+++
Gab*W129F*	W129F	−52	141	39	+++

Spectrophotometric Analysis of Flash-Induced Kinetics of Cytochrome b_H. The kinetic single-beam spectrophotometer used has been described elsewhere (Crofts & Wang, 1989). The kinetics of redox changes in the cytochrome components of the bc_1 complex were measured as previously described (Crofts et al., 1983; Meinhardt & Crofts, 1983; Yun et al., 1991a). All experiments were performed at pH 7.0 with chromatophores suspended in a 100 mM KCl and 50 mM MOPS buffer. The mediators used in each experiment were as previously described (Yun et al., 1991a).

RESULTS

Growth Properties of Mutant Strains. The following mutants were characterized: R114A, R114Q, R114K, W129A, and W129F. The characteristics of each of the mutant–bc_1 complexes were examined after expressing the mutant *fbc* alleles in a strain of *Rb. sphaeroides* (BC17) from which the wild-type chromosomal *fbc* operon had been deleted (Yun et al., 1990). With the exception of R114Q and R114A, the mutant–bc_1 variants confer upon strain BC17 the ability to grow photosynthetically (Table 1). All of the strains are able to grow aerobically and under microaerophilic conditions.

Spectroscopic and Electrochemical Properties of the Cytochromes. Full-spectrum redox titrations were performed with membrane preparations from strains expressing each of the mutant–bc_1 complexes. For these experiments, all strains were grown under microaerophilic conditions. Mutant R114Q lacks all of the cytochromes of the bc_1 complex (cytochromes c_1, b_L, and b_H) and is unable to grow photosynthetically, suggesting that the complex is not assembled in the membrane. It was, therefore, not examined further. A second mutant that could not grow photosynthetically, R114A, does contain cytochrome *c* and possibly *b*-type components, in addition to those observed in the *fbc*⁻ control strain (BC17). Although cytochrome c_1 appears to be fully present, the cytochrome *b* is only about 20% of that in a control strain (BC17C), and the spectroscopic and redox properties measured in membranes are not those typically associated with cytochromes b_H and b_L of the bc_1 complex. This reflects the presence of one or more additional *b*-type cytochromes in the *fbc*⁻ strain BC17, which are minor components in strains with a normal complement of bc_1 complex, but which contribute an increasingly dominant fraction of the mid-potential *b* cytochrome complement when the bc_1 complex (or its cytochrome *b* subunit) is weakly expressed. The main component of this extra complement titrates in the range 200–100 mV and therefore overlaps with cytochrome b_{150}.

As shown in Table 1, the wild-type complex in strain BC17C has three electrochemically defined cytochrome *b* components. Cytochrome b_L normally has a midpoint potential (pH 7) at −90 mV, and cytochrome b_H shows two components, referred to as cytochrome b_{50} and cytochrome b_{150}, where the subscripts

Table 2: Characteristics of Cytochrome b_H

mutation	total potentiometric cyt b_H ($b_{50} + b_{150}$) (nmol/mg of protein)	potentiometrically determined $b_{150}/(b_{50} + b_{150})$	antimycin-induced oxidation $b_{150}/(b_{50} + b_{150})$	% total cyt b_H reduced by flash kinetics
BC17C	1.96	0.262	0.148	10.7
R114K	1.5	0.074	0.013	3.1
W129A	0.58	0.119	0.058	2.2
W129F	0.72	0.35	0.207	2.9

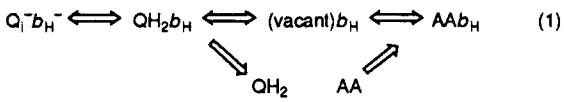
indicate the approximate apparent midpoint potentials on titration (Dutton & Jackson, 1972; Meinhardt & Crofts, 1983). In the presence of antimycin, cytochrome b_H titrates as a simple species with E_m = 50 mV; antimycin is thought to bind at the quinone reductase site and displace a bound semiquinone species involved in reactions, leading to the apparent change in E_m giving rise to cytochrome b_{150} (Meinhardt & Crofts, 1984; Crofts, 1985; Rich et al., 1989; Salerno et al., 1989). As shown in Table 1, the R114A mutant has a component with a remarkably low potential (−150 mV). We are not able to say on the basis of these titrations whether this represents the cytochrome b_L component. The midpoint potentials of the two cytochrome components in the mid-potential range are near normal for cytochrome b_H. However, the reduced-minus-oxidized spectra of cytochrome titrating over the ranges of cytochromes b_H and b_L are atypical (not shown). For example, the low-potential cytochrome (b_L) has a split α-band in the wild type, but this is not observed for the component titrating at −150 mV in the R114A mutant (not shown). These data suggest that the small amount of the mutant bc_1 complex that is present has a severely perturbed structure or that the cytochrome b species observed in the membranes (Table 1) may not even be associated with the complex.

The two mutants that are the least perturbed are R114K and W129A. In each case, the midpoint potential of cytochrome b_H (the b_{50} component) is shifted down to 12 and 16 mV, respectively. The bc_1 complex of the R114K mutant is present in the membrane at close to the level observed for the wild type (77% of $b_{50} + b_{150}$ when normalized per milligram of membrane protein), whereas the amount of the complex in W129A is reduced (37%). Surprisingly, the W129F mutant apparently is more perturbed than when alanine is substituted at this position. The W129F mutation results in a substantial perturbation to the midpoint potential of cytochrome b_L, which is located on the opposite side of the membrane in structural models (Crofts et al., 1987), although the spectrum is unperturbed compared to the wild type and the enzyme is sufficiently active to support photosynthetic growth of the strain expressing this allele. Hence, the W129F mutant displays some structural perturbation that is reported by the cytochrome component on the other side of the membrane, implying global rather than local effects.

