Guanidine Hydrochloride and Acid Denaturation of Horse, Cow, and *Candida krusei* Cytochromes *c*†

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ABSTRACT: Horse and cow cytochrome *c* differ in amino acid sequence by three residues. *Candida krusei* cytochrome *c* contains 5 more residues than horse cytochrome *c* and differs in sequence at 45 of the 103 residues common to both proteins. The denaturation by guanidine hydrochloride (Gdn·HCl) and acid has been studied to determine the effect of the differences in sequence on the relative stabilities of these homologous proteins. Denaturation was followed using optical rotation measurements at 300 nm. At pH 6.5, 25°, the midpoints of the Gdn·HCl denaturation curves are 1.89 M Gdn·HCl for *Candida*, 2.42 M for horse, and 2.63 M for cow ferricytochromes *c*. An analysis of these data indicates that cow is a minimum of 650 cal/mol more stable than horse ferricytochrome *c* and 2300 cal/mol more stable than *Candida* ferricytochrome *c*, in the presence of 2–3 M Gdn·HCl. An analysis of the acid denaturation curves shows that near pH 2.5 at low ionic strength, cow is a minimum of 720 cal/mol more stable than the other two proteins which have similar stabilities. Under physiological conditions, it appears that *Candida* will be somewhat (0–1 kcal/mol) less stable than horse ferricytochrome and that horse will be approximately 700 cal/mol less stable than cow ferricytochrome *c*. Most of the factors which might contribute to the difference in stability suggest that horse should be more stable than cow ferricytochrome *c*. The midpoints of the acid denaturation curves occur at lower pH values when the reaction is monitored using the absorbancy at 395 nm. This is a clear demonstration that the mechanism of denaturation for ferricytochrome *c* is more complex than a simple two-state mechanism. The optical rotatory properties of the Gdn·HCl-denatured proteins suggest that they have similar, randomly coiled conformations. In contrast, acid-denatured cow ferricytochrome *c* appears to be less unfolded than the other two proteins.

A study of the denaturation of members of a set of homologous proteins may prove useful in at least two ways. First, it may lead to a better understanding of the forces which contribute to globular protein stability. By determining the difference in stability between two proteins differing only slightly in amino acid sequence, it should be possible to estimate the contribution of certain interactions, such as hydrogen bonds, to the stability of the protein. This requires measuring small differences in stability; however, in a previous study of three genetic variants of β-lactoglobin, we showed that differences in stability as small as 100 cal/mol can be readily determined from a denaturation study (Alexander and Pace, 1971).

Second, these studies may provide information on the role of protein stability in globular protein evolution. For a set of homologous proteins such as the cytochromes *c* where the functional properties of the molecules appear almost unaffected by large differences in amino acid sequence (Margoliash, 1971), it will be interesting to see if the stability of the proteins is similarly unaffected.

The cytochromes *c* are the best characterized set of homologous proteins. Amino acid sequences are available for over 50 different cytochromes *c* (Dayhoff, 1972) and the three-dimensional structures of three of these have been determined (Takano *et al.*, 1973). By far the best-characterized cytochrome *c* is that from horse. Studies of the denaturation of horse cytochrome *c* by heat (Urry, 1965), acid (Babul and Stellwagen, 1972), alcohols (Kaminsky *et al.*, 1973), urea (Myer, 1968; Stellwagen, 1968), and Gdn·HCl[1] (Ikai *et al.*, 1973) have been reported. In addition, the mechanism of denaturation has been investigated in several recent kinetic studies (Tsong, 1973; Henkens and Turner, 1973; Ikai *et al.*, 1973).

We report here studies of the Gdn·HCl and acid denaturation of the cytochrome *c* from horse, cow, and *Candida krusei*. The proteins from horse and cow are very similar, differing in amino acid sequence at only three out of 104 residues. In contrast, the cytochrome *c* from *Candida* differs from horse at 45 out of the 103 residues common to both proteins and contains five additional residues (Dayhoff, 1972).

Experimental Section

Materials. Cytochrome *c* from horse heart (Type VI) and beef heart (Type V) was obtained from Sigma Chemical Co. *Candida* cytochrome *c* was a twice-crystallized sample from Sankyo Co. The proteins were further purified by gel filtration on a 3 × 110 cm Sephadex G-75 column (Flatmark, 1964a). Complete oxidation to ferricytochrome *c* was achieved by adding 0.1 g/l. of potassium ferricyanide. The oxidant and other salts were removed by dialysis and protein solutions were concentrated with an Amicon ultrafiltration cell. Protein concentrations were determined on a Cary 15 spectrophotometer using a molar extinction coefficient of 8.5×10^8 at 550 nm for horse (Margalit and Schejter, 1970) and cow (Flatmark, 1966a) ferricytochrome *c* and a value of 8.4×10^8 at 549 nm for *Candida* ferricytochrome *c* (Yamanaka *et al.*, 1964).

