Stopped-Flow NMR Measurement of Hydrogen Exchange Rates in Reduced Horse Cytochrome *c* Under Strongly Destabilizing Conditions

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ABSTRACT A procedure to measure exchange rates of fast exchanging protein amide hydrogens by time-resolved NMR spectroscopy following in situ initiation of the reaction by diluting a native protein solution into an exchanging deuterated buffer is described. The method has been used to measure exchange rates of a small set of amide hydrogens of reduced cytochrome *c*, maintained in a strictly anaerobic atmosphere, in the presence of an otherwise inaccessible range of guanidinium deuterochloride concentrations. The results for the measured protons indicate that hydrogen exchange in the unfolding transition region of cytochrome *c* reach the EX2 limit, but emphasize the difficulty in interpretation of the exchange mechanism in protein hydrogen exchange studies. Comparison of free energies of structure opening for the measured hydrogens with the global unfolding free energy monitored by far-UV CD measurements has indicated the presence of at least one partially unfolded equilibrium species of reduced cytochrome *c*. The results provide the first report of measurement of free energy of opening of structure to exchange in the 0–2-kcal/mol range. Proteins 32:241–247, 1998. © 1998 Wiley-Liss, Inc.

Key words: hydrogen exchange; stopped-flow NMR; time-resolved NMR; reduced cytochrome c

INTRODUCTION

Detection of partially unfolded states of proteins in the presence of subdenaturing concentrations of denaturants has been described in recent hydrogen exchange (HX) studies on oxidized cytochrome *c*,[1] RnaseH,[2] and barstar.[3] An important query to follow up is whether such states are detectable even under strongly destabilizing conditions. A direct approach to the problem involves the measurement of HX rates in the presence of unfolding concentrations of denaturants. In unfolding conditions the kinetics of HX reactions are too fast, typically a few tens of seconds or less, and are immeasurable if the exchange reaction is initiated by the conventional solvent exchange technique. Thus, a method for initiating hydrogen exchange in the subminute regime is highly desirable.

In this article we address the possibility of initiating hydrogen exchange in a few seconds by mixing two solutions within the nuclear magnetic resonance (NMR) magnet. Rapid mixing and stopped-flow NMR have been employed successfully in a number of protein folding studies. Frieden et al.[4–6] have incorporated a stopped-flow device into the NMR spectrometer to follow folding and unfolding of *E. coli* dihydrofolate reductase in real time. Adler and Scheraga[7] have used a continuous recycled flow method to probe a folding intermediate of bovine pancreatic ribonuclease A. Studies have also been carried out without the use of a stopped-flow device. Examples include observation of unfolding of RnaseA,[8,9] and folding of apoplastocyanin[10] and α-lactalbumin.[11] We have now employed a similar mixing method to initiate hydrogen exchange in the NMR tube positioned within the magnet. The procedure is simple and involves simultaneous delivery of an aqueous protein solution and the deuterated buffer, contained separately in two gas-tight syringes, into the NMR tube via two flow lines.

The application of the method has been demonstrated by measuring HX rates of a set of mainchain amide hydrogens of reduced cytochrome *c* (cyt *c*) held near the unfolding transition midpoint where HX rates are greatly accelerated. The reduced form of horse cyt *c* was chosen for the following reasons. One, unlike the oxidized form, very little is known about the folding reaction of reduced cyt *c*, in both equilibrium and kinetics. Two, recent HX studies of oxidized cyt *c* have shown the presence of partially unfolded forms at equilibrium.[1] We want to ask if similar forms exist in the reduced state as well, given that the crystal structures of cyt *c* in the two oxidation states are very similar,[12,13] and that the reduced protein is overwhelmingly more stable than the oxidized form.[14,15] Three, by virtue of being one of

the most stable proteins, the reduced form of horse cyt *c* provides an opportunity to demonstrate the possibility of achieving large jumps in denaturant concentration by the use of the mixing procedure described here. In the present set of experiments, measurements have been done up to 4.6-M GdnDCl. Four, by using the reduced form of cyt *c* under a constant flow of argon, the feasibility of performing experiments in an inert atmosphere within the NMR tube is demonstrated.

