Role of Arginine-38 in Regulation of the Cytochrome *c* Oxidation-Reduction Equilibrium†

Robert L. Cutler,‡ Anne M. Davies,§ Steve Creighton,∥ Arieh Warshel,∥ Geoffrey R. Moore,*,§ Michael Smith,*,‡ and A. Grant Mauk*,‡

Department of Biochemistry, University of British Columbia, Vancouver, British Columbia V6T 1W5, Canada, School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, U.K., and Department of Chemistry, University of Southern California, Los Angeles, California 90089

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ABSTRACT: Arg-38 is an internal residue of mitochondrial cytochrome *c* that is close to heme propionate-7. Previous work comparing the behavior of cytochromes *c* from several species [Moore, G. R., Harris, D. E., Leitch, F. A., & Pettigrew, G. W. (1984) *Biochim. Biophys. Acta 764*, 331-342] has suggested that Arg-38 lowers the pK_a of this propionate group and thereby accounts for the relative pH independence of the cytochrome *c* reduction potential from pH 5 to pH 8. The influence of Arg-38 on the oxidation-reduction equilibrium of yeast iso-1-cytochrome *c* has now been investigated by electrochemical, NMR, and theoretical analysis of six specifically mutated forms of this protein in which Arg has been replaced by Lys, His, Gln, Asn, Leu, or Ala. As the electron-withdrawing character of the residue at position 38 decreases, the reduction potential of the protein also decreases, with the largest decrease (ca. 50 mV) observed for the Ala variant. However, the variation in the reduction potentials of the mutants as a function of pH was similar to that observed for the wild-type protein. The effects of some of these mutations on the pK_a values of His-33 and His-39 have been determined by NMR spectroscopy and found to be minimal. Calculations of the electrostatic free energy for the Leu-38 variant predict a decrease in the reduction potential of this mutant that is remarkably close to that observed experimentally. This work establishes that while Arg-38 contributes to the relatively high reduction potential of cytochrome *c*, this residue does not appear to be the sole functionality responsible for lowering the heme propionate-7 pK_a.

Since the first study of the cytochrome *c* oxidation-reduction equilibrium was reported over 50 years ago (Coolidge, 1932), much has been learned concerning general structural factors that determine the reduction potential of heme proteins (Kassner, 1972; Moore & Williams, 1977; Moore et al., 1986; Churg & Warshel, 1986). Nevertheless, many important questions remain concerning the involvement of several strategically located residues in determining the oxidation-reduction properties of cytochrome *c*. With the application of site-directed mutagenesis to the study of this protein [e.g., Pielak et al. (1985), Liang, et al. (1987), and Holzschu et al. (1987)], many of these mechanistic issues can now be addressed directly.

One residue of cytochrome *c* that has attracted considerable attention in recent years is Arg-38. The basis for this interest has been 3-fold. First, Moore, Pettigrew, and co-workers (Moore et al., 1984) have advanced a model in which Arg-38 is proposed to lower the pK_a of heme propionate 7, with which it could form a hydrogen bond (Takano & Dickerson, 1980; Louie et al., 1988), and thereby greatly reduce the variation of reduction potential as a function of pH over the physiological range (5.5-8.5). Second, Arg-38 has been proposed to be one of the key residues for transmitting the redox-state conformational change away from the iron and toward the periphery of the protein (Moore, 1983). Third, Churg and Warshel (1986) have recently presented an analysis of the effect of placing a negative electrostatic charge on heme propionate-7 on the reduction potential of the heme group which has led them to conclude that the environment of this functional group is, in fact, more polar in nature than previously acknowledged. Systematic variation of the residue at position 38 as permitted by site-directed mutagenesis should permit extension of these lines of investigation.

Consequently, in the present study we have prepared mutants of yeast iso-1-cytochrome *c* in which the arginine residue at position 38 has been replaced by six different residues: Lys, His, Gln, Asn, Leu, and Ala. The resulting mutants have been studied by electronic and NMR spectroscopies and by spectroelectrochemical analysis. In addition, microscopic electrostatics calculations were carried out to investigate the dielectric properties of the heme-arginine interaction. If such methods are sufficiently reliable, they can be used to explore fundamental problems ranging from the control of reduction potentials in proteins to the energetics of photosynthetic electron-transfer processes (Creighton et al., 1988).

Experimental Procedures

Bacteria, Yeast and DNA. Bacteria and yeast strains and the conditions used to grow and transform them were as described previously (Cutler et al., 1987). Mutagenic oligonucleotides and sequencing primers were synthesized by the solid-phase phosphoramidite method (Atkinson & Smith, 1984) on an Applied Biosystems 380A DNA synthesizer. All oligonucleotides were purified by gel electrophoresis before use.

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‡ University of British Columbia.
§ University of East Anglia.
∥ University of Southern California.

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Site-Directed Mutagenesis of Iso-1-cytochrome c. A 2.5-kb *Bam*HI/*Hin*dIII yeast DNA fragment of known sequence, which includes the iso-1-cytochrome *c* (CYC1) gene (Smith et al., 1979; D. W. Leung, K. Mehta, and M. Smith, unpublished results), was inserted into M13mp8 (Messing, 1983). Several strategies of mutagenesis were used to construct the mutants at position 38. To alter the codon from that of arginine to that of leucine, two-primer mutagenesis was employed as described by Zoller and Smith (1984). To construct the glutamine mutant, a gapped-duplex procedure similar to that described by Kramer et al. (1984) was used. Briefly, 2 μg of the 6.1-kb *Ava*I/*Bgl*II fragment of M13mp8 RF DNA was annealed with 0.4 μg of single-stranded DNA containing the CYC1 insert at 100 °C for 5 min in a sealed capillary in 20 μL of annealing buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol), incubated for 5 min at 65 °C, and then allowed to cool to room temperature. One picomole of 5′-phosphorylated mutagenic oligonucleotide was then annealed to the gapped duplex, and extension and ligation reactions were carried out as described by Zoller and Smith (1983). The construction of the other mutants used biological selection against the parental strand according to Kunkel (1985). Recombinant phage were grown in *Escherichia coli* strain RZ1032 to obtain template DNA in which a portion of the thymidine residues were replaced with uracil. A fresh overnight culture of strain RZ1032 was used to inoculate 100 mL of YT medium. Two hours after inoculation (early log phase), the culture was infected with the supernatant from a 1-mL culture of *E. coli* strain JM101 which had been used to propagate the recombinant phase. Growth of infected strain RZ1032 was continued for 6 h, and single-stranded DNA was prepared from isolated phage (Messing, 1983). One picomole of 5′-phosphorylated oligonucleotide was annealed to approximately 0.5 pmol of single-stranded DNA in annealing buffer at 65 °C for 5 min and then allowed to cool to room temperature. The extension and ligation reactions were carried out at 16 °C overnight as described by Zoller and Smith (1983). One-fourth of the mutagenesis mix was used to transform JM101. Plaques were picked at random, and 1-mL cultures of phage were prepared and screened for mutants by dot blots (Zoller & Smith, 1983). Mutations were confirmed by sequencing the complete coding region of the iso-1-cytochrome *c* gene.

To construct double mutants possessing a threonyl residue at position 102, the *Kpn*I/*Hin*dIII fragment from pEMBL8+ containing the CYC1 gene with the mutation at position 102 (Cutler et al., 1987) was used to replace the *Kpn*I/*Hin*dII fragment of each of the mutants at position 38. The reconstructed genes were sequenced completely to ensure that no mutations arose from the digestion and ligation procedures. The mutant genes were then subcloned into the YEp13 yeast shuttle vector and amplified in *E. coli* strain MM294 before being used to transform the *Saccharomyces cerevisiae* strain GM-3C2, a strain of yeast which does not produce any cytochrome *c*.

