Spectroscopic Properties of a Mitochondrial Cytochrome *c* with a Single Thioether Bond to the Heme Prosthetic Group†

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ABSTRACT: The yeast *iso*-1-cytochrome *c* variant Cys14Ser has been prepared in which one of the two thioether bonds by which the heme prosthetic group is normally bound to the protein has been eliminated. Comparison of the properties of this variant with those of the wild-type cytochrome provides insight into the role of this covalent attachment of the heme group to the apo-protein toward the functional properties of the wild-type cytochrome. Although NMR and EPR spectra indicate that the Cys14Ser variant ferricytochrome adopts the native conformation characteristic of the wild-type protein with His18 and Met80 coordinated to the heme iron (Met80 ϵ-CH −23.6 ppm; g_z, g_y, g_x, at 3.01, 2.29, ∼1.3, respectively), the electronic spectrum of the variant does not exhibit the 695 nm CT band that is characteristic of native ferricytochromes *c* with these axial ligands. Chromatographic and spectropolarimetric comparison of the variant and wild-type ferricytochromes suggests that the structure of the variant is more disordered, particularly in the region of the sole tryptophanyl residue (Trp59). Upon reduction, the electronic spectrum of the variant exhibits a symmetrically broadened α-band that is shifted ∼3 nm to the ultraviolet relative to its position in the spectrum in the wild-type protein. In the MCD spectrum, a band appears above 390 nm that is more intense than the Soret *A*-term which is also shifted to lower energy.

The process by which protoheme IX is attached to apo-cytochrome *c* has been the subject of recent, intense interest and has been reviewed in detail (*1*−*3*). In eukaryotes, heme attachment occurs posttranslationally, after the translocation of the protein from the cytoplasm into the mitochondrial intermembrane space (*4*, *5*). Once attached, the polypeptide chain folds around the porphyrin macrocycle (*5*), and the holo-protein can begin to shuttle electrons between a variety of proteins as required, for example, for cellular respiration (*6*). With the exception of a few examples in which heme attachment to the protein backbone occurs spontaneously (*7*−*10*), covalent attachment of the heme to apo-cytochrome *c* requires the enzyme cytochrome *c* heme lyase or related catalytic assistance (*2*) to recognize a specific heme binding motif of the apo-protein (generally -Cys-Xxx-Zzz-Cys-His-) (*11*, *12*) and to catalyze heme attachment (Figure 1) (*13*).

Two protozoan cytochromes *c*, cytochrome c_{557} from *Crithidia oncopelti* and cytochrome c_{558} from *Euglena gracilis*, constitute a subgroup of *c*-type cytochromes in which the heme is bound to the polypeptide chain through a single thioether bond (*14*). In these proteins, the vinyl group of pyrrole ring I remains unsaturated, and the amino acid at position 14 is an alanyl residue. Pettigrew, Stellwagen, and their co-workers found that the native conformations of the ferric forms of these cytochromes are not significantly less stable than the equine protein despite their lack of one thioether bond (*14*−*16*). With the increased area over which electron spin density can be distributed in the porphyrin ring, these protozoan cytochromes have a lower midpoint reduction potential (c_{557}: 254 mV; c_{558}: 244 mV versus SHE) than the equine protein (260 mV) (*14*), and their spectroscopic properties are intermediate between those of a conventional *c*-type and those of a *b*-type cytochrome with equivalent axial coordination. In the reduced state, for example, the Soret, α-, and *β*-bands occur at lower energies relative to the equivalent features in horse heart ferrocytochrome *c* (*14*, *15*) but at higher energies than those of ferrocytochrome b_5. More detailed study of these protozoan

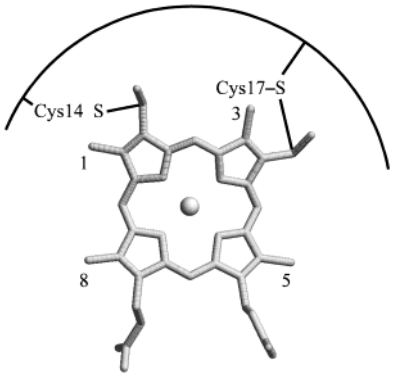

FIGURE 1: Schematic diagram of the covalent attachment of a heme group to yeast *iso*-1-cytochrome *c* through thioether bonds formed between the α-carbon atoms of the ethylene groups on the porphyrin ring and the thiol groups of Cys14 and Cys17 (vertebrate numbering scheme). The structure of the heme is taken from the protein coordinates (2YCC.PDB).

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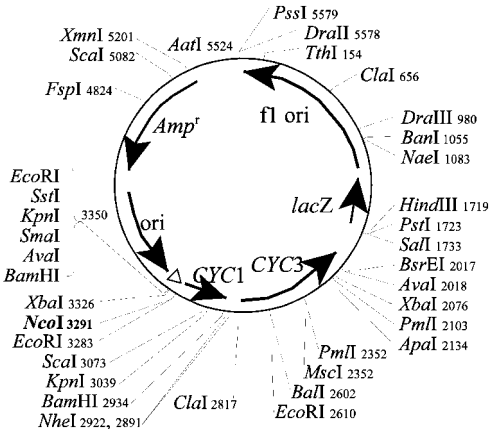

FIGURE 2: Functional map of the 5.6 kbp phagemid pBTR. Selected restriction sites are shown roughly to scale as determined from the published sequence of pEMBL18+ (*60*), pGYM (*22*), and the sequenced *Nco*I-*Hin*dIII fragment that contains the genes *CYC*1 and *CYC*3 (B. Pollock, unpublished results). The genes *CYC*1 and *CYC*3 encode yeast *iso*-1-cytochrome *c* and yeast cytochrome *c* heme lyase, respectively, *Amp*^{r} encodes a β-lactamase, and ori and f1 ori are orgins of replication. The open triangle denotes the *lac*Z promoter.

cytochromes is currently limited, however, by lack of expression systems for producing site-directed variants of these proteins and by unavailability of three-dimensional structures for them.

Previous attempts to express yeast cytochrome *c* variants in which Cys14 or Cys17 is replaced by another amino acid residue have not resulted in a recombinant protein suitable for functional characterization. Dumont and co-workers, for example, found that when yeast apo-cytochrome *c* variants in which Ala or Ser replaced the Cys residues at positions 14 and/or 17 are expressed in yeast they accumulate in the intermembrane space of mitochondria, but heme incorporation does not occur (*17, 18*). Based partly on this result, Cys14 and Cys17 are regarded as essential components of the pentapeptide motif -Cys-Xxx-Zzz-Cys-His- and for the expression of mitochondrial cytochromes *c*. Tanaka et al., on the other hand, have reported electronic spectra of the Cys14Ala variant of human cytochrome *c* expressed in yeast (*19*), but more detailed characterization of this interesting protein is not available.

