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Ionic Strength Dependence of Cytochrome *c* Structure and Trp-59 H/D Exchange from Ultraviolet Resonance Raman Spectroscopy†

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ABSTRACT: Ultraviolet resonance Raman spectra are reported for cytochrome *c* (cyt *c*) in Fe^{II} and Fe^{III} oxidation states at low (0.005 M) and high (0.9-1.5 M) ionic strength. With 200-nm excitation the amide band intensities are shown to remain constant, establishing that redox state and ionic strength have no influence on the α-helical content. The tyrosine 830/850-cm^{-1} doublet, however, shows a loss in 830-cm^{-1} intensity at $I = 0.005$ M for the Fe^{III} protein, suggesting a weakening or a loss of H-bonding from an internal tyrosine, probably Tyr-48, which is H-bonded to a heme propionate group in cyt *c* crystals. Excitation profiles of tryptophan peak at ~229 nm for both Fe^{II} and Fe^{III} forms of cyt *c*, but at ~218 nm for aqueous tryptophan. The ~2200-cm^{-1} red shift of the resonant electronic transition is attributed to the Trp-59 residue being buried and H-bonded. Consistent with this Trp environment, the H-bond-sensitive 877-cm^{-1} Trp band is strong and sharp, and the 1357/1341-cm^{-1} doublet has a large intensity ratio, ~1.5, for both Fe^{II} and Fe^{III} cyt *c*. The 877-cm^{-1}-band frequency shifts to 860 cm^{-1} when the Trp indole proton is replaced by a deuteron. This band was used to show that Trp H/D exchange in D_2O is much faster for Fe^{III} than Fe^{II} cyt *c*. The half-time for exchange at room temperature is estimated to be ~30 and ~5 h, respectively, for Fe^{II} and Fe^{III} when examined at $I = 0.005$. Increasing the ionic strength to 1.5 M, however, raises the half-time to ~30 h for Fe^{III} cyt *c* and to a much larger value for the Fe^{II} cyt *c*. This variation in the protein dynamics is consistent with recent evidence that the radius of gyration of the Fe^{III} protein increases with decreasing ionic strength (Trewhella et al., 1988).

It is now well recognized that proteins are not rigid arrays of atoms whose positions are frozen at their crystallographically derived coordinates. Proteins may undergo a variety of internal motions of a character that is not always evident from an examination of the crystal structure. A clear illustration is offered by cytochrome *c* (cyt *c*), whose crystallographically determined tertiary structure is essentially the same for the

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reduced and oxidized forms; only small atomic displacements are seen when high-resolution diffraction maps are compared (Takano & Dickerson, 1981). Yet the oxidized form is much more readily denatured in solution (Margoliash & Schejter, 1966), and its amide protons undergo H/D exchange an order of magnitude faster than they do in the reduced form (Ulmer & Kagi, 1968; Kagi & Ulmer, 1968; Patel & Canuel, 1976; Wand et al., 1986). It has also been shown that the compressibility increases by 2% upon oxidation of the protein (Kharakoz & Mkhitaryan, 1986).

Small-angle X-ray-scattering experiments have recently revealed an ionic strength dependence of the Fe^{III} cyt c structure (Trewhella et al., 1988). The radius of gyration was found to decrease by ~8% upon addition of 0.2 M NaCl to a solution of the protein in 5 mM phosphate buffer. Moreover, the rate of Fe^{II} cyt c oxidation by Co^{III} complexes increases at low ionic strength, a process suggested by Rush et al. (1988) to involve a conformation change to a form with an open heme crevice. These interesting results raise the possibility that, since cyt c crystals are obtained from concentrated ammonium sulfate solutions, the close similarity of the X-ray-derived structures for the Fe^{II} and Fe^{III} proteins may stem from the high ionic strength that crystallization requires and that larger differences might be encountered if the comparison could be made at low ionic strength. We have therefore applied ultraviolet resonance Raman (UVRR) spectroscopy to Fe^{II} and Fe^{III} cyt c solutions at low and high ionic strength to probe for structural alterations. UVRR signals from the aromatic residues tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) (Johnson et al., 1984; Rava & Spiro, 1984, 1985a,b; Hildebrandt et al., 1988) have been shown to be sensitive to the environment of the side chains, and the amide signals (Mayne et al., 1985; Dudik et al., 1985) reflect variations in the protein secondary structure (Copeland & Spiro, 1986, 1987). The protein states resulting from pH variations in Fe^{III} cyt c solutions were previously characterized by UVRR spectroscopy (Copeland & Spiro, 1985). In the present study of neutral solutions we find no evidence for alterations in secondary structure, indicating that the α-helical segments seen in the crystal structure are preserved in solution at low as well as high ionic strength. The aromatic residues do, however, give evidence for alterations in the tertiary contacts depending on ionic strength as well as redox state.

