Increasing the redox potential of isoform 1 of yeast cytochrome *c* through the modification of select haem interactions

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The oxidation–reduction potential of eukaryotic cytochromes *c* varies very little from species to species. We have introduced point mutations into isoform 1 of yeast cytochrome *c* (iso-1-cytochrome *c*) to selectively engineer a protein with a higher redox potential. Of the ten different mutant proteins generated for the present investigation Y67R, Y67K and W59H were found to be non-functional. Three other mutant proteins, L32M, L32T and T49K, were functional, but too unstable for biophysical studies. Mutant cytochromes *c* K79S, K79T, Y48H and Y48K

were purified and characterized. The Y48K mutant is the only one that exhibits a significant increase of +117 mV in redox potential compared with the wild-type protein while still supporting oxidative phosphorylation *in vivo*. Low temperature difference spectroscopy confirmed the formation of the holo-protein, while adsorption and CD spectroscopy reveal perturbations in the structure of Y48K iso-1-cytochrome *c*.

Key words: formal potential, mutagenesis, spectroscopy.

INTRODUCTION

Eukaryotic cytochromes *c* are a class of haem proteins that contain a protoporphyrin IX covalently bound to the protein through two thioether linkages. Acting as electron intermediates between cytochrome *c*₁ and *aa*₃ in the mitochondrial inter-membrane space, they have a highly conserved redox potential of $+260 \pm 20$ mV [1]. This is in contrast with the wide range of potentials of their bacterial counterparts, which demonstrate redox potentials of -400 mV to $+400$ mV. This wide range of potentials is due to the various metabolic processes of each of the bacterial cytochromes and also to their varied structures.

Mitochondrial cytochromes *c* are the most thoroughly characterized *c*-type cytochromes. There are over 100 reported primary sequences for mitochondrial cytochromes *c* and five high resolution crystal structures, which exhibit an evolutionarily conserved structure. The relatively constant redox potential of these cytochromes is due to the protein component that surrounds the common haem prosthetic group. The modulation of the redox potential of *c*-type cytochromes is proposed to be due to several factors, including the polarity of the haem environment [2], the accessibility of the haem to the solvent [3], the strength of the axial ligand field [4,5], and the electrostatic interactions between the haem and its propionates [6].

The redox potential of cytochrome *c* is dependent upon the relative stability of the oxidized and reduced forms of the protein. Of pivotal importance to the redox potential is the charge distribution on the haem macrocycle. Ferricytochrome *c* holds a +1 charge on the haem, whereas the net charge of the ferrocyanochrome *c* redox centre is zero [1]. Stabilization of this positive charge in the oxidized state leads to a lower redox potential, while its destabilization should lead to an increase in the redox potential. This charge stabilization may be brought about in several ways. Modification of the haem axial ligands or their immediate environment alters the electron density of the haem macrocycle, leading to the most drastic changes in the stability of the cytochrome. Recently, semi-synthetic methods

and site-directed mutagenesis have been used to modify both axial ligands [7–11]. Mutation of these two invariant residues, however, almost always yields non-functional proteins. Other mutations to residues that modulate the properties of the axial ligands have been attempted. Some success was demonstrated by the increased stability of the Y67F mutant [12–15], but not with different Pro³⁰ mutants [16], which lead to haem-crevice destabilization.

Other ways to modify the charge on the haem macrocycle lie in the interaction of the haem propionates with residues in the lower haem pocket (see Figure 1). Introducing residues that add or remove hydrogen bonds to the haem propionates leads to alteration of the electron density of the haem macrocycle and a change of the redox potential. The introduction of electron withdrawing residues near propionates may also lead to destabilization of the net positive charge on the oxidized haem prosthetic group. Site-directed mutagenesis to residues Tyr⁶⁷, Asn⁵² and Arg³⁸ [14,15,17,18] has been performed to investigate the roles of these residues in modifying the redox potential of cytochrome *c*. Each mutation leads to a marked decrease in the redox potential of the cytochrome, with the greatest decrease (of 123 mV) compared with the wild-type protein shown in the tri-substituted isoform 1 of yeast cytochrome *c* (iso-1-cytochrome *c*) (R38A/N52I/F82S) [19]. The overall decrease of 123 mV is not equal to the sum of the independent changes in redox potential. The exception to simple addition of the independent changes in redox potentials is possibly due to electrostatic or structural interactions between the three mutated sites [19].

