Conformational Transitions of Frog Heart Ferricytochrome *c*†

David N. Brems, Robert Cass, and Earle Stellwagen*

ABSTRACT: A monomeric cytochrome *c* containing an intramolecular disulfide bond linking residues 20 and 102 was obtained from bullfrog hearts, as previously reported by Chan et al. [Chan, S. K., Walasek, O. F., Barlow, G. H., & Margoliask, E. (1967) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 26*, 723]. The stability of the native globular conformation of this protein is enhanced relative to that of other cytochromes *c*; e.g., it is stable to 0.01 N HCl in the absence of neutral salts at 25 °C, and its denaturation transition in guanidine at neutral pH and 25 °C is centered at 3.8 M. Guanidine-denatured frog cytochrome *c* renatures in two kinetic phases whose time constants differ by about 2 orders of magnitude as observed when using other cytochromes *c*. However, the absolute values for the time constants of the frog protein are notably smaller. We note that the time constants for cytochromes *c* are inversely related to the midpoints of their guanidine transitions, suggesting that within a homologous series of proteins the more stable the conformation the faster it folds.

Both sequence (Dickerson & Timkovich, 1975) and crystallographic (Almassy & Dickerson, 1978) comparisons suggest that the cytochromes *c* form a homologous series of proteins having a common polypeptide fold. Of all known sequences of cytochromes *c*, only that of bullfrog contains two cysteine residues in addition to the two cysteinyl residues linking the heme to the polypeptide chain by thioether bridges. Chan et al. (1967) have shown that the two additional cysteinyl residues in bullfrog cytochrome located at positions 20 and 102 can form an intramolecular disulfide bond. The crystallographic model of tuna cytochrome *c* (Takano et al., 1973) indicates that such an intramolecular disulfide bond should not distort the polypeptide fold of the native structure. This report describes an investigation of the contribution of the intramolecular disulfide bond linking the N and C termini of cytochrome *c* to the kinetics of protein folding and to the stability of the folded product.

Experimental Procedures

Materials. Bullfrog hearts were purchased from Gulf-South Biologicals, Inc., Ponchatoula, LA. Cytochrome *c* was prepared from these hearts by using the procedure of Margoliash & Walasek (1967). About 2 μmol of cytochrome *c* was obtained/kg wet weight of frog hearts. The amount of frog cytochrome *c* available to us was rather limited since a typical bullfrog heart weighs about 0.4 g, necessitating judicious programming of the experimentation to be pursued. Cytochrome *c* purified from horse heart (type VI), cow heart (type V), and tuna heart (type XI) and from the yeast *Saccharomyces cerevisiae* (type VIII) was purchased from Sigma. The disulfide dimer of *Saccharomyces* cytochrome *c* was obtained by treatment with oxidized dithiothreitol prior to fractionation from the monomeric form using Sephadex G-50. *Candida krusei* cytochrome *c* was purchased from Sankyo, Tokyo. The concentration of all cytochromes was measured spectrophotometrically by using the extinctions reported by Margoliash & Frohwirt (1959).

Methods. Absorbance spectra were obtained by using a Cary Model 15 recording spectrophotometer. Fluorescence emission spectra were obtained by using a Hitachi Model MPF-2A fluorometer and an excitation wavelength of 280 nm. Circular dichroic spectra were obtained by using a Cary Model 60 spectropolarimeter having a circular dichroic attachment. Kinetic measurements were made by using a Durrum-Gibson stopped-flow spectrophotometer having a fluorescence attachment. Kinetic measurements are reported as time constants which are reciprocals of the apparent rate constants. Polyacrylamide slab gel electrophoresis was done in solvents containing both urea and sodium dodecyl sulfate as described by Swank & Munkres (1971). The reactivity of cysteine sulfhydryl groups was examined by reaction of cytochrome with a 28-fold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid) in 100 mM Tris-HCl buffer, pH 8.1, at 25 °C in both the presence and absence of 10% sodium dodecyl sulfate. The course of the reaction was observed at 412 nm. Samples of cytochrome for amino acid analysis were subjected to performic acid oxidation (Hirs, 1956) prior to hydrolysis in 6 N HCl for 24 h at 110 °C.

