Glycine-231 Residue of the Mouse Mitochondrial Protonmotive Cytochrome *b*: Mutation to Aspartic Acid Deranges Electron Transport†

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ABSTRACT: The mouse LA9 HQN-R11 cytochrome *b* mutant, in which the glycine residue at position 231 is replaced by aspartic acid, has increased resistance to all inhibitors of the Q_n redox center. It is shown here that this single amino acid alteration has multiple and unexpectedly diverse effects upon the mitochondrial protonmotive bc_1 complex. (1) The specific activities of both succinate– and ubiquinol–cytochrome *c* oxidoreductases in isolated mitochondria are reduced by approximately 65% in the mutant. The parallel reductions in both oxidoreductase activities are not compatible with simple Q pool kinetics for mitochondrial electron transport. (2) There is also a reduction in the relative concentration of cytochrome *b* in the mutant when calculated on the basis of mitochondrial protein; this decrease does not account for more than a small portion of the reduced catalytic fluxes. (3) The increased antimycin resistance of the mutant is lost upon solubilization by the detergent dodecyl maltoside of the bc_1 complex from mitochondria. (4) In pre-steady-state assays of cytochrome *b* reduction by quinol, the mutant shows a reduced extent of reduction. It was observed in other experiments that there was less oxidant-induced extrareduction of cytochrome *b* in the mutant. These results could arise from a lowering of the midpoint potentials of both the cytochrome *b*-562 and cytochrome *b*-566 heme groups. Alternatively, these effects may reflect changes at the Q_p and Q_n quinone/quinol binding sites. (5) An unexplained observation for the mutant is the increased rate of cytochrome c_1 reduction in the presence of myxothiazol. (6) These functional alterations in the LA9 HQN-R11 mutant are not accompanied by detectable changes in the spectral properties of the cytochrome *b* or c_1 heme groups.

The best available description of the catalytic role of the bisheme cytochrome *b* in mitochondrial complex III (ubiquinol–cytochrome *c* oxidoreductase, bc_1 complex) is that provided by the protonmotive Q cycle originally formulated by Mitchell (1976, 1987) and subsequently refined by several groups [reviewed in Bowyer and Trumpower (1981), Rich (1984), Berry and Trumpower (1985), and Crofts (1985)]. Unfortunately, mechanistic details of the redox reactions and proton translocation pathways at the molecular level have remained elusive as there is little definitive structural information available for cytochrome *b* beyond comparative amino acid sequence analyses and hydropathy profiles. The consensus view is that cytochrome *b* folds into *eight* hydrophobic transmembrane domains with a ninth hydrophobic domain being extramembrane (Crofts et al., 1987; Brasseur, 1988; Howell & Gilbert, 1988; Howell, 1989). The two heme groups are ligated between transmembrane domains II and V via two pairs of invariant histidine residues. These structural predictions have been nicely complemented by EPR studies (Ohnishi et al., 1989) and by analyses of the electrogenic events accompanying Q cycle turnover (Robertson & Dutton, 1988), both of which indicate that the low-potential *b*-566 heme is near the electropositive side of the membrane while the high-potential *b*-562 heme lies near the center of the membrane. Standard approaches of structural analysis have not been applicable as separation of cytochrome *b* from other components of complex III results in gross changes in the reduced absorption maxima and/or midpoint potentials of the heme groups (Tsai & Palmer, 1982; Salerno et al., 1986a). There is accumulating evidence that a phospholipid-rich or membrane environment is necessary to maintain native conformation and function of the cytochrome (Yu & Yu, 1980; Salerno et al., 1986a).

The presence of two topologically and functionally distinct heme redox centers in cytochrome *b*—a fundamental tenet of the Q cycle—has been strongly supported by the use of inhibitors which block reactions at one redox center without affecting the other (Edwards et al., 1982; von Jagow & Link, 1986). The ubiquinol oxidizing center is located near the electropositive side of the membrane and is designated Q_p while the ubiquinone reducing center is at the electronegative side and is designated Q_n.[1] Antimycin A, funiculosin, diuron, and HQNO (2-*n*-heptylhydroxyquinoline-*N*-oxide) block the Q_n center while myxothiazol, mucidin (strobilurin), and stigmatellin act at the Q_p center. To define which regions of the cytochrome *b* protein are involved in inhibitor binding or interaction, mutants resistant to these inhibitors have been isolated and the sequence changes in the gene encoding the apocytochrome determined in mouse cells (Howell et al., 1987; Howell & Gilbert, 1988), the yeasts *Saccharomyces cerevisiae* (di Rago et al., 1986, 1989; di Rago & Colson, 1988) and *Schizosaccharomyces pombe* (Weber & Wolf, 1988), and *Rhodobacter capsulatus* (Daldal et al., 1989).

Certain structural features of Q_p and Q_n inhibitors, and their ability to abolish quinone binding at Q_p and semiquinone binding at Q_n, suggest that they may bind to regions within the cytochrome *b* protein which overlap those for quinone/quinol binding [discussed in Howell and Gilbert (1988) but see Brandt et al. (1988) for a more cautionary view]. If this is so, then the amino acid replacements leading to inhibitor resistance may deleteriously affect the catalytic properties of

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[1] This terminology is being adopted in an effort to be more accurate and consistent. Previous designations for the Q_p site included Q_o and Q_z while those for the Q_n site were Q_i and Q_c.

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the protein. Support for this interrelationship comes from the observation that mutation of the highly conserved Gly-142 residue to alanine or serine in the mouse cytochrome *b* (Howell & Gilbert, 1988; Howell, unpublished observations) produces resistance to the Q_p inhibitors myxothiozol and mucidin. However, when the homologous residue (Gly-158) mutates to aspartic acid in the *Rhodobacter* cytochrome *b*, the catalytic function of the Q_p center (but not Q_n) is abolished (Daldal et al., 1989). The inhibitor-resistant mutants isolated in this laboratory have been screened, therefore, to determine which might have functional alterations in electron transport.

