Journal of Bioenergetics and Biomembranes, Vol. 31, No. 3, 1999

The Role of Various Domains of the Iron–Sulfur Protein in the Assembly and Activity of the Cytochrome bc_1 Complex of Yeast Mitochondria

Diana S. Beattie[1,2] Yudong Wang,[1] and Victor H. Obungu[1]

Received March 31, 1999

Assembly studies *in vitro* of deletion mutants of the iron–sulfur protein into the cytochrome bc_1 complex revealed that mutants localized in the extramembranous regions of the protein were not assembled into the complex in contrast to the efficient assembly of mutants in the membrane-spanning region. Charged amino acids located in the extramembranous α1-β4 loop and the α1 helix were mutated and expressed in yeast cells lacking the gene for the iron–sulfur protein. Mutating the charged amino acid residues H124, E125, R146, K148, and D149 as well as V132 and W152 resulted in loss of enzymatic activity due to the loss of iron–sulfur protein suggesting that these amino acids are required to maintain protein stability. By contrast, no loss of iron–sulfur protein accompanied the 30–50% loss of bc_1 complex activity in mutants of three conserved alanine residues, A86, A90, and A92, suggesting that these residues may be involved in the proposed movement of the flexible tether of the iron–sulfur protein during catalysis.

KEY WORDS: Cytochrome bc_1 complex; complex III; iron–sulfur protein; yeast mitochondria; ubiquinol; cytochrome *c* reductase.

INTRODUCTION

The cytochrome bc_1 complex is an integral multiprotein complex of the inner mitochondrial membrane that catalyzes the transfer of electrons from ubiquinol to cytochrome *c* coupled to the translocation of protons across the membrane (Brandt and Trumpower, 1994; Trumpower and Gennis, 1994). The bc_1 complex of yeast mitochondria consists of ten subunits, of which three have prosthetic groups that serve as redox centers, cytochromes *b* and c_1, and the Rieske iron–sulfur protein. According to the Q-cycle hypothesis, the generally accepted model for electron transfer and proton translocation in the bc_1 complex, two separate ubiquinone or ubiquinol-binding sites are present in the complex (Brandt and Trumpower, 1994). A ubiquinol-oxidizing site (Q_O) is located at the *p*-side of the membrane and a ubiquinone-reducing site (Q_i) at the *n*-side of the membrane. The oxidation of ubiquinol at the Q_O site results in the transfer of one electron to the [2Fe–2S] cluster of the iron–sulfur protein that subsequently is oxidized by transfer of an electron to the heme of cytochrome c_1. The strongly reducing ubisemiquinone anion formed during ubiquinol oxidation in the Q_O-site reduces the low potential cytochrome b_L that rapidly transfers an electron to the high-potential cytochrome b_H. The reduced cytochrome b_H is then oxidized by transfer of an electron to either ubiquinone or ubisemiquinone at the Q_i site.

The recent resolution of the crystal structure of the cytochrome bc_1 complex isolated from beef (Xia *et al.*, 1997; Iwata *et al.*, 1998) and chicken (Zhang *et al.*, 1998) heart mitochondria has revealed that the bc_1 complex exists as a dimer with the eight membrane-spanning helices of cytochrome *b* comprising the core

[1] Department of Biochemistry, West Virginia University School of Medicine, Morgantown, West Virginia 26506-9142.
[2] Author to whom correspondence should be addressed. email: dbeattie2@wvvhsc1.hsc.wvv.edy

0145-479X/99/0600-0215$16.00/0

of the complex. The location of the Q_O and Q_i sites in the bc_1 complex was established by determining the site of binding of specific inhibitors of quinol oxidation and reduction in these sites (Kim *et al.*, 1998). In the crystal structure, the iron–sulfur protein consists of three separate domains, a membrane-spanning α-helix at its N-terminus passing through the membrane at an angle, a soluble extramembranous domain containing the bulk of the iron–sulfur protein including the [2Fe–2S] cluster, and a "tether" of 7 to 9 amino acids connecting the extrinsic domain to the membrane-spanning α-helix (Iwata *et al.*, 1998; Zhang *et al.*, 1998).

With the exception of cytochrome *b*, the sole mitochondrial gene product of the bc_1 complex, all the subunits of the complex are synthesized on free cytoplasmic ribosomes and in a subsequent step imported into mitochondria where they are assembled into a functional complex in the membrane (Neupert, 1994). The iron–sulfur protein of *Saccharomyces cerevisiae* is synthesized as a precursor protein with a molecular weight of 29 kDa and possesses a 30 amino acid leader sequence at the amino terminus of the protein. The precursor form of the iron–sulfur protein is processed *in vivo* into the mature form through an intermediate form in two distinct processing events observed both *in vitro* and *in vivo* (Sidhu and Beattie, 1983; Fu *et al.*, 1990). The mechanism of assembly of the subunits of the cytochrome bc_1 complex and, in particular, that of the iron–sulfur protein has been the subject of several studies. Earlier studies had suggested that the assembly of the different subunits of the cytochrome bc_1 complex occurred at different rates in an ordered manner (Sidhu *et al.*, 1983; Crivellone *et al.*, 1988). This experimental approach had indicated that the iron–sulfur protein might be one of the last proteins to become associated with a postulated "core" membrane-bound complex, during mitochondrial biogenesis. In more recent studies in our laboratory, the assembly of the iron–sulfur protein into the bc_1 complex was investigated *in vitro* by using selective immunoprecipitation with antiserum against either the iron–sulfur protein or the intact bc_1 complex after import of radiolabeled precursor into mitochondria lacking the iron–sulfur protein (Fu and Beattie, 1991).