Results

Polymerization. Our preparations of frog heart cytochrome *c* are frequently resolved into two components by exclusion chromatography with Sephadex G-50 using either 100 mM phosphate buffer, pH 7.0, or 7% formic acid as the equilibration solvent. A typical elution profile is shown in Figure 1 with the components labeled D and M in order of elution. The relative areas associated with these two components in the elution profiles of different preparations of frog cytochrome *c* vary considerably. The visible and ultraviolet absorbance spectra of both the ferri and ferro forms of components D and M are equivalent with respect to the wavelength of absorbance maxima and the ratios of absorbances for the maxima of a given form at neutral pH. As shown in Table I, the elution volumes for components M and D of frog cytochrome in 100 mM phosphate, pH 7.0, correspond with those observed by using the monomer and disulfide dimer forms of *Saccharomyces* iso-1 cytochrome *c*. In contrast to the frog protein which has cysteine residues at positions 20 and 102, the yeast protein has only a cysteine at position 102 (Narita & Titani, 1969). Accordingly, the yeast protein can only form a disulfide dimer. While the yeast and frog cytochromes *c* have common elution profiles using Sephadex G-50 chromatography at pH 7.0, the elution profile of the yeast protein but not that of the frog protein is displaced toward smaller volumes in acid solvents as shown in Table I. While both the yeast monomer and disulfide dimer are denatured in these acidic solvents (Y. C.

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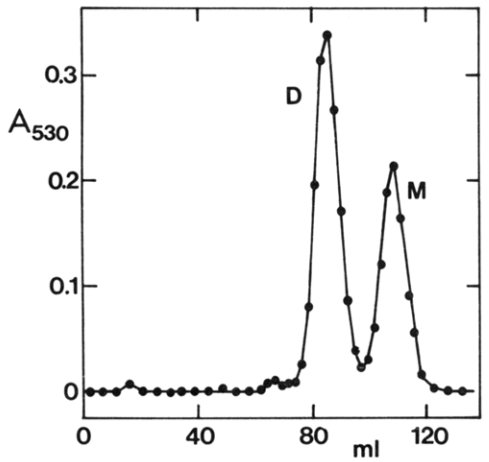

FIGURE 1: Exclusion chromatographic elution profile for frog cytochrome *c*. About 6 mg of the frog protein obtained from a dialyzed concentrated supernatant containing 70% ammonium sulfate was applied to a 1.5 × 118 cm column of Sephadex G-50 equilibrated and developed by using 100 mM phosphate buffer, pH 7.0, at room temperature. Material that contained between 76 and 92 mL inclusive was pooled, concentrated, and termed component D. Material that contained between 101 and 115 mL was pooled, concentrated, and termed component M.

Table I: Elution Volumes for Sephadex G-50 Chromatography[a]

cytochrome *c*	component	pH	elution vol (mL)
frog	M	neutral	113
	D		88
yeast	monomer		113
	dimer		89
frog	M	acidic	106
	D		84
yeast	monomer		85
	dimer		62

[a] All chromatography was done by using a 1.5 × 118 cm column of Sephadex G-50 equilibrated and developed with either 100 mM phosphate buffer, pH 7.0 (neutral pH), or 7% formic acid (acidic pH).

Liu, R. Cass, and E. Stellwagen, unpublished experiments), components M and D of the frog protein are not, as will be shown below.

Component M isolated from the Sephadex G-50 elution profile of the frog protein migrates as a single electrophoretic band when examined by polyacrylamide gel electrophoresis in a denaturing solvent devoid of a reducing reagent. As shown in Figure 2, the mobility of component M is slightly greater than that of horse heart cytochrome *c*, a protein having the same chain length as monomeric frog cytochrome *c*, a covalently attached heme, but unlike the frog protein, no potential for an intramolecular disulfide bond. The increased mobility of frog cytochrome *c* relative to that of the horse protein is consistent with the presence of an intramolecular disulfide bond which would prevent attainment of as random a structure as that achieved by the horse protein, thereby giving an artificially low molecular weight of 9.5×10^3. A similar situation pertains in the electrophoretic analysis of component D in the frog heart cytochrome preparation. The mobility of this component corresponds to a molecular weight of 21×10^3 and is somewhat greater than that observed for the disulfide dimer of yeast iso-1 cytochrome *c*. The increased relative mobility is likely due in part to the extra five residues in each chain of the yeast protein and in part to the conformational constraint of two intermolecular disulfide bonds available to the frog dimer as opposed to a single intermolecular disulfide in the yeast protein.

