Westheimer, F. H. (1968), *Accounts Chem. Res. 1*, 70.
Wieker, J. H., and Witzel, H. (1967), *Eur. J. Biochem. 1*, 251.
Wigler, P. W. (1968), *Biochem. Prep. 12*, 107.
Wilkinson, G. N. (1961), *Biochem. J. 80*, 324.
Witzel, H., and Barnard, E. A. (1962), *Biochem. Biophys. Res. Commun. 7*, 295.

Kinetics of Refolding of Guanidine Hydrochloride Denatured Cytochrome *c*. Temperature Dependence†

Robert W. Henkens* and Stephen R. Turner

ABSTRACT: Horse heart ferricytochrome *c* is denatured by guanidine hydrochloride and refolds upon removal of the denaturing conditions. The circular dichroism of the native and refolded protein is virtually identical, indicating that secondary structure and dissymmetrical interactions of heme and chromophoric side chains are recovered on refolding. Ikai and Tanford (Ikai, A., and Tanford, C. (1971), *Nature (London) 230*, 100) have recently reported kinetic evidence for the mechanism $N \rightleftharpoons X_1 \rightleftharpoons D \rightleftharpoons X_2$, in which the reversible interconversion of the native (N) and denatured (D) states involves two intermediates (X_1 and X_2). The intermediate X_2 is interpreted as representing an incorrectly folded form. Under the conditions of our experiments (25°, 2 M guanidine hydrochloride, pH 6.5), the first step in the ultimate conversion of D to N is the rapid formation of the incorrectly folded form. The absorbance changes associated with this step indicate that X_2 is highly folded. The temperature dependence of the rate constant for this step, $D \rightarrow X_2$, corresponds to the activation parameters $\Delta H^{\pm} = 15$ kcal mol^{-1} and $\Delta S^{\pm} = -6$ cal deg^{-1} mol^{-1}. Depletion of the incorrectly folded state and ultimate conversion of all the protein to the native state occur with apparent activation parameters of $\Delta H^{\pm} = 8$ kcal mol^{-1} and $\Delta S^{\pm} = -38$ cal deg^{-1} mol^{-1}. This shows that an unfavorable entropy change is a major barrier to conversion of the incorrectly folded form to N.

Cytochrome *c* is a small compactly folded protein molecule, consisting of a single polypeptide chain with no disulfide cross-links. The structure of crystalline horse heart ferricytochrome *c* has been determined by X-ray methods to a resolution of 2.8 Å (Dickerson *et al.*, 1971). In a detailed study of the kinetics of the reversible denaturation of this protein by guanidine hydrochloride, Ikai *et al.* (1973) report observations consistent with the view that transformation of the disordered polypeptide to the native protein involves rapid formation of a relatively highly ordered but incorrectly folded state; depletion of this incorrectly folded state and ultimate conversion of all the protein to the native state occur more slowly. We report here studies of the temperature dependence of the kinetics of refolding of horse heart ferricytochrome *c*. Activation parameters, $\Delta H^{\pm}$ and $\Delta S^{\pm}$, associated with the conversion of the random coil to the incorrectly folded form and with the conversion of the incorrectly folded form to the native protein, are deduced from these studies.

Experimental Section

Horse heart ferricytochrome *c*, crystalline protein type VI, was purchased from Sigma. Protein concentrations were determined from the absorbance at 550 nm, after reduction with sodium dithionite, using a molar absorptivity $\epsilon_{550} = 27.7 \times 10^4$ M^{-1} cm^{-1} (Margoliash and Frohwirt, 1959). Guanidine hydrochloride was purchased from Heico Inc. (Delaware Water Gap, Pa.). Guanidine hydrochloride stock solution concentrations were determined from the refractive index. All solutions were prepared with deionized water provided by Continental Water which had been filtered through a 0.45-μm Gelman membrane.

Spectral measurements were made with a Cary 15 spectrophotometer, periodically checked for absorbance linearity and excessive stray light, and a Durrum-Jasco J-15, standardized with *d*-10-camphorsulfonic acid using a specific ellipticity $[\psi]_{290} = 3.00 \times 10^3$ deg cm^3 g^{-1} dm^{-1}.

