Stabilization of the Globular Structure of Ferricytochrome *c* by Chloride in Acidic Solvents†

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ABSTRACT: Increasing concentrations of chloride were found to increase the resolution between two visible absorbance spectral transitions associated with acidification of ferricytochrome *c*. Analysis of a variety of spectral and viscosity measurements indicates that protonation of a single group having an apparent p*K* of 2.1 ± 0.2 and an intrinsic p*K* of about 5.3 displaces the methionine ligand without significantly perturbing the native globular conformation. Analysis of methylated ferricytochrome *c* suggests that protonation of a carboxylate ion, most likely a heme propionate residue, is responsible for displacement of the methionine ligand. Addition of a proton to a second group having an apparent p*K* of 1.2 ± 0.1 displaces the histidine ligand and unfolds the protein from a globular conformation into a random coil. It is most likely that the second protonation occurs on the imidazole ring of the histidine ligand itself. Chloride is proposed to perturb these transitions by ligation in the fifth coordination position of the heme ion. Such ligation stabilizes a globular conformation of ferricytochrome *c* at pH 0.0 and 25°.

Acidification of solutions of ferricytochrome *c* in the absence of added salt produces a single cooperative transition having an apparent p*K* of 2.5 involving both the gross unfolding of the globular native conformation and the replacement of the two protein axial ligands for the heme iron by solvent (Babul and Stellwagen, 1972). In the presence of NaCl, the single transition is separated into two steps whose resolution is increased with increasing NaCl concentration (Boeri et al., 1953; Fung and Vinogradov, 1968; Greenwood and Wilson, 1971). We have reinvestigated the combined effects of chloride and proton concentrations on the properties of ferricytochrome *c* to identify the ionizable protein groups responsible for the individual steps, to measure the conformational changes associated with each step, and to describe the mechanism of the chloride effect.

Experimental Procedure

Horse heart cytochrome *c*, type VI, was purchased from the Sigma Chemical Co. Porphyrin cytochrome *c* and yeast iso-2-cytochrome *c* were gifts from Drs. A. B. Robinson and E. Margoliash, respectively. All protein concentrations were determined spectrophotometrically.

Spectral titrations were performed at room temperature using either a Gilford or a Cary spectrophotometer. Absorbance values were corrected for dilution resulting from the addition of concentrated HCl or KOH solutions. Kinetic spectral measurements were performed using a Durram stopped-flow spectrophotometer. Viscosity measurements were obtained using Ostwald capillary viscometers having outflow times of 77.68 and 81.99 sec for water at 25.00°. Preliminary measurements indicated that the acid transitions measured by absorbance spectroscopy and by viscometry are reversible at constant chloride concentrations. It was found most convenient to maintain a constant chloride concentration by addition of HCl to the desired chloride concentration followed by titration with 10 *M* KOH to the desired pH values. Solutions containing greater than 1.0 *M* chloride were adjusted to pH 0.0 with concentrated HCl in the presence of sufficient NaCl to achieve the desired total chloride concentration.

Ferricytochrome *c* was methylated at room temperature by dissolving 100 mg of the protein in 10 ml of *dry* methanol containing 40 μl of concentrated HCl. The reaction was terminated by dilution with 10 volumes of water. The methylated protein was then dialyzed against 1 m*M* HCl and concentrated by ultrafiltration. The heme was cleaved from the protein using $AgSO_4$ as described previously (Stellwagen et al., 1972), extracted with ether, and subjected to paper chromatography using chloroform as the mobile solvent.

Results

Equilibrium Absorbance Measurements. Figure 1 illustrates the combined effects of acidity and chloride concentration on the Soret absorbance of horse heart ferricytochrome *c* measured at 395 nm. As the chloride concentration of the protein solvent is increased, the resolution between the two pH dependent spectral changes appears to increase as observed previously (Boeri et al., 1953). We shall refer to the spectral transition shown in Figure 1 having the higher apparent p*K* value as transition I and that having the lower apparent p*K* value as transition II.