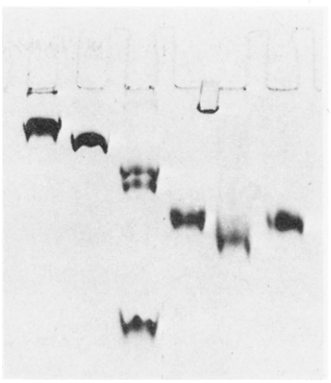

FIGURE 2: Polyacrylamide electrophoresis. The polyacrylamide slab was polymerized from a solution containing 12.5% acrylamide and 1.25% bis(acrylamide). All solvents contained urea and 0.1% sodium dodecyl sulfate. The samples reading from left to right contained the following: lane 1, the disulfide dimer of yeast iso-1 cytochrome *c*; lane 2, component D of frog cytochrome *c*; lane 3, β-lactoglobulin, ribonuclease, and the 66–104 fragment from horse cytochrome *c*; lane 4, monomeric yeast iso-1 cytochrome *c*; lane 5, component M of frog cytochrome *c*; lane 6, horse cytochrome *c*. The apparent band between lanes 4 and 5 is a photographic artifact resulting from the position of the sample well divider.

Chemical Analyses. Two separate preparations of bullfrog cytochrome *c* were individually subjected to performic acid oxidation, acid hydrolysis, and amino acid analysis. Horse cytochrome *c* was subjected to the same protocol to serve as an analytical control. Each protein hydrolysate was analyzed in triplicate, and the mean value for the number of each residue per chain and its standard deviation were calculated for each protein. The mean value for the content of each amino acid stable to performic acid oxidation and acid hydrolysis was within one standard deviation of the reported composition for each protein (Margoliash et al., 1961; Chan et al., 1972). In particular, the frog protein was found to contain 4.0 ± 0.4 cysteic acids per chain by this procedure, providing for two cysteine residues in addition to the two linked to the heme in thioether bridges.

Reaction of component M with a 25-fold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid) failed to increase the absorbance at 412 nm in the presence or absence of detergent. These results indicate that cysteines-20 and -102 are bridged by an intramolecular disulfide bond. Since conformational analysis (see below) predicts that the intramolecular disulfide bond in the frog protein is buried, we attempted to reduce the disulfide in a denaturing solvent. However, it was observed that the Soret absorbance of frog ferricytochrome *c* rapidly (minutes) disappeared upon incubation of the protein in 100 mM β-mercaptoethanol, 6 M guanidine hydrochloride, and 100 mM Tris-HCl buffer, pH 8.2, at 25 °C. A similar result was obtained by using horse ferricytochrome *c*, indicating a direct reaction involving an exposed heme. Since limited quantities of frog cytochrome *c* are availabe to us, we have not had opportunity to search for conditions which will selectively reduce the disulfide bond.

Absorbance Spectral Measurements. The visible absorbance spectra of both the ferri and ferro forms of frog cytochrome *c* at neutral pH are identical with those of horse heart cytochrome *c* (Margoliash & Frohwirt, 1959) with respect to the wavelengths of maximal absorbance and the absorbance ratio of various maxima. Accordingly, native frog cytochrome *c* is a low spin coordination complex having two strong field protein ligands in the axial coordination positions of the heme iron. The ferri form exhibits a weak, $\epsilon = 10^3\ M^{-1}\ cm^{-1}$, charge transfer band centered at 698 nm, indicating that one of the

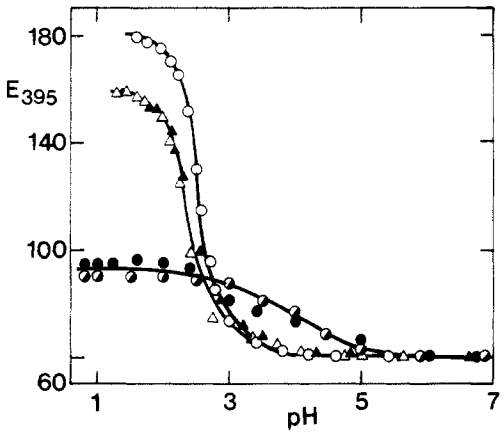

FIGURE 3: Change in Soret absorbance accompanying acidification. Incremental additions of HCl were made to solutions of cytochromes *c* at 25 °C, and the absorbance at 395 nm was measured. (○) Horse protein in water; (◑) horse protein in 3 M NaCl; (●) frog protein in water; (△) cow protein in water; (▲) tuna protein in water.