ASSEMBLY OF DELETION MUTANTS INTO THE bc_1 COMPLEX *IN VITRO*

Our initial approach to understanding the assembly of the iron–sulfur protein into the cytochrome bc_1 complex focused on establishing which regions of the protein are required for its proper assembly. A series of eight deletion mutants were constructed located in both the membrane-spanning and extramembranous regions of the entire iron–sulfur protein (Fig. 1). All of the deletion mutants were imported efficiently into yeast mitochondria *in vitro* and processed to the mature form in the identical two-step process as the wild-type iron–sulfur protein (Japa and Beattie, 1989, Obungu *et al.*, 1998). The assembly of the mature form of the iron–sulfur protein into the bc_1 complex after import *in vitro* has been studied in our laboratory using specific antisera against the iron–sulfur protein and the intact bc_1 complex (Fu and Beattie, 1991; Ramabadran *et al.*, 1997; Obungu *et al.*, 1998). The labeled mature form of the iron–sulfur protein after import was immunoprecipitated from mitochondria solubilized with Triton X-100 using the antiserum against the intact bc_1 complex (complex III). The complex III antiserum recognized core proteins I and II, cytochromes *b* and c_1, as well as subunits 6 and 7 in immunoblots of the bc_1 complex, *but did not recognize the iron–sulfur protein*. Moreover, this antiserum did not recognize the precursor form of the iron–sulfur protein after synthesis in reticulocyte lysates (Obungu *et al.*, 1998).

The efficiency of assembly of the wild-type and mutant iron–sulfur proteins into the bc_1 complex was determined by assuming that the radioactivity formed with the antiserum against the iron–sulfur protein represents the total imported iron–sulfur protein. The percentage of assembly was then estimated by dividing the radioactivity in the immunoprecipitate obtained with the antibody against the bc_1 complex by the radioactivity immunoprecipitated with the antiserum against the iron–sulfur protein. Using this calculation, we estimated that 72–95% of the mature wild-type iron–sulfur protein imported into mitochondria was assembled into the bc_1 complex (Obungu *et al.*, 1998). Three of the mutants containing deletions in the membrane spanning region of the iron–sulfur protein Rip(Δ41–55), Rip(Δ55–66), and Rip(Δ66–78) were assembled 50–90% as well as the wild-type protein as was the mutant containing a deletion of the C-terminus of the protein, Rip(Δ182–196), suggesting that these regions may not be critical for the assembly of the iron–sulfur protein into the bc_1 complex (Fig. 1). Interestingly, the mutant, Rip(Δ55–66) was assembled into the bc_1 complex as efficiently as the wild type *in vitro*. Moreover, this mutant protein was also assembled *in vivo* into the bc_1 complex of JPJ1 transformed with Rip(Δ55–66), suggesting that this hydrophobic

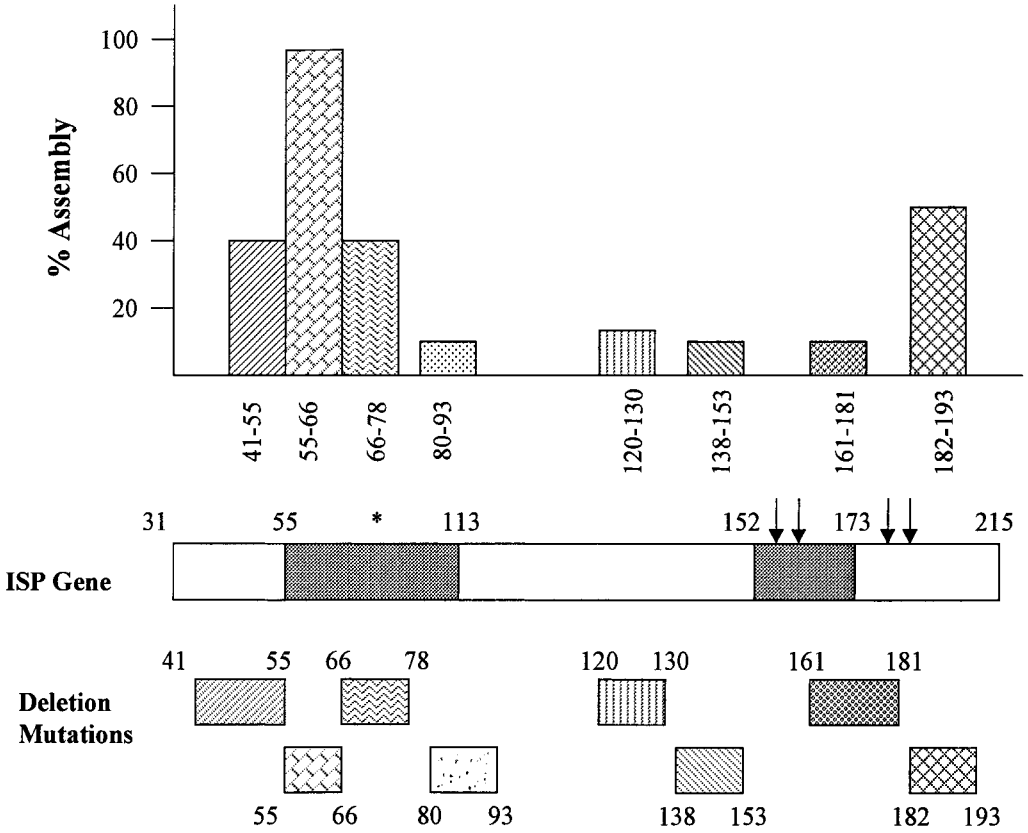

Fig. 1. Assembly of deletion mutants of the iron–sulfur protein into the bc_1 complex after import *in vitro* into mitochondria of yeast strain JPJ1. The percentage assembly in the top panel was calculated by dividing the radioactivity in the iron–sulfur protein immunoprecipitated by the complex III antiserum by the radioactivity in the iron–sulfur protein immunoprecipitated by the iron–sulfur protein antiserum. The percentage efficiency of assembly of the wild-type iron–sulfur protein was set at 100%. The middle bar shows a model of the mature iron–sulfur protein numbered for the precursor form. The open areas represent hydrophilic regions of the protein and the shaded areas hydrophobic regions (Brandt and Trumpower, 1994). The arrows above the bar indicate the sites of binding of the [2Fe–2S] cluster. Thermolysin cleaves the complete beef heart iron–sulfur protein at residue 87 (indicated by the asterisk). The boxes below the bar indicate the site of the deletion mutants used in this study.

region of the iron–sulfur protein is not required for its assembly into the bc_1 complex (Ramabadran *et al.*, 1997).

