The structure and function of omega loop A replacements in cytochrome *c*

MICHAEL E.P. MURPHY,^{1,3} JACQUELYN S. FETROW,² RANDALL E. BURTON,² AND GARY D. BRAYER¹

¹ Department of Biochemistry, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

² Department of Biological Sciences, State University of New York at Albany, 1400 Washington Avenue, Albany, New York 12222

(RECEIVED March 29, 1993; REVISED MANUSCRIPT RECEIVED June 22, 1993)

Abstract

The structural and functional consequences of replacing Ω -loop A (residues 18–32) in yeast iso-1-cytochrome *c* with the corresponding loop of *Rhodospirillum rubrum* cytochrome *c*₂ have been examined. The three-dimensional structure of this loop replacement mutant RepA2 cytochrome *c*, and a second mutant RepA2(Val 20) cytochrome *c* in which residue 20 was back substituted to valine, were determined using X-ray diffraction techniques. A change in the molecular packing is evident in the RepA2 mutant protein, which has a phenylalanine at position 20, a residue considerably larger than the valine found in wild-type yeast iso-1-cytochrome *c*. The side chain of Phe 20 is re-directed toward the molecular surface, altering the packing of this region of Ω -loop A with the hydrophobic core of the protein. In the RepA2(Val 20) structure, Ω -loop A contains a valine at position 20, which restores the original wild-type packing arrangement of the hydrophobic core. Also, as a result of Ω -loop A replacement, residue 26 is changed from a histidine to asparagine, which results in displacements of the main-chain atoms near residue 44 to which residue 26 is hydrogen bonded. In vivo studies of the growth rate of the mutant strains on nonfermentable media indicate that the RepA2(Val 20) cytochrome *c* behaves much like the wild-type yeast iso-1 protein, whereas the stability and function of the RepA2 cytochrome *c* showed a temperature dependence. The midpoint reduction potential measured by cyclic voltammetry of the RepA2 mutant is 271 mV at 25 °C. This is 19 mV less than the wild-type and RepA2(Val 20) proteins (290 mV) and may result from disruption of the hydrophobic packing in the heme pocket and increased mobility of Ω -loop A in RepA2 cytochrome *c*. The temperature dependence of the reduction potential is also greatly enhanced in the RepA2 protein.

Keywords: composite protein; crystallography; cytochrome *c*; electron transfer; heme; omega loop; protein folding; reduction potential

The omega (Ω)-loop, a novel class of secondary structure, has been defined as a protein segment having a small distance between segment termini but containing no other regular secondary structure (Leszczynski & Rose, 1986). An examination of these structures has shown that although Ω -loops lack regular hydrogen-bonding patterns, they fold into compact globular domains and may be independent folding units. Ω -Loops are generally found at the protein surface and are often sites of amino acid insertions and deletions in homologous proteins. As a result, these loops are thought to be important in determining

such protein functions as protein–protein interactions and substrate binding.

Because segment termini are close in space and these structures are at the protein surface, Ω -loops should be interchangeable without greatly distorting the overall three-dimensional structure of a protein. Yeast iso-1-cytochrome *c* has been used as a model system to study the role of Ω -loops by replacement and deletion of such loops (Fetrow et al., 1989). The yeast protein was chosen because there is both a high-resolution X-ray structure available (Louie & Brayer, 1990) and a well-developed system to generate mutants by site-directed mutagenesis (Pielak et al., 1985; Holzchu et al., 1987).

The first Ω -loop (Ω -loop A) of cytochrome *c* is defined as those residues from the heme iron ligand, His 18 to Leu 32 (Fig. 1). As shown in Figure 2, this surface loop

Reprint requests to: Gary D. Brayer, Department of Biochemistry, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada.

³ Present address: Department of Biological Structure SM-20, University of Washington, Seattle, Washington 98195.

cytochrome *c*. Cytochrome *c* function, as determined from yeast growth rates on nonfermentable media, shows that only one of the five loop replacements is completely nonfunctional.

One of the yeast Ω -loop A replacements studied, RepA2 cytochrome *c*, has the corresponding loop from cytochrome *c*₂ of the nonsulfur purple bacterium *Rhodospirillum rubrum*. The RepA2 mutant was selected for our studies because the structure of cytochrome *c*₂ from *R. rubrum* is also known to high resolution (Salemme et al., 1973; Salemme, 1977). Furthermore, cytochrome *c*₂ is a phylogenetically distant cytochrome *c* with a distinct functional role. This replacement loop is of the same residue length, but contains five amino acid replacements relative to yeast iso-1-cytochrome *c* (Fig. 1). A preliminary model of the RepA2 structure showed the likelihood of a large overlap between residues Phe 20 and Phe 10 in this composite protein. Using site-directed mutagenesis, a further substitution of valine for a phenylalanine at position 20 was introduced into the RepA2 protein, producing a new mutant named RepA2(Val 20) cytochrome *c*. Structural studies by X-ray crystallography were carried out on both RepA2 and RepA2(Val 20) cytochromes *c* to study the effects of an Ω -loop A replacement on protein structure. In addition, the midpoint electrochemical potential of both proteins was determined as a probe of their heme environments and in vitro function. Growth curves in liquid lactate media were done to measure the in vivo function of these hybrid proteins in yeast.

Results

Comparison of the structures of RepA2, RepA2(Val 20), and iso-1-cytochromes c

Ω -Loop A is located adjacent to the heme prosthetic group and is an integral component of the heme pocket

Fig. 3. Stereo plot of iso-1-cytochrome *c* with Ω -loop A drawn with thick lines. Every 10th residue is labeled with the one-letter amino acid code. The orientation of this plot is similar to that of Figure 2.

(Figs. 2, 3; Kinemage 1). Part of this loop is adjacent to the solvent-exposed edge of the heme and may be involved in the binding of redox partners. The structural differences present were examined by the superposition of the main-chain and heme-atom coordinates of RepA2 and RepA2(Val 20) cytochromes *c* onto those of iso-1-cytochrome *c* by a least-squares fit. The two amino-terminal residues (Thr -5 and Glu -4) were not included in this process because they are substantially disordered. The average main-chain positional deviation between the three superimposed structures was computed on a per residue basis and plotted as shown in Figure 4. The average deviations of the Ω -loop A main-chain atoms from the iso-1 structure are 0.21 Å and 0.16 Å for the RepA2 and RepA2(Val 20) cytochrome *c*, respectively. Both of these average deviations are similar to the overall averages for

all main-chain atoms, which are 0.22 Å and 0.17 Å for the two mutant proteins, respectively (Fig. 4).