The high-resolution crystal structures of cyt c which permit detailed comparisons of the Fe^{II} and Fe^{III} forms are for the protein from tuna (Takano & Dickerson, 1981). Horse heart cyt c has a very similar structure, however (Dickerson et al., 1971). We chose horse heart cyt c for the present study because it has only one Trp and four Tyr residues, while the tuna protein has two Trp and five Tyr residues. The smaller number of these aromatic residues, which are the main focus of the study, makes spectral interpretation more straightforward.

Experimental Procedures

Horse heart cytochrome c was purchased from Sigma and was of the highest grade available (>96%). Absorption spectra show that the material as received contains about 90% oxidized and 10% reduced protein. Pure oxidized or reduced cyt c was prepared by dissolving the protein in 5 mM MOPS buffer (pH 7) and then adding excess $Na_3[Fe(CN)_6]$ or $Na_2S_2O_4$. The excess oxidant or reductant was removed by passage of the solution through a Sephadex G-25 column. Sodium sulfate was added to adjust the ionic strength of the solution.

For the H/D exchange experiments, the protein solutions were lyophilized and then dissolved quickly in 5 mM MOPS/D_2O buffer (pD 7.6) and monitored as a function of time.

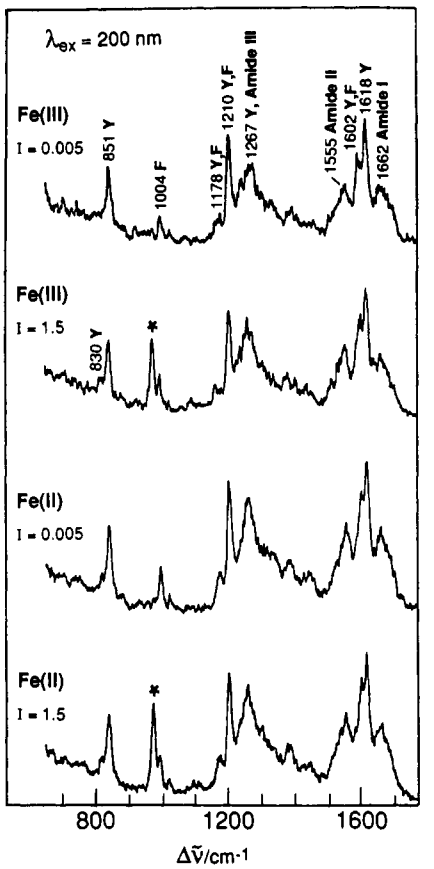

FIGURE 1: Ultraviolet resonance Raman spectra, with 200-nm excitation, for Fe^{II} and Fe^{III} cytochrome c (200 μM) at low (0.005 M) and high (1.5 M) ionic strengths. Each spectrum is the average of 10–15 scans. The bands are labeled at the top by frequency and assignment; letters refer to the amino acid side chains that contribute to the bands. No changes are seen except for the disappearance of the 830-cm^{-1} band in the top spectrum. S marks the ν_1 peak of sulfate, which was present in the high ionic strength solutions.

UVRR spectra were obtained by focusing the output of a H_2 Raman-shifted doubled or quadrupled Nd:YAG laser onto a flowing stream of recirculating sample and collecting the backscattered Raman signal with a Spex 1269 single monochromator equipped with a solar blind photomultiplier and gated integrator electronics (Fodor et al., 1986). Absorption spectra were monitored before and after scanning to make sure that the oxidation state was preserved and that the protein was not denatured. The 3-mL sample, containing 0.2 mM protein, was generally discarded after two scans and replaced with fresh solution.