The LA9 HQN-R11 mutant is the subject of the present investigation: it was selected for resistance to HQNO but shows low-level resistance to all Q_n inhibitors tested in assays of mitochondrial succinate–cytochrome *c* oxidoreductase (Howell et al., 1983). The resistance phenotype is due to the substitution of aspartic acid for glycine at position 231 of cytochrome *b* (Howell et al., 1987). Comparison of mitochondrial-type cytochrome *b* proteins suggests that there has been selective pressure to maintain a small amino acid residue at this position: glycine is found in all higher eukaryotes, including plants, threonine in the fungi, and alanine in prokaryotes (Howell, 1989). The observation which stimulated the present investigation was that mitochondria isolated from this mutant showed a significantly reduced specific activity of succinate–cytochrome *c* oxidoreductase (Howell & Nalty, 1987), an indication that this amino acid substitution may alter electron transport through cytochrome *b*. The aim of these studies was to ascertain at which point(s) in the catalytic pathway that function was deranged.

Experimental Procedures

Cell Lines and Preparation of Mitochondria. The LA9 HQN-R11 mutant and LA9TK wild-type lines were passaged in Dulbecco's modification of MEM containing 4.5 mg/mL glucose, 2 mM L-glutamine, and 10% calf serum (Colorado Serum Co., Denver) as detailed elsewhere (Howell & Nalty, 1987). Mitochondria were isolated from digitonin-solubilized cells by using the procedure of Moreadith and Fiskum (1984) as detailed in Howell and Nalty (1987). Mitochondrial suspensions were diluted with glycerol to a final concentration of 50% and stored at −80 °C. Storage for several weeks under these conditions results in no decrease in the specific activity of succinate–cytochrome oxidoreductase or cytochrome oxidase; often a 10–20% increase was noted (data not shown).

Dodecyl Maltoside Extracts of Mitochondria. All steps were carried out at 0–4 °C. Mitochondria were thawed, pelleted (10000 rpm for 10 min; GS-34 rotor and Sorvall RC-5B centrifuge), and then washed 2–3 times with 50 mM Tris-HCl/1 mM $MgSO_4$ (pH 7.5). The mitochondria were then washed sequentially with 0.015 and 0.15 M KCl to remove cytochrome *c*, and other soluble proteins, as described by Jacobs and Sanadi (1960). The KCl-washed mitochondria were resuspended in Tris/Mg buffer to a protein concentration of 10 mg/mL and dodecyl maltoside (Boehringer Mannheim Biochemicals) was added to a final concentration of 1 mg/mg of mitochondrial protein. The solubilization process was allowed to proceed on ice for 1 h and the extract then clarified by centrifugation (10000 rpm for 20 min). These conditions are sufficient to solubilize about 90% of the total cytochrome *b*. Mitochondrial extracts were routinely used the same day, but it was observed that thay could be stored for 2–3 days at 4 °C without loss of catalytic activity or change in the spectra of the cytochrome *b* and c_1 heme groups. Concentrations of cytochromes *b* and c_1 in the extracts were estimated by using the molar extinction coefficients of Vanneste (1966).

Biochemical Assays. Ubiquinol–cytochrome *c* oxidoreductase (QCR) was assayed at 30 °C in a buffer containing 40 mM NaP_i (pH 7.5), 0.5 mM EDTA, 20 mM sodium malonate, 0.5 mM KCN, 50 μM horse heart cytochrome *c*, and 5 μM DBH or NBH (2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinol or the nonyl analogue, respectively). The reduced quinol substrate was prepared as described in Trumpower and Edwards (1979); no difference was observed in activity with DBH or NBH as substrate. Succinate–cytochrome *c* oxidoreductase (SCR) was measured by using the same buffer except that sodium malonate was omitted and 20 mM sodium succinate replaced DBH/NBH. For both assays, cytochrome *c* reduction was measured in a dual-wavelength spectrophotometer with a stirred reaction cuvette using the 550–539-nm wavelength pair. Specific activity is expressed as micromoles of cytochrome *c* reduced per minute per milligram of mitochondrial protein and calculated by using a molar extinction coefficient for reduced cytochrome *c* of 21.5. In each assay, the nonenzymic rate of cytochrome *c* reduction was determined and the mitochondrial suspension or dodecyl maltoside extract then added; all values shown here are corrected for nonenzymic activity.

The SCR specific activities obtained with this assay are uniformly about 4-fold higher than those obtained in our previous studies (Howell & Nalty, 1987) using the assay method of Tisdale (1967). The assays performed here are carried out at 30 °C (versus 24–25 °C in our previous studies) but assume a higher molar extinction coefficient for reduced cytochrome *c* (21.5 versus 18.5). The dual-wavelength/stirred reaction cuvette procedure used here is likely to yield greater and more accurate initial rates.

Assays of pre-steady-state reduction of cytochromes *b* and c_1 were carried out with dodecyl maltoside extracts of cytochrome *c* depleted mitochondria as described previously (von Jagow et al., 1984). Extract was diluted to a final concentration of 0.4 μM cytochrome c_1 or 0.8 μm cytochrome *b* in 100 mM NaP_i, 0.5 mM EDTA, 0.5 mM KCN, and 0.5% sodium cholate (pH 7.5) in a stirred reaction cuvette maintained at 30 °C. To ensure that the cytochromes were completely oxidized prior to the start of the experiment, freshly prepared $K_3Fe(CN)_6$ was added to a final concentration of no more than 2 μM. The oxidation–reduction status of the cytochromes was determined prior to and after each reaction. Cytochrome *b* reduction was assayed by using the wavelength pair 562.5–575 nm while cytochrome c_1 reduction was followed with a 553–539-nm wavelength pair. Additional experimental details are given in the legends of the appropriate figures.

These experiments were carried out with an Aminco DW2a dual-wavelength spectrophotometer with either a 2 or a 1-nm band-pass (catalytic assays or absorption spectra, respectively). Data were collected on a Nicolet Explorer III storage oscilloscope/memory system. The spectrophotometer was interfaced to the oscilloscope through an optical encoder attached to the monochromometer; data points were collected at 0.1-nm intervals.

