Mutations in the tether region of the iron–sulfur protein affect the activity and assembly of the cytochrome bc1 complex of yeast mitochondria

ARTICLE *in* BIOCHIMICA ET BIOPHYSICA ACTA · MARCH 2000

Impact Factor: 4.66 · DOI: 10.1016/S0005-2728(99)00116-4 · Source: PubMed

5 AUTHORS, INCLUDING:

Victor H Obungu
Eli Lilly
17 PUBLICATIONS **336** CITATIONS

SEE PROFILE

ELSEVIER

Biochimica et Biophysica Acta 1457 (2000) 36–44

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Mutations in the tether region of the iron–sulfur protein affect the activity and assembly of the cytochrome bc_1 complex of yeast mitochondria

Victor H. Obungu, Yudong Wang, Suzelle M. Amyot, Christian B. Gocke, Diana S. Beattie *

Department of Biochemistry, P.O. Box 9142, West Virginia University School of Medicine, Morgantown, WV 26506-9142, USA

Received 21 October 1999; received in revised form 14 December 1999; accepted 23 December 1999

Abstract

Resolution of the crystal structure of the mitochondrial cytochrome bc_1 complex has indicated that the extra-membranous extrinsic domain of the iron–sulfur protein containing the 2Fe2S cluster is connected by a tether to the transmembrane helix that anchors the iron–sulfur protein to the complex. To investigate the role of this tether in the cytochrome bc_1 complex, we have mutated the conserved amino acid residues Ala-86, Ala-90, Ala-92, Lys-93 and Glu-95 and constructed deletion mutants ΔVLA(88–90) and ΔAMA(90–92) and an insertion mutant I87AAA88 in the iron–sulfur protein of the yeast, *Saccharomyces cerevisiae*. In cells grown at 30°C, enzymatic activities of the bc_1 complex were reduced 22–56% in mutants A86L, A90I, A92C, A92R and E95R, and the deletion mutants, ΔVLA(88–90) and ΔAMA(90–92), while activity of the insertion mutant was reduced 90%. No loss of cytochromes *b* or c–c_1, detected spectrally, or the iron–sulfur protein, determined by quantitative immunoblotting, was observed in these mutants with the exception of the mutants of Ala-92 in which the loss of activity paralleled a loss in the amount of the iron–sulfur protein. EPR spectroscopy revealed no changes in the iron–sulfur cluster of mutants A86L, A90I, A92R or the deletion mutant ΔVLA(88–90). Greater losses of both protein and activity were observed in all of the mutants of Ala-92 as well as in A90F grown at 37°C. suggesting that these conserved alanine residues may be involved in maintaining the stability of the iron–sulfur protein and its assembly into the bc_1 complex. By contrast, no significant loss of iron–sulfur protein was observed in the mutants of Ala-86 in cells grown at either 30°C or 37°C despite the 50–70% loss of enzymatic activity suggesting that Ala-86 may play a critical role in catalysis in the bc_1 complex.

Keywords: Iron-sulphur protein; Cytochrome bc_1; Yeast; Mitochondria

1. Introduction

The cytochrome bc_1 complex is an integral multiprotein complex of the inner mitochondrial membrane which catalyzes the transfer of electrons from ubiquinol to cytochrome *c* coupled to the translocation of protons across the membrane [1–3]. The bc_1 complex of yeast mitochondria consists of 10 sub-

Abbreviations: Cytochrome bc_1 complex, ubiquinol:cytochrome *c* oxidoreductase; DBH_2, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RIP, wild-type iron–sulfur protein gene; rip, mutant iron–sulfur protein gene

* Corresponding author. Fax: +1-304-293-6846;
E-mail: dbeattie@hsc.wvu.edu

0005-2728/00/$ – see front matter
PII: S0005-2728(99)00116-4

units, 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 [1,3]. 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 2Fe2S 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 [4,5] and chicken [6] heart mitochondria has revealed that the bc_1 complex exists as a dimer with the 8 membrane spanning helices of cytochrome *b* comprising the core of the complex. 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 extra-membranous domain containing the bulk of the iron–sulfur protein including the 2Fe2S cluster, and a 'tether' of 7–9 amino acids connecting the extrinsic domain to the membrane-spanning α-helix [5,6].

The subsequent resolution of three different conformations of the iron–sulfur protein in the bc_1 complex has suggested the possibility that the extrinsic domain of the iron–sulfur protein might undergo movement during electron transfer [3,5,7]. 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 2Fe2S cluster to heme c_1 occurs [8].

