The Unfolding of the Cytochromes c in Methanol and Acid*

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The cytochromes c are a family of hemoproteins that share a number of structural features: a thioether linkage between the protein and the heme, histidine and methionine as the fifth and sixth iron ligands, and a tertiary structure known as the "cytochrome fold." These proteins follow a common mechanism of equilibrium unfolding in methanol and acid, differing only in their reactivity to the denaturing conditions. The reduced cytochromes c exhibit an increased conformational stability which is consistent with the presence of a strengthened iron-methionine linkage in the reduced state.

Hemoproteins participate in a variety of biological reactions, based on the abilities of the heme iron to transfer electrons reversibly and to bind small molecules. The isolated heme moiety is useless for many of its biological functions, since it is rapidly oxidized by molecular oxygen. The hydrophobic environment provided by an associated protein chain in a hemoprotein serves to protect the reduced heme from oxidation by nonbiological sources. Furthermore, specific interactions between protein and heme, such as the coordination of amino acid side chains by the heme iron, are essential to the development of specialized roles for particular hemoproteins.

In order to understand these protein-heme interactions better, it is useful to characterize the conformational states which appear during the unfolding of hemoproteins. The cytochromes c are particularly suited to this kind of study. As an easily purified component of respiratory and photosynthetic energy systems, they have been extensively studied (1). X-ray analysis has shown that these proteins share a native tertiary structure known as the "cytochrome fold" (2-7). Accordingly, differences in unfolding among the cytochrome c might be expected to reflect subtle variations in the nature of the protein heme interaction.

Considerable research has been done on the unfolding of horse ferricytochrome c. At neutral pH, treatment with 3 M urea or heating to 53°C causes part of the polypeptide chain to become uncoupled from the heme, while the native methionine and histidine ligands remain bound to the heme iron (6, 9). This is monitored by a slight increase in absorbance at 407 to 409 nm, which is attributed to an increase in the hydrophilicity of the heme environment. Again at neutral pH, raising the urea concentration to 6.5 M or raising the temperature to 82°C causes the methionine ligand to become displaced from the heme iron, as evidenced by the disappearance of the native 605 nm absorption band associated with an intact iron-methionine linkage (8, 9a, 10). At pH 2 in water, or pH 4 in 9 M urea, the histidine ligand becomes protonated and can no longer coordinate to the iron. Replacement of the histidine ligand by a weak field solvent molecule shifts the Soret maximum to 395 to 400 nm, and produces a new band at 620 nm, a consequence of the formation of a high spin heme complex (11, 12). The Soret spectra of the known conformational states of horse ferricytochrome c are shown in Fig. 1.

The object of this paper is to further characterize the unfolding of horse ferricytochrome c, and to compare this process with the unfolding of cytochromes c from several microorganisms. The results of this study suggest that all of these cytochromes c unfold by approximately the same mechanism. Quantitative differences between the unfolding of the oxidized and reduced forms may be accounted for by the presence of a stronger iron-methionine linkage in the reduced proteins.

**EXPERIMENTAL PROCEDURES**

Horse heart cytochrome c was prepared by T. Takano and O. B. Kallai of this laboratory. Candida krusei cytochrome c was the product of Sankyo Co. (Tokyo). Pseudomonas aeruginosa cytochrome c550 was supplied by R. Almassy of this laboratory. Paracoccus denitrificans cytochrome c550 was the gift of G. McLain and L. Smith of Dartmouth. Rhodosporillum rubrum cytochrome c550 was provided by R. Bartsch. These proteins will be referred to by abbreviated names: horse, C. krusei, and P. aeruginosa cytochromes c550, and R. rubrum c550.

Purification proceeded as follows: horse and C. krusei were each adsorbed on Bio-Rex-70, washed with 50 mM acetate buffer, pH 6, then eluted with 20% ammonium sulfate, adjusted to pH 7 with ammonia; C. krusei was adsorbed and washed on Sephadex SP-C25 with 50 mM ammonium acetate buffer, pH 3.8, then eluted with the same buffer at pH 4.72; C. krusei and C. aeruginosa were used as received. In preparation for spectroscopic measurements, the proteins were oxidized with a slight excess of potassium ferricyanide and dialyzed at 4°C into the desired buffer. To prepare the reduced proteins, and to protect against autoxidation, the final spectroscopic solutions were adjusted to 0.1% v/v mercaptoethanol immediately prior to use.