Gdn·HCl was purchased from Heico Inc. The concentration of stock solutions was determined by refractive index measurements (Pace, 1966).

Methods. Optical rotations were measured at 300 nm with a Cary 60 spectropolarimeter and are presented as [*m'*], the reduced mean residue rotation (Fasman, 1963). Each point

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[1] Abbreviation used is: Gdn·HCl, guanidine hydrochloride.

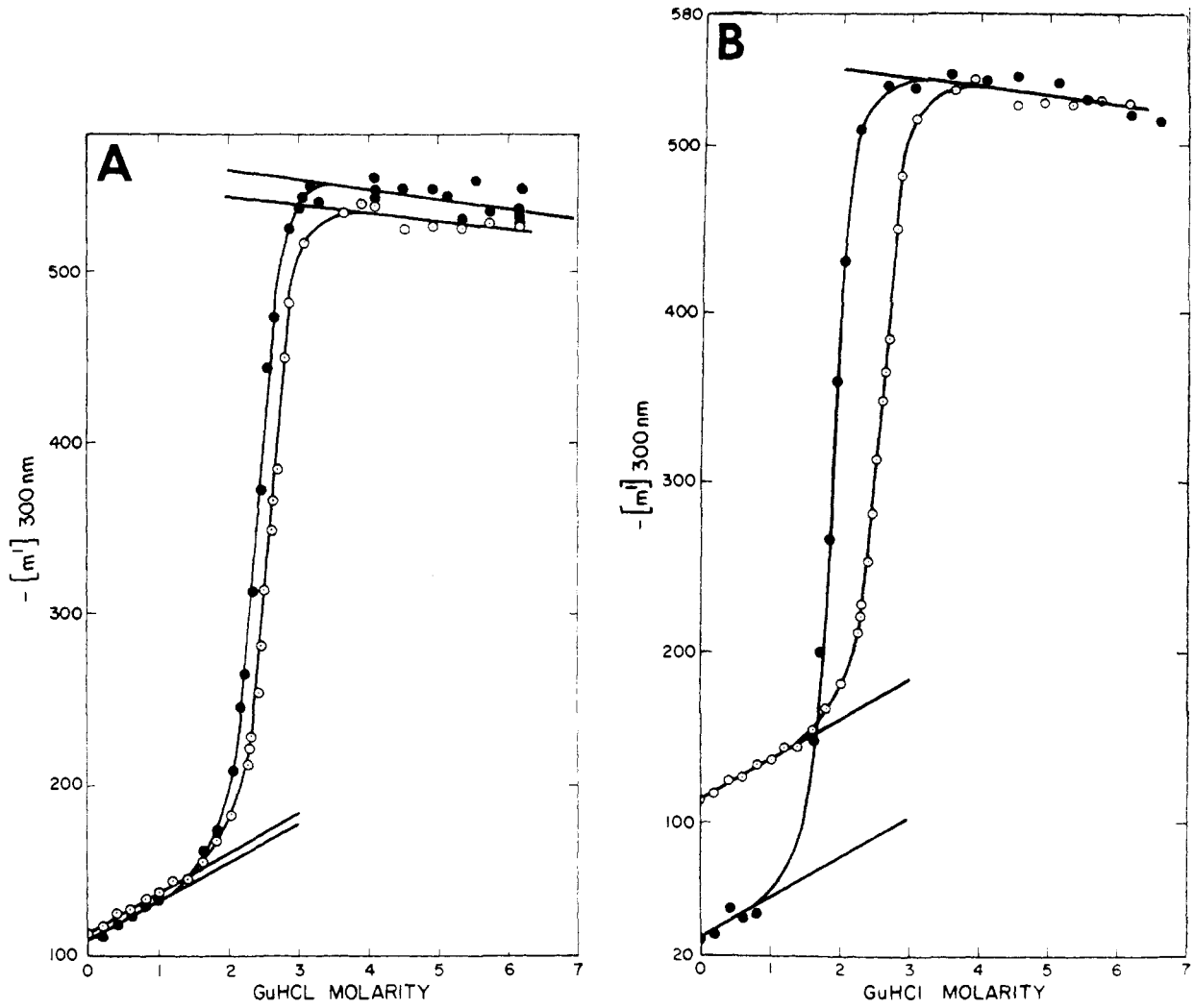

FIGURE 1: Guanidine hydrochloride denaturation curves at pH 6.5 and 25°: (A) horse (●) and cow (⊙) ferricytochrome *c*; (B) *Candida* (●) and cow (⊙) ferricytochrome *c*.