With our recent development of a bacterial expression system for eukaryotic cytochromes *c* (*20*), it has become possible to express mitochondrial cytochrome *c* variants that do not function adequately to support respiration for growth of the host organism. With this system, we have now been able to express and characterize the spectroscopic properties of the Cys14Ser variant of yeast *iso*-1-cytochrome *c* for the purpose of evaluating the contribution of the thioether bond between Cys14 and the heme 2-vinyl group to the spectroscopic properties of the wild-type protein (*21*).

EXPERIMENTAL PROCEDURES

Construction of pBTR1 and Mutation of CYC1. The 1.6 kbp *Hin*dIII−*Nco*I fragment which contains the genes *CYC*1 and *CYC*3 was excised from pBPCYC1(T-5A)/3 (*20*), purified by agarose gel electrophoresis, and ligated to the 4 kbp *Nco*I−*Hin*dIII fragment of pGYM (*22*). The resulting phagemid (pBTR; Figure 2) was analyzed by restriction mapping. The gene encoding yeast *iso*-1-cytochrome *c* in this vector (*CYC*1) was mutated by the method of Deng and Nickoloff (*23*) to revert the Thr5Ala mutation and to encode for Ala in place of the wild-type Lys72 residue (*20*). For the purposes of this work, this variant is used as the reference cytochrome and is referred to as wild-type (WT).[1] Without replacement of Lys72 with Ala, yeast cytochrome *c* expressed in bacteria undergoes an alkaline isomerization with a pK_{app} of 7.9 (*20*). *CYC*1 was further mutated by the method of Kunkel (*24, 25*) to encode for Ser in place of the cysteine residue at either position 14 or position 17. These mutations were verified by the elimination of the *Acc*I or *Pfl*MI restriction sites, respectively, prior to automated sequencing (Applied Biosystems 180A) of the entire gene and the upstream ribosome binding site. All three cytochromes also include the mutation Cys102Thr to curtail protein autoreduction and dimerization (*26*).

Expression and Purification of Cytochrome c Variants. Freshly transformed *E. coli* BL21DE3::pBTR were incubated 36−48 h in multiple 2 L Erlenmeyer flasks containing 1 L of Terrific Broth (*27*) which was supplemented with ampicillin (100 mg/L). The cells were collected by centrifugation (Sorvall GS3 rotor, 12 min, 8000 rpm) and resuspended in a minimal volume of sodium phosphate buffer (460 mM, pH 7.2). Lysozyme, DNaseI, and RNaseI were added (~100, 5, and 2 mg, respectively, per 100 g of cell pellet), and the mixture was incubated on ice for 1 h with gentle shaking before two cycles of flash-freezing in liquid nitrogen and thawing at 4 °C. The cell lysate was cleared by centrifugation (Sorvall SS34 rotor, 20 min, 13 000 rpm), and the resulting solution was collected. The pellet was resuspended in chilled Buffer A (46 mM sodium phosphate, pH 7.2) and centrifuged repeatedly until the supernatant fluid was no longer pink. The colored fractions were pooled, and $(NH_4)_2SO_4$ (20% w/v) was added over a period of 30 min while stirring gently on ice. The resulting suspension was centrifuged before dialyzing the supernatant fluid overnight in distilled water (4 °C). The dialysate was centrifuged again, and the protein in the cleared solution was batch-extracted with CM-Sepharose CL-6B (Pharmacia Biotech; ~20 mL of resin equilibrated in Buffer A). This resin was transferred to a chromatography column (2.5 cm × 4 cm) and washed with 5 column volumes of buffer A, and the cytochrome was eluted with the same buffer containing 1 M NaCl. The eluate was dialyzed overnight (4 °C) in 20 mM sodium phosphate buffer, pH 7.2, and the precipitated material was removed by centrifugation. DTT was added to the protein solution (~1 mM final concentration) at least 30 min before purifying the protein by cation exchange chromatography with a Pharmacia Mono-S (HR 10/10) column equilibrated with MES buffer (20 mM, pH 6.0). The protein was eluted with a linear NaCl gradient (1 mM/mL) that was initiated at 170 mM NaCl.

The purity of the resulting protein and its chromatographic characteristics in the oxidized and reduced states were assessed by analytical cation exchange chromatography with a Pharmacia Mono-S (HR 5/5) column under conditions similar to those for purification. Cytochrome *c* samples (1−5 μM protein, 100 μL) were reduced (DTT) or oxidized [with $K_3Fe(CN)_6$ or $Co(dipic)_2{\cdot}NH_4$ (*28*)] 5−10 min before ap-

[1] Abbreviations: CT, charge transfer; WT, wild-type; dipic, dipicolinato; DTT, dithiothreitol; MCD, magnetically induced circular dichroism; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

plication to the column. The column was developed with a NaCl gradient, and the resulting elution profiles were monitored at 405 nm and recorded. A chromatography asymmetry factor was calculated according to eq 1:

$$\text{asymmetry factor} = \frac{a}{b} \quad (1)$$

where *a* refers to the volume between the rear of the elution profile (measured at 10% full peak height) and the point of maximum elution and *b* is the volume between the front of the profile and the point of maximum elution.

Spectrophotometry. Electronic absorption spectra (260−750 nm) were recorded with a Cary 300 spectrophotometer at 25.0 °C. Spectra of Cys14Ser ferricytochrome variant (∼15 μM cytochrome in 0.1 M KCl) were obtained as a function of pH between pH 5.5 and 10.5. The pH was varied by addition of 0.1 M NaOH or HCl and measured, after equilibration, with a Radiometer model PHM84 pH meter which was equipped with a Radiometer model 2321 combination electrode. The resulting titration data were analyzed at multiple wavelengths with the program Scientist version 2.01 (Micromath Scientific Software, Orem, UT), and by singular value decomposition analysis (Specfit, Spectrum Associates, Chapel Hill, NC). Ferrocytochromes *c* were treated with hydrazine hydrate and heated at 100 °C in the presence of pyridine for 15 min as described elsewhere (*14*) to assay for unsaturated heme vinyl groups. The resulting solutions were cleared by centrifugation, and the visible electronic spectrum of the product was recorded.

Circular dichroism (CD) spectra were collected at 25.0 °C from 190 to 300 nm and from 280 to 700 nm (0.1 and 1.0 cm path length, respectively) with a Jasco model J720 spectropolarimeter which was equipped with a computer-controlled Neslab RTE-110 water bath. Magnetic circular dichroism (MCD) spectra were collected at 25.0 °C with the same spectropolarimeter fitted with a 1.5 T electromagnet (Alpha Magnetics). This magnet exerted a field $\boldsymbol{B}_0$ at the sample of ∼1 T. The spectra reported here are the average of five scans that have been corrected for the baseline but not smoothed.