Fast kinetic measurements were made with a Durrum-Gibson stopped flow spectrophotometer. In most cases the optical pathlength was 2 mm, although in a few instances we used 2 cm to obtain data over a wider concentration range. The instrument is prone to a number of flow and optical artifacts, detected as anomalous transmittance changes in mixing experiments, and in actual kinetic experiments (*cf.* Ikai, 1971; Turner, 1971). We frequently checked for instrument error by mixing water with water and water with cytochrome *c* solution. Most of the errors could be detected by these simple mixing experiments, and were usually eliminated by removing trapped bubbles or realigning the optical system. In our experiments the reactant solutions differed markedly in refractive index. In the Durrum instrument, local refractive index gradients persist after mixing. This gives rise to Schlieren effects which cause oscillations in the transmittance. We found that working with shorter pathlengths and higher protein concentrations tended to reduce these oscillations to negligible proportions. Reaction solutions were degassed under house vacuum to reduce bubble formation during mixing. All components of the flow system were constructed of Kel F, Teflon, or glass. Temperature control was pro-

† From the Paul M. Gross Chemical Laboratory, Department of Chemistry, Duke University, Durham, North Carolina 27706. *Received August 24, 1972.* This work was aided by a Biomedical Science Support Grant from the National Institutes of Health to Duke University, and by funds from the National Science Foundation (GB-36050) and the Duke Endowment.

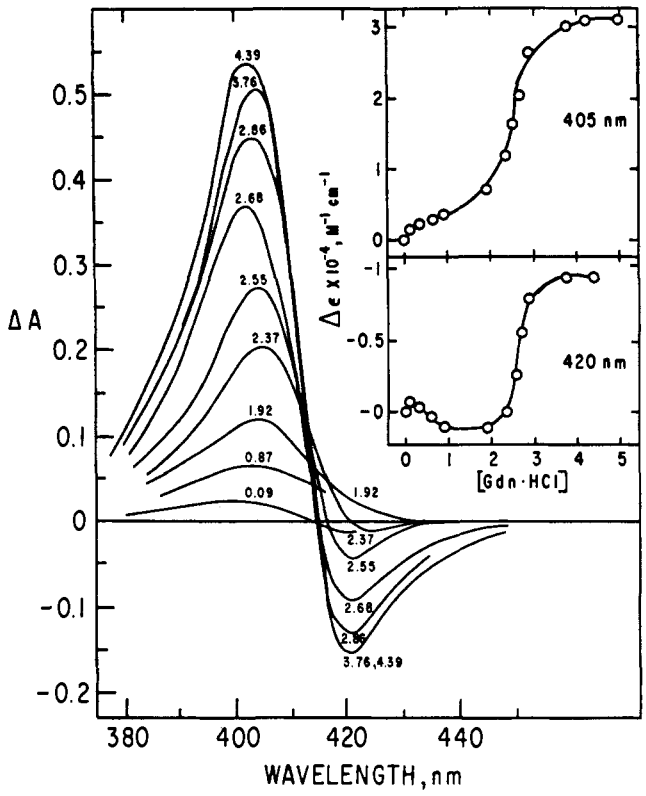

FIGURE 1: Denaturation transition of ferricytochrome *c* determined from changes in the difference spectrum. Both sample and reference cell (1-cm pathlength) contained the same concentration of protein (0.20 mg/ml) in an unbuffered solution, near pH 6.5, 25°; in addition the sample cell contained the molar concentration of guanidine hydrochloride (Gdn·HCl) indicated by the numbers next to the curves. The inserts give plots of molar absorptivity near extrema in the difference spectrum *vs.* Gdn·HCl concentration.