in Figures 1 and 2 was determined on a freshly prepared solution after equilibrium had been attained. Protein concentrations were near 1.0 mg/ml. The temperature of the solutions was 25.0 ± 0.05°. All pH measurements were made at room temperature with a Radiometer Model 26 pH meter. Analytical polyacrylamide gel electrophoresis was carried out as described by Flatmark (1964b).

Results

The Gdn·HCl denaturation curves for horse, cow, and *Candida* ferricytochrome *c* are shown in Figure 1, and the acid denaturation curves in Figure 2. A wavelength of 300 nm is particularly convenient for following the denaturation of cytochrome *c*. There is a fivefold decrease in the rotation accompanying denaturation and, in addition, this wavelength corresponds to a minimum on the absorption spectrum so the noise in measuring the rotation is minimized. Our primary goal is to analyze and interpret the substantial dissimilarities in the denaturation curves in terms of the differences in amino acid sequence among these proteins.

Reversibility. The Gdn·HCl (Ikai *et al.*, 1973), urea (Stellwagen, 1968) and acid (Babul and Stellwagen, 1972) denaturation of horse cytochrome *c* have been shown to be reversible. We find that the Gdn·HCl and acid denaturation of cow and *Candida* cytochrome *c* are also completely reversible.

Denatured Cytochrome c. The intrinsic viscosity of Gdn·HCl denatured horse cytochrome *c* is 14.6 ml/g which indicates that the protein approaches a randomly coiled conformation (Ikai *et al.*, 1973). Babul and Stellwagen (1971) have presented good evidence, however, that the fifth and sixth positions of the heme iron are coordinated to histidyl residues in cytochrome *c* denatured by 9 M urea or 6 M Gdn·HCl above pH 6. All three of the histidyl residues are near the heme (positions 18, 26, and 33) and coordination must not restrict the flexibility of the chain to a great extent.

Figure 1 shows that the [*m'*] value for Gdn·HCl-denatured horse cytochrome *c* is about 15° more negative than for cow and *Candida*. It has been shown that the differences in rotation between Gdn·HCl-denatured proteins can be accounted for reasonably well on the basis of differences in amino acid composition (Tanford, 1968). Using estimates of the contribution of individual residues to the rotation given by Tanford (1968), we would predict a difference in rotation of 12° between cow and horse cytochrome *c* and of 11° between *Candida* and horse, both in good agreement with the experimental results. This suggests that the cow and *Candida* cytochromes *c* also approach a randomly coiled conformation when denatured by Gdn·HCl.

It can be seen in Figure 2 that the [*m'*] value for acid denatured cow cytochrome *c* is about 40° less than for the other two proteins. This was surprising in view of the results for the Gdn·HCl-denatured proteins. To investigate it further we