The complement of different activities of cytochrome b in each mutant is summarized in Table 2. It can be seen that the fraction of cytochrome b_H present as cytochrome b_{150} and the fraction connected to reaction centers to provide a complete electron transfer chain vary considerably between strains. In all strains (including the BC17C control), the fraction of complexes connected to reaction centers was small; this reflects the semiaerobic conditions used for growth. However, in the control strain and most mutants, the photosynthetic electron transport chain is expressed with a fixed stoichiometry of approximately 2 reaction centers:1 bc_1 complex. In the W129F strain, the stoichiometry was approximately 10 reaction centers:1 bc_1 complex, making precise kinetic measurements difficult.

Flash Kinetics and the Effects of Binding Antimycin. Further effects of the mutations were characterized by monitoring the changes in flash-induced kinetics of cytochrome b_H and by examining the binding of the inhibitor antimycin. For the flash kinetic experiments, turnover of the bc_1 complex is initiated by a 90%-saturating flash of 5 μs duration. The chromatophore samples were poised at 100 mV, so that cytochromes b_H and b_L were oxidized before the flash. Antimycin was used as an inhibitor of electron flow from cytochrome b_H to the quinone electron acceptor at the Q_i site (Crofts et al., 1983; Mitchell, 1976). The resulting redox changes were monitored at selected wavelengths on a millisecond time scale.

Antimycin binding to the complex can be titrated by monitoring the effect of the inhibitor on the flash-induced kinetics of cytochrome b_H reduction or, independently, by following the antimycin-induced oxidation of cytochrome b_{150}. At a solution potential (E_h) of 110 mV, the population of cytochrome b_H with a midpoint potential of about 150 mV is mostly reduced. We have suggested that cytochrome b_{150} is formed upon reduction of cytochrome b_H by QH_2 at the Q_i site to give a complex with $Q_i^- b_H^-$ and that the equilibrium mix is shifted upon binding of antimycin, as shown by the following reaction (Crofts, 1985; Glaser et al., 1984; Meinhardt & Crofts, 1984; Rich et al., 1989; Robertson & Dutton, 1988; Salerno et al., 1989):



(1)

Mutations at Position 114. The substitution of R114 by lysine is the least perturbing of all the mutations examined. Figure 2 shows the flash-induced kinetics of cytochrome b_H in the absence (Figure 2A) and presence (Figure 2B) of antimycin. In the absence of antimycin, R114K shows a very small transient reduction of cytochrome b_H. The reoxidation of cytochrome b_H in the wild-type control (strain BC17C) is also shown in Figure 2A. In the presence of antimycin, the full extent of reduction of the kinetically competent cytochrome b_H is observed to be about double for the R114 mutant compared to the wild-type control (BC17C). However, the concentration of cytochrome b_H, as determined by the redox titration (cytochrome b_{50} + cytochrome b_{150}) in the cuvette, was about 4.9-fold higher for the R114K mutant than for the wild type. Hence, the fraction of bc_1 complex that is kinetically coupled to the flash-activated reaction centers is about one-third for the R114K mutant in comparison to the wild-type control (see Table 2). Since the extent to which the activity of the bc_1 complexes could be detected kinetically in these flash experiments depends on factors beyond the structure of the bc_1 complex, the magnitude of the observed changes is difficult to interpret. More significant are the rates that are observed.