We report the measurement of structure-opening reactions in a protein in substantially denatured forms with free energy of opening of structure to exchange in the 0–2-kcal/mol range. The results for all the measured protons indicate that HX in cyt *c* achieves the EX2 limit even under global unfolding situations. The available data also indicate the presence of more than one partially unfolded equilibrium state of the protein.

RESULTS AND DISCUSSION
Mixing Method and Time Evolution of NMR Spectra

The mixing procedure involves recording a series of one-dimensional (1D) NMR spectra in a time-resolved manner after a rapid change in the solvent condition from an aqueous solution to a deuterated buffer of desired composition where hydrogen exchange occurs. We initiated hydrogen exchange by simultaneous injection of one part of a 12–15-mM aqueous solution of native reduced cyt *c* and nine parts of the exchanging D_2O buffer containing deuterated guanidinium chloride directly into the NMR tube positioned in the magnet. The simple assembly required for driving solutions into the NMR tube consisted of two gas-tight syringes, which contained the protein solution and the buffer, and two plastic or Teflon transfer lines for delivering solutions to the NMR tube. At one end the transfer lines were tightly attached to the syringes, and at the other they were introduced into the NMR tube to a depth of about 75 mm from the tip. In this configuration the distance between the tip of the flow lines and the surface of the mixed solution is ~55 cm. The two flow lines were loosely tied to the NMR tube to facilitate their lowering into the magnet. To meet the requirement of an oxygen-free atmosphere for the present set of experiments, a third flow line, which carried a stream of argon gas, was directly introduced to a depth of ~50 cm from the tip of the NMR tube. Although the native cyt *c* solution and the exchanging buffer contained sodium dithionite for oxygen-scavenging, a constant and gentle flow of argon over the mixed solution ensured a complete reductive atmosphere throughout. Since the mixed solution experienced no turbulence because of argon flow, the field homogeneity is not expected to be significantly

affected. For injection of solutions, syringe plungers were evenly driven with the help of a Teflon block. In this way, a final hydrogen exchanging medium composed of ~1–1.5-mM reduced cyt *c*, 90% D_2O, ~23-mM sodium dithionite, and 100-mM phosphate buffer containing GdnDCl in the concentration range of 4–4.6 M at pH 6 was obtained.

The experimental parameters, including the pulse width and receiver gain, were optimized prior to setting up the flow lines by the use of a standard cyt *c* sample matching the HX solution conditions. A series of 1D NMR spectra in arrayed mode without preacquisition delay was recorded within 1–3 seconds of mixing the solutions. To determine the mixing efficiency, a control experiment was performed where an aqueous solution of L-serine was mixed with the exchanging buffer containing ~3-M GdnDCl under conditions identical with those used for hydrogen exchange measurements. By following spectral intensities of the mixed solution of L-serine as a function of time, the mixing time was found to be ~7 seconds. A stack plot of a small expanded amide region showing the decay of several resonance intensities of backbone amide protons of reduced cytochrome *c* in 4-M GdnDCl is presented in Figure 1. Chemical shifts and intensities of other resonances in the spectrum remain constant throughout the course of the experiment. The meso protons of the heme porphyrin ring also do not appear to exchange in the range of GdnDCl used (Fig 1).

Hydrogen Exchange Rates in Substantially Denaturing Conditions

Amide hydrogen exchange rates for reduced cyt *c* denatured in the presence of 4-, 4.2-, 4.4-, and 4.6-M GdnDCl were measured. Under the present exchanging conditions, a number of backbone hydrogens are well resolved in the 1D spectrum and can be identified with their native-state resonance assignments.[16] Representative rate profiles for six amide hydrogens, and the GdnDCl dependence of rate curves for V11 are plotted in Figure 2. Since the exchanging medium contains 10% water, a residual fraction of intensity is expected to persist at infinite exchanging time. This, however, does not affect the rates obtained by fitting the data to a single-exponential function. That cyt *c* stayed reduced and that no secondary process took place during the course of HX experiments are indicated by the constant intensity of the M80 ϵCH_3 resonance (-3.26 ppm) plotted on a per-proton basis in the panel for V11 in Figure 2.