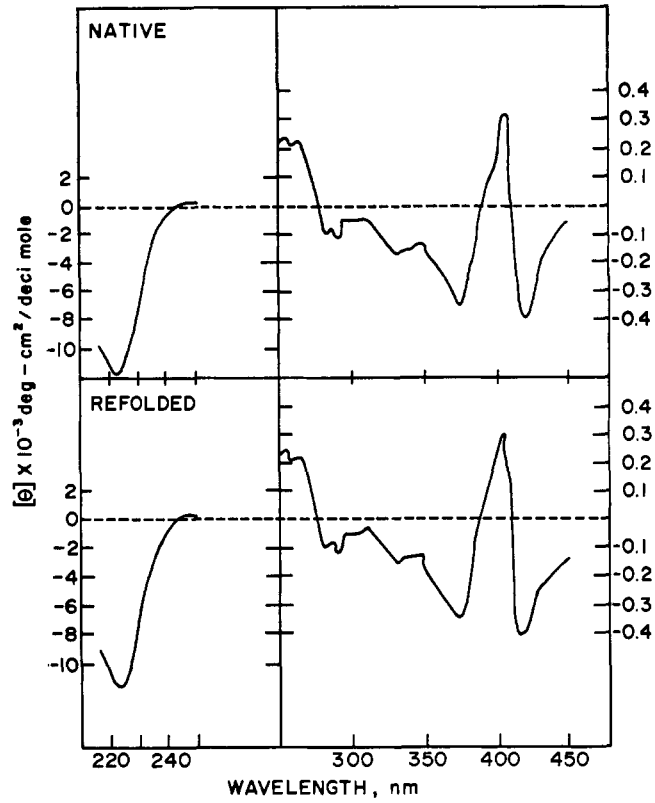

FIGURE 2: Circular dichroism of native and refolded ferricytochrome *c* in 2.0 M guanidine hydrochloride, 0.1 M phosphate buffer, pH 7.2, 22°. Refolded protein originally denatured in 4.0 M guanidine hydrochloride. Protein concentration usually *ca.* 0.5 mg/ml.

vided by circulating thermostated water around the drive syringes and observation cell.

In most experiments, protein originally denatured in 4 M guanidine hydrochloride was mixed with an equal volume of water. Refolding was monitored, at 420 nm, through changes in transmittance (T), which is related to absorbance by $A = \log (1/T)$. We were generally interested in the quantity needed for first-order kinetic plots, which is $\ln (A_\infty - A) = \ln [\log (T/T_\infty)]$, where A and A_∞ are the absorbances at time t and ∞, respectively, and T and T_∞ are the corresponding transmittances.

Results

Reversible Denaturation. The unfolding and refolding were monitored by difference absorption spectroscopy and circular dichroism. The denaturation transition, determined from changes in the difference spectrum at 420 and 405 nm, is shown in Figure 1. The major change, resulting from a blue shift in the Soret band, occurs between 2 and 3 M guanidine hydrochloride. Smaller changes observed below 2 M guanidine hydrochloride are due mainly to an increase in Soret band absorbance. The results are similar to those reported by Ikai *et al.* (1973). We assume, as do Ikai *et al.*, that the changes below 2 M guanidine hydrochloride are solvent effects on the heme, without a major change in protein conformation. Consistent with this, we observe that the circular dichroism of ferricytochrome *c* in 2 M guanidine hydrochloride (Figure 2) is very similar to published spectra in dilute buffer (Myer, 1968; Flatmark and Robinson, 1968; Zand and Vinogradov, 1967; Aki *et al.*, 1966).

On dilution of the guanidine hydrochloride from 4.0 to 2.0 M the denatured protein refolds, recovering its native structure as judged by its absorption spectrum and circular dichroism. In particular, disappearance of the difference spectrum in the Soret region suggests that the heme group has returned to its original environment within the protein. We have used this region of the spectrum to monitor the kinetics of refolding.

The circular dichroic spectra of the native and of the refolded proteins, in the region 210–450 nm, shown in Figure 2, are virtually identical, indicating that secondary structure as well as dissymmetrical interactions of heme and chromophoric side chains are the same in native and refolded forms. Ikai *et al.* (1973) give additional evidence that the denaturation process is thermodynamically reversible, and evidence that the state of the protein on completion of the denaturation transition is a random coil with no residual noncovalent structure.

Kinetics of Refolding. Except where indicated, all kinetic experiments were carried out in unbuffered solutions near pH 6.5. Typical kinetic data for the refolding of denatured cytochrome *c* are shown in Figure 3. First-order kinetic plots are curved implying that intermediate species accumulate during the reaction. However, the slopes of the plots (Figure 3) do not vary significantly, albeit within a fairly wide experimental range of uncertainty, over more than a 25-fold change in protein concentration, indicating that reaction steps involving two or more protein molecules do not occur.