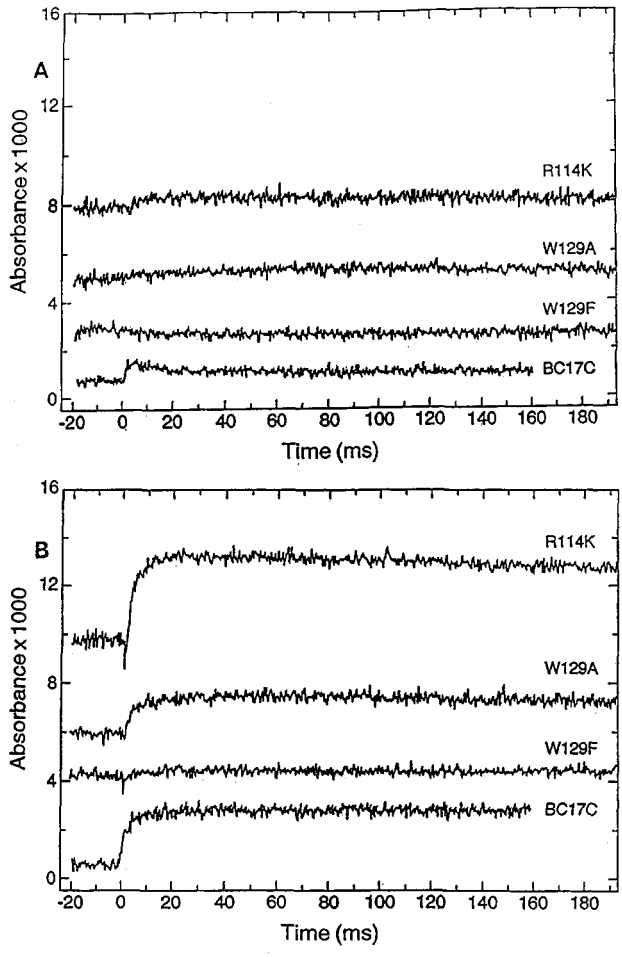


FIGURE 2: Flash-induced kinetics showing the reduction of cytochrome b_H for the wild-type BC17C and for mutants in the absence (A) and presence (B) of antimycin. The figure shows the difference between absorbance changes at 561 and 569 nm. Measurements were made using chromatophore preparations adjusted to contain the same chlorophyll concentrations (20 μM BChl). The final relative concentrations of cytochrome b_H ($b_{50} + b_{150}$) in comparison to BC17C (wild-type control) are R114K 4.9, R114A 2.7, W129A 2.5, and W129F 1. Because of differences in photosynthetic expression, the protein concentration was different in each sample.

In order to better observe the rate at which cytochrome b_H is reoxidized, the electrogenic reactions of the bc_1 complex were monitored by following the time course of the carotenoid band shift. The absorption spectrum of the membrane-bound carotenoids responds to charge movements through the bc_1 complex, and the antimycin-sensitive component, shown in Figure 3, reflects the additional electron transfer that can occur in the uninhibited complex (Glaser & Crofts, 1984; Jackson & Crofts, 1971). In the presence of antimycin, only a single electron passes from the Q_o site to cytochrome b_H; in the uninhibited complex, two electrons traverse the complete span from the Q_o site to the quinone at the quinone reductase (Q_i) site. The results show that the rates of reoxidation of cytochrome b_H are comparable for the R114K mutant and for the wild-type control (BC17C). Because the amplitude of the electrochromic change reflects not only the concentration of bc_1 complex but also the concentration of light-harvesting complexes (which contain the carotenoids that show the electrochromic shift), the amplitude of the change is not comparable between strains. For example, R114K had more cytochrome b_H/BChl than wild type (Figure 2 legend) and therefore less light-harvesting complex/cytochrome b_H, and this accounts for the relatively small electrochromic change.

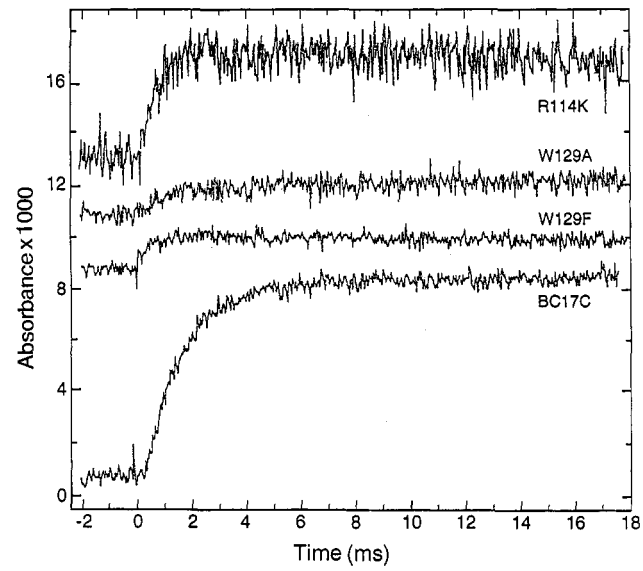


FIGURE 3: Kinetics of the flash-induced carotenoid band shift as seen in the wild type (BC17C) and in the mutants. The traces show the antimycin-sensitive component of the total carotenoid absorption change measured at 503 nm. This antimycin-sensitive portion reflects additional electrogenic events that occur when the oxidation of cytochrome b_H is unimpeded and are the differences between traces with and without antimycin in which electrogenic contributions from the photochemical reactions have been canceled.