Concentrated protein solutions (1−2 mM cytochrome *c*) were oxidized or reduced in a gel filtration chromatography column (0.5 cm × 20 cm, BioRad SP6-DG resin) equilibrated in deoxygenated sodium phosphate buffer (5 mM with 100 mM NaCl, pH 7.4) immediately before recording the electronic, CD, and MCD spectra. All electronic spectra are normalized with respect to the absorbance at 280 nm, and in most cases, samples from the same solution were used to measure the various types of spectra.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were recorded with a Bruker MSL-200 spectrometer operating in the quadrature detection mode at 200.13 MHz. A sample of the Cys14Ser variant (∼0.5 mM, 400 μL) was prepared in deuterated sodium phosphate buffer [5 mM with 100 mM NaCl, pH* 7.4 (value uncorrected for the isotope effect)]. Data were collected with a superWeft pulse sequence (*29*) with a recycle delay of 220 ms and consisted of 10 000 transients. The spectral resolution was enhanced by multiplication of the FID with a cosine squared function prior to Fourier transform, and the baseline was corrected with an interactive polynomial. All chemical shifts are referenced with respect to the residual water resonance which is assigned a chemical shift of 4.63 ppm relative to DSS.

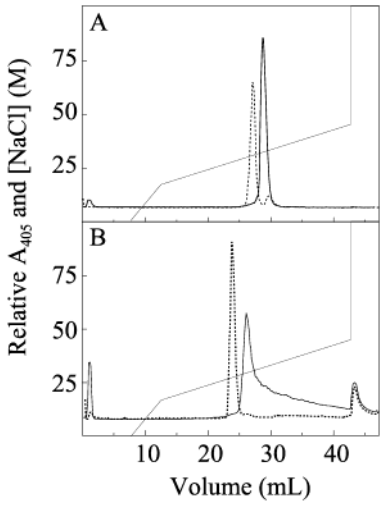

FIGURE 3: FPLC elution profiles (405 nm) of (A) the Lys72Ala variant of cytochrome *c* (referred to in this work as the WT protein) and (B) the Cys14Ser/Lys72Ala variant. The dotted and continuous line profiles depict the elution of the proteins in the reduced and the oxidized state, respectively. The chromatography was performed with a Pharmacia Mono-S HR 5/5 column equilibrated with MES buffer (20 mM, pH 6.0) and developed with a linear NaCl gradient as shown.

EPR Spectroscopy. X-band (∼9.5 GHz) EPR spectra were obtained at 10 K with a Bruker ESP300E spectrometer which was equipped with an Oxford Instruments ESR900 continuous flow helium cryostat, an Oxford Instruments ITC4 temperature controller, and a Hewlett-Packard Model 5352B microwave frequency counter. Data were collected with a microwave power of 0.63 mW and with modulation frequency and amplitude of 100 kHz and 1 mT, respectively. Protein samples (1−2 mM, in 5 mM sodium phosphate buffer, pH 7.4) included glycerol (50% v/v) as a glassing agent and were frozen in liquid nitrogen prior to loading in the cavity.

RESULTS

Protein Expression. The cytochrome *c* expression system described here (*E. coli* BL21DE3::pBTR) typically yields ∼20 mg of purified wild-type cytochrome *c* per liter of Terrific Broth culture. This yield represents an improvement over the expression system we reported previously with *E. coli* HB2151::pBPCYC1/3 [7 mg/L; (*20*)]. We attribute the consistently lower yield of the Cys14Ser variant (∼2 mg/L) either to poor recognition of the apo-protein as a substrate by cytochrome *c* heme lyase or to a decreased efficiency in the reaction(s) leading to heme attachment.

Purification of the Cys14Ser Variant. At pH 6.0, both the reduced and oxidized forms of the WT cytochrome elute from an analytical cation exchange chromatography column as a single, sharp peak (Figure 3A). The ferrous form of the Cys14Ser variant also elutes in this manner, but the elution profile of the oxidized protein exhibits considerable asymmetry (asymmetry factor ∼12.1 vs 1.86 for WT ferricytochrome *c*; Figure 3B). The tailing of the protein occurs independently of the oxidizing agent used [$K_3Fe(CN)_6$ or $KCo(dipic)_2Cl$], suggesting that the elution asymmetry is not a consequence of adventitious binding of the oxidizing agent to the protein surface. The asymmetry is also observed at

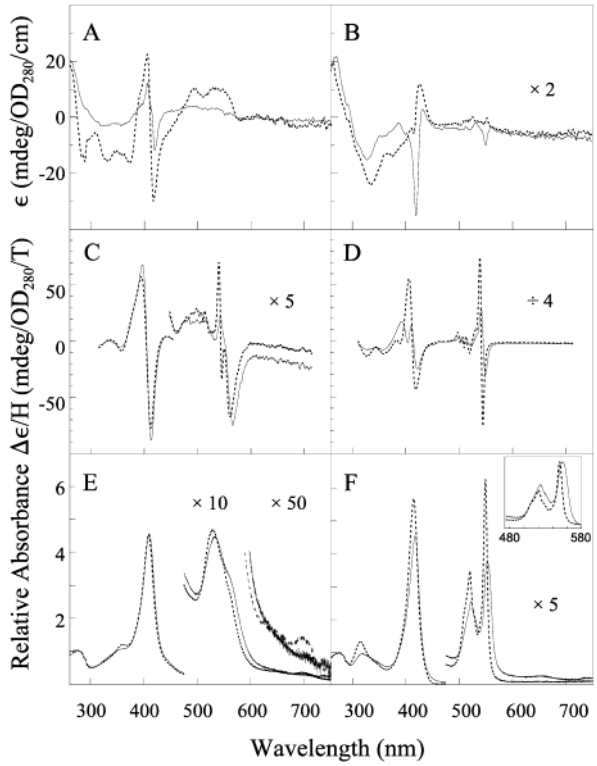

FIGURE 4: Comparison of (A and B) the CD, (C and D) MCD, and (E and F) electronic absorption spectra of WT yeast *iso*-1-cytochrome *c* (···) and the Cys14Ser variant (—) in the oxidized (A, C, and E) and reduced (B, D, and F) states. These spectra were obtained with protein samples (~10 μM) prepared in sodium phosphate buffer [5 mM, pH 7.4, containing sodium chloride (100 mM), 25 °C]. The inset in (F) illustrates the α-band-normalized spectra of the ferrous proteins.

higher pH (e.g., 7.2), and it is reversible. Rechromatography of the pooled fractions eluting between 280 and 400 mM NaCl following reduction with DTT yields an elution profile that is indistinguishable from that of protein that was not oxidized previously. This behavior suggests that Cys14Ser ferricytochrome *c* undergoes structural fluctuations on the time scale of the chromatographic elution.