Previous investigations have found that the replacement of an amino acid in cytochrome *c* generally results in a decrease in redox potential. Being that cytochrome *c* is one of the most thoroughly characterized proteins, we attempted to use this information to rationally design mutations into the protein that would lead to an increase in redox potential. The ten mutant forms of cytochrome *c* generated for the present study were engineered to selectively reduce the relative stability of the oxidized form of the protein.

Abbreviations used: iso-1-cytochrome *c*, isoform 1 of yeast cytochrome *c*; SHE, standard hydrogen electrode.

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Figure 1 Positions of the side-chains of the mutated residues relative to the haem and its propionates in the three-dimensional structure of yeast iso-1-ferri cytochrome *c*

The side chains of the haem ligands and the amino acid residues modified by site-directed mutagenesis in the present study are shown as thick lines. The haem prosthetic group is represented as a wireframe structure. Hydrogen bonds are shown with broken lines. Wat¹⁶⁶ and the haem iron are represented as large dark grey and light grey spheres respectively. The haem environment is represented in (a) standard and (b) bottom orientations to show the interactions between the haem and amino-acid side chains. The Figures were created using the coordinates from the Brookhaven Protein Data Bank and the RASMOL modelling program [30]. HP, haem propionate.

MATERIALS AND METHODS

Mutagenesis and protein expression

Site-directed mutagenesis [20,21], protein expression and purification [22,23] of iso-1-cytochrome *c* were performed as described previously. Iso-1-cytochrome *c* was expressed in the multicopy plasmid pING4 [20] in yeast (*Saccharomyces cerevisiae*) strain GM3C-2 (a strain deficient in both isoforms of yeast cytochrome *c*) [24]. The mutations were carried out on a modified version of the naturally occurring iso-1-cytochrome *c* gene in which the codon for cysteine at position 102 was replaced with that for threonine. The resulting product does not have problems with dimerization and auto-reduction during purification [22]. For the purposes of the present study, the C102T variant is referred to as the wild-type protein. In order to confirm the identity and integrity of the mutant genes after their introduction into yeast, plasmid DNA was re-isolated from yeast cells and re-introduced into bacteria for nucleotide sequencing as described previously [20]. The incorporation of the desired mutation into the protein purified from yeast was further verified by electrospray ionization MS on a Fisons Instrument VG Quattro II triple quadrupole mass spectrometer using the Mass Lynx program. Purified proteins were further characterized using scanning adsorption spectroscopy before being stored at -80°C in cryovials (Nalgene) until needed. Purified cytochromes *c* were oxidized by the addition of ammonium cobalt (II) bis(picolate) [25], desalted using gel-filtration chromatography (PD-10; Pharmacia) to remove the oxidant, and exchanged into either potassium phosphate buffer (20 mM; pH 6.4) or buffer A (5 mM Mops, 5 mM Mes, 5 mM Taps, 5 mM Ches, 5 mM Caps and 100 mM NaCl) by diafiltration (Centricon-10; Amicon). Protein concentrations were obtained on a Cary 1 Bio UV-visible spectrophotometer using quartz cuvettes with 1-cm optical pathlengths. The absorbance at 410 nm was measured and the protein

concentration was calculated, using a molar absorption coefficient (ϵ_{410}) of $106.1 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [26].

Iso-1-cytochrome *c* assays

Each mutant form of cytochrome *c* was assayed *in vivo* for function based on its ability to support oxidative phosphorylation in yeast cells (GM3C-2) that do not produce either isoform of cytochrome *c* [20,27]. Yeast cells expressing mutant cytochromes were streaked on to synthetic medium supplemented with fermentable and non-fermentable carbon sources for growth. Plasmids expressing functional cytochromes *c* are capable of supporting growth on non-fermentable carbon sources, such as glycerol or lactate, that can only be metabolized via the tricarboxylic acid cycle with the involvement of the terminal electron transport system.