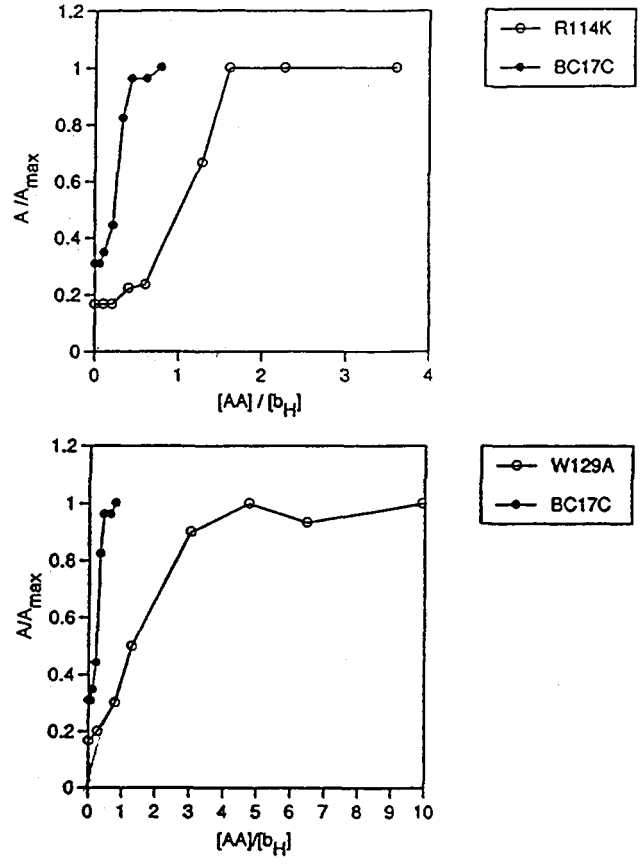


FIGURE 4: Effect of antimycin on the inhibition of flash-induced reduction of cytochrome b_H. Chromatophores were poised at 100 mV and illuminated by a single flash. The amplitude of the change 10 ms after the flash was measured to assay the degree of inhibition shown upon titration with aliquots of antimycin. The absorbance change due to cytochrome b_H reduction (561–569 nm) was measured and normalized to account for differences in cytochrome b_H expression. Sample conditions were as for Figure 2.

Figures 4 and 5 show that the R114K mutant has a somewhat reduced affinity for antimycin. In Figure 4, the

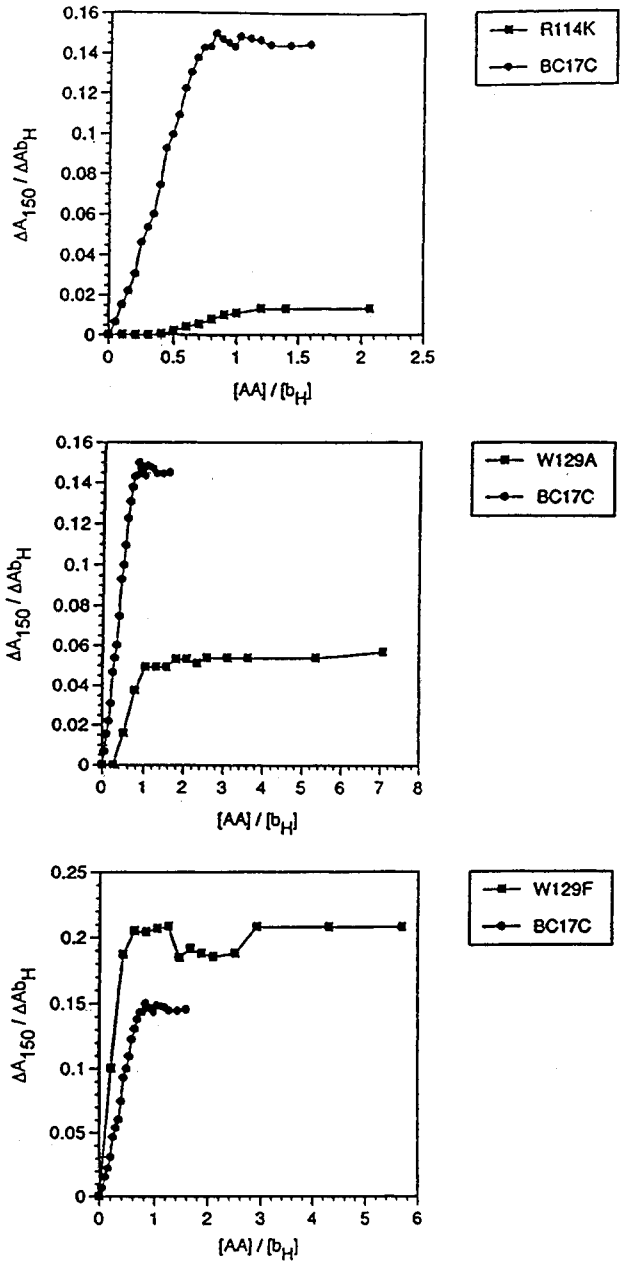


FIGURE 5: Titration of the antimycin-induced oxidation of the cytochrome b_{150} component of cytochrome b_H. Samples were poised at 110 mV, where cytochrome b_{50} is predominantly oxidized and cytochrome b_{150} is predominantly reduced. Each sample was adjusted to the same protein concentration of 2 mg/mL. The addition of antimycin converts cytochrome b_{150} to the cytochrome b_{50} form, giving rise to an absorbance decrease centered at 560 nm and reflecting loss of reduced cytochrome b_{150}. This absorbance change was normalized using total cytochrome b_{50} + b_{150} to allow comparison between the wild type (BC17C) and the mutants, which have different complements of cytochrome b on a protein basis.

amount of antimycin required to block the flash-induced reduction of cytochrome b_H is greater for the R114K mutant than for the wild-type control (BC17C). In Figure 5, it is shown that the addition of antimycin does induce the oxidation of cytochrome b_{150} under the experimental conditions employed, but the amount of antimycin required to observe the full effect is somewhat larger than that for the wild-type control.

More dramatic is the lower amount of antimycin-induced oxidation, indicating a reduced amount of cytochrome b_{150}. This would be expected in the above mechanism, since the equilibrium constant for the formation of cytochrome b_{150} depends on the ΔE_m between the cytochrome and the quinone pool (see Discussion). The lower E_m in this strain would be expected to disfavor the formation of cytochrome b_{150}.

The most significant perturbations due to the R114K mutation therefore appear to be a lower midpoint potential of cytochrome b_{50}, a somewhat weaker affinity for antimycin, and a lower fraction of cytochrome b_H in the form of cytochrome b_{150}. These effects are localized at the cytochrome b_H/quinol reductase site, located on the cytoplasmic side of the membrane in our model.