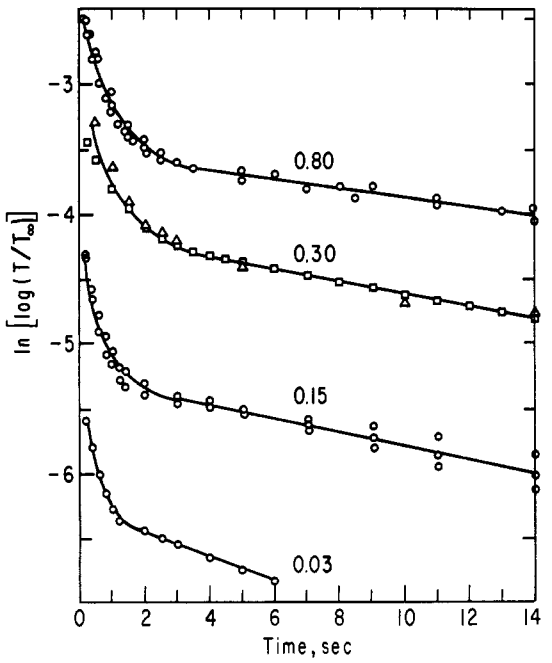

FIGURE 3: Kinetics of refolding of guanidine hydrochloride denatured ferricytochrome *c* at 25°, following dilution of the guanidine hydrochloride from 4.0 to 2.0 M. Protein concentration, in milligrams per milliliter, indicated by numbers next to the curves. All experiments were carried out in unbuffered solutions, near pH 6.5, and monitored at 420 nm.

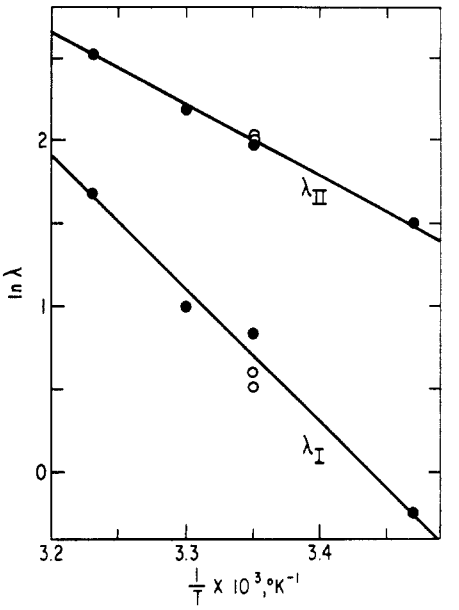

FIGURE 5: Arrhenius plots of λ_I and λ_{II}: (●) obtained from experiments illustrated in Figure 4 together with additional experiments not included in Figure 4 but carried out under the same conditions with the same sample of protein; (○) experiments performed at widely separated times near pH 5.5 and 6.5 (one value of λ_{II} at 25° obtained using a Cary 15 spectrophotometer). Each point is the average of from two to four values of ln λ. The curve for λ_{II} is displaced upward by five units on the ordinate.

Variation of the pH from 5.5 to 8.1 had no detectable effect on the kinetics.

The analysis of unimolecular reaction kinetics has been recently discussed in detail by Ikai and Tanford (1973). We will closely follow their method. The kinetic data on refolding of cytochrome *c* can be fitted to an equation for unimolecular reactions which has two exponential decay terms

$$A - A_\infty = (A_0 - A_\infty)P_I e^{-\lambda_I t} + (A_0 - A_\infty)P_{II} e^{-\lambda_{II} t} \quad (1)$$

where A_0, A, and A_∞ are the protein absorbances at $t = 0$, t, and ∞, respectively, P_I and P_{II} are constants, which in our case represent the fraction of the total absorbance change, $A_0 - A_\infty$, associated with decay terms I and II, and λ_I and λ_{II} are time constants (macroscopic rate constants) which are functions only of the microscopic rate constants for the reaction. The ordinate in Figure 3 is equal to ln $(A - A_\infty)$. We will adopt the convention that I refers to the faster of the two terms.