This peak is one of several well-resolved marker resonances for the reduced form of cyt *c*.

Exchange Mechanism: EX1 or EX2?

In spite of appreciable progress in the field of hydrogen exchange methodologies, there has been
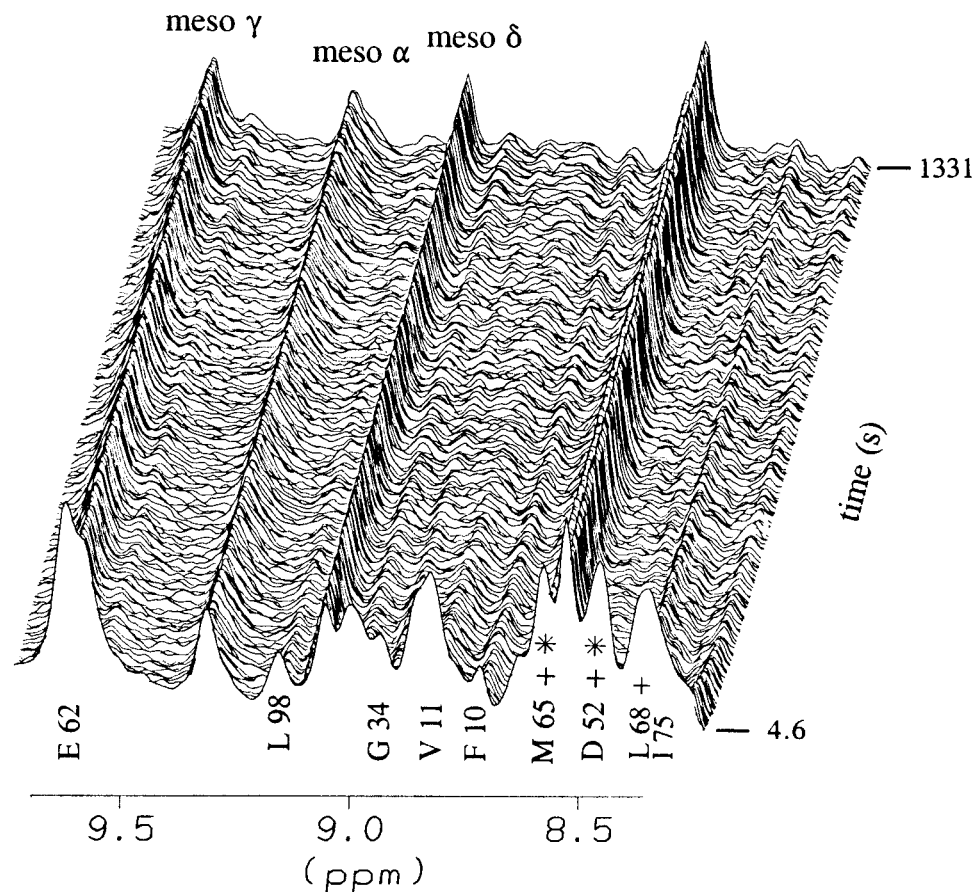
Fig. 1. Stack plot display of time-resolved 600-MHz NMR spectra of reduced cyt *c* following initiation of hydrogen exchange in 4-M GdnDCl at 24°C. Spectra up to the 256th FID are shown with a row increment of 2. Chemical shifts and intensities of other resonances in the spectra remain constant throughout the course of the experiment. The meso protons of the heme porphyrin ring also do not appear to exchange in the range of GdnDCl used. After 1:9 mixing of the dithionite-reduced aqueous protein solution and the deuterated buffer, both prepared in 0.1-M phosphate buffer and the latter containing GdnDCl, the final medium for HX contained ~1.2–1.5-mM cytochrome *c*, 90% D_2O, ~23-mM sodium dithionite, and 4-M GdnDCl at pH 6. The reported pH value is the corrected glass electrode reading recorded after HX measurements. Spectra were recorded in a Varian Unity plus spectrometer operating at a ^{1}H frequency of 600.051 MHz. The spectral width was 10,101 Hz. Each series of spectra was arrayed to 512 FIDs with no preacquisition delay in between. An FID was an average of two or four scans. Water suppression was achieved by on-resonance irradiation of the signal during the relaxation delay of 1 sec. All experiments were done at 24°C. Data were processed using Felix 95 (BIOSYM, San Diego, CA) and VNMR software provided by Varian Associates, Palo Alto, CA. The inaccuracy in intensity measurement was approximately ±7%.