The substitution of either glutamine or alanine at position 114 clearly is extremely deleterious. The R114Q mutant appears not to assemble, and the R114A mutation results in the low expression of an inactive and abnormal complex, in which no reduction of cytochrome b_H is seen in the presence of antimycin, and there is also no rapid rereduction of cytochrome c_2 (not shown). This suggests that the bc_1 complex is not functional, either because no photosynthetic chains have a complex or because the quinol oxidase site is not functioning. In either case, the fact that no antimycin-induced oxidation of cytochrome b_{150} is observed (not shown) suggests that the quinone reductase site (Q_i) is abnormal. Clearly, the lack of a positive charge at position 114 results in an altered conformation or poor assembly of the cytochrome b subunit.

Mutations at Position 129. Although tryptophan is invariant at this position in all known sequences of the cytochrome b subunit, it seems that either alanine or phenylalanine is tolerated, judging from the fact that both the W129A and W129F mutants have an assembled bc_1 complex that is capable of supporting photosynthetic growth. As will be shown here, the activity of the bc_1 complex in these strains was severely reduced; however, we have previously observed that complexes that turnover at significantly less than 20% of the rate of the wild type can be physiologically competent under the conditions for photosynthetic growth employed. This has been demonstrated by the previously reported D252N mutant (Hacker et al., 1993), which has very slow turnover, yet supports photosynthetic growth.

Further analysis of the W129A and W129F mutants shows that these substitutions are not benign. In the W129A mutant, the midpoint potential of cytochrome b_H is lowered compared to that of the wild-type control, whereas the electrochemical properties of cytochrome b_L are not altered (Table 1). Figure 2 shows that both W129A and W129F exhibit a small flash-induced reduction of cytochrome b_H in the presence of antimycin, although no transient reduction was detectable in the absence of the inhibitor. In the experiment with W129A, the total concentration of the bc_1 complex was 2.5–fold higher than that in the wild-type control, but the small magnitudes of the changes observed in Figure 2B suggests a smaller portion is kinetically coupled to the reaction center. Note that in W129A, the half-time for cytochrome b_H reduction is not substantially altered by the mutation. Figure 3 shows the antimycin-sensitive component of the kinetics of the carotenoid band shift, which reflects the electron transfer between the Q_o and Q_i sites and depends on electron transfer from cytochrome b_H to the quinone acceptor at the Q_i site. In this case, a marked reduction in amplitude of the antimycin-sensitive slow phase was apparent in strain W129A, but the half-time for the small residual slow phase was similar to that of the control. The small amplitude is readily explained by the small fraction of complexes coupled to reaction centers in the photosynthetic chain, and the kinetics suggests that the complex is not severely inhibited. The electrogenic processes in R114K were relatively unperturbed.

The titration with antimycin of the extent of flash-induced reduction of cytochrome b_H (indicating inhibition of oxidation through the Q_i site) of strain W129A is shown in Figure 4. Although the results suggest an apparent weaker affinity for antimycin similar to that observed with the R114K mutant, the small amplitudes of the kinetic changes made precise measurements difficult and less reliable than the titrations of antimycin-induced oxidation (see Discussion). Similar results were obtained with strain W129F, with similar difficulties (not shown). In Figure 5, titrations of the antimycin-induced oxidation of cytochrome b_{150} of the W129A mutant are shown. This mutant has a significantly lower portion of cytochrome b_H in the high-potential form than the wild-type control. As in the case of the R114K mutation, this is consistent with the lowered E_m for cytochrome b_H in this strain. The sharp end point of the titration using W129A does not show the lower affinity for antimycin seen in the titration of flash kinetics (Figure 4). The difference most probably reflects the difficulty of measuring the inhibition accurately in the kinetic experiments where the changes were relatively small. The difference was not explored further, and we do not regard it as significant. The main point is that the changes resulting from the W129A mutation (mainly the lowered E_m of cytochrome b_H and the low fraction of cytochrome b_{150}) are substantially localized to sites expected to be on the cytoplasmic side of the membrane, similar to the observations with the R114K mutant.

In contrast, the W129F mutant exhibits more global defects. The midpoint potential of cytochrome b_L is significantly higher than that of the wild-type control, whereas little electrochemical perturbation of cytochrome b_H is observed. W129F has normal spectra for both cytochrome *b* components (not shown), and the complex is sufficiently active to allow photosynthetic growth. In the absence of antimycin, no transient reduction of cytochrome b_H is observed (Figure 2A), and in the presence of the inhibitor, only a small reduction of cytochrome b_H could be detected. Although the change is small, we conclude from this and a number of similar experiments (not shown) that the half-time for the reduction of cytochrome b_H is somewhat slower (5–7 ms) than that for wild-type controls (~1.5 ms). In the experiments shown, the concentration of the bc_1 complex for the W129F strain in the sample cuvette (as assayed by redox titration of cytochrome $b_{50} + b_{150}$) was the same as that for the wild-type control, although the amount present in the membrane (per milligram of membrane protein) was only about 37% of the wild type. Since antimycin appears to bind with high affinity, we may assume that it blocks the oxidation of cytochrome b_H in this strain, as in the wild type (see following). The small amplitude of cytochrome b_H reduction therefore indicates either a blocked quinol oxidizing site or disproportionately low coupling between bc_1 complex and reaction centers.