Despite the changes in the position of the 2Fe2S cluster of the iron–sulfur protein, the membrane-spanning region and head group of the protein remains essentially unchanged in all the reported structures [4,6,9] with the exception of a minor 2A displacement of the 2Fe2S cluster in the cluster-binding fold observed in the 'intermediate' form described by Iwata et al. [5]. This observation suggests that movement of the 2FeE2S cluster during electron transfer results mainly from the rotation of the entire head domain of the iron–sulfur protein. The flexibility to allow this rotation must come from the 6–8 amino acid tether connecting the head domain to the transmembrane anchor. The tether domain, a highly conserved region of the iron–sulfur protein (Fig. 1), has the sequence TADVLAMA in yeast, amino acids 85–92 [10]. 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 proline 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* [11]. Further evidence for the suggested flexibility in the tether of the iron–sulfur protein has been obtained in double cysteine mutants of *R. sphaeroides*, in which the loss of enzymatic activity was shown to result from disulfide bond formation, which would increase the rigidity of the tether region [12].

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 Ala-86, Ala-90 and Ala-92 were mutated to residues with more bulky side chains that might interfere with the proposed movement of the tether. Two charged amino acids, Lys-93 and Glu-95, adjacent to the 'tether' region and conserved in the bc_1 complexes of mitochondria were also mutated (Fig. 1A), since previous studies from our laboratory had indicated that charged amino acids in other regions

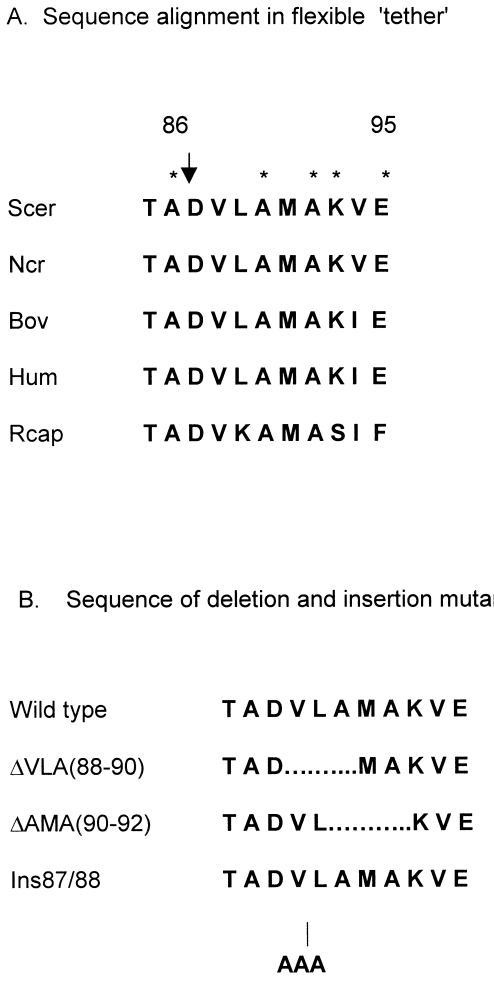

Fig. 1. (A) Sequence alignment of amino acid residues in the flexible 'tether' of the Rieske iron–sulfur proteins from different species [23]. The arrow indicates the site of cleavage by thermolysin [24]. The sequences are those from *Saccharomyces cerevisiae* (Scer), *Neurospora crassa* (Ncr), bovine heart (bov), human (hum), and *Rhodobacter capsulatus* (Rcap). The asterisks indicate the amino acid residues that were mutated in this study. (B) Structure of deletion and insertion mutants. Deletion and insertion mutants were constructed as described in Section 2. In the deletion mutant Δ88–90, residues Val–Leu–Ala (VLA) were deleted; in the mutant Δ90–92, residues Ala–Met–Ala (AMA) were deleted; in the deletion mutant Δ87–93, residues Asp–Val–Leu–Ala–Met–Ala–Lys (DVLAMAK) were deleted. Three alanine (AAA) residues were inserted between Asp-87 and Val-88 to construct the insertion mutant I87/88.

of the iron–sulfur protein contributed to the stability of the iron–sulfur protein [13,14]. The underlying assumption in this experimental approach is that the yeast iron–sulfur protein has a similar, if not identical, conformation as the protein in beef and chicken heart mitochondria. In addition, two deletion mutants that removed one or two alanine residues were constructed as well as an insertion mutant in which three alanine residues were added (Fig. 1B). A yeast cell line lacking the gene for the iron–sulfur protein (JPJ1) [15] was transformed with these mutant constructs and the effect of these mutations on growth, enzyme activity, and protein expression was investigated.