Purification and Amino Acid Analysis of Mutant Cytochromes. The mutant cytochromes were purified according to the procedure described by Cutler et al. (1987). Amino acid compositions were determined for protein samples (31 μg) prepared by lyophilization followed by hydrolysis in 1 mL of constant-boiling HCl (Pierce Chemical Co.) at 110 °C under vacuum. Cysteine was quantified as cysteic acid after performic acid oxidation. Amino acid analysis was performed with a Durrum Model DF550 amino acid analyzer. All of the residues except for threonine and serine were determined from analysis of a single, 24-h hydrolysate. Correction for threonine and serine degradation was performed by analysis of 24, 36, and 48-h hydrolysates and by extrapolation to zero time to determine the original amounts of these residues.

Electronic Spectroscopy. UV–visible spectra were recorded on either a Cary-219 spectrophotometer or a Shimadzu Model UV-260 spectrophotometer in 150 mM potassium phosphate buffer, pH 7.2 (25 °C). Cytochromes were reduced with 2-mercaptoethanol or oxidized with $[Co(dipicolinate)_2]NH_3$ (Mauk et al., 1979). Both the oxidizing and reducing agents were removed from the protein sample by gel filtration. Protein solutions were passed through a 0.45-μm filter (Millipore) prior to recording of spectra.

Kinetics of Mutant Cytochrome Reactions with Cytochrome c Peroxidase. The rate of oxidation of each of the mutant cytochromes by hydrogen peroxide as catalyzed by yeast cytochrome *c* peroxidase was measured in 150 mM potassium phosphate, pH 6.0 (25 °C), with substrate concentrations of 100 mM for hydrogen peroxide and 20 mM for cytochrome *c*. Reactions were initiated by the addition of hydrogen peroxide and were monitored at 550 nm. Calculations of turnover numbers were performed according to Kang and Erman (1982). Cytochrome *c* peroxidase was isolated as described by Nelson et al. (1978).

Spectroelectrochemistry. Electrochemical measurements were made with an optically transparent thin-layer electrode (OTTLE) that employed a gold minigrid working electrode and a miniature saturated calomel reference electrode (Radiometer) in the configuration that we have reported previously (Reid et al., 1982). Cytochrome *c* concentration used in these measurements was 2×10^{-4} M, and the mediator $[Ru(NH_3)_5pyr^{2+/3+}$ (Cummins & Gray, 1977)] concentration varied between 2 and 10 mM. The potentials reported were converted to the saturated hydrogen electrode (SHE) standard as described by Dutton (1978).

NMR Spectroscopy. Samples of the ferricytochromes were prepared for NMR after three cycles of lyophilization and dissolution in D_2O to effect the exchange of NH protons for deuterons. The final protein concentrations were approximately 1 mM. Samples of ferrocytochrome *c* were obtained by reduction of ferricytochrome samples prepared as described above with stoichiometric amounts of sodium ascorbate. pH values were monitored with a Radiometer Model PHM 82 pH meter equipped with a Russell glass electrode and adjusted by the addition of NaOD and DCl. Quoted pH values are direct meter readings uncorrected for the small isotope effect and are therefore designated by pH*. ^1H NMR spectra were recorded at 25 °C with a JEOL GX-400 NMR spectrometer. Resolution-enhanced spectra were obtained with the standard Gaussian multiplication routine of the JEOL PLEXUS program. Nuclear Overhauser enhancement (NOE) spectra were obtained as described previously (Moore & Williams, 1984). 1,4-Dioxane was used as an internal standard, but all chemical shifts are quoted in parts per million (ppm) downfield from the methyl resonance of 2,2-dimethyl-2-silapentane-5-sulfonate.

Calculations of Electrostatic Free Energy. Computer simulations of the mutation of Arg to Leu, both in the presence and absence of a charge on the heme group, have been performed. The relevant thermodynamic cycle is shown in Figure 1. The quantity of interest is $\Delta G_3 - \Delta G_4$, the energy difference between charging the heme in the native and mutant forms of the protein. In the present calculations, we have evaluated the difference $\Delta G_2 - \Delta G_4$, which is the same as the quantity of interest because the thermodynamic cycle is closed.

Table I: Oligonucleotide Mutagenesis of Arginine-38 of Yeast Iso-1-cytochrome *c* (Thr-102 Variant)

residue	oligonucleotide (5′ to 3′)	method[a]	yield (%)
Arg	GACCAGAGTGTCTGCCAAAGAT		
Ala	CAGAGTGAGCGCCAAAGAT	Kunkel; SP	2
His	CAGAGTGGTGGCCAAAGAT	Kunkel; SP	5
Lys	CAGAGTGCTTGCCAA	Kunkel; SP	3
Leu	CAGAGTGTAGGCCAA	two primer	14
Asn	GACCAGAGTGATTGCCAAAG	Kunkel; SP	15
Gln	GACCAGAGTGTTGGCCAAAG	gapped duplex	27

[a] SP indicates use of single primer. The two-primer and gapped-duplex procedures did not employ any biological selection.

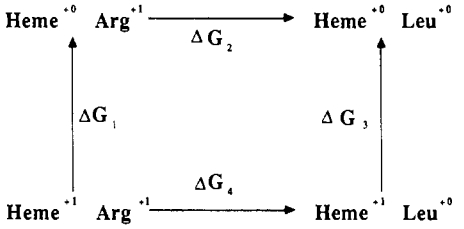

FIGURE 1: Born–Haber cycle depicting the effect of substituting a leucyl residue for the arginyl residue at position-38 of yeast iso-1-cytochrome *c*.

The free energy difference between the native and mutants proteins is divided into two parts: (1) the free energy contribution of the protein and nearby water and (2) the contribution of the remaining water. The free energy differences from each of these contributions have been evaluated by different methods. To evaluate the first contribution, we use a free energy perturbation method [as described in detail in early studies (Warshel & Russell, 1984; Warshel & Sussman, 1986; Warshel et al., 1986; Hwang & Warshel, 1987) [for related work, see Singh et al. (1987)]]. In this approach, we propagate trajectories of the protein and water system using the program MOLARIS with constraint potential of the form

$$V_m = (1 - \lambda_m)V_1 + \lambda_m V_2 \quad (1)$$

where V_1 and V_2 are respectively the force fields of the native and mutant protein in the given redox state. Changing the parameter λ in small increments from 0 to 1 gives the free energy associated with the mutation by

$$\delta G(\lambda_m \rightarrow \lambda_{m'}) = -(1/\beta) \ln [\langle \exp[-(V_{m'} - V_m)\beta] \rangle_m] \quad (2)$$

$$\Delta G(\lambda_0 \rightarrow \lambda_N) = \sum_m \Delta G(\lambda_m \rightarrow \lambda_{m'}) \quad (3)$$

where $\beta = 1/k_B T$ and k_B is the Boltzmann constant. The system simulated by this approach contains the cytochrome *c* protein and 80 nearby water molecules. The cytochrome coordinates were taken from the X-ray study of tuna cytochrome *c* (Takano & Dickerson, 1980) which has a significant homology to the yeast cytochrome (Louie et al., 1988).