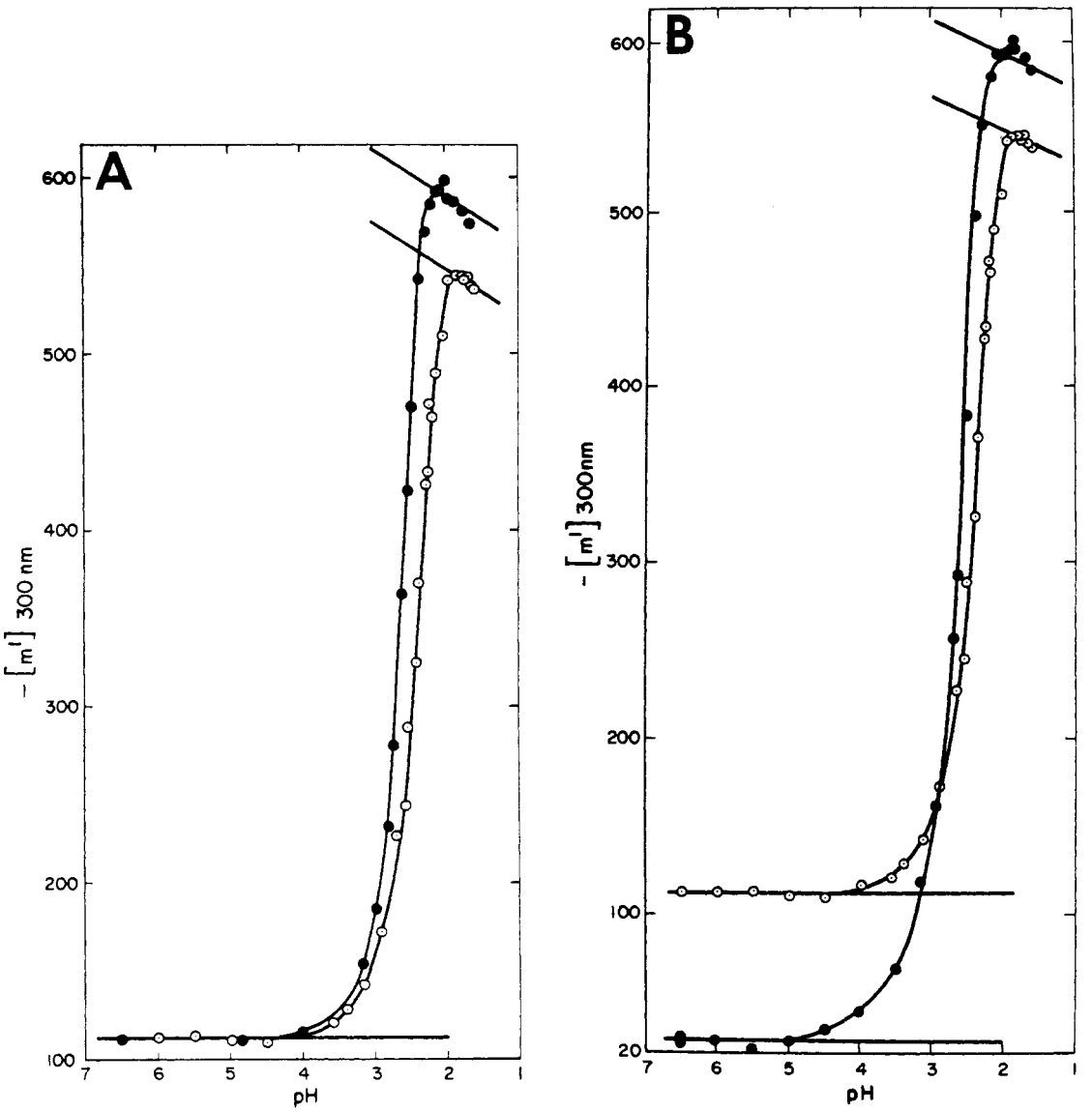

FIGURE 2: Acid denaturation curves at 25°: (A) horse (●) and cow (⊙) ferricytochrome *c*; (B) *Candida* (●) and cow (⊙) ferricytochrome *c*. Salt-free solutions of the protein were adjusted to the pH indicated on the curve using 0.1 M HCl.

determined the $[m']$ value expected for the Gdn·HCl-denatured proteins at low pH. This was done by determining the dependence of $[m']$ on Gdn·HCl concentration at various pH values and extrapolating the results to 0 M Gdn·HCl concentration.[2] For horse cytochrome *c* the $[m']$ value obtained at pH 2 is −594° which is identical with the $[m']$ value for the acid-denatured protein. Similar results were obtained for *Candida* cytochrome *c*. For cow cytochrome *c* the $[m']$ value obtained at pH 2 is −580° which is 30° more negative than the $[m']$ value for the acid-denatured protein. Thus, it appears that the conformation of acid-denatured cow cytochrome *c* differs from that of the other two proteins, perhaps because the cow cytochrome *c* retains some structure after acid denaturation. Aune *et al.* (1967) have shown that for chymotrypsinogen, ribonuclease, and lysozyme, acid and thermal denaturation leads to a product which can be further unfolded by adding Gdn·HCl. Our results suggest that this may be the case for cow but not for horse or *Candida* cytochrome *c*. This is a remarkable result in view of the small difference in sequence between horse and cow cytochrome *c*.