2. Experimental procedures

2.1. Site-directed mutagenesis and transformation of yeast cells

Site-directed mutagenesis and construction of deletion and insertion mutants were performed using the Stratagene Quick Change site-directed mutagenesis kit as described previously [13]. The mutant DNA, thus constructed, was analyzed by restriction analysis and by sequencing the mutated gene. DNA containing the wild-type iron–sulfur protein (RIP) gene or the mutant rip genes was used to transform yeast cells (JPJ1), in which the RIP gene had been deleted, by the lithium acetate method [16] as modified [17]. Mutant yeast cells were selected by their ability to grow on a medium lacking uracil. To test for respiratory competence, the transformed JPJI colonies from the uracil minus plates were streaked on plates containing 1% yeast extract, 2% peptone, 3% glycerol and 4% ethanol (pH 5.0) in 1.5% agar and incubated at 30°C and 37°C. Subsequently, the transformed yeast cells were grown in the same liquid medium at 30°C and 37°C and the rate of growth monitored as described [13].

2.2. Growth of yeast cells, preparation of mitochondria and enzyme assays

For determination of enzymatic activity in the yeast cells containing the mutated iron–sulfur protein, yeast cells were grown aerobically at 30°C and 37°C in a semi-synthetic medium with galactose as carbon source. Mitochondria were isolated as described previously [18]. The activity of the cytochrome bc_1 complex was determined at 30°C by mea-

Table 1
Growth characteristics, enzymatic activities, content of cytochromes *b* and c–c_1 and the iron–sulfur protein in JPJ1 yeast cells transformed with the wild-type RIP and mutant rip genes (30°C)

Mutant	Doubling time (h)	Enzymatic activity		Content (nmol/mg)		
		μmol/min/mg	% Control	Cytochrome *b*	Cytochrome c–c_1	ISP[a]
Wild type	2.0	1.75	100	0.083	0.092	1.00
A86C	2.0	1.67	95	0.083	0.096	0.95
A86L	3.0	0.77	44	0.081	0.091	0.93
A90F	2.0	1.60	91	0.085	0.092	0.97
A90I	2.0	1.25	71	0.083	0.091	0.90
A92C	2.5	1.15	66	0.085	0.098	0.76
A92F	2.0	1.59	91	0.083	0.090	0.82
A92R	2.0	1.29	74	0.086	0.092	0.82
K93L	2.3	1.79	100	0.068	0.079	0.95
E95R	2.2	1.37	78	0.081	0.097	1.00
Δ88–90	2.0	1.17	67	0.083	0.087	0.98
Δ90–92	2.0	1.37	78	0.073	0.090	0.93
Ins87/88	>10.0	0.16	9	0.059	0.070	0.59
JPJ1	No growth	0		0.041	0.074	0

Site-directed and deletion mutants were constructed and used to transform JPJ1 yeast cells as described in Section 2. Growth of the transformed cells on the non-fermentable carbon source glycerol/ethanol was monitored at 30°C as described [12]. To determine enzymatic activity and cytochrome content, wild-type and mutant cells were grown in a medium containing galactose and mitochondria prepared as described [12]. Enzymatic activity and cytochrome content were determined as described in Section 2 and [12].
[a]The content of iron–sulfur protein (ISP) was determined by laser densitometry of immunoblots of mitochondria probed with antisera specific for the iron–sulfur protein and calculated relative to the wild-type protein set as 1.00.

suring the reduction of 40 μM horse heart ferricytochrome *c* at 550–540 nm using 150 μM of the ubiquinol analog decylbenzoquinol (DBH_2) as electron donor [19]. Each enzymatic assay was performed in triplicate. The values in the tables are representative of at least three different mitochondrial preparations. To confirm the enzymatic nature of the observed cytochrome *c* reductase activity, the inhibitory effects of the specific inhibitors of the cytochrome bc_1 complex, antimycin A and myxothiazol, were determined. For every mitochondrial preparation, antimycin and myxothiazol completely inhibited the reductase activity.

2.3. Optical absorption spectroscopy

Optical spectra were recorded using the dual wavelength mode of the Aminco DW-2A spectrophotometer coupled to a recorder with reference beam set at 539 nm and the contents of cytochrome *b* and c–c_1 determined as described [13].

2.4. Immunoblotting

Immunoblotting was performed using the ECL Western blotting system from Amersham/Pharmacia as earlier described [13]. The amount of the iron–sulfur protein present in the immunoprecipitate was quantitated using laser densitometry.

2.5. EPR spectroscopy

Mitochondria for EPR studies were isolated from JPJ1 cells transformed with RIP gene (wild type = W.T.), with the mutant rip genes (A86L, A90I, A92R, and ΔVLA (88–90) as described above. Mitochondrial membranes were prepared after sonication of the mitochondria and centrifugation at $100\,000 \times g$ for 45 min. The mitochondrial membrane suspensions at 50 mg/ml were reduced with ascorbate before freezing at 14 K. EPR spectra were recorded using a Bruker ESP-300E spectrometer equipped with an Oxford Instruments ESR-9 helium cryostat.