At large t, term I becomes negligible and the limiting slope of a semilog plot (Figure 3) yields λ_{II}. To obtain λ_I, the contribution of the slow exponential decay term, $(A_0 - A_\infty) \cdot P_{II} e^{-\lambda_{II} t}$, is subtracted from $A - A_\infty$, and the logarithm of the residue R plotted against time. The slope of this plot gives λ_I.

Data showing the temperature dependence of refolding, treated in the manner outlined, are shown in Figure 4. The logarithm of R against t gave satisfactory straight-line plots from which values of λ_I were determined. The second part of Figure 4 shows only the portion of the first-order plots from which λ_{II} values were determined (the portion at large values of t).

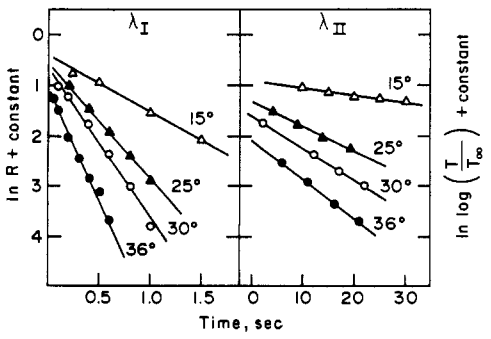

FIGURE 4: Temperature dependence of the kinetics of refolding of guanidine hydrochloride denatured ferricytochrome *c*. All experiments carried out in unbuffered 2.0 M guanidine hydrochloride solutions, near pH 6.5, at a protein concentration of 0.15 mg/ml. The data are treated in the manner outlined in the text. The slope of the lines gives λ_I and λ_{II} at the indicated temperatures.

TABLE I: Activation Parameters for the Refolding of Guanidine Hydrochloride (Gdn·HCl) Denatured Ferricytochrome *c* at 25°, 2.0 M Gdn·HCl, pH 6.5.

λ (sec^{-1})	E_a (kcal mol^{-1})	$\Delta G^{\pm}$ (kcal mol^{-1})	$\Delta H^{\pm}$ (kcal mol^{-1})	$\Delta S^{\pm}$ (cal deg^{-1} mol^{-1})	P
		Fast Phase (I)			
2.02	15.8	17.0	15.2	−6	0.7
		Slow Phase (II)			
0.050	8.6	19.2	8.0	−38	0.3

Arrhenius plots of λ_I and λ_{II} are shown in Figure 5. The energies of activation are E_I = 15.8 kcal mol^{-1} and E_{II} = 8.6 kcal mol^{-1}. The corresponding enthalpies, $\Delta H^{\pm}$, and entropies, $\Delta S^{\pm}$, are given in Table I.

Discussion

Like many other proteins, cytochrome *c* is reversibly denatured by guanidine hydrochloride. Our data show that at least one partly folded state of the protein accumulates during the folding of the randomly coiled denatured form (D) to the native structure (N). The more extensive kinetic studies of Ikai *et al.* (1972) indicate that at least two partly folded states (X_1 and X_2) are involved. The suggested mechanism is

$$N \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} X_1 \underset{k_{42}}{\overset{k_{24}}{\rightleftharpoons}} D \underset{k_{34}}{\overset{k_{43}}{\rightleftharpoons}} X_2 \qquad (2)$$

An important feature of this mechanism is that X_2 is an incorrectly folded form on a dead-end pathway. Optical parameters indicate that X_1 is quite highly unfolded, close to D, while X_2 is more folded, with optical properties closer to those of the native protein (Ikai *et al.*, 1973). This mechanism can account for experimental results in the transition region with a set of self-consistent microscopic rate constants. Under the conditions of our studies (25°, 2 M guanidine hydrochloride, pH 6.5) k_{43} is larger than the other k_{ij} (Ikai, 1971; Ikai *et al.*, 1973) so that λ_I becomes essentially equal to k_{43} and P_I becomes equal to the optical parameter $(A_X - A_D)/(A_N - A_D)$ where A_X, A_D, and A_N refer to the absorbance of the intermediate X_2, the randomly coiled form D, and the native state N, respectively (*cf.* Ikai and Tanford, 1971, 1973; Ikai *et al.*, 1973; Ikai, 1971). Therefore, the temperature dependence of the macroscopic rate constant λ_I corresponds to the activation parameters, $\Delta H^{\pm}$ = 15.2 kcal mol^{-1} and $\Delta S^{\pm}$ = −6 cal deg^{-1} mol^{-1}, for the conversion of the randomly coiled to the incorrectly folded form of the protein (Table I).