Gdn·HCl Denaturation. Kinetic studies (Ikai *et al.*, 1973) have shown clearly that the Gdn·HCl denaturation of horse ferricytochrome *c* is not a two-state process. Nevertheless, it will prove useful to analyze our data in terms of a two-state mechanism. The data in Figure 1 were used to calculate an apparent equilibrium constant, K_{app}, and from this an apparent free energy of denaturation, ΔG_{app}, using

$$K_{app} = e^{-\Delta G_{app}/RT} = ([m']_{eq} - [m']_n)/([m']_d - [m']_{eq}) \quad (1)$$

where $[m']_{eq}$ is the observed rotation, and $[m']_n$ and $[m']_d$ represent the rotations which the native and denatured states would have under the conditions where $[m']_{eq}$ is measured. The values of $[m']_n$ and $[m']_d$ were obtained by extrapolating the linear portions of the denaturation curves into the transition region. Values of ΔG_{app} calculated in this way are shown plotted as a function of Gdn·HCl concentration in Figure 3. For all three proteins, ΔG_{app} varies linearly with Gdn·HCl concentration. A least-squares analysis was used to fit the data to the equation

$$\Delta G_{app} = \Delta G_{app}^{H_2O} - m(\text{Gdn·HCl}) \quad (2)$$

Values of $\Delta G_{app}^{H_2O}$ and m are given in Table I.

[2] The dependence of $[m']_{300\,nm}$ on pH for GuHCl denatured ferricytochrome *c* is interesting. The value of $[m']$ goes through a minimum near pH 4.5 and increases at both higher and lower pH values. Ionization of the histidyl ligands of the heme iron and possibly the heme carboxyls may contribute to the observed variation.

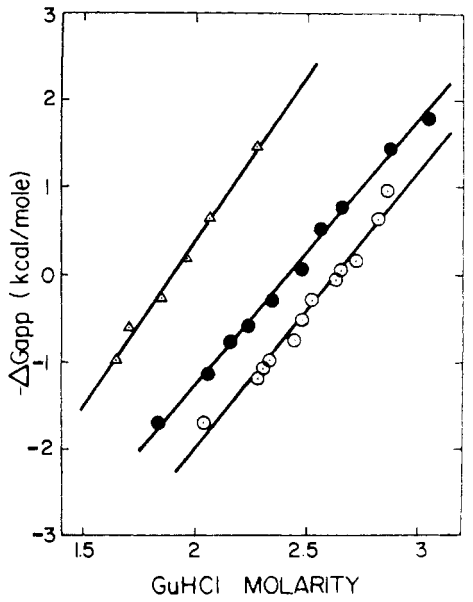

FIGURE 3: ΔG_{app} for the denaturation of candida (△), horse (●), and cow (○) ferricytochrome *c* as a function of Gdn·HCl molarity. ΔG_{app} was calculated with eq 1 using the data in Figure 1.

If we were able to correct for the presence of intermediates and calculate ΔG_D, the free-energy change for a two-state mechanism, how would the plots compare with those in Figure 3? Tanford (1968) has shown that if, as seems likely, the properties of the intermediates vary in rough proportions to the extent of unfolding, then the slope of the plots in Figure 3 would be less than the slope of plots for a two-state mechanism. Thus, the values of $\Delta G_{app}^{H_2O}$ and *m* are lower limits for the values expected for a two-state transition.

We have estimated the difference in stability between the proteins by multiplying the difference between midpoints of the denaturation curves by the values of *m* given in Table I. We find that cow ferricytochrome *c* is 650 cal/mol more stable than horse and 2300–2800 cal/mol more stable than *Candida*. For the reasons discussed above, these estimates represent the minimum difference in stability between the proteins. It is important to note that these are the differences in stability under conditions near the midpoint of the transition, *i.e.*, at moderately high Gdn·HCl concentrations. The high ionic strength would tend to minimize differences in stability arising from electrostatic interactions.

We have designated the intercepts of the plots in Figure 3 as $\Delta G_{app}^{H_2O}$. This is just one method of estimating the free energy of denaturation from this kind of data and it assumes that the linear variation observed in the transition region will extend to zero concentration of denaturant. Another method of estimating $\Delta G_{app}^{H_2O}$ assumes that denaturation results from denaturant binding which leads to the equation

$$\Delta G_{app} = \Delta G_{app}^{H_2O} - (\Delta n)RT \ln (1 + ka_{\pm}) \quad (3)$$

