Chapter 2

Two-state Folding of Horse Ferrocytochrome c

2.1 Abstract

Ferrocytchrome c is a classic two-state, single domain, fast folder. This assurance comes from extensive equilibrium and kinetic folding studies carried out under strictly anaerobic conditions at 22°C. The GdnHCl-induced unfolding transitions monitored by the use of a sizable set of optical probes do not suggest the accumulation of any intermediate to a detectable level. The GdnHCl dependence of unfolding free energy ($\Delta G_\text{D}$) is linear over the full range of the denaturant concentration. The GdnHCl folding chevron is characterized by curvatures in both folding and unfolding limbs. However, refolding rates as a function of urea in the presence of different concentrations of GdnHCl yield $m_f^\dagger$ values (the kinetic m-value) that are quantitatively identical. This result, analyzed in terms of the denaturant dependence of the difference in the extent of solvent exposure between a relatively fixed transition state and the preceding state involved in the transition, suggests that the chevron curvature is not related to differential accumulation of a folding intermediate with varying concentration of GdnHCl in the refolding medium. Denaturant dependence of stopped-flow burst signals recorded in normal refolding experiments (pH 7, 22°C) is essentially identical with that recorded in simulating experiments where the protein stays steadily unfolded even in the denaturant-diluted medium (pH 1.5-2, 22 or 43°C depending on the use of urea or GdnHCl), and they match the denaturant dependence of equilibrium signals for the unfolded protein. The results
demonstrate that the burst phase does not involve an early folding intermediate. Rather, the folding kinetics are essentially two-state.

2.2 Introduction

Ever since the pioneering work of Tanford and coworkers (39) and others (40-42), horse cyt c has served as a paradigm in the pursuit of the protein folding code. Indeed, some of the fundamental concepts in this area have evolved from extensive work with ferricyt c (43-46). There have, however, been serious problems from the viewpoint of interpretation of results, most of the conflicts arising from undesirable modulations of heme-polypeptide contacts across the folding-unfolding equilibrium. The chemistry involved is very well understood (25, 45, 47-49). Briefly, the native-state axial ligation of M80 to the oxidized heme is replaced by H26 and H33 under destabilizing conditions. Even though non-native, the Fe$^{3+}$-H26 and Fe$^{3+}$-H33 contacts are stable, so the dissociation rates of these two ligands from the iron are far smaller than the protein folding rate. Consequently, the polypeptide collapses with persisting Fe$^{3+}$-H26 and Fe$^{3+}$-H33 contacts. This act of misfolding generates chain misconfiguration at later stages of folding. Folding is then retarded because the frustrated chain organization stays in a kinetically frozen intermediate state until thermal dissociation of non-native histidines from the heme iron gives way to the ligation of M80, thus facilitating polypeptide reconfiguration. Such fortuitous events not only obscure the processes that are of fundamental interest but also lead to subtly conflicting interpretations. For example, in order to achieve correct chain configuration at a later stage of folding, an unfolding event that would disperse the incorrect organization, is necessary. In this sense, the intermediate could be
regarded as a trapped misfolded intermediate (47), a prototype of late intermediates predicted by theoretical studies (50). On the other hand, the intermediate may be described just at the level of the relevant N- and C-terminal helices without paying any attention to wrong chain organizations in other parts of the molecule (51-52).

The problem of kinetic trap or chain misorganization in the folding run of ferricyt c can be mitigated by allowing refolding at low pH or in the presence of an extrinsic heme ligand that blocks non-native histidine ligation. Folding kinetics is then modeled as a two-state reaction (43, 47) or by invoking intermediates (44), again a matter of conflict.

Interpretational ambiguities are avoided with ferrocyt c, since non-native heme ligands do not interfere with inherent accelerated folding of this redox state of cyt c. Aqueous stability of ferrocyt c is extremely high (~18 kcal mol\(^{-1}\) at 22°C), and stopped-flow observable millisecond kinetics are fast, simple, and essentially monophasic (5, 14, 25-28). These virtues render ferrocyt c even more attractive a system for exploring the fundamental principles that govern protein folding (5).

Studies have projected ferrocyt c as a prototype of a two-state protein (5, 25-28). However, recently, Roder and coworkers state that folding of ferrocyt c is not two-state (53). Despite the simplicity of observed folding kinetics, the rates roll over in the folding chevron of ferrocyt c (25). Further, a submillisecond burst change in optical signals characterizes the stopped-flow kinetics (5). These two criteria would appear to disqualify ferrocyt c for a two-state system, since one of the interpretations of chevron curvature is that accumulation of an early intermediate, presumably formed in the burst phase, limits the overall rate of
refolding (54-56). The phenomenon of chevron curvature coupled with burst signals, observed for a number of proteins, is often used as a strong evidence for a general three-state (or even higher) protein folding mechanism (56-58, for example). But careful studies have also shown that a number of small proteins, folding kinetics of which were earlier described as three-state on the basis of observed chevron curvature and submillisecond burst kinetics, are actually two-state (59-63).