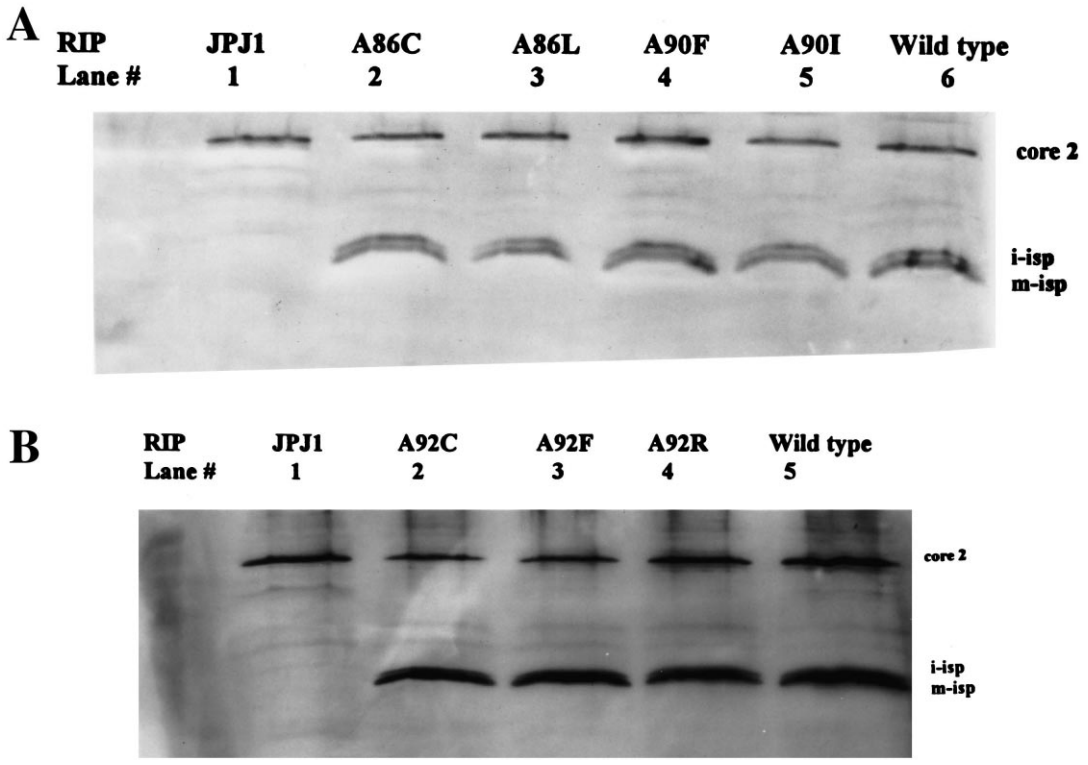

Fig. 2. Representative immunoblots of mitochondrial membranes from yeast cells expressing wild-type RIP and mutant rip genes. Mitochondria were isolated from yeast grown at 30°C. Mitochondrial proteins were separated on SDS–PAGE and blotted with the antisera against the iron–sulfur protein and core 2 protein. Each panel represents a single experiment with mitochondria from wild-type cells (JPJ1 transformed with the RIP gene), the mutants (JPJ1 transformed with the indicated rip genes) and JPJ1, the strain in which the RIP gene was deleted [14]. The positions of core 2, the intermediate form of the iron–sulfur protein (i-isp), and the mature form of the iron–sulfur protein (m-isp) are indicated. (A) Mutants of Ala-86 and Ala-90. (B) Mutants of Ala-92.

3. Results

3.1. Growth characteristics of yeast containing mutant iron–sulfur proteins

Transformed yeast cells containing single site mutations of the three conserved alanine residues, Ala-86, Ala-90 and Ala-92 and the charged amino acids Lys-93 and Glu-95, as well as the two deletion mutants and the insertion mutant (Fig. 1B) were grown on the non-fermentable carbon source glycerol/ethanol in liquid medium at 30°C. JPJ1 cells transformed with the wild-type RIP gene, indicated as wild type in Table 1, grew with a doubling time of 2 h at 30°C. Four of the mutants cells, A86L, A92C, K93L, and E95R grew slightly more slowly than the wild-type cells at 30°C with doubling times of 3.0, 2.5, 2.3, and 2.2 h, respectively. The insertion mutant I87AAA88 had a barely detectable growth rate with a doubling time greater than 10 h. The remaining mutant cells including the deletion mutants, ΔVLA(88–90) and ΔAMA(90–920, grew as well as the wild-type cells.

