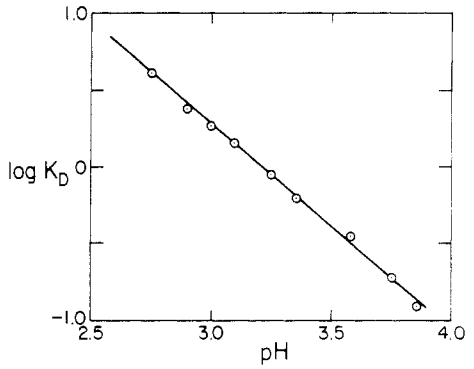

FIGURE 9: Plot of log K_D vs. pH for $N \rightarrow I_{2.2}$.

C is found to be 4.30 by substituting $K_D = 1$ at pH 3.21, and ΔG_D is found in the usual way:

$$\Delta G_D = -RT \ln K$$
$$= -1.36\ (-1.34\ \text{pH} + 4.30)$$
$$= 1.82\ \text{pH} - 5.85\ (\text{kcal mol}^{-1})$$

The transition is essentially complete between pH 4.5 and 2.2; at these values, $\Delta G_D = 2.34$ and -1.87 kcal mol^{-1}, respectively. The difference between them, 4.2 kcal mol^{-1}, is an estimate of $\Delta G_D^{H_2O}$ for the transition $N_{5.5} \rightarrow I_{2.2}$. It can only be regarded as an estimate because there will be a contribution to the total observed ΔG from the protonation reactions which take place during the titration.

Nevertheless, we have entered the figure 4.2 kcal mol^{-1} in Figure 8. This allows us to estimate the other values shown in parentheses. Thus it has been possible to describe five conformational states of the α-lactalbumin molecule and to measure or estimate the standard free energy changes (in water) that accompany the processes among them.

References

Aune, K. (1968) Ph.D. Dissertation, Duke University, Durham, NC.
Aune, K., & Tanford, C. (1969) *Biochemistry 8*, 4586.
Bigelow, C. C. (1964) *J. Mol. Biol. 8*, 696.
Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108.
Hermans, J., & Scheraga, H. A. (1961) *J. Am. Chem. Soc. 83*, 3283.
Kronman, M. T., Cerankowski, L., & Holmes, L. G. (1965) *Biochemistry 4*, 518.
Kugimiya, M., & Bigelow, C. C. (1973) *Can. J. Biochem. 51*, 581.
Kuwajima, K. (1977) *J. Mol. Biol. 114*, 241.
Pace, C. N. (1975) *CRC Crit. Rev. Biochem. 3*, 1.
Sharma, R. N., & Bigelow, C. C. (1974) *J. Mol. Biol. 88*, 247.

Nature of the Fast and Slow Refolding Reactions of Iron(III) Cytochrome *c*†

John A. Ridge,‡ Robert L. Baldwin,* and Alexander M. Labhardt

ABSTRACT: The fast and slow refolding reactions of iron(III) cytochrome *c* (Fe(III) cyt *c*), previously studied by Ikai et al. (Ikai, A., Fish, W. W., & Tanford, C. (1973) *J. Mol. Biol. 73*, 165–184), have been reinvestigated. The fast reaction has the major amplitude (78%) and is 100-fold faster than the slow reaction in these conditions (pH 7.2, 25 °C, 1.75 M guanidine hydrochloride). We show here that native cyt *c* is the product formed in the fast reaction as well as in the slow reaction. Two probes have been used to test for formation of native cyt *c*: absorbance in the 695-nm band and rate of reduction by L-ascorbate. Different unfolded species (U_F, U_S) give rise to the fast and slow refolding reactions, as shown both by refolding assays at different times after unfolding ("double-jump" experiments) and by the formation of native cyt *c* in each of the fast and slow refolding reactions. Thus the fast refolding reaction is $U_F \rightarrow N$ and the slow refolding reaction is $U_S \rightarrow N$, where N is native cyt *c*, and there is a $U_S \leftrightarrow U_F$ equilibrium in unfolded cyt *c*. The results are consistent with the $U_F \leftrightarrow U_S$ reaction being proline isomerization, but this has not yet been tested in detail. Folding intermediates have been detected in both reactions. In the $U_F \rightarrow N$ reaction, the Soret absorbance change precedes the recovery of the native 695-nm band spectrum, showing that Soret absorbance monitors the formation of a folding intermediate. In the $U_S \rightarrow N$ reaction an ascorbate-reducible intermediate has been found at an early stage in folding and the Soret absorbance change occurs together with the change at 695 nm as N is formed in the final stage of folding.

Cytochrome *c* (cyt *c*)[1] was the subject of the first systematic study, in modern terms, of the kinetics of a protein folding transition. After finding conditions for reversible unfolding and refolding, Ikai et al. (1973) analyzed the kinetics by the theory of Ikai & Tanford (1973), which assumes that only one form of the unfolded protein is present. They concluded that the fast-folding reaction (0.1–0.2 s, pH 6.5, 25 °C, 0.5–2.7 M Gdn-HCl) probably represents the formation of one or more dead-end or abortive intermediates. The discovery of two forms of unfolded RNase A cast doubt on this conclusion. There is a slow equilibrium between a fast-folding form U_F and a slow-folding form U_S (Garel & Baldwin, 1973, 1975a,b; Brandts et al., 1975; Hagerman & Baldwin, 1976; Garel et al., 1976). If the same is true of cyt *c*, it could explain the

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[1] Abbreviations used: cyt *c*, ferricytochrome *c* (horse heart); RNase A, bovine pancreatic ribonuclease A; Gdn-HCl, guanidine hydrochloride; U_S and U_F, slow-folding and fast-folding forms of an unfolded protein; N, native form; τ, time constant of a reaction (reciprocal of the apparent rate constant).

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fast and slow refolding reactions observed by Ikai et al. (1973). Because they assumed only one unfolded form, they required a four-species mechanism to represent the biphasic kinetics of unfolding and refolding. However, Hagerman (1977) showed that their results could be represented by the three-species mechanism $U_S \leftrightarrow U_F \leftrightarrow N$ if there are two unfolded forms, as in the case of RNase A (Hagerman & Baldwin, 1976). Brandts et al. (1975) suggested that the folding kinetics of cyt *c* could be represented by a proline isomerism model in which the U_F form has the correct trans isomer in each of the four trans proline residues found in native cyt *c* (Dickerson et al., 1971) and there are multiple U_S forms each having at least one incorrect cis proline isomer.

Additional experiments have been presented by later workers (Tsong, 1977; Babul et al., 1978; Henkens & Turner, 1979; Stellwagen, 1979; Creighton, 1980). Some of these (see Discussion) are aimed at testing the specific model of Brandts et al. (1975) for the refolding of U_S species with wrong proline isomers, and, until now, there is no compelling evidence to decide whether the fast and slow refolding reactions of Ikai et al. (1973) are produced by different forms of the unfolded protein. To address this question, we use two different approaches in order to check that both give the same answer. First, in unfolding experiments, we ask whether the species produced in the Soret-detected unfolding reaction is exclusively a fast-folding species and if there is also a separate slow-folding species which is produced slowly in unfolding by the pathway

$$N \xrightarrow{\text{fast}} U_F \underset{\text{slow}}{\rightleftharpoons} U_S$$

as in the case of RNase A. The second approach is to take the unfolded protein at equilibrium and to ask if there are separate U_F and U_S species, each of which produces native cyt *c* in separate fast and slow refolding reactions ($U_F \rightarrow N$ and $U_S \rightarrow N$). Two probes are used which are specific for N: the rate of reduction by L-ascorbate and the 695-nm band spectrum.