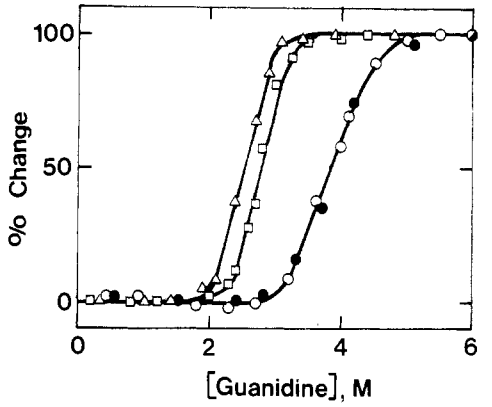

FIGURE 4: Fluorescence intensity changes. The open symbols denote mixtures of ferricytochrome *c* in 100 mM phosphate buffer, pH 7.0, with concentrated guanidine hydrochloride in the buffer to give the indicated final concentrations of denaturant. The closed symbols denote mixtures of ferricytochrome *c* in 6 M guanidine hydrochloride and 100 mM phosphate buffer, pH 7.0, with buffer to give the indicated final concentration of denaturant. (○; ●) Frog ferricytochrome *c*; (△) horse cytochrome *c*; (□) tuna cytochrome *c*. All measurements were made at 25 °C at equilibrium.

axial ligands is the sulfur of methionine-80 (Schechter & Saludjian, 1967).

Acidification of an aqueous solution of frog ferricytochrome *c* in the absence of salt systematically shifts the Soret maximum from 409 to 401 nm over the pH range 2–6. Spectra collected within this range exhibit an isosbestic point at 407 nm, indicating a two-state transition. This spectral change was measured at 395 nm as a function of pH in order to corrrelate it with changes so observed for other cytochromes *c*. The increase in absorbance of frog cytochrome *c* solutions with acidification describes a single transition having a midpoint at pH 3.8 and a $\Delta\epsilon_{395}$ of 35 mM^{-1} cm^{-1}, as shown in Figure 3. By contrast, acidification of solutions of horse ferricytochrome *c* in the absence of neutral salt shifts the Soret maximum from 409 nm at neutral pH to 395 nm at low pH. The observed increase in absorbance at 395 nm describes a large cooperative transition having a midpoint at pH 2.5 and a $\Delta\epsilon_{395}$ of 100 mM^{-1} cm^{-1}, as shown in Figure 3 and reported previously (Babul & Stellwagen, 1972). Similar changes are observed by using cow (Knapp & Pace, 1974) and tuna ferricytochromes *c* as illustrated in Figure 3. The pH-dependent increase in 395-nm absorbance of horse ferricytochrome in the absence of neutral salt is coincident with the gross denaturation of the native conformation and with the dissociation of both protein axial ligands for the heme iron, histidine-18 and methionine-80 (Babul & Stellwagen, 1972). However, acidification of horse ferricytochrome solutions in the presence of relatively high concentrations of neutral salt limits the extent of the Soret blue shift to 401 nm, markedly diminishes the $\Delta\epsilon_{395}$, and increases the midpoint of the spectral transition observed at 395 nm as shown in Figure 3. This less cooperative transition, involving a single proton, increases the exchange rate of the methionine-80 ligand but neither perturbs the histidine-18 ligation nor significantly alters the native conformation (Stellwagen & Babul, 1975; Dyson & Beattie, 1981). As shown in Figure 3, the 395-nm transition of the frog protein in the absence of neutral salt is virtually coincident with that of the horse protein in the presence of neutral salt. Since the far-ultraviolet circular dichroic spectrum and tryptophan fluorescence of frog ferricytochrome *c* are not altered by acidification in the absence of neutral salt as will be shown below, we conclude that the gross features of the native conformation of the frog protein are particularly stable to acidification. This stability includes exposure to 7% formic acid since the Soret maximum in this solvent occurs at 401 nm. By contrast, addition of 6 M guanidine to a solution of frog ferricytochrome *c* produces a blue shift in the Soret absorbance characteristically observed for other cytochromes *c* such as the horse protein (Ikai et al., 1973).

Fluorescence Measurements. The fluorescence intensity of the single tryptophan in frog cytochrome *c*, tryptophan-59, measured in 100 mM phosphate buffer, pH 7.0, is over 75% quenched. Such quenching is commonly observed for native cytochromes *c* and results from the proximity and orientation of the buried tryptophan-59 and the buried heme in the native structure (Fisher et al., 1973). In contrast to the horse protein, acidification of a solution of frog ferricytochrome *c* to pH 2.0 in the absence of salt does not increase the tryptophan fluorescence. However, the presence of high concentrations of guanidine hydrochloride in buffered solutions of frog ferricytochrome *c* at neutral pH increases tryptophan fluorescence. The change in fluorescence intensity is reversible, having a midpoint at 3.8 M guanidine hydrochloride as shown in Figure 4. By contrast, the midpoint for the horse cytochrome *c* intensity change occurs at 2.6 M guanidine hydrochloride as shown in Figure 4 and as reported previously (Tsong, 1976). The midpoint for the conformational transition of cow ferricytochrome *c* occurs at the same concentration (Knapp & Pace, 1974). The midpoint for the conformational transition of tuna ferricytochrome *c* occurs at 2.8 M as shown in Figure 4.