Other than the disordered amino-terminal residues -5 and -4, the region of Figure 4 with the most significant deviation is centered at position 44. The structures of RepA2 and iso-1-cytochromes *c* in this region are plotted in Figure 5 and Kinemage 2. Note that as a result of Ω -loop A substitution, residue 26 is changed from a histidine to an asparagine in the RepA2 protein. The histidine side chain in iso-1-cytochrome *c* forms two hydrogen bonds to Asn 31 N and Glu 44 O, bridging these two sections of polypeptide chain. The OD1 atom of Asn 26 in RepA2 is able to occupy the same location as His 26 ND1 in iso-1 and forms the first hydrogen bond. However, due to a shorter side-chain length, Asn 26 ND2 cannot occupy the same location as the original His 26 NE2 atom of the

Fig. 4. The average positional deviations of main-chain atoms between RepA2 and wild-type yeast iso-1 (thick line), RepA2(Val 20) and wild-type yeast iso-1 (thin line), and RepA2 and RepA2(Val 20) (dotted line) cytochromes *c* are plotted along the course of the polypeptide chain. Horizontal lines represent the overall average deviations for main-chain atoms of residues -3 to 103 for each comparison: RepA2 and iso-1, 0.22 Å; RepA2(Val 20) and iso-1, 0.17 Å; RepA2(Val 20) and RepA2, 0.18 Å.

Fig. 5. The structures of wild-type yeast iso-1 (thin lines), RepA2 (thick lines), and RepA2(Val 20) (medium lines) cytochromes *c* are plotted in the region of residue 26. In the iso-1 structure, His 26 bridges these two segments of polypeptide chain by forming two hydrogen bonds (dashed lines). In the RepA2 and RepA2(Val 20) structures, the main chain surrounding Glu 44 shifts to form the same two hydrogen bonds to the shorter replacement Asn 26 side chain.

iso-1 protein. As a result, the polypeptide chain from Gln 42 to Gly 45 shifts so that Glu 44 O is positioned within hydrogen-bonding distance.

A further analysis of the positional deviation of well-ordered side chains with average thermal factors less than $25 \text{ \AA}^2$ reveals three side chains with different conformations between RepA2 and iso-1-cytochrome *c*. These residues are Tyr 97, Leu 98, and Ala 101, all of which pack against the side chain at position 20. As can be seen in Figure 6 and Kinemage 3, the side chain of Phe 20 in the RepA2 structure is positioned in a direction defined by the vector between atoms Val 20 CB and Val 20 CG2 of the wild-type iso-1-cytochrome *c*. This conformation has the phenyl ring directed toward the protein surface. Although the presence of a phenylalanine at position 20 does not markedly perturb the main-chain atoms of Ω -loop A (Fig. 4), the side-chain atoms of Tyr 97, Leu 98, and Ala 101 are displaced on average by 0.8, 0.7, and 0.7 $\text{\AA}$, respectively (Fig. 6; Kinemage 3).

In the RepA2(Val 20) structure, Leu 98 is one of the residues that line an internal hydrophobic buried cavity large enough to contain a solvent molecule. A similar cavity is observed in the iso-1-cytochrome *c* structure (Louie & Brayer, 1990). The structural and functional role of this cavity is unknown, but amino acid substitutions in the hydrophobic core of the protein have been shown to affect

the size of this cavity (Murphy et al., 1992). The RepA2 structure does not contain an internal cavity observable with a 1.4- $\text{\AA}$ -radius probe. The loss of this well-defined cavity appears to be a result of the repositioning of the Leu 98 side chain. None of the atoms of the Phe 20 side chain occupy the space of the original Val 20 CG1 methyl group, which is buried in the iso-1 structure. Movement of the side chain of Leu 98 in the RepA2 structure, however, partially fills the space vacated by Val 20 CG1.

The substitution of valine for phenylalanine at position 20 in the RepA2(Val 20) protein eliminates most of the structural changes described above. Residues Val 20, Tyr 97, Leu 98, and Ala 101 are all positioned in a manner similar to that observed in the iso-1 structure (Fig. 6; Kinemage 3). However, as observed in the RepA2 structure, the substitution of asparagine for histidine at position 26 results in similar shifts of the main-chain atoms of residues Gln 42 through Gly 45 (Fig. 4).

Electrochemistry of RepA2 and RepA2(Val 20) cytochromes c

Direct electrochemistry of RepA2 and RepA2(Val 20) cytochromes *c* appears to be reversible with a peak-to-peak separation of $62 \pm 7 \text{ mV}$ for all measurements, over a temperature range of 10–25 $^{\circ}\text{C}$. However, measurements

Fig. 6. Superimposed structures showing the packing of residue 20 against the nearby carboxy-terminal α -helix in wild-type yeast iso-1 (thin lines), RepA2 (thick lines), and RepA2(Val 20) (medium lines) cytochrome *c*. In the RepA2 structure, three side chains (Tyr 97, Leu 98, Ala 101) are repositioned to accommodate the larger phenylalanine side chain at position 20.

above 25 °C could not be made because protein solutions became turbid, resulting in greatly increased peak-to-peak separation in the voltammograms and a loss of reversibility. A plot of the temperature dependence of the midpoint potential of these two Ω -loop replacement proteins and iso-1-cytochrome *c* is presented in Figure 7. Thermodynamic parameters derived from these data are listed in Table 1 along with midpoint potentials under standard conditions. The midpoint potential for the RepA2(Val 20) protein at 25 °C is the same as that observed for wild-type iso-1-cytochrome *c* within experimental error. In contrast, the midpoint potential of RepA2 differs markedly and is 19 mV lower than that of the wild-type cytochrome *c*. The enthalpy and entropy of reduction of the RepA2 and RepA2(Val 20) proteins are less than that of wild-type iso-1-cytochrome *c* and partially compensate for each other.