In contrast to the unfolding reactions of other small proteins, for which equilibrium intermediates are detected with difficulty if at all, the equilibrium unfolding transition of cyt *c* shows spectrally measurable intermediates (Schejter & George, 1964; Myer, 1968; Stellwagen, 1968; Kaminsky et al., 1973; Drew & Dickerson, 1978) even though the calorimetric criterion for a two-state ($N \leftrightarrow U$) transition is closely obeyed (Privalov & Khechinashvili, 1974). As summarized by Drew & Dickerson (1978), the following intermediates are recognized: I, a nearly native form in which the polypeptide chain is loosened around the heme and the bonding between Met-80 and the heme iron is weakened; Ia, a variant of I in which the 695-nm band (dependent on Met-80 being a heme ligand; see below) is lost; II, the product of cooperative unfolding in which His-18 remains as an axial ligand but Met-80 is replaced, probably by another histidine side chain; and III, the unfolded protein which no longer contains amino acid side chains as axial ligands. The nearly native species I is formed below 1 M Gdn-HCl at pH 6.5 (Ikai et al., 1973) and in their refolding conditions as in ours the product of refolding is I. For convenience in discussing the refolding kinetics in these conditions, we refer to I as a native form and denote it by N, as did Ikai et al. (1973).

The two probes used here to follow recovery of the native conformation have the following properties. The 695-nm band is present only when methionine is an axial ligand of the heme and the band can be produced in a heme undecapeptide containing His-18 by adding *N*-acetyl-DL-methionine (Schechter & Saludjian, 1967). The usefulness of the 695-nm band as a test for native conformation rests on the fact that this band disappears [or decreases strongly in intensity; see Myer et al., (1980)] at an intermediate stage in unfolding [see discussion by Drew & Dickerson (1978)].

The rate constant for reduction of cyt *c* by L-ascorbate depends on the relative oxidation–reduction potentials of L-ascorbate and cyt *c*, in addition to other factors. Oxidation–reduction potentials of different cytochromes and also of heme fragments with different axial ligands (Henderson & Morton, 1970; Loach, 1970; Moore & Williams, 1977) show that the oxidation–reduction potential of the heme drops below that of ascorbate, making it difficult for ascorbate to reduce the heme, either when methionine is replaced by another axial ligand such as histidine or when unfolding of cyt *c* occurs, with or without change in liganding. A good example of the effect of replacing Met-80 by another ligand is provided by the alkaline isomerization of cyt *c* (Greenwood & Palmer, 1965; Brandt et al., 1966; Gupta & Koenig, 1971). The apparent rate constant for ascorbate reduction of the alkaline form of cyt *c* is at least 10^5-fold less than that of the neutral pH form at pH 8, 1 M ascorbate (Greenwood & Palmer, 1965). On unfolding cyt *c* in 3.8 M Gdn-HCl, pH 8, there is a 10^3-fold drop in rate of ascorbate reduction, and the major decrease follows the unfolding transition curve (Ridge, 1978).

The Soret absorbance change (405 nm) on folding, which was measured by Ikai et al. (1973), is also measured here. The intensity and λ_{max} of the Soret band depend on the spin state of the heme, which in turn depends on the axial heme ligands and on the hydrophilic or hydrophobic environment of the heme [cf. Babul & Stellwagen (1971); Drew & Dickerson (1978)]. When cyt *c* unfolds in 6 M Gdn-HCl, pH 7, there is a small blue shift of λ_{max} (Ikai et al., 1973; Tsong, 1975) resulting from a more hydrophilic heme environment [see Drew & Dickerson (1978)]. His-18 remains as an axial ligand (Babul & Stellwagen, 1971; Tsong, 1975).

In conditions similar to ours, but inside the folding transition zone (pH 7, 25 °C, 2.75 M Gdn-HCl), the refolding of cyt *c* has also been studied by the fluorescence emission of Trp-59 (Tsong, 1976), which is quenched by energy transfer to the heme and thus monitors the overall dimensions of the polypeptide chain. Both fast and slow refolding reactions are observed, with kinetic properties similar to the ones reported here for Soret-detected folding, but the difference in folding conditions may affect this comparison.

Materials and Methods

Materials. Fe(III) cyt *c* (type VI from horse heart) was purchased from Sigma Chemical Co. and further purified by chromatography on SP Sephadex (Pharmacia) by the procedure of Fisher et al. (1973). Ultrapure Gdn-HCl was purchased from Schwarz/Mann and L-ascorbic acid from J. T. Baker. All other compounds were reagent grade.

Methods. Stopped-flow measurements were made with a dual-beam Gibson-Durrum instrument with extensively modified mechanics and optics. Two monochromators were used in tandem to minimize stray light. The photomultiplier voltages were processed by a dividing amplifier and stored digitally in a Datalab DL 905 interfaced to a PDP 11/40 computer. In some cases, 20 repeated experiments were averaged and subsequently smoothed by convoluting the data points with a rectangular time window.

Results

(a) Biphasic Kinetics of Refolding Observed by Soret Band Absorbance. In conditions (pH 7.2, 25 °C, 1.75 M Gdn-HCl) similar to those studied by Ikai et al. (1973) (pH 6.5, 25 °C,

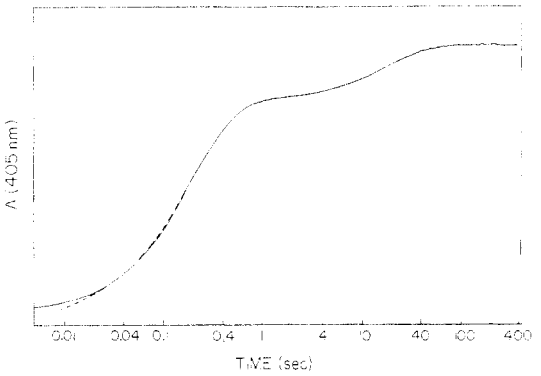


FIGURE 1: The kinetics of refolding of cyt *c* monitored by Soret band absorbance (405 nm) can be fitted almost exactly to a biphasic curve (τ_1 = 19 s, relative amplitude 0.22; τ_2 = 0.195 s, relative amplitude, 0.78; pH 7.2, 0.1 M sodium cacodylate buffer, 25 °C, 1.75 M Gdn-HCl, protein concentration 0.1 mg/mL).

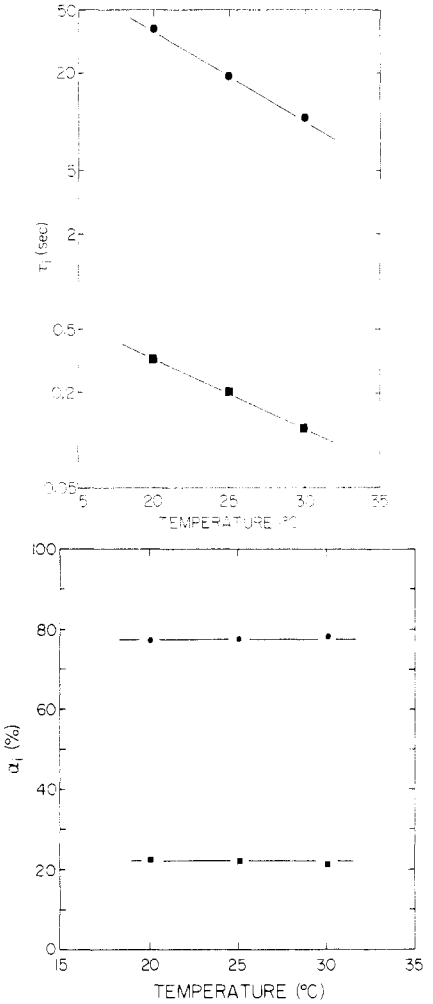


FIGURE 2: Dependence on temperature of the time constants (a, upper) and of the relative amplitudes (b, lower) of the fast- and slow-refolding reactions monitored at 405 nm. See Figure 1 for conditions. The data are taken at 1.75 M Gdn-HCl, below the folding transition zone, where it is possible to interpret the temperature dependences in a simple manner [cf. Hagerman & Baldwin (1976)].