This work presents some basic equilibrium and kinetic folding experiments to demonstrate further that the folding kinetics of ferrocyt c are indeed two-state (28). Since kinetic intermediates are best described when equilibrium experiments hint at their accumulation, an exhaustive search for neutral-pH equilibrium intermediate was made. Transitions probed by the use of circular dichroism at several wavelengths ranging from far-UV to the near-infrared region, heme absorbance at Soret, visible, and near-infrared bands, and heme-tryptophan fluorescence excitation energy transfer, are all coincident and two-state. Kinetic experiments, in which refolding in the presence of different concentrations of GdnHCl along the chevron were measured as a function of urea, indicate that the chevron curvature is not due to the accumulation of a kinetic intermediate. Analyses of GdnHCl and urea distributions of burst relaxation signals of persisting unfolded cyt c at pH 1.5-2 and normal cyt c at pH 7 suggest that the submillisecond burst phase does not entail a structural intermediate. These demonstrations are essential in order to be able to use ferrocyt c kinetics for a mechanistic description of protein folding.
2.3 Materials and Methods

Horse heart cyt c (Type VI) was from Sigma. Denaturants were obtained from Gibco BRL. Other chemicals were analytical grade. All experiments were done in 0.1 M sodium phosphate buffer at 22°C unless indicated otherwise. Extreme care was taken to avoid air exposure of solutions at all stages of experiments.

2.3.1 Measurements of equilibrium unfolding

The protein concentration varied from 8 µM to 2.1 mM depending on the transition dipole strength of the spectroscopic probe used. Stock solutions of the native and the unfolded protein (5-8 M GdnHCl or 10 M Urea) were mixed in appropriate ratios to obtain protein samples containing different concentrations of denaturants. To prepare ferrocyt c, solutions were deaerated under nitrogen before addition of a small volume of a concentrated aqueous solution of sodium dithionite to obtain a final reductant concentration of 1-3 mM. The sample was sealed under nitrogen and equilibrated for ~45 minutes at 22°C. The pH and denaturant concentrations of samples were checked before and after measurements. The reported GdnHCl concentrations are those determined after measurements. Tryptophan fluorescence excited at 280 nm (slit width 1.5 nm) was measured at 358 nm (slit width 8.0 nm) by using a 1 cm square quartz cuvette. Optical absorption measurements were done as usual (1 cm cuvette). For CD measurements, a JASCO J715 instrument was used (1 mm cuvette). Corrections for buffer signal, including the contribution of denaturants, were done routinely.
2.3.2 Measurement and primary analysis of folding-unfolding kinetics

The procedure for kinetic experiments under controlled anaerobic condition has been described earlier (26). Briefly, cyt c initially unfolded in 6.5 M GdnHCl, pH 7, was reduced under nitrogen by adding a concentrated solution of sodium dithionite to a final concentration of ~3 mM. The final protein concentration in the refolding mixture was 44 µM when experiments involved GdnHCl alone. The final protein concentration for those experiments where refolding was carried out in the presence of various concentrations of urea was 16 µM. Use of the same protein concentration in all experiments involving urea facilitated data normalization. Unfolding experiments involved only GdnHCl, and were performed following the same procedure of two-syringe mixing. The final protein concentration in unfolding experiments was ~28 µM. Gas-tight syringes were employed to transfer protein and buffer solutions into stopped-flow mixing head (SFM 400, Biologic). Excitation wavelength was 280 nm, and emission was measured using a 335 nm cut-off filter. Typically, 10-20 shots were averaged. Kinetic traces were analyzed using single- or double-exponential functions to extract apparent rates, $\lambda_i$, the initial signal, $S_0$, which corresponds to the ‘zero-time’ signal in the stopped-flow time window, the observed signal, $S_{obs}$, and the final equilibrium signal, $S_\infty$, corresponding to the signal value at $t = t_\infty$. The $S_0$, $S_{obs}$, and $S_\infty$ signals were subjected to initial normalization by first subtracting the buffer fluorescence signals, and then dividing by the recorded signal of the unfolded protein in the highest GdnHCl concentration. In the unfolding set of measurements the $S_\infty$ value of the kinetic trace at the highest concentration of GdnHCl employed was used to divide the fluorescence signals.
2.4 Results

2.4.1 Two-state equilibrium unfolding

In search of equilibrium population of structural intermediates of ferrocyt c, the GdnHCl-induced unfolding transition was probed by employing CD at several wavelengths in the far-UV to the near-infrared region, heme absorbance at Soret, visible and near-infrared bands, and tryptophan fluorescence (Figure 2.4.1). Since optical properties of different spectroscopic probes are different in both pre- and post-transition regimes, the unfolding curves shown were normalized individually according to the observed functional dependence of the optical property on GdnHCl. The pre- and post-unfolding baselines in the raw data were fitted to first-order polynomial in order to extract normalized values of fraction unfolded as a function of the denaturant. Figure 2.4.1 shows the simple result that the transitions measured by different optical probes are, within error, nearly superimposable and cooperative. The observation of indistinct transition curves fails the basic test for the accumulation of equilibrium intermediate to a detectable level. The solid lines in the figure is a two-state fit of the data assuming a linear functional dependence of $\Delta G_D$ on GdnHCl according to (64-65),

$$\Delta G_D = \Delta G_D^o - m_g [\text{GdnHCl}] = m_g (C_m - [\text{GdnHCl}])$$