Results

Evidence for Preserved Secondary Structure but Tyr H-Bond Breaking at Low I for Fe^{III} cyt c. Figure 1 shows 200-nm-excited UVRR spectra for Fe^{II} and Fe^{III} cyt c at I = 0.005 and 1.5 M. At this wavelength one sees resonance-enhanced amide bonds (Mayne et al., 1985; Dudik et al., 1985; Copeland & Spiro, 1986) I, II, and III, arising from C=O and C—N bond stretching and N—H bond bending motions of the polypeptide backbone. Their frequencies and intensities are sensitive to the secondary structure. In particular, the amide II intensity is strongly depressed in α-helical polypeptides because of the hypochromism of the resonant π–π^* electronic transition due to the alignment of the amide bonds (Copeland & Spiro, 1986, 1987). The moderate intensity of the amide II mode in Figure 1 has been shown (Copeland &

Spiro, 1985) to be in quantitative accord with the 35% α-helical content of cyt *c* (Takano & Dickerson, 1981). None of the amide bonds show any signifiant variation with oxidation state or ionic strength. Thus, the α-helical content remains unaltered in all of these samples.

The remaining major bands in the spectra arise from vibrations of the phenylalanine (Phe) and tyrosine (Tyr) residues, of which there are four each. None of these bands show a significant alteration except for the 830-cm^{-1} shoulder. This band is clearly absent for Fe^{III} cyt *c* at low ionic strength, but is present and its intensity is constant for the other three samples. The 830/850-cm^{-1} Tyr doublet results from a Fermi resonance interaction between the tyrosine ring breathing mode, ν_1, and the overtone of an out-of-plane deformation mode, $2\nu_{16a}$ (Siamwaza et al., 1975). The intensity ratio varies with the status of tyrosine H-bonding, H-bond donation correlating with a stronger 830-cm^{-1} component (Rava & Spiro, 1985b; Hildebrandt et al., 1988; Copeland & Spiro, 1985).

Horse heart cyt *c* has two surface tyrosines, in contact with solvent, and two that are buried (Takano & Dickerson, 1981). One of the latter, Tyr-67, is involved in H-bond donation to the sulfur atom of Met-80 and a water molecule, which is in turn H-bonded to Thr-78. These are expected to be relatively weak H-bonds. The other buried tyrosine, Tyr-48, donates an H-bond to a negatively charged heme propionate group, which is expected to produce a strong interaction. We tentatively ascribe the loss of 830-cm^{-1} intensity in the $I = 0.005$ M Fe^{III} cyt *c* solution to a weakening of the Tyr-48 → propionate H-bond. There is no indication that the buried tyrosines are exposed to solvent in the low ionic strength solution. If this were to occur, then the 1210-cm^{-1} Tyr band would be expected to weaken relative to the other UVRR features, as it does when Fe^{III} cyt *c* is exposed to low pH, resulting in a significant unfolding of the protein (Copeland & Spiro, 1985). The low ionic strength effect is more subtle, apparently involving only a weakening of a strong H-bond. [The 850-cm^{-1}-band intensity is also sensitive to the environment (Hildebrandt et al., 1988). An increase in the 830/850-cm^{-1} ratio might indicate a *more* hydrophobic environment, but in the present case the 850-cm^{-1} intensity does not change. Rather the 830-cm^{-1} band weakens, consistent with a weakened H-bond.]

UVRR Signatures of Buried, H-Bonded Trp-59. Horse heart cyt *c* contains a single tryptophan residue, at position 59. It is in a hydrophobic environment, and the indole NH proton is H-bonded to the same heme propionate group that receives an H-bond from Tyr-48 (Takano & Dickerson, 1981). In Figure 2 we plot the intensities of Trp UVRR bands, measured relative to sodium sulfate internal standard, as a function of the excitation wavelength. These excitation profiles are quite different for cyt *c* from those for tryptophan in aqueous solution (Fodor et al., 1989) taken under the same conditions. The latter maximize at ~218 nm (see Figure 2), coinciding with the peak wavelength of the strongest UV absorption band of aqueous tryptophan (Fodor et al., 1989). For cyt *c*, the Trp excitation profiles are considerably red-shifted, maximizing at ~229 nm. We infer a corresponding red shift in the Trp electronic absorption band; its position cannot be determined from the protein absorption spectrum because of interfering contributions from the heme group. In addition to the red shift, the protein intensity data show augmentation of the maximum Trp Raman cross section, from ~10% for the 1555-cm^{-1} band to ~100% for the 760-cm^{-1} band.