Electrochemistry

The direct electrochemistry of all the cytochromes was performed at a gold-modified electrode [28]. The gold-disk electrode (0.16 cm^2) was prepared by polishing with a fine alumina ($0.3\text{-}\mu\text{m}$ diameter particles)/water slurry on a polishing cloth (Bioanalytical Systems, West Lafayette, IN, U.S.A.). After rinsing the polished electrode with deionized water and sonication in a water bath to completely remove any residual particles, the electrode was immersed in a 2 mM solution of aldrithiol-4 (Aldrich) for 2 min with any excess solution on the sides of the electrode being removed prior to use.

Cyclic voltammetry was carried out in a micro-cell assembly, consisting of a three-electrode, two-compartment glass cell (Bioanalytical Systems). Protein solutions (0.20 ml; 0.3 mM) exchanged into buffer A were placed into the sample compartment and deaerated by passage of a stream of water-saturated

purified argon through the sample, then over the surface of the solution for the duration of the experiment to maintain anaerobic conditions. The Ag/AgCl reference electrode with 3.5 M KCl filling solution and platinum wire auxiliary electrode were both inserted into the reference compartment, and the gold-disk electrode was inserted into the sample compartment. All electrodes used in the present study were purchased from Bio-analytical Systems. The sample temperature was maintained at 25 °C by inserting the electrochemical cell into a thermostatically regulated glass jacket (VWR/Canlab, Montreal, QC, Canada) connected to a refrigerated circulating water bath. The electrode potential and sweep rate were controlled by an EG & G Princeton Applied Research potentiostat (Model 273). A potential range of –400 to 500 mV compared with a standard hydrogen electrode (SHE) was scanned at a rate of 20 mV · s⁻¹.

UV-vis and CD spectroscopy

The absorption spectra of the oxidized and reduced proteins were recorded at 25 °C using a Cary 1 Bio UV-visible spectrophotometer. Proteins were reduced by adding small amounts of either dithiothreitol or sodium dithionite.

CD spectra measurements were conducted with a Jasco J-715 spectropolarimeter. The CD spectra were collected in the far-UV (250–180 nm) and Soret (450–350 nm) regions using protein concentrations of 9 and 35 µM respectively. The experiments were performed on cytochrome *c* samples prepared in 20 mM potassium phosphate buffer, and placed in a 0.1-cm cell at 25 °C (an average of four scans were taken). Concentrations for proteins scanned in the Soret region were based on the absorption coefficient at 410 nm as mentioned previously, whereas in the far-UV region, the concentrations of the proteins were calculated using a Bradford Assay (Bio-Rad), standardized using wild-type iso-1-cytochrome *c*. All of the data are expressed in terms of mean residue (molar) ellipticity, $[\theta]$, calculated by the equation:

$$[\theta] = \theta / (10lc n)$$

where θ is the observed ellipticity in millidegrees, l is the length of the cell in cm, c is the molar concentration of the protein and n is the number of amino acids.

RESULTS

Cytochrome *c* mutant proteins

The ten different mutant cytochromes *c* generated for the present investigation are listed in Table 1 along with the rationale for their production. They are grouped as modifications that should affect either the haem ligand environment or the haem propionate

environment. Figure 1 represents the residues modified in the present study by showing the lower region around the haem propionates and the surrounding areas of both axial ligands in yeast iso-1-ferricytochrome *c*. Invariant residues Tyr⁴⁸, Asn⁵² and Trp⁵⁹ form hydrogen bonds to haem propionate-7, whereas residue Thr⁴⁹ hydrogen bonds to haem propionate-6. The side-chain of Lys⁷⁹ can also be seen to be in close proximity to haem propionate-6. The residue Tyr⁶⁷ is involved in the stabilization of the iron–sulphur bond between the haem and the Met⁸⁰ axial ligand. Tyr⁶⁷ also forms part of a hydrogen-bonding network, including an internal water molecule (Wat¹⁶⁶), Asn⁵² and Thr⁷⁸ (not shown). Pro³⁰ provides the stabilization of the N-terminal ligand His¹⁸ through a hydrogen bond to the His¹⁸-δ2 nitrogen. The close proximity of Leu³² to Pro³⁰ makes it a good site for mutations that could hydrogen bond to Pro³⁰ and disrupt the His¹⁸ to Pro³⁰ hydrogen bond.