Direct measurements from the amplitude of the reaction center change and the reduction of cytochrome b_H following multiple flashes in the presence of antimycin, show that the stoichiometry was approximately 12 reaction centers:1 cytochrome b_H reduced, indicating a stoichiometry 6-fold less than that in wild-type (and most mutant) strains. Because of this low stoichiometry, the electron transfer activity of the W129F mutant could be more clearly observed by monitoring the rereduction of cytochrome *c*, which required multiple turnovers of the complex (Figure 6). The slow rate observed (half-time of ~60 ms, compared to 2–3 ms in wild type) is further inhibited by antimycin, showing that although the activity of the complex is low, the complex is turning over in an antimycin-sensitive reaction. The antimycin-sensitive

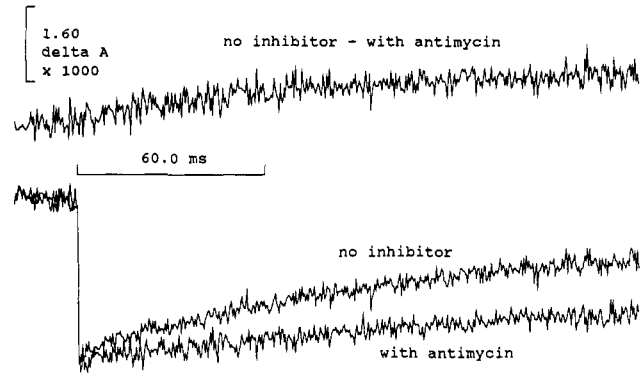


FIGURE 6: Flash-induced kinetics showing the rereduction of cytochrome *c* ($c_1 + c_2$) in the mutant strain W129F. The kinetics of cytochrome *c*, measured from the difference between kinetics measured at 551 and 542 nm, was recorded following a single flash with and without antimycin, and the difference kinetics showing the antimycin-sensitive reduction was obtained by subtraction. Chromatophores were poised at E_h = 100 mV.

component of the carotenoid band shift also shows clear evidence that electron transfer is occurring in the W129F mutant. Note that the half-time of the slow (antimycin-sensitive) electrogenic step is not much altered from that observed for the wild type, but the amplitude is much reduced (Figure 3). While in wild type the slow phase is comparable in magnitude with the fast phase, in this mutant it was ~10% of the fast phase (not shown), reflecting the relative stoichiometry of complex and reaction center. Since the slow electrogenic phase requires the turnover of both the Q_o and Q_i sites of the bc_1 complex, it seems likely that the complex is functional, but that only a small fraction of centers is connected in a functional chain. The very slow electrogenic phase expected from the multiple turnovers, leading to reduction of the high-potential chain (as seen in the cytochrome *c* reduction), is likely lost in the decay due to dissipation of the membrane potential. These data are consistent with the ability of W129F to grow photosynthetically. The turnover of the complex indicated by the relatively rapid electrogenic events suggests that the higher midpoint potential of cytochrome b_L does not greatly perturb the turnover of either site. Nevertheless, apart from the poor connection to reaction centers, this change in E_m for cytochrome b_L is the main observable defect and presumably reflects a long range structural effect, since the cytochrome b_L heme is thought to be on the opposite side of the membrane from the site of the lesion.

The titration of the antimycin-induced oxidation of cytochrome b_{150} shows an amplitude in W129F similar to that in the wild-type control (Figure 5), as might be expected from the similar E_m values for cytochrome b_H. To further examine the functional integrity of the quinone reductase site, an experiment was performed to study the flash-induced electron flow from the quinol pool directly into cytochrome b_H (reversal of the normal forward reaction) in the presence of myxothiazol to block the function of the quinol oxidase (Q_o) site (Glaser et al., 1984; Gray et al., 1994; Robertson et al., 1990). The results (not shown) indicated behavior similar to that of the wild-type control, consistent with a functional quinol reductase (Q_i) site. Hence, this mutant appears to have a fully functional Q_i site, but this is unusual among strains examined to date with mutations on the cytoplasmic side of the subunit since the main phenotypic effect is on cytochrome b_L, which is located on the opposite side of the membrane from the site of mutation.

DISCUSSION

The work presented here continues the exploration of the roles of highly conserved residues predicted to be near the cytoplasmic boundaries of transmembrane spans of the cytochrome *b* subunit of the bc_1 complex. Previous studies revealed that substitutions for G48, H217, K251, or D252 resulted in defects localized to cytochrome b_H and/or the quinone reductase (Q_i) site (Hacker et al., 1993; Yun et al., 1992). A common feature observed with these mutants is a significantly reduced rate of electron transfer from cytochrome b_H to the quinone acceptor at the Q_i site due to mutations at these positions. Especially dramatic effects are observed for mutants at positions 217 and 252, which indicate direct functional roles for H217 and D252 at the Q_i site. The residues implicated as being at the Q_i site are located at the cytoplasmic boundaries of transmembrane helices A, D, and E. The experimental results previously obtained are part of the basis for a proposed model defining the position of these helices, which places G48, H217, K251, and D252 within a common volume that is the proposed Q_i site. This is shown in Figure 7A. Helices B and C also have very highly conserved residues located at their putative cytoplasmic boundaries. R114 in helix B is highly conserved phylogenetically and is substituted by lysine only. W129 in helix C is invariant. In the model shown in Figure 7B, these residues would not be expected to share the same volume previously proposed as the Q_i site, although R114 clearly is close by. The fact that none of the mutational substitutions for either R114 or W129 exhibit the dramatic inhibition of cytochrome b_H reoxidation that is observed for the D252 and H217 mutants substantiates this conclusion. Although the variety of phenotypes cannot be explained in detail, some useful general conclusions can be drawn from the results of this work.