The force-field parameters used are identical with those employed in previous studies (Warshel et al., 1986b; Creighton et al., 1988). The residual charges of the reduced and oxidized heme were evaluated by the QCFF/PI method (Warshel, 1977). Using the free energy perturbation method gives the energy contribution associated with the protein and the nearby water molecules. Here, however, we deal with long-range electrostatic interactions that require one to evaluate the effect of the surrounding bulk water molecules. This second contribution can be evaluated by including thousands of water molecules in the simulation, but such an approach is very expensive. Instead, we used the Protein Dipoles Langevin Dipoles (PDLD) method [e.g., Warshel and Russell (1984)] which was found to be effective even in calculating electrostatic free energies associated with bound water molecules (Churg & Warshel, 1986). This approach represents the water molecules as a grid of polarizable Langevin-type dipoles (in this case the grid radius was taken as 25 Å around the protein and inner water molecules). To implement this method with the free energy perturbation method, one should add to the potential V_m of eq 1 the energy of the Langevin dipole, allowing the dipoles to relax at selected configurations generated by the trajectory on V_m and determining the Langevin dipoles contribution to $V_{m'} - V_m$ in eq 2 by fixing these dipoles at equilibrium arrangement for the V_m potential and evaluating their energy at the $V_{m'}$ charge distribution. The present study evaluated the PDLD contribution by considering the change in charge in two increments ($\lambda = 0 \rightarrow \lambda = 0.5$ and $\lambda = 0.5 \rightarrow \lambda = 1$) using the protein trajectories for $\lambda = 0.5$.

The charge states of heme propionates-6 and -7 have not been firmly established. The assumption in this and previous work is that heme propionate-7 has a low pK_a and is therefore ionized at pH 7. However, heme propionate-6 may have a high pK_a and be uncharged at pH 7.0 (Hartshorn & Moore, 1989; Tonge et al., 1989). As previous electrostatic calculations of the reduction potential of cytochrome *c* have taken both heme propionate groups to be dissociated at pH 7 (Churg & Warshel, 1986), we have also adopted this scheme for the present calculations.

To evaluate the relevant changes in free energy ($\Delta G_2 - \Delta G_4$), one has to calculate the free energy associated with two mutation processes. The first (ΔG_4) involves the $Arg^+ \rightarrow Leu$ mutation in the presence of the ionized propionate groups and a +1 charge on the heme, and the second (ΔG_2) involves the $Arg^+ \rightarrow$ Leu mutation in the presence of the ionized propionate groups but with no charge on the heme. The two processes involve different charge distributions ($0 \rightarrow -1$ in the first and $-1 \rightarrow -2$ in the second). Thus, the calculated difference $\Delta G_2 - \Delta G_4$ may involve significant error as the bulk contribution is very different in both cases (the contribution of the bulk is related to the square of the charge of the inner system). The uncertainties in the radius for the bulk around the Langevin grid could lead to an error of a few kilocalories per mole which will exclude any quantitative conclusion. To overcome this problem, we include in the second case a +1 charge on a distant lysyl residue (16 Å away) that is completely accessible to the solvent to keep the overall change in charge the same for both simulations. The thermodynamic cycle was then completed by subtracting the electrostatic interaction of the solvated lysine with the arginine by use of an effective dielectric constant of 60. This macroscopic approximation is quite reliable when at least one of the interacting residues is a solvated surface group; in the present case since the distance between the charges is large, the estimated error for ϵ between 30 and 80 is 0.25 kcal/mol (Warshel & Russell, 1984).

RESULTS

Construction and Expression of Mutant Cytochromes in Yeast. All mutants at position 38 were constructed in a

Table II: Amino Acid Analyses of Mutant Cytochromes c[a]

residue	no. of residues/molecule						
	WT	Ala-38	His-38	Lys-38	Leu-38	Asn-38	Gln-38
Ala	7.0	8.2	7.1	7.0	7.0	7.0	7.0
Arg	3.4	2.3	2.3	2.2	2.3	2.3	2.3
Asx	11.3	11.7	11.3	11.3	11.3	12.1	11.4
Glx	9.1	9.4	9.0	9.0	9.0	9.0	10.1
Gly	12.0	11.7	11.8	11.8	11.6	11.7	11.8
His	3.9	3.6	4.9	4.0	3.9	4.0	4.0
Ile	3.6	3.7	3.5	3.6	3.6	3.5	3.6
Leu	8.1	8.3	8.2	8.3	9.2	8.1	8.2
Lys	14.4	14.2	14.4	15.2	14.4	15.1	14.9
Met	1.6	1.6	1.6	1.6	1.7	1.7	1.7
Phe	4.0	4.0	3.9	4.0	3.9	3.9	3.9
Pro	4.3	4.7	4.6	4.5	4.5	4.4	4.3
Ser	3.1	2.7	2.8	2.9	3.0	3.1	3.0
Thr	8.0	7.9	7.6	7.7	7.8	7.8	7.9
Tyr	4.7	4.9	4.6	4.7	4.6	4.6	4.7
Val	2.8	2.9	2.8	2.8	2.8	2.8	2.8
tLys	0.9	0.9	0.9	0.9	0.9	0.9	0.9

[a] Each form of the cytochrome, including the one referred to as wild type (WT), possessed a threonyl residue at position 102. tLys refers to N^6,N^6,N^6-trimethyllysine.

Table III: Steady-State Activities for Oxidation of Mutant Cytochromes c by H_2O_2 and Cytochome c Peroxidase[a]

cytochrome	V_0/E (s^{-1})	relative V_0/E
Arg-38/Thr-102	1122	1.00
Lys-38/Thr-102	1166	1.04
His-38/Thr-102	1089	0.97
Gln-38/Thr-102	1089	0.97
Asn-38/Thr-102	1058	0.95
Leu-38/Thr-102	1105	0.98
Ala-38/Thr-102	1116	0.99

[a] 20 μM ferrocytochrome c, 0.15 M potassium phosphate, pH 6.0, and 0.1 M H_2O_2, 25 °C.

Table IV: Reduction Potentials of Iso-1-cytochrome c Position 38 Variants[a]

cytochrome	$E°'$ (mV vs. SHE)	$\Delta E°'$ (mV)
Arg-38/Thr-102	272	0
Lys-38/Thr-102	249	−23
His-38/Thr-102	245	−27
Gln-38/Thr-102	242	−30
Asn-38/Thr-102	238	−34
Leu-38/Thr-102	231	−41
Ala-38/Thr-102	225	−47

[a] 0.1 M NaCl and 0.1 M sodium phosphate (pH 7.0), 25 °C. The uncertainty in the reduction potentials is ±2 mV.

background where Cys-102 had been replaced by Thr. Table I summarizes the results of the mutagenesis experiments. The low yields of mutants obtained with the procedure of Kunkel (1985) are unusual; however, these mutants were not obtainable at all with any other mutagenic procedure for unknown reasons. Conversely, the gapped-duplex procedure did not yield mutants for the Leu, Ala, or Asn substitutions. All of the mutant cytochromes were able to support the growth of the yeast on the nonfermentable carbon sources glycerol and lactate, which indicates that the mutant cytochromes are at least partially functional in vivo. However, because the YEp13 vector exists at a copy number of 20–30 in yeast cells, overproduction of an inefficient mutant may compensate for its deficiency.

Cytochrome Purification and Amino Acid Analyses. Approximately 1.5–2 kg of yeast was obtained from 40 L of culture, which in turn yielded 100–120 mg of purified cytochrome c. All of the purified mutant cytochromes had A_{550}/A_{280} ratios of 1.3 in the reduced form. The amino acid analyses of the purified mutant cytochromes are shown in Table II. We note that, despite the presence of two mutations in each of these cytochromes, we find evidence for a trimethyllysyl residue in each case.

Electronic Spectroscopy. Within experimental error, the visible spectra (340–750 nm) of both the reduced and oxidized forms of the position 38 mutants prepared in the present study are identical with those obtained for the wild-type protein. This identity includes the sulfur to iron charge transfer band at 695 nm, which is generally regarded as signifying integrity of methionine ligation to the heme iron (Schechter & Saludjian, 1976).