Results

SCR and QCR Specific Activities in Mitochondria. The data in Table I confirm the results obtained previously which showed that mitochondria from the LA9 HQN-R11 mutant have a reduced specific activity of succinate–cytochrome *c* oxidoreductase (Howell & Nalty, 1987). This assay measures the flux of reducing equivalents through both succinate–ubiquinone oxidoreductase (complex II) and ubiquinol–cytochrome *c* oxidoreductase (complex III). To more directly measure the steady-state rate of cytochrome *b* electron

Table I: Specific Activities of SCR and QCR

	LA9TK	LA9 HQN-R11
SCR	0.48 ± 0.29 (4)[a]	0.16 ± 0.03 (13)
QCR	5.58 ± 0.91 (6)	2.08 ± 0.40 (14)

[a] Data are expressed as micromoles of cytochrome *c* reduced per minute per milligram of protein ± 95% confidence interval (number of independent preparations). SCR and QCR are abbreviations for succinate- and ubiquinol-cytochrome *c* oxidoreductase, respectively.

Table II: Antimycin Sensitivity of SCR and QCR

assay	LA9TK[a]	LA9 HQN-R11	mutant/ wild type
SCR[b]	1.0×10^{-10}	1.4×10^{-9}	14.0
SCR[c]	6.1×10^{-11}	2.5×10^{-10}	4.1
QCR[c]	4.3×10^{-11}	8.0×10^{-11}	1.9
QCR/DM[d]	1.8×10^{-10}	4.2×10^{-11}	0.23

[a] Data are expressed as I_{50} values: the moles of antimycin per milligram of protein which inhibits activity 50%. [b] Mean values taken from Howell and Gilbert (1988). [c] SCR and QCR were assayed with thawed mitochondria which had been stored frozen at −80 °C in 50% glycerol. [d] QCR assays done with dodecyl maltoside extracts.

transport, ubiquinol–cytochrome *c* oxidoreductase was assayed directly (Table I). SCR and QCR specific activities in the mutant were approximately one-third those of wild-type mitochondria. The lower specific activity of SCR relative to QCR for mitochondria from the LA9TK wild-type cell line is in accord with earlier observations that in the SCR span of the respiratory chain, the succinate–ubiquinone oxidoreductase (complex II) is kinetically rate-limiting [e.g., see Schneider et al. (1982)].

Antimycin Inhibition of SCR and QCR. The LA9 HQN-R11 mutant was selected for resistance to HQNO at the cellular level and was subsequently shown to have increased resistance to all Q_n inhibitors tested in assays of mitochondrial SCR (Howell et al., 1983; Howell & Nalty, 1987). It has been observed here that inhibitor resistance in this mutant is more complex than initially realized. The antimycin I_{50} value for SCR in LA9TK whole mitochondria (Table II) is very similar to that determined previously (Howell & Gilbert, 1988). The antimycin I_{50} value for LA9TK QCR activity in dodecyl maltoside extracts was about 3-fold higher than that with whole mitochondria, but this was not unexpected since the specific activity of QCR and the concentration of cytochrome *b* in the extracts assayed were also increased about 3-fold (data not shown). In marked contrast, the antimycin I_{50} values for LA9 HQN-R11 mitochondria thawed after frozen storage in 50% glycerol (Table II) were substantially lower than those obtained previously. This trend is particularly accentuated when antimycin resistance of QCR in detergent extracts is assayed. Representative inhibition curves are shown in Figure 1: under these conditions, the QCR of the mutant is more *sensitive* to antimycin. It should be noted that the QCR of the mutant was more sensitive irrespective of whether the data are calculated upon the basis of mitochondrial protein (Table II), cytochrome *b* (Figure 2), or cytochrome c_1 (data not shown). More extensive experiments will be conducted to verify this result and extend this approach to other Q_n inhibitors and to partially purified bc_1 complex.

Room Temperature Cytochrome Spectra. The room temperature spectra of the cytochrome *b* and c_1 heme groups in dodecyl maltoside extracts of LA9TK and LA9 HQN-R11 mitochondria are shown in Figure 2. Under these experimental conditions, the spectral characteristics of the two cytochrome *b* heme groups cannot be resolved, and a single peak centered at about 561 nm is observed. The reduced absorption maxima for the two cell lines are the same within experimental error

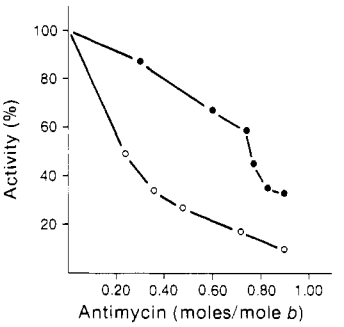

FIGURE 1: Antimycin titrations of QCR activity in dodecyl maltoside extracts of LA9TK (●) and LA9 HQN-R11 (○) mitochondria. Note that the antimycin concentration is expressed as moles per mole of cytochrome *b* heme.

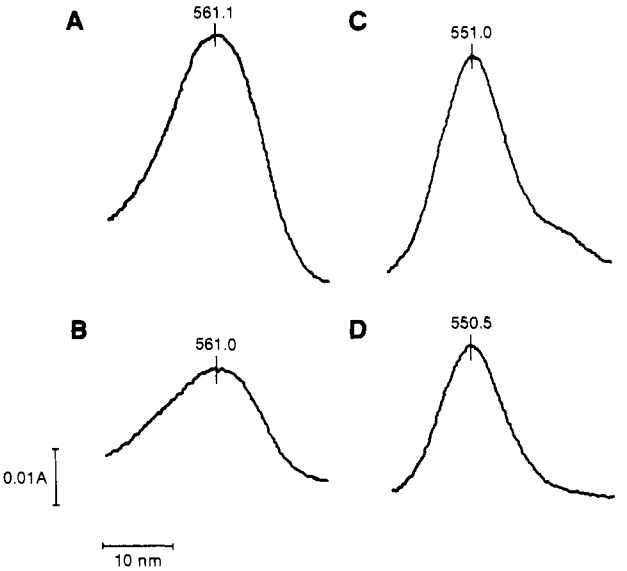

FIGURE 2: Room temperature spectra of cytochrome *b* and c_1 heme groups. Dodecyl maltoside extracts of LA9TK (A, C) and LA9 HQN-R11 (B, D) were assayed. The cytochrome *b* spectra presented in panels A and B are dithionite − ascorbate difference spectra. The cytochrome c_1 spectra shown in panels C and D were obtained from ascorbate reduced − ferricyanide oxidized difference spectra.