Circular Dichroic Measurements. The far-ultraviolet circular dichroic spectrum of frog ferricytochrome *c* in 100 mM phosphate buffer, pH 7.0, is characteristic for cytochromes *c* having about 50% helicity and no β structure. Acidification of an aqueous solution of the frog protein to pH 2.0 with HCl in the absence of salt does not change the circular dichroic spectrum as shown in Figure 5. By contrast, the dichroic spectrum of the horse protein in 0.01 N HCl is that typical of a random coil (Babul & Stellwagen, 1972). Such a spectrum is observed for the frog protein in the presence of 6 M guanidine hydrochloride at neutral pH as shown in Figure 5.

Kinetic Measurements. The kinetics of the refolding of frog ferricytochrome were measured in 2.5 M guanidine hydrochloride, a concentration outside the equilibrium transition zone shown in Figure 4. Two kinetic phases were observed by monitoring changes in either Soret absorbance or tryptophan fluorescence upon rapid dilution of the guanidine-denatured protein. A typical result is illustrated in Figure 6. The time constants for each phase and the fractional change

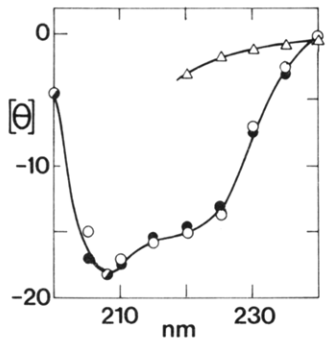

FIGURE 5: Far-ultraviolet circular dichroic spectra of frog cytochrome *c*. The molar ellipticity, [θ], has the units deg cm^2 dmol^{-1} 10^{-3}. (○) In water, pH 7.0; (●) in water adjusted to pH 1.6 with HCl; (△) in 6 M guanidine hydrochloride and 100 mM phosphate buffer, pH 7.0. The line connecting the open and filled circles represents the dichroic spectrum obtained from neutralization of the pH 1.6 sample using NaOH. The (△) spectrum could not be obtained below 220 nm because of the contribution by guanidine.

Table II: Kinetic Measurements

cytochrome *c*	measurement	time constant (s) fast phase	time constant (s) slow phase	fast phase (%)
frog	fluorescence[a]	0.040	5.8	89
	absorbance[a]	0.015	12.7	85
	average	0.027 ± 0.013	9.2 ± 3.5	87 ± 2
horse	fluorescence[a]	0.210	11.9	84
	absorbance[a]	0.253	14.8	73
	average	0.232 ± 0.022	13.3 ± 1.5	78 ± 6
horse	fluorescence[b]	0.160	22	86
	absorbance[c]	0.176 ± 0.039	17 ± 2	77 ± 4

[a] Measurements were made by rapid dilution of a micromolar solution of ferricytochrome *c* in 100 mM phosphate buffer, pH 7.0, containing 5.0 M guanidine hydrochloride with an equal volume of 100 mM phosphate buffer, pH 7.0. [b] Measured in 100 mM Tris buffer, pH 7.0, at 25 °C in 1.6 M guanidine (Tsong, 1976). [c] Measured in 1.0–1.6 M guanidine at 25 °C and pH 6.5 (Ikai et al., 1973).

associated with the fast phase are given in Table II. The magnitude of these values observed by using horse cytochrome *c* in the same protocol is also shown in Table II. Since 2.5 M guanidine places the horse protein nearly in the midst of its transition zone, we also list the kinetic values measured for the horse protein in 1.0–1.6 M guanidine, a concentration range as remote from its transition zone as 2.5 M guanidine is from the transition zone of the frog protein. These comparisons indicate that the kinetic pattern characteristic for the refolding of guanidine-denatured horse cytochrome *c* is also observed by the frog protein. The time constant for the fast kinetic phase in the refolding of the frog protein is about 5–10 times shorter than that for the horse protein, while the time constant for the slow phase is only twice as short.