Absorbances were measured at 24°C on a Beckman model 25 UV-VIS spectrophotometer. The pH values of acidic solutions were determined after spectroscopic measurement with a Beckman SS-2 pH meter, calibrated at pH 4, and deviating from linearity less than 0.03 unit between pH 2 and pH 4. The pH values of methanol/water solutions are given as the pH which the same buffer would produce in the absence of methanol. Below pH 6.4, solutions were buffered with an acetic acid-sodium acetate mixture. Formic acid was substituted for acetic acid below pH 3. Above pH 6.4, solutions were buffered with a Tris-HCl/Tris base mixture. Ionic strength was adjusted by the sodium acetate and Tris-HCl concentrations, rather than by adding sodium chloride, since chloride ion may form a salt bridge with the cytochromes c at low pH (12, 13). Unless otherwise specified, all solutions were set to a final ionic strength of 0.10.

In calculations on the acid-induced spectral transitions of the cytochromes c, it is postulated that there are only two forms of the protein: an unprotonated form, designated as $P_0$, and a protonated...
A plot of log ($K_{eq}$) against (-pH) yields a slope of $n$, the order of positions of the major visible absorption bands and the spin state, of which are given in Table I, along with the spin state, where the spin state of ferric heme (17) is known from experiments in progress. In cases where the spin state of the heme iron is not included here. Methanol was found to have little effect on the spectra of hemin and cytochrome c in the $S = 3/2$ and $S = 5/2$ states.

2. The $S = 3/2$ state is characterized by a Soret band at 401 ± 1 nm, and visible bands at about 525 nm (variable) and 630 ± 10 nm.

3. The $S = 1/2$ state is characterized by a Soret band at 410 ± 3 nm, and a visible band at 530 ± 3 nm.

These relations are intended to serve only as guides in interpreting the structure of unfolded hemoproteins. Far more data would be needed to establish a rigorous relation between spectra and spin state.

Conformational Transitions of Horse Ferricytochrome c

As explained above, horse ferricytochrome c is known to proceed through three stages of denaturation: 1) unfolding without displacement of either native ligand from the heme iron; 2) displacement of the methionine ligand; and 3) protonation and displacement of the histidine ligand. The extent of coordination of the methionine ligand may be followed by the 409 nm absorbance, which is associated with an intact iron-methionine linkage in ferricytochrome c (9a, 10). The method of Kaminsky et al. (9a) was used to correct for the contribution to the 685 nm band of absorption bands at lower wavelengths. The extent of coordination of the histidine ligand may be followed by the 695 nm absorbance, which is sensitive to the spin state of the heme iron.

Methanol Unfolding—Since the unfolding of horse ferricytochrome c in methanol at neutral pH results in displacement of methionine, the 695 nm band absorbance is a sensitive measure of the extent of unfolding. The effect of methanol on the equilibrium value of the 695 nm band (20 min of denaturation) is shown in Fig. 2. At pH 5, the iron-methionine linkage remains intact to 35% methanol, at which point methionine is displaced cooperatively from the heme iron. However, at pH 6, a decrease in the 695 nm band is observed prior to the cooperative transition at 35% methanol.
Unfolding of the Cytochromes c in Methanol and Acid

FIG. 2. Fraction of 695 nm band of horse ferricytochrome c remaining after 20 min of reaction, as a function of methanol concentration.

FIG. 3. Decrease with time in 695 nm band of horse ferricytochrome c, in 33.3% methanol (ionic strength of 0.08).

change exhibits a first order dependence on methanol concentration, it probably involves the equilibrium binding of methanol to a single site on the protein molecule. The displacement of methionine as a consequence of this methanol binding is time-dependent, as evidenced by the slow decay of the 695 nm band presented in Fig. 3. It is accompanied by an increase in the overall long wavelength equilibrium absorbance, as may be seen in Fig. 4, but produces little change in the visible absorption bands at 410 and 530 nm.