Determination of *in vivo* cytochrome *c* function and levels

Results of the growth curve experiments in liquid lactate media are shown in Figure 8. Control strains containing 200% (B7533) or 100% (B7534) of the normal protein grow at the same rate at all temperatures, thus the presence of additional cytochrome *c* has little effect on the growth of the cells. A control strain, B7537, with no cytochrome *c*, does not grow at any temperature in synthetic lactate medium. At all temperatures, the RepA2(Val 20) strain (B7819) grows as well as the two wild-type B7533 and B7534 strains, demonstrating that this protein is functionally equivalent to the wild-type protein. On the other hand, the RepA2 strain (B7709) grows slower than the wild-type and RepA2(Val 20) strains at 25 and 30 °C. At 37 °C the RepA2 strain grows only slightly better than the

Table 1. Electrochemical properties of the yeast iso-1, RepA2, and RepA2(Val 20) cytochromes *c*^a

Cytochrome <i>c</i>	E_m (mV)	ΔH° (kcal/mol)	ΔS_{cyt}° (e.u.)	ΔG° (kcal/mol)
Iso-1	290 ± 2	-14.0 ± 0.2	-9.1 ± 0.4	-6.7 ± 0.1
RepA2	271 ± 2	-18.2 ± 0.7	-24 ± 2	-6.3 ± 0.1
RepA2(Val 20)	290 ± 2	-15.2 ± 0.1	-13.0 ± 0.2	-6.7 ± 0.1

^a Experimental conditions were 25 °C, pH 6.0, $\mu = 0.1$ M, and standard hydrogen electrode reference. Data for iso-1-cytochrome *c* are from Rafferty et al. (1990).

control strain B7537, which does not contain a gene for cytochrome *c*. Note that because all other factors are constant, it appears that the single amino acid difference between RepA2 and RepA2(Val 20) cytochromes *c* (Fig. 1) causes this drastic change in functional properties.

Fig. 7. The midpoint reduction potential of the RepA2 (□), RepA2(Val 20) (◇), and wild-type iso-1 (△) cytochromes *c* are plotted as a function of temperature. Data for iso-1-cytochrome *c* are from Rafferty (1992).

Fig. 8. Growth curves of yeast strains in liquid lactate media containing RepA2 (■), RepA2(Val 20) (●), 200% wild-type iso-1 (□), 100% wild-type iso-1 (◇), and the absence of (△) cytochrome *c* are plotted at three different temperatures.

The difference in the functionality of RepA2 cytochrome *c* at various temperatures could be caused by a decreased level of intact cytochrome *c* present within the cell. To examine this possibility, levels of holo-cytochrome *c* present within the cell were measured spectroscopically by monitoring a heme band (550 nm) at liquid nitrogen temperatures. The control strains B7533, B7534, and B7537 contain two, one, and no copies of the wild-type iso-1-cytochrome *c* gene, respectively. At all temperatures, these strains contain 200%, 100%, and 0% of the normal levels of this protein, respectively (Fig. 9). At 25 and 30 °C, RepA2(Val 20) cytochrome *c* is present at the same or slightly lower quantities than found in the wild-type strain (B7534). However, at 37 °C, RepA2(Val 20) cytochrome *c* is present at a substantially lower level. RepA2 cytochrome *c* is present in diminished but substantial amounts at both 25 and 30 °C. At 37 °C, the RepA2

protein level is barely detectable. These results correspond well with the growth curves presented in Figure 8, where strain growth is related to the functionality of mutant and wild-type cytochrome *c*. Note that this spectroscopic technique can only determine levels of holo-protein, that is, protein with a heme group covalently attached. Levels of apo-protein are not determined using this technique.

Discussion

Comparison of the wild-type yeast iso-1 and *R. rubrum* structures

Overall, the amino acid sequence identity between yeast iso-1-cytochrome *c* and *R. rubrum* cytochrome *c*₂ is 38% as shown by the sequence alignment in Figure 1. A comparison of the amino acid sequences of their respective Ω-loop A segments (residues 18–32) reveals that 10 of the 15 residues present are identical (67% of the total). There are 18 additional residues from other polypeptide chain segments that contact Ω-loop A in iso-1-cytochrome *c*. Nine of these (50%) are identical with those of *R. rubrum* cytochrome *c*₂. The main-chain and heme coordinates of *R. rubrum* cytochrome *c*₂ can be superimposed onto the iso-1 structure with an average deviation of 1.1 Å, excluding residues 57 and 76, which differ greatly in conformation between these two proteins. The Ω-loop A portion of these superimposed structures is illustrated in Figure 10. If only the main-chain atoms for Ω-loop A are considered, the average deviation observed is 0.53 Å.

Many of the residues of Ω-loop A conserved between the yeast iso-1 and *R. rubrum* cytochromes form intra-loop hydrogen bonds that stabilize this folded structure (Table 2). Note that two of the four intra-loop hydrogen bond differences listed in Table 2 are equivalent because the hydrogen bond between the side chain of residue 26 and the main-chain amide of residue 31 is conserved despite a change in amino acid sequence. The other two hydrogen bond differences are a result of an amino acid difference at position 21 and the absence of a γ-turn in the *R. rubrum* structure. A further understanding of the structural heterogeneity in Ω-loop A can be found by examining the interaction of this loop with the remainder of the protein molecule. Hydrogen bond interactions involving only one loop atom are more varied due to amino acid substitutions occurring at positions outside of Ω-loop A (Table 2). Substitutions at positions outside of Ω-loop A particularly affect the packing of buried loop side chains.

One prominent example of packing differences in a side-chain group between the yeast iso-1-cytochrome *c* and *R. rubrum* cytochrome *c*₂ involves residue 20, which forms part of the hydrophobic core of both molecules. This residue is a valine in the iso-1 structure and a much larger phenylalanine in *R. rubrum* cytochrome *c*₂. The larger side chain of the *R. rubrum* protein is accommo-

Fig. 9. Spectra of intact cells from the same yeast strains as in Figure 8 at the same three growth temperatures. The legend in this figure identifies the various yeast strains according to line type. The peak at 550 nm (indicated with an arrow) is one of the heme absorbance peaks in reduced cytochrome *c* and was used to assess the amount of protein present in each yeast strain.