Structural Comparisons. Of the known cytochrome *c* sequences, the sequence of cow cytochrome *c* is most similar to that of the frog protein, differing at the 11 positions listed in Table III. The sequence of horse cytochrome *c* differs from the frog sequence at an additional 3 positions as shown in Table III. The sequence of tuna cytochrome *c* differs from that of the frog protein at 15 positions, 7 of which are positional differences between the frog and horse proteins as shown in Table III. Since (1) the cow and horse proteins have very similar stability to acid and guanidine denaturation (Knapp & Pace, 1974), (2) the most detailed solution measurements have been done by using the horse protein, and (3) a high-resolution crystallographic model is only available for the tuna protein (Takano et al., 1977), we will confine our comparisons

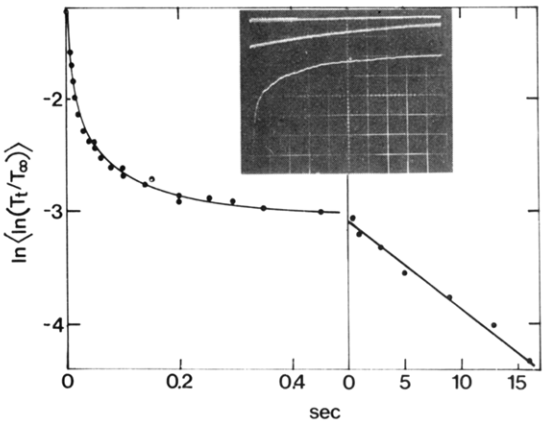

FIGURE 6: Kinetics of protein refolding. A solution of frog ferricytochrome *c* in 5.0 M guanidine hydrochloride and 100 mM phosphate buffer, pH 7.0, was rapidly mixed with an equal volume of buffer, and the percent transmission at 405 nm was observed as a function of time. The ordinate on the photograph represents a potential of 20 mV/division. The lower trace on the photograph was obtained at 50 ms/division and the intermediate trace at 2 s/division. The upper trace represents the percent transmission at the completion of the reaction. First-order plots of these transmission (*T*) changes are shown in the major portion of the figure.

Table III: Sequence Comparisons[a]

sequence position	residue in cytochrome sequence frog	cow	horse	tuna	side-chain exposure Å^2	%	net Δg_{tr} (kcal/mol)
20	C	V	V	V	7	5	−1.5
28	V	T	T		57	43	+1.5
33	Y	H	T	W	83	36	+2.5
36	I	F	F	F	21	11	0
44	A	P	P	E	109	73	−2.1
47	S		T		75	91	0
60	G		K	N	56	46	−1.5
62	D	E	E		81	70	0
89	G		T		–	–	0
92	Q	E	E		75	50	0
100	S	K	K		46	56	−1.5
102	C	T	T	T	0	0	0
103	S	N	N		89	100	0
104	K	E	E	· –	–	–	+1.5

[a] Residues are identified in the sequences of cow (Nakashima et al., 1966), horse (Margoliash et al., 1961), and tuna (Kreil, 1965) cytochromes *c* only if they differ from that in the aligned sequence of frog cytochrome *c* (Chan et al., 1972). It should be noted that the tuna cytochrome *c* sequence differs from that of frog cytochrome *c* at eight additional positions. Side-chain exposure was calculated (Stellwagen, 1978) from the crystallographic coordinates of the tuna protein. Percentage exposure indicates the exposure of the side chains listed in the tuna protein relative to the exposure of the same amino acid occupying the central position in the tripeptide GXG as reported by Shrake & Rupley (1973). The net free energy of transfer, net Δg_{tr}, indicates the net contribution of the residue in the frog sequence relative to that of the horse sequence toward stabilization of the native structure by assuming optimal side-chain exposure. A positive sign predicts preferential stability of the frog protein. Δg_{tr} values were taken from Nall & Landers (1981).

to the frog, horse, and tuna proteins. For these comparisons, we assume that all native cytochromes *c* have the same polypeptide fold (Almassy & Dickerson, 1978). Using free energy of transfer values in the manner of Nall & Landers (1981), we would expect that the sequence differences between the horse and frog proteins would preferentially stabilize the horse conformation by about 1 kcal/mol, assuming all the side chains considered are maximally buried. However, analysis (Stellwagen, 1978) of the crystallographic model of tuna cytochrome *c* indicates that the side chains of only 3 of the 14