By contrast, the other four deletion mutants, Rip(Δ80–95), Rip(Δ122–135), Rip(Δ138–153), and Rip(Δ161–182) were not efficiently assembled into the bc_1 complex (Fig. 1). The lack of assembly may have resulted because these deletions resulted in improper folding of the mature iron–sulfur protein in the mitochondria such that it cannot assemble with other proteins of the bc_1 complex. It should be noted that all of these deletion mutants were efficiently imported into mitochondria and processed to the mature form (Obungu *et al.*, 1998). These results suggest that during assembly of the bc_1 complex, the iron–sulfur protein may interact with other subunits of the complex through hydrophilic and hydrophobic interactions of regions of the protein localized outside the membrane rather than through protein–protein interactions of regions of the membrane-spanning domain (Obungu *et al.*, 1998).

ROLE OF CHARGED AMINO ACIDS IN THE α1-HELIX AND α1–β4 LOOP IN THE ASSEMBLY OF THE IRON–SULFUR PROTEIN INTO THE CYTOCHROME bc_1 COMPLEX

The assembly studies involving the assembly of the deletion mutants of the iron–sulfur protein into the

bc_1 complex suggested that the amino acids located in the extramembranous regions of the iron–sulfur protein might be involved in specific interactions with other subunits of the bc_1 complex during the assembly process. To provide more precise information about these proposed interactions, site-directed mutagenesis of amino acid residues in the hydrophilic extramembranous regions of the iron–sulfur protein was performed. The recently described X-ray crystal structure of a water-soluble fragment of the iron–sulfur protein with an intact [2Fe–2S] cluster indicated that the C terminal domain of the iron–sulfur protein is a flat spherical molecule containing three layers of anti-parallel β-sheets comprising a total of ten β-strands (β1–β10) (Iwata *et al.*, 1998; Link *et al.*, 1996). A single α-helix (α-1) connected to a long loop (α1–β4) is inserted between the β3 and β4 strands of the protein. In their structure determination, Link *et al.* (1996) proposed that the highly conserved region of the iron–sulfur fragment flanking the α-1 helical loop might be involved in the interaction of the iron–sulfur protein with cytochrome c_1. It should be noted that both the α1 helix and the α1–β4 loop are located within the deleted regions (Δ122–135) and (Δ138–153), that did not assemble efficiently into the bc_1 complex *in vitro* (Fig. 1).

Further examination of one of these deletion mutants that did not assemble into the bc_1 complex *in vitro*, that containing amino acid residues 138–153, revealed the presence of six charged amino acids present in the loop connecting the α1 helix and the β4 sheet. To determine the possible role of these charged amino acids in this region of the yeast iron–sulfur protein, which we assume has a similar if not identical structure to the beef heart iron–sulfur protein, site-directed mutagenesis of the charged amino acids present in this region was performed (Obungu *et al.*, 1998). The acidic amino acids, Asp139, Asp143, and Asp149 were mutated to alanine (D139A, D143A, D149A) and Asp145 was mutated to leucine (D145L), while the basic amino acids, Arg146 and Lys148, were mutated to isoleucine (R146I, K148I). As a control, two uncharged amino acid residues Gln141 and Val147 were mutated to isoleucine and serine, respectively (Q141I, V147S). In addition, Trp152 was mutated to phenylalanine (W152F), as a previous study had reported that the W152R mutation resulted in a loss of enzymatic activity (Graham *et al.*, 1993).

In a similar study, several amino acids in the single extra-membranous α-helix (α1) were also mutated (Obungu and Beattie, 1998). The charged amino acids, His24, Glu125, and Glu128, were mutated to leucine and glutamine (H124L, E125Q, and E128Q). The moderately conserved amino acid Thr122 was mutated to alanine (T122A), while Val132 was mutated to leucine (V132L). All of the mutations were confirmed by restriction analysis and DNA sequencing.

The wild-type RIP genes and each of the mutant rip genes were used to transform JPJ1, the yeast strain lacking the RIP genes (Beckmann *et al.*, 1989), and growth determined on the nonfermentable carbon source, glycerol/ethanol. The enzymatic activity of the cytochrome bc_1 complex was determined as cytochrome *c* reductase with the decyl analog of ubiquinol (DBH_2) using mitochondria isolated from the mutated yeast strains grown in medium containing galactose as carbon source. To confirm the enzymatic nature of the observed cytochrome *c* reductase activity, the inhibitory effects of both antimycin and myxothiazol were determined. The cytochrome *c* reductase activity of two mutants, R146I and W152F, was less than 10%, while that of the mutants D143A, K148I, and D149A was 50, 33, and 11% of the wild-type mitochondria, respectively. The activity of the remaining mutants D139A, Q141I, D145L, and V147S was identical to that of the wild-type mitochondria.