These dramatic alterations are attributed to the special Trp environment in cyt *c*. Both the H-bond donation and the hydrophobic environment are expected to lead to red shifts and intensification of the Trp electronic absorption (Demchenko, 1986). Except for a slight difference in the maximum intensity of the 760-cm^{-1} band, the effects are the same for the Fe^{II} and Fe^{III} forms of the protein.

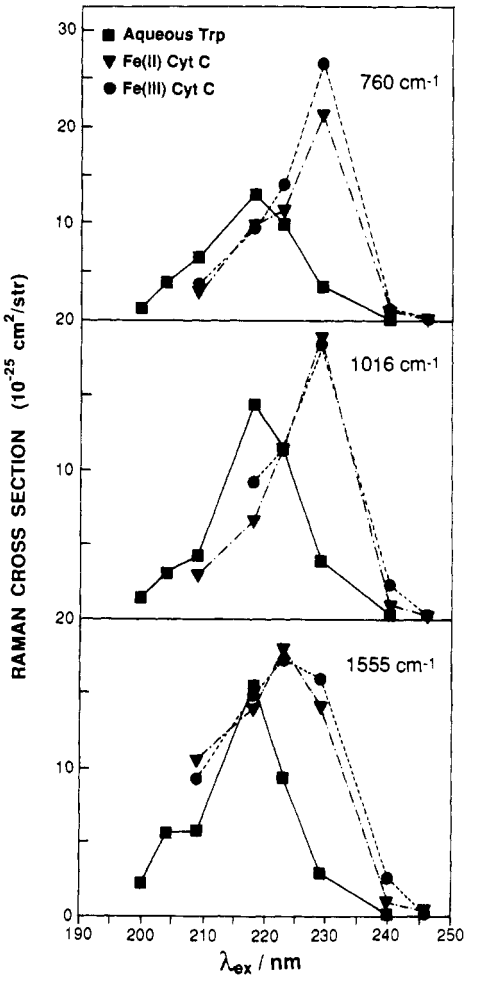

FIGURE 2: Ultraviolet resonance Raman excitation profiles for three vibrational bands of Trp-59 in Fe^{II} and Fe^{III} cytochrome *c*. The corresponding profile for aqueous Trp is shown for comparison. Raman intensities were measured relative to sodium sulfate and the cross sections calculated as in Fodor et al. (1989).

Figure 3 compares UVRR spectra in the two cyt *c* oxidation states at low ionic strength for excitation at 229 nm, the peak wavelength of the Trp excitation profiles. Strongly enhanced Trp bands are seen, in addition to the bands of Tyr, for which this is also a favorable wavelength (Hildebrandt et al., 1988; Fodor et al., 1988). We note particularly the doublet (Rava & Spiro, 1985b) at 1357/1341 cm^{-1} which has been assigned (Takashi et al., 1988) to a Fermi resonance interaction in Trp. The 1341-cm^{-1} component is stronger for aqueous Trp, but the intensities are reversed when Trp is buried (Rava & Spiro, 1985b). Figure 3 shows a ~1.5 intensity ratio consistent with Trp-59 being buried. There is no signficant change in the shape of the band envelope between the Fe^{II} and Fe^{III} proteins. Thus, the Trp residue is not exposed to solvent even at low ionic strength for Fe^{III} cyt *c*. The 877-cm^{-1} band, which is sensitive to the H-bonding status of the Trp indole proton, is strong and sharp, as is expected when there is strong H-bond donation (Takahashi et al., 1988). Again there is no change between the two samples, indicating that the Trp environment is not altered upon cyt *c* oxidation, even when the ionic strength is low.

Trp-59 H/D Exchange Varies with Oxidation State and

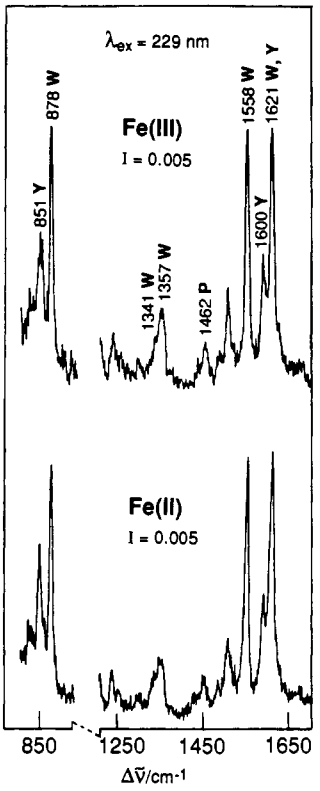

FIGURE 3: Ultraviolet resonance Raman spectra, with 229-nm excitation, for Fe^{II} and Fe^{III} cytochrome *c* at low (0.005 M) ionic strength (average of 10–15 scans). The two panels are not shown on the same intensity scale; no frequency differences are seen.