Fig. 10. A stereo plot of Ω -loop A from yeast iso-1-cytochrome *c* (thick lines) superimposed on that of cytochrome *c*₂ from *R. rubrum* (thin lines). The overall path of the polypeptide chain is similar in both loops, but the precise conformation differs. The termini of this loop, His 18 and Leu 32, and every fifth residue of yeast iso-1-cytochrome *c* are labeled.

dated in the hydrophobic core by the complementary substitution of Phe 10 Ser (Fig. 1). In addition, the presence of Phe 33 eliminates a hydrogen bond to the carbonyl oxygen of residue 20 formed in the iso-1 structure by His 33 ND1. These factors serve to optimize the fit of the phenyl group of residue 20 into the hydrophobic core of the *R. rubrum* protein.

Another major packing difference involves residue 26. In one instance, the hydrogen bond between the side chain

of residue 26 and the main chain of residue 31 is in fact conserved between the two proteins. However, the interaction of this residue with the nearby polypeptide chain segment (involving primarily residues 44 and 45) is substantially different. In the iso-1 structure, a hydrogen bond is formed between His 26 NE2 and Glu 44 O. In the *R. rubrum* structure, Asn 26 ND2 interacts with the side chain of Asn 45, leading to a different folding of the polypeptide chain in this area.

Table 2. Hydrogen bond interactions of Ω -loop A in yeast iso-1, RepA2, RepA2(Val 20) cytochromes *c*, and *R. rubrum* cytochrome *c*₂^a

Cytochrome	Intra- Ω -loop A interactions	Involving other protein residues
Yeast iso-1	His 18 ND1-30 O Thr 19 OG1-25 O Thr 19 OG1-Asn 31 ND2 20 N-Glu 21 OE1 24 N-21 O 27 N-29 O 31 N-His 26 ND1 Asn 31 ND2-Thr 19 OG1 Asn 31 ND2-21 O 32 N-19 O	18 N-14 O His 26 NE2-44 O Lys 27 NZ-15 O 29 N-17 O 33 N-Asn 31 OD1 His 33 ND1-20 O 35 N-32 O Tyr 46 OH-28 O
Yeast RepA2	⊖ 20 N-Glu 21 OE1 ⊕ 31 N-Asn 26 OD1 ⊖ 31 N-His 26 ND1	⊕ Asn 26 ND2-44 O ⊖ His 26 NE2-44 O
Yeast RepA2(Val 20)	⊖ 20 N-Glu 21 OE1 ⊕ 31 N-Asn 26 OD1 ⊖ 31 N-His 26 ND1	⊕ Asn 26 ND2-44 O ⊖ His 26 NE2-44 O ⊖ 33 N-Asn 31 OD1
<i>R. rubrum</i>	⊖ 20 N-Glu 21 OE1 ⊖ 27 N-29 O ⊕ 31 N-Asn 26 OD1 ⊖ 31 N-His 26 ND1	⊕ Asn 26 ND2-Asn 45 ND2 ^b ⊖ His 26 NE2-44 O ⊖ 28 N-17 O ⊖ His 33 ND1-Asn 31 OD1 ⊕ Lys 43 NZ-31 O ⊕ Lys 43 NZ-Asn 31 OD1

^a Ω -Loop A is composed of residues His 18 through Leu 32. Where main-chain atoms are involved only the sequence number and atom name are given. All hydrogen bonds are listed for wild-type iso-1-cytochrome *c*. For the other three structures, the symbol ⊕ indicates an additional hydrogen bond in a particular structure, whereas ⊖ indicates the absence of a hydrogen bond relative to the iso-1 structure. Hydrogen bonds are defined by the following criteria: a H...A distance <2.60 Å, 2.70 Å, or 3.05 Å, where A is an oxygen, nitrogen, or sulfur atom, respectively; a D-H...A angle >120°; and a C-A...H angle >90°.

^b In the structure determined for *R. rubrum* cytochrome *c*₂ (Salemme et al., 1973), if the side-chain amide group of Asn 45 is rotated 180°, the OD1 atom would be positioned within hydrogen-bonding distance.

Structural effects of Ω -loop A replacements

In RepA2 cytochrome *c*, Ω -loop A from *R. rubrum* cytochrome *c*₂ has been spliced into the yeast iso-1-cytochrome structure in place of the normally resident residues. An examination of this structure reveals that the new Ω -loop A has an overall main-chain conformation very similar to that observed for the original loop in the iso-1 structure (Fig. 4). In some cases, such as the Ω -loop A amino acid differences at positions 21 and 22 on the surface of the protein, these substitutions are easily accommodated into the yeast iso-1 structure without any obvious structural perturbations. However, in other cases, such as the side-chain substitutions at positions 20 and 26, which occur in the molecular interior where these residues form close molecular interactions, the Ω -loop A substitution has had a significant structural effect. For example, if the *R. rubrum* loop is inserted without adjustment of the new phenylalanine side chain at position 20, a steric clash with the side chain of Phe 10 results. This conflict is relieved in the RepA2 structure by redirecting the Phe 20 side chain toward the molecular surface by a 120° rotation about the χ_1 torsional angle of this residue. Nonetheless, this reorientation is not entirely sufficient to avoid disruption of the packing in the hydrophobic core of the yeast iso-1-cytochrome *c* structure and, as a consequence, the side chain of Leu 98 shifts to fill some of the internal empty space generated by the Val 20 Phe substitution. At the molecular surface, the large phenyl group at position 20 in the RepA2 mutant also displaces the side chains of Tyr 97 and Ala 101, which pack against the original Val 20 side chain (Fig. 6; Kinemage 3). The end result is that residues not part of Ω -loop A, along with the Phe 20 side chain, undertake positional adjustments that allow the swapped loop to have a main-chain conformation nearly identical to the loop it replaces.

In the RepA2(Val 20) mutant, residue 20 has been substituted back to a valine as in the wild-type iso-1-cytochrome *c*. The side chain of Val 20 in the RepA2(Val 20) structure is in a conformation comparable to that of the iso-1 protein (Fig. 6; Kinemage 3). As a result, this substitution restores the packing of the hydrophobic core and does not lead to the displacement of Tyr 92, Leu 98, or Ala 101.