As shown in Figure 1, transition I appears to be resolved from transition II in protein solvents containing at least 1 *M* chloride. At the completion of transition I in such solvents, pH 2.2, the Soret maximum of ferricytochrome *c* occurs at 402 nm, characteristic of coordination of the partially buried heme iron with one strong field and one weak field ligand (Nanzyo and Sano, 1968; Wainio et al., 1970). The $\Delta\epsilon_{395}$ of 32 mM^{-1} cm^{-1} observed for transition I is comparable to the $\Delta\epsilon_{395}$ of 38 mM^{-1} cm^{-1} resulting from coordination of a strong field ligand, either imidazole or *N*-acetylmethionine in place of a weak field ligand in cytochrome *c* ferrihemopeptide 14-21 (Babul and Stellwagen, 1972). If transition I is considered to represent a proton-dependent equilibrium between two isomeric forms of the protein dif-

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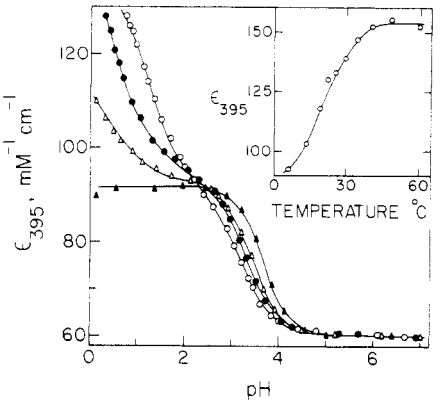

FIGURE 1: The effect of pH on the extinction of horse heart ferricytochrome *c* measured at 395 nm. All measurements shown in the main portion of the figure were made at room temperature in the presence of (○) 0.2 *M*; (●) 0.5 *M*; (△) 1.0 *M*; and (▲) 3.0 *M* chloride. The insert shows the effect of temperature on the extinction of the protein measured at 395 nm in the presence of 0.2 *M* HCl. Protein concentrations ranged from 0.3 to 11 μM.

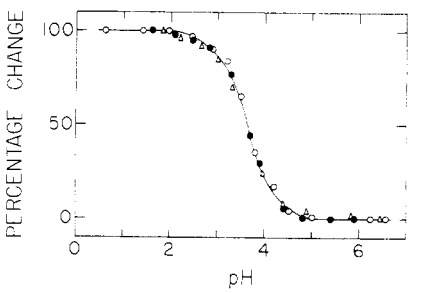

FIGURE 2: The effect of pH on the extinction of horse heart ferricytochrome *c* measured in 3 *M* chloride. Measurements were made at (○) 395 nm; (△) 620 nm; and (●) 695 nm, all at room temperature. Protein concentrations ranged from 0.011 to 0.706 m*M*.

fering in the ligand occupying one axial coordination position, then the spectral changes associated with transition I may be analyzed according to

$$\log\left(\frac{\epsilon_{\text{protonated}} - \epsilon_{\text{obsd}}}{\epsilon_{\text{obsd}} - \epsilon_{\text{dissociated}}}\right) = \text{p}K_{\text{app}} - n\text{pH} \qquad (1)$$

where n is the number of protons involved in spectral transition I. Analysis of the data shown in Figure 1 indicates that transition I involves 1.2 ± 0.2 protons whose apparent pK value ranges from 3.2 to 3.7 depending on the chloride concentration.