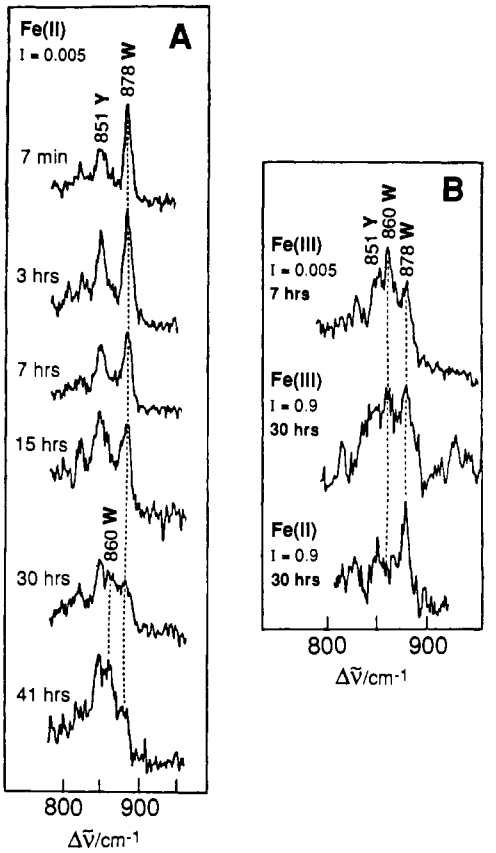

FIGURE 4: Ultraviolet resonance Raman spectra at 229-nm excitation of the 870-cm^{-1} region, showing H/D exchange at 25 °C for the indole proton of Trp-59 for cytochrome *c* in D_2O at low (0.005 M) and high (0.9 M) ionic strengths. The 878-cm^{-1} Trp band shifts down to 860 cm^{-1} when a deuteron is substituted for the NH proton. The panel on the left shows the time course of exchange for the reduced protein at I = 0.005 (half-time about 30 h). The panel on the right shows spectra corresponding to roughly 50% exchange for Fe^{III} at low and high ionic strength. For Fe^{II} at high ionic strength exchange is not observed even at 30 h.

Ionic Strength. The Trp 877-cm^{-1} band shifts down ~20 cm^{-1} when the indole NH proton is replaced by a deuteron, due to significant involvement of N–H bond bending in this mode (Rava & Spiro, 1985a). This shift can be used to monitor the tryptophan H/D exchange in D_2O, as Harada and co-workers have demonstrated (Takashi et al., 1988). In Figure 4 we show expanded views of the 870-cm^{-1} region at various times following dissolution of lyophilized cyt *c* in D_2O buffer. The 877-cm^{-1} band is gradually replaced by the 860-cm^{-1} band. Although the data from these experiments were somewhat noisy, and there is interference from the Tyr 850-cm^{-1} band, it can readily be seen that there are large variations in the rate of H/D exchange depending on oxidation state and ionic strength. The half-time for exchange can be estimated roughly as ~30 and ~4 h for Fe^{II} and Fe^{III} cyt *c*, respectively, at I = 0.005 M. At I = 0.9 M, the Fe^{III} exchange half-time increases, to ~30 h, while the Fe^{II} exchange rate is very low, there being no clear evidence of exchange at 30 h.

The tyrosine bands were also examined in the D_2O solutions in an attempt to monitor H/D exchange of the –OH proton. The ν_{8b} band shifts from 1601 to 1586 cm^{-1} in Tyr–OD and becomes clearly separated from ν_{8a} at 1614 cm^{-1} (Copeland & Spiro, 1985). This effect was clearly seen in the cyt *c* D_2O spectra at the earliest measurement times, 10 min, and there was no subsequent change at later times. Thus, all the Tyr residues appear to undergo exchange with solvent within 10 min.

Discussion

Englander and Kallenbach (1984) argue convincingly for a model of proton exchange in which the observed rate is the product of an intrinsic rate, characteristic of the chemical group undergoing exchange when it is exposed to the solvent, and the rate of the local protein fluctuations that permit this exposure. Thus, the enhanced rate of amide protons in the Fe^{III} form relative to the Fe^{II} form of cyt *c* (Ulmer & Kagi, 1968; Kagi & Ulmer, 1968; Patel & Canuel, 1976; Wand et al., 1986) implies a larger protein fluctuation rate.