Although the substitution of asparagine for histidine at position 26 in both loop replacement proteins is conservative, as Figures 4 and 5 (Kinemage 2) show, it does result in alterations in the main-chain fold centered at the adjacent residue Glu 44. An analysis shows that the main-chain atomic positions of residue 26 appear to be more tightly fixed by hydrogen bond interactions with other groups than residues 42 through 45, which occupy a more solvent-exposed position. As a result, this latter segment of polypeptide chain has greater flexibility to shift position in order to maintain a hydrogen bond between the side chain of residue 26 and the carbonyl group of resi-

due 44. It is of interest that in yeast iso-2-cytochrome *c* there is also an asparagine at position 26 (Murphy et al., 1992). Although the polypeptide chain in the region of residue 44 is slightly displaced relative to that of the iso-1 structure, the hydrogen bond between Asn 26 ND2 and Glu 44 O is not formed. This appears to be a consequence of the replacement of alanine at the adjacent residue 43 by a valine, which fills the space vacated as a result of the substitution of the smaller asparagine side chain for the histidine side chain at position 26. This second substitution therefore appears to prevent the type of displacement observed in the polypeptide chain of RepA2 and RepA2(Val 20) cytochromes *c*, which is required to form a hydrogen bond to the carbonyl group of Glu 44.

In vivo functional consequences of Ω -loop A replacement

The presence of significant amounts of RepA2 protein in intact cells at 25 and 30 °C suggests that Ω -loop A replacement has not interfered with protein production at these temperatures (Fig. 9); however, the protein that is produced at these temperatures is not fully functional as measured by cell growth on liquid lactate media (Fig. 8). At 37 °C, both the amount of RepA2 cytochrome *c* in intact cells and the growth rate on lactate media decrease significantly. At this higher temperature, a loss of protein stability appears to diminish the amount of protein, thereby limiting cell growth. Although the amount of RepA2(Val 20) protein in intact cells decreases at 37 °C, the growth rate remains unaffected. These results suggest that the presence of a phenylalanine at position 20 decreases the stability and function of RepA2 cytochrome *c*.

Inspection of the structure of the RepA2 cytochrome *c* has shown that Phe 20 disrupts both the hydrophobic core as well as groups located at the protein surface (Fig. 6; Kinemage 3). These changes in atomic interactions could account for a loss of stability at 37 °C of the RepA2 protein. Also observed is that the average main-chain thermal factors of Ω -loop A in the RepA2 structure are higher than in the RepA2(Val 20) and iso-1 structures (Fig. 11). These elevated thermal factors also suggest that the interactions between Ω -loop A and the protein are weakened.

The relationship between structure and heme reduction potential

The RepA2(Val 20) cytochrome *c* has a midpoint potential comparable to that of wild-type yeast iso-1-cytochrome *c* (Table 1). It therefore appears that the other amino acid substitutions present in Ω -loop A in comparison to wild-type iso-1-cytochrome *c* have no effect on this functional parameter. This includes the Lys 22 Gln substitution, which has caused a change in the surface charge of the molecule. However, the single substitution of valine for a phenylalanine at position 20 in the RepA2(Val 20) cy-

Fig. 11. The average thermal factor of the four main-chain atoms of each amino acid in RepA2 (thin line), RepA2(Val 20) (dotted line), and wild-type iso-1 (thick line) cytochromes *c* are plotted as a function of residue number for Ω -loop A. The thermal factors have been normalized over all protein atoms by an additive factor. The greatest main-chain thermal factor difference between the RepA2 structure and the other two structures is within the Ω -loop A region.

tochrome *c* did restore the midpoint potential of the RepA2 cytochrome *c* to comparable values found for the wild-type iso-1 protein at 25 °C (Fig. 7). Clearly, Val 20 is important for the maintenance of the heme reduction potential in wild-type iso-1-cytochrome *c*. Nonetheless, the mechanism by which residue 20 modulates midpoint potential is not readily apparent. Previous studies have linked a change in the solvent accessibility of the heme (Stellwagen, 1978; Louie & Brayer, 1988b) or polarity of the heme environment (Kassner, 1973; Louie & Brayer, 1989) to a change in reduction potential. However, the solvent accessibilities of the porphyrin ring atoms of the heme are the same for RepA2, RepA2(Val 20), and iso-1-cytochrome *c*. Furthermore, no other changes in the position of polar groups near the heme are observed in either the RepA2 or RepA2(Val 20) structures relative to the wild-type protein.

The thermodynamic parameters derived from the temperature dependence of the midpoint reduction potential may provide insight into the mechanism of reduction potential modulation by residue 20 (Table 1). The larger decrease in the entropy of reduction is partially offset by a decrease in the enthalpic contribution. A change in the polypeptide contribution to the thermodynamic properties of cytochrome *c* reduction has been suggested to be primarily due to electrostatic effects (Schejter et al., 1982); however, the substitution at position 20 does not involve a change in net molecular charge. The change in enthalpy could be accounted for by the increase in hydrophobic bulk of the protein (Kassner, 1973). Taniguchi et al. (1980) have suggested that polypeptide chain order-

ing and protein-solvent interactions are also likely important in determining the entropy of reduction. The Phe 20 side chain is largely solvent accessible in the RepA2 structure, which may alter solvent ordering upon reduction. The increased mobility of Ω -loop A as seen in temperature factor values (Fig. 11) may also contribute to this effect. In addition, the increase in thermal factors may result in an increase in dynamic heme solvent exposure.

Materials and methods

Yeast strains, mutagenesis, and protein purification

Mutagenesis and construction of yeast strains containing mutant iso-1 cytochrome *c* (CYC1) genes were carried out as described previously (Fetrow et al., 1989). The genotype of the parent strain, B-6748, is *MATa cyc1-Δ::lacZ cyc7-Δ1::CYH2 ura3-52 his3-Δ1 leu2-3 leu2-112 trp1-289 can1-100 cyh2* (Holzchu et al., 1987). The control strain, B-7533, has the same genotype, except the wild-type *CYC1*⁺ gene is present. Protein was purified by a variation on methods described previously (Sherman et al., 1968; Nall & Landers, 1981).

Structure determination

RepA2 crystals were produced by hair seeding liquid/liquid diffusion capillaries with crushed iso-1-cytochrome *c* crystals. Crystals of yeast RepA2(Val 20) cytochrome *c* were grown initially by hair-seeding hanging drops with crushed yeast iso-1-cytochrome *c* crystals as described previously (Leung et al., 1989). Crystals used for diffraction analyses were subsequently produced by macro-seeding hanging drops with small RepA2(Val 20) crystals. For both proteins, the final crystallization conditions were 95% (NH₄)₂SO₄, 0.07 M Na₂S₂O₄, and 0.1 M sodium phosphate buffer adjusted to pH 5.5. Crystals used in diffraction analyses were first transferred into a freshly prepared solution of 97% (NH₄)₂SO₄, 0.1 M sodium phosphate buffer at pH 5.5, and 0.04 M dithiothreitol, to maintain the reduced state of the heme group. These were then mounted in thin-wall glass capillaries.