Our next approach was to investigate whether changes in the spectral properties or content of cytochromes *b* and c–c_1 had occurred as a result of the mutations introduced into the iron–sulfur protein; spectral analyses were performed. The concentration of cytochromes *b* and c–c_1 in the wild-type mitochondria was determined to be 0.088 and 0.095 nmol/mg of protein, respectively. Examination of the spectra of the mutants revealed the presence of cytochromes c–c_1 at the same level as that of the wild-type cells in all of the mutants with the exception of W152F in which the cytochrome c–c_1 content was diminished by 30%. By contrast, spectral analysis of mitochondria obtained from several of the mutants revealed significant changes in the spectra of cytochrome *b* leading to calculated decreases in the amount of cytochrome *b* present in the mitochondria. For example, the iron–sulfur protein mutants E125Q, D139A, Q141I, and D149A had 15–17% less cytochrome *b* heme than the wild-type, while the cytochrome *b* heme content was diminished by 32% in R1461I and 41% in W152F. These reductions in the cytochrome *b* levels may reflect damage to the environment of the *b* hemes due either to the absence of the iron–sulfur protein or to

a change in its conformation as a result of the specific amino acid mutation (Graham and Trumpower, 1991).

The decreased levels of cytochrome *b* observed in several of these mutants rather than the actual mutation in the iron–sulfur protein may be responsible for the observed lowered enzymatic activity of the bc_1 complex in these mutants. To make this determination, the turnover numbers of the bc_1 complex in the various mutants were calculated as enzymatic activity (nmol of cytochrome *c* reduced mg^{-1} of mitochondrial protein min^{-1}) / the content of cytochrome *b* (nmol mg^{-1} of mitochondrial protein). In the mutants located in the α-1 helix, the decrease in turnover number was most pronounced in mutants H124L and V132L (60 and 80%, respectively) with smaller decreases in mutants T122A, E125Q, and E128Q (Fig. 2). In the mutants located in the α1–β4 loop, a 90% decrease in turnover number was observed in mutants R146I, D149A, and W152F. The turnover numbers of mutants D143A and K148I were also significantly decreased (50 and 65%, respectively); however, the turnover numbers for the remaining mutants were not affected. These results indicate that the decreased cytochrome *c* reductase activity observed in these mutants resulted from the mutations introduced into the iron–sulfur protein and not from a decreased content of either of cytochromes *b* or c–c_1.

We next investigated whether site-directed mutagenesis of several charged amino acids in the α1-helix and the α1–β4 loop plus Val132 and Trp152 of the iron–sulfur protein resulted in decreased expression of the iron–sulfur protein in the mitochondria of these mutants. The presence of the iron–sulfur protein in mitochondria isolated from these mutants was deter-

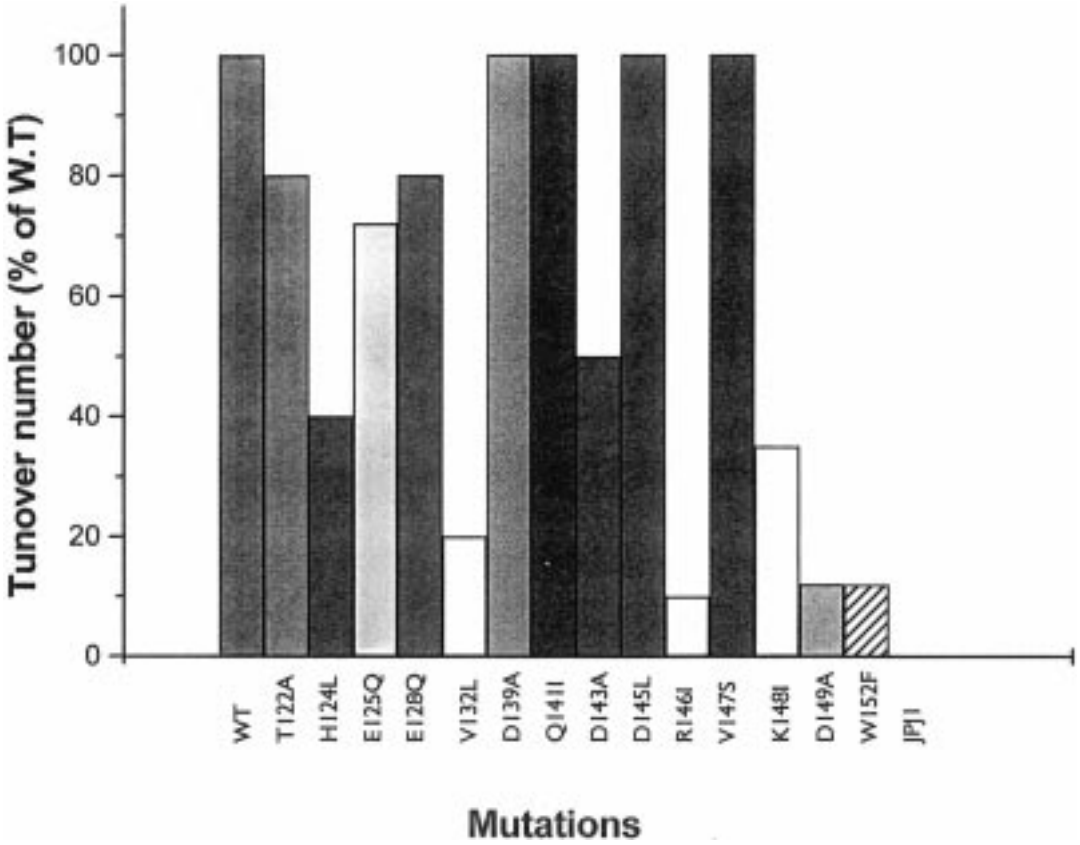

Fig. 2. Turnover numbers of cytochrome bc_1 complex in mitochondria isolated from yeast strain JPJ1 and JPJ1 expressing mutant iron–sulfur proteins. Turnover numbers were determined by dividing the specific enzymatic activity in μmoles of cytochrome *c* reduced per milligram of mitochondrial protein by the concentration of cytochrome *b* in nmoles per milligram of mitochondrial protein. The turnover numbers are expressed as a percentage of the wild-type set at 100%.