The kinetic behavior of the isomerization suggests a reasonable molecular interpretation of the data. The rate of increase of the total absorbance at 695 nm (not the baseline-corrected 695 nm band) was measured as a function of methanol concentration (Fig. 5A) and of pH (Fig. 5B). The first order dependence of the rate on methanol concentration supports the conclusion drawn from the equilibrium data that isomerization results from the binding of methanol to a single site on the protein molecule. The inhibition of the rate by hydrogen ion suggests that protonation of one or more sites on the protein prevents interconversion to the isomer. It may be shown that protonation of a single residue bearing a pKₐ of 5.4 adequately accounts for the observed pH dependence.

It appears that low concentrations of methanol disrupt native structural aromatic ring interactions in horse ferricytochrome c, and may result in substitution of a basic residue, such as lysine, for methionine 80 as the sixth iron ligand.

FIG. 4. Change in long wavelength absorption of horse ferricytochrome c, after 20 min of reaction at pH 6.0. ---, no methanol; ---, 20% methanol; ---, 30% methanol; ---, 40% methanol.

FIG. 5. Rate of increase in total 695 nm absorbance of horse ferricytochrome c, as function of: A, methanol, pH 6.0; B, pH, 33.3% methanol (ionic strength of 0.10 in acetate and 0.08 in Tris). The rate constant is defined as \( k = \frac{\text{initial rate (absorbance units/min)}}{\text{initial absorbance}} \).

Implicit in this proposal is the concept that the native coordination of methionine in ferricytochrome c is imposed by the protein folding, so that weakening of the native protein conformation favors coordination of lysine, which is known to be a much stronger ligand for ferric heme when unprotonated (16). Its pKₐ may be shifted from 10.5 for free lysine to below 6 by its coordination to the heme iron. When this lysine becomes protonated at low pH, and can no longer bind the iron, the weaker iron-methionine linkage may be restored.

The evidence for this mechanism comes from chemical modification studies of horse cytochrome c. Bromination of tryptophan 59 (18) and nitration of tyrosine 67 (19) affect the
stability of the iron-methionine linkage in the same way as do low concentrations of methanol. As the result of each of these modifications, methionine becomes displaced from the ferric iron as the pH is raised from 5 to 7, with a pKₐ for the conformational change of 5.4 to 5.9. A similar pH dependence for coordination of the sixth ligand results from carboxymethylation (20) or photooxidation (10) of the native methionine ligand. The sixth ligand of methionine-modified cytochrome c becomes displaced from the iron by protonation with a pKₐ of 5.2, as marked by a change in the visible spectrum from low spin (S = 1/2) to intermediate spin (S = 3/2).

As a whole, these chemical modifications studies (10, M-20) point to the existence of a stable conformation of ferricytochrome c which is not far removed from the native structure. This "near-native" structure exhibits a loosened folding of the protein about the heme and a weakened iron-methionine linkage. Low concentrations of methanol shift the conformational equilibrium from the native to the less tightly folded form. High concentrations of methanol displace the methionine ligand from the heme iron regardless of pH. The high order of methanol dependence (-10) for this conformational change suggests that it is associated with cooperative unfolding on the methionine side of the heme. The ferric iron remains low spin after unfolding, as evidenced by new visible maxima at 407 and 528 nm. If water were to replace methionine as the sixth ligand, the iron would become high spin (S = 5/2) as in metmyoglobin. Hence, the low spin spectrum of horse ferricytochrome c argues that its sixth ligand remains occupied by a protein residue even after unfolding.

Acid Unfolding—The effect of acid on the visible spectrum of horse ferricytochrome c is shown in Fig. 6, A and B. From the pH dependence of the total absorbances at 409, 530, 620, and 695 nm, and the intensity of the baseline-corrected 695 nm band, an acidity constant (pKₐ) and an order of hydrogen ion binding (n(H⁺)) were calculated at each wavelength. These are listed in Table II. The shift in the Soret maximum from 410 nm at pH 4 to 396 nm at pH 2 is a measure of the conformational change from the native, low spin protein to the acid-unfolded, high spin protein. This transition, monitored by the decrease in absorbance at 409 nm, has a pKₐ of 2.5 and involves the protonation of 2.4 sites on the protein molecule.