although it appears that the spectrum of the *b* heme groups of the mutant may be broader. The reduced absorption maxima—centered about 551 nm—of the cytochrome c_1 heme groups are also very similar although we consistently note that the LA9TK ascorbate-oxidized difference spectra frequently contain a small shoulder at 562–563 nm. Such a shoulder has not yet been observed with dodecyl maltoside extracts of mutant mitochondria. These results suggest that in detergent extracts of wild-type mitochondria, a small amount of cytochrome *b* can be reduced with ascorbate; this may be a consequence of delipidation/denaturation (Salerno et al., 1986b).

A basic characteristic of mammalian cytochrome *b* is that antimycin induces a 1–2-nm red-shift of the reduced absorption maximum of the *b*-562 heme group (von Jagow & Link, 1986). In dodecyl maltoside extracts of LA9TK mitochondria, a 1.5–2.0-fold molar excess of antimycin induces such a shift as shown by the +antimycin/−antimycin fully reduced difference spectra in which there was a trough at 559.0 nm and a peak at 565.0 nm (mean of five assays; data not shown). Identical results were obtained with extracts of LA9 HQN-R11 mitochondria, yielding values of 558.9 and 565.0 nm, respectively (mean of three assays). The amplitude of the shift was similar between the two types of preparation, and no additional spectral changes were observed by increasing the concentration of antimycin. These results complement and

Table III: Concentration of Cytochrome Heme Groups in Dodecyl Maltoside Extracts

	LA9TK	LA9 HQN-R11
cytochrome *b*	0.25 ± 0.04 (5)[a]	0.17 ± 0.02 (4)
cytochrome c_1	0.22 ± 0.02 (5)	0.18 ± 0.05 (4)

[a] The units are nanomoles of cytochrome per milligram of protein, and the data are expressed as mean ± standard deviation (number of independent preparations).

Table IV: Pre-Steady-State Assays of Cytochrome *b* Reduction

assay condition[a]	LA9TK	LA9 HQN-R11
+ANT, +MYXO[b]	0	0
-ANT, -MYXO	28	12
-ANT, +MYXO	32	16
+ANT, -MYXO (+oxidant)[c]	41, 20 (extra)	33, 12 (extra)

[a] All assays were performed with DBH as quinol substrate. The levels of cytochrome *b* reduction are expressed as the percent of total dithionite-reducible cytochrome *b*. The values shown are the average of two to five independent assays and represent the cytochrome *b* which is rapidly reduced (viz., within the ≤1-s mixing time of the cuvette). The final concentrations of antimycin and myxothiazol are 1.0 and 2.0 μM, respectively. [b] In the "double-kill" experiment, there is a slow reduction of cytochrome *b* which for the mitochondria of both cell lines is about 8–9% of the total cytochrome *b* per minute. [c] The second values given are the amounts of oxidant-induced (ferricyanide) extrareduction.

confirm the antimycin inhibition assays of Table II: detergent-solubilized extracts of mutant mitochondria do not show antimycin resistance.

The concentrations of cytochrome *b* and c_1 heme groups were determined and the results summarized in Table III. The mutant line contains about 30% less cytochrome *b* on a protein basis. There may also be some reduction in the concentration of cytochrome c_1, but, if so, it is less marked.

Pre-Steady-State Reduction of Cytochrome b. The pre-steady-state reduction of cytochrome *b* and c_1 heme groups in dodecyl maltoside extracts of LA9TK and LA9 HQN-R11 mitochondria was assayed to derive information about the functional integrity of the Q_n and Q_p redox reaction centers and the flow of electrons into the high-potential pathway of the Q cycle: the Rieske iron–sulfur center and cytochrome c_1. As predicted by the Q cycle (von Jagow et al., 1984; von Jagow & Link, 1986), in the presence of both antimycin and myxothiazol (the "double-kill" experiment), the quinol substrate does not rapidly reduce cytochrome *b* (Table IV). There is, however, for extracts of both wild-type and mutant mitochondria, a very slow cytochrome *b* reduction which may represent a "leak" at one or both sites of inhibition.

In the absence of inhibitor, DBH addition results in the reduction of 28% of the total cytochrome *b* in LA9TK extracts but only a 12% reduction in LA9 HQN-R11 extracts (Table IV and Figure 3). When myxothiazol is used to block electron transfer through the Q_p center, essentially the same results are obtained although there is a slight increase in the extent of cytochrome *b* reduction for both types of extracts (Table IV). This cytochrome *b* reduction is very rapid, being within the mixing time of the cuvette (about 1 s; B. Trumpower, personal communication). The extent of reduction for both extracts is stable for at least 10 min and is not increased by the addition of an electron mediator such as phenazine methosulfate (data not shown). Difference spectra showed that the absorption maximum of the cytochrome *b* reduced by DBH has a wavelength maximum in the range 561–562 nm, indicating, as expected, that it is the high-potential *b*-562 heme group in both wild-type and mutant extracts which is reduced under these conditions.