considerable difficulty in the prediction, analysis, and interpretation of denaturant dependence of protein hydrogen exchange in the two kinetic limits, EX1 and EX2. In the description of exchange data in the presence of lower concentration of denaturants near neutral pH, the EX2 mechanism is often assumed mainly because in mildly denaturing medium the reclosing rate of an amide site is likely to be much faster than its opening rate and its chemical exchange rate. The validity of the EX2 limit in the pretransition region has in fact been shown in a number of studies.[1,2,17,18] Such analysis predicts that HX would occur by the EX2 mechanism in the unfolding region as well. That this prediction is not entirely fulfilled for the amide HX reactions in

RNase A under unfolding conditions has been pointed out.[19] A change in mechanism from EX2 to EX1 for the globally exchanging residues of barnase under mildly denaturing conditions has also been reported.[20] Furthermore, hydrogen exchange in some special cases of local unfolding does not completely satisfy the EX2 condition.[21] Thus, it is not clear if the EX2 reaction continues to occur beyond the pretransition region where denaturants promote large structural unfolding.

For the Linderstrøm-Lang scheme[22]

$$\text{closed(H)} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \text{open(H)} \overset{k_{ch}}{\rightarrow} \text{open(D)} \qquad (1)$$
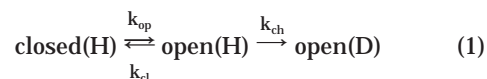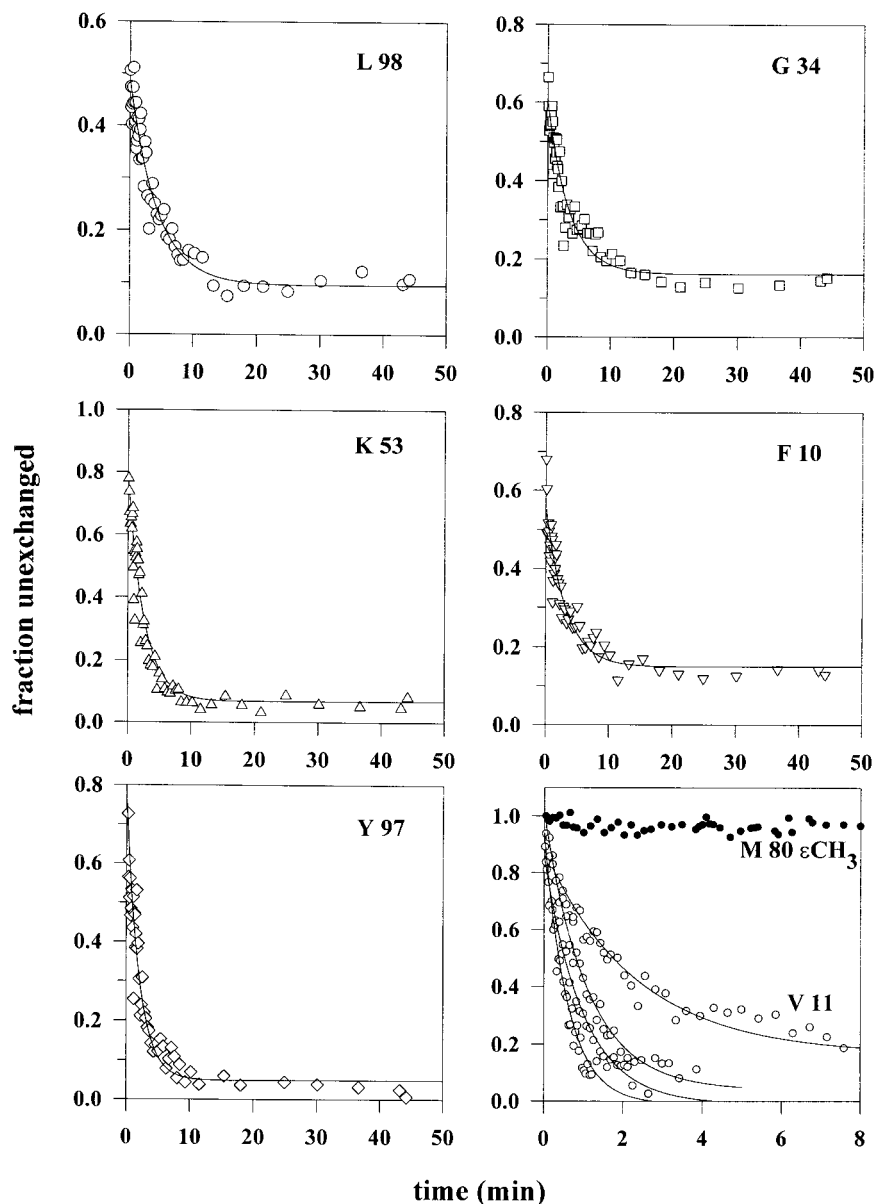
Fig. 2. Representative traces showing proton-to-deuterium exchange kinetics for the backbone amides of reduced cytochrome *c* in the presence of 4-M GdnDCl. The GdnDCl dependence of the observed kinetics for V11 is shown in the panel at the **right bottom**. The rate increases in the order of: 4-, 4.2-, 4.4-, and 4.6 M GdnDCl. This panel also shows the constant intensity of the M80 ϵCH_3 resonance (-3.26 ppm) plotted on a single proton basis, which indicates that the protein stayed reduced and that the spectral changes are due only to HX.