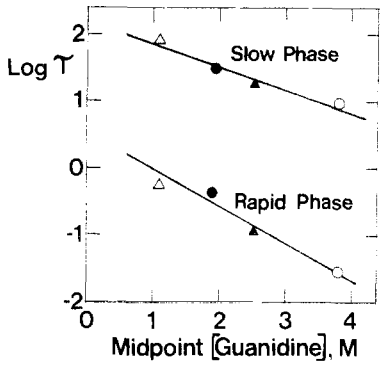

FIGURE 7: Dependence of refolding kinetics on equilibrium stability. The time constants of horse (Ikai et al., 1973) (▲) and *Saccharomyces* iso-2 (△) (Nall & Landers, 1981) cytochromes *c* are average values below the transition region measured in the absence of buffer at pH 6.5 and 25 °C and in 100 mM phosphate buffer, pH 7.2, at 20 °C, respectively. Time constants for frog (○) and *Candida* (●) cytochromes were measured in 100 mM phosphate buffer, pH 7.0, at 25 °C and were obtained just outside the transition region in 2.5 and 1.0 M guanidine hydrochloride, respectively. The guanidine hydrochloride concentration required to denature 50% of each protein was measured under the same conditions except for the *Candida* value which was obtained either in the absence of buffer at pH 6.5 and 25 °C (Knapp & Pace, 1974) or in the presence of 20 mM phosphate buffer, pH 7.0, at 25 °C (Kawaguchi & Noda, 1977). Filled circles indicate measurements made by using Soret absorbance changes, and open circles indicate measurements made by using fluorescence changes.

variable sequence positions in the horse and frog proteins are two-thirds buried and that only 8 of 14 are one-third buried as shown in Table III. Considering only these three and eight sequence differences, the horse protein would be expected to be stabilized by −1.5 and −0.5 kcal/mol, respectively. These comparisons suggest that the sequence differences between the frog and horse proteins would preferentially stabilize the horse protein but by only a small amount.

It is of interest to note that of the 14 sequence differences between the frog and horse proteins, the 2 positions at which the cysteine residues occur in the frog protein are the most buried in the tuna model. This observation predicts that the intramolecular disulfide bond in the frog protein should not be reactive.

Discussion

We find that the globular structure of frog heart ferricytochrome *c* differs from that of horse heart ferricytochrome in at least three respects: (1) the frog cytochrome *c* structure is stable to acidification by HCl at 25 °C in the absence of neutral salts; (2) it is more stable to guanidine hydrochloride denaturation at neutral pH; (3) it is more rapidly refolded following dilution of denaturating guanidine concentrations. We propose that the intramolecular disulfide bond in the frog protein is chiefly responsible for these effects.

The equation of Flory (1956) predicts that the disulfide bond in frog cytochrome *c* encompassing 81 amino acids would reduce the entropy of the denatured protein by 13.2 eu, thereby contributing 3.9 kcal/mol toward the stability of the native conformation at 25 °C. While this value is small relative to the anticipated total free energy of the native or denatured protein, it is large relative to the difference between the native and denatured free energies. As an estimation of the magnitude of this difference, if the equilibrium transition of the horse protein in guanidine hydrochloride is treated as a two-state transition, the equation of Schellman (1978) predicts that the ΔG_0 for unfolding of the native conformation is 7.3 kcal/mol. The incremental addition of −3.9 kcal/mol from the disulfide bond in the frog protein could then offset the small decrease in ΔG_0 predicted from sequential differences and still significantly enhance the stability of the frog protein to denaturants.

While the restriction in the flexibility of denatured frog cytochrome *c* could expedite refolding by reducing the number of potential conformations to be searched by the folding chain, the enhanced folding rate of the frog protein may be an example of a more general phenomenon. All guanidine-denatured cytochromes *c* examined refold in two kinetic phases with about 80–90% of the change in the fast phase. This suggests a common folding pathway for homologous proteins. In Figure 7, we plot two observables, the midpoint of the guanidine transition and the time constants for the refolding phases for the cytochromes reported thus far, horse, yeast iso-2, and frog. In an effort to explore this relationship further, we have measured the time constants for the biphasic folding of guanidine-denatured *C. krusei* ferricytochrome *c* outside its transition zone. We find that this protein has time constants predicted by the relationship described by the three other cytochromes *c*. This relationship suggests that for a homologous series of proteins, the greater the stability of the native conformation, the faster it folds. If the free energy of the denatured forms of a homologous series of proteins is assumed to a first approximation to be equivalent, then a constant activation free energy of unfolding for the homologous globular structures would yield the relationship shown in Figure 6. We do not know this to be the case but submit that the intriguing relationship shown in Figure 6 merits further examination.