The present results show that the indole NH proton of Trp-59 likewise exchanges faster in the Fe^{III} form and also reveal a large ionic strength dependence of the exchange rate in both oxidation states. The rate decreases by a factor of ~7.5 between I = 0.005 and I = 0.9 in the Fe^{III} form, and by an unknown factor in the Fe^{II} form, the high ionic strength rate being slower than the current measurements can detect. Thus, the protein fluctuations associated with the Trp-59 indole H/D exchange are inhibited at high ionic strength in both oxidation states. Presumably this effect is produced by screening of the surface charges. We note that there is evidence for specific anion effects on the rate of cyt *c* oxidation (Osherhoff et al., 1980) and that chemical modification of Lys-13 or Lys-72 abolishes the ionic strength dependence of the rate of oxidation by tris(1,10-phenanthroline)cobalt(III) (Rush et al., 1988). It seems likely that the slowing of the Trp-59 NH exchange rate at high sodium sulfate concentrations is due to screening of the lysine and arginine surface charges by SO_4^{2-}, thereby reducing the lysine–lysine repulsions and suppressing the protein fluctuations leading to exchange.

Is this strictly a dynamic effect, or does the equilibrium structure of the protein also change with ionic strength? The observation that the radius of gyration of Fe^{III} cyt *c* decreases 8% upon addition of 0.2 M NaCl (Trewhella et al., 1988) points to a change in structure, as does the decrease in com-

pressibility upon reduction (Kharakoz & Mkhitaryan, 1986). Yet the UVRR spectra show that the structure change is subtle. The constancy of the amide bands establishes that the α-helical content is unaltered upon change of the oxidation state of the ionic strength. Since the amide II intensity has been shown (Copeland & Spiro, 1987) to be consistent with the α-helical content determined from X-ray crystallography, we infer that the helices seen in the crystal structure are fully preserved in solution and do not unravel at low ionic strength. Moreover, the Trp-59 environment remains unaltered, despite the large variations seen in its indole NH exchange rate. The Trp-59 UVRR bands are the same for Fe^{II} and Fe^{III} cyt *c*, even at low ionic strength, and the excitation profiles, strongly red-shifted by the indole H-bonding and hydrophobic environment, are essentially the same for both forms. Thus, the changes in the protein fluctuations leading to the indole NH exchange are not reflected in a detectable alteration in the equilibrium structure around Trp-59.

The one static UVRR change that is detected is an intensity loss of the 830-cm^{-1} component of the 830/850-cm^{-1} tyrosine Fermi doublet specifically in the Fe^{III} form at low ionic strength. This change is attributable to a loss or weakening of a strong H-bond, tentatively identified as the H-bond between the OH group of Tyr-48 and a negatively charged carboxylate side chain of the heme (Takano & Dickerson, 1981). We note that this is the same carboxylate to which Trp-59 is H-bonded and that the two residues are on either side of an intervening short helix, involving residues 49–55. This helic contains two lysine residues, 53 and 55. Moreover, residue 60, just on the other side of Trp-59, is also a lysine. It may be that Lys-60 repels the Lys-53,55 pair sufficiently to shift the 49–55 helix at low ionic strength, when the charges are poorly screened. This shift would be at the expense of the Tyr-48–propionate H-bond, which ancors the helix. It is easy to see how such a helix displacement could facilitate the protein fluctuations leading to Trp-59 H/D exchange.

It seems likely that the structure change reflected in the increased radius of gyration at low ionic strength likewise involves relative displacements of polypeptide segments resulting from mutual repulsions of like charges, principally the lysine and arginine residues scattered over the protein surface (Takano & Dickerson, 1981). At high ionic strength these repulsions are screened and the protein collapses to a more compact arrangement, the one seen in the crystal structure. The reason that ionic strength effects are expressed more strongly in the Fe^{III} forms, which also has increased compressibility, may simply be that the uncompensated positive charge at the center of the protein augments the electrostatic repulsions of the surface positive charges, increasing the driving force for mutual displacement of the polypeptide segments.

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Registry No. Cyt *c*, 9007-43-6; Fe, 7439-89-6.

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