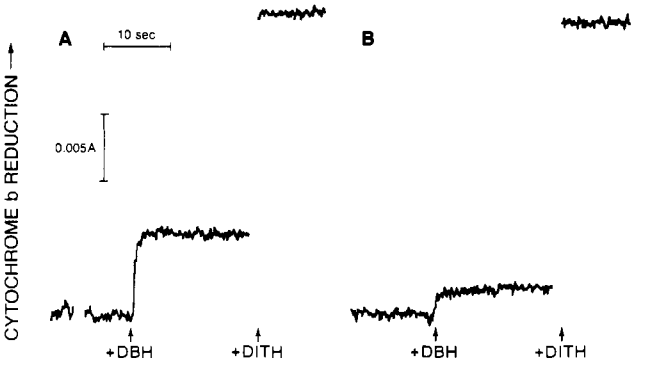

FIGURE 3: Pre-steady-state reduction of cytochrome *b* reduction by DBH in dodecyl maltoside extracts of LA9TK (A) and LA9 HQN-R11 mitochondria (B). The additions are shown at the bottom of the traces. Note that discontinuities in the traces represent time breaks in the experiment; for example, the level of cytochrome *b* reduction was stable for several minutes under these conditions, but only the initial portion of the kinetic trace is shown. The absorbance and time scales shown here are also used in the subsequent figures. Assays were carried out with 0.8 μM cytochrome *b*.

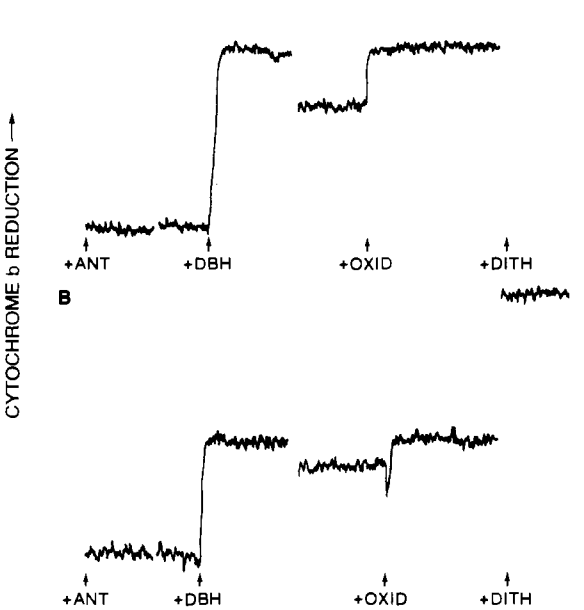

FIGURE 4: Oxidant-induced extrareduction of cytochrome *b* under pre-steady-state conditions in extracts of LA9TK (A) and LA9 HQN-R11 (B) mitochondria. The conditions used in this experiment were the same as those used in Figure 3 (0.8 μM cytochrome *b*) except for the initial addition of 2 μM antimycin and a pulse of ferricyanide where indicated. The oxidant was not added until the level of cytochrome *b* reduction had stabilized (about 5 min) after the initial overshoot when DBH was added.

To determine whether the Q_p redox center of the LA9 HQN-R11 cytochrome *b* was functional, oxidant-induced extrareduction was assayed (Table IV and Figure 4). With dodecyl maltoside extracts of LA9TK mitochondria (Figure 4A), addition of substrate in the presence of antimycin causes an "overshoot" of cytochrome *b* reduction which decreases to a stable level within 5–7 min. Under these conditions in which the cytochrome *b* heme groups equilibrate with the Q pool through the Q_p center only, the mutant again shows less cytochrome *b* reduction. At this point, addition of oxidant (ferricyanide in these experiments) causes additional reduction of cytochrome *b*, about 20% of the total reducible cytochrome *b* in LA9TK extracts (Table IV). Oxidant-induced extrare-

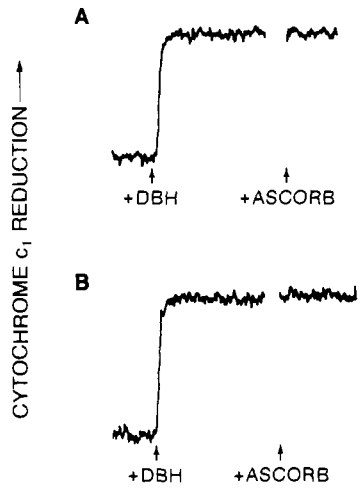

FIGURE 5: Pre-steady-state reduction of cytochrome c_1 in dodecyl maltoside extracts of LA9TK (A) and LA9 HQN-R11 (B) mitochondria. Assays were carried out with 0.4 μM cytochrome c_1.

duction occurs with LA9 HQN-R11 extracts (Figure 4B) although the amount of extrareduction averages only 12% of the total cytochrome *b* heme (Table IV). For either LA9TK or LA9 HQN-R11 extracts, omission of antimycin or substitution with myxothiazol results in no oxidant-induced extrareduction (data not shown), results predicted by the Q cycle (von Jagow et al., 1984).

Cytochrome spectra were taken immediately before and after oxidant addition. The resulting difference spectra for wild-type extracts had a trough averaging 552.4 nmn and a peak at 565.1 nm. For the experiments with the mutant extracts, the corresponding values were 551.6 and 565.1 nm (data not shown). The trough reflects the oxidation of cytochrome c_1, while the peak value indicates it is the low-potential *b*-566 heme group which shows extrareduction. These analyses suggest that the G231D mutation does not markedly alter the spectral properties of the *b*-566 heme group. This interpretation should be treated with caution, however, pending higher resolution spectral analyses. Oxidation of cytochrome c_1—as in these assays—produces a small *increase* in absorbance in the 565–570-nm region, and this could obscure differences in the reduced absorption maxima of the cytochrome *b*-566 heme groups in these extracts [see Figure 7 of Robertson and Dutton (1988)].