Cytochrome c Peroxidase Kinetics. The relative abilities of the Arg-38 variants to serve as substrates in the cytochrome c peroxidase reaction are tabulated in Table III. These results demonstrate that the effects of substitutions at this position in the protein have little or no consequence on the reaction with the peroxidase as revealed by a steady-state kinetic analysis. This result is not altogether surprising inasmuch as the site of modification is remote from the proposed cytochrome c peroxidase interaction domain on the surface of the cytochrome (Poulos & Kraut, 1980).

Electrochemical Studies. Each of the mutant cytochromes was electrochemically well behaved between pH 5.5 and pH 8.5 as we have previously reported for the Thr-102 protein at pH 7 (Cutler et al., 1987). The midpoint reduction potentials at pH 7 ($E_{m,7}$) for each of these proteins are shown in Table IV. From these results, it is clear that as less basic (less electron withdrawing) residues are substituted at position 38 there is a systematic decrease in the observed potential. The pH dependencies of the reduction potentials of the mutant cytochromes are illustrated in Figure 2. For those mutants that have not been studied by NMR spectroscopy (the Lys-38, His-38, and Leu-38 variants), these data have been fitted to the relationship derived by Moore et al. (1983) (eq 4) to

$$E_m = \tilde{E} + \frac{RT}{nF} \ln \frac{[H^+]^2 + K_r[H^+]}{[H^+]^2 + K_{01}[H^+] + K_{01}K_{02}} \quad (4)$$

generate the solid lines shown in Figure 2. For the other forms of the cytochrome, all of which have been studied by NMR spectroscopy, the pK values derived from these experiments have been substituted into this relationship to generate the solid lines illustrated in the figure. The pK values arising either

Table V: Heme Resonance Assignments of Ferricytochromes c[a]

proton	horse	tuna	yeast position 38 mutants				
			Arg	Lys	His	Gln	Ala
heme CH_3-8	34.8	35.15	34.85	35.65	35.55	35.75	35.75
heme CH_3-3	32.0	32.4	31.75	31.50	32.0	31.6	31.6
heme CH_3-5	10.1	10.2	10.65	10.95	10.7	10.9	10.9
HP-7β	19.0	19.7	16.1	17.7	16.8	17.1	17.1
HP-7β	11.5	12.3	12.95	12.5	13.2	12.95	12.95
Met-80 CH_3	−24.8	−23.8	−23.10	−23.2	−23.5	−23.5	−23.5
Trp-59 C-4/C-7	7.58	7.53	7.52	7.6	7.55	nd	7.52

[a] pH* 7.0, 25 °C.

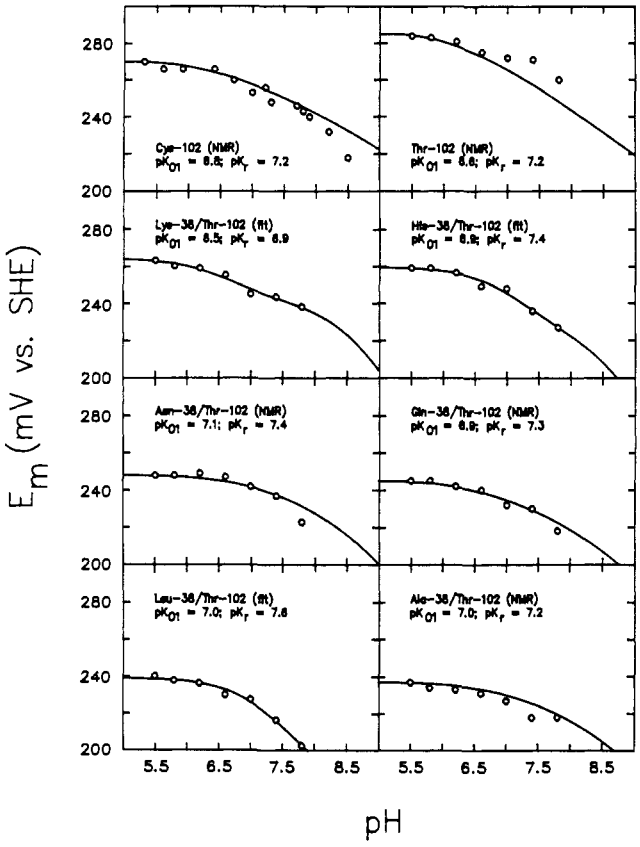

FIGURE 2: Variation of yeast iso-1-cytochrome *c* variant reduction potentials (vs. SHE) with pH [25 °C, μ = 0.1 M (phosphate)]. For those forms of the cytochrome for which NMR spectra were studied as a function of pH, the plots are marked (NMR), and the solid lines were generated as described in the text. For those forms of the cytochrome that were not studied by NMR spectroscopy, the plots are marked (FIT), and the solid lines were generated as described in the text. The values of pK_{01} and pK_r values derived from both methods are indicated in the appropriate panel for each form of the protein. The NMR-determined p*K* values are accurate to ±0.1 p*K* unit, the fitted pK_{01} values have uncertainties of ±0.1 to ±0.3 p*K* unit, and the fitted pK_r values have uncertainties of ±0.2 to ±0.3 p*K* unit. Fitted p*K* values calculated for those proteins studied by NMR are within error of the NMR-determined p*K* values. The curves representing the fitted and experimental p*K* values were calculated with the assumption that pK_{02} is 8.5 except for the Leu-38 (8.1) and His-38 (8.3) variants, which exhibit lower values for this p*K* (as indicated) (A. L. Gärtner and A. G. Mauk, unpublished results).

from the NMR experiments or from the fits to the electrochemical data are indicated for each form of the cytochrome in Figure 2.

NMR Spectroscopy. The NMR spectrum of Thr-102 ferricytochrome *c* is highly similar to those of other mitochondrial cytochromes *c*. Chemical shifts of heme and selected amino acid resonances are given in Table V. Assignments of the ring methyls are taken from Keller and Wüthrich (1978) and Senn et al. (1983), and the assignments of the heme propionate-7 resonances are derived from NOE difference spectra following the procedure of Moore and Williams (1984). The assignment of the resonance of Trp-59 was obtained from a 2D COSY experiment (Davies et al., 1988; Pielak et al., 1988).

Figure 3 shows the NOE difference spectra which relate heme methyl-8, heme propionate-7, and Trp-59. All the mutants had similar NOE difference spectra to those of Figure 3. This similarity indicates that heme methyl-8, heme propionate-7, and Trp-59 have a similar spatial arrangement in all of the proteins. None of the heme resonances monitored were significantly shifted by varying pH* in a similar way to

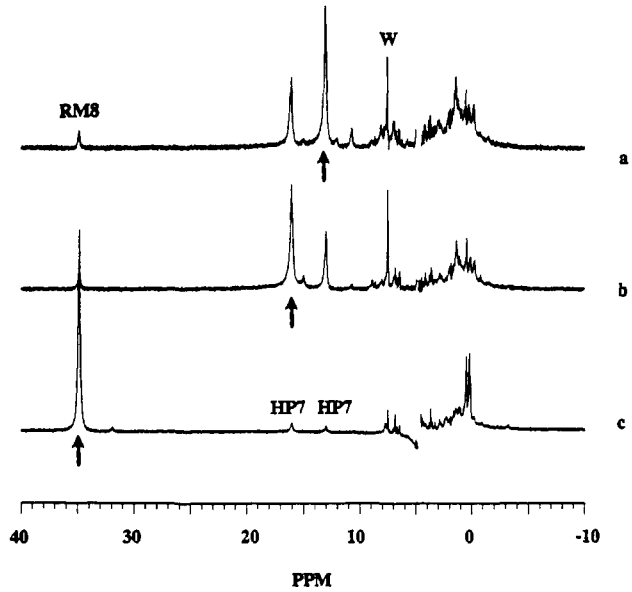

FIGURE 3: NMR spectra of Thr-102 ferricytochrome *c*. (a–c) NOE difference spectra at pH* 6.1 obtained after irradiation of the protein (indicated by ↓) with a 0.5-s presaturation pulse. The difference spectra are the result of 1200 scans with the second irradiation on-resonance and the same number of scans with it off-resonance. RM8 indicates a heme methyl resonance, HP-7 indicates the β heme propionate resonance of heme propionate-7, and W indicates a resonance of Trp-59.