mined by Western blotting with an antiserum against the iron–sulfur protein. As a control, the expression of either core protein 2 or cytochrome c_1 in these mitochondria was compared on the same gel. Immunoblot analysis revealed the presence of the iron–sulfur protein in all of the mutants; however, the levels of expression of the iron–sulfur protein were considerably lower in the mutants H124L, V132L, D143A, R146I, K148I, D149A, and W152F, when compared to the wild-type levels (Obungu *et al.*, 1998; Obungu and Beattie, 1998). By contrast, the levels of core protein 2 or cytochrome c_1 determined by immunoblotting with the antisera against these proteins were unaffected in the mutants. These results indicate that mutating a histidine, H124L, a hydrophobic amino acid V132L, two acidic amino acids, Asp143 and Asp149, two basic amino acids, Arg146 and Lys148, or the aromatic residue, Trp152, located in the extramembranous region of the iron–sulfur protein results in varying decreases in the levels of the iron–sulfur protein present in mitochondria *in vivo*. The loss of the iron–sulfur protein in these mutants resulted in a comparable loss in enzymatic activity as this protein plays an essential role in electron transfer in the bc_1 complex. Lowered amounts of the iron–sulfur protein in these mutants might result from a decreased expression of the protein, an increased instability of the newly synthesized protein or a less efficient assembly of the protein into the bc_1 complex. For example, removal of the charges in this region of the iron–sulfur protein or changing the valine or tryptophan moiety through mutagenesis may result in the formation of an unstable protein that is degraded soon after translation. It is clear that these mutations affect the amount of the iron–sulfur protein in the bc_1 complex but do not affect directly the catalytic activity of the protein.

The decreased amounts of the iron–sulfur protein observed in several of the mutants prompted us to investigate whether the import into mitochondria of these mutant proteins and their subsequent assembly into the bc_1 complex were affected by the mutations. After coupled transcription/translation *in vitro*, the radiolabeled precursors of the mutant and the wild-type iron–sulfur protein were all imported efficiently into the mitochondria isolated from yeast strain JPJ1 where they underwent the identical two-step cleavage process (Obungu *et al.*, 1998). The assembly of the wild type and mutant iron–sulfur proteins into the cytochrome bc_1 complex was investigated *in vitro* using the selective immunoprecipitation with specific antibodies described above. Slight variations in the efficiency of assembly of the various mutants were observed (Fig. 3); however, the most significant effects were noted with the following mutants: an 80% decrease in R1461 and a 60% decrease in both D149A and W152F. We suggest that Arg146, Asp149, and Trp152 may be involved in establishing the conformation of the iron–sulfur protein necessary for its efficient assembly with the other proteins in the bc_1 complex. The charged amino acids may form hydrogen bonds or salt bridges with other charged amino acids to maintain the α1–β4 loop in its extended conformation, while the location of Trp152 at the base of the β4 sheet may be critical for the overall conformation of the protein.

MUTATION IN THE TETHER REGION OF THE IRON–SULFUR PROTEIN DO AFFECT THE ACTIVITY OF THE CYTOCHROME bc_1 COMPLEX

The recent resolution of the crystal structure of the beef and chicken heart bc_1 complexes has indicated that the iron–sulfur protein consists of three separate domains, a membrane-spanning α-helix at its N-terminus, passing through the membrane at an angle, a soluble extramembranous domain containing the bulk of the iron–sulfur protein including the [2Fe–2S] cluster, and a "tether" of 7 to 9 amino acids connecting the extrinsic domain to the membrane-spanning α-helix (Iwata *et al.*, 1998; Zhang *et al.*, 1998). The subsequent resolution of three different conformations of the iron–sulfur protein in the bc_1 complex in the presence of inhibitors has suggested the possibility that the extrinsic domain of the iron–sulfur protein might undergo movement during electron transfer (Yu *et al.*, 1998; Zhang *et al.*, 1998; Iwata *et al.*, 1998; Crofts and Berry, 1998). A model to explain electron transfer through the bc_1 complex suggests that when ubiquinol binds in the Q_O site, formed by the end of helix C, helix cd_1, and loop ef of cytochrome *b*, and is deprotonated, the iron–sulfur protein moves closer to cytochrome *b* and assumes the so-called "*b*" state. One electron is then transferred to the iron–sulfur protein with the stabilization of the resulting ubisemiquinone bound to the Q_O site. Transfer of the second electron from the bound ubisemiquinone to heme b_L destabilizes the binding of the oxidized ubiquinone resulting in the movement of the reduced iron–sulfur protein to the "c_1" state where rapid electron transfer from the [2Fe–2S] cluster to heme c_1 occurs (Brandt, 1998).