3.2. Ubiquinol:cytochrome c oxidoreductase

The enzymatic activity of the cytochrome bc_1 complex was determined as DBH_2:cytochrome *c* reductase to determine whether the mutations had an effect on the activity of the bc_1 complex. 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 (Table 1). Similar reductions of 33% and 22% were observed in the deletion mutants ΔVDA(88–90) and ΔAMA(90–92), while the cytochrome *c* reductase activity of the insertion mutant, I87AAA88 was reduced more than

90%. These results suggest that the three conserved alanine residues, Ala-86, Ala-90, and Ala-92, located in the tether region of the iron–sulfur protein and Glu-95 located at the beginning of the β1 sheet are required to maintain optimal rates of electron transport through the bc_1 complex. Removal of three amino acids from this region resulted in a comparable loss of enzymatic activity as the single-site mutations, while insertion of three amino acids resulted in a complete loss of enzymatic activity.

3.3. Spectral analysis of mitochondria from mutants

We next investigated 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. The concentrations of cytochromes *b* and c–c_1 in the wild-type mitochondria isolated from cells grown at 30°C were determined to be 0.083 and 0.092 nmol/mg of protein, respectively (Table 1). Examination of the spectra of mitochondria isolated from the mutants revealed the presence of cytochromes *b* and c–c_1 at the same level as that of the wild-type cells with the exception of the insertion mutant I87AAA88 in which the content of cytochromes *b* and c–c_1 were reduced 20–29% compared to the wild-type cells. These observations suggest that single-site and small deletion mutations in the 'tether' region of the iron–sulfur protein do not significantly damage the environment of the *b* heme and/or the expression of cytochrome *b* as well as cytochrome c_1.

3.4. Western blot analysis

The observation that mutating Ala-86, Ala-90 and Ala-92 located in the 'tether' region of the iron–sulfur protein as well as Glu-95 resulted in significant reductions in the enzymatic activity of the bc_1 complex prompted us to investigate the levels of the iron–sulfur protein in these mutants. The presence of the iron–sulfur protein in mitochondria was determined by Western blotting with an antibody against the iron–sulfur protein. As a control, the expression of core 2 protein in these mitochondria was compared on the same gel (Fig. 2). Quantitative analysis of the immunoblots by laser densitometry revealed the presence of the iron–sulfur protein in all the mutants at the same level as the wild type except for an approximately 20% reduction in all of the mutants of Ala-92 and a 40% reduction in the insertion mutant, I87AAA88 (Table 1). No significant loss of iron–sulfur protein was observed in mutants of Ala-86, Ala90, Lys-93, Glu-95 or the deletion mutants, suggesting that these mutations do not affect either the stability of the protein or its assembly into the bc_1 complex in yeast grown at 30°C.

Table 2
Growth characteristics, enzymatic activities and content of cytochromes *b* and c–c_1 and the iron–sulfur protein in JPJ1 yeast cells transformed with the wild-type RIP and mutant rip genes (37°C)

Mutant	Doubling time (h)	Enzymatic activity		Content (nmol/mg)		
		μmol/min/mg	% Control	Cytochrome *b*	Cytochrome c–c_1	ISP[a]
Wild type	4.0	1.16	100	0.065	0.084	1.00
A86C	4.0	1.04	90	0.066	0.087	1.04
A86L	4.5	0.46	40	0.064	0.083	0.97
A90F	5.0	0.51	49	0.056	0.070	0.52
A90I	4.5	0.80	70	0.064	0.081	0.90
A92C	7.0	0.47	41	0.048	0.098	0.47
A92F	4.5	0.61	53	0.075	0.055	0.44
A92R	8.0	0.32	28	0.060	0.048	0.34

Site-directed and deletion mutants were constructed and used to transform JPJ1 yeast cells as described in Section 2. Growth of the transformed cells on non-fermentable carbon source glycerol/ethanol was monitored as described at 37°C as described [12]. Wild-type and mutant cells were grown in a medium containing galactose at 37°C and mitochondria prepared as described [12]. Enzymatic activity and cytochrome content were determined as described in Section 2 and [12].[a]The content of the iron–sulfur protein (ISP) was determined by laser densitometry of immunoblots of mitochondria probed with antisera specific for the iron–sulfur protein relative to the wild-type protein set at 1.00.