A single RepA2 crystal was utilized to collect diffraction data to 2.25 Å resolution (91% complete) on a Rigaku R-AXIS II imaging plate detector system. Each frame had a ϕ oscillation of 1.0° and was exposed for 15 min to CuK α X-rays from a rotating anode generator operating at 80 mA and 50 kV. A total of 99 frames were collected after 35 h of X-ray exposure time. The resulting images were processed to structure factor amplitudes, and the merging *R*-factor for both full and partial intensities was 7.3%. Other data collection parameters are listed in Table 3. The absolute scale of the resultant structure factors was derived by a linear rescale against a wild-type iso-1-cytochrome *c* data set having the same resolution range. An overall thermal factor of 20 Å² was

Table 3. Data collection statistics

Parameter	RepA2	RepA2(Val 20)	Iso-1 ^a
Crystal size (mm)	0.1 × 0.1 × 0.05	0.48 × 0.38 × 0.2	
Space group	P4 ₃ 2 ₁ 2	P4 ₃ 2 ₁ 2	P4 ₃ 2 ₁ 2
Unit cell parameters (Å)			
<i>a</i> , <i>b</i>	36.38	36.37	36.46
<i>c</i>	137.63	137.64	136.86
Resolution (Å)	2.25	1.90	
No. of reflections			
measured	25,731	9,290	
No. of unique reflections	4,498	7,918	
Merging <i>R</i> -factor on <i>I</i>	0.073	0.068	
Completeness (%) ^b	86	65	
$\langle I \rangle / \langle \sigma(I) \rangle$ ^c	3.6	3.7	
No. reflections included			
in refinement	4,197	5,802	
Final <i>R</i> -factor	0.183	0.191	

^a The cell dimensions and space group of wild-type yeast iso-1-cytochrome *c* are provided for comparison purposes (Louie & Brayer, 1990).

^b Percentage of reflections with $I > \sigma(I)$ in the resolution shells of 2.50–2.25 Å and 2.10–1.90 Å for the RepA2 and RepA2(Val 20) data sets, respectively.

^c Average values for reflections with $I > \sigma(I)$ in the resolution ranges indicated for the completeness parameter.

determined for these data by inspection of a Wilson (1942) plot.

From a single RepA2(Val 20) crystal, a complete diffraction data set to 1.9 Å was collected on an Enraf Nonius CAD4-F11 diffractometer using continuous Ω scans of 0.6° at 0.55°/min. The incident CuK α radiation was Ni filtered and generated from an X-ray tube operated at 26 mA and 40 kV. Intensity backgrounds were measured by extending the scans by 25% on either side. Three standard reflections were measured every 8 h of X-ray exposure time to monitor crystal decay and slippage. An average of background measurements of neighboring reflections within a sphere of reciprocal space centered on the current reflection was used for background correction (Murphy et al., 1992). Diffraction intensities were corrected for absorption, decay, Lorentz, and polarization effects as described previously (Louie et al., 1988a). Crystal-specific data collection statistics are presented in Table 3. The initial scale and an overall thermal factor of 16 Å² were determined as described for RepA2 cytochrome *c*.

A starting model of RepA2(Val 20) cytochrome *c* was constructed from the coordinates of wild-type yeast iso-1-cytochrome *c* (Louie & Brayer, 1990). At each position of differing amino acid sequence, the residue present was initially modeled as an alanine (Fig. 1). A sulfate ion and a selection of 52 water molecules from the iso-1-cytochrome *c* structure were included in the initial model. This model was refined by a restrained parameter least-squares method (Hendrickson & Konnert, 1981) using reflections with $F > 2\sigma(F)$ and a resolution greater than

6 Å. Fragment deleted, $F_o - F_c$, $2F_o - F_c$, and $3F_o - 2F_c$ maps were examined at intervals during the course of refinement as a guide for the addition and manual adjustment of side chains in the structure. Water molecules were added to the refinement model if at least one hydrogen bond was formed to the existing structure and if $2F_o - F_c$ or $3F_o - 2F_c$ maps had significant electron density at the water position. At the end of refinement, 74 water molecules (average thermal factor of 27 Å²) were included in the model and the conventional *R*-factor was 19.1%.

The final RepA2(Val 20) structure was used as an initial model for the refinement of the RepA2 structure, with residue Val 20 converted to an alanine by deletion of the coordinates of the two γ -carbons. The same selection of 52 water molecules and a sulfate ion from the wild-type iso-1 structure were used as the initial solvent model. This model was refined as described for RepA2(Val 20), with the eventual inclusion of the Phe 20 side chain to a final *R*-factor of 18.3%. The final RepA2 structure contained 51 water molecules and a sulfate ion and had an average thermal factor of 34 Å² for solvent atoms.

The agreement of the RepA2 and RepA2(Val 20) structures with ideal stereochemical geometry and the corresponding refinement weights used in the final refinement cycles are outlined in Table 4. To estimate coordinate errors, a plot of the conventional *R*-factor as a function of resolution along with theoretical curves calculated by assuming various root mean squared (rms) coordinate errors (Luzzati, 1952) was constructed. Inspection of this plot suggests a coordinate error for both structures of approximately 0.2 Å. This value is a statistical estimate of the overall coordinate error, whereas the actual positional error of an individual atomic coordinate will depend on the location of the atom in the structure. Additional

Table 4. Final stereochemistry of the RepA2 and RepA2(Val 20) cytochromes *c*

Restraint	rms deviation from ideal values		Restraint weight
	RepA2	RepA2(Val 20)	
Distances (Å)			
Bond	0.018	0.018	0.020
Angle	0.042	0.039	0.030
Planar 1–4	0.053	0.048	0.050
Plane (Å)	0.013	0.015	0.020
Planar torsional (deg.)	2.3	2.5	2.5
Chiral volume (Å ³)	0.210	0.213	0.160
Nonbonded contact (Å) ^a			
Single torsion	0.217	0.209	0.250
Multiple torsion	0.195	0.195	0.250
Possible hydrogen bonds	0.224	0.192	0.250

^a The root mean square (rms) deviations from ideality for this class of restraint incorporates a reduction of 0.2 Å from the radius of each atom involved in a contact.

analyses by an individual atom method (Cruickshank, 1949; Chambers & Stroud, 1979) suggests rms coordinate errors of 0.16 Å for both RepA2 and RepA2(Val 20) cytochrome *c*.