where *k*, $a_{\pm}$, and Δn represent, respectively, the binding constant of the denaturant to the protein, the mean ion activity of the denaturant, and the difference in the number of denaturant molecules bound to the denatured and native states of the molecule (Aune and Tanford, 1969). We have determined the values of $\Delta G_{app}^{H_2O}$ and Δn which give the best fit to our experimental data using $a_{\pm}$ values from Aune and Tanford (1969) and $k = 1.20$, a value which has been used in evaluating $\Delta G_{app}^{H_2O}$ by this procedure for lysozyme (Aune and Tanford, 1969), ribonuclease (Salahuddin and Tanford, 1970), myoglobin (Puett, 1973), and α-chymotrypsin (R. F. Greene, personal communication). The results of this analysis are given in Table II along with similar data for the proteins above mentioned. It is interesting that for this diverse group of proteins the estimates of $\Delta G_{app}^{H_2O}$ all fall within a few kilocalories per mole of one another. There is as much variation among the homologous proteins as among the proteins which are unrelated in amino acid sequence. The similarity between the values for the cytochromes *c* and the other proteins offers some hope that our analysis in terms of a two-state mechanism does not introduce large errors. For lysozyme, ribonuclease, and myoglobin, Gdn·HCl denaturation is known to approach a two-state mechanism (Tanford, 1970).

Acid Denaturation. The midpoints of the acid denaturation curves in Figure 2 occur at pH 2.44 for cow, pH 2.62 for horse, and pH 2.63 for *Candida* ferricytochrome *c*. The data were analyzed as described above and the results are shown in Figure 4 where ΔG_{app} is plotted as a function of pH. Near the midpoint of the transitions ($\Delta G_{app} = 0$), cow ferricytochrome *c* is about 720 cal/mol more stable than the other two proteins which have very similar stabilities.

The conditions here differ considerably from the conditions in the Gdn·HCl denaturation experiments. Here the molecules have a large net positive charge, approaching 20, and the ionic strength is very low. The agreement between the estimates of the difference in stability between horse and cow cytochrome *c* suggests that the difference in stability does not result from electrostatic effects.

Both the Gdn·HCl (Ikai *et al.*, 1973) and acid (Babul and Stellwagen, 1972) denaturation of horse ferricytochrome *c*

TABLE I: Parameters Characterizing the Gdn·HCl Denaturation of Ferricytochromes *c* at pH 6.5, 25°.

Protein	$(Gdn \cdot HCl)_{1/2}$[a] (M)	$\Delta G_{app}^{H_2O}$[b] (kcal/mol)	*m*[b] (cal/mol M^{-1})
Cow	2.63	8.38	3190
Horse	2.42	7.27	3010
Candida	1.89	7.24	3820

[a] The midpoint of the denaturation curve ($\Delta G_{app} = 0$). [b] From eq 2.

TABLE II: Analysis of the Gdn·HCl Denaturation of Several Proteins by Eq 3.

Protein	$\Delta G_{app}^{H_2O}$[a] (kcal/mol)	Δn[a]
Cytochrome *c*		
Cow	15.4	30.4
Horse	12.7	26.4
Candida	14.0	32.9
Myoglobin[b]		
Horse	11.8	30
Sperm whale	14.3	30
Ribonuclease[c]	13.0	24.4
Lysozyme[d]	14.2	21.5
α-Chymotrypsin[e]	12.3	29.3

[a] From eq 3. [b] Puett (1973). [c] Salahuddin and Tanford (1970). [d] Aune and Tanford (1969). [e] R. F. Greene, personal communication.

have been studied under conditions identical to ours but employing different properties for following denaturation. In both cases the midpoints of the curves, 2.50 M Gdn·HCl and pH 2.50, indicated that their protein might be slightly more stable than ours. This, plus the difficulty encountered in explaining why cow is more stable than horse cytochrome *c*, prompted us to take a careful look at our protein samples.

Analytical polyacrylamide gel electrophoresis revealed that our samples of horse and cow cytochrome *c* contained the small amounts of altered, probably deamidated (Flatmark, 1966b) cytochrome *c* normally observed (Flatmark, 1964b). No other proteins could be detected even when samples of over 100 μg were applied to the gels. In addition, for both horse and cow cytochrome *c* identical results were obtained on two different lots of each protein. Thus, we feel the discrepancies noted above probably represent real differences.

For non two-state transition there is no guarantee that the midpoints will coincide when different physical properties are used for following denaturation (Lumry *et al.*, 1966). To investigate this possibility we followed the acid denaturation of the three proteins by measuring the absorbance at 395 nm. The midpoints of these curves were found to be pH 2.32 for cow, pH 2.47 for horse, and pH 2.50 for *Candida*. All of these midpoints are from 0.12 to 0.15 pH unit lower than those found from the curves in Figure 2. Note that the midpoint for horse cytochrome *c* is now in good agreement with the value obtained by Babul and Stellwagen (1972) by the same technique. Thus, it appears that this is a case where the presence of stable intermediate states can be detected by using different physical properties for following denaturation.