(1)

where $\Delta G_D^o$ is the free energy of unfolding in the absence of denaturant, $m_g$ (= $2.3RT \partial \log K / \partial [\text{GdnHCl}]$); the subscript ‘g’ in $m_g$ stands for global) is a parameter related to the change in surface area in global unfolding of the protein, and $C_m$ is the GdnHCl concentration at which $\Delta G_D = 0$. Values of $\Delta G_D^o$ and $m_g$ obtained from this analysis are 17.9(±1) kcal mol$^{-1}$ and 3.54(±0.05) kcal mol$^{-1}$ M$^{-1}$, respectively, indicating enormous aqueous stability of ferrocyt c.
The equilibrium parameters for the unfolding of ferrocyt c are independent of protein concentration. In the present study, the protein concentration used for various spectroscopic probes differed widely, in the 0.008-2.1 mM range. In spite of a 260-fold difference in the protein concentration, the two-state unfolding parameters did not change. The coincidence of various transition curves for different protein concentrations provides a reason to rule out the possible accumulation of associating intermediates of the kind reported for certain solvent-induced unfolding of proteins (66-67).

**Figure 2.4.1** Plots of fraction of ferrocyt c unfolded as a function of GdnHCl probed by CD signals at 222 nm (◊), 290 nm (●), 413 nm (■), 525 nm (▲), and 603 nm (▲), tryptophan fluorescence at 358 nm (△), and heme absorbance at 430 nm (▼), 549 nm (▼), and 643 nm (♦). The origins of these optical transitions are well understood. The 643-nm band associated with the unfolding of ferrocyt c has not been described before. The solid line represents the iterated least squares fit of the data to a two-state unfolding transition. The fit yields $\Delta G_D^{\circ}=17.9(\pm1)$ kcal mol$^{-1}$ and $m_g=3.54(\pm0.05)$ kcal mol$^{-1}$ M$^{-1}$. All transitions were measured at 22(±1)°C, 0.1 M phosphate, pH 7.

### 2.4.2 Dependence of the free energy of unfolding, $\Delta G_D$, on GdnHCl

But how reliable is the value of $\Delta G_D^{\circ}$? A simple approach to validate the linear extrapolation method is to analyze unfolding transitions induced by one denaturant in the presence of several lower concentrations of another (68). The
strategy used in this study is to extract $\Delta G_D^\circ$ values from a series of urea-induced unfolding transitions, one transition in the presence of a given GdnHCl concentration, and then to test if they are mapped linearly onto the $\Delta G_D$ values already obtained from the data shown in Figure 2.4.1. Therefore, urea-induced transition curves of ferrocyt $c$ in the presence of 0, 0.9, 1.4, 1.9, 2.3, 2.7, and 3.3 M GdnHCl were recorded under identical conditions of pH and temperature. Figure 2.4.2 shows these data. It is clear that within the limit of the aqueous solubility of urea, complete unfolding is achieved only in the presence of 3.3 M GdnHCl. Observation of incomplete unfolding in the presence of other concentrations of GdnHCl is not surprising, given the extraordinary thermodynamic stability of ferrocyt $c$. To make progress, all data sets were normalized with respect to the fluorescence observed in 6 M urea and 3.3 M GdnHCl (i.e., the last point in the transition labeled 7). Transition 7, that shows complete unfolding, was then analyzed using eq 2 (ref 24).

$$S_{\text{obs}} = \frac{(c_f + m_f [D]) + (c_u + m_u [D]) \exp(-\frac{\Delta G_D^\circ + m_u [D]}{RT})}{1 + \exp(-\frac{\Delta G_D^\circ + m_u [D]}{RT})}$$

(2)

where $S_{\text{obs}}$ is the observed signal, $c_f$ and $c_u$, and $m_f$ and $m_u$ represent intercepts and slopes of native and unfolded baselines, respectively, and [D] represents urea concentration. All parameters were floated during fitting. The iterated fit parameters are given in the legend to Figure 1.4.2.

Since the urea dependence of post unfolding baselines is expected to be same for all transitions, the values of $c_u$ and $m_u$ obtained from the analysis of transition 7 can be used to constrain the fits to transitions 1-6. To search for other constraints, the same series of experiments was repeated with ferricyt $c$ (data not
shown). Due to its less stability \( (\Delta G_D^\circ = 8 \pm 1 \text{ kcal mol}^{-1}) \) ferricyt \( c \) is completely unfolded by urea even in the absence of GdnHCl. Analysis of these data (not shown), indicated that the value of \( m_g \) \( (1.7(\pm0.06) \text{ kcal mol}^{-1} \text{ M}^{-1}) \) does not vary at all with different concentrations of GdnHCl in the unfolding medium, consistent with results of similar studies reported earlier \((68)\). This observation provided a basis for constraining the value of \( m_g \) in the analysis of ferrocyan c unfolding curves. Hence, values of \( c_u, m_u, \) and \( m_g \) determined from transition 7 were used in eq 2 to constrain the fits to transitions 1-6. Thermodynamic parameters thus obtained are given in the legend to Figure 2.4.2.