Direct electrochemistry

The direct electrochemistry of RepA2 and RepA2(Val 20) cytochrome *c* by cyclic voltammetry was achieved at a polished gold surface modified with 4,4'-dithiodipyridine following the method described by Barker et al. (1989) and Rafferty et al. (1990). All samples were first oxidized by passing them through $\text{NH}_4(\text{Co}(\text{dipicolinate}))$ on a desalting gel filtration column. Experiments were performed on 0.5 mL of a 0.4 mM protein solution in $\mu = 0.1$ M buffer (50 mM KCl with the remaining ionic strength provided by sodium phosphate), at pH 6.0. A potential range of 44–544 mV (standard hydrogen electrode) was studied at sweep rates of 10–100 mV/s. The temperature dependence of the midpoint potentials was also measured at 5° intervals from 10 to 25 °C for both the RepA2 and RepA2(Val 20) proteins.

Growth curves for the determination of in vivo protein function

The functional capacity of mutant and wild-type cytochromes *c* was determined by growth in liquid lactate media of yeast strains containing these genes. Either iso-1 or iso-2 cytochrome *c* protein must be functional for yeast to be able to grow on this nonfermentable carbon source. Our strains are lacking iso-2 cytochrome *c*, so growth in this medium is a good measure of the function of mutant iso-1-cytochrome *c*.

Yeast strains were first made diploid to remove the possibility of random background recessive mutations, which could interfere with measurements (Schweingruber et al., 1977). Growth curves were determined by a variation on the method of Schweingruber et al. (1979). Each strain was grown at 30 °C for 16–20 h in 10 mL YPD (1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose) and then placed in the refrigerator for 24 h to bring all cells to the same stage in the cell cycle. Ten-milliliter aliquots of liquid lactate media (0.67% Bacto-yeast nitrogen base without amino acids, 0.05% Bacto-yeast extract, 1% DL-lactate) were placed in 300-mL side-arm flasks. These flasks were inoculated with cells from the refrigerated overnight cultures to an initial culture density of precisely eight Klett units. Cultures were then grown for 24–26 h at the appropriate temperatures (25, 30, or 37 °C). Culture growth was monitored by measuring the culture density every 2 h with a Klett–Summerson colorimeter fitted with a No. 54 filter. Each curve is the average of at least two independent cultures.

Low-temperature spectroscopy to determine the in vivo level of intact iso-1-cytochrome c

The level of wild-type and mutant iso-1-cytochrome *c* within intact yeast cells was measured by spectroscopic determination of the 550-nm α -heme band at liquid nitrogen temperatures. Appropriate yeast strains were grown in 5 mL YPD at 30 °C overnight and placed at 4 °C for 8 h. YPD medium (25 mL) was inoculated with 1 mL of the overnight culture and grown at the appropriate temperature (25, 30, or 37 °C) for 16–18 h. Cells were spun down for 3 min at 3,700 rpm, 5 °C, in a Beckman tabletop centrifuge and rinsed twice with 10 mL of 100 mM sodium phosphate, pH 7. Cells were resuspended in 1 mL of 100 mM sodium phosphate buffer and placed on ice. A total of 250 mL of cells were added to 10 mL sodium phosphate buffer, and the cell density was measured using a Klett–Summerson colorimeter fitted with a No. 54 filter. The original cell suspensions were then diluted with buffer such that all samples had the same cell density as that of the least dense sample.

For each spectrum, two 250- μ L samples of cells were then removed to separate tubes. One sample was reduced with 25 μ L of 13 mg/mL dithionite and one sample was oxidized with 25 μ L of 5% H_2O_2 . Each sample was then mixed with 250 μ L of saturated sucrose. The reduced sample was placed in the sample compartment, and the oxidized sample was placed in the reference compartment of a 1-mm-pathlength cuvette and samples were frozen in liquid nitrogen.

Spectra were measured from 500 to 650 nm using an SLM-Aminco DW2000 dual wavelength spectrophotometer fitted with a low-temperature apparatus. Because these cells lack iso-2-cytochrome *c*, the absorbance at 550 nm is a measure of the amount of reduced wild-type or mutant iso-1-protein present. To directly compare the absorbance from one spectrum to another, a constant was added to or subtracted from all spectra so that the absorbance at 540 nm was identical.

Acknowledgments

We thank Theresa Yang for determining the crystallization conditions for RepA2 and RepA2(Val 20) cytochrome *c*. Professor Grant Mauk and Dr. Paul Barker assisted with the midpoint potential measurements by cyclic voltammetry. Dr. Yaoguang Luo collected the RepA2 cytochrome *c* data with an R-Axis II area detector generously made available by Professor Daniel Yang (McMaster University). Albert Berghuis assisted by reviewing the manuscript. The coordinates and structure factors of both RepA2 and RepA2(Val 20) cytochromes *c* have been deposited in the Protein Data Bank under the identification codes 1RAP and 1RAQ, respectively (Bernstein et al., 1977). These studies were supported by a grant (MA-7975) from the Medical Research Council of Canada to G.D.B. and a grant (GM44829) from the National Institutes of Health to J.S.F. M.E.P.M. is the recipient of a Medical Research Council of Canada Graduate Studentship.