Table VI: p*K* Values for Yeast Cytochromes *c*[a]

protein	His-33		His-39	
	Fe^{3+}	Fe^{2+}	Fe^{3+}	Fe^{2+}
Cys-102/Arg-38	6.7	6.7	6.8	7.2
Thr-102/Arg-38	6.8	6.8	6.6	7.2
Thr-102/Ala-38	6.8	6.8	7.0	7.2
Thr-102/Gln-38	7.0	7.0	6.9	7.3
Thr-102/Asn-38	7.0	7.0	7.1	7.4

[a] 25 °C. The p*K* values stated are accurate to ±0.1 p*K* unit except for those of the Cys-102/Arg-38 protein which were previously reported to be accurate to ±0.2 p*K* unit (Robinson et al., 1983).

resonances of bacterial cytochromes whose heme propionate substituents titrated (Moore et al., 1984; Leitch et al., 1984). Therefore, the pK_a values of the heme propionates are outside the range 4.5–9.0.

The spectra of the ferrocytochromes were very similar to each other and to previously reported spectra of other mitochondrial ferrocytochromes (Robinson et al., 1983; Moore et al., 1985). Selected regions of the resolution-enhanced spectra are shown in Figure 4. The resonance assignments of the Thr-102 ferrocytochrome *c* were obtained by comparison of spectra with those of horse and tuna ferrocytochromes *c* (Moore et al., 1985) and from 2D NMR studies (Pielak et al., 1988). A striking feature of Figure 4 is that a number of resonances have been shifted as a result of the modification at position 38. Until the detailed assignments have been obtained for the mutant proteins, the extent of the chemical shift changes will not become apparent. However, some features are obvious. The Leu-32 resonance of the Thr-102 protein at −0.85 ppm shifts on substitution at position 38 by up to 0.07 ppm. The region 0.2–0.7 ppm of the spectra differs considerably among Arg-38 mutants, but it is not possible to assign resonances in this region by inspection only. However, the resonance of Leu-58 at 0.3 ppm is clearly shifted in some of the mutants. Thus, Figure 4 indicates strongly that the environments of Leu-32 and Leu-58 are perturbed by the substitutions at position 38.

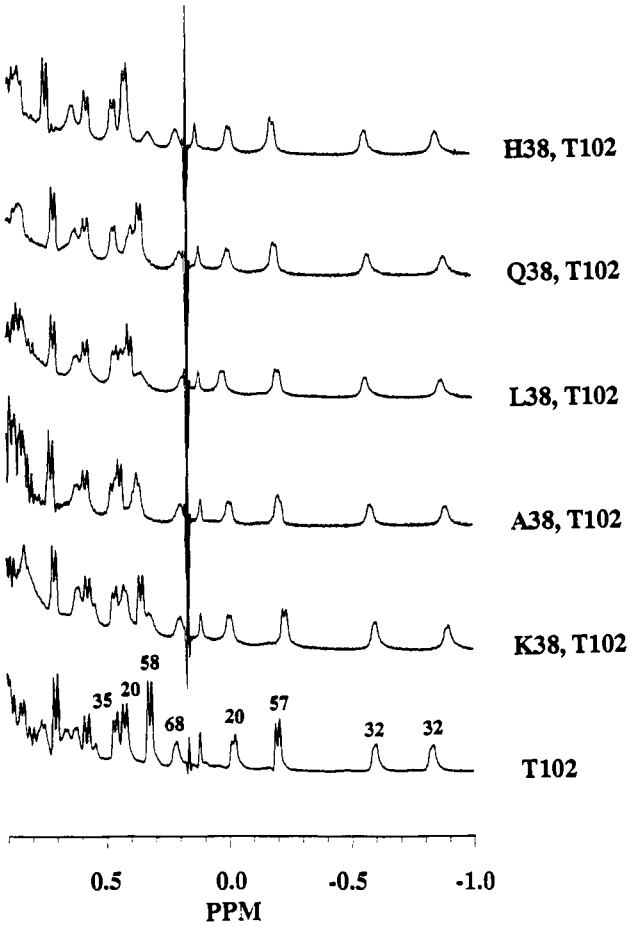

FIGURE 4: Regions of the resolution-enhanced NMR spectra of wild-type and mutant yeast ferrocytochromes *c* at pH* 7 and 25 °C. The singlet resonance at 0.14 ppm is an impurity present in the ^2H_2O. Resonances indicated by 20, 32, 35, 57, 58, and 68 come from Val-20, Leu-32, Ile-35, Val-57, Leu-58 and Leu-68.

Over the pH* range 4.3–8.5, singlet resonances arising from the C-2 protons of His-33 and His-39 shifted with pH*. Typical spectra are shown in Figure 5. Plots of chemical shifts as a function of pH* (Figure 6) allowed the pK_a values to be determined (Table VI). Identification of the C-4 resonances by pH* difference spectra was obtained for the Ala-38 protein. The pK_{01} values were found to be 6.86 ± 0.04 and 6.84 ± 0.08.

Definitive assignments of the His-33 and His-39 resonances, and hence pK_a values, were not made. However, by comparison with wild-type *Saccharomyces* and *Candida* cytochromes *c* (Robinson et al., 1983), His-39 has a relatively large oxidation state dependent shift in its pK_a as indicated by the proposed assignments given in Table VI. This oxidation state dependent shift arises from the conformational change that accompanies change in protein oxidation state.

A singlet resonance at 8.64 ppm in the spectrum of the His-38 ferricytochrome *c* mutant had no obvious counterpart in the spectra of the other proteins, and thus, it probably comes from the His-38 C-2 proton. This resonance was pH* independent over the range 6.0–7.5, and since its chemical shift indicates the histidine is in its protonated form, His-38 must have a $pK_a > 7.5$. This is consistent with the histidine interacting with heme propionate-7 in a way similar to that of Arg-38.