Of the ten different mutants expressed in yeast, three variants, W59H, Y67K and Y67R, did not support oxidative phosphorylation. We intended to destabilize the oxidized state of the protein when we designed the Y67K and Y67R mutations which introduce positive charges near the haem iron. The replacement of Tyr⁶⁷ by charged amino acids results in non-functional proteins that are probably improperly processed within the cell, as confirmed by low temperature difference spectroscopy which shows no formation of the holoprotein (C. M. Lett and J. G. Guillemette, unpublished work). The burial of a charged group in this region of the protein is obviously detrimental to the biogenesis of a functional cytochrome *c*.

All other mutant cytochromes transformed into yeast grew on non-fermentable carbon sources, which establishes the presence of a functional cytochrome *c* in the electron transport assembly of proteins in the mitochondria. Proteins carrying mutations at either residue 32 or 49 were extremely unstable. Modification of the purification procedure [23] and the addition of protease inhibitors did not result in the purification of appreciable amounts of protein. Low temperature spectroscopy indicated that the covalently attached cytochrome *c* holoproteins were being expressed (C. M. Lett and J. G. Guillemette, unpublished work) as expected, since the proteins supported oxidative phosphorylation. The decrease in stability of our mutants is also consistent with a previous report studying the role of the invariant Leu³² of cytochrome *c* [29]. Juillera and Taniuchi [29] proposed that the Leu³² residue strongly influences the electronic state of the porphyrin ring and is essential for stabilization of the Met⁸⁰-sulphur to haem-Fe bond. In either case, our L32M and L32T mutations adversely affect the stability of the protein probably due to altered haem–ligand interactions.

The Thr⁴⁹-nitrogen and -γ1 oxygen form hydrogen bonds with the haem propionate-6. In addition, Thr⁴⁹ is the N-terminal

Table 1 Rationale for the generation of mutant iso-1-cytochrome *c* proteins

Mutation	Rationale
Haem ligand environment	
L32M and L32T	Disrupt the hydrogen bond between Pro ³⁰ and His ¹⁸ by introduction of an additional hydrogen bond acceptor to the δ1 nitrogen of Pro ³⁰
Y67K and Y67R	Raise the redox potential by introducing a buried positive charge next to the haem iron to destabilize ferricytochrome <i>c</i>
Haem propionate-6	
T49K	Raise the redox potential by introducing an electron withdrawing residue next to haem propionate-6
K79S and K79T	Introduce a hydrogen bond to haem propionate-6 and decrease the electron density of the haem macrocycle
Haem propionate-7	
Y48H*, Y48K and W59H*	Raise the redox potential by introducing an electron withdrawing residue next to haem propionate-7

* When the histidine side-chain is present in the form of an imidazolium ion.

Figure 2 Low temperature difference spectrum of yeast cells expressing iso-1-cytochromes *c*

Low temperature conditions (-196°C) were used to monitor the production of holocytochrome *c* within intact yeast cells, as indicated by a peak at 548 nm. The spectra of yeast cells expressing (a) wild-type, (b) Y48K and (c) W59H cytochromes *c* are presented. The spectrum of yeast cells not expressing cytochrome *c* is also shown (d).

residue of an α -helix that stretches from residue 49 to residue 55 at the bottom of the protein [30]. The introduction of a positively charged lysine residue at the N-terminus of the helix may cause its destabilization due to unfavourable electrostatic interactions. The loss of a hydrogen bond and the destabilization of the α -helix may significantly perturb the bottom of the protein while still maintaining function *in vivo*. As appreciable amounts of protein were not obtained, no results were obtained for proteins with mutations at either residue 32 or 49. A different type of expression system (and/or purification technique) will be required to investigate these functional, but unstable, proteins.

Protein characterization

Only cytochromes *c* with mutations at residues 48 and 79 yielded reasonable amounts of purified protein. The MS results confirmed the identity of each of the mutant proteins. Expression of the Y48K mutant resulted in a low yield of less than 1 mg of protein/litre. Most of the Y48K protein is probably lost during the purification procedure, since it does support oxidative phosphorylation and low temperature spectroscopy shows a peak at 548 nm indicating that it is produced in the cell as a holoprotein (Figure 2).