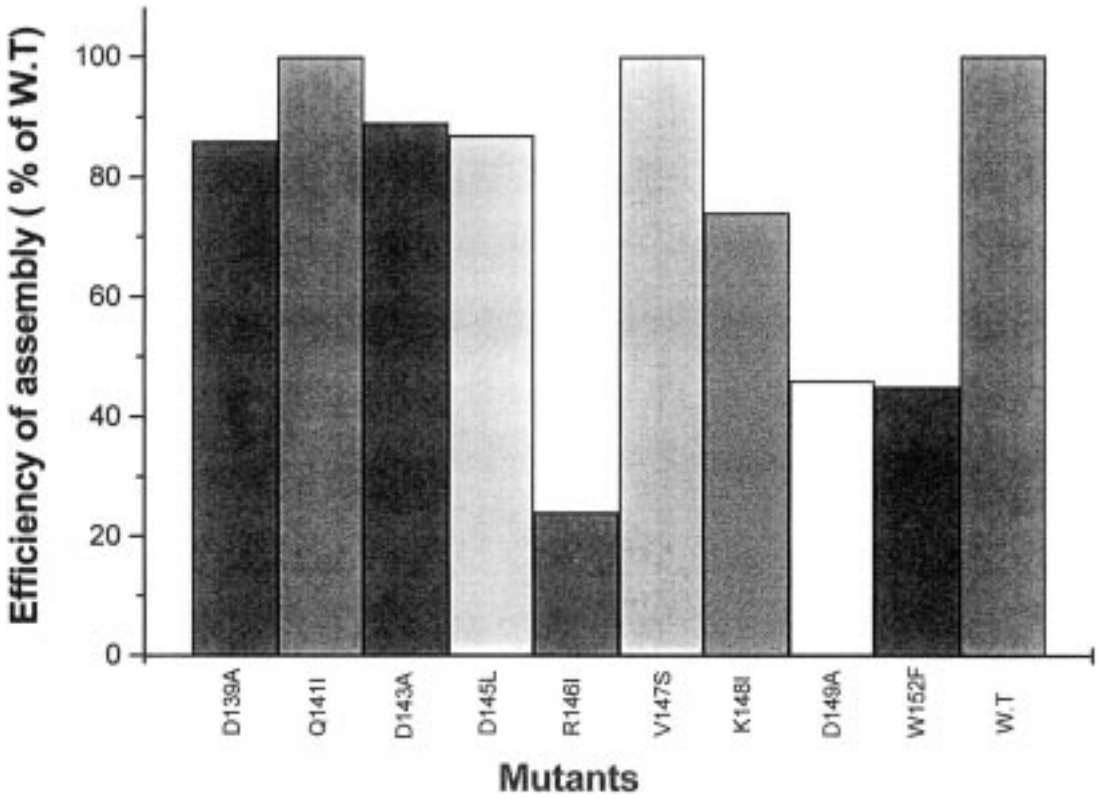

Fig. 3. Efficiency of assembly *in vitro* of wild-type and mutant iron–sulfur proteins into the cytochrome bc_1 complex of mitochondria from JPJ1. The relative radioactivity of the mutant and wild-type iron–sulfur proteins into the bc_1 complex were quantified by Phosphorimager analysis, as described in the legend to Fig. 1. The percentages indicate the value of normalized immunoprecipitates divided by that of the wild type.

Despite the changes in the position of the [2Fe–2S] cluster of the iron–sulfur protein, the structure of the extrinsic head and the membrane-anchoring domains of the iron–sulfur protein remain unchanged in all of the crystal structures. This observation suggests that the 6–8 amino acid tether connecting these two domains may stretch and bend to permit the rotation of the extrinsic globular domain during electron transfer. The tether domain, a highly conserved region of the iron–sulfur protein has the sequence TADVLAMA in yeast (residues 85–92). The presence of three highly conserved alanine residues in this region of the protein suggests that these small amino acid residues may provide the needed flexibility for the proposed stretching of the "tether." Indeed, a recent report has indicated that substituting two prolines for two alanine residues, 90 and 92, or three prolines for residues 86–88 resulted in an almost complete absence of electron transfer through the bc_1 complex of *Rhodobacter sphaeroides* (Tian *et al.*, 1998). Our approach to determine the role of these three conserved alanine residues in the assembly and function of the bc_1 complex was to make substitutions in which Ala86, Ala90, and Ala92 were mutated to residues with larger side chains. Two charged amino acids, Lys93 and Glu95, adjacent to the "tether" region and conserved in the bc_1 complexes of mitochondria were also mutated. Yeast strain JPJ1 lacking the iron–sulfur protein was transformed with plasmids containing these mutant constructs and the effect of these mutations on growth, enzyme activity of the bc_1 complex, and expression of the iron–sulfur protein was investigated.

Growth of yeast transformed with these mutant constructs in the nonfermentable carbon source glycerol/ethanol was not affected. The cytochrome *c* reductase activity of the bc_1 complex of the mitochondria isolated from mutants A86L, A90I, A92C, A92R, and E95R was reduced 56, 29, 34, 26, and 21%, respectively, compared to the activity of the wild-type cells (Fig. 4). These results suggest that the three conserved