0.5–2.7 M Gdn-HCl), our refolding kinetics monitored by Soret absorbance (405 nm) are the same as theirs and also similar to those of Henkens & Turner (1973). The kinetics fit a biphasic curve rather closely (Figure 1), with 78% of the amplitude in the fast-folding reaction and 22% in the slow-refolding reaction. The temperature dependences of the time constants are shown in Figure 2a and of the amplitudes in Figure 2b. The activation enthalpy for the slow refolding

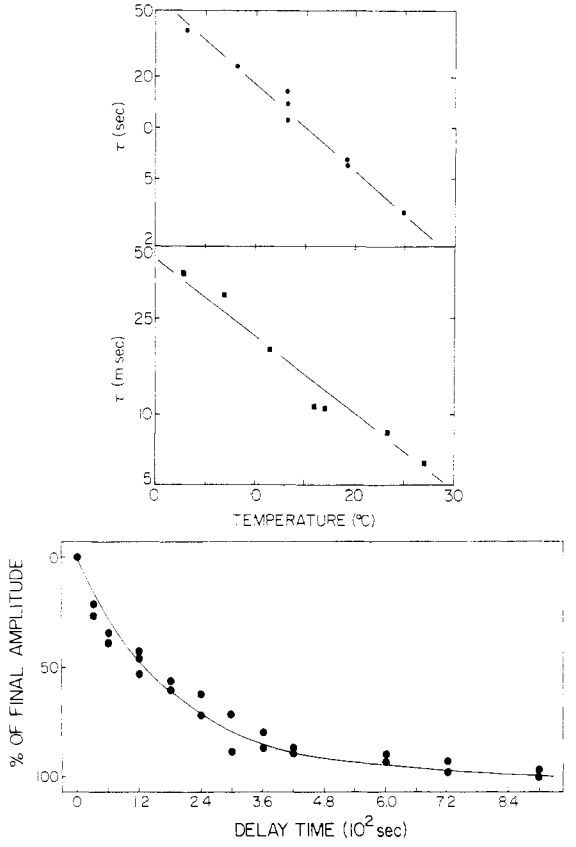


FIGURE 3: (a, upper) Temperature dependences of the time constants for the two unfolding reactions measured by Soret absorbance (405 nm); the lower line shows the major fast phase and the upper line shows a minor (3.3%) slow phase. Conditions: 5.5 M Gdn-HCl, pH 7.2, and 0.1 M sodium cacodylate buffer. (b, lower) A "double-jump" experiment monitoring the formation of a slow-folding species (U_S) which gives rise to the slow-refolding reaction shown in Figure 1. Unfolding is carried out under the conditions of (a) at 5 °C and at 2 mg/mL cyt *c*. After various delay times, aliquots are taken and diluted 1:4 with 0.1 M sodium cacodylate buffer to initiate refolding at 1.375 M Gdn-HCl, pH 7.2, 5 °C, 405 nm.

reaction is 21 kcal/mol (1.75 M Gdn-HCl, pH 7.2, 20–30 °C). The lower value reported by Henkens & Turner (1973) at 2 M Gdn-HCl, pH 6.5, may reflect the effect of including some measurements made within the folding transition zone [cf. Hagerman & Baldwin (1976)]. In other refolding conditions (pH 8.0, 0.5 M Gdn-HCl), the relative amplitude of the fast refolding reaction is temperature-dependent (J. A. Ridge, unpublished data), perhaps caused by the presence of the N ↔ I reaction (Drew & Dickerson, 1978).

(*b*) *Kinetics of Unfolding and the Slow Formation of a Slow-Folding Species.* Stopped-flow measurements of unfolding in 5.5 M Gdn-HCl, pH 7.2, 405 nm, have been made from 1 to 30 °C. The kinetics are biphasic: there is a major fast decrease in absorbance ($\Delta\epsilon$ = −2.2 mL/mg) followed by a 1000-fold slower, minor increase in absorbance of opposite sign ($\Delta\epsilon$ = +0.072 mL/mg). The relative amplitude of the slow phase is −3.3%. The time constants of the two reactions are shown as a function of temperature in Figure 3a. The slow unfolding reaction has an activation enthalpy equal to 20 kcal/mol.

"Double-jump" measurements have been made after unfolding at 5 °C, in the same conditions as above, to find out if the slow-refolding reaction monitored by Soret absorbance (405 nm) is produced by a U_S species according to a

$$N \xrightarrow[\text{(fast)}]{\text{unfold}} U_F \underset{\text{(slow)}}{\overset{\text{isomerize}}{\rightleftharpoons}} U_S$$

unfolding mechanism. Refolding assays have been made at

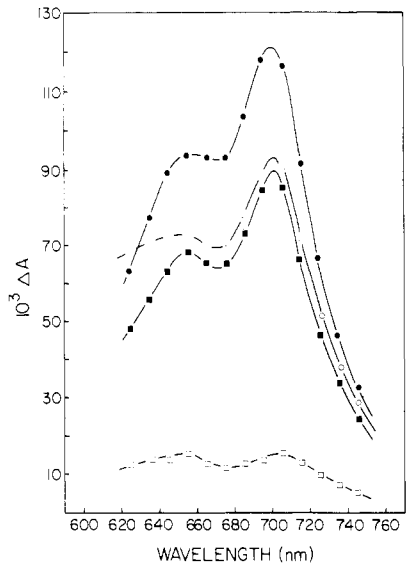


FIGURE 4: Kinetics of refolding monitored by the difference spectrum of the 695-nm band. The reference state for the difference spectrum is cyt *c* unfolded in 3.5 M Gdn-HCl, at 5 mg/mL cyt *c*; other conditions are given in Figure 1. The figure shows the cumulative difference spectrum at the end of each kinetic phase. The spectrum closely matches that of native cyt *c* at the end of the 1.6-s phase and again at the end of the 21-s phase. The time constant of each phase, reading from bottom to top, is: τ = 60 ms (□), 1.6 s (■), 320 ms (○), 21 s (●).

various times after unfolding, and the amplitude of the slow refolding reaction has been measured in 1.375 M Gdn-HCl, pH 7.2, 5 °C. The results are shown in Figure 3b. The species responsible for the slow-refolding reaction is produced slowly in a $U_F \leftrightarrow U_S$ reaction. The time constant of the $U_F \leftrightarrow U_S$ reaction is 190 s, more than 5 times larger than the time constant of the slow phase measured optically at the same temperature (see Figure 3a). Consequently, the minor slow reaction detected by Soret absorbance cannot be the $U_F \leftrightarrow U_S$ reaction of Figure 3b.

(*c*) *Kinetic Phases in the Formation of Native Cyt c Monitored by Absorbance in the 695-nm Band.* The refolding kinetics monitored by the 695-nm band at 25 °C, pH 7.2, 1.75 M Gdn-HCl (Figure 4), are more complex than those seen by Soret absorbance, and there are both similarities and differences between the kinetics monitored by the two probes.

(1) There are three fast phases in refolding: a minor absorbance increase at 60 ms, a major increase at 320 ms, and a further minor decrease at 1.6 s. In addition, there is a slow increase at 21 s.

(2) The total amplitude of the three fast phases (76%) agrees with that of the single phase seen by Soret absorbance (78%).

(3) The difference spectrum of native cyt *c* is found at the end of the three fast phases (after the 1.6-s phase) and again at the end of the slow reaction. The difference spectrum at the end of the 320-ms phase resembles that of native cyt *c* at wavelengths above 670 nm. Since it is not known whether the 60-ms phase results from the folding of U_F or of U_S, there is some ambiguity as to whether the $U_F \rightarrow N$ reaction is complete at 0.32 or at 1.6 s, as measured by the 695-nm band.

(4) The time constant of the single fast reaction seen by Soret absorbance (195 ms) is considerably smaller than that of either the major phase (320 ms) or the final fast phase (1.6 s) seen by the 695-nm band, indicating that these two probes detect different conformations during folding. On the other hand, in the final slow phase of folding the time constant of the reaction detected by each probe (19 s, 405 nm; 21 s, 695 nm) is the same within experimental error.