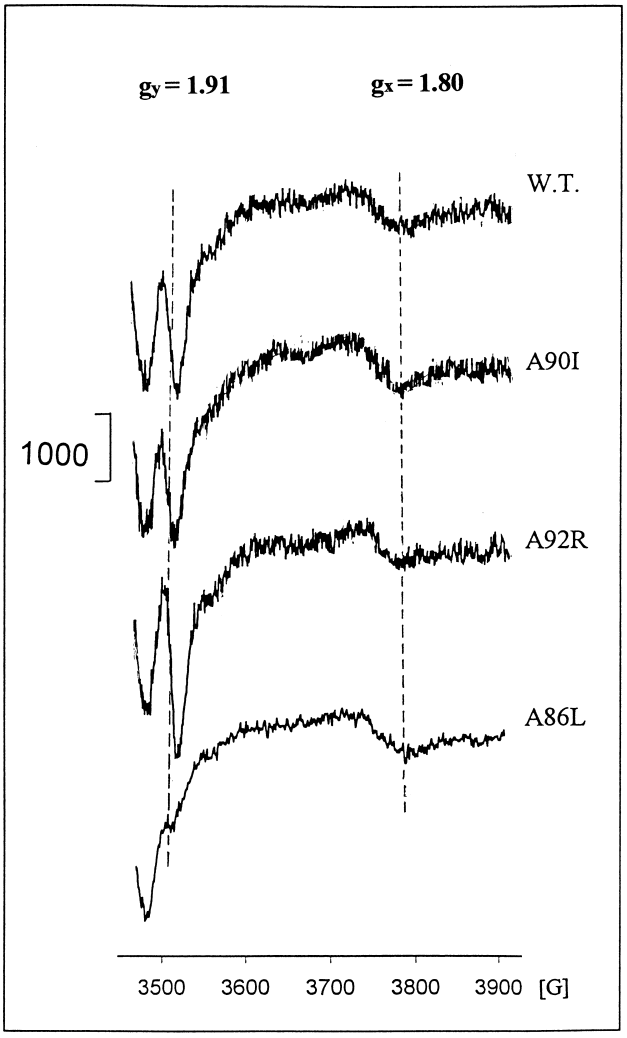

Fig. 3. EPR spectroscopy of iron–sulfur clusters of the iron–sulfur protein of the cytochrome bc_1 complex. Mitochondria suspensions (50 mg/ml) were reduced with ascorbate before freezing at 14 K. Top trace: JPJ1 cells transformed with the wild-type RIP gene (indicated as W.T.). Other traces are of mitochondria transformed with the mutated rip genes, as indicated as A86L, A90I, and A92R. EPR spectra were recorded at 14 K with the following instrument settings: microwave frequency, 9.426 Hz; microwave power, 20 mW; modulation amplitude, 30 G; modulation frequency, 100 kHz; time constant, 5.12 ms; scan rate, 67 G/s.

3.5. *Effects of these mutations in yeast cells grown at the non-permissive temperature (37°C)*

The 20% loss of iron–sulfur protein observed in mutants of Ala-92 suggested that mutating this amino acid might result in formation of an unstable iron–sulfur protein. To test this hypothesis, the growth rate and enzymatic activities of these mutants were determined in yeast cells grown at the non-permissive temperature, 37°C. The wild-type cells, JPJ1 transformed with the wild-type RIP gene, grew more slowly in the non-fermentable carbon source at 37°C than at 30°C with a doubling time of 4 h (Table 2). The mutant cells A86L, A90F and A92F had slightly lower rates of growth than the wild-type cells with respective doubling times of 4.5, 5.0 and 4.5 h, while mutants A92C and A92R grew at 37°C with doubling times of 7.0 and 8.0 h, respectively.

The enzymatic activity of the cytochrome bc_1 complex in the mutants grown at 37°C. was decreased from 30% to 70% in mutants A86L, A90F, A90I, A92C, A92F and A92R (Table 2). The loss of activity in the mutants of Ala-92 (A92C, A92F and A92R) and one mutant of Ala-90 (A90F) was accompanied by a similar reduction in the amount of the iron–sulfur protein present in the mitochondrial membranes as determined by quantitative immunoblotting. We suggest that substituting the large amino acids, Phe or Arg, for Ala-92 and Phe for Ala-90 results in an unstable iron–sulfur protein that does not assemble efficiently into the cytochrome bc_1 complex in cells grown at 37°C, the non-permissive temperature. By contrast, the loss of activity observed in the mutant in which leucine was substituted for Ala-86 and isoleucine for Ala-90 was not accompanied by a loss of iron–sulfur protein, suggesting that these mutations result in a change in the catalytic properties of the iron–sulfur protein.