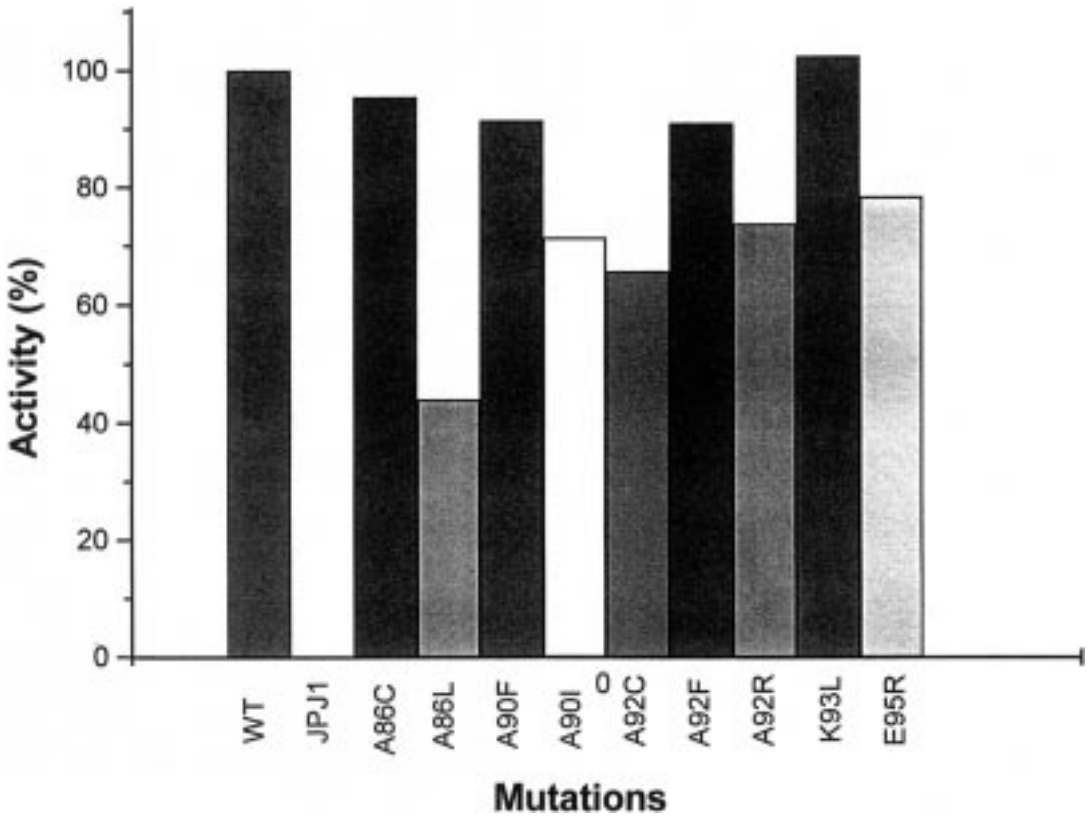

Fig. 4. Enzymatic activity of the cytochrome bc_1 complex in mitochondria isolated from yeast strain JPJ1 and JPJ1 expressing mutant iron–sulfur proteins. Mitochondria were isolated from mutant cells grown in a medium containing galactose as carbon source. Mitochondria were isolated and the enzymatic activity of the bc_1 complex determined as cytochrome *c* reductase activity with the decyl analog of ubiquinol as substrate. Activities are expressed as a percentage of the wild type set at 100%. Wild-type activity was 1.75 μmol cytochrome *c* reduced per minute per milligram of protein.

alanine residues, Ala86, Ala90, and Ala92, located in the tether region of the iron–sulfur protein and Glu95 located at the beginning of the β_1 sheet are required to maintain optimal rates of electron transport through the bc_1 complex. Difference spectroscopy revealed no decrease in the levels of cytochromes *b* or c–c_1, EPR spectroscopy indicated no changes in the environment of the [2Fe–2S] cluster, and immunoblotting with specific antisera showed no loss of iron–sulfur protein in all of these mutants. These results have lead us to conclude that these amino acid substitutions in the tether region of the iron–sulfur protein must affect the catalytic activity of the bc_1 complex as no other changes in the components of the cytochrome bc_1 complex were observed.

To localize the defect in electron transfer through the bc_1 complex in these mutants, single turnover studies were performed. The rate of reduction of cytochromes *b* and c–c_1 in the absence of an electron acceptor and in the presence of cyanide was determined independently at the appropriate wavelengths, 562–570 nm for cytochrome *b* and 554–540 nm for cytochrome c–c_1. In mitochondria from the wild-type cells, maximum reduction of both cytochrome *b* and c–c_1 was complete within 1.5 s after addition of exogenous succinate as electron donor (Fig. 5). The initial rate of cytochrome *b* reduction was not significantly reduced in the mutants A90I and A92R, located in the midregion of the putative tether at a distance from the membrane. The rate of cytochrome c–c_1 reduction, however, in these mutants was reduced 27 and 30% respectively, a decrease comparable to the loss in overall rate of cytochrome *c* reduction with DBH_2 as substrate. By contrast, the rate of reduction of cytochrome *b* was reduced 47% and that of cytochrome c–c_1 was reduced 42% in mutant A86L, located in the putative

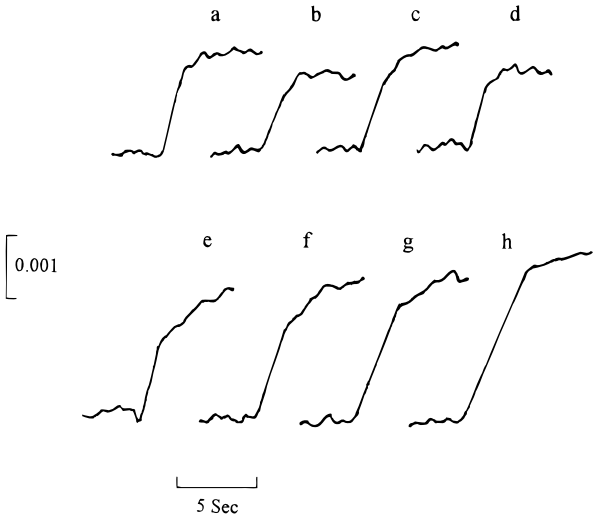

Fig. 5. Single turnover numbers of mitochondria isolated from yeast JPJ1 expressing wild-type and mutant iron–sulfur proteins. The initial rates of reduction of cytochrome *b* (traces a–d) and cytochrome c–c_1 (traces e–h) were determined at the wave length pairs 562–570 m and 550–540 nm, respectively, after addition of 15 m*M* succinate. Traces a and e, wild type; b and f, mutant A86L; c and g, mutant A90I; and d and h, mutant A92R.

tether at or near the insertion of the iron–sulfur protein into the membrane. The overall rate of cytochrome *c* reduction was lowered 56% in this mutant.