To test the validity of linear functional dependence of Gibbs free energy of unfolding on GdnHCl, the values of \( \Delta G_D \) determined from urea unfolding curves are plotted in Figure 2.4.2b together with the values of \( \Delta G_D \) \( (= -RT \ln K) \) calculated from the curves shown in Figure 2.4.1. A linear least square fit of the data \( (r = 0.99) \) yields \( \Delta G_D^\circ = 18.36(\pm0.45) \text{ kcal mol}^{-1} \) and \( m_g = -3.6 \text{ kcal mol}^{-1} \text{ M}^{-1} \) for ferrocyan c, and \( \Delta G_D^\circ = 7.07 \text{ kcal mol}^{-1} \) and \( m_g = 3.0(\pm0.1) \text{ kcal mol}^{-1} \text{ M}^{-1} \) for ferricyt \( c \). These values of \( \Delta G_D^\circ \) and \( m_g \) are in excellent agreement with those obtained from the equilibrium curves shown in Figure 2.4.1. The results thus validate the linear functional dependence of \( \Delta G_D \) on GdnHCl in the full range of the denaturant concentration.
Figure 2.4.2 (a) Urea-induced denaturation of ferrocyt c in the presence of different concentrations of GdnHCl, 22(±1)°C, 0.1 M phosphate, pH 7. Curve labels 1 to 7 correspond to 0, 0.9, 1.4, 1.9, 2.3, 2.7, and 3.35 M GdnHCl, respectively. Values of $\Delta G^\circ$ (in kcal mol$^{-1}$) for curves 7 to 1 are: 6.49(±0.75), 10.54(±0.8), 11.87(±1.0), 12.49(±0.4), 13.77(±1.2), 15.29(±1.33), and 18.73 (±1.21), respectively. The value of 1.76(±0.4) kcal mol$^{-1}$ M$^{-1}$ for $m_g$, obtained from the fit to curve 7 (eq. 2), was used to constrain the fits to curves 1-6 (see text). (b) Plot of $\Delta G^\circ$ (○, extracted from data in Figure 2.4.1) and $\Delta G^\circ$° (●, determined from curves 1-7 shown in panel a) as a function of GdnHCl. Values of $\Delta G^\circ$° and $m_g$, obtained as the y-intercept and the slope of the linear least square fit (r=0.99), are 18.36(±0.45) kcal mol$^{-1}$, and 3.61 kcal mol$^{-1}$ M$^{-1}$, respectively.

2.4.3 The curvatures in folding chevron and burst relaxation

Stopped-flow kinetics of the folding-unfolding reactions of ferrocyt c were recorded as a function of GdnHCl to check for possible accumulation of kinetic intermediate. For refolding, ferrocyt c was initially unfolded in 6.5 M GdnHCl, pH 7. Figure 2.4.3a shows the initial 100 ms of a representative kinetic trace that is best described by two exponentials: a major phase with rate constant, $k_{obs} = 276$ s$^{-1}$ and a minor phase with rate constant, $k_{obs} = 39$ s$^{-1}$. The slow minor phase, the amplitude of which averages to no more than 10(±2)% of the total observed amplitude, is due most likely to a small heterogeneous population and is excluded from further analysis. The kinetic trace also reports on the loss of 70% of the total expected fluorescence in a burst kinetic phase that is complete within the dead time of the stopped flow (~2.6 ms). Figure 2.4.3b shows the denaturant dependence of the logarithm of the observed folding and unfolding rates. The relaxation minimum in the chevron plot corresponds to the transition midpoint ($C_m \approx 5$ M GdnHCl, see Figure 2.4.1a). The observed folding rate increases
progressively as the GdnHCl concentration in the refolding medium is lowered below the transition midpoint. The rate-denaturant relationship is apparently not linear, however; the rates rollover as increasingly native-like conditions are approached. Similar rollover of unfolding rates is observed as strongly unfolding conditions are approached. This is atypical of a kinetically two-state protein, especially when the dependence of ΔG_D on GdnHCl has been found to be linear (Figure 2.4.2b). To account for curvatures in both folding and unfolding limbs the chevron is fitted within the constraint of a two-state N ⇌ U model by assuming the following quadratic relationship between log k_f(u) and GdnHCl (69).

\[
\begin{align*}
\log k_f &= \log k_{f}^\circ - m_{1,f}[D] - m_{2,f}[D]^2 \\
\log k_u &= \log k_{u}^\circ + m_{1,u}[D] - m_{2,u}[D]^2
\end{align*}
\]

An earlier interpretation of chevron curvature is the transient accumulation of kinetic intermediates (54-55). Under strongly nativelike conditions, where structural intermediates are expected to accumulate due to their increased stability, the observed refolding rate is limited by the rate of \( \text{I} \rightarrow \text{N} \) conversion according to a \( \text{U} \leftrightarrow \text{I} \leftrightarrow \text{N} \) scheme. The chevron rollover may appear coupled to burst refolding of ferrocyt \( \text{c} \). Figure 2.4.3c shows the denaturant distribution of the normalized burst relaxation signals. The ‘zero-time’ fluorescence signal in the stopped-flow time window \( S_o \), quantified by extrapolating the fitted kinetic traces to \( t = 0 \), decreases as the final GdnHCl concentration in the refolding medium is lowered. The area between the \( S_o \) values and the equilibrium post unfolding baseline extrapolated linearly to native-like conditions contains the unobservable fluorescence changes associated with burst folding kinetics. Burst refolding is also seen in the folding kinetics of ferricyt \( \text{c} \), and the GdnHCl
dependence of the $S_0$ values has often been assumed to be the reminiscence of a
distinct phase transition of a refolding intermediate that is fully formed within
the dead time of stopped-flow (52). It may then appear that the rollover in the
folding limb of ferrocyt $c$ chevron is also
due to accumulation of an intermediate.