Changes in the optical rotation at 300 nm are expected when any part of the polypeptide chain unfolds, while changes in the absorbancy at 395 nm will reflect more specifically only changes in the environment of the heme. Thus, it is probably reasonable that the percentage unfolding as revealed by the absorbancy measurements lags behind the percentage unfolding as revealed by the optical rotation measurements.

The absorbancy data are less accurate than the data in Figure 2, but when they are analyzed by the same procedures very similar results are obtained. The stabilities of horse and *Candida* cytochrome *c* are very similar, while cow cytochrome *c* is about 720 to 800 cal per mol more stable than the other two proteins.

Discussion

The analysis of the Gdn·HCl denaturation curves led to an estimate of the differences in stability under the conditions which exist near the midpoint of the transition, *i.e.*, pH 6.5, 2–3 M Gdn·HCl, high ionic strength. We are most interested in obtaining an estimate of the differences in stability in water, at neutral pH, and moderate ionic strengths. The major effect of the high ionic strength will be to minimize electrostatic interactions among the charged groups on the surface of the proteins. However, even physiological ionic strengths (≅0.15 M) are high enough to largely eliminate these interactions and it is unlikely that the differences in stability would be changed appreciably by lowering the ionic strength from 2–3 to 0.15 M (Tanford and Roxby, 1972).

The estimates of $\Delta G_{app}^{H_2O}$ from either eq 2 (Table I) or eq 3 (Table II) suggest that in the absence of denaturant *Candida* ferricytochrome *c* may be as stable or more stable than horse ferricytochrome *c*. This results because the dependence of ΔG_{app} on denaturant concentration, the *m* values in Table I, is much greater for *Candida* than for the other two proteins.

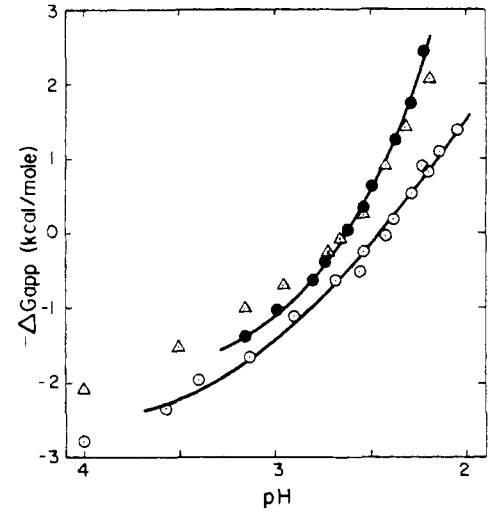

FIGURE 4: ΔG_{app} for the denaturation of *Candida* (△), horse (●), and cow (○) ferricytochrome *c* as a function of pH. ΔG_{app} was calculated with eq 1 using the data in Figure 2.

At least in part, this larger value of *m* is observed because in *Candida* more residues are freshly exposed to denaturant by unfolding. Denaturation by Gdn·HCl occurs because almost all of the constituent parts of a protein have a lower free energy in Gdn·HCl solution than in water (Nozaki and Tanford, 1970). Thus, the larger the portion of the polypeptide chain that becomes freshly exposed to denaturant, the larger the value of *m* expected.

The similarity of the functional properties of *Candida* cytochrome *c* to those of the other two proteins suggests that it possesses a similar conformation. If so, the five additional residues it contains can contribute to an enhanced value of *m* directly, and also by burying some residues on the surface which might otherwise have been exposed to solvent. An idea of the magnitude of the effect on *m* can be obtained by procedures outlined by Tanford (1970) using model compound data reported by Nozaki and Tanford (1970). If 5 more peptide units were being exposed to solvent in the denaturation of *Candida* than in the denaturation of the other two proteins, the value of *m* would be increased by about 250 cal/mol M^{-1}. Since there are likely to be some side chains buried along with the peptide groups, it seems apparent that this could explain the larger value of *m* observed for *Candida* cytochrome *c*.

Another factor which may contribute to the variation in the *m* values is a difference in mechanism. If the mechanism of denaturation for *Candida* cytochrome *c* is closer to a two-state mechanism than for the other two proteins, then, as explained in Results, a larger value of *m* would be expected.