Cyclic voltammetry was performed on all of the purified mutants to determine their redox potentials (Table 2). The electrochemistry at the aldrithiol-4-modified gold electrode for all the functional cytochromes was found to be reversible at 25°C . The peak-to-peak separations at $20\text{ mV} \cdot \text{s}^{-1}$ were 60 mV, as expected for a reversible one electron transfer. The redox potential for the wild-type protein was determined to be +290 mV compared with a SHE (ionic strength = 0.1 M; pH 6.0; 25°C), which is in agreement with reported values using this technique [17,23].

Table 2 Mutant cytochrome *c* functionality and redox potential

Redox potentials were measured in buffer A (ionic strength = 0.1 M; pH 6.0; 25°C). Values have an error of $\pm 2\text{ mV}$.

Mutant	Functional	Redox potential (mV)
Wild-type	+	290
L32M and L32T*	+	—
Y48F†	+	263
Y48H	+	210
Y48K	+	407
T49K*	+	—
N52A	+	262
N52I‡	+	232
W59F	+	253
W59F/Y48F	+	220
W59H	—	—
Y67A*	+	—
Y67F‡	+	234
Y67K and Y67R	—	—
K79A§	+	292
K79S	+	234
K79T	+	258

* Although functional, these mutant proteins were unstable and not available for physical studies.

† Originally reported in [17].

‡ Originally reported in [14].

§ Originally reported in [34].

Figure 3 Cyclic voltammogram of yeast iso-1-ferricytochromes *c*

Wild-type (broken line) and Y48K (continuous line) were scanned in buffer A (pH 6.4; 25°C) at a protein concentration of 0.3 mM from 400 to -300 mV and 500 to -100 mV respectively, at a rate of $20\text{ mV} \cdot \text{s}^{-1}$. Redox potentials were found to be +290 mV for wild-type cytochrome *c* and +407 mV for the Y48K mutant protein.

While the other three mutants (Y48H, K79S, K79T) had decreased redox potentials, only the Y48K mutant displayed an elevated value of +407 mV compared with the SHE. The cyclic voltammogram of Y48K shown in Figure 3 depicts the increased redox potentials compared with wild-type iso-1-cytochrome *c*. This result prompted us to further characterize this protein.

Except for the Y48K mutant, the oxidized and reduced UV-visible spectral characteristics of the variants were similar to the wild-type protein. For the Y48K mutant protein, the Soret peak shifted from 410 nm to 406 nm in the oxidized state (Figure 4, top panel), and from 416 nm to 418 nm in the fully reduced state

Figure 4 Visible spectra of the wild-type and Y48K mutant cytochromes *c*

The oxidized (top panel) and reduced (bottom panel) forms of the wild-type (broken line) and Y48K (continuous line) proteins were scanned from 700 to 300 nm in potassium phosphate buffer (20 mM; pH 6.0; 25 °C).

(Figure 4, bottom panel). The α and β peaks in the reduced state are reduced in size relative to the wild-type protein, with a ratio of 1.35 for the α -peak₅₅₀/ β -peak₅₂₀ (wild-type iso-1-cytochrome *c* has a ratio of 1.81, which is similar to the published value of 1.87 for horse heart cytochrome *c*) [31]. Both the oxidized and reduced states exhibit a small peak around 640 nm, which is characteristic of a high-spin form of haem.

The CD spectra of both wild-type and Y48K ferricytochromes *c* were performed in the Soret and far-UV regions (Figure 5). The Soret region (450–350 nm) of the wild-type protein exhibits the characteristic negative Cotton effect previously reported in cytochrome *c* [23,32]. Replacement of Tyr⁴⁸ with a lysine residue reduces the negative Cotton effect, and gives a Soret spectrum similar to that of a partially unfolded cytochrome [33]. Far-UV (250–180 nm) CD for the wild-type protein shows characteristic α -helical content with troughs at 222 and 208 nm, and a peak around 190 nm, while the Y48K mutant shows a decreased trough at 222 nm and an increased trough at 208 nm, indicative of a perturbed α -helical structure. The Tyr⁴⁸ residue is adjacent to the N-terminus of the short α -helix stretching from residues 49–55 [30]. The introduction of a positively charged lysine at residue 48 should destabilize the dipole moment of this α -helix and possibly the entire region at the bottom of the protein, which would be consistent with our spectropolarimetry results.