Heme Attachment to the Protein. Incubation of the Cys14Ser variant at 100 °C in hydrazine hydrate and pyridine resulted in a 3.1 nm shift of the α-band of the ferrous protein to the ultraviolet while identical treatment of the WT cytochrome produced essentially no spectroscopic changes. In contrast, heme groups with two unsaturated vinyl groups have been reported to exhibit shifts of 8−9 nm following reaction with hydrazine and pyridine under these conditions (*14*). The result for the Cys14Ser ferrocytochrome variant indicates that the 2-vinyl group in this protein is free to react with hydrazine in this assay and that, therefore, a bond does not form between Ser14 and the heme 2-vinyl group.

Electronic Absorption Spectroscopy. The spectrum of the Cys14Ser ferricytochrome variant measured at pH 7.4 is compared to that of the WT ferricytochrome in Figure 4E following normalization of the spectra at 280 nm. While the Soret bands of the two proteins are nearly superimposable, the α/β region is less intense, noticeably broader (~15% broader fwhm), and shifted ~4 nm to lower energy in the spectrum of the variant. These spectroscopic differences and the loss of intensity at ~360 nm may result from pH-linked conformational equilibria which perturb the immediate

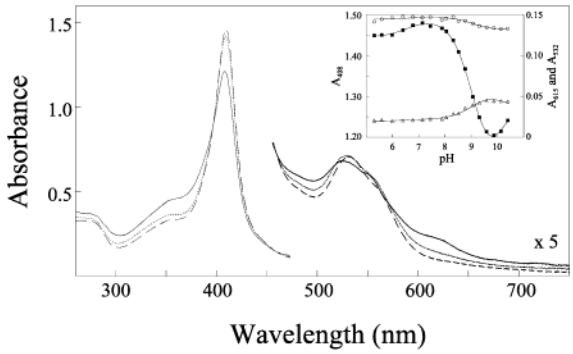

FIGURE 5: Electronic absorption spectra of the Cys14Ser variant of yeast *iso*-1-ferricytochrome *c* measured at pH 5.3 (- - -), 8.5 (···), and 9.6 (—). The inset illustrates the pH-dependence of the absorbance at 408, 532, and 615 nm (■, ○, and △, respectively), and the lines drawn through the points correspond to the simultaneous, least-squares fit of the three wavelengths to a model describing three consecutive deprotonations.

environment of the heme group. The weak absorption band near 695 nm is also absent from the spectrum of the Cys14Ser variant between pH 5.3 and 10.4 (Figure 5). While the band at 695 nm is often regarded as a diagnostic indicator of the integrity of the Met80−Fe(III) bond and the native conformation of ferricytochromes *c*, its absence alone does not provide evidence for disruption of this bond (vide infra) (*6*).

At neutral and mildly acidic pH, the Soret and α/β maxima of the Cys14Ser ferricytochrome variant occur at 409.6 and 532 nm, respectively. The ratios of absorbance between these bands and that at 280 nm are typical for yeast ferricytochrome *c* with values of ~4.5 and ~2.1, respectively. With increasing pH, both the Soret and visible bands decrease in intensity, and a new feature appears at ~620 nm that reflects the formation of a high-spin heme iron species. The alkaline conformational transition of *Euglena* cytochrome c_{558} [pK_{app} ~ 10.3; (*15*)] is also accompanied by the appearance of a high-spin form (620 nm). Moreover, elevated temperature leads to bleaching of the band at 695 nm in the spectra of protozoan cytochromes [T_m ~ 52 and 68 °C for cytochromes c_{557} and c_{558}, respectively; (*14*)] with the concomitant appearance of another band near ~615 nm. The spectroscopic changes observed with increasing pH of the Cys14Ser variant are reversible; the high-spin character of the protein is diminished on acidification, and the initial spectrum is reestablished. The corresponding titration data are best fit to a model that includes three proton binding equilibria with pK_{app} values of 6.61(9), 9.12(3), and 10.0(3) (Figure 5 inset). Singular value decomposition analysis of these data yields pK_{app} values that are in good agreement with these results.

The electronic spectra of WT ferrocytochrome *c* and the Cys14Ser variant measured at pH 7.4 (Figure 4F) reveal more significant differences between the two proteins. In the spectrum of the variant, for example, the α- and β-bands shift to the red ~4 and ~2.5 nm to 553 and 522 nm, respectively. The Soret and δ-bands also shift to the red (3 and ~4 nm, respectively), and the intensities of all four maxima are significantly lower than observed for the corresponding maxima in the spectrum of the WT protein (following normalization at 280 nm). The spectrum of the Cys14Ser ferrocytochrome variant thus resembles the spectra of the protozoan ferrocytochromes c_{557} from *Chrithidia* and

c_{558} from *Euglena* with respect to the red-shifted α-band, but it differs in that this feature in the spectrum of the variant is symmetrical (Figure 4F, inset) and the intensity ratio of the Soret band with respect to the A_{280} is lower [4.6 vs 5.65 and 6.0 for cytochromes c_{557} and c_{558}, respectively (*14*)]. Interestingly, two weak bands appear at ∼660 and 730 nm in the spectrum of the Cys14Ser ferrocytochrome variant that are not observed in the spectrum of the WT cytochrome *c*.

Visible CD and MCD Spectroscopy. The MCD spectrum of the ferricytochrome variant at pH 7.4 is similar to that of the WT protein (Figure 4C). The *A*-terms in the Soret regions of the two spectra are almost superimposable, but the variant appears to be more sensitive to the magnetic field. The α/*β* region also exhibits the broadening and apparent shift of the Soret band to lower energy. Note that the sharp Cotton effects between 500 and 540 nm in the MCD spectrum of the WT ferricytochrome correspond to a small fraction of the sample which is present in the ferrous form. In contrast, the CD spectra of the two proteins differ significantly (Figure 4A). The maxima just above 400 nm are less intense in the spectrum of the variant, and the two negative Cotton effects at ∼320 and 360 nm are absent. These differences may reflect a weaker Met80−Fe^{III} bond in the variant (vide supra), particularly in view of the sensitivity of the Soret CD spectrum of cytochromes *c* to this bond (*30*).