Microscopic Electrostatic Calculations. The procedure described under Experimental Procedures has been used to calculate $\Delta G_3 - \Delta G_1$ for the Arg-38 and Leu-38 cytochromes. The results of the calculations are summarized in Table VII. The calculated value of $\Delta G_3 - \Delta G_1$ and, therefore, of $\Delta G_2 - \Delta G_4$ is around 1 kcal/mol with an estimated convergence error of about 1 kcal/mol. From the calculated free energy difference, we can estimate a free energy difference of 43 mV (4.14 kJ/mol), which is in excellent agreement with observed

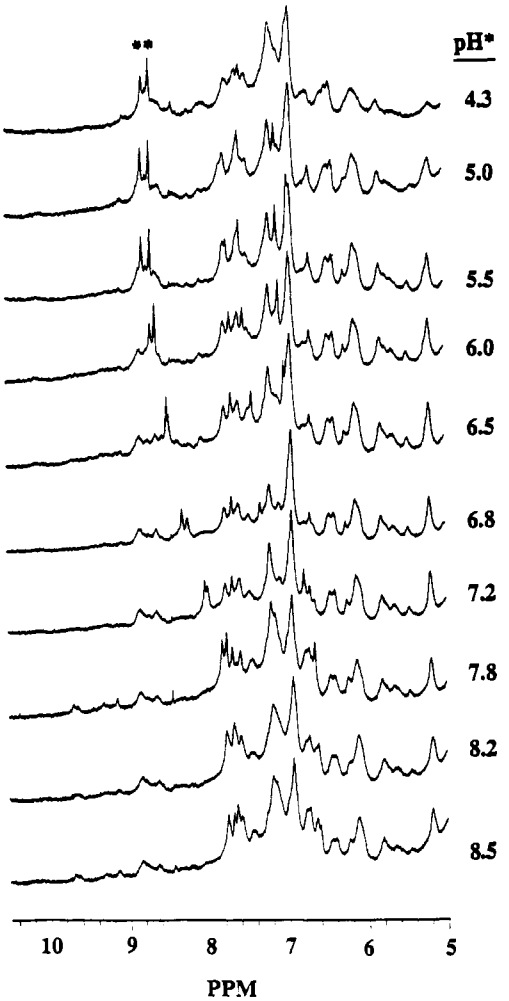

FIGURE 5: pH* dependence of the aromatic region of the NMR spectrum of yeast Ala-38/Thr-102 ferrocytochrome *c*. The C-2 resonances of His-33 and His-39 are indicated by (*) in the pH* 4.3 spectrum.

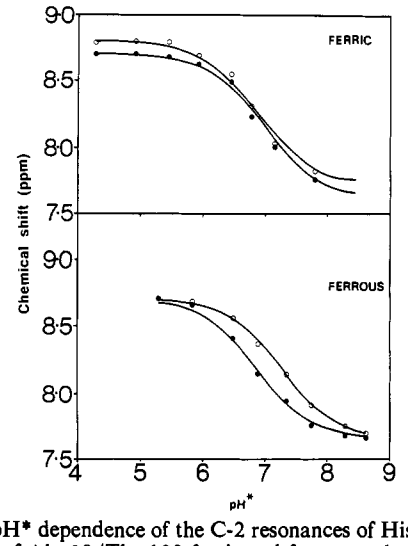

FIGURE 6: pH* dependence of the C-2 resonances of His-33 (●) and His-39 (○) of Ala-38/Thr-102 ferri- and ferrocytochromes *c*. The circles are experimental points, and the solid lines are theoretical curves for one proton ionization with the appropriate pK_a values from Figure 2.

Table VII: Calculation of the Difference between Free Energy of the Mutation Arg-38 to Leu-38 in the Presence and Absence of a Charge on the Heme[a]

mapping parameter (λ)	0.2	0.4	0.6	0.8	1.0
$\Delta\Delta G(\lambda)$ forward	1.6	+1.8	+2.6	+4.3	+5.9
$\Delta\Delta G(\lambda)$ reverse	+1.9	+1.7	+2.8	+3.5	+3.9
average mapping	+4.9				
average PDLD	−3.5				
Lys–Arg interaction	−0.3[b]				
calcd difference	1.1 kcal/mol or 48 mV				
exptl difference	+0.9 kcal/mol or 41 mV				

[a] The numbers reported are $[\Delta G_2 - \Delta G_4](\lambda)$. All energies are in kcal/mol. [b] 16-Å distance with assumed dielectric of 60.

value of 41 mV. While the uncertainty in the calculated free energy difference is approximately equal in magnitude to the calculated value, the result of the calculation nevertheless represents promising agreement between experiment and theory. This correlation demonstrates the ability of microscopic calculations to reproduce in an a priori manner the energy compensation required to account for the observed heme–arginine interactions.

Discussion

The model developed by Moore, Pettigrew, and colleagues for the differences observed in the dependence of the cytochrome *c* reduction potential on pH was based on data collected for several species of cytochromes *c* and c_2. The intrinsic limitations of this approach are 2-fold. First, comparison of proteins from different species by necessity involves consideration of proteins that have multiple amino acid substitutions, and second, refined three-dimensional structures are available for only a few *c*-type cytochromes. One consequence of these limitations is that subtle structural differences may exist between different species of cytochromes that cannot be anticipated on the basis of the limited structural information that is available.

In the present study, we have attempted to eliminate these difficulties through the use of site-directed mutagenesis to change the identity of a single, critical residue in yeast iso-1-cytochrome *c*, one of the proteins investigated in the original study. The feasibility of this approach is further enhanced by the recent determination of the three-dimensional structure of wild-type iso-1-cytochrome *c* by Louie et al. (1988). Figure 7 presents a schematic view of the heme propionate-7 environment that illustrates the presence of Tyr-48, Trp-59, and Arg-38 in this region.

Electrochemical Studies. The electrochemical results from this work indicate that the principal effect of Arg-38 on the reduction potential of cytochrome *c* is to elevate the potential by about 50 mV relative to the most drastic substitution studied (Ala-38). Interestingly, as the electron-withdrawing character of the residue at position-38 decreases, the reduction potential of the protein also decreases. Within experimental error, the variation of reduction potentials of the mutant cytochromes with pH is identical with that determined for the wild-type protein. Evidently, this residue is not required for the suppression of the propionate-7 pK_a below the physiological pH range 4.5–9.0. As Tyr-48, Asn-52, and Trp-59 are close to this propionate, any one or all or some combination of these residues may be responsible for this effect. For this reason, we have initiated work to substitute a phenylalanyl residue for Tyr-48 to determine the influence of this residue on the electrochemical behavior of the cytochrome. We note that Sherman and co-workers have reported mutants of Trp-59, which also hydrogen bonds to heme propionate-7 [summarized in Hampsey et al. (1985)]. Most of these mutants are thermally unstable, and none of them has yet been subjected to electrochemical analysis.

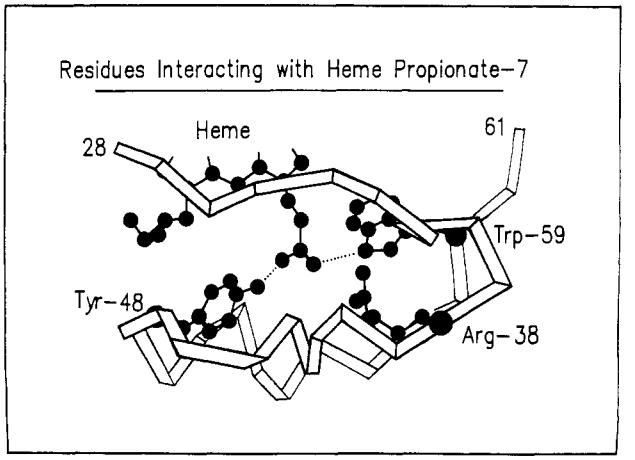

FIGURE 7: Schematic illustration of the environment of Arg-38 in yeast iso-1-cytochrome *c* with hydrogen-bonding interactions indicated.

NMR Studies. The NMR spectra in Figure 3 demonstrate that the three-dimensional structures of the mutant ferricytochromes are only slightly perturbed at most by the substitutions at position 38. It is particularly significant that the conformations of heme propionate-7 and its interaction with Trp-59 are not materially affected since Arg-38 is close to heme propionate-7.

The NMR spectra of the ferrocytochromes show that some structural perturbations do accompany the substitutions (Figure 4). Resonances of residues within 10 Å of the substitution site, namely, Leu-32 and Leu-58, for example, are clearly shifted. Translation of these shifts into changes in atomic coordinates is not possible at present, and thus, the full extent of the conformational perturbations has not been established. However, most of the assigned shifts are relatively small, so changes in atomic positions are unlikely to be greater than 1 Å.

