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

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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 c1 and aα in the mitochondrial intermembrane space, they have a highly conserved redox potential of +260 ± 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 ferrocytochrome 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 Pro80 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 Tyr45, Asn52, and Arg88 [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 (123 mV) compared with the wild-type protein shown in the trisubstituted 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.

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 holoprotein, while adsorption and CD spectroscopy reveal perturbations in the structure of Y48K iso-1-cytochrome c.

Key words: formal potential, mutagenesis, spectroscopy.
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-ferrocytochrome 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 quadropole mass spectrometer using the Mass Lynx program. Purified proteins were further characterized using scanning absorption spectroscopy before being stored at −80 °C in cryovials (Nalgene) until needed. 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 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 cm²) was prepared by polishing with a fine alumina (0.3-µm diameter particles)/water slurry on a polishing cloth (Bioanalytical Systems, West Lafayette, IN, USA). 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 gas.
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 Bioanalytical 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 yeast iso-1-ferricytochrome c. Invariant residues Tyr¹⁸, Asn⁵² and Trp⁵⁸ form hydrogen bonds to haem propionate-7, whereas residue Thr¹⁹ 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 Met⁶⁰ axial ligand. Tyr⁶⁷ also forms part of a hydrogen-bonding network, including an internal water molecule (Wat¹⁶⁴), Asn⁵² and Thr⁷⁸ (not shown). Pro⁸⁰ provides the stabilization of the N-terminal ligand His¹⁴ through a hydrogen bond to the His¹⁸–δ₂ nitrogen. The close proximity of Leu⁸⁵ to Pro⁸⁰ makes it a good site for mutations that could hydrogen bond to Pro⁸⁰ and disrupt the His¹⁸ to Pro⁸⁰ 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 Leu⁵² of cytochrome c [29]. Juilera and Tanizumi [29] proposed that the Leu⁵² residue strongly influences the electronic state of the porphyrin ring and is essential for stabilization of the Met⁶⁰–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

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haem ligand environment</td>
<td>Disrupt the hydrogen bond between Pro⁵⁰ and His⁵⁸ by introduction of an additional hydrogen bond acceptor to the δ¹ nitrogen of Pro⁵⁰</td>
</tr>
<tr>
<td>L32M and L32T</td>
<td>Raise the redox potential by introducing a buried positive charge next to the haem iron to destabilize ferricytochrome c</td>
</tr>
<tr>
<td>Y67K and Y67R</td>
<td>Raise the redox potential by introducing an electron withdrawing residue next to haem propionate-6</td>
</tr>
<tr>
<td>Haem propionate-6</td>
<td>Introduce a hydrogen bond to haem propionate-6 and decrease the electron density of the haem macrocycle</td>
</tr>
<tr>
<td>T49K</td>
<td>Raise the redox potential by introducing an electron withdrawing residue next to haem propionate-6</td>
</tr>
<tr>
<td>K79G and K79T</td>
<td>Raise the redox potential by introducing an electron withdrawing residue next to haem propionate-6</td>
</tr>
<tr>
<td>Haem propionate-7</td>
<td>Raise the redox potential by introducing an electron withdrawing residue next to haem propionate-7</td>
</tr>
</tbody>
</table>

* When the histidine side-chain is present in the form of an imidazolium ion.
residue of an \( \alpha \)-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 \( \alpha \)-helix may significantly perturb the bottom of the protein while still maintaining function in \( \text{S} \text{E} \text{H} \). 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 mV \( \cdot \) 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 \( \text{SHE} \) (ionic strength = 0.1 M; pH 6.0; 25 °C), which is in agreement with reported values using this technique [17,23].

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 \( \text{SHE} \). 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.

![Table 2 Mutant cytochrome c functionality and redox potential](image)

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Functional</th>
<th>Redox potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>+</td>
<td>+290</td>
</tr>
<tr>
<td>L32M and L32T*</td>
<td>+</td>
<td>263</td>
</tr>
<tr>
<td>Y48F</td>
<td>+</td>
<td>210</td>
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<tr>
<td>Y48K</td>
<td>+</td>
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<td>T49K*</td>
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<tr>
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<td>+</td>
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<tr>
<td>N521†</td>
<td>+</td>
<td>232</td>
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<tr>
<td>W59F</td>
<td>+</td>
<td>253</td>
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<td>220</td>
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<td>–</td>
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<td>+</td>
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<td>Y67F†</td>
<td>+</td>
<td>234</td>
</tr>
<tr>
<td>Y67K and Y67R</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>K79A</td>
<td>+</td>
<td>292</td>
</tr>
<tr>
<td>K79S</td>
<td>+</td>
<td>234</td>
</tr>
<tr>
<td>K79T</td>
<td>+</td>
<td>258</td>
</tr>
</tbody>
</table>

* 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](image)

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 mV \( \cdot \) s\(^{-1} \). Redox potentials were found to be +290 mV for wild-type cytochrome \( c \) and +407 mV for the Y48K mutant protein.
Redox potential of cytochrome 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 \(\alpha\) and \(\beta\) peaks in the reduced state are reduced in size relative to the wild-type protein, with a ratio of 1.35 for the \(\alpha\)-peak/\(\beta\)-peak (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 Tyr\(\\textsuperscript{48}\) 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 \(\alpha\)-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 \(\alpha\)-helical structure. The Tyr\(\\textsuperscript{48}\) residue is adjacent to the N-terminus of the short \(\alpha\)-helix stretching from residues 49–55 [30]. The introduction of a positively charged lysine at residue 48 should destabilize the dipole moment of this \(\alpha\)-helix and possibly the entire region at the bottom of the protein, which would be consistent with our spectropolarimetry results.

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 Lys\(\\textsuperscript{79}\) 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 Met\(\\textsuperscript{79}\) 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 \(c\)
reduced hydrogen bonds and electron density of the haem macrocycle, in ligands and the haem propionates. Structural features, such as those affecting 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, from haem 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 indicating that either the structure of the purified protein is 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.

We wish to thank Dr Jack Kornblatt of Concordia University for access to the low temperature spectroscopy equipment, and Dr Linda Nazar at the University of Waterloo for the use of the EG & G potentiostat. This work was supported by the National Sciences and Engineering Research Council of Canada.

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Received 17 October 2001/13 November 2001; accepted 10 December 2001