The CD and MCD spectra of the Cys14Ser ferrocytochrome variant in the near-UV, Soret, and visible regions are compared to those of the WT cytochrome in Figure 4B,D. Although replacement of Cys14 with Ser does not appear to induce significant changes in the secondary structure of the ferrous form of the variant (see far-UV CD results below), it does affect the electronic properties of the heme group. In the Soret region, for example, the CD spectrum of the variant (Figure 4B) exhibits positive ellipticity at 432 nm and a sharp negative Cotton effect just below 420 nm that resembles more closely that of a *b*-type cytochrome [e.g., cytochrome b_2 (*31*)] or of the noncovalent apo-cytochrome/heme complex (*32*). In contrast, the spectrum of the WT protein exhibits a typical line shape for mitochondrial cytochromes *c* with positive Cotton effects near 427 and 411 nm, and a minimum at 415.5 nm. The CD spectra of the two ferrocytochromes also differ significantly in the region of the α/*β*-bands. Whereas the spectrum of the WT protein exhibits positive ellipticity at 552 nm, that of the variant exhibits a negative Cotton effect at 550 nm. Myer has shown that the spectra of ferrocytochromes *c* from higher eukaryotes exhibit a high degree of variation in this region (*33*), but the origin of this variation is not understood.

Significant differences are also encountered in the MCD spectra of the two ferrocytochromes (Figure 4D). For the WT protein, the Soret band exhibits a line-shape that is similar to that of the ferricytochrome. In the spectrum of the Cys14Ser variant, on the other hand, the Soret band appears to be the superposition of two bands of similar shape but which are centered a few nanometers apart. Alternatively, the positive Cotton effect corresponding to the *δ*-band has gained intensity at the expense of the Soret feature. The *δ*-band is believed to arise from the heme porphyrin ring (*33*), so it may be sensitive to structural changes of the heme that result from changes in the number of thioether bonds formed by the heme with the apo-protein. Finally, the α/*β* region of the two spectra exhibit very similar line shapes.

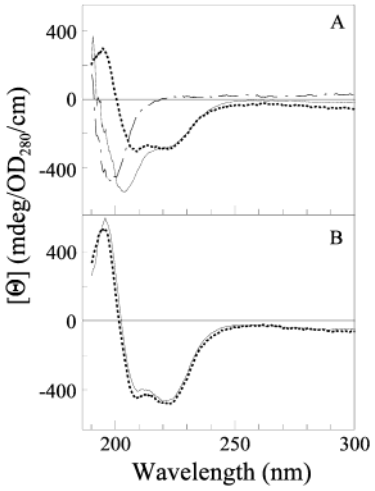

FIGURE 6: Far-UV CD spectra of (A) the Cys14Ser variant of yeast *iso*-1-cytochrome *c* and (B) the WT protein (Lys72Ala variant) in the reduced (···) and oxidized (—) states. The oxidized minus reduced difference spectrum spectra is also shown (panel A, — · —).

In this region, the only differences exhibited by the variant are a ∼4 nm shift to longer wavelength accompanied by loss of intensity and fine structure near 500 nm.

Ultraviolet CD Spectroscopy. The far-UV CD spectrum of the Cys14Ser ferrocytochrome variant (Figure 6A) exhibits negative ellipticity at 222 and 208 nm, indicating that, like the WT protein (Figure 6B), the variant contains a high percentage of α-helical structure. Interestingly, comparison of the spectrum of the WT and variant proteins following normalization at 280 nm demonstrates that the ellipticity in the spectrum of the variant is only ∼75% as great as that of the WT protein. This reduced ellipticity may reflect a mutation-induced perturbation of the protein environment in the immediate vicinity of Trp59 (vide infra) which is manifested by increased absorptivity at 280 nm. As a result, the absolute magnitude of the normalized CD spectrum in the far-UV region appears to be lower than that of the WT protein.

Extensive structural and spectroscopic work concerning cytochromes *c* from a variety of species has established that subtle structural changes accompany changes in oxidation state (*34*−*39*). These changes are reflected in the far-UV CD spectrum by a small loss of ellipticity at 208 and 222 nm (*40*) as confirmed by the spectra of WT cytochrome (Figure 6B). In contrast, the spectrum of the Cys14Ser variant changes more dramatically upon oxidation. Specifically, a sharp feature appears at 203 nm while the ellipticity at 222 nm remains almost unchanged. A similar spectroscopic feature at the shorter wavelength has been encountered, for example, following chemical modification of the protein (*41*, *42*), upon addition of propanol [50% (v/v) (*43*)], and in the noncovalent heme/apo-cytochrome complex (*5*). In the present case, the ferricytochrome *c* minus ferrocytochrome *c* difference spectrum exhibits a negative band at ∼198 nm that resembles the signature of a polypeptide predominantly in a disordered conformation, such as apo-cytochrome *c* (*5*, *12*, *15*, *44*).

NMR and EPR Spectroscopy. The hyperfine-shifted resonances of the axial ligands and substituent groups of the heme are sensitive reporters of structural changes in the active site. Observation of the resonance at ∼ −23.5 ppm in the ^{1}H

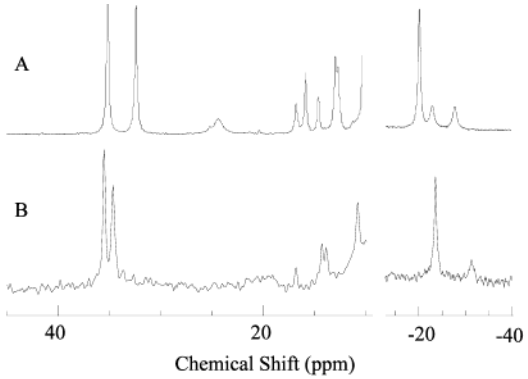

FIGURE 7: Hyperfine-shifted, 200 MHz ^{1}H NMR spectra of (A) WT yeast ferricytochrome *c* [Lys72Ala variant; ~2 mM protein in sodium phosphate−sodium borate buffer (25 mM each, pH* 7.4) (*20*)], and (B) the Cys14Ser ferricytochrome variant [~0.5 mM protein in sodium phosphate buffer (5 mM, pH* 7.4, with 100 mM sodium chloride].

NMR spectrum of the Cys14Ser ferricytochrome variant (Figure 7) provides compelling evidence that this variant adopts a native conformation that is comparable to that of other ferricytochromes *c*. This resonance corresponds to the ϵ-CH_3 protons of Met80, and it is found this far upfield only when this residue is coordinated to the heme iron (*45*, *46*). Nevertheless, the ~3.4 ppm change in chemical shift of this resonance in the spectrum of the variant indicates that the Cys14Ser substitution does perturb the heme environment. In the proton NMR spectrum of cytochrome c_{557} from *Crithidia oncopelti*, the ϵ-CH_3 protons of Met80 also resonate closer to −20 ppm where they overlap the signal from a single proton (*47*).