The 695-nm band results indicate that native cyt *c* is formed in a complex fast phase (amplitude 76%, final time constant either 0.32 or 1.6 s) and also in a slow phase (amplitude 24%, time constant, 21 s).

(*d*) *Refolding Kinetics Monitored by Reduction with* L-*Ascorbate.* The basic aim of the ascorbate reduction experiments is to find out if the species formed in the fast phase of folding is reduced by L-ascorbate at the rate expected for native cyt *c*. These experiments are based on the following considerations. (1) The pseudo-first-order rate constant for ascorbate reduction (with [ascorbate] $\gg$ [cyt *c*]) drops 1000-fold between 0 and 3.8 M Gdn-HCl at pH 8 (Ridge, 1978), indicating that the folded structure of native cyt *c* is needed for a high specific rate of reduction. (2) Reduction of Fe(III) to Fe(II) cyt *c* is monitored by the increase in absorbance at 550 nm. The experiment is to use the stopped-flow apparatus to mix equal volumes of cyt *c* in 3.5 M Gdn-HCl and 2*c* M ascorbate, where *c* is varied from 1 to 0.001, and to observe the kinetics of reduction during refolding at *c* M ascorbate and 1.75 M Gdn-HCl, just below the folding transition zone at pH 7.2. In a control experiment, the ascorbate is omitted and the small change in absorbance at 550 nm during refolding of Fe(III) cyt *c* is subtracted by

$$h(t) = g(t) - f(t)\left[\frac{g(\infty) - g(t)}{g(\infty) - g(0)}\right] \qquad (1)$$

where $f(t)$ and $g(t)$ are the kinetic progress curves monitored in the absence and presence, respectively, of ascorbate. It is assumed that unfolded Fe(III) cyt *c* in 1.75 M Gdn-HCl is not reduced by ascorbate at a significant rate and that, if any partially folded intermediates of Fe(III) cyt *c* are reduced by ascorbate within the time range of refolding, they will have the absorbance of native Fe(II) cyt *c* at 550 nm. (3) Reduction of native cyt *c*, or of any reducible intermediate i, follows an exponential curve whose argument is $k_i ct$ (see Appendix), k_i being the pseudo-first-order rate constant for reduction in excess ascorbate and *c* being the ascorbate molarity. We assume that the presence of ascorbate has little effect on the refolding kinetics of Fe(III) cyt *c* unless ascorbate-reducible intermediates are formed during refolding. The kinetics of reduction during refolding are analyzed by varying the ascorbate concentration and by measuring the extent of reduction at a given time of refolding as a function of ascorbate concentration.

Figure 5 shows the kinetic progress curves for ascorbate reduction during refolding of Fe(III) cyt *c* plotted against log *t* in order to span a broad time range. At low ascorbate concentrations (e.g., *c* = 0.001 M), refolding is complete before much reduction occurs and a single sigmoid curve is observed, characteristic of the reduction of native cyt *c*. As the ascorbate concentration is increased and reduction occurs at earlier times of refolding, the curves become complex, noticeably so above *c* = 0.01 M.

In Figure 6a, the $t_{1/2}$ of ascorbate reduction is plotted against log *c*. At low values of *c* the curve is linear, as expected for reduction of a single species [$(1/t_{1/2}) = k_1 c/\ln 2$], and the rate constant (k_1 = 13 M^{-1} s^{-1}) agrees with that for reduction of native cyt *c*. The line curves at higher ascorbate concentrations, indicating that species other than native cyt *c* are present at shorter times of refolding, and that these species are reduced at lower rates or not at all.

In order to find out if there is a transient species in the slow-refolding reaction which is reduced at a measurable low rate, the fraction of Fe(III) cyt *c* remaining at 2.5 s of refolding

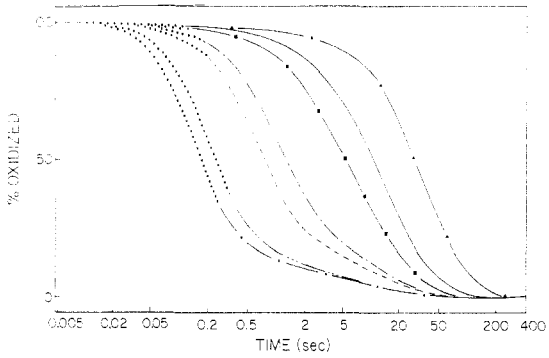


FIGURE 5: Kinetics of reduction of Fe(III) cyt *c*, during the refolding process, by different concentrations of L-ascorbate (0.001–1.0 M), 550 nm; refolding conditions, which are given in Figure 1, include 1.75 M Gdn-HCl. The kinetic progress curve for refolding without reduction (i.e., in the absence of ascorbate) has been measured separately and used to correct the results for a small absorbance change at 550 nm which occurs in the refolding of Fe(III) cyt *c*. From right to left, the concentration of L-ascorbate is: c = 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 1.0 M.

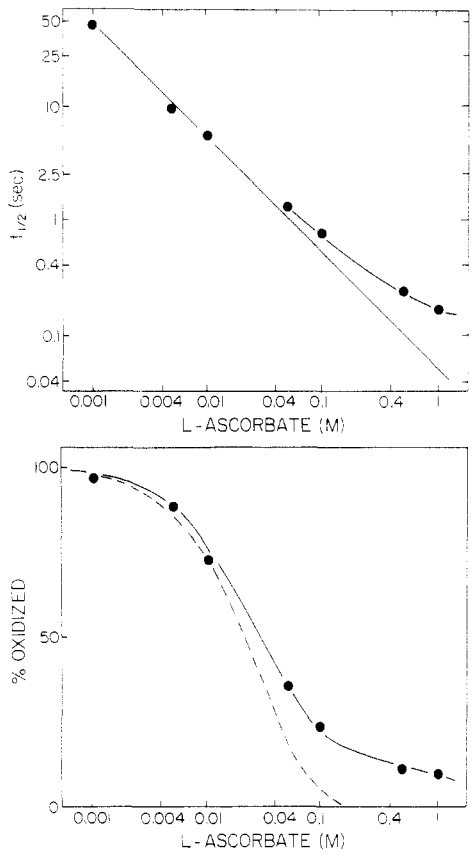


FIGURE 6: Analysis of the ascorbate reduction data shown in Figure 5. (a, upper) ln $t_{1/2}$ for reduction is plotted against ln c, where c is the ascorbate molarity. In the reduction of native cyt c, $t_{1/2} = (\ln 2)/(k_1 c$; k_1 = 13 M^{-1} s^{-1} is found from the initial slope at low ascorbate concentrations. (b, lower) Amount of Fe(III) cyt c remaining after 2.5-s reduction during refolding is plotted against ln c, where c is the ascorbate molarity. In the absence of ascorbate and after 2.5-s refolding, the $U_F \rightarrow N$ reaction is complete and 12% of the $U_S \rightarrow N$ reaction has taken place, giving 80% native cyt c. The dashed line shows the reduction curve expected if only native cyt c can be reduced by ascorbate, with k_1 = 13 M^{-1} s^{-1} (a). The solid line through the experimental points is the fit of the reduction curve to a sum of two exponentials $[\alpha \exp(-tck_1) + (1 - \alpha) \exp(-tck_2)]$, where α = 0.80, k_1 = 13 M^{-1} s^{-1}, and k_2 = 0.36 M^{-1} s^{-1}.

time is plotted against log c (Figure 6b). At 2.5 s, the fast-folding reaction is complete, as monitored either at 405 or at 695 nm, and about 12% of the slow-refolding reaction has occurred. The dashed line shows the reduction curve expected