**Figure 2.4.3** (a) Refolding trace in the presence
of 0.95 M GdnHCl, 0.1 M phosphate, 22°C. The
amplitude is normalized with reference to the
fluorescence of the unfolded protein (6.8 M
GdnHCl). A prominent burst amplitude (70%) is
apparent. Kinetics are best described by two
exponentials; 276 s$^{-1}$ (90% amplitude) and 39 s$^{-1}$
(10% amplitude). The slower phase is due likely
to heterogeneity or a small fraction of the
oxidized protein in the unfolded sample. (b) The
chevron: refolding (●) and unfolding (○). The
solid lines are quadratic fits according to eq 3.
(c) GdnHCl dependence of the burst amplitude.
Gray solid circles (○) are the normalized
amplitude of the major kinetic phase. The broken
line through these points is an empirical fit to $y =
\begin{align*}
a + bx + cx(\exp(-dx)) \end{align*}$, where $a$, $b$, $c$, $d$ are constants. The equilibrium transition (●) yields
$\Delta G_0^\circ = 17.99$ kcal mol$^{-1}$ and $m_g = 3.54$ kcal mol$^{-1}$ M$^{-1}$. 
2.4.4 A test on the interpretation of chevron curvature

To test if the chevron rollover indeed originates from accumulation of a transient intermediate, an extended series of kinetic experiments was carried out where refolding of ferrocyt c in the presence of 0.9, 1.5, 1.9, 2.3, 2.7, and 3.0 M GdnHCl was studied as a function of urea. The rationale is that if an intermediate is involved, and it is populated increasingly with more native-like conditions, then the gradients of \( \log k_f \) versus urea for different concentrations of GdnHCl in the refolding medium are not expected to be uniform.

Figure 2.4.4a samples the initial 40 ms of kinetic traces showing refolding of ferrocyt c to 0.2(±0.05) M urea in the presence of GdnHCl concentrations indicated. The traces are described by single-exponential, and they show the burst behavior discussed earlier. Figure 2.4.4b shows the plots of \( \log k_f \) against urea for the six different concentrations of GdnHCl. While the lowest concentration of GdnHCl (0.9 M, the uppermost plot) offers a strongly native-like condition where the chevron curvature is accentuated (Figure 2.4.3b), the highest one (3.0 M, the lowermost plot) approaches the transition region where the protein is expected to operate in a two-state \( N \iff U \) manner. The relationship between \( \log k_f \) and urea is not absolutely linear. The data are best described by

\[
\log k_f = \log k_f^0 - m_f[\text{urea}] - 0.0103[\text{urea}]^{\frac{1}{2}}
\]

(4)

where \( m_f^0 = 2.303RT(m_f^0 - 0.0103[\text{urea}]) \). Note that the kinetic \( m \)-value under linear rate-denaturant assumption is given by \( m_{f(0)}^0 = 2.3RT (\partial \log k_f/\partial[GdnHCl]) \). This conclusion is further supported by observations that the burst phase represents the earliest collapse of the unfolded state to a state (\( U' \)) that is devoid of any considerable native-like structure. These derivations constrain the folding. The normalized quantity \( \alpha = m_f^0/m_g \), where \( m_g \) is the change in surface
area in urea-induced equilibrium unfolding, provides a measure of the amount of surface area buried upon folding from the initial denatured state to the transition state in a given concentration of GdnHCl. As the slopes of the plots suggest, the value of $\frac{m_f}{m_g}$ is found to be uniform for all the concentrations of GdnHCl used. At 2 M urea, for example, $\frac{m_f}{m_g} = 0.27 \pm 0.02$. This observation indicates that the initial denatured state that is created by rapid dilution of the unfolded protein is not a kinetic intermediate, and the chevron rollover is, therefore, not due to the accumulation of a kinetic intermediate under native-like conditions.

Figure 2.4.4 (a) Representative traces for refolding to 0.2 (±0.05) M urea in the presence of 0.9 (○), 1.5 (□), 1.9 (∆) and 2.31 (∇) M GdnHCl. (b) $k_f$ versus urea plots for refolding in the presence of (from top to bottom) 0.9, 1.5, 1.9, 2.3, 2.7, and 3.0 M GdnHCl.

2.4.5 Testing on the origin of urea dependence of burst relaxation signal

Since the ultrafast signals are associated with the incipient event in folding, it would be of interest to know if these optical signals are related to those that originate from the initial transient denatured states. Let the denatured state be the unfolded state under low denaturant (or refolding) conditions. The check requires finding conditions under which the protein is steadily unfolded even in the absence of a denaturant. One then compares the denaturant dependence of its
equilibrium signal with the ‘zero time’ burst signal under identical experimental conditions (70-71). In earlier experiments, a fragment of ferricyt c was used to model the unfolded protein (70). It is, however, desirable to use the whole protein rather than a fragment. The present study primarily deals with ferrocyt c. But due to its large stability no condition exists, not even at very high temperature, under which ferrocyt c is unfolded in the absence of high concentrations of denaturant. Experiments were therefore designed to interrogate the denaturant dependence of burst relaxation signals of intact and persistently unfolded ferricyt c.