3.6. *EPR Spectroscopy*

The results described above indicate that significant reductions of enzymatic activity of the bc_1 complex occur without large losses in the amount of the iron–sulfur protein in mitochondria from these cells grown at 30°C, with the exception of the mutants of Ala-92 and the insertion mutant (Table 1). These observations prompted us to examine whether changes in the environment of the iron–sulfur cluster had occurred in these mutants, resulting in the loss of enzymatic activity. The EPR spectra of mitochondrial membranes isolated from JPJ1 transformed with the wild-type RIP gene reduced with ascorbate revealed the typical spectrum of the 2Fe2S cluster of the yeast Rieske iron–sulfur protein with the charac-

teristic resonance at $g_x = 1.80$ and $g_y = 1.91$ (Fig. 3). The EPR spectra of the mutant mitochondrial membranes were similar to those of the wild-type cells. In the EPR spectrum of the A86L mutant, the $g_x = 1.80$ peak is prominent; however, the g_y peak is masked by a large peak observed at $g_z = 2.02$ (not shown in the spectra).

4. Discussion

In the current study, we have investigated the role of three conserved alanine residues and two charged amino acids located in the tether connecting the extrinsic domain of the iron–sulfur protein with the membrane-anchoring α-helix in the yeast cytochrome bc_1 complex. The tether region of the iron–sulfur protein, residues 65–73 in the beef heart enzyme (85–93 in the yeast numbering system), has been suggested to act as a 'flexible loop' that extends and retracts during electron transfer by the bc_1 complex [3,5,6]. Extension of the tether would result in movement of the extrinsic domain of the iron–sulfur protein containing the 2Fe2S cluster from a position near the Q_O site on cytochrome *b* where transfer of electrons from ubiquinol occurs to a position near cytochrome c_1 where efficient transfer of electrons from the 2Fe2S cluster to heme c_1 occurs [3]. In the current study, selected mutations of the three conserved alanine residues, Ala-86, Ala-90 and Ala-92, the charged residue Glu-95 as well as deletion of three amino acid residues (ΔVLA and ΔAMA) resulted in significant (22–56%) decreases in enzymatic activity of the cytochrome bc_1 complex. No loss of heme content of either cytochrome *b* or c–c_1 was detected spectrally, suggesting that drastic changes in the cytochrome bc_1 complex did not occur as a result of these mutations of the iron–sulfur protein.

Quantitative immunoblotting revealed that certain conserved amino acid residues are important for maintaining stability of the iron–sulfur protein. For example, a loss of iron–sulfur protein, which paralleled the loss of cytochrome *c* reductase activity was observed in all of the mutants of Ala-92 grown at 30°C, including a cysteine substitution. Even greater loss of iron–sulfur protein and activity was observed in these mutants of Ala-92 grown at 37°C, the non-permissive temperature for yeast (Table 2), providing additional evidence that Ala-92 plays an important role in maintaining stability of the iron–sulfur protein. In addition, substituting the bulky phenylalanine residue for Ala-90, also resulted in a loss of the iron–sulfur protein concomitant with loss of enzymatic activity in cells grown at 37°C. We conclude that the highly conserved alanine residues, Ala-90 and Ala-92 in the yeast numbering system, may contribute to the stability of the iron–sulfur protein and hence its proper assembly into the cytochrome bc_1 complex.

By contrast, mutations of the conserved alanine residue at position 86 of the yeast iron–sulfur protein did not result in a loss of protein. For example, mutating Ala-86 to leucine resulted in 56–70% decreases in cytochrome *c* reductase activity in cells grown at 30°C and 37°C, respectively, without any loss of iron–sulfur protein. Moreover, no changes were observed in the $g_x = 1.8$ peak in the EPR spectra of mutant A86L suggesting that substituting leucine for alanine did not affect the environment of the 2Fe2S cluster. The $g_x = 1.80$ peak in the EPR spectrum of the iron–sulfur protein has been shown to indicate formation of a complex between quinone bound in the Q_O site and reduced iron–sulfur protein [20]. The crystal structure has revealed the location of Ala-86 at the membrane-spanning helix near the cd helices of cytochrome *b*, which cover the quinol-binding pocket [6,11,21]. Thus, converting the conserved alanine at residue 86 to the bulky leucine residue may reduce the catalytic activity of the bc_1 complex either directly or by impeding the flexibility of the tether region of the iron–sulfur protein.

Interestingly, deleting three amino acids from the putative tether region of the iron–sulfur protein resulted in 20–30% losses of enzymatic activity of the bc_1 complex without any loss in iron–sulfur protein in cells grown at 30°C. These results suggest that if movement of the tether of the iron–sulfur protein is required for enzymatic activity, considerable flexibility must exist in this region of the protein. By contrast, the loss of both enzymatic activity and protein content as well as the hemes of cytochromes *b* and c_1 in the insertion mutant suggests that the mutant iron–sulfur protein containing three additional amino acids does not assemble properly into the bc_1 complex.