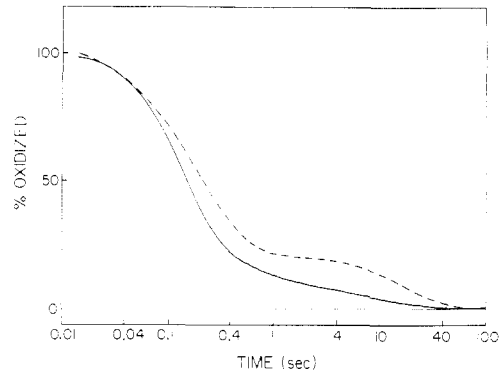


FIGURE 7: Comparison between the refolding kinetics of Fe(III) cyt *c* monitored by Soret absorbance (405 nm, dashed line) and by reduction with 1 M L-ascorbate (550 nm, solid line) at pH 8.0 0.1 M sodium cacodylate buffer and 1.75 M Gdn-HCl, 25 °C. The data indicate that an ascorbate-reducible intermediate is formed at an early stage in the fast-refolding reaction.

if all the Fe(III) cyt *c* remaining at 2.5 s is native. The solid line through the experimental points shows the reduction curve predicted if 80% of the Fe(III) cyt *c* remaining at 2.5 s is native and the other 20% is also reducible but at a lower rate (k_2 = 0.36 M^{-1} s^{-1} vs. k_1 = 13 M^{-1} s^{-1} for native cyt *c*). The data show clearly that the 20% nonnative cyt *c* present at 2.5 s is reducible by ascorbate at a low rate.

When the ascorbate reduction experiment is repeated at pH 8, where reduction is faster than at pH 7.2, the presence of a reducible intermediate in the fast-folding reaction can also be demonstrated. Figure 7 shows refolding monitored by Soret absorbance, in the absence of ascorbate, as compared to reduction by 1 M ascorbate during refolding. Even at the earliest observed times, reduction is faster than the folding process monitored by Soret absorbance.

Discussion

(*a*) *Fast-Folding and Slow-Folding Species of Unfolded Cyt c.* Until now the only direct test for U_F and U_S species of cyt *c* was that of Creighton (1980), who was not able to resolve them in urea-gradient electrophoresis at 4 °C (see below). Several workers have pointed out that the slow refolding reaction studied by Ikai et al. (1973) might result from slow ligand exchange during folding. Tsong (1977) demonstrated that slow as well as fast ligand exchange reactions can be observed in unfolded cyt *c* by using a pH jump to perturb the liganding equilibria. Babul et al. (1978) showed that the slow phase of cyt *c* refolding has quite different kinetic properties at acidic pH's than at the pH 6.5 conditions of Ikai et al. (1973), and they suggested that changes in liganding are responsible. Henkens et al. (1979) showed that the refolding of iron-free porphyrin cyt *c* occurs in a single fast phase (14 ms), and they concluded that slow ligand exchange probably causes the slow-refolding reaction of Ikai et al. (1973). The exchange of Met-80 for a different ligand is known to be a slow reaction (~20 s) in the case of the alkaline isomerization of cyt *c* (Greenwood & Palmer, 1965; Brandt et al., 1966; Davis et al., 1974). These experiments show that slow ligand exchange occurs in some cases, but it is not known whether ligand exchange during refolding can produce a slow refolding reaction.

Our experiments show that there are U_F and U_S forms of unfolded cyt *c* and that they account for the fast- and slow-refolding reactions of Ikai et al. (1973). First, unfolding has been shown to occur by a

$$N \xrightarrow{\text{fast}} U_F \overset{\text{slow}}{\rightleftharpoons} U_S$$

pathway. The major (97%) Soret-detected unfolding reaction reaction is more than 1000-fold faster than the reaction in which U_S is formed. Although there is a small slow change in Soret absorbance after unfolding, it does not yield a slow-folding species: it is more than 5 times faster than the $U_F \leftrightarrow U_S$ reaction. Second, the species formed in the fast-folding reaction is native cyt *c* as judged by its rate of reduction with L-ascorbate (Figure 6a,b). Finally, the 695-nm band spectrum of native cyt *c* is recovered in two separate phases, a fast phase and a slow phase (intermediates are also detected; Figure 4), and the yield of native cyt *c* in each reaction is the same as that measured by Soret absorbance.

As suggested by Creighton (1980), we suppose that urea-gradient electrophoresis at 4 °C fails to resolve the U_S species because it folds too rapidly. He estimates from computer simulations that $t_{1/2}$ for the $U_S \rightarrow N$ reaction should be at least 1 min to be observed as a slow reaction. At 1.75 M Gdn-HCl, 4 °C, the half-time of the $U_S \rightarrow N$ reaction is about 170 s (from Figure 2a).

(*b*) *Proline Isomerization as an Explanation for the U_F and U_S Species.* Brandts et al. (1975) suggested that proline isomerization is a possible explanation for the fast and slow refolding reactions of Ikai et al. (1973). Babul et al. (1978) tested Brandts' model for the refolding kinetics, in which a wrong isomer of any proline residue creates a U_S species, by comparing the refolding kinetics of tuna cyt *c* (three prolines) with horse (four prolines). They found no increase in the proportion of the slow-refolding reaction, measured by Soret absorbance at acidic pH's, for horse over tuna, and they concluded that Brandts' model does not explain these results [cf. Stellwagen (1979)]. Henkens et al. (1979) concluded that wrong proline isomers are not likely to be the explanation for the slow-refolding reaction because porphyrin cyt *c* folds in a single fast phase. However, this may be explained by a weakening of the constraints on the folded conformation when the iron is removed from the heme, so that wrong proline isomers can then be accommodated in a native-like structure [cf. Levitt (1981)].

Our results give only limited information about proline isomerization as the explanation for the $U_F \leftrightarrow U_S$ reaction of unfolded cyt *c*, but they agree with proline isomerization. First, the rate (τ = 190 s, 5 °C) is reasonable: for RNase A, τ = 400 s at 5 °C (Schmid & Baldwin, 1978), and the rate of proline isomerization varies with the bulkiness of the preceding residue (Brandts et al., 1975). Second, the U_S species is formed slowly after unfolding

$$N \xrightarrow{\text{fast}} U_F \underset{}{\overset{\text{slow}}{\rightleftharpoons}} U_S$$

as expected if U_S contains wrong proline isomers introduced after unfolding.

If proline isomerization accounts for the $U_F \leftrightarrow U_S$ reaction, then it is useful to compare the rate of the $U_S \rightarrow N$ reaction with that of the $U_F \leftrightarrow U_S$ reaction [cf. Cook et al. (1979); Jullien & Baldwin (1981)]. If the native protein contains a single trans proline residue and if the cis isomer of this residue creates a U_S species of the unfolded protein which must isomerize ($U_S \rightarrow U_F$) before folding ($U_F \rightarrow N$) can occur, then

$$\tau(R)/\tau(DJ) = 1 + K_{tc} \qquad (2)$$

where τ(R) is the time constant of the refolding ($U_S \rightarrow N$) reaction, τ(DJ) is that of the reaction studied by double-jump measurements ($U_F \leftrightarrow U_S$), and K_{tc} is the cis:trans ratio of the proline residue in the unfolded protein. Since τ(DJ) is found to be independent of Gdn-HCl concentration in the case of RNase A (Schmid & Baldwin, 1979), as expected for proline

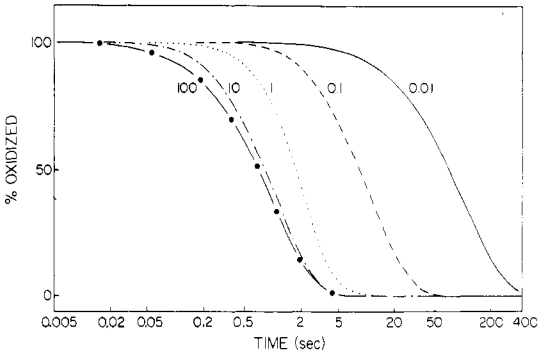


FIGURE 8: Progress curve for the appearance of reduced cyt *c* during refolding for the mechanism formulated in eq A1 and calculated by eq A 3. It is assumed that only fast-folding material exists and the rate of folding k_f is set equal to 1 s^{-1}. The ratio x of the halftime for folding to halftime for reduction has the value shown on the curve.

isomerization, it may be compared directly with τ(R) which is measured at a low Gdn-HCl concentration. The value of τ(R) extrapolated to 5 °C (Figure 2a) is 245 s and τ(DJ) is 190 s, giving K_{tc} = 0.29, which is not unreasonable for the cis:trans isomer ratio. This cannot be an accurate way of estimating K_{tc} [cf. Cook et al. (1979)], but it serves to show that, if proline isomerization is rate limiting, then the rate constant for isomerization during folding (cis → trans) is close to the value calculated from the reaction in unfolded cyt *c*.