where k_{op} and k_{cl} are the rate constants for opening and closing an amide site, and k_{ch} is the chemical exchange rate of the same amide proton in the random chain, the experimentally observed hydrogen exchange rate, k_{ex}^{obs}, under denaturing conditions is given by

$$k_{ex}^{obs} = \frac{k_{op}k_{ch}}{k_{op} + k_{cl} + k_{ch}}. \qquad (2)$$

In the EX2 limit, when k_{cl} is greater than both k_{op} and k_{ch}, k_{ex}^{obs} will be given by

$$k_{ex}^{obs} \approx K_{op}k_{ch} = \frac{1}{P} k_{ch} \qquad (3)$$

where $K_{op} = k_{op}/k_{cl}$, and where P represents the protection offered by structural effects to an amide proton against exchange.

In the EX1 limit, expected under strongly destabilizing conditions where the magnitude of k_{op} approaches that of k_{cl}, k_{ch} may be taken to exceed both of these rates. Thus, k_{ex}^{obs} is given by

$$k_{ex}^{obs} \approx k_{op}. \qquad (4)$$

If the HX mechanism does not switch over from EX2 to EX1 on approaching substantially unfolding conditions, the protection factor must converge to unity near the midpoint of the equilibrium unfolding transition of reduced cyt *c*, where $K_u = 1$ (see Fig. 3).
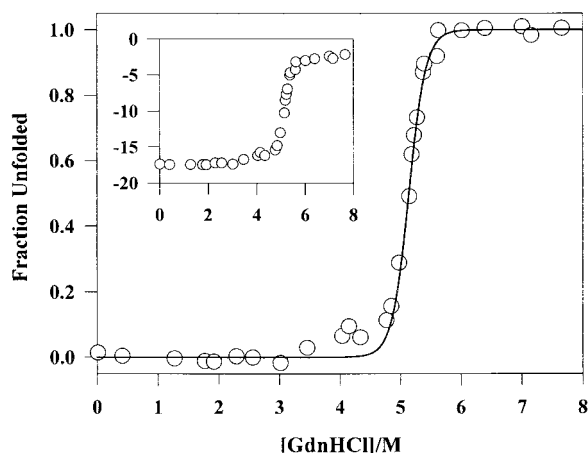
Fig. 3. GdnHCl-induced equilibrium unfolding transition of reduced cytochrome *c* in H_2O solutions monitored by far-UV CD at 24°C ($\pm$1). Raw data plotted as ellipticity at 222 nm as a function of GdnHCl concentration are shown in the **inset**. The curve is the least squares fit to the data assuming a two-state model with a linear dependence of the free energy change as a function of GdnHCl concentration, i.e., $-RT \ln K = m(C_m - [GdnHCl])$, where m = 3.51 kcal mol^{-1}M^{-1}, and C_m = 5.15 M. The free energy of unfolding in water, $\Delta G(H_2O)$, is 18 kcal/mol. The protein solutions (~15 µM in 100-mM phosphate buffer, pH 6, ~500-µM dithionite) contained in tightly capped quartz cuvettes were incubated in a nitrogen atmosphere for 90 min. Ellipticity values were measured using a Jasco J720 spectropolarimeter. After CD measurements, absorption spectra were recorded in a Carey instrument to ensure that the protein stayed reduced during the measurements.

To obtain information about the presence of partially unfolded states in terms of ΔG_{op}, we have analyzed the available data in the EX2 limit (see below). Using the k_{ex}^{obs} values obtained from rate curves like those shown in Figure 2, along with k_{ch} values determined for the residues from the calibrated procedure of Bai et al.,[23] free energies of structure opening, ΔG_{op}, for each residue were fit to the 'two-process' model.[24–26] The pertinent equation for analysis is given by[3,26]