The ionizations of the His-33 and His-39 residues of the mutant proteins mirror closely their behavior in the wild-type protein (Table VI). The His-39 pK_a values are important because their redox state shift is a reflection of the oxidation state conformational change (Robinson et al., 1983). Thus, all the proteins undergo an oxidation state linked conformational change.

The NMR chemical shifts of the heme propionate resonances are given in Table V. These chemical shifts are strongly influenced by the unpaired electron of the Fe^{3+} ions with both scalar and dipolar effects occurring (Wüthrich, 1976). It is notable that whereas the corresponding heme methyl chemical shifts of the horse, tuna, and yeast cytochromes differ by less than 1 ppm, the heme propionate-7 chemical shifts differ by up to 3.6 ppm. Furthermore, the heme propionate-7 chemical shifts for the yeast proteins vary considerably more than do their heme methyl chemical shifts, although there is no obvious pattern to the shift. The dipolar contribution to the shift will remain approximately constant since the propionate conformation is unchanged by mutation. Thus, the scalar contribution to the chemical shift is changing. The heme propionate-7 chemical shifts strongly suggest that the neighboring positive charge in some of the yeast proteins does not materially affect the electron distribution within heme propionate-7.

Electrostatics Calculations. The electrostatics calculations presented here have demonstrated that the free energy perturbation method can qualitatively reproduce the surprisingly

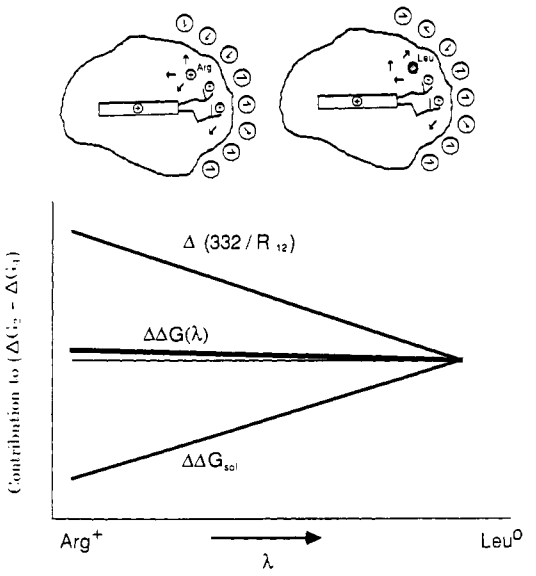

FIGURE 8: Schematic description of the compensation between the charge–charge interaction and the solvation free energy in the apparent electrostatic interaction between the heme and Arg-38. $332/R_{12}$ designates the vacuum charge–charge interaction between residue 38 and the oxidized heme. $\Delta\Delta G_{sol}$ is the change in the solvation free energy of the system upon gradual mutation of residue 38 from Arg to Leu (the change in λ). As illustrated by the figure, the reduction in vacuum electrostatic repulsion upon mutation is compensated by the corresponding change in solvation energy.

small effect of substituting an uncharged residue for a positively charged residue on the reduction potential of cytochrome *c*. Clearly, despite whatever limitations this method may have, it does succeed in reproducing the high dielectric of this region of the protein.

In this context, the term "high dielectric" refers to the effective interaction between two charges separated by a distance R_{12} (Å), which can be written (Warshel & Russell, 1984) as

$$\Delta G = 332Q_1Q_2/[\epsilon(R)R_{12}] = 332Q_1Q_2/R_{12} + \Delta\Delta G_{sol} \quad (5)$$

where ΔG is given in kcal/mol, Q refers to the charges in electron charge units, and $\epsilon(R)$ is the effective dielectric constant. $\Delta\Delta G_{sol}$ is the change in solvation free energy of the system upon change of R from its given value to infinity. When the parameter ϵ is large, as in the present case, then the free energy associated with changing R from its given value to infinity is small. This could occur only if the interaction beween the two positive charges and their "solvation" by the entire protein system is as strong as the solvation of the infinitely separated charges when at least one of them is in water [see Figures 27 and 29 of Warshel and Russell (1984)]. The same is true for the process of switching Q_2 from 0 to 1, which corresponds to the present experiment and is described schematically in Figure 8. The effective mode of solvation of the heme$^+$–Arg$^+$ system involves the protein, bound water molecules, and the bulk water molecules. An important part of this solvation effect is associated with the ionized propionate which provides a part of the high dielectric constant for the heme–Arg interaction. In the same way, the Arg$^+$ provides a part of the dielectric for the heme–propionate electrostatic interaction. Thus, the high effective dielectric constant for the Arg–heme interaction is obtained because the environment is polar.

One puzzling finding of the present study is that the mutation of Arg-38 to Lys gives almost the same effect as substitution of Leu for Arg-38, despite the positive charge on the lysyl residue. In the absence of microscopic simulation, we interpret this finding as follows. The ionic radii of Arg and Lys are substantially different, and the solvation energy of ionized Arg in water is about −45 kcal/mol while that of Lysine is about −80 kcal/mol (Warshel & Russell, 1984). To keep an ionized amino acid side chain inside the protein, the protein must have a greater ΔG_{sol} for the side chain than water does (otherwise the side chain will leave the protein interior to be solvated by the exterior water). This means that the protein site must provide much more electrostatic stabilization to the ionized ε-amino group of the Lysyl residue than to the much larger guanidino group of the arginyl residue (water solvates Lys$^+$ by ca. 35 kcal more than Arg$^+$). If the protein site cannot accomplish this, then the lysyl residue would not stay at the same site as the arginyl residue but would place its ε-amino group in water. In this case, the interaction between the lysyl and the heme charges will be much smaller than that of the arginine and the heme because the protein dipoles would be more polarized by the Lys$^+$ than the Arg$^+$ and thus would be more effective at screening the Lys$^+$–heme$^+$ interaction. This interesting problem requires further study by both structural determination and computer simulations.

Role of Arg-38 in Cytochrome c Oxidation–Reduction Behavior. Arginine-38 is present in all wild-type mitochondrial cytochromes *c* and in all but two of the homologous bacterial cytochromes c_2 (Meyer & Kamen, 1982; Cutler et al., 1987; Dayhoff & Schwartz, 1978). In both cytochromes c_2 lacking Arg-38, a heme propionate ionizes with an oxidation state dependent pK in the pH range 6–7.5, while in the other cytochromes studied the heme propionates do not ionize in the pH range 5–8. Moore et al. (1984) therefore proposed that the role of Arg-38 was to suppress the pK_a of heme propionate-7 to below 5. Clearly, the data presented in the present paper do not support this proposal.