U_A, the acid-unfolded state of ferricyt c (pH 1.5-2, without Cl⁻), closely simulates the denaturant-unfolded state of the protein at pH 7 (72-73). Although assumed to be structureless, U_A shows some changes in tryptophan fluorescence when titrated with urea (Figure 2.4.5a). Nevertheless, U_A is truly structureless in urea concentrations >2 M. Figure 2.4.5b presents a few kinetic traces to show what happens to the fluorescence of U_A when it is transferred rapidly from a solution of 10 M urea to ones of lower concentration of the denaturant in a stopped-flow. Each denaturant jump sets the fluorescence signal to a new value, S_o (signal at time t = 0), within the dead-time of the stopped flow, and the signal continues thereafter at the same level without any detectable change, S_∞ (the signal at time t = ∞). For all refolding jumps, S_o = S_∞. In the presence of 0.8 M urea the reset value is ~60% less than the fluorescence of U_A in the presence of 10 M urea. To compare these results with those derivable from actual folding-unfolding reactions, equilibrium and kinetic experiments were conducted with ferricyt c at pH 7, 22°C. Figure 2.4.5c maps the urea dependence of normalized values of burst and equilibrium signals for the U_A state (pH 1.5) on to those obtained from normal refolding experiments (pH 7). It is apparent that (i) the urea
dependencies of the two sets of burst signals overlap, (ii) urea distribution of both sets of burst signals are in agreement with that of the equilibrium fluorescence of the $U_A$ state, and (iii) in higher concentrations of urea these signals appear to merge with the post unfolding baseline of the equilibrium unfolding transition recorded at pH 7. These observations indicate that the burst folding fluorescence of cyt $c$ originates from the unfolded state of the protein under denaturant-diluted refolding conditions, and that the urea-dependence of burst signals represents most likely an extension of the post unfolding baseline to native-like conditions rather than the titration of an early kinetic intermediate.

**Figure 2.4.5** (a) Fluorescence of ferricyt $c$ as a function of urea, pH 1.5, 22°C. The fluorescence values were normalized by $(F_x - F_0) / (F_{10} - F_0)$, where $F_x$, $F_0$, and $F_{10}$ are values in the presence of $x$, 0, and 10 M urea, respectively. (b) Typical time-resolved fluorescence signals of ferricyt $c$ (pH 1.5, 22°C) recorded by stopped-flow dilution of urea from 10 M to different final concentrations indicated. (c) Urea dependence of burst signals quantified from normal refolding kinetics at pH 7, 22°C (●) is compared with burst signals recorded at pH 1.5 as shown in panel b (▲), equilibrium fluorescence signals of cyt $c$ at pH 1.5 as shown in panel a (■), and at pH 7 (●).
2.4.6 Testing on the origin of GdnHCl dependence of burst fluorescence of cyt c

Since GdnHCl is more relevant for kinetic studies with cyt c, it is desirable to probe into GdnHCl dependence of burst fluorescence changes. The $U_A$ state of ferricyt c (pH 2) was used for this set of experiments also. But $U_A$ is known to assume a molten globule-like structure in the presence of increasing concentrations of GdnHCl up to ~1 M (72). A temperature melt of $U_A$ in the presence of 0.9 M GdnHCl (data not shown) suggested that it is largely unstructured at temperatures $>40^\circ$C. The effect of GdnHCl on fluorescence signals of the $U_A$ state was therefore studied at 42(±2)$^\circ$C. Experiments involved measurement of (i) equilibrium fluorescence of $U_A$ as a function of GdnHCl, (ii) burst change in fluorescence of $U_A$ in stopped-flow GdnHCl jumps, (iii) equilibrium unfolding of ferricyt c at 10 and 40$^\circ$C, pH 7, and (iv) stopped-flow refolding kinetics at 10$^\circ$C, pH 7.

Figure 2.4.6 shows the results. The equilibrium fluorescence of cyt c at pH 2 ($U_A$), and the $t = 0$ fluorescence signals determined from kinetic experiments at pH 2 ($U_A$) and at pH 7, all show the same dependence on GdnHCl within the limitations of experimental constraints (see the Figure legend), and these three sets of fluorescence values merge with the post unfolding baseline of the GdnHCl-induced transition of cyt c (pH 7). Thus, the GdnHCl distribution of burst signals recorded in normal refolding experiments may be taken as the extension of the post unfolding baseline to native-like conditions. These results again indicate that the burst fluorescence of cyt c is due to the transient denatured state generated when the denaturant is diluted rapidly in order to drive the protein to fold.
2.5 Discussion

Because the unfolded polypeptide cannot sample out the possible configurations within the Levinthal limit of even the smallest of all real proteins, it is generally believed that folding is hierarchic, and progresses through discrete pathways consisting of one or more intermediates (74). Possible kinetic intermediates most often escape detection, and are not amenable to structural characterization due to their transient lifetime and accumulation to an insufficient level. Kinetic folding intermediates are described best if their equilibrium accumulation is demonstrated. Implication of one or more intermediates in both equilibrium and kinetic processes would, therefore, strengthen the pathway concept of folding.