The broad peak just below 20 ppm corresponds to the His18-ϵ1 proton. The width and shift upfield relative to its position in the spectrum of the WT protein ($\Delta\delta \sim 5$ ppm) is also consistent with a perturbed heme environment. Further downfield, the peaks at 35.4 and 34.5 ppm correspond to heme methyl group protons, but it is not possible at this time to assign these features to specific methyl groups. With a vinyl group at position 2 of the porphyrin, the electronic distribution on the ring may shift sufficiently to deshield the methyl groups at positions 1 and 5 more than those at positions 3 and 8. The peak at 35.4 ppm corresponds to either the 1- or the 8-methyl group protons based on a distinct nOe connectivity pattern observed between this resonance, that of the δ-meso proton (7.51 ppm), and another heme methyl group resonance (13.8 ppm; data not shown).

The EPR spectrum of the Cys14Ser variant (Figure 8) is similar to those of other cytochromes *c* in which Met80 is coordinated to the heme iron. From the principal *g* values ($g_z = 3.01$, $g_y = 2.29$, and $g_z \sim 1.13$), the tetragonality and rhombicity of the metal center (2.120 and 0.649) place this cytochrome within the boundaries of group C in the Blumberg and Peisach "truth" diagrams (*48*). Heme proteins in this region typically possess a low-spin heme center ($S = {}^1/_2$) with His/Met coordination. This observation is consistent with the NMR and Soret CD spectra (vide supra). The rhombic signals at $g = 6.03$ and ~2 suggest that a fraction of the variant is in a high-spin electronic configuration. This component may be associated with a pH-linked conformational transition of the protein that occurs under mildly alkaline conditions (vide supra). Finally, the signal at $g =$

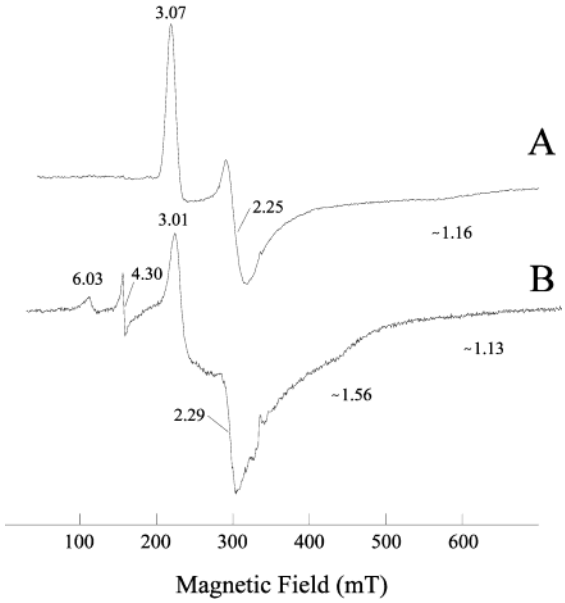

FIGURE 8: X-band EPR spectra of (A) WT yeast ferricytochrome *c* (Lys72Ala variant) and (B) the Cys14Ser variant. Protein samples (1−2 mM) were prepared in sodium phosphate buffer (5 mM, pH 7.4) with 50% (v/v) glycerol.

4.3 presumably corresponds to nonspecifically associated iron that is commonly observed in the spectrum of this and other metalloproteins.

DISCUSSION

As noted above, our bacterial expression system is capable of producing the Cys14Ser variant for physical studies even though the level of expression is significantly lower than that routinely achieved for the WT cytochrome. This reduced efficiency in expression may result from poor recognition of the apo-protein as a substrate for the cytochrome *c* heme lyase, from reduced efficiency of catalysis of heme attachment, or from decreased stability of the variant in vivo. A seryl residue was selected in the design of this variant because this substitution introduces the smallest structural perturbation that could be achieved, i.e., the replacement of an oxygen atom with a sulfur atom. Despite the conservative nature of this mutation, it is evident that the hydroxyl group of the seryl residue is inert in the reaction of heme attachment. Although there was a remote possibility that this substitution could result in formation of an ether bond between Ser14 and the heme 2-vinyl group, the reactivity of the variant with hydrazine rules out this possibility. Interestingly, our efforts to express the Cys17Ser variant and thus selectively eliminate the other thioether bond resulted in even lower expression levels (<0.1 mg/L) than achieved with the Cys14Ser variant. Modifications to fermentation conditions such as media composition, pH, salt concentrations, culture duration, and incubation temperature did not improve the yield of isolated holo-protein. This result is consistent with the present lack of examples of naturally occurring cytochromes possessing only the Cys14−heme 2-vinyl thioether bond.

Recently, Tomlinson and Ferguson reported studies conducted on analogous variants of cytochrome c_{552} from the thermophilic bacterium *H. thermophilus* (*10*) that differ in some respects from our results obtained with the yeast

mitochondrial cytochrome. Perhaps the primary difference between our results is that even without the coexpression of a enzymatic system for covalent attachment of the heme prosthetic group, *E. coli* cytochrome c_{552} variants could be expressed in which either or both of the relevant Cys residues were replaced with alanine. The level of expression of these proteins was comparable to that observed for the WT protein with the exception of the Cys17Ala variant analogue, which was isolated at about one-third the level of the others. Our inability to isolate this variant can be rationalized in terms of the extent to which the two proteins attain a nativelike conformation and the requirement for a lyase. Whereas apocytochrome from the thermophilic bacterium exhibits a relatively well-organized secondary structure (*49*), the mitochondrial apo-cytochrome exhibits little or no secondary structure (*5*). The required interaction of yeast apo-cytochrome with its cognate lyase to produce the holo-protein may also introduce additional complications. Poor recognition of the heme-binding motif (i.e., if Cys17 is involved in docking with the lyase) or inefficient attachment of the heme (i.e., if Cys17 forms the first thioether) are potential obstacles to cytochrome *c* maturation that cannot be overcome when Cys17 is replaced with any other residue.

Structural Implications of the Cys14−Heme 2-Vinyl Thioether Bond. The nonplanarity of the heme prosthetic group that is apparent in the three-dimensional structures of cytochromes *c* [e.g., (*34*)] has been suggested to result from structural constraints imposed on the heme group by the polypeptide. On the basis of theoretical considerations and resonance Raman experiments involving native and metal-substituted cytochromes *c* and model porphyrins, Shelnutt and co-workers attribute deformations of the heme group in cytochromes *c* specifically to the thioether bonds (*50*) and to the five residues from Cys14 to His18 (*51*−*53*). This pentapeptide and the covalently attached heme have been suggested to serve as a foundation around which the remainder of the cytochrome folds (*15*). The availability of a cytochrome variant lacking one of the two thioether bonds that attach the heme group to the apo-protein provides a valuable means of evaluating these hypotheses.