As shown in Figure 1, the apparent pK for transition II decreases with increasing chloride concentration. This decrease together with the constraint of maintaining a fixed chloride concentration at low pH values makes it impossible to observe the totality of transition II at 25°. Accordingly, we measured the ϵ_{395} of the protein in 0.2 *M* HCl as a function of temperature. As shown in the insert of Figure 1, ferricytochrome *c* attains a maximal value for ϵ_{395} of 152 mM^{-1} cm^{-1} at temperatures in excess of 40°. The Soret absorbance at these temperatures has a maximum at 395 nm above 40° in 0.2 *M* HCl, indicating the coordination of two weak field ligands in the axial positions of the heme iron (Nanzyo and Sano, 1968; Babul and Stellwagen, 1971, 1972). The effect of temperature on the Soret absorbance of such a high spin iron complex was evaluated by observing the response of the Soret absorbance of ferrihemopeptide 14-21 in 0.2 *M* HCl. The Soret maximum of this peptide remained at 393 nm and its extinction decreased by about 6% between 25 and 40°. We anticipate that the ϵ_{395} for ferricytochrome *c* at the completion of transition II free of thermal effects would be 143 mM^{-1} cm^{-1}. Alternatively, we subjected the experimental values shown in Figure 1 for transition II of ferricytochrome measured in 0.2, 0.5, and 1.0 *M* chloride to a double reciprocal analysis plotting $1/\epsilon_{395}$ vs. $1/[H^+]$. The experimental values extrapolate to a common y intercept corresponding to an ϵ_{395} at the completion of the transition of 144 mM^{-1} cm^{-1}. The $\Delta\epsilon_{395}$ associated with transition II is therefore 52 mM^{-1} cm^{-1}. This value is comparable to a $\Delta\epsilon_{395}$ of 44 mM^{-1} cm^{-1} associated with the pH-dependent ligation of an intrinsic histidyl residue with the heme iron of ferrihemopeptide 14-21 measured in 0.5 *M* KCl (Babul, 1971). Assuming that transition II represents a proton-dependent equilibrium between two isomeric forms differing in the ligand occupying the other axial position, the spectral changes associated with transition II in Figure 1 may also be analyzed using eq 1. Using ϵ_{395} values of 144 and 92 mM^{-1} cm^{-1} for the totally protonated and dissociated isomers, respectively, the experimental values indicate that transition II involves 0.8 ± 0.1 proton whose apparent pK value ranges from 1.1 to <0 depending on the chloride ion concentration.

As shown in Figure 2, the increase in absorbance at 395 nm which describes transition I is coincident with an increase in absorbance at 620 nm and a decrease in absorbance at 695 nm. An increase in absorbance at 620 nm is characteristic of replacement of a strong field axial ligand by a weak field ligand (Morton, 1973) while a decrease in absorbance at 695 nm is characteristic of replacement of the methionine-80 axial ligand (Shechter and Saludjian, 1967). However, the $\Delta\epsilon_{695}$ associated with transition I measured in 2 *M* NaCl is 340 M^{-1} cm^{-1} which is only a portion of the $\Delta\epsilon_{695}$ of about 650 M^{-1} cm^{-1} associated with the replacement of the methionine ligand during the alkaline isomerization of ferricytochrome *c* (Greenwood and Wilson, 1971). A double reciprocal analysis of that portion of transition II detectable in 0.2 *M* chloride indicates that the absorbance of the 695-nm band observed at the completion of transition I decreases upon further acidification giving a $\Delta\epsilon_{695}$ of 210 M^{-1} cm^{-1} associated with transition II.

The spectral changes observed at 395 nm upon acidification of yeast iso-2-ferricytochrome *c* in 1 *M* chloride are compared with those observed with the horse heart protein in Figure 3. The changes observed for yeast ferricytochrome *c* were found to parallel those observed with the horse heart protein. Analysis of spectral transition I of the yeast protein indicates a $\Delta\epsilon_{395}$ of 48 mM^{-1} cm^{-1} involving a single proton having an apparent pK of 3.3 in 1 *M* chloride.

In contrast to ferricytochrome *c*, the Soret absorbance maximum of porphyrin cytochrome *c* shifts from 404 nm at neutral pH to 406 nm at pH 0.6 giving a $\Delta\epsilon_{406}$ of 170 mM^{-1} cm^{-1} upon acidification. This increase occurs almost entirely in a single step transition involving two protons having an apparent pK of about 1.6 in 0.4 *M* chloride. It is most likely that this spectral transition results from formation of the dicationic form of the porphyrin (Falk, 1964).

Kinetic Spectral Measurements. The kinetics of changes in the absorbance of ferricytochrome *c* at 395 nm associated with spectral transitions I and II were examined both individually and coincidentally by performing stopped-flow spectrophotometric measurements in solvents having variable chloride concentrations. At low chloride concentrations, about 0.01 *M*, where the two spectral transitions are

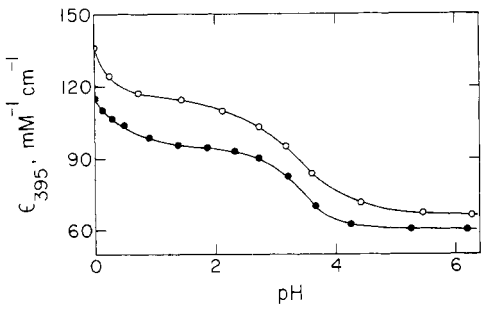

FIGURE 3: Comparison of the spectral transitions of yeast iso-2-ferricytochrome *c* (○) and horse heart ferricytochrome *c* (●) in 1 *M* chloride. Protein concentrations ranged from 3.2 to 10.0 μM.