2.5.1 Two-state equilibrium of ferrocyan c at neutral pH

A part of the present study concerns with whether or not structural intermediates accumulate to a level detectable at neutral pH in the equilibrium
unfolding of ferrocyt c. The basic experimental test to ascertain the operation of a protein according to the two-state equilibrium, $N \rightleftharpoons U$, is the coincidence of unfolding transitions for multiple spectroscopic probes (75), although observation of distinct transition curves of two different properties for the denaturation does not necessarily provide a proof of the existence of an intermediate (76). By this criterion (Figure 2.4.1), no structural intermediate of substantial thermodynamic stability populates across the unfolding transition of ferrocyt c. Another evidence for the $N \rightleftharpoons U$ equilibrium is found from the ratio of calorimetric to effective enthalpies at various transition temperatures. Although horse ferrocyt c is not directly amenable to the usual thermal unfolding studies due to its unusually large conformational stability, extensive calorimetric studies of Privalov and coworkers (41, 77) with oxidized cyt c have indicated many years ago that the ratio of calorimetric enthalpy to van’t Hoff enthalpy is close to unity, suggesting the existence of insignificant populations of intermediates that are thermodynamically stable. It is noted that thermal reversibility and calorimetric data for yeast iso-1 and iso-2 ferrocyt c have been reported (78-79).

The tests conducted here thus provide no evidence for a neutral-pH equilibrium intermediate of horse cyt c. Well-defined equilibrium intermediate at physiological pH for proteins the size of cyt c or smaller has been rarely described. In fact, to a very good approximation, unfolding transitions of small globular proteins are representatives of a cooperative two-state transition (77). On the other hand, the accumulation of detectable intermediates in equilibrium transitions of proteins relatively larger or moderate in size has been shown many years ago in several cases, including $\alpha$-lactalbumin (80-81), chicken cystatin (82), pituitary growth hormone (83), bovine carbonic anhydrase (84), penicillinase
Two-state transition of small proteins at physiological pH could be a consequence of their size, and the lack of disulfides or interactions essential for stabilization of intermediates. Stabilizing interactions, introduced by adding ions for eliminating charge-charge repulsions in acid or alkaline solutions, for example, lead to accumulation of partly folded conformations. For ferricyt c, the formation of a molten globule at pH ~2 and high salt, also called the “A state”, has been well documented (73, 86-88). Demonstration of the possible existence of the acid molten globule of ferrocyt c has not been possible due to severe experimental constraints imposed by higher autooxidation rate of the protein at acid pH.

2.5.2 Origin and interpretation of curved chevron plot of ferrocyt c

The idea that the downward curvature in the folding limb of chevron plots could be related to transient accumulation of submillisecond folding intermediates is due to the early work of Fersht and coworkers with barnase (54). The implication of folding intermediates in chevron curvatures received major support from observations that a sizable set of proteins whose refolding rates roll over under nativelike conditions also display varying degrees of loss of optical signals in a burst refolding phase. Based mainly on these facts, it has been generalized that chevron curvatures reflect accumulation of folding intermediates (56). It is a general belief that the stopped-flow rates roll over because the burst product limits the overall rate of folding to the native state. The rollover is accentuated with increasing stabilization, and therefore accumulation, of the burst product as more nativelike conditions are approached. As such, this interpretation appears rational. However, chevron curvature is also seen in the unfolding limb of ferrocyt c.
(Figure 2.4.3b), even though no burst-unfolding signal is recorded (Figure 2.4.3c). Further, loss of optical signals in the submillisecond regime need not necessarily mean accumulation of an intermediate. Assignment of the burst product to a structural intermediate requires knowledge of its attribute. It is therefore necessary to analyze in detail the chevron properties and denaturant dependences of burst optical signals.

A subset of refolding experiments, where kinetics of folding of the GdnHCl-unfolded protein to different final concentrations of GdnHCl are recorded as a function of a weaker denaturant like urea (Figure 2.4.4b), provide some clues to chevron curvature. The parameter of main interest is $m_{\ell}^{\ddagger}$ that provides a measure of surface area that is buried on going from the $U'$ state to the transition state, $\ddagger$, according to the following scheme

$$U \rightarrow U' \rightarrow \ddagger \rightarrow N$$

$U'$ forms incipiently when the denaturant is diluted out, and is the initial denatured protein or an early folding intermediate, depending on the extent of its structure. Within error, the same value of $m_{\ell}^{\ddagger}$ is obtained for different concentrations of GdnHCl. This result is not expected if the attribute of $U'$ is a structural intermediate. If it were so, the value of $m_{\ell}^{\ddagger}$ obtained from rate-urea space for a low GdnHCl concentration (0.9 M) that strongly supports structure would have been different from that for a higher concentration of GdnHCl (3.0 M) where intermediates are not expected to accumulate. According to this interpretation, the folding transition state of ferrocyt $c$ is largely immobile.

Chevron curvature in other two-state proteins has been explained by assuming mobile transition states (ref 69, for example). Movement of the transition state ensemble toward the unfolded state under conditions of increased
protein stability is envisaged if Hammond behavior is invoked (89-90). According to this model a broad energy barrier separates the native state from the unfolded one. The rate-limiting transition state ensemble which is the highest point on the barrier energy profile moves toward the unfolded state upon approaching native-like conditions, giving rise to rate rollover in the refolding limb of the chevron. Similarly, movement of the rate-limiting ensemble toward the native state at high denaturant concentrations can cause a curvature in the unfolding limb of the chevron. This model was used to explain the curvatures in both folding and unfolding limbs of the chevron plot of the two-state protein U1A (69).