Changes in Structural Dynamics and the Electronic Spectrum Resulting from the Cys14Ser Substitution. The first indication that the structure of the Cys14Ser variant is subject to greater structural flexibility than the WT ferricytochrome was manifested in the unusual asymmetry with which the variant elutes from the cation-exchange resin during purification (Figure 3). The observation of three pH-linked transitions in the electronic spectrum of the ferricytochrome between pH 5 and 10 (Figure 5), however, provides a more detailed and quantitative demonstration of the relative instability of the active site of this variant. Further evidence for increased disorder around the active site is provided by the small, negative Cotton effects at 282 and 289 nm in the CD spectrum of the variant which are associated with the single tryptophanyl residue (*54*). Formation of the molten globule conformation of the protein upon addition of methanol and the associated lability of the polypeptide in the vicinity of Trp59 have also been reported to decrease the optical activity at these wavelengths (*55, 56*).

All ferricytochromes possessing an absorption maximum at 695 nm also possess a methionine ligand to the heme iron. The absence of a band at 695 nm, however, does not establish that a cytochrome lacks a methionine ligand. For example, mitochondrial ferricytochrome *c* lacks a band at 695 nm at 50 °C even though NMR spectroscopy demonstrates that the methionine ligand remains coordinated under these conditions (*57*). Similarly, deprotonation of the imidazole group coordinated trans to the methionine causes a shift of the 695 nm band to higher energy where it may be obscured by the greater intensity of the α/β region of the spectrum as reported for ferricytochrome b_{562} (*58*).

The coordination of Met80 to the heme iron in the native form of the Cys14Ser variant is established by the positive and negative ellipticities at 405.4 and 418.6 nm of the CD spectrum measured near neutral pH (*30, 59*), by the EPR characteristics commonly associated with His/Met, low-spin, ferriheme coordination, and particularly by the paramagnetically shifted resonance of the Met80 methyl group that is characteristic of an Fe^{III}-coordinated methionyl residue. The lack of a 695 nm band even at mildly acidic pH makes it unlikely that deprotonation of His18 is relevant in this case, but in the absence of additional information we cannot rule out the possibility that the electronic perturbation resulting from elimination of a thioether bond to the heme shifts this transition to higher energy where it is more difficult to detect. In view of these observations, it seems reasonable to conclude, therefore, that for yeast cytochrome *c* both the coordination of the Met80 residue to Fe(III) and the presence of the thioether bond between Cys14 and the heme 2-vinyl are required for observation of the weak charge-transfer band near 695 nm. The overall insight that these spectroscopic data provide into the pH-dependence of axial ligation exhibited by the Cys14Ser variant is consistent with the conclusion that the Met80 axial ligand is coordinated to the heme iron at neutral and slightly alkaline pH and that the transition with a $pK_a \sim 9.1$ involves a change in ligation to produce a high-spin, hydroxide-coordinated heme iron species with an absorption maximum at 620 nm. Presumably, the transition corresponding to $pK_a \sim 10$ corresponds to replacement of the putative hydroxide ligand by one or more lysyl residues.

While the effects of the Cys14Ser substitution on the properties of the yeast protein imply a significant structural role for the corresponding thioether bond, it is apparent that sequence differences in the naturally occurring, protozoan cytochromes act to compensate for the stability that is lost in the absence of this link. For example, the ferricytochromes of *Crithidia* and *Euglena* exhibit a charge-transfer band at 698 and 702 nm, respectively, in the electronic absorption spectrum. Alkaline titration of this feature reveals a single, pH-linked transition with apparent pK_a values of 8.6 and 10.3, respectively. Similarly, these proteins are not significantly more susceptible to thermal denaturation than the equine cytochrome (*14*−*16*). Evidently, organisms that have evolved with cytochromes possessing a single thioether bond have also experienced selective pressure to produce other compensatory mutations in the sequence to overcome any loss in stability. In a sense, the cytochrome c_{552} variants generated by Tomlinson and Ferguson have also benefitted from similar evolutionary pressures. Because cytochrome c_{552} is from a thermophilic bacterium, the protein scaffold is already optimized to withstand extreme environmental conditions that proteins from yeast, for example, may not tolerate. Nevertheless, although Tomlinson and Ferguson do not regard as

significant a decrease in the guanidine hydrochloride concentration required to denature their single Cys to Ala variants of up to 40−50%, the decreased stabilities are in general agreement with the conclusions presented here. Namely, without a thioether linkage to the porphyrin ring, the overall structure of the yeast cytochrome is destabilized by the lack of this critical bond.