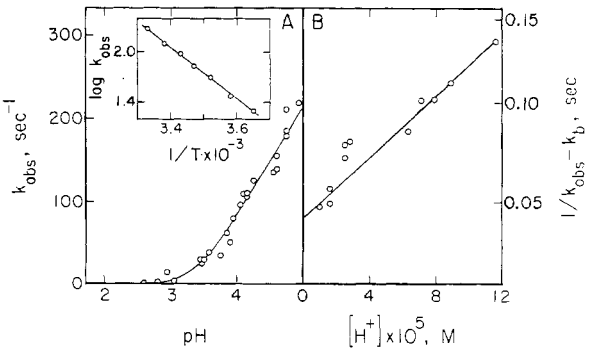

FIGURE 4: Kinetic measurements of the increase in absorbance of acidified ferricytochrome *c* following addition of KOH. (A) The observed rate constant for the increase in absorbance at 695 nm as a function of the final pH of the protein solution. Solutions of horse heart ferricytochrome *c*, 0.1–0.2 m*M*, in 50 m*M* succinate adjusted to pH 2.0 were rapidly mixed with an equal volume of KOH solutions of variable concentration to give the indicated final pH values. All measurements shown in the main portion of the figure were made at 6°. The insert illustrates the effect of temperature on the observed rate measured at pH 3.55. (B) Analysis of the kinetic data collected at 6° shown in A according to eq 2.

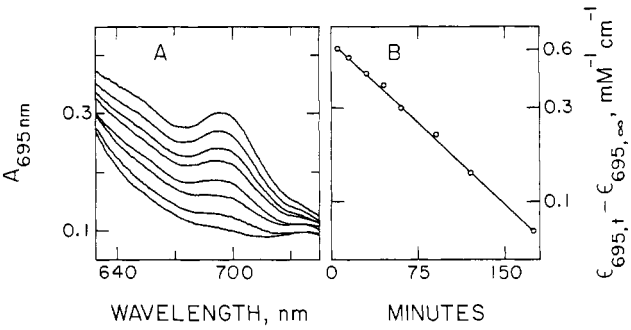

FIGURE 5: Effect of methylation on the 695-nm absorbance band of ferricytochrome *c*. Horse heart ferricytochrome *c* was dissolved in dry methanol containing 10 m*M* HCl at room temperature. Aliquots were removed at the indicated times, diluted in 1 m*M* HCl, and dialyzed against this solvent. (A) Spectra of the methylated protein in 1 m*M* HCl adjust to pH 3.8 with NaOH after methylation for 5, 15, 30, 45, 60, 90, 120, and 175 min reading downward. (B) First-order plot of the loss of absorbance at 695 nm.

virtually coincident, at least three simultaneous pH-dependent first-order reactions are detected at 25° upon rapid addition of base to solutions of the acidified protein (Babul, 1971). Similarly, multiple reactions are detected when similar measurements are made in 0.5 *M* chloride over the pH ranges corresponding to spectral changes I and II individually. However, if the reactions are monitored at 695 nm in the nonbonding buffer, succinate (Margoliash et al., 1970), a single pH-dependent first-order reaction is observed having a $\Delta\epsilon$ characteristic of spectral transition I. The dependence of the rate constant on pH associated with the reappearance of the species having a 695-nm absorbance following addition of base to the acidified protein is shown in Figure 4A. Measurements were done at 6° to extend the measurements to higher final pH values. By contrast, the reverse reaction, that is the loss of 695-nm absorbance resulting from acidification of the native protein, is pH independent having a rate constant of 6.8 sec^{-1} at 6° in the same solvent.