1. Conservation of a positive charge at position 114 is important. Substitution of either glutamine or alanine at this position results in either no assembly of the complex (R114Q) or low expression of a nonfunctional complex with global defects. A lysine substituted at this position changes the E_m value for cytochrome b_H, and this modifies the quinol reductase (Q_i) site proposed to be located nearby. Possible roles for a positively charged residue at this position are to form a salt bridge to one of the propionate side chains of cytochrome b_H, to help anchor helix B properly in the membrane, or both. The effect of the R114Q mutation suggests that a positive charge is important: if the group interacts with the propionate side chain, it is likely an electrostatic interaction, providing an important charge compensation, rather than an H-bond. The lowered E_m in the mutant strain might reflect a weaker electrostatic interaction because of the shorter reach of the lysine than the arginine.

Previous work has demonstrated that heme b_H can be eliminated entirely by mutating H111 or H212, without preventing assembly of the complex (Yun et al., 1991a). Since helix B contains axial ligands to both cytochrome b_H and cytochrome b_L, it is not difficult to rationalize effects extending to both hemes due to a mutation that might influence positioning of this helix in the membrane.

2. The characteristics of the W129A mutant are consistent with the location of this residue near the Q_i site and cytochrome b_H, but not necessarily directly at this site. Although the phenotype is quite different from that of the wild type, the main functional perturbations (lowered E_m of cytochrome b_H and decreased fraction of antimycin-induced oxidation of cytochrome b_{150}) appear rather local to the cytoplasmic side and the Q_i site, and the complex shows near normal kinetics of turnover. The phenotypic effects largely reflect the fact that the amount of bc_1 complex present in the membrane is substantially lower than that in the wild-type control, and a smaller fraction is coupled to reaction centers. This might indicate an assembly or stability problem. One might expect that substitution of phenylalanine for W129 might be more conservative and more benign than placing a small aliphatic side chain in this position. However, this clearly is not the case. Since W129 is invariant in all of the reported sequences, there is no reason based on phylogeny to believe that a phenylalanine would be tolerated any better at this position. It seems possible that the polarity or hydrogen-bonding capacity of tryptophan might be more important than its aromatic nature.

3. The W129F mutant is particularly unusual, since the main phenotypic effect was observed at cytochrome b_L and not at the more proximal Q_i site. As with W129A, the amount of bc_1 complex present in the membrane is substantially lower than that in the wild-type control, and the major phenotypic effects largely reflect this and the small fraction that is coupled to reaction center. This might indicate an assembly or stability problem. This suggests that the highly conserved residues near the cytoplasmic boundary are important to stabilize the overall structure of the subunit.

4. In these mutants, the fraction of cytochrome b_H present in the b_{150} form seems to correlate with a lowered E_m for the cytochrome. This is consistent with the mechanism proposed. In the mechanism outlined in eq 1 earlier, the stability of the cytochrome b_{150} form is determined by the following terms:

overall reaction

$$QH_2(\text{pool}) + \text{cyt } b_HQ \Leftrightarrow Q(\text{pool}) + \text{cyt } b_H^-Q^-(2H^+) \quad (2)$$

partial reaction

$$QH_2(\text{pool}) + \text{cyt } b_HQ \Leftrightarrow Q(\text{pool}) + \text{cyt } b_HQH_2 \quad (2a)$$

$$\text{cyt } b_HQH_2 \Leftrightarrow \text{cyt } b_H^-Q^-(2H^+) \quad (2b)$$

At any defined pH, the equilibrium constant for the formation of [cyt $b_H^-Q^-(2H^+)$] (the cytochrome b_{150} form) according to eq 2 is given by

$$K_{eq} = K_{(2a)}K_{(2b)} = K_{a(QH_2)}/K_{a(Q)} \exp\{(E_{m(\text{cyt } b_H)} - E_{m(QH^\cdot/QH_2)})F/RT\} \quad (3)$$

where K_a values are association (binding) constants for QH_2 and Q, and $E_{m(\text{cyt } b_H)}$ and $E_{m(QH^\cdot/QH_2)}$ refer to the midpoint potentials of cytochrome b_H and the semiquinone/quinol couple at the site, respectively. Computer simulations show that the redox titration curves for cytochrome b_{150} and antimycin-induced oxidation (showing [cyt $b_H^-Q^-(2H^+)$]) can be well modeled in wild-type strains using the assumption that reaction 2 is the pathway preferred.

Examination of eq 3 shows that a lower value of K_{eq} could be expected if $E_{m(\text{cyt } b_H)}$ is decreased or $E_{m(QH^\cdot/QH_2)}$ is increased ($K_{(2b)}$) or if the binding of quinol is decreased or the binding of quinone is increased ($K_{(2a)}$). In the two mutants characterized here (R114K and W129A), in which the E_m of cytochrome b_H was lowered but by residue changes removed from the catalytic domain, the amount of cytochrome b_{150} was substantially decreased, in line with this conclusion.