Absorbance changes at other wavelengths reflect a less than complete unfolding of cytochrome c. The decrease in intensity of the baseline-corrected 695 nm band exhibits a pKₐ of 3.2, involves the protonation of 1.7 sites, and is due to displacement of methionine from the heme iron. It is difficult to determine whether the iron remains low spin in acid after rupture of the iron-methionine linkage, as is the case during methanol unfolding. Changes in the total absorbance at 530, 620, and 695 nm may result from a complex series of conformational changes from low spin to high spin protein. These transitions share a pKₐ of 2.8 ± 0.1.

One point is clear from comparison of these pKₐ values: in acid as well as in methanol, displacement of methionine from the heme iron precedes displacement of histidine. Certainly, this common sequence of unfolding in methanol and acid arises from a conformational preference of the cytochrome c molecule.

Effect of Methanol on Acid Unfolding—The effect of methanol on the acid unfolding of horse ferricytochrome c is shown in Fig. 7. Based on the change in absorbance at 409 nm, an acidity constant and an order of hydrogen ion binding were calculated at a series of methanol concentrations. These data show that the native coordination of methionine inhibits acid denaturation of cytochrome c. Below 35% methanol (log % methanol = 1.5), where the iron-methionine linkage is initially intact at neutral pH, changes in methanol concentration affect the pKₐ only moderately. Above 35% methanol, where methionine begins to be displaced at neutral pH, the pKₐ be-

![Fig. 6. Visible spectra of horse ferricytochrome c as function of pH. ---, pH 3.89; --, pH 2.82; ----, pH 1.86.](http://www.jbc.org/)

![Fig. 7. The pKₐ and n(H⁺) (order of hydrogen ion binding) of horse ferricytochrome c as function of methanol concentration.](http://www.jbc.org/)
comes more sensitive to changes in methanol concentration. Apparently, rupture of the iron-methionine linkage, accompanied by cooperative uncoupling of the protein from the heme, labilizes the iron-histidine linkage. Complete displacement of methionine by 60% methanol raises the pK_a of cytochrome c to 3.7, a value comparable to the pK_a of 3.8 observed for the iron-histidine linkage of hemopeptide 14-21 (11).

The order of hydrogen ion binding describes the number of sites on the protein molecule which must be protonated to effect acid unfolding. The hydrogen ion binding order by cytochrome c decreases from 2.4 in the absence of methanol to 1.0 in 35% methanol. Protonation of a single residue, the histidine ligand, is sufficient to achieve acid unfolding of the protein in 35% methanol, since 1.4 protonation sites have been disorganized by the denaturant. It was proposed above that binding of methanol to a single site on horse cytochrome c leads to disruption of the native structural aromatic ring interactions, resulting in a tendency for replacement of the native methionine ligand by another residue. It may be deduced from the effect of methanol on acid unfolding that this binding also results in disruption of several native structural interactions involving unspecified acidic residues.

**Mechanism of Unfolding of Horse Ferricytochrome c**—It is useful to define symbols for the protein conformations observed during the unfolding of horse ferricytochrome c. **N**, the native protein (Soret maximum at 410 nm, 695 nm band intact); **I**, the less tightly folded native protein, bound at a single site by methanol (slightly increased intensity at 409 nm, 695 nm band intact); **Ia**, an isomer of I, with the methionine ligand displaced (slightly increased intensity at 409 nm, 695 nm band absent); **II**, the cooperatively unfolded protein, with the methionine ligand displaced (increased intensity at 407 nm, 695 nm band absent); **III**, the cooperatively unfolded protein, with both the methionine and histidine ligands displaced (increased intensity at 396 nm, 695 nm band absent, new band at 620 nm).