The results of these single turnover studies in which the rates of reduction of cytochrome *b* and cytochrome c_1 were determined independently are consistent with the suggested role of the tether in catalysis. In two mutants, A90I and A92R, the rate of cytochrome c_1 reduction was decreased significantly, while the rate of cytochrome *b* reduction remained at the wild-type level. A mutation in which a bulky amino acid, such as isoleucine or arginine, is substituted for a conserved alanine may restrict the flexibility of the loop such that the movement of the extrinsic domain of the iron–sulfur protein, after its reduction by ubiquinol, is impaired. Consequently, the reduction of cytochrome c_1 would be slowed; however, the transfer of an electron to cytochrome b_L and then to b_H from ubisemiquinone in the Q_O site would be unaffected by restricted motion of the tether region. Changing the conserved Ala86, located in the loop region close to the cd_1 loop of cytochrome *b* (Zhang *et al.*, 1998; Tian *et al.*, 1998; Saribas *et al.*, 1998) to the bulky leucine residue resulted in a decrease in the rate of reduction of cytochrome *b* concomitant with the decrease in the rate of cytochrome c_1 reduction. In addition to a restriction in the flexibility of the tether in the A86L mutant, substituting leucine for alanine may affect the site of cytochrome *b* resulting in a lowered rate of electron transfer to cytochrome b_L and b_H.

We suggest that the loss of ubiquinol:cytochrome *c* reductase activity in selected mutants of conserved alanine and charged residues in the tether region of the iron–sulfur protein is consistent with the suggested requirement for flexibility in this region of the protein necessary for movement during catalysis. The loss of enzymatic activity in the mutants of these three conserved alanine residues does not occur because of a loss of the iron–sulfur protein or of cytochromes *b* and c–c_1 or to a change in the environment of the [2Fe–2S] cluster. Moreover, these residues are located in the protein far removed from the active site of the iron–sulfur protein or the regions of the protein involved in interaction with cytochrome *b* (Zhang *et al.*, 1998). More direct experimental evidence is required to prove that movement of the iron–sulfur protein does occur during catalysis.

REFERENCES

Beckmann, J. D., Ljungdahl, and Trumpower, B. L. (1989). *J. Biol. Chem.* **264**, 3713–3722.
Brandt, U. (1998). *Biochim. Biophys. Acta* **1365**, 261–268.
Brandt, U., and Trumpower, B. (1994). *Crit. Rev. Biochem. Mol. Biol.* **29**, 165–197.
Crivellone, M. D., Wu, M. A., and Tzagoloff, A. (1988). *J. Biol. Chem.* **263**, 14323–14357.
Crofts, A. R. and Berry, E. A. (1998). *Curr. Opin Struct. Biol.* **8**, 501–509.
Fu, W., and Beattie, D. S. (1991). *J. Biol. Chem.* **266**, 16212–16218.
Fu, W. Japa, S., and Beattie, D. S (1990). *J. Biol. Chem.* **265**, 16541–16547.
Japa, S., and Beattie, D. S (1989). *Arch. Biochem. Biophys.* **268**, 716–720.
Graham, L. A., and Trumpower, B. L (1991). *J. Biol. Chem.* **266**, 22485–22492.
Graham, L. A., Brandt, U., Sargent, J. S., and Trumpower, B. L (1993). *J. Bioenerg. Biomembr.* **25**, 245–257.
Iwata, S., Lee, J. W., Okada, K., Lee, J. K., Iwata, M., Rasmussen, B., Link, T. A., Ramaswamy, S., and Jap, B. K. (1998). *Science* **281**, 64–71.
Kim, H., Xia, D., Yu, C. A., Xia, J. Z., Kachurin, A. M., Zhang, L., Yu, L., and Deisenhofer, J. (1998). *Proc. Natl. Acad. Sci. U.S.* **95**, 8026–8033.
Link, T. A., Saynovtis, M., Assman, C., Iwata, S., Ohnishi, T., and Von Jagow, G. (1996). *Eur. J. Biochem.* **237**, 71–75.
Neupert, W. (1994). *Clin. Invest.* **72**, 251–261.
Obungu, V. H. and Beattie, D. S. (1998). *Arch. Biochem. Biophys.* **359**, 128–132.
Obungu, V. H., Wang, Y., and Beattie, D. S. (1998). *J. Biol. Chem.* **273**, 11917–11922.
Ramabadran, R. S., Japa, S., and Beattie, D. S. (1997). *J. Bioenerg. Biomembr.* **29**, 45–54.
Saribas, A. S., Valchanova, M. V., Tokito, M. K., Zhang, Z. Berry, E. A., and Daldal, F. (1998). *Biochemistry* **37**, 8105–8114.

Sidhu, A., Clejan, L., and Beattie, D. S (1983). *J. Biol. Chem.* **258**, 12308–12314.
Tian, H., Yu, L., Mather, M. W., and Yu, C. A. (1998). *J. Biol. Chem.* **273**, 27953–27959.
Trumpower, B. L., and Gennis, B. (1994). *Annu. Rev. Biochem.* **63**, 675–716.
Xia, D., Yu, C. A., Kim, H., Xia. J. Z., Kachurin, A. M., Zhang, L., Yu, L., and Deisenhofer, J. (1997). *Science* **277**, 60–66.
Yu, C.-A., Xia, D., Kim, H., Deisenhofer, J., Zhang, L., Kachurin, A. M., and Yu, L. (1998). *Biochim. Biophys. Acta* **1365**, 151–158.
Zhang, Z., Huang, L., Shulmeister, V. M., Chi, Y. I., Kim, K. K., Hung, L. W., Crofts, A.R., Berry, E. A., and Kim, S. H. (1998). *Nature* **392**, 677–684.