These kinetic results have been analyzed in terms of a rapid protonation reaction which precedes ligation of methionine-80 according to the following mechanism:

$$NCH \underset{}{\overset{K_H}{\rightleftharpoons}} NC + H$$

$$NC \underset{k_b}{\overset{k_f}{\rightleftharpoons}} C$$

where NCH and NC represent the protonated and dissociated forms of the protein having a weak field ligand in the sixth ligation position and C represents the form of the protein having methionine ligated in this position, the only species considered having a 695-nm band. As shown by Davis et al. (1974), the constants for this mechanism can be obtained by plotting the kinetic values according to

$$\frac{1}{k_{obsd} - k_b} = \frac{1}{k_f} + \frac{[H^+]}{k_f K_H} \qquad (2)$$

Analysis of the observed kinetic values in terms of eq 2, Figure 4B, indicates that k_f is 2.5×10^2 sec^{-1}, and K_H is 4.8×10^{-6} *M*. Since k_b is 6.8 sec^{-1}, the equilibrium constant, K_i, for NC $\rightleftharpoons$ C is then about 3.7×10^2. The product $K_H K_i$ should equal the K_{app} for transition I measured in the absence of chloride. The pK_{app} of 2.7 so calculated agrees favorably with the observed pK_{app} of 2.6 (Babul and Stellwagen, 1972a).

The effect of temperature on the observed rate for the forward reaction measured at pH 3.55 is shown in the insert of Figure 4A. An activation enthalpy of 13.8 kcal/mol was calculated from the slope. A free energy of activation of 14.5 kcal/mol was calculated from the k_{obsd} at 25°. Combination of these values gives an entropy of activation of 2.4 cal/(deg mol) for the pH-dependent isomerization involving ligation of methionine-80.

Methylation. Ferricytochrome *c* was esterified by incubating the protein in absolute methanol containing 0.05 *M* HCl for 7.5 hr at room temperature. The protein was then dialyzed against 1 m*M* HCl and its visible absorbance spectrum measured at several pH values. At pH 3.8, the methylated protein exhibits a maximum at 620 nm with no evidence of a maximum at 695 nm. Raising the pH to 6.0 results in the disappearance of the maximum at 620 nm but no reappearance of a maximum at 695 nm. These changes are characteristic of replacement of a weak field ligand, likely solvent, by a primary amine ligand in an axial position on the heme iron.

The kinetics of the loss of absorbance at 695 nm resulting from methylation were observed by removing aliquots from the methylation reaction at intervals and measuring their absorbance at 695 nm upon neutralization to pH 7.0. As shown in Figure 5, the loss of absorbance at 695 nm with time describes a pseudo-first-order reaction having a half-time of 47 min under the conditions employed. These re-

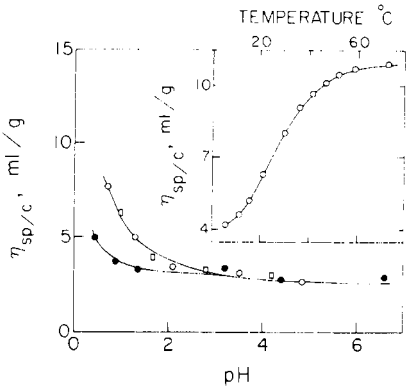

FIGURE 6: The effect of pH on the reduced viscosity of horse heart ferricytochrome *c*. Measurements were made at 25.00° in the presence of (○,□) 0.2 *M* chloride and (●) 0.5 *M* chloride. The insert shows the effect of temperature on the reduced viscosity of the protein in 0.2 *M* HCl.

sults indicate that the loss of 695-nm absorbance is essentially completed after reaction for 3.5 hr.

The number of carboxyl groups methylated after 3.5 hr reaction was determined by differential potentiometric titration. Titration of the native protein in water between pH 7 and 2 required addition of 16.6 protons per molecule of protein. This value agrees with the 17 protons expected to protonate the 12 aspartate and glutamate side chains, two imidazole side chains, two heme propionate side chains, and the one C-terminal carboxylate. After methylation for 3.5 hr, 10.6 protons per molecule of cytochrome *c* were consumed between pH 7 and 2. Since only carboxyl groups are methylated by the conditions employed (Fraenkel-Conrat and Olcott, 1945), we conclude that six carboxy groups have been esterified by this procedure.