References

- Barker, P.D., Hill, A.O., & Walton, N.J. (1989). Fast second order electron transfer reactions coupled to redox protein electrochemistry. *J. Electroanal. Chem.* 260, 303-326.
- Bernstein, F.C., Koetzle, T.F., Williams, G.J.B., Meyer, E.F., Jr., Brice, M.D., Rodgers, J.R., Kennard, O., Shimanouchi, T., & Tasmui, M. (1977). The Protein Data Bank: A computer-based archival file for macromolecular structures. *J. Mol. Biol.* 112, 535-542.
- Chambers, J.L. & Stroud, R.M. (1979). The accuracy of refined protein structures: Comparison of two independently refined models of bovine trypsin. *Acta Crystallogr.* 35, 1861-1874.
- Cruickshank, D.W.J. (1949). The accuracy of electron-density maps in X-ray analysis with special reference to dibenzyl. *Acta Crystallogr.* 2, 65-82.
- Dus, K., Sletten, K., & Kamen, M.D. (1968). Cytochrome c_2 of *Rhodospirillum rubrum*, complete amino acid sequence and phylogenetic relationships. *J. Biol. Chem.* 243, 5507-5518.
- Fetrow, J.S., Cardillo, T.S., & Sherman, F. (1989). Deletions and replacements of omega loops in yeast iso-1-cytochrome c . *Proteins Struct. Funct. Genet.* 6, 372-381.
- Hendrickson, W.A. & Konnert, J. (1981). Stereochemically restrained crystallographic least-squares refinement of macromolecular structures. In *Biomolecular Structure, Function, Conformation and Evolution*, Vol. 1 (Sunivasan, R., Ed.), pp. 43-57. Pergamon Press, Oxford, UK.
- Holzchu, D., Principio, L., Conklin, K.T., Hickey, D.P., Short, J., Rao, R., McLendon, G., & Sherman, F. (1987). Replacement of invariant lysine 77 by arginine in yeast iso-1-cytochrome c results in enhanced and normal activities in vitro and in vivo. *J. Biol. Chem.* 262, 7125-7131.
- Kassner, R.J. (1973). A theoretical model for the effects of local non-polar heme environments on the redox potentials of cytochromes. *J. Am. Chem. Soc.* 95, 2674-2677.
- Leszczynski, J.F. & Rose, G.D. (1986). Loops in globular proteins: A novel category of secondary structure. *Science* 234, 849-855.
- Leung, C.J., Nall, B.T., & Brayer, G.D. (1989). Crystallization of yeast iso-2-cytochrome c using a novel hair seeding technique. *J. Mol. Biol.* 206, 783-785.
- Louie, G.V. & Brayer, G.D. (1989). A polypeptide chain-refolding event occurs in the Gly 82 variant of yeast iso-1-cytochrome c . *J. Mol. Biol.* 210, 313-322.
- Louie, G.V. & Brayer, G.D. (1990). High-resolution refinement of yeast iso-1-cytochrome c and comparisons with other eukaryotic cytochromes c . *J. Mol. Biol.* 214, 527-555.
- Louie, G.V., Hutcheon, W.L.B., & Brayer, G.D. (1988a). Yeast iso-1-cytochrome c : A 2.8 Å resolution three-dimensional structure determination. *J. Mol. Biol.* 199, 295-314.
- Louie, G.V., Pielak, G.J., Smith, M., & Brayer, G.D. (1988b). The role of Phe-82 in yeast iso-1-cytochrome c and remote conformational changes induced by a serine residue at this position. *Biochemistry* 27, 7870-7876.
- Luzzati, V. (1952). Traitement statistique des erreurs dans la détermination des structures cristallines. *Acta Crystallogr.* 5, 802-810.
- Murphy, M.E.P., Nall, B.T., & Brayer, G.D. (1992). Structure determination and analysis of yeast iso-2-cytochrome c and a composite mutant protein. *J. Mol. Biol.* 227, 160-176.
- Nall, B.T. & Landers, T.A. (1981). Guanidine hydrochloride induced unfolding of yeast iso-2 cytochrome c . *Biochemistry* 20, 5403-5411.
- Pielak, G.J., Mauk, A.G., & Smith, M. (1985). Site-directed mutagenesis of cytochrome c shows that an invariant Phe is not essential for function. *Nature (Lond.)* 313, 152-153.
- Rafferty, S.P. (1992). Functional properties of active site variants of yeast cytochrome c . Ph.D. Thesis. University of British Columbia, Vancouver.
- Rafferty, S.P., Pearce, L.L., Barker, P.D., Guillemette, J.G., Kay, C.M., Smith, M., & Mauk, A.G. (1990). Electrochemical, kinetic, and circular dichroic consequences of mutations at position 82 of yeast iso-1-cytochrome c . *Biochemistry* 29, 9365-9369.
- Salemme, F.R. (1977). Structure and function of cytochromes c . *Annu. Rev. Biochem.* 46, 299-329.
- Salemme, F.R., Freer, S.T., Xuong, N.H., Alden, R.A., & Kraut, J. (1973). The structure of oxidized cytochrome c_2 of *Rhodospirillum rubrum*. *J. Biol. Chem.* 248, 3910-3921.
- Schejter, A., Aviram, I., & Goldkorn, T. (1982). The contribution of electrostatic factors to the oxidation-reduction potentials of c -type cytochromes. In *Electron Transport and Oxygen Utilization* (Ho, C., Ed.), pp. 95-99. Elsevier, Amsterdam.
- Schweingruber, M.E., Sherman, F., & Stewart, J.W. (1977). Altered absorption spectra of iso-1 cytochromes c from mutants of yeast. *J. Biol. Chem.* 252, 6577-6580.
- Schweingruber, M.E., Stewart, J.W., & Sherman, F. (1979). Primary site and second site revertants of missense mutants of the evolutionary invariant tryptophan 64 in iso-1-cytochrome c from yeast. *J. Biol. Chem.* 254, 4132-4143.
- Sherman, F., Stewart, J.W., Parker, J.H., Inhaber, E., Shipman, N.A., Putterman, G.J., Gardisky, R.L., & Margoliash, E. (1968). The mutational alteration of the primary structure of yeast iso-1-cytochrome c . *J. Biol. Chem.* 243, 5446-5456.
- Smith, M., Leung, D.W., Gillam, S., Astell, C.R., Montgomery, D.L., & Hall, B.D. (1979). Sequence of the gene for iso-1-cytochrome c in *Saccharomyces cerevisiae*. *Cell* 16, 753-761.
- Stellwagen, E. (1978). Haem exposure as the determinate of oxidation-reduction potential of haem proteins. *Nature (Lond.)* 330, 86-88.
- Taniguchi, V.T., Sailasuta-Scott, N., Anson, F.C., & Gray, H.B. (1980). Thermodynamics of metalloprotein electron transfer reactions. *Pure Appl. Chem.* 52, 2275-2281.
- Wilson, A.J.C. (1942). Determination of absolute scale from relative X-ray intensity data. *Nature (Lond.)* 150, 151-152.