$$\Delta G_{op} = -RT \ln[K_1 + K_u^\circ e^{m[GdnDCl]/RT} (1 + K_1)] \quad (5)$$

where K_1 and K_u° are equilibrium constants for local structure opening in the presence of a given concentration of GdnDCl and the global unfolding event in the absence of denaturant, respectively.

The value for the rate of refolding of reduced cyt *c*, k_f, near the midpoint, C_m, of the global unfolding transition determined from kinetic folding studies using the heme absorbance, has been reported to be ~1.5/s at 22°C.[27] Similar values have been measured at both higher[28] and lower (unpublished results) temperatures. The k_{cl} value in the Linderstrøm-Lang scheme is expected to correspond to k_f. Similarly, the rate of unfolding of the protein, k_u, is expected to correspond to k_{op}. Thus at 24°C, the condition of present measurements, k_{cl} is greater than k_{ch}. In addition, k_f is greater than k_u (and,

hence $k_{cl} > k_{op}$) for concentrations of GdnHCl below the C_m of 5.0 M, which corresponds to the minimum of the chevron plot of folding and unfolding (unpublished results). Therefore, both conditions for EX2 exchange appear to be met.

It is important to realize that the possibility that some protons not measured in our study may exchange via the EX1 mechanism can not be excluded. Moreover, for all amide protons, the exchange mechanism is expected to change from EX2 to EX1 at a GdnHCl concentration where k_f becomes smaller than k_{ch}. This is expected to happen only at GdnHCl concentrations greater than 5.0 M, although it is possible that transiently formed structures in much faster time scale[29,30] might prevent a transition from EX2 to EX1 near the C_m.

Validity of the EX2 Mechanism

Figure 4 presents a comparison of GdnDCl-dependent behavior of ΔG_{op} for nine protons and the far-UV CD-monitored ΔG_u (see Fig. 3) in the unfolding transition region of reduced cyt *c*. Regarding the exchange mechanism, two features of these curves are notable. One, all of the ΔG_{op} curves, although having some dispersion among themselves (see below), approach zero in the transition region of global unfolding, indicating that within the error of measurements, the hydrogen exchange reactions have satisfied the EX2 limit. Two, the ΔG_{op} values calculated for various measured protons having different k_{ch} values (in the range ~0.03–0.4/s) are in close agreement, implying EX2 behavior of hydrogen exchange.[1] If HX were occurring in the EX1 limit, where the HX rate is independent of k_{ch}, correcting the various protons for their different k_{ch} values would result in considerable disagreement between the ΔG_{op} values, even though they may be related by the same fluctuation motions.