The extent of methylation of the heme propionyl side chains by this procedure was investigated by cleaving the heme from the polypeptide chain using Ag_2SO_4 and subjecting the cleaved heme to paper chromatographic analysis using chloroform as the mobile solvent. In contrast to heme cleaved from native cytochrome *c* which remains at the origin, the heme obtained from the methylated protein travelled with the solvent front, indicating that both propionyl groups were esterified (Falk, 1964).

Viscosity Measurements. The effect of chloride concentration on the pH dependence of the reduced viscosity of ferricytochrome *c* is shown in Figure 6. Measurement of the reduced viscosity of the protein in higher chloride concentrations could not be done owing to the appearance of insoluble material at low pH values. The low reduced viscosity values observed between pH 2 and pH 7 indicate that a globular conformation persists over the pH range characteristic for spectral transition I. However, the reduced viscosity increases in the pH range characteristic of spectral transition II. As shown in the insert of Figure 6, the reduced viscosity measured in 0.2 *M* HCl increases to a maximal value of 12.0 ml/g at elevated temperatures. Since the reduced viscosity of randomly coiled ovalbumin decreases about 30% between 25 and 55° (Ahmad and Salahuddin, 1974), we propose that the maximal reduced viscosity observed at 60° is equivalent to the value expected for cytochrome *c* having a randomly coiled conformation at 25°, 15.1 ml/g (Stellwagen, 1968). Analysis of the pH dependence of the reduced viscosity in terms of an isomerization between a globular and a randomly coiled conformation using the viscosity analog of eq 1 indicates the involvement of a single proton whose apparent p*K* values is about 0.4 pH lower than the apparent p*K* values calculated for spectral transition II measured in comparable chloride concentrations. As will be noted below, it is likely that the offset in apparent p*K* values is due to the differences in the protein concentrations employed in the spectral and viscosity measurements.

The reduced viscosity of porphyrin cytochrome *c* in 0.2 *M* chloride attains a limiting value of 14.9 ml/g at pH 1.2, indicating a transition from a globular conformation at neutral pH (Fisher et al., 1973) to that of a random coil at pH 1.2. Reduced viscosity measurements of porphyrin cytochrome *c* in 0.2 *M* chloride as a function of pH indicate a single transition with a midpoint at pH 3.2, significantly different from the value of 0.8 estimated for the hemoprotein. The reduced viscosity of ferricytochrome *c* containing six methylated carboxyl groups is 3.0 ml/g when measured in 0.2 *M* KCl at pH 6.2. Thus, loss of the 695-nm absorbance band by methylation does not perturb the globular conformations of the protein.

Discussion

At neutral pH, ferricytochrome *c* is a globular protein having two strong field protein ligands, an imidazole nitrogen of histidine-18 and the sulfur of methionine-80, coordinated in the axial positions of the heme iron (Dickerson et al., 1971). In the presence of 1 *M* chloride or greater, acidification of the native protein to pH 2 produced an alternative globular conformation termed CH in which the methionine ligand is replaced by a weak field ligand presumably supplied by the solvent. Continued acidification, most evident in solvents having lower chloride concentrations, converts CH into a randomly coiled conformation, termed CH_2, and simultaneously replaces the other protein ligand, histidine-18, with a weak field ligand presumably also contributed by the solvent.

A variety of spectral evidence supports these conclusions. At the acidic end of transition I, the absorbance spectrum of the protein exhibits maxima at 402 and 620 nm characteristic of the ligation of one strong field and one weak field ligand in the axial heme coordination positions. The $\Delta\epsilon_{395}$ associated with transition I is comparable to the value observed upon displacement of a weak field axial ligand on cytochrome *c* hemopeptides by an intrinsic or extrinsic strong field ligand. The hyperfine electron paramagnetic resonance (EPR) spectrum (Peisach et al., 1971) of ferricytochrome *c* measured under conditions which stabilize CH are characteristic of the EPR spectrum of hemoglobin which has a histidine ligand in one axial coordination position and a solvent molecule in the other position. High-resolution ^1H NMR spectra (Gupta and Koenig, 1971) indicate the loss of the resonance at 2.2 ppm below pH 4 in solvents of moderate ionic strength. This resonance has been assigned to the methyl protons of methionine-80 when coordinated to the heme. Formation of CH results in a significant loss of the extinction of the 695-nm band which is characteristic for ligation of methionine-80 (Shechter and Saludjian, 1967). Finally, it should be noted that the sulfur of methionine-80, which is not chemically reactive in C at neutral pH, is readily alkylated at pH 3 (Ando et al., 1965; Tsai and Williams, 1965), indicating that its coordination is weakened if not lost in CH. Alkylation of methionine-80 does not increase the reduced viscosity of the protein while alkylation of the other ligand histidine-18 produces a marked increase in reduced viscosity (Babul and Stellwag-