Based on the data presented in Figs. 2, 3, 4, 5A, 5B, and 7, the methanol unfolding of horse ferricytochrome c may be represented as:

\[
\text{N} \quad \text{I} \quad \text{II} \quad \text{III}
\]

Similarly, from the data in Table II and Fig. 7, the acid unfolding may be given by either of two schemes:

\[
\text{N} \quad \text{I} \quad \text{II} \quad \text{III}
\]

**Comparative Unfolding of the Cytochrome c**

Having characterized the conformational states of horse ferricytochrome c, it is desirable to extend these results to cytochromes C. *krusei*, C_2_, C_550_, and C_551_.

**Mechanol Unfolding of the Ferricytochromes c**—The effect of methanol on the relative absorbances of the different ferricytochromes c at 409 nm is shown in Fig. 8, A and B. The Soret maxima and spectrally observed spin states of these proteins in 60% methanol are listed in Table III. At low concentrations of methanol, all five proteins display a slightly increased absorbance at 409 nm, characteristic of a less tightly folded native structure (conformation I, as defined above). Isomerization to conformation Ia was observed only for the proteins from horse and C. *krusei*, but conditions under which isomerization might occur were not thoroughly investigated for the other three proteins.

Further addition of methanol to horse, C. *krusei*, and C_2_ (Fig. 8A) increases their 409 nm absorbance by 25% and shifts their Soret maximum to 407 nm. These spectral changes are consistent with cooperative unfolding to conformation II. Horse, C. *krusei*, and C_2_ remain low spin in methanol, suggesting that the sixth ligand position of these unfolded proteins continues to be occupied by a protein residue at pH 6.

Further addition of methanol to C_550_ and C_551_ (Fig. 8B) decreases the 409 nm absorbance by shifting the Soret maximum to 400 ± 1 nm. The visible spectra suggest that this is due to a change in the spin state of the iron upon displacement of methionine from S = 1/2 to S = 3/2 for C_550_ and to a mixture of S = 3/2 and S = 5/2 for C_551_. The iron-histidine linkage in high spin C_550_ and C_551_ remains intact, as evidenced by the ligand binding behavior of homoe iron: imidazole was found to coordinate to a single site in each of these unfolded cytochromes c with a decrease in free energy of 5 to 6 kcal/mol.
This binding produces a low spin spectrum, with sixth ligand position, while histidine continues to occupy the fifth. It is apparent that c550 and c651 unfold only to conformation II in methanol, rather than conformation III. The high spin character of the iron in conformation II of c550 and c651 suggests that their sixth ligand positions are not occupied by a protein residue at pH 6.

At pH 10 in 60% methanol, all five ferricytochrome c exhibit a low spin spectrum, with visible maxima at 406 ± 1 and 529 ± 1 nm. The appearance of low spin character in the spectrum of conformation II of c550 and c651 suggests that the unprotonated form of a residue such as lysine or asparagine occupies the sixth ligand position of these proteins at pH 10. Protonation of this basic residue at pH 6 may prevent its coordination to the heme iron.

These interpretations of spectral changes in the Soret region are consistent with studies at longer wavelengths. The relative intensity of the long wavelength band in the spectrum of ferricytochrome c (695 nm for horse cytochrome c) is sensitive to the extent of coordination of methionine. Disappearance of this band in methanol, as shown in Fig. 9, A and B, is due to cooperative unfolding from conformation I to conformation II. The iron-methionine linkage, and likewise conformation I, remains fully intact to 35% methanol for horse, 40% methanol for C. krusei, 30% methanol for c2, 25% methanol for c650, and 40% methanol for c651. As expected, since methionine is not an ionizable residue, the iron-methionine linkage in c651 is equally resistant to rupture by methanol at pH 6 and pH 8 (Fig. 9 B).

Acid Unfolding of the Ferricytochromes c—From the pH dependence of the 409 nm absorbance, an acidity constant and an order of hydrogen ion binding were calculated for the acid unfolding of each cytochrome c. Since these values were found to exhibit a significant dependence on salt concentration, measurements were made at a series of buffer concentrations and extrapolated to zero ionic strength. The extrapolated values are listed in Table IV.