Recently, we have successfully isolated the bc_1

complex from mutants A86L, A90I, and A92R. No loss of iron–sulfur protein, cytochromes *b* or c_1, or ancillary subunits was observed in gels of these purified preparations suggesting that these mutations do not result in instability of the bc_1 complex [22]. The cytochrome *c* reductase activity of the purified preparations was decreased 50% in A86L and 20% in A90I compared to the wild type but was unchanged in A92R. Further characterization of the purified bc_1 complexes from these mutants is currently under way in our laboratory.

Acknowledgements

We would like to thank Dr. Andrew Shiemke of the Department of Biochemistry, Dr. Larry Halliburton and his graduate students, Kevin Stevens and Nelson Garces, from the Department of Physics at West Virginia University for their contributions to the experiments involving EPR spectroscopy and Mr. David Howell for his help in constructing the deletion mutants. This work was supported, in part, by National Institutes of Health Grant GM 57213.

References

[1] U. Brandt, B. Trumpower, Crit. Rev. Biochem. Mol. Biol. 29 (1994) 165–197.
[2] J.P.O. Di Rago, C. Bruel, L. Graham, P. Slonimski, B.L. Trumpower, J. Biol. Chem. 271 (1996) 15341–15345.
[3] C.-A. Yu, D. Xia, H. Kim, J. Deisenhofer, L. Zhang, A.M. Kachurin, L. Yu, Biochim. Biophys. Acta 1365 (1998) 151–158.
[4] D. Xia, C.A. Yu, H. Kim, J.Z. Xia, A.M. Kachurin, L. Zhang, L. Yu, J. Deisenhofer, Science 277 (1997) 60–66.
[5] S. Iwata, J.W. Lee, K. Okada, J.K. Lee, M. Iwata, B. Rasmussen, T.A. Link, S. Ramaswamy, B.K. Jap, Science 281 (1998) 64–71.
[6] Z. Zhang, L. Huang, V.M. Shulmeister, Y.I. Chi, K.K. Kim, L.W. Hung, A.R. Crofts, E.A. Berry, S.H. Kim, Nature 392 (1998) 677–684.
[7] A.R. Crofts, E.A. Berry, Curr. Opin. Struct. Biol. 8 (1998) 501–509.
[8] U. Brandt, Biochim. Biophys. Acta 1365 (1998) 261–268.
[9] H. Kim, D. Xia, C.A. Yu, J.Z. Xia, A.M. Kachurin, L. Zhang, L. Yu, J. Deisenhofer, Proc. Natl. Acad. Sci. USA 95 (1998) 8026–8033.
[10] L.A. Graham, B. Trumpower L, J. Biol. Chem. 266 (1991) 22485–22492.
[11] H. Tian, L. Yu, M.W. Mather, C.A. Yu, J. Biol. Chem. 273 (1998) 27953–27959.
[12] H. Tian, S. White, L. Yu, C.-A. Yu, J. Biol. Chem. 274 (1999) 7146–7152.
[13] V.H. Obungu, D.S. Beattie, Arch. Biochem. Biophys. 359 (1998) 128–132.
[14] V.H. Obungu, Y. Wang, D.S. Beattie, J. Biol. Chem. 273 (1998) 11917–11922.
[15] J.D. Beckmann, P.O. Ljungdahl, B.L. Trumpower, J. Biol. Chem. 264 (1989) 3713–3722.
[16] H. Ito, Y. Fukuda, K. Murata, A. Kimura, J. Bacteriol. 153 (1983) 158–163.
[17] B. Dunn, P. Szauter, L. Purdue, J.W. Szotak, Cell 39 (1984) 191–201.
[18] W. Fu, D.S. Beattie, J. Biol. Chem. 266 (1991) 16212–16218.
[19] M.E. Schmitt, B.L. Trumpower, J. Biol. Chem. 266 (1991) 14958–14963.
[20] A.S. Saribas, M.V. Valchanova, M.K. Tokito, Z. Zhang, E.A. Berry, F. Daldal, Biochemistry 37 (1998) 8105–8114.
[21] A.R. Crofts, B. Barquera, R.B. Gennis, R. Kuras, M. Guergova-Kuras, E.A. Berry, in: G.A. Peschek, W. Loeffelhardt, G. Schmetterer (Eds.), The Phototrophic Prokaryotes, Plenum, New York, 1999, pp. 229–239.
[22] M. Ghosh, Y. Wang, V.H. Obungu, D.S. Beattie, Biophys. J. (1999) 274.
[23] L.A. Graham, U. Brandt, J.S. Sargent, B.L. Trumpower, J. Bioenerg. Biomembr. 25 (1993) 245–257.
[24] T.A. Link, M. Saynovtis, C. Assman, S. Iwata, T. Ohnishi, G. Von Jagow, Eur. J. Biochem. 237 (1996) 71–75.