en, 1972). These observations taken together indicate that spectral transition I involves the methionine and not the histidine axial ligand.

The magnitude of the $\Delta\epsilon_{395}$ of spectral transition II indicates that this transition also involves a ligand exchange rather than a change in heme solvation resulting from the coincident conformational unfolding. The increased extinction at 620 nm of CH_2 relative to CH supports this view. Finally, proton NMR measurements indicate that the apparent pK of one of the three imidazole side chains in the protein occurs below pH 2 (Cohen et al., 1974), which is compatible with the continued ligation of histidine-18 to at least this pH in solvents of moderate ionic strength. Accordingly, we have assigned spectral transition II to the ligation of histidine-18.

Having made these assignments, let us now consider why each of these two ligand replacements is pH dependent in chloride concentrations sufficient to eliminate electrostatic interactions. The potentiometric titration curve for ferricytochrome c in 0.2 M chloride (Bull and Breese, 1966) indicates that 24 protons are placed on the protein between the isoelectric point, 10.1, and pH 1.7. This leaves only one acidic pK value unaccounted for which must have an apparent pK value below 1.7 in this solvent. Analysis of spectral transition II in 0.2 M chloride (Figure 1) by eq 1 indicates that the displacement of the histidine-18 ligand involves a single proton having an apparent pK of 1.1. Since an imidazole cannot be protonated while ligated to a ferriheme iron, the single apparent pK at 1.1 must be assigned to the protonation of histidine-18 coincident with its displacement from an axial coordination position. This assignment is compatible with the very low apparent pK for one of the three histidyl residues in ferricytochrome c measured by proton NMR titrations (Cohen et al., 1974). Since the pH dependent gross unfolding of the globular conformation of ferricytochrome c essentially parallels spectral transition II, ligation of histidine-18 and complexation of a single chloride, to be discussed below, must play critical roles in maintaining the globular conformation. In support of this conclusion, it should be noted that the pH-dependent unfolding of globular porphyrin cytochrome c occurs about 2.4 pH units higher than that of ferricytochrome in 0.2 M chloride. This difference must be due to the contributions of the axial protein ligands, particularly histidine-18, to the stability of the hemoprotein.

Since a methionine residue has no ionizable group in water, the pH dependence for methionine-80 ligation must reflect the proton dissociation of another group, most likely either a carboxyl or an imidazole. Proton NMR measurements (Stellwagen and Shulman, 1973; Cohen and Hayes, 1974) indicate that the imidazole of histidine-33 has an apparent pK of 6.5 ± 0.1, that of histidine-26 a pK of about 3.2, and that of histidine-18 a pK below 2. Since yeast iso-2-ferricytochrome c, which has a glutamine residue at position 26, exhibits a normal spectral transition I (Figure 3), the ionizing group which facilitates the methionine ligation must be a carboxyl. Inspection of the crystallographic models of horse heart ferricytochrome c indicates that all 12 glutamate and aspartate residues are located on the protein surface. Most of these extend into the solvent, although a few may be involved in electrostatic interactions with adjacent lysine protonated ϵ-amine groups (Stellwagen and Shulman, 1973). However, the crystallographic model (Dickerson et al., 1971) indicates that both heme propionic carboxylate groups are involved in defined noncovalent protein–protein interactions. The outer propionic residue attached to tetrapyrrole ring 3 is positioned at the bottom of the heme crevice and serves as a hydrogen bond acceptor for the hydroxyl of threonine-49 and the amide of asparagine-52. The inner propionic residue attached to tetrapyrrole ring 4 is buried in the protein interior and serves as the hydrogen bond acceptor for the indole of tryptophan-59 and the phenolic hydroxyl of tyrosine-48. As noted above, methylation of six of the 14 carboxyl groups of cytochrome c prevents the ligation of methionine-80 without perturbing the globular structure of the protein. Among the six carboxyl groups methylated were both propionic carboxyls. Analysis of the kinetics of spectral transition I suggested an intrinsic pK of 5.3 for the ionizing group controlling methionine ligation, a value in the pK range for porphyrin dicarboxylic acids (Falk, 1964). We propose that the ionization of a single carboxyl controls the ligation of methionine-80, that formation of the carboxylate stabilizes a noncovalent interaction near the left side of the heme pocket, and that the carboxyl is located on one of the two heme propionic groups.