This type of agreement between τ(R) and τ(DJ) has not been found for RNase A, especially in strongly native folding conditions (low temperatures, low Gdn-HCl) (Cook et al., 1979). Two points should be noted. First, our refolding conditions (1.75 M Gdn-HCl) are not strongly native conditions. A change in the mechanism of the $U_S \rightarrow N$ reaction of RNase A is found between 0 and 2 M Gdn-HCl (Nall et al., 1978; F. X. Schmid, unpublished results). Second, although eq 2 assumes that there are no folding intermediates before proline isomerization, nevertheless the ascorbate reduction experiments indicate that there is such an intermediate.

If the $U_S \rightarrow N$ reaction of cyt *c* results from wrong proline isomers, one would like to know how many (and which) proline residues are involved. The small proportion of U_S (0.23) could be consistent with only one or two essential proline residues. An essential proline is one which gives rise to a slow-folding species (Schmid & Baldwin, 1978). The fraction of U_S increases with the number (n) of essential proline residues, in a protein with only trans prolines, as $(1-p^n)$, where p is the fraction trans of any proline residue in the unfolded state. If p = 0.90, then only two proline residues would be essential, and if p = 0.80, one essential proline residue would most nearly explain 0.23 as the fraction of U_S. Values of p = 0.8–0.9 have been found commonly (Brandts et al., 1975; Grathwohl & Wüthrich, 1976), but more data on this point are needed.

(*b*) *Transient Folding Intermediates Detected by the 695-nm Band and by Ascorbate Reduction.* The 695-nm band is recovered during folding in four distinct kinetic phases (Figure 4). The cumulative difference spectrum agrees with that of native cyt *c* in only two of the four phases (τ = 1.6 and 21 s). This shows the presence of two folding intermediates since only two of the four reactions can be attributed to the presence of two forms (U_F, U_S) of the unfolded protein. Since the native 695-nm band spectrum is found at 1.6 s when the relative amplitude (0.76) agrees with the fraction of U_F (0.78) calculated from Soret-detected folding, the simplest explanation of the four phases is that the first three belong to the fast refolding reaction ($U_F \rightarrow N$) and the fourth phase is the slow refolding reaction. However, it is possible that the first fast reaction at 60 ms and/or the third reaction at 1.6 s represent

intermediate stages in the $U_S \rightarrow N$ reaction. In any case, the difference between the τ of the second major reaction (320 ms) and the τ of Soret-detected folding (195 ms) shows that the Soret absorbance change monitors the formation of an intermediate in the fast refolding reaction.

There is an ascorbate-reducible intermediate present in the $U_F \rightarrow N$ and in the $U_S \rightarrow N$ reaction, but these experiments do not give the rate of formation of the intermediate.[2] At pH 8, reduction of Fe(III) cyt *c* by 1 M L-ascorbate precedes the Soret absorbance change both in the $U_F \rightarrow N$ and in the $U_S \rightarrow N$ reactions (Figure 7). The experiments in Figure 6a,b give the ascorbate reduction rate of the intermediate in the $U_S \rightarrow N$ reaction as being 35-fold less than that of N in our standard refolding conditions (pH 7.2, 1.75 M Gdn-HCl).

It is surprising that an ascorbate-reducible intermediate is present at a stage in the $U_S \rightarrow N$ reaction when very little, if any, of the intensity of the 695-nm band has been recovered: the 695-nm band is believed to measure the liganding of Met-80, and Met-80 liganding is thought to be necessary for a rapid rate of ascorbate reduction (see introduction). Kaminsky & Miller (1972) have reported a similar observation: ascorbate reduction still occurs after disappearance of the 695-nm band in equilibrium studies of the unfolding of cyt *c* by urea or by 1-propanol. Whether Met-80 liganding can persist after a protein conformational change obliterates the 695-nm band or whether some ligand other than Met-80 can permit a rapid rate of ascorbate reduction is not known.

Appendix

This appendix describes the quantitative treatment of the simultaneous refolding–reduction experiments. The aims of this analysis are twofold.

(1) Unfolded cyt *c* exists in two forms, a fast- and a slow-folding species. Both of these may refold and be reduced during a refolding experiment. Therefore, at least four kinetic phases are expected. The first aim of the theoretical treatment is to formulate conditions for which fewer phases are seen, when there are no populated folding intermediates, and to provide a framework for the quantitative analysis of the experimental data.

(2) Reducible intermediates may also be formed during folding. They will be exposed to L-ascorbate for varying times and, in pseudo-first-order conditions (c = [L-ascorbate] $\gg$ [cyt *c*]), their reduction will not always follow an exponential curve with the single argument $k_i ct$ (t is time, k_i is the reduction rate of intermediate i). The second aim of the theoretical treatment is to find conditions for which the error is negligible in assuming a purely exponential time course for reduction of each intermediate.

The kinetic equations are readily solved if (1) the folding kinetics are measured outside the folding transition range, so that the rates of unfolding may be neglected, and (2) reduction of Fe(III) cyt *c* is carried out under pseudo-first-order conditions.

Both conditions are fulfilled in the experiments described here. Since the fast and slow folding reactions may be considered as kinetically decoupled outside the transition region [cf. Hagerman & Baldwin (1976)], we treat the two reactions separately.

Turning first to the U_f species, folding followed by reduction can, in its most simple case, be described by the sequential scheme

$$U_f \xrightarrow{k_f} O_f \xrightarrow{ck_1} R_f \qquad \text{(A1)}$$

U, O, and R are the unfolded, the reducible oxidized and the reduced species of cyt *c*, c is the concentration of L-ascorbate, and k_f and k_1 are the first- and second-order rate constants. The corresponding kinetic equations read

$$(d[U_f]/dt = -k_f[U_f] \qquad \text{(A2a)}$$

$$d[O_f]/dt = k_f[U_f] - ck_1[O_f] \qquad \text{(A2b)}$$

$$d[R_f]/dt = ck_1[O_f] \qquad \text{(A2c)}$$

Equation A2a is solved by an exponential decay, which, inserted into eq A2b, allows one to write down readily the solution for $[O_f]$ by using the method of Lagrangian multipliers. The concentration $[R_f]$ of material reduced in the fast reaction is then obtained from $[O_f]$ simply by integration. The result reads

$$[R_f] = [U_f]_0\{1 - [[1/(1-x)]\exp(-ck_1t)] + [[x/(1-x)]\exp(-k_ft)]\} \qquad \text{(A3)}$$

$[U_f]_0$ is the starting concentration of fast-folding cyt *c* and x ($x \neq 1$) is the ratio of second-order to first-order rates:

$$x = k_1c/k_f \qquad \text{(A4)}$$

As is seen, doubling the reduction time ($t \rightarrow 2t$) will not necessarily change the amount of reduced cyt *c* in the same way as doubling the concentration c of L-ascorbate ($c \rightarrow 2c$). This, however, is what has been assumed in the text. The effect is described by the preexponential factors $1/(1-x)$ and $x/(1-x)$, respectively, and is caused by the variation in exposure time of reducible cyt *c* to L-ascorbate during folding. The effect is most pronounced if $k_1c = k_f$ (i.e., $x = 1$), for which condition eq A3 reads