The five ferricytochromes c share a pK_a for the acidic spin state transition of 2.5 ± 0.2. Thus, in terms of their resistance to acid unfolding, these proteins behave in a similar fashion. On the other hand, the number of residues which must be protonated to achieve acid unfolding ranges from 1.3 for

Table IV

<table>
<thead>
<tr>
<th>Protein</th>
<th>pK_a</th>
<th>n(H⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>2.50 ± 0.09</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>C. krusei</td>
<td>2.61 ± 0.04</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>c2</td>
<td>2.51 ± 0.03</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>c550</td>
<td>2.31 ± 0.05</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>c651</td>
<td>2.70 ± 0.08</td>
<td>3.8 ± 0.7</td>
</tr>
</tbody>
</table>

Fig. 9. Fraction of long wavelength band remaining after 20 min of reaction, as function of methanol concentration, for: A, oxidized horse, C. krusei, and c2, pH 5.0; B, oxidized c550 and c651, pH 6.0 (c651 also at pH 8.0).

Fig. 10. Relative absorbances at respective Soret maxima, pH 6.0, as function of methanol concentration, for: A, reduced horse, C. krusei, and c2; B, reduced c550 and c651 (also at pH 8.0).
cytochrome c₂ to 4.7 for cytochrome c₅₅₀. We have not detected any relationship between these orders of hydrogen ion binding and the comparative physical or crystallographic properties of the cytochromes c.

**Mechanistic Unfolding of the Ferrocytochromes c**—The effect of methanol on the relative absorbances of the different ferrocytochrome c at their respective Soret maxima is shown in Fig. 10. A and B. The Soret maxima and spectrally observed spin states of these proteins in 80% methanol are listed in Table V. While limited exposure to mercaptoethanol does not noticeably affect native cytochrome c, it induces aggregation in unfolded cytochrome c. At high methanol concentrations, where the curves in Fig. 10, A and B end, all of the reduced proteins exhibit a time-dependent decrease in the overall Soret absorbance. This suppression is characteristic of heme-heme aggregation (21). Use of dithionite as a reducing agent was found to produce a similar effect.

Treatment of horse cytochrome c with 5% v/v mercaptoethanol in 80% ethanol at pH 6, was found to cleave the heme from the protein, presumably by reduction of the covalent thioether linkages to sulfhydryl groups. The free heme elutes before the apoprotein on Sephadex G-25 in 10% acetic acid. A possible mechanism for this reaction might be that exposure of the thioether linkages to the solvent during unfolding leads to cleavage of the heme, which is then followed by expulsion of the heme from its pocket and subsequent aggregation of free heme in solution. No data were recorded at a higher methanol concentration than that at which aggregation was first observed for each protein.

At low concentrations of methanol, all five ferrocytochrome c display a slightly increased absorbance at their respective Soret maxima. This spectral change in the reduced cytochromes c is analogous to the slight increase in the 409 nm absorbance of every oxidized cytochrome c produced by low concentrations of methanol, and is associated with unfolding of the native structure to conformation I.

Further addition of methanol to the ferrocytochrome c decreases the measured absorbance by shifting the Soret band to shorter wavelengths. In 80% methanol, horse and C. krusei cytochrome c (Fig. 10A) exhibit a reduced, high spin (S = 2) spectrum, with visible maxima at 409 ± 1 nm and 531 ± 1 nm (see Williams (17) for a correlation of visible maxima with the spectrum, with visible maxima at 409 ± 1 nm and 531 ± 1 nm). This high spin spectrum has been observed previously for carboxymethylated horse cytochrome c (22) and carboxymethylated cytochrome c₅₅₀ (29). It is associated with rupture of the iron-methionine linkage and subsequent formation of a five-coordinate heme complex, as is found in deoxymyoglobin (22). Ferrocytochromes c₉₀ and c₅₅₀ (Fig. 10B) also show signs of a transition from low spin to high spin heme, but aggregation due to mercaptoethanol ensues before this spectral change is complete. Ferrocytochrome c₂ remains low spin to 85% methanol, and thus exhibits a remarkably stable reduced iron-methionine linkage.