In Figure 4A, ΔG_{op} = 0 for E62 at ~4.4-M GdnDCl, and becomes negative in higher concentrations of denaturant, implying that the amide hydrogen of E62 exchanges out at a rate faster than the random coil rate. This may, however, not be the case because k_{ch} values used here are not corrected for the effect of GdnDCl on the base catalyzed rate, k_b ($k_{ch} \approx k_b[OH^-]$). The small but complicated effects of GdnHCl on k_{ch} values have been addressed by Loftus et al.[31] Accordingly, in the presence of 4–5-M GdnHCl, the values of k_{ch} would increase 1.5- to 1.7-fold, corresponding to an increase in apparent ΔG_{op} by ~0.3 kcal/mol. Another possible reason for somewhat lower ΔG_{op} value relative to ΔG_u apparent for all the measured protons could be the use of a very high initial concentration of the protein for the HX experiments, even though we have no evidence for oligomerization of reduced cytochrome *c* in the native state.
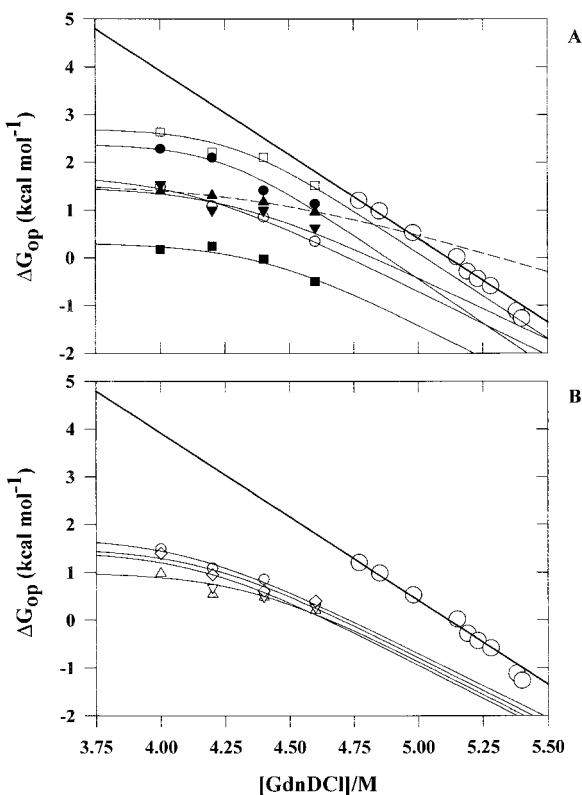
Fig. 4. Comparison of free energy of equilibrium unfolding of reduced cytochrome c, ΔG_u, and free energies of structure-opening events, ΔG_{op}, that lead to hydrogen exchange. **A:** Dependence of ΔG_{op} for L98 (small open circle), G34 (open square), K53 (filled circle), E62 (filled square), K79+I81 (up-pointing triangle), and I85/K87 (down-pointing triangle) on the concentration of GdnDCl. ΔG_u values (= $\Delta G(H_2O) - m[GdnHCl]$) calculated from the equilibrium curve shown in Figure 3 are plotted as large open circles. The solid boldface line through these points was calculated by using fractions of unfolded and folded proteins ($\Delta G_u = -RT \ln K$). The HX curves are fits to the data assuming a "two-process" model.[25,26] **B:** Guanidine dependence of ΔG_{op} for L98 (open circle), V11 (up-pointing triangle), F10 (down-pointing triangle), and Y97 (diamond). These residues form the amino and the carboxyl terminal helix of cytochrome c. On the basis of strikingly similar exchange energies and m values for these residues, the two terminal helices appear to define a subglobal structural unit displaying a common cooperative structure-opening reaction.