In summary, the data are consistent with the propositions that both R114 and W129 are located near the heme of

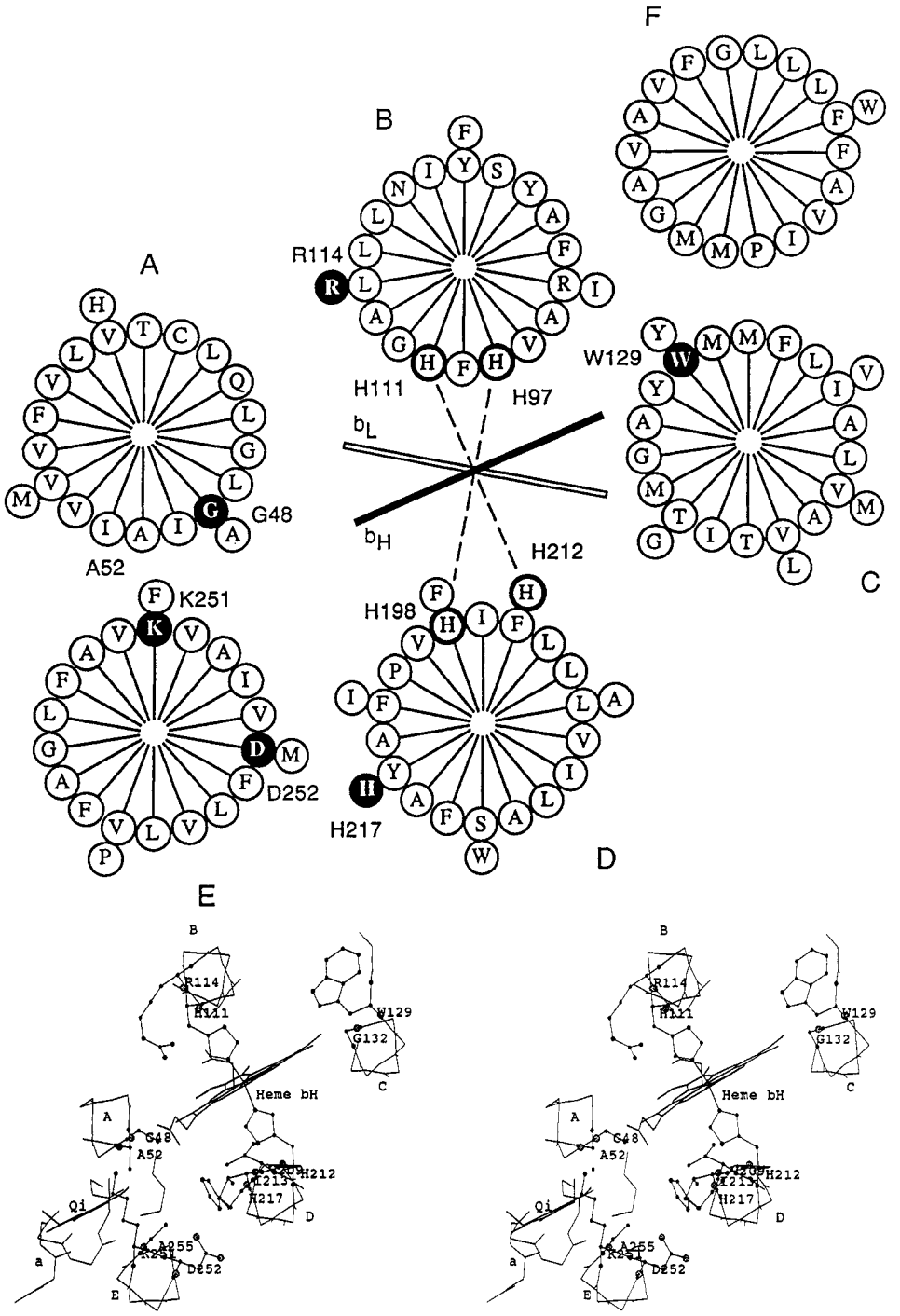


FIGURE 7: (A, top) Helical wheel projection of six of the eight helices that comprise the cytochrome *b* subunit of the bc_1 complex. The hemes are shown along with their respective histidine ligands located in helix B and helix D. Residues discussed in the text are shown as filled circles. G48, H217, K251, and D252 are proposed to define the Q_i site, while R114 and W129 are more important for the global assembly and stability of the cytochrome *b* subunit. (B, bottom) Hypothetical atomic model of the cytochrome *b* subunit showing a 15 Å thick slice through the structure at the membrane/water interface on the cytoplasmic side. The view is from the aqueous phase, looking down along an axis perpendicular to the membrane. The figure shows a stereopair for cross-eyed viewing. The orientation is similar to that of the helical wheel diagram of part A. Side chains for residues mentioned in the text are shown together with the peptide chain backbone, with the heme of cytochrome b_H and a ubiquinone indicating the approximate location of the Q_i site. Letters A–E indicate putative helices identified in structural prediction studies (Crofts et al., 1992). This picture was generated using pdVWIN software (A. R. Crofts, University of Illinois).

cytochrome b_H at the cytoplasmic boundary of transmembrane helices B and C and that these residues are important for both the assembly of the bc_1 complex and the maintenance of the correct conformation of the cytochrome *b* subunit.

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