These data show that the reduced cytochromes c follow the same path of unfolding as do their oxidized counterparts. For the ferroproteins, low concentrations of methanol favor conformation I over the native structure, while further addition of methanol favors conformation II over conformation I. However, as is evident from the data summarized in Table VI, conformation I is more resistant to methanol unfolding in the reduced proteins. This is consistent with the presence of a stronger iron-methionine linkage in the reduced molecule. It is known that methionine binds more tightly to reduced than to oxidized heme (17), and that the methionine ligand of horse cytochrome c is more resistant to chemical modification when the iron is reduced (24).

**DISCUSSION**

The unfolding of horse ferriprochrome c in methanol and acid involves a minimum of three conformational intermediates, designated in this paper as: I, a less tightly folded form of the native protein, with both native ligands still coordinated to the heme iron; II, the cooperatively unfolded protein, with the methionine ligand displaced from the iron; and III, the cooperatively unfolded protein, with both the methionine and histidine ligands displaced from the iron. These conformations appear in the sequence (where N is the native protein):

\[ N \rightarrow I \rightarrow II \rightarrow III \]

This scheme of unfolding applies equally well to both the oxidized and reduced forms of every cytochrome c examined here, and is consistent with the crystallographic results which show that these proteins share a common tertiary structure known as the "cytochrome fold" (25).

This unfolding mechanism, which is derived from equilibrium measurements of protein conformation, may also be consistent with the kinetically derived results of other investigators. Ikai et al. (26) have reported that the unfolding of horse ferricytochrome c by guanidine hydrochloride is a bi- phasic process. This is suggestive of conformations I and II observed here for the same protein in methanol. Tsong (27) has detected three kinetic phases for the thermal unfolding of horse ferricytochrome c in acid. These might be conformations I and II, and III observed here for the same protein in acid. It would be of interest to relate the unfolding of cytochrome c to its electron transfer properties. Typically, protein denaturation is a two-state phenomenon (for a recent review of protein unfolding, see Baldwin (28)). Proteins often unfold in a single cooperative step from native to denatured protein (conformation II of cytochrome c). Thus, the question arises as to whether conformation I of cytochrome c represents an unimportant exception to the two-step unfolding principle, or a biologically significant conformation of the native molecule. Although this is an intriguing idea, present characterization of the in vivo conformations of cytochrome c does not suggest any functional role for conformation I.

**Acknowledgments**—We wish to thank T. Takano and R. Almasy for their helpful assistance in preparation and purification of the

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**Table V.** Visible maxima of the ferrocytochromes c, pH 6.0

<table>
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<th>Protein</th>
<th>Native</th>
<th>In 80% methanol</th>
<th>Spin state in 80% methanol</th>
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</thead>
<tbody>
<tr>
<td>Horse</td>
<td>416, 521, 550</td>
<td>409, 533</td>
<td>2</td>
</tr>
<tr>
<td>C. krusei</td>
<td>416, 521, 549</td>
<td>408, 529</td>
<td>2</td>
</tr>
<tr>
<td>C₂</td>
<td>417, 522, 551</td>
<td>414, 522, 551</td>
<td>0</td>
</tr>
<tr>
<td>c₉₀</td>
<td>416, 522, 550</td>
<td>(Aggregates)</td>
<td>?</td>
</tr>
<tr>
<td>c₅₅₀</td>
<td>417, 521, 551</td>
<td>(Aggregates)</td>
<td>?</td>
</tr>
</tbody>
</table>

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**Table VI.** Stability of the iron-methionine linkage in the cytochromes c, pH 6.0

<table>
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<tr>
<th>Protein</th>
<th>% methanol to which iron-methionine bond remains fully intact</th>
</tr>
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<tbody>
<tr>
<td>Oxidized</td>
<td>Reduced</td>
</tr>
<tr>
<td>Horse</td>
<td>85</td>
</tr>
<tr>
<td>C. krusei</td>
<td>85</td>
</tr>
<tr>
<td>c₉₀</td>
<td>40</td>
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<tr>
<td>c₅₅₀</td>
<td>40</td>
</tr>
<tr>
<td>c₅₅₁</td>
<td>40</td>
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proteins. We are also indebted to R. Bartsch, G. McLain, and L. Smith for supplying several varieties of cytochrome c used here. We express further appreciation to L. Casler for the preparation of figures.

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H R Drew and R E Dickerson


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