Pre-Steady-State Reduction of Cytochrome c_1. To further analyze the functional integrity of the Q_p center, donation of the first electron from quinol substrate to cytochrome c_1 (via the Rieske iron–sulfur protein) was assayed under pre-steady-state conditions. As shown in Figure 5, for both LA9TK and LA9 HQN-R11 mitochondrial detergent extracts, addition of DBH substrate produces a rapid and complete reduction of cytochrome c_1 (the total amount being defined as that reducible by ascorbate). Additional experiments, however, indicate a significant difference between wild-type and mutant bc_1 complexes. Using myxothiazol to block redox events at the Q_p center, there is no rapid reduction of cytochrome c_1 in extracts of LA9TK mitochondria: instead, there is a slow reduction amounting to about 20% of the total cytochrome c_1 reduced per minute (Figure 6A). In marked contrast, the rate of cytochrome c_1 reduction is about 60% per minute with LA9 HQN-R11 extracts in the presence of myxothiazol (Figure 6B). Doubling the amount of inhibitor does not change the rate of reduction for either type of extract. In previous studies, the LA9 HQN-R11 mutant showed no significant resistance to Q_p inhibitors (Howell & Gilbert,

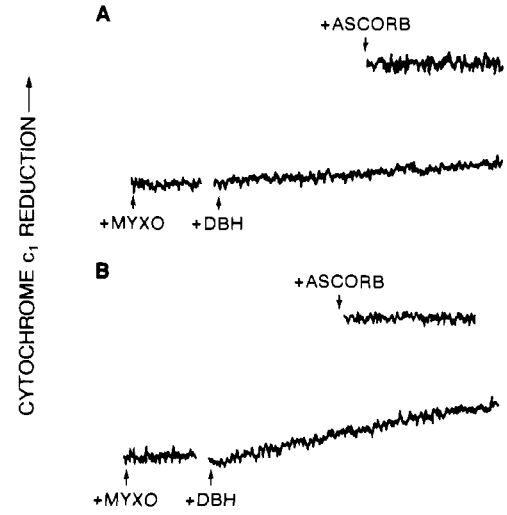

FIGURE 6: Pre-steady-state reduction of cytochrome c_1 in LA9TK (A) and LA9 (B) HQN-R11 extracts in the presence of myxothiazol. The experimental conditions are the same as those in Figure 5 (0.4 μM cytochrome c_1) except that myxothiazol was added to a final concentration of 3 μM. To conserve space, the trace showing the level of cytochrome c_1 reduction after ascorbate addition is shown above the kinetic trace of reduction after DBH addition.

1988). Thus, this rate differences cannot be due to differences in the inhibition of electron flow through the low-potential *b*-566 heme group.

Discussion

Replacement of the glycine residue at position 231 of mouse cytochrome *b* with aspartic acid produces increased resistance of mitochondrial succinate–cytochrome *c* oxidoreductase (SCR) to inhibitors of the Q_n site (Howell et al., 1987). It was observed here that this single amino acid substitution has multiple and complex effects upon the mitochondrial electron transport chain. The preliminary evidence (Howell & Nalty, 1987) that the LA9 HQN-R11 mutant has a functional deficit in cytochrome *b* electron transport was strengthened with the finding that the specific activity of mitochondrial ubiquinol–cytochrome *c* oxidoreductase (QCR) in the mutant is 37% of that in the wild-type parental line when expressed on the basis of mitochondrial protein (Table I). Some of this decrease is due to a reduction in the relative concentration of cytochrome *b* in the mutant (Table III). When the QCR activities are calculated on the basis of detergent-extractable cytochrome *b*, however, the specific activity of the mutant is still no more than half that of wild-type mitochondria.

The reduced concentration of cytochrome *b* (and perhaps cytochrome c_1) in mitochondria isolated from the LA9 HQN-R11 mutant may be a nonspecific, secondary consequence of reduced mitochondrial electron transport. It has been observed in earlier studies from this laboratory that a mutant lacking mitochondrial complex I activity (NADH-ubiquinol oxidoreductase) also had a partial—but significant—reduction in SCR specific activity but normal levels of cytochrome oxidase (Howell & Nalty, 1987). However, it cannot be ruled out that the mutation in the cytochrome *b* gene affects the structure of the protein so that it is less stable or less efficiently integrated into complex III, and, as discussed below, other results also indicate that the cytochrome *b* protein of the mutant may be more labile.

The results in Table I also demonstrate that in the mutant, there are parallel decreases in the specific activities of SCR and QCR. This behavior is not that predicted on the basis of Q pool kinetics for flux through the mitochondrial respir-

atory chain (Kroger & Klingenberg, 1973). If quinone is a mobile carrier of reducing equivalents among mitochondrial respiratory chain complexes, the steady-state rate of flux through the chain is given by eq 1. In this case, v is the flux

$$v = \frac{V_{red} V_{ox}}{V_{red} + V_{ox}} \quad (1)$$

of reducing equivalents from succinate to cytochrome *c*, V_{red} the rate of quinone reduction or succinate–ubiquinone oxidoreductase (complex II), and V_{ox} the rate of ubiquinol oxidation or ubiquinol–cytochrome *c* oxidoreductase (complex III). By use of the activities of SCR and QCR of LA9TK mitochondria to estimate V_{red}, and assuming that this activity is unchanged in the mutant, the value for v in the mutant should be reduced about 13% rather than the 60% observed. Correcting for the difference in the concentrations of cytochrome *b* does not resolve the issue. It is possible that the mutation in the cytochrome *b* protein pleiotropically affects complex II assembly or stability. The cytochrome *b* mutation might also alter the kinetics of complex II turnover through allosteric effects. In this regard, an intriguing observation is that in isolated succinate–cytochrome *c* oxidoreductase, antimycin abolishes the semiquinone EPR signal at the Q_n center but *increases* the intensity of a complex II semiquinone signal (Ohnishi & Trumpower, 1980).

An alternative possibility, however, merits serious consideration. As discussed in detail by Rich (1984), Q pool or liquid-state kinetic behavior is not predicted for all experimental conditions. For example, if there is a high ratio of k_{on}/k_{off} rate constants for quinone/quinol binding to complex III, and if the rates are slow compared to the rate of electron transport, then succinate–cytochrome *c* oxidoreductase could exhibit "solid-state" kinetics in which complexes II and III would apparently behave as a stoichiometric unit (Rich, 1984). Therefore, if an amino acid change that decreases inhibitor binding or interaction concomitantly alters quinone/quinol binding, Q pool kinetics may no longer operate. More detailed kinetic analysis of complex II and complex III electron transport in the LA9 HQN-R11 mutant should clarify the situation.