Chevron rollover in the absence of kinetic intermediates can also be explained by invoking a continuum of rapidly interconverting collapsed states such that the rates of interconversion are irresolvable in the time-scale of conversion of each individual collapsed state to the native state (91). Denaturant dependent redistribution of the collapsed ensemble may give rise to rate rollover. The continuum model was used to explain the chevron curvature for half a dozen proteins (91).

These alternative interpretations of chevron curvature are context specific. They do not anull the interpretation relating to the accumulation of kinetic intermediates. To make further progress, the nature of the burst product the accumulation of which supposedly gives rise to curvatures needs to be ascertained.

2.5.3 Possible origin of burst refolding phase of cyt c

Both dimensional and configurational properties of denatured proteins are highly sensitive to changing solvent conditions. The density of unfolded chains,
for example, increases linearly as the ionic strength of the denaturing medium increases (76). Similarly, polypeptide chains expand as the solvent quality becomes better, but contract when diluted into a poorer solvent that supports folding. Non-native states are also characterized by ultrafast reorganization of backbone dihedral angles at the level of individual residues (92), and the average distribution of $\phi-\psi$ angles across the backbone changes in a solvent-specific manner (92-93). These solvent dependent chain geometries are known to generate optical activity not bona fide to extended secondary structural elements that characterize native or native-like structures (94). Solvent dependent chain contraction or expansion also alters fluorescence and extinction coefficients of chromophores depending on the extent of readjustments in their spatial locations and environments. The stopped-flow burst signals that are produced in response to a transfer of the unfolded polypeptide from higher to lower concentrations of a denaturant must then be considered from this perspective.

That burst signals in stopped-flow refolding experiments could be due to a fast contraction of the polypeptide and readjustments of the average backbone geometry was first suggested by Sosnick et al. (70). By modeling two large unstructured peptide fragments of cyt c (F1-65 and F1-80) for the unfolded protein, they found that burst changes in both fluorescence and CD signals recorded in normal refolding experiments with holocyt c are quantitatively identical with the corresponding signals for the unfolded protein. The same conclusion was reached with Rnase A (71). They suggested that the product of the submillisecond burst phase is simply the transient unfolded state under low GdnHCl refolding condition where both geometry and dimension of the polypeptide readjust to the new solvent milieu. Hence, the burst signals are due to
the overall response of the unfolded polypeptide under the new solvent conditions rather than to a structured intermediate. This conclusion was found inconsistent with the real-time partial resolution of the burst phase as an exponential process characterized by a modest energy barrier between the initial unfolded state and the burst product (44). Accordingly, it has been argued that the burst signals originate from a specific process whereby the unfolded state collapses to a structured intermediate. There have also been concerns whether the fragments of cyt c missing as much as a third of the protein and ~45% of the hydrophobic side-chains can be considered as representative models for the denatured state of the whole protein (56).

Results show that both equilibrium and kinetic burst fluorescence signals of the intact and unstructured cyt c under persisting unfolding conditions vary with denaturant in a quantitatively identical manner with the denaturant dependence burst amplitudes recorded in normal refolding kinetics of cyt c at pH 7. Further, as Figures 2.4.5 and 2.4.6 show, the phenomenon is observed with both GdnHCl and urea, suggesting that the submillisecond process is associated with U→U' process where U' is the unfolded state under refolding conditions. Identical results are obtained when these experiments are repeated with CD (data not shown). More direct evidence for the lack of extended secondary structure in the collapsed U' state comes from CD-monitored real-time submillisecond kinetics of cyt c refolding. The data of Akiyama et al. (95) show that the helix content in the burst product is some 8% of that for native cyt c. A further analysis of their data has shown that the burst CD amplitude closely matches the equilibrium CD signal of unfolded cyt c (96).
Since the objective of the present study is to examine the refolding kinetics of ferrocyt c, it is desirable to interrogate the burst phenomenon directly on the reduced state of the protein, even though the heme oxidation state of the same protein is not expected to make a great difference in the early collapse. Unfortunately, due to both very high aqueous stability and enhanced autooxidation rate at lower pH, a solvent condition where ferrocyt c can be held unfolded steadily is hard to find. Nevertheless, a CD-monitored ultrafast refolding study shows that the unstructured attribute of the burst product is true for ferrocyt c as well. Chen et al. (97) triggered refolding of ferrocyt c in the presence of 3.5 M GdnHCl, a solvent condition where ferricyt c is largely unfolded but ferrocyt c is completely native, by photoinduced electron transfer from NADH to the oxidized protein. Using time-resolved CD spectroscopy they found that just about 20% of the total expected ellipticity in the far-UV region is recovered in the submillisecond regime. Actually, the number would be much smaller than 20% when the allowance for some residual helicity of oxidized cyt c in the presence of 3.5 M GdnHCl is given.

2.6 Summary and conclusion

The results and interpretations presented weigh in rather substantially toward the view that chevron rollover in ferrocyt c (Figure 2.4.3b) is inconsistent with accumulation of a folding intermediate mechanism of ferrocyt c to the simple U→U′→N scheme shown at the left, where U→U’ represents the unobservable submillisecond relaxation of the unfolded state, and the U’→N is the stopped-flow observable single-exponential refolding. For two-state proteins like ferrocytochrome c, \( \Upsilon \rightarrow N \) is a downhill-biased run. Accumulation of post-
transition intermediates \((I_n)\) is conditional to the adequacy of their lifetimes. If they populate, folding kinetics are not modeled as two-state.