Thus, in the absence of denaturant we would expect the difference in stability between *Candida* and the other two proteins to be considerably smaller than the value of 2300 cal/mol found in Gdn·HCl. Recall that the analysis of the acid denaturation curves showed that the stabilities of horse and *Candida* cytochrome *c* were very similar at low pH and low ionic strength. *Candida* cytochrome *c* has the same number of acidic groups as horse and cow cytochrome *c*, but has 4 and 3, respectively, less basic groups (Dayhoff, 1972). Consequently, at low pH the electrostatic repulsion between the positively charged groups on *Candida* cytochrome *c* should be less than for the other two proteins. This suggests that at neutral pH horse should be more stable than *Candida* ferricytochrome *c*. Considering all of the evidence, we think that under physiological conditions *Candida* is less stable than horse ferricytochrome *c* but probably not by more than 1 kcal/mol. The fact

TABLE III: Differences in Sequence between Horse and Cow Cytochrome *c*.

	Sequence Position		
Cytochrome *c*	47	60	89
Horse	Thr	Lys	Thr
Cow	Ser	Gly	Gly

that the stability of *Candida* cytochrome *c* does not differ markedly from the stability of the other two proteins, despite the large differences in amino acid sequence, surely indicates that the amino acid substitutions compensate one another to keep the stability within tolerable limits.

For horse and cow cytochrome *c* similar estimates of the difference in stability are obtained from both the Gdn·HCl and the acid denaturation studies. Here the difference in the *m* values in Table I probably reflects a difference in mechanism since the three differences in amino acid sequence all occur at residues on the surface of the molecules exposed to solvent. Thus, it seems likely that under physiological conditions cow is about 700 cal/mol more stable than horse ferricytochrome *c*.

Horse, bonito, and tuna ferricytochrome *c* have very similar conformations with the differences among the molecules confined to those expected on the basis of the amino acid side-chain differences (Takano *et al.*, 1973). Since cow cytochrome *c* differs less in sequence from horse than do the bonito and tuna proteins, it seems likely that the conformation of cow ferricytochrome *c* will be similar to that of horse. The three differences in sequence between horse and cow cytochrome *c* are shown in Table III. As noted, all three of these residues are found on the surface of the molecule in horse ferricytochrome *c* (Takano *et al.*, 1973). The two Thr residues and the Lys residue do not appear to form intramolecular hydrogen bonds on the basis of the crystallographic structure. However, nuclear magnetic resonance studies have suggested that Lys-60 is hydrogen bonded, perhaps to Glu-62, in horse ferrocytochrome *c* (Stellwagen and Shulman, 1973) and, with less certainty, in ferricytochrome *c* (E. Stellwagen, personal communication).

With the detailed information available it seemed that a reasonable explanation for the difference in stability between these two proteins should be possible; this has not proven to be the case. We have considered the following: (1) steric effects in the native proteins; (2) steric effects in the denatured proteins (*e.g.*, the two Gly residues in cow cytochrome *c* will increase the flexibility and, hence, the conformational entropy (Némethy *et al.*, 1966) of the denatured state); (3) hydrogen bonding; (4) electrostatic interactions involving the ϵ-amino of Lys-60. All of the factors considered either suggest that horse should be more stable than cow ferricytochrome or that they will have no effect on the stability. At present we can offer no reasonable explanation for the fact that cow cytochrome *c* is found experimentally to be a minimum of 700 cal/mol more stable than horse cytochrome *c*. Donkey cytochrome *c* is intermediate in structure between horse and cow cytochrome *c* and a study of its denaturation might provide a clue to the explanation.

On the basis of a limited number of studies, it appears that the native, globular state of proteins is only from 5 to 15 kcal per mol more stable than unfolded states under physiological conditions (Tanford, 1970). If the proteolytic degradation involved in protein turnover is controlled in part by the equilibrium between native and unfolded states, then differences in stability, such as those observed here, could cause a significant change in the steady-state concentration of a protein. In this way, an amino acid substitution might influence the evolution of the protein even though it exerts no direct effect on the function of the protein. Two of the differences in sequence between horse and cow cytochrome *c* occur at hypervariable positions (Margoliash, 1971). For 51 different cytochromes *c*, eight different amino acids have been found at position 60 and nine at position 89 (Margoliash, 1971). It is very unlikely that these residues are directly involved in the function of the protein but our studies suggest that substitutions at these positions may influence the stability of cytochrome *c*.

Acknowledgments

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