The Q_n inhibitor resistance of the mutant appears to be conditional upon mitochondrial integrity. Differences in SCR and QCR antimycin resistance between wild-type LA9TK and LA9 HQN-R11 mitochondria are reduced in preparations which have been stored frozen in 50% glycerol (Table II). These storage conditions uncouple mitochondrial respiratory control and release soluble proteins (Howell, unpublished observations). Most striking, however, is the complete loss of antimycin sensitivity in QCR assays with LA9 HQN-R11 mitochondria solubilized with dodecyl maltoside. The normal cytochrome spectra in detergent extracts of the mutant suggest that gross physical disruption of the bc_1 complex has not occurred. One speculative explanation is offered below.

The Gly-231 residue of the mouse cytochrome *b* is positioned within hydrophobic transmembrane domain VI (Crofts et al., 1987; Brasseur, 1988; Howell & Gilbert, 1988). In yeast, mutations altering residues Phe-225 and Lys-228 also increase resistance to Q_n inhibitors (di Rago & Colson, 1988); these residues are located in the loop connecting transmembrane hydrophobic domains V and VI. A second Q_n inhibitor binding/interaction region is located in the N-terminal hydrophilic stretch of residues and extends to include the Gly-37 residue within transmembrane domain I (di Rago & Colson, 1988; Howell & Gilbert, 1988). Mutations altering Gly-37 result in very high level antimycin resistance but *no* increase in HQNO resistance (Howell et al., 1987; Howell & Gilbert, 1988). Antimycin has a very high affinity for cytochrome *b* (von Jagow & Link, 1986), about 1000-fold greater than that of HQNO. Considering these various results together, it seems plausible that the region of cytochrome *b* including the Gly-231 residue and the domain V–VI extramembrane loop constitutes a relatively low-affinity binding site for Q_n inhibitors. Certain amino acid substitutions would alter the conformation of this region, thereby increasing resistance to all Q_n inhibitors in intact mitochondria. However, conditions such as those which exist in detergent extracts could cause local "loosening" of protein structure, disruption or weakening of the "low" affinity site, and thereby allow unobstructed antimycin binding to the high-affinity site.

The pre-steady-state assays of cytochrome *b* and c_1 reduction demonstrate that the Gly-231 → Asp mutation alters the redox equilibration of the bc_1 complex with the Q pool. Assays with inhibitors which block either the Q_n or the Q_p center suggest that events at both redox sites are deranged in the mutant. In the presence of myxothiazol, the high-potential *b*-562 heme group will equilibrate with the Q pool via the Q_n site only. Under these conditions, the extent of cytochrome *b* reduction for the mutant is less than half that observed for reduction of cytochrome *b* from the wild type. This is a true equilibrium difference as shown by the stability of the level of cytochrome *b* reduction over a period of several minutes and by the lack of effect of adding the redox mediator phenazine methosulfate.

One explanation for these results is that the mutation results in a lowering of the E_m (midpoint potential) of the *b*-562 heme group. By use of the Nernst equation (eq 2), an estimate of

$$E_h = E_o + RT/nF \ln [ox]/[red] \quad (2)$$

the change in the E_m can be derived with the following assumptions: (a) The E_h established by the quinol substrate under the assay conditions is in the range of +60 to +90 mV (Rich, 1984). (b) Only the high-potential *b*-562 heme group will be reduced in this E_h range.

In eq 2, R, T, F, and n are, respectively, the Boltzmann constant, the absolute temperature, the Faraday constant, and the number of electrons transferred (equal to 1 for cytochrome *b*). Under the assay conditions used, eq 3 is a reasonable approximation (Dutton, 1978):

$$E_h(\text{DBH}) = E_{m,7}(b\text{-}562) + 0.06 \log [\text{ox cyt } b]/[\text{red cyt } b] \quad (3)$$

Solution of this equation indicates that the $E_{m,7}$ of the *b*-562 heme of the mutant could be lowered relative to that for the wild-type heme group by about 35 mV. As a point of reference, when eq 3 is solved by using the values for cytochrome *b* reduction from the assays with extracts of wild-type mitochondria, the predicted $E_{m,7}$ is in the range of +65 to +95 mV. These values are compatible with those determined by potentiometric titration for the *b*-562 heme group of mammalian mitochondria (Rieske, 1976).

The evidence that redox events at the low-potential *b*-566 heme group are altered in the mutant is more indirect. The results in Table IV show that there is less oxidant-induced extrareduction of cytochrome *b* for the mutant as well as less pre-steady-state reduction when equilibration with the Q pool is limited to the Q_p center. As interpreted within the framework of the protonmotive Q cycle (Berry & Trumpower, 1985), the "extra" cytochrome *b* which is reduced results from a transient increase in the concentration of the Q_p semiquinone anion when cytochrome c_1 and the iron–sulfur protein are oxidized and electron flow from the *b*-566 heme to the *b*-562

heme is blocked by antimycin. Under these conditions, the low-potential *b*-566 heme group is reduced by the Q_p semiquinone. It was confirmed for both the mutant and wild-type preparations that the additional cytochrome *b* reduced had the spectral characteristics of the *b*-566 heme group. Although this type of assay is a nonequilibrium one, one interpretation of the results would be that there is also a decrease in the $E_{m,7}$ of the *b*-566 heme group in the mutant.

An alternative interpretation of these results is that the effective midpoint potentials of the $QH_2/Q^{\bullet-}$ and $Q^{\bullet-}/Q$ semiquinone couples at both the Q_p and Q_n centers are raised in the mutant. This could occur through appropriate changes in the binding constants for quinone, quinol, or semiquinone itself, leading to a net relative increase in the stability of quinone binding. It should be possible to distinguish between these alternatives by potentiometric titration of the cytochrome *b* heme groups of bc_1 complexes isolated from mutant and wild-type mitochondria, and such experiments are currently underway and will be reported separately. Whatever the explanation for these results, the derangements in the redox properties of the bc_1 complex of the LA9 HQN-R11 mutant are not accompanied by detectable changes in the reduced absorption maxima of the cytochrome *b* and c_1 heme groups at room temperature. Within the relatively low limits of resolution of these spectra, therefore, there is no evidence for a gross distortion of the heme electronic environment.