Figure 5 CD spectra of the wild-type and Y48K mutant cytochromes *c*

Wild-type (broken line) and Y48K (continuous line) iso-1-ferricytochromes *c* were scanned in the (top panel) far-UV (250–180 nm) and (bottom panel) Soret (450–350 nm) regions at 20 nm · min⁻¹ in potassium phosphate buffer (20 mM; pH 6.0; 25 °C).

Two additional mutants (K79S and K79T) were designed to decrease the electron density of the haem macrocycle (Table 1). Our modelling studies indicate that residue 79 is in the proper position to form a hydrogen bond with propionate-6 if the lysine residue is replaced by a serine or threonine residue. Both mutations resulted in decreased redox potentials, suggesting that hydrogen bonds had not been formed between the mutant residue and haem propionate-6 in either of the proteins. It is noteworthy that in a previous study we reported that replacement of Lys⁷⁹ with an alanine residue had no effect on the redox potential [34]. The decreased redox potentials for K79S and K79T may be due to interactions between their hydroxy groups and adjacent residues, such as the Met⁸⁰ ligand.

DISCUSSION

In the present investigation we have attempted to selectively increase the redox potential of iso-1-cytochrome *c*. Based on our current understanding of protein structure and function, we introduced a series of single-site mutations that would either affect the haem axial ligands or the propionate environment. In only one of the ten mutant proteins designed for this purpose were we able to show an increased redox potential. Eukaryotic cytochromes *c* act to shuttle electrons between cytochrome *c*

reductase (c_1) and cytochrome c oxidase (aa_3) in the inner mitochondrial space. The redox potential for Y48K iso-1-cytochrome c , as determined by cyclic voltammetry, seems extremely high for a functional eukaryotic c -type cytochrome, considering the redox potentials of the respiratory complexes of the electron transport chain. For example, haems a and aa_3 from cytochrome c oxidase have redox potentials of +220 and +380 mV respectively. It is therefore thermodynamically unfavourable for the Y48K mutant cytochrome c with a redox potential of +407 mV to shuttle electrons between its two redox partners. However, cells expressing the mutant do grow on non-fermentable carbon sources, indicating that the Y48K mutant does support oxidative phosphorylation. In addition, low temperature spectroscopy shows that the mutant is produced as a protein with a covalently bound haem within the cell (Figure 2). The possibility exists that the *in vivo* redox potential is different from that determined by cyclic voltammetry, since the electrochemistry is performed in an *in vitro* anaerobic environment.

Surprisingly, the Y48K mutant auto-oxidizes rapidly, which indicates that either the structure of the purified protein is perturbed or more likely that there is a change in stability. This is further supported by the CD results and the presence of a band around 640 nm in the visible spectrum, which is indicative of a high-spin state of the haem iron (Figure 4). As previously noted [17], unlike Y48F, the substitution of Tyr⁴⁸ with a positively charged residue, such as lysine, is an extreme structural modification and is likely to result in a significant destabilization of the native conformation. The replacement of Tyr⁴⁸ with an aspartate does not result in a functional protein [35], which makes the Y48K mutant protein all the more intriguing. Furthermore, the polypeptide chain segment composed of residues 47–59 was reported to be one of the focal points of oxidation state-dependent structural alterations [36]. The loss of the hydrogen bond afforded by Tyr⁴⁸ upon mutagenesis of this residue must cause a reorganization of the hydrogen-bonding network around propionate-7, which is reflected in the changes in redox potential and structural stability as seen for the Y48K mutant cytochrome c .

We conclude that the redox potential of cytochrome c is affected not only by the haem axial ligands, but by a complex infrastructure consisting of the environments of the haem axial ligands and the haem propionates. Structural features, such as hydrogen bonds and electron density of the haem macrocycle, in these important regions affect the differential stabilities of both Fe²⁺ and Fe³⁺ forms of the protein, which translates into the observed changes in the redox potential.

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