Previous studies involving modification of the Arg-38 environment of horse heart cytochrome *c* fall into two categories. The first group involves the introduction of large blocking groups either by conventional chemical modification (Pande & Myer, 1980) or by a semisynthetic approach (Wallace & Rose, 1983). In the former case, the electrochemical and detailed spectroscopic properties of the modified protein were not reported. In the latter case, the structure of the modified protein deviated substantially from that of the native protein as indicated by the absence of a 695-nm band. The first application of the second type of study involved the preparation of acetimidylated derivatives in which the peptide band on either the C-terminal side or the N-terminal side of Arg-38 was selectively hydrolyzed (Moore et al., 1984; Proudfoot et al., 1986). The derivative with a disruption of the peptide bond on the C-terminal side of Arg-38 was found to have structural (D. E. Harris and G. R. Moore, unpublished results) and functional (Moore et al., 1984; Proudfoot et al., 1986) properties substantially different from those of the native protein while the derivative in which the peptide bond on the N-terminal side of Arg-38 was hydrolyzed exhibited functional properties closer to those of the native protein. However, even with this latter system, interpretation of functional differences is not straightforward. A recent report by Proudfoot and Wallace (1987) illustrates this point. These workers present studies of complexes in which the peptide bond between residues 37 and 38 was hydrolyzed and in which residue 38 was replaced by either Lys or Gln. The hydrolysis of the 37–38 peptide bond was shown to reduce the potential of the protein by 45 mV (to 215 mV), and the substitution of Lys was found to reduce the potential an additional 50 mV (to 166 mV) while

the substitution of Gln reduced the potential by an additional 67 mV (to 148 mV). These observations suggest that disruption of the peptide bond between residues 37 and 38 introduces a greater perturbation into the structure of the resulting complex than previously appreciated (Proudfoot et al., 1986). Furthermore, substitutions at position 38 within such a complex produce greater perturbations of the reduction potential than we have observed for the same substitutions through site-directed mutagenesis. Advantages of the site-directed mutagenesis approach rest in (1) the subtlety of the substitutions that can be introduced relative to chemical modification, (2) the greater range of substitutions employed, (3) the reduced uncertainty regarding the conformational status of the proteins studied, and (4) the use of detailed spectroscopic analysis and crystallographic techniques (Louie et al., 1988; G. V. Louie and G. D. Brayer, unpublished results) to determine the structural consequences of our mutations.

The heme propionate pK_a reflects the nature of the environment of the carboxylic acid, so all neighboring groups could influence its pK_a. Thus, although the presence of Arg-38 may result in the reduction of the pK_a, it may not be solely responsible, and it may not even be largely responsible. Other polar groups that may contribute to lowering the pK_a are Tyr-48, Asn-52, and Trp-59. Mutants of these sites are currently being constructed.

ACKNOWLEDGMENTS

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Registry No. GACCAGAGTGTCTGCCAAAGAT, 118681-46-2; CAGAGTGAGCGCCAAAGAT, 118681-42-8; CAGAGTGGTGGCCAAAGAT, 118681-43-9; CAGAGTGCTTGCCAA, 118681-40-6; CAGAGTGTAGGCCAA, 118681-41-7; GACCAGAGTGATTGCCAAAG, 118681-44-0; GACCAGAGTGTTGGCCAAAG, 118681-45-1; L-Arg, 74-79-3; L-Ala, 56-41-7; L-His, 71-00-1; L-Lys, 56-87-1; L-Leu, 61-90-5; L-Asn, 70-47-3; L-Gln, 56-85-9; H_2O_2, 7722-84-1; cytochrome *c*, 9007-43-6; heme, 14875-96-8; cytochrome *c* peroxidase, 9029-53-2.

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Role of Second Metal Ion in Establishing Active Conformations of Concanavalin A†

Annamma Sadhu*,‡ and James A. Magnuson§
Biochemistry/Biophysics Program, Washington State University, Pullman, Washington 99164-4660
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ABSTRACT: The stoichiometry of Mn^{2+} binding to concanavalin A was found to be influenced by temperature, pH, and the presence or absence of saccharide. Demetalized concanavalin A binds one Mn^{2+} (S1 site) at 5 °C, pH 6.5, and two Mn^{2+} at 25 °C (S1 and S2 sites). The association constants for Mn^{2+} are 6.2×10^5 and 3.7×10^4 M^{-1} for the S1 and S2 sites, respectively, at 25 °C. Concanavalin A with one Mn^{2+} bound per monomer remains in an open conformation and exhibits a relatively high water proton relaxation rate. Concanavalin A with two Mn^{2+} ions remains in a closed conformation characterized by a lower relaxation rate. The rate of binding of the second Mn^{2+} to concanavalin A as determined by ESR and the rate of conversion of open form to closed form (folding over) as determined by proton relaxation rate measurements gave an identical rate constant of 80.0 ± 5.8 M^{-1} h^{-1} at 17 °C. Ca^{2+}, Sr^{2+}, and high levels of methyl α-D-mannopyranoside also induce folding of concanavalin A. Ca^{2+} is not catalytic but stoichiometric in causing the folding. Mn^{2+} in the S1 site can be displaced by Ni^{2+}, Co^{2+}, and Zn^{2+}, and Mn^{2+} in the S2 site can be displaced by Ca^{2+} and Sr^{2+}. Concanavalin A with Ni^{2+}, Co^{2+}, Zn^{2+}, or Mn^{2+} in the S1 site and Ca^{2+} or Sr^{2+} in the S2 site has a higher affinity for methylumbelliferyl α-D-mannopyranoside than Ni–Mn–, Co–Mn–, Zn–Mn–, and Cd–Cd–concanavalin A.

Concanavalin A (Con A),[1] the lectin isolated from jack bean (*Canavalia ensiformis*) (Sumner & Howell, 1936), has been widely used in the affinity purification of glycoproteins, glycopeptides, and polysaccharides and in the separation of viruses and bacteria (Bittinger & Schnebli, 1976). Con A has been used extensively to study cell surface architecture; differences occurring during cell growth and division and following transformation are commonly monitored by Con A binding (Lis & Sharon, 1973; Krach et al., 1974; Ruddon, 1983). The mitogenic response of lymphocytes elicited by Con A is used extensively as a model for antigen stimulation (Powell & Leon, 1970; Yahara & Edelman, 1973; Rosenberg et al., 1982; Fathman & Frelinger, 1983; Sharon, 1983). The interaction of Con A with cell surface components occurs because the lectin binds specifically to sugar moieties with the α-D-arabinopyranoside configuration at the C-3, C-4, and C-6 positions (Goldstein et al., 1973). Because of the usefulness of this lectin, it has been the subject of many structural studies.

Con A exists in two pH-dependent forms, each composed of identical subunits of molecular weight 25 500 (Wang et al., 1971; Edmundson et al., 1971). The form found near physiological pH is predominantly tetrameric (McKenzie et al., 1972; Kalb & Lustig, 1968). Each subunit possesses one specific carbohydrate binding site and two metal ion binding sites; the S1 site is usually occupied by a transition metal ion, and the S2 site is occupied by a Ca^{2+} ion. X-ray crystallographic studies of the protein (Mn–Ca–Con A) show that the S1 and S2 sites are only 4.25 Å apart (Hardman et al., 1982) and that the two metal ions are bridged by the same carboxyl groups of two aspartic acid residues (Hardman & Ainsworth, 1972; Edelman et al., 1972). A variety of divalent metal ions such as Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+} can bind to the S1 site, whereas only Ca^{2+} and Cd^{2+} have been shown to bind to the S2 site (Shoham et al., 1973).

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* Address correspondence to this author.
‡ Present address: Department of Molecular Genetics and Cell Biology, Cummings Life Science Center, 920 E. 58th St., University of Chicago, Chicago, IL 60637.
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[1] Abbreviations: Con A, concanavalin A; ESR, electron spin resonance; Mops, 3-(*N*-morpholino)propanesulfonate; α-MDM, methyl α-D-mannopyranoside; MUM, 4-methylumbelliferyl α-D-mannopyranoside; apo-Con A, demetalized Con A; Mn–, Ni–, Co–, Zn–, Ni–Mn–, Co–Mn–, Zn–Mn–, Mn–Sr–, Mn–Ca–, Mn–Mn–Con A, apo-Con A remetalized with one or both of the respective metal ions. In M–Con A, M is in the S1 site, and in M_1–M_2–Con A, M_1 is in the S1 site and M_2 is in the S2 site.

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