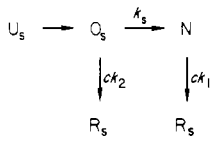
$$[R_f] = [U_f]_0\{[1 - [(k_ft + 1)\exp(-k_ft)]]\} \qquad \text{(A5)}$$

Figure 8 shows the reduction progress curves for the fast-folding form assuming $k_f = 1\ s^{-1}$, calculated by eq A3 or A5. The curves from left to right correspond to x = 100, 10, 1, 0.1, and 0.01. These two facts are obvious: (1) The progress curves for x = 0.01 and 100 are single exponentials for all practical purposes. The deviation from a pure exponential for $x = 1$ is not serious. (2) With increasing x (in the text, with increasing concentration of L-ascorbate; compare eq 4) the reduction progress curves cluster around the progress curve of the fast-folding reaction (assumed in Figure 8 to have a time constant of 1 s). Comparison with the measured curves (Figure 5), which show much more rapid reduction by 1 M L-ascorbate, proves that the rate-limiting folding step which precedes reduction at pH 7.2 is faster than either of the two fast-folding reactions measured at 695 nm (τ_f = 0.32 and 1.6 s) which give a difference spectrum close to that of native cyt

[2] However, the rate of formation of native Fe(III) cyt *c* in the slow ($U_S \rightarrow N$) folding reaction can be determined from the ascorbate reduction/refolding results. After completion of this paper, the data given in Figures 5 and 6, for the reduction of Fe(III) cyt *c* during refolding, were analyzed in a different way, by computer fitting the data for refolding at different ascorbate concentrations to one second-order reaction (reduction of native cyt *c*) plus a set of first-order reactions (reduction which is rate limited by folding to give either native cyt *c* or a reducible intermediate). At low ascorbate concentrations (e.g., 0.001 M), refolding is nearly complete before much reduction occurs, and the major reaction is the second-order reduction of native cyt *c*. The rate constant of the second-order reaction determined by computer optimization agrees with the known rate constant for reduction of native cyt *c* (13 $M^{-1}\ s^{-1}$). When computer fitting is used, the time constant for the slowest first-order reaction can be determined with better accuracy than those of the faster first-order reactions: the time constant of the slowest reaction is found to be 19.7 s, in agreement with the time constant of the slow refolding reaction measured either by Soret absorbance or by the 695-nm band. Three faster first-order reactions are also detected, with less accuracy; a careful analysis of these reactions and their amplitudes requires further work.

c. The appearance of the 695-nm band is believed to monitor ligation of the heme group by Met-80. The reducible state of O_f in the fast-folding pathway is therefore not the state with the native 695-nm band. Inspection of Figure 7, which compares the reduction kinetics to the Soret band folding kinetics, further indicates that O_f is also not the product of the fast-folding reaction detected in the Soret region ($\tau_f = 0.20$ s) since the reduction kinetics precede the Soret band folding kinetics.

Evidence has been presented in the text showing why a reducible intermediate O_s must be postulated on the slow-folding pathway. Schematically:



(A6)

U, O, and R are again unfolded, oxidized, and reduced states, N is native cyt *c*, k_s is the folding rate constant on this pathway, and k_1 and k_2 are the two second-order reduction rates. To keep the number of new variables small we assume (1) that the conversion of U_s to O_s is rapid as compared to both the slow-folding reaction (k_s) and the reduction of O_s (ck_2) and (2) that the typical γ band of reduced cytochrome *c* at 550 nm is displayed by either reduction product R_s (starting from O_s or N).

With these simplifications the rate equations read

$$d[O_s]/dt = -(k_s + ck_2)[O_s] \quad \text{(A7a)}$$

$$d[N]/dt = k_s[O_s] - ck_1[N] \quad \text{(A7b)}$$

$$d[R_s]/dt = ck_2[O_s] + ck_1[N] \quad \text{(A7c)}$$

The three steps outlined above for the solution of eq A2a–c lead to the result:

$$[R_s] = [U_s]_0\{1 - [[1/(1 - x')] \exp(-ck_1t)] + [[x'/(1 - x')] \exp(-k_st - ck_2t)]\} \quad \text{(A8)}$$

where $[U_s]_0$ is the initial concentration of slow folding cyt *c* and x' is given by

$$x' = \frac{(k_1 - k_2)c}{k_s} \quad \text{(A9)}$$

Experimentally, the sum of eq A8 and A3 is measured

$$[R]_{tot} = [R_s] + [R_f] \quad \text{(A10)}$$

For comparison with experimental data, the following two cases are of interest.

(1) $x, x' \ll 1$. For these conditions, eq (A10) reduces to

$$[R]_{tot} = [U]_{tot}[1 - \exp(-ck_1t)] \quad \text{(A11)}$$

with $[U]_{tot} = [U_s]_0 + [U_f]_0$. Inspection of the definitions of x and x' (eq A4 and A9) shows that the above condition is met if both folding reactions are rapid as compared to the reduction rate of native cyt *c*. This is the case (compare Figure 6b) for concentrations of L-ascorbate smaller than 2 mM. The rate k_1 can then be calculated from the halftime $t_{1/2}$:

$$k_1 = (\ln 2)/(t_{1/2}c) \quad \text{(A12)}$$

Relation A12 was used in the text to calculate the value $k_1 = 13$ M^{-1} s^{-1} from the straight line in Figure 6b.

(2) For higher concentrations of L-ascorbate, the kinetic progress curves become complex (see eq A10). For the analysis presented in Figure 6b the following simplifications are possible: (a) At the time point $t_0 = 2.5$ s, the fast exponential term $\exp(-k_ft_0)$ in eq A3 will vanish for all concentrations. (b) For $x \gg 1$ in eq A8 the fast exponential $\exp(-ck_1t_0)$ vanishes also and the amplitude of the second exponential $\exp(-k_st_0 - ck_2t_0)$ equals −1. The condition $x' \gg 1$ holds for L-ascorbate concentration $c > 10$ mM (compare Figure 6a).

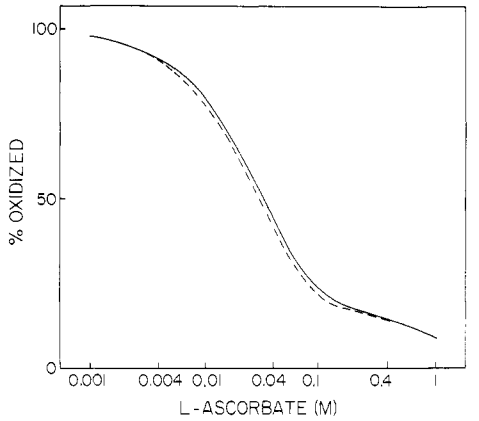


FIGURE 9: Comparison of the full theoretical expression (full line; see eq A3, A8, and A10) with the simplified eq A13 (dashed line). The lines show the amount of reduced cytochrome *c* after $t_0 = 2.5$-s refolding as a function of the concentration of L-ascorbate. See text for details.

With these simplifications, eq A10 reduces to

$$[R]_{tot} = [U]_{tot} - \{[U_f] \exp(-ck_1t_0)\} - \{[U_s]\exp(-ck_2t_0)\} \quad \text{(A13)}$$

with $[U_s] = [U_s]_0 \exp(-k_st_0)$ and $[U_f] = [U]_{tot} - [U_s]$. Note that the preexponential $1/(1 - x)$ from eq A3 was also set equal to unity, which at first sight appears to be correct only if $x \ll 1$, i.e., at low concentrations. At higher concentrations, however, the exponential $\exp(-ck_1t_0)$ itself goes to zero ($k_1 = 13$ M^{-1}), so that expression A13 holds for any value of x. Figure 9 compares the full expression for $[R]_{tot}$ (using $k_f = 5$ s^{-1}, $k_1 = 13$ M^{-1} s^{-1}, $k_s = 0.05$ s^{-1}, $k_2 = 0.36$ M^{-1} s^{-1}, $t_0 = 2.5$ s, $[U_f]_0 = 76\%$, and $[U_s]_0 = 24\%$) to the simplified expression A13 with $k_1 = 13$ M^{-1} s^{-1}, $k_2 = 0.36$ M^{-1} s^{-1}, and $[U_s] = 20\%$. The agreement is excellent over the *entire* concentration range. In the text eq A13 was used to fit by least-squares the data in Figure 6b using k_1 and k_2 as variables with a fixed and preset value of $[U_s] = 20\%$.