The value of the optical parameter (Table I) indicates that X_2 is highly folded; in particular, since absorbance measurements were made in the Soret band, it suggests that the heme group is removed from contact with the aqueous solvent. Folding of a polypeptide into a compact structure results in a decrease of the configurational entropy, which for a small protein, the size of cytochrome *c*, may correspond to about 100 kcal mol^{-1} in free energy (Tanford, 1970). This must be overcome by opposite effects.

The entropy of activation for the conversion of D to X_2 is very small, corresponding to about 2 kcal mol^{-1} in free energy, at 25°. This suggests that decreases in configurational entropy occurring during folding to the activated complex are largely compensated by other increases in entropy (*cf.* Tanford, 1970; Timasheff, 1970).

The temperature dependence of λ_{II} does not have as simple an interpretation as that of λ_I. Under the conditions of our studies, λ_{II} is approximately equal to $k_{42}k_{34}/k_{43}$ (Ikai, 1971) so that the measured activation parameters (Table I) are given by $\Delta H^{\pm}_{II} = \Delta H^{\pm}_{42} + \Delta H^{\pm}_{34} - \Delta H^{\pm}_{43}$ and $\Delta S^{\pm}_{II} = \Delta S^{\pm}_{42} + \Delta S^{\pm}_{34} - \Delta S^{\pm}_{43}$. Since the equilibrium constant for the interconversion of X_2 to D is k_{34}/k_{43}, the measured activation parameters, $\Delta H^{\pm}$ = 8.0 kcal mol^{-1} and $\Delta S^{\pm}$ = −38 cal deg^{-1} mol^{-1}, are equal to the sum: equilibrium thermodynamic parameter for the reaction X_2 to D plus activation parameter for the conversion of D to X_1. This shows that an unfavorable entropy change is a major barrier to conversion of X_2 to N, even though mounting the barrier evidently involves a net unfolding of the polypeptide chain.

Although the temperature dependence of the rate constants λ_I and λ_{II} corresponds to widely different values of the corresponding activation parameters $\Delta H^{\pm}$ and $\Delta S^{\pm}$, the values of $\Delta G^{\pm}$ are relatively close; increases in $\Delta H^{\pm}$ are largely compensated by increases in $\Delta S^{\pm}$. Such compensation of entropy and enthalpy is widely observed in conformational transitions of proteins (Lumry and Biltonen, 1969).

References

Aki, K., Takagi, T., Isemura, T., and Yamano, T. (1966), *Biochim. Biophys. Acta 122*, 193.

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

Flatmark, T., and Robinson, A. B. (1968), *in* Structure and Function of Cytochromes, Okunuki, K., Kamen, M. D., and Sekuzu, I., Ed., Tokyo, Japan, University of Tokyo Press, p 318.

Ikai, A. (1971), Ph.D. Thesis, Duke University.

Ikai, A., Fish, W. W., and Tanford, C. (1973), *J. Mol. Biol. 73*, 165.

Ikai, A., and Tanford, C. (1971), *Nature (London) 230*, 100.

Ikai, A., and Tanford, C. (1973), *J. Mol. Biol. 73*, 145.

Lumry, R., and Biltonen, R. (1969), *in* Structure and Stability of Biological Macromolecules, Timasheff, S. N., and Fasman, G. D., Ed., New York, N. Y., Academic Press, p 65.

Margoliash, E., and Frohwirt, N. (1959), *Biochem. J. 71*, 570.

Myer, Y. P. (1968), *J. Biol. Chem. 243*, 2115.

Tanford, C. (1970), *Advan. Protein Chem. 24*, 1.

Timasheff, S. N. (1970), *Accounts Chem. Res. 3*, 62.

Turner, S. R. (1971), Ph.D. Thesis, Duke University.

Zand, R., and Vinogradov, S. (1967), *Biochem. Biophys. Res. Commun. 26*, 121.