One final result from the pre-steady-state assays merits comment. It was observed that quinol reduction of the cytochrome c_1 heme was normal for both the mutant and wild type when the assays were performed in the absence of a Q_p inhibitor (Figure 5). However, in the mutant, there was a substantially increased rate of reduction in the presence of myxothiazol (Figure 6). The nature of the "leak" around the myxothiazol block begs for an explanation. However, the increased rate of reduction in the mutant is not associated with less efficient myxothiazol inhibition of electron donation to the low-potential cytochrome *b* pathway as the concentration used is sufficient to block cytochrome *b* reduction in the double-kill experiment when both antimycin and myxothiazol are present (footnote *b* in Table IV). One possibility is that, in the presence of myxothiazol, quinol can bind at the Q_p center and that there is an aborted circuit whereby *both* electrons are shunted into the high-potential ISP/cytochrome c_1 branch of the electron transfer pathway under these conditions. For reasons which are not clear, there would be an increased probability of this occurring in the mutant. Formally, it cannot be excluded that quinol is *directly* reducing cytochrome c_1, at a slow rate, but it is less clear how the cytochrome *b* mutation would increase the rate of this reaction.

In summary, the results presented here show that substitution of the Gly-231 residue by aspartic acid in the protonmotive cytochrome *b* produces multiple and unanticipated functional changes in the bc_1 complex. This may not be surprising as redox centers, such as heme groups, need to be protected by protein folding and membrane insertion from solvent (viz., water) caging effects and from the detrimental effects of highly polarizable groups (Larsson, 1988). The mutational substitution of an aspartic acid residue at position 231, which lies within a transmembrane hydrophobic domain, severely violates this principle. The substitution will destabilize transmembrane helix VI, thereby altering the Q_n inhibitor binding domain. As this domain may overlap the Q_n quinone binding site (Howell & Gilbert, 1988), redox catalysis may be directly altered. Furthermore, this local conformational change in the cytochrome *b* protein destabilizes the structure such that the observed inhibitor resistance is lost upon detergent solubilization. It also appears, however, that this amino acid substitution has more "global" structural effects as evidenced by the changes in redox catalysis at the Q_p center; the altered complex II–complex III interaction; the enhanced myxothiazol-resistant cytochrome c_1 reduction; and, possibly, lowering the midpoint potentials of the *b*-562 and *b*-566 heme groups. Further analysis of this mutation, and others which alter the inhibitor binding domains, should provide new insights into the structure–function relationships of cytochrome *b* and additional mechanistic details of electron transport in redox proteins.

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Registry No. Cytochrome *b*, 9035-37-4; ubiquinol–cytochrome *c* reductase, 9027-03-6; succinate–cytochrome *c* reductase, 9028-10-8; glycine, 56-40-6; aspartic acid, 56-84-8.

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Electron-Transfer Reactions in Manganese-Depleted Photosystem II†

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ABSTRACT: We have used flash-detection optical and electron paramagnetic resonance spectroscopy to measure the kinetics and yield per flash of the photooxidation of cytochrome b_{559} and the yield per flash of the photooxidation of the tyrosine residue Y_D in Mn-depleted photosystem II (PSII) membranes at room temperature. The initial charge separation forms $Y_Z^+ Q_A^-$. Following this, cytochrome b_{559} is oxidized on a time scale of the same order and with the same pH dependence as is observed for the decay of Y_Z^+; under the conditions of our experiments, the decay of Y_Z^+ is determined by the lifetime of $Y_Z^+ Q_A^-$. In order to explain this observation, we have constructed a model for electron donation in which Y_Z^+ and $P680^+$ are in redox equilibrium and cytochrome b_{559} and Y_D are oxidized via $P680^+$. Using our results, together with data from earlier investigations of the kinetics of electron transfer from Y_Z to $P680^+$ and charge recombination of $Y_Z^+ Q_A^-$, we have obtained the first global fit for electron donation in Mn-depleted PSII that accounts for the data over the pH range from 5 to 7.5. From these calculations, we have obtained the intrinsic rate constants of all the electron-donation reactions in Mn-depleted PSII. These rate constants allow us to calculate the free energy difference between Y_Z^+ P680 and Y_Z $P680^+$, which is found to increase by 47 ± 4 mV/pH from pH 5 to 6 and is observed to increase more slowly per pH unit for pH > 6. An important conclusion of our experimental work is that the rates of photooxidation of cytochrome b_{559} and Y_D are determined by the lifetime of the oxidizing equivalent on Y_Z/P680. Extension of our model to oxygen-evolving PSII samples leads to the prediction that the kinetics and yields of electron donation from cytochrome b_{559} and Y_D to $P680^+$ will depend on the S_2- or S_3-state lifetime.

The crystal structure of the reaction center from the purple nonsulfur bacterium *Rhodopseudomonas viridis* and its analogy to PSII[1] has significantly advanced our understanding of the electron-transfer pathway from the primary electron donor, P680, to plastoquinone along the electron-acceptor side of PSII (Michel & Deisenhofer, 1988, and references cited therein). However, the analogy between the bacterial reaction center and PSII does not extend to the electron-donor side. The ability of PSII to oxidize water and several components present only in the water oxidation system emphasize the structural and functional differences of electron donation in

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[1] Abbreviations: chl, chlorophyll; cyt b_{559}(ox, red), cytochrome b_{559}(oxidized, reduced); DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; E_m, reduction potential; EPR, electron paramagnetic resonance; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; kDa, kilodaltons; MES, 2-(*N*-morpholino)ethanesulfonic acid; PSII, photosystem II; Tris, tris(hydroxymethyl)aminomethane.

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