References

Babul, J., & Stellwagen, E. (1971) *Biopolymers 10*, 2359–2361.

Babul, J., Nakagawa, A., & Stellwagen, E. (1978) *J. Mol. Biol. 126*, 117–121.

Brandt, K. G., Parks, P. C., Czerlinski, G. H., & Hess, G. P. (1966) *J. Biol. Chem. 241*, 4180–4185.

Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14*, 4953–4963.

Cook, K. H., Schmid, F. X., & Baldwin, R. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6157–6161.

Creighton, T. E. (1980) *J. Mol. Biol. 137*, 61–80.

Davis, L. A., Schejter, A., & Hess, G. P. (1974) *J. Biol. Chem. 249*, 2624–2632.

Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A., & Margoliash, E. (1971) *J. Biol. Chem. 246*, 1511–1535.

Drew, H. R., & Dickerson, R. E. (1978) *J. Biol. Chem. 253*, 8420–8427.

Fisher, W. R., Taniuchi, H., & Anfinsen, C. B. (1973) *J. Biol. Chem. 248*, 3188–3195.

Garel, J.-R., & Baldwin, R. L. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3347–3351.

Garel, J.-R., & Baldwin, R. L. (1975a) *J. Mol. Biol. 94*, 611–620.

Garel, J.-R., & Baldwin, R. L. (1975b) *J. Mol. Biol. 94*, 621–632.

Garel, J.-R., Nall, B. T., & Baldwin, R. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1853–1857.
Grathwohl, C., & Wüthrich, K. (1976) *Biopolymers 15*, 2025–2041.
Greenwood, C., & Palmer, G. (1965) *J. Biol. Chem. 240*, 3660–3663.
Gupta, R. K., & Koenig, S. H. (1971) *Biochem. Biophys. Res. Commun. 45*, 1134–1143.
Hagerman, P. J. (1977) *Biopolymers 16*, 731–747.
Hagerman, P. J., & Baldwin, R. L. (1976) *Biochemistry 15*, 1462–1473.
Henderson, R. W., & Morton, T. C. (1970) in *Handbook of Biochemistry* (Sober, H. A., Ed.) 2nd ed., pp J41–J57, CRC Press, Cleveland, OH.
Henkens, R. W., & Turner, S. R. (1973) *Biochemistry 12*, 1618–1621.
Henkens, R. W., & Turner, S. R. (1979) *J. Mol. Biol. 254*, 8110–8112.
Ikai, A., & Tanford, C. (1973) *J. Mol. Biol. 73*, 145–164.
Ikai, A., Fish, W. W., & Tanford, C. (1973) *J. Mol. Biol. 73*, 165–184.
Jullien, M., & Baldwin, R. L. (1981) *J. Mol. Biol. 145*, 265–280.
Kaminsky, L. S., & Miller, V. J. (1972) *Biochem. Biophys. Res. Commun. 49*, 252–256.
Kaminsky, L. S., Miller, V. J., & Davison, A. J. (1973) *Biochemistry 12*, 2215–2221.
Levitt, M. (1981) *J. Mol. Biol. 145*, 251–263.
Loach, P. A. (1970) in *Handbook of Biochemistry* (Sober, H. A., Ed.) 2nd ed., pp J33–J40, CRC Press, Cleveland, OH.
Moore, G. R., & Williams, R. J. P. (1977) *FEBS Lett. 79*, 229–232.
Myer, Y. P. (1968) *Biochemistry 7*, 765–776.
Myer, Y. P., MacDonald, L. H., Verma, B. C., & Pande, A. (1980) *Biochemistry 19*, 199–207.
Nall, B. T., Garel, J.-R., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 317–330.
Parr, G. R., & Taniuchi, H. (1980) *J. Biol. Chem. 255*, 8914–8918.
Privalov, P. L., & Khechinashvili, N. N. (1974) *Biofizika 19*, 10–15.
Ridge, J. A. (1978) Ph.D. Thesis, Stanford University.
Schechter, E., & Saludjian, P. (1967) *Biopolymers 5*, 788–790.
Schejter, A., & George, P. (1964) *Biochemistry 3*, 1045–1949.
Schmid, F. X., & Baldwin, R. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4764–4768.
Schmid, F. X., & Baldwin, R. L. (1979) *J. Mol. Biol. 133*, 285–287.
Stellwagen, E. (1968) *Biochemistry 7*, 2893–2898.
Stellwagen, E. (1979) *J. Mol. Biol. 135*, 217–229.
Tsong, T. Y. (1975) *Biochemistry 14*, 1542–1547.
Tsong, T. Y. (1976) *Biochemistry 15*, 5467–5473.
Tsong, T. Y. (1977) *J. Biol. Chem. 252*, 8778–8780.

Effect of Long-Chain Alkyl Sulfate Binding on Circular Dichroism and Conformation of Soybean Trypsin Inhibitor†

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ABSTRACT: The disorganization and helix formation process of "Kunitz" soybean trypsin inhibitor (STI) effected by sodium dodecyl sulfate binding was investigated by the circular dichroism (CD) probe. The binding isotherms of dodecyl sulfate to STI were determined at the ionic strength of 0.033, 0.12, and 0.25 at pH 7.3, 25 °C. The perturbation and disorganization of this nonhelical protein were observed at an early binding stage ($\bar{\nu}$, the average molar ratio of bound detergent to STI, up to about 7 in the case of the isotherm at $I = 0.12$). The disappearance of a positive CD peak at 226 nm and appearance of a negative CD band at 239 nm took place at this step and were affected by the number of carbon atoms in the alkyl group of detergents. The transition of the polypeptide backbone into a more ordered conformation proceeded gradually during cooperative binding of dodecyl sulfate molecules. An abrupt increase of detergent binding occurred near the critical micelle concentration of the detergent. The helix formation was completed prior to this step ($\bar{\nu} = 30$, at $I = 0.12$).

Many studies have been carried out with the aim of understanding interactions between protein and detergent, especially sodium dodecyl sulfate ($NaDodSO_4$),[1] for four decades. Despite accumulation of a wealth of knowledge on this subject [for reviews, see Tanford (1968), Steinhardt & Reynolds (1969), and Lapanje (1978)], the exact nature of protein–dodecyl sulfate complex has not been conclusively elucidated. The increase in the helix content of some proteins upon complexing with sodium dodecyl sulfate has been observed by optical activity measurements (Meyer & Kauzmann, 1962; Jirgensons, 1966; Reynolds & Tanford, 1970a; Visser & Blout, 1971). Recently, conformational properties of the complexes of 15 proteins were analyzed by circular dichroism. The complexes were shown to be a mixture of random coil and α helix (Mattice et al., 1976). However, not much attention has been paid to the process of helix formation induced by $NaDodSO_4$ binding.

Soybean trypsin inhibitor (Kunitz) is a well-characterized stable protein [for review, see Laskowski & Sealock (1971)].

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; STI, soybean trypsin inhibitor (Kunitz); CD, circular dichroism; UV, ultraviolet; I, ionic strength; $\bar{\nu}$, average molal ratio of bound $NaDodSO_4$ to STI. The values reported in this paper were calculated by using the binding isotherm at $I = 0.12$ (Figure 1).

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