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Viscosity-Dependent Conformational Relaxation of Ribonuclease A in the Thermal Unfolding Zone†

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ABSTRACT: Previous studies monitoring tyrosine absorption changes have failed to detect rapid unfolding reaction of ribonuclease A at pH 7 by the temperature jump method, although these reactions (in the 50-ms time range, or τ_2 reaction) have been observed by the stopped-flow pH jump in both unfolding (pH 7 → 2) and refolding (pH 2 → 7) directions. When the unfolding reaction is coupled to the color change of a colorimetric indicator, phenol red, in an unbuffered protein solution, at least three relaxations ranging from submilliseconds to seconds have been resolved with a 4 °C temperature jump over the range 10–80 °C, at pH 7. The fast reaction (τ_f = 0.9 ms at 25 °C), which detects a proton uptake with a pK_a of about 6.0, was reported earlier by French and Hammes [French, T. C., & Hammes, G. G. (1965) *J. Am. Chem. Soc. 87*, 4669–4673]. This reaction, observable in the entire folding–unfolding transition zone, has been found to depend strongly on viscosity additives to the solution such as ethylene glycol, glucose, and sucrose. The reaction was obscured by a substrate-induced conformational relaxation in the same time range, when cytidine 2′-monophosphate was present, but it could be separated from the latter in a viscous medium because of its stronger dependence on solvent viscosity. Thus, it is unlikely that it is a part of the substrate binding reaction. The other two slower relaxations also detect a proton uptake, but with a pK_a of about 8.0, are independent of the solvent viscosity, and have similar relaxation times for the folding–unfolding of ribonuclease A monitored by the tyrosine absorption, under similar experimental conditions ($\tau_2 \sim 80$ ms, $\tau_1 \sim 10$ s at pH 7, 25 °C). As is true for the τ_f reaction, both the τ_2 and τ_1 reactions were present even at 25 °C, i.e., a temperature more than 35 °C below the thermal melting temperature (T_m = 61.5 °C), or at a temperature near 80 °C, i.e., after the thermal transition is presumably complete. However, all three reactions were abolished in a 5 M guanidine hydrochloride solution. These observations are consistent with earlier results that thermally unfolded ribonuclease A retains residual structures which can be removed by guanidine hydrochloride. Two possible sources of the viscosity-dependent relaxation are suggested. If all of the protein molecules are in the folded state at 25 °C, the τ_f reaction could reflect solvent permeation into the protein structure. Conversely, if fractions of protein molecules are unfolded, or are in a dynamic equilibrium between the folded and the unfolded states, the τ_f reaction could have detected an early step in the protein chain folding. In both cases, the reaction would be expected to depend on the microscopic viscosity of the solution.

Different approaches have been used to investigate the mechanisms of protein chain folding (Baldwin & Creighton, 1980; Privalov, 1979; Anfinsen & Scheraga, 1975). Among these methods, kinetic measurement seems uniquely suitable for obaining dynamic information about the chain folding process (Baldwin & Creighton, 1980). For example, it is now clear that the recovery, from the unfolded form, of the active, native conformation of small proteins, whether they are disulfide containing or not, does not take more than seconds (Schechter et al., 1970; Tsong et al., 1971; Ikai & Tanford, 1971; Garel & Baldwin, 1973; Leutzinger & Beychok, 1981). Recent kinetic analyses of ribonuclease A (RNase A)[1] (Garel & Baldwin, 1973; Schmid & Baldwin, 1978; Kim & Baldwin, 1980), carp parvalbumin (Brandts et al., 1977), and a derivative of pancreatic trypsin inhibitor (Jullien & Baldwin, 1981) have shown that the unfolded state of these proteins exists in different isomeric forms, and transformation among these forms is slow compared to a major folding reaction (Baldwin & Creighton, 1980; Brandts et al., 1975). As a result, the overall kinetics of folding reflect these isomerizations in addition to the chain folding process. Identification and characterization of each kinetic phase are, thus, important steps in the kinetic study of protein chain folding.

In the case of RNase A, it has now become clear that proline isomerization in the unfolded state of the protein results in the separation of the population into two kinetic species, the fast-folding and the slow-folding species (Garel & Baldwin, 1973; Brandts et al., 1975). However, the kinetics of the chain folding from neither of the two species seem rate limited by the proline isomerization reaction (Nall et al., 1978; Cook et al., 1979; Kim & Baldwin, 1980). In an earlier study (Tsong & Baldwin, 1978), we have attempted to identify reactions

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[1] Abbreviations: 2′-CMP, cytidine 2′-monophosphate; Gdn·HCl, guanidine hydrochloride; RNase A, bovine pancreatic ribonuclease A.

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