Finally let us consider the effect of chloride on the pH dependence of the ligation of each axial ligand. As shown in Figure 1, increasing chloride concentrations increase the apparent pK of spectral transition II at 25°. These changes occur well above 0.2 M NaCl, a concentration which should eliminate any electrostatic effects, suggesting that the protein may bind chloride. Analysis of the chloride dependence of spectral transition I in terms of formation of a protein-chloride complex using the expression:

$$\log\left(\frac{\epsilon_{\text{no Cl}} - \epsilon_{\text{obsd}}}{\epsilon_{\text{obsd}} - \epsilon_{\infty\ \text{Cl}}}\right)_{395} = \text{p}K_{\text{Cl}} - n \log [\text{Cl}] \qquad (3)$$

indicates that one chloride is bound per ferricytochrome c. Remembering that only a single proton is involved in each spectral transition, the simplist mechanism consistent with the experimental values shown in Figure 1 is

$$\text{C} + \text{H} \overset{K_{\text{H}}}{\rightleftharpoons} \text{CH}$$

$$\text{CH} + \text{Cl} \overset{K_{\text{Cl}}}{\rightleftharpoons} \text{CHCl}$$

$$\text{CH} + \text{H} \overset{K_{2\text{H}}}{\rightleftharpoons} \text{CH}_2$$

Computer-assisted fitting of this mechanism to the experimental values indicates that pK_{H} = 2.1 ± 0.2, p$K_{2\text{H}}$ = 1.7 ± 0.1, and K_{Cl} = 0.037 ± 0.019 M^{-1}. The $\Delta\epsilon_{395}$ values calculated using these constants have an average deviation of 2.8% and a range from −3.8 to 9.5% from all the experimental values shown in Figure 1. Consideration of the presence of significant quantities of additional species such as CCl and CH_2Cl produced distinctly inferior fits with the experimental values.

The proposed mechanism agrees with the experimental observation that the pH dependence dissociations of both axial ligands in the presence of minimal anion concentrations are nearly identical. The apparent common pK = 2.5 for acidification of ferricytochrome c in water with HCl is expected to be greater than the intrinsic pK values due to the extensive charge repulsions of the 21 surface cationic groups. The mechanism indicates that only a single effective chloride binds to the CH species and the viscosity results indicate that it does so without disrupting the globular conformation. Such an effect could be achieved if the chlo-

ride were itself ligated to the heme and stabilized by functioning as an electrostatic bridge between the cationic ferric heme and a cationic ϵ-amino group such as that of the alternative ligand lysine-79 which should be located close to the heme in the native globular structure, Fe^{+}-Cl^{-}···NH_3^{+}. This ferricytochrome *c*–chloride complex differs from that proposed by Boeri et al. (1953) in that a single chloride occupies the fifth coordination position rather than ligation of chlorides to both the fifth and sixth coordination positions, necessitating displacement of the histidine-18 ligand. Electrostatic stabilization of the chloride complex proposed here would be weakened by the conformational unfolding of the globular structure coincident with spectral transition II. Accordingly, we suggest that chloride can serve as an axial ligand for the heme iron in place of methionine-80 and that such ligation stabilizes the globular protein conformation to the unfolding coincident with the removal of histidine-18 from the other axial coordination position.

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