Residual Protection to K79 and I81

Since the resonances of amide hydrogens of these two residues overlap, the ΔG_{op} values plotted in Figure 4A were calculated using the average k_{ch} value for these two residues. The fit to the equation for ΔG_{op} predicts that ΔG_{op} would cross the global unfolding curve at the transition midpoint and converge to zero at about 5.5-M GdnDCl. ΔG_{op} may appear to be higher than ΔG_u if k_{ch} is overestimated because of the presence of residual structure in the unfolded state of the protein. We do not know of the presence of such HX blocking residual structure in unfolded reduced cyt c. A possible explanation could be the following. In cytochrome c, K79 and I81 are in immediate primary structural proximity of M80,

which forms a covalent bond with the heme iron. NMR exchange spectroscopy in the equilibrium transition midpoint has indicated that in the unfolded state of reduced cyt c, M80 dissociates from the heme iron (A.K.B., unpublished result) and establishes transient contact with the ferrous iron with association and dissociation time constants of 40 μs and 4 μs, respectively.[28,32] Clearly, the rate of contact formation is faster than the chemical exchange for these two residues. The polypeptide loop formed by virtue of the Fe^{2+}–M80 contact makes it possible to offer protection to K79 and I81 against exchange. This provides an example as to how transient contact formation, irresolvable in the HX time scale, can protect an amide site from exchange.

Multiple Structure-Opening Reactions

For some proteins, HX studies carried out in subdenaturing conditions have shown considerable dispersion in ΔG_{op} curves. Data for RNaseH[2] and oxidized cyt c[1] have shown that as GdnDCl concentration increases the dispersion narrows down and various fast exchanging hydrogens merge to define more than one partially folded structural unit. The ΔG_{op} curves of reduced cyt c presented in Figure 4A show persistent dispersion within ~2 kcal/mol, indicating that several structure-opening equilibria, representing multiple protein conformations closely spaced in energy, rather than a single global opening, are operative in the exchange of protein amide hydrogens even under relatively strong unfolding conditions. Thus, considering the accuracy in the measurement of k_{ex}^{obs} and assuming a complete calibration of primary structural effects on k_{ch} values,[23] we attribute the dispersion to the presence of more than one partially unfolded form. The available data for reduced cyt c easily identify the presence of at least one such form comprised of the N- and C-terminal helices represented by residues F10, V11, Y97, and L98, all four showing isoenergetic behavior with uniform m values (Fig. 4B). This partially unfolded form has been described for the oxidized state of cyt c as well.[1,33,34] G34 and K53 may represent, respectively, the omega loop (residues 20–35) and the bottom loop of residues 36–61, both of which have been identified as equilibrium partially unfolded forms of the oxidized form of cytochrome c.[1] The data available are not sufficient for a detailed comparison of oxidation-state-dependent residue-specific HX behavior. The protection against exchange experienced by K79+I81, as discussed in the preceding section, appears specific to the reduced form of cytochrome c. In the oxidized state of the protein such marked protection is observed for the residues in the proximal side of H18.[1] As reflected by lower ΔG_{op} and smaller m value, relative to the global unfolding process monitored by far-UV CD, the partially unfolded forms are less stable than the native cyt c molecule.

Advantages and Drawbacks of the Rapid Mixing HX Method

The main advantage of the in situ procedure is that it is simple and provides an opportunity to measure HX reactions occurring in tens of seconds even without the access to a rapid mixing apparatus for using quenched flow protocols. The present set of experiments required three flow lines. Flow lines may be added or removed according to the need. For example, recording the kinetics of the slow folding–unfolding phase of barstar required the use of two lines (unpublished result). The presence of the flow tubes were not found to change the line shape and the line width. The method, however, does suffer from the requirement of high protein concentrations. Being a real-time experiment, the NMR spectral resolution achievable under the in situ condition, relative to that obtainable through the use of quenched flow methods, is somewhat poor even when enhanced by pulsed field gradients.

ACKNOWLEDGMENTS

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