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REFERENCES

1. Neupert, W. (1997) *Annu. Rev. Biochem. 66*, 863−917.
2. Thöny-Meyer, L. (1997) *Microbiol. Mol. Biol. Rev. 61*, 337−376.
3. Kranz, R., Lill, R., Goldman, B., Bonnard, G., and Merchant, S. (1998) *Mol. Microbiol. 29*, 383−396.
4. Nicholson, D. W., Hergersberg, C., and Neupert, W. (1998) *J. Biol. Chem. 263*, 19034−19042.
5. Dumont, M. E., Corin, A. F., and Campbell, G. A. (1994) *Biochemistry 33*, 7368−7378.
6. Moore, G. R., and Pettigrew, G. W. (1990) *Cytochromes c. Evolutionary, Structural and Physicochemical Aspects*, Springer-Verlag, New York.
7. Barker, P. D., Ferrer, J. C., Mylrajan, M., Loehr, T. M., Feng, R., Konishi, Y., Funk, W. D., MacGillivray, R. T. A., and Mauk, A. G. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6542−6546.
8. Sambongi, Y., and Ferguson, S. J. (1994) *FEBS Lett. 340*, 65−70.
9. Barker, P. D., Nerou, E. P., Freund, S. M., and Fearnley, I. M. (1995) *Biochemistry 34*, 15191−15203.
10. Tomlinson, E. J., and Ferguson, S. J. (2000) *J. Biol. Chem. 275*, 32530−32540.
11. Hampsey, D. M., Das, G., and Sherman, F. (1988) *FEBS Lett. 231*, 275−283.
12. Fisher, W. R., Taniuchi, H., and Anfinsen, C. B. (1973) *J. Biol. Chem. 248*, 3188−3195.
13. Dumont, M. E., Cardillo, T. S., Hayes, M. K., and Sherman, F. (1991) *Mol. Cell. Biol. 11*, 5487−5496.
14. Pettigrew, G. W., Leaver, J. L., Meyer, T. E., and Ryle, A. P. (1975) *Biochem. J. 147*, 291−302.
15. Stellwagen, E., and Cass, R. (1974) *Biochem. Biophys. Res. Commun. 60*, 371−375.
16. Brems, D. N., and Stellwagen, E. (1983) *J. Biol. Chem. 258*, 10919−10923.
17. Dumont, M. D., Mathews, A. J., Nall, B. T., Baim, S. B., Eustice, D. C., and Sherman, F. (1990) *J. Biol. Chem. 265*, 2733−2739.
18. Wang, X., Dumont, M. E., and Sherman, F. (1996) *J. Biol. Chem. 271*, 6594−6604.
19. Tanaka, Y., Kubota, I., Amachi, T., Yoshizumi, H., and Matsubara, H. (1990) *J. Biochem. (Tokyo) 108*, 7−8.
20. Pollock, W. B. R., Rosell, F. I., Twitchett, M. B., Dumont, M. E., and Mauk, A. G. (1998) *Biochemistry 37*, 6124−6131.
21. Rosell, F. I., and Mauk, A. G. (2000) *Biochemistry 39*, 71.
22. Guillemette, J. G., Matsushima-Hibiya, Y., Atkinson, T., and Smith, M. (1991) *Protein Eng. 4*, 585−592.
23. Deng, W. P., and Nickoloff, J. A. (1992) *Anal. Biochem. 20*, 81−88.
24. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
25. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.
26. Cutler, R. L., Pielak, G. J., Mauk, A. G., and Smith, M. (1987) *Protein Eng. 1*, 95−99.
27. Tartof, K. D., and Hobbs, C. A. (1987) *Bethesda Res. Lab. Focus 9*, 12.
28. Mauk, A. G., Coyle, C. L., Brodignon, E., and Gray, H. B. (1979) *J. Am. Chem. Soc. 101*, 5054−5056.
29. Inubishi, T., and Becker, E. D. J. (1983) *J. Magn. Reson. 51*, 128−133.
30. Santucci, R., and Ascoli, F. (1997) *J. Inorg. Biochem. 68*, 211−214.
31. Sturtevant, J. M., and Tsong, T. Y. (1969) *J. Biol. Chem. 244*, 4942−4950.
32. Goldberg, M. E., Schaeffer, F., Guillou, Y., and Djavadi-Ohaniance, L. (1999) *J. Biol. Chem. 274*, 16052−16061.
33. Myer, Y. P. (1970) *Biochim. Biophys. Acta 221*, 94−106.
34. Berghuis, A. M., and Brayer, G. D. (1992) *J. Mol. Biol. 223*, 959−976.
35. Dong, A., Huang, P., and Caughey, W. S. (1992) *Biochemistry 31*, 182−189.
36. Baistrocchi, P., Banci, L., Bertini, I., Turano, P., Bren, K. L., and Gray, H. B. (1996) *Biochemistry 35*, 13788−13796.
37. Schejter, A. (1996) in *Cytochrome C* (Scott, R. A., and Mauk, A. G., Eds.) pp 335−345, University Science Books, Sausalito, CA.
38. Banci, L., Bertini, I., Bren, K. L., Gray, H. B., Sompornpisut, P., and Turano, P. (1997) *Biochemistry 36*, 8992−9001.
39. Barker, P. D., and Ferguson, S. J. (1999) *Struct. Fold. Des. 7*, R281−290.
40. Myer, Y. P. (1985) *Curr. Top. Bioenerg. 14*, 149−188.
41. O'Hern, D. J., Pal, P. K., and Myer, Y. P. (1975) *Biochemistry 14*, 382−391.
42. Pal, P. K., Verma, B., and Myer, Y. P. (1975) *Biochemistry 14*, 4325−4334.
43. Kaminsky, L. S., Yong, F. C., and King, T. E. J. B. C. (1972) *J. Biol. Chem. 247*, 1354−1359.
44. Kang, X., and Carey, J. (1999) *J. Mol. Biol. 285*, 463−468.
45. Wüthrich, K. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 1071−1078.
46. Redfield, A. G., and Gupta, R. K. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 405−411.
47. Keller, R. M., Pettigrew, G. W., and Wuthrich, K. (1973) *FEBS Lett. 36*, 151−156.
48. Blumberg, W. E., and Peisach, J. (1971) in *Probes of Structure and Function of Macromolecules and Membranes* (Chance, B., Yonetani, T., and Mildvan, A. S., Eds.) pp 215−228, Academic Press, New York.
49. Tomlinson, E. J., and Ferguson, S. J. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 5156−5160.
50. Hobbs, J. D., and Shelnutt, J. A. (1995) *J. Protein Chem. 14*, 19−25.
51. Jentzen, W., Ma, J.-G., and Shelnutt, J. A. (1998) *Biophys J. 74*, 753−763.
52. Ma, J.-G., Laberge, M., Song, X. Z., Jentzen, W., Jia, S. L., Zhang, J., Vanderkooi, J. M., and Ja, J. A. S. (1998) *Biochemistry 37*, 5118−5128.
53. Ma, J.-G., Zhang, J., Franco, R., Jia, S. L., Moura, I., Moura, J. J., Kroneck, P. M., and Shelnutt, J. A. (1998) *Biochemistry 37*, 12431−12442.
54. Davies, A. M., Guillemette, J. G., Smith, M., Greenwood, C., Thurgood, A. G. P., Mauk, A. G., and Moore, G. R. (1993) *Biochemistry 32*, 5431−5435.
55. Bychkova, V. E., Dujsekina, A. E., Klenin, S. I., Tiktopulo, E. I., Uversky, V. N., and Ptitsyn, O. B. (1996) *Biochemistry 35*, 6058−6063.
56. Kamatari, Y. O., Konno, T., Kataoka, M., and Akasaka, K. (1996) *J. Mol. Biol. 259*, 512−523.
57. Angstrom, J., Moore, G. R., and Williams, R. J. P. (1982) *Biochim. Biophys. Acta 703*, 87−94.
58. Moore, G. R., Williams, R. J., Peterson, J., Thomson, A. J., and Mathews, F. S. (1985) *Biochim. Biophys. Acta 829*, 83−96.
59. Looze, Y., Polastro, E., Deconinck, M., and Léonis, J. (1978) *Int. J. Pept. Protein Res. 12*, 233−236.
60. Dente, L., Cesareni, G., and Cortese, R. (1983) *Nucleic Acids Res. 11*, 1645−1655.

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