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Structural Description of Acid-Denatured Cytochrome *c* by Hydrogen Exchange and 2D NMR†

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ABSTRACT: Hydrogen exchange and two-dimensional nuclear magnetic resonance (2D NMR) techniques were used to characterize the structure of oxidized horse cytochrome *c* at acid pH and high ionic strength. Under these conditions, cytochrome *c* is known to assume a globular conformation (A state) with properties resembling those of the molten globule state described for other proteins. In order to measure the rate of hydrogen–deuterium exchange for individual backbone amide protons in the A state, samples of oxidized cytochrome *c* were incubated at 20 °C in D_2O buffer (pD 2.2, 1.5 M NaCl) for time periods ranging from 2 min to 500 h. The exchange reaction was then quenched by transferring the protein to native conditions (pD 5.3). The extent of exchange for 44 amide protons trapped in the refolded protein was measured by 2D NMR spectroscopy. The results show that this approach can provide detailed information on H-bonded secondary and tertiary structure in partially folded equilibrium forms of a protein. All of the slowly exchanging amide protons in the three major helices of native cytochrome *c* are strongly protected from exchange at acid pH, indicating that the A state contains native-like elements of helical secondary structure. By contrast, a number of amide protons involved in irregular tertiary H-bonds of the native structure (Gly37, Arg38, Gln42, Ile57, Lys79, and Met80) are only marginally protected in the A state, indicating that these H-bonds are unstable or absent. The H-exchange results suggest that the major helices of cytochrome *c* and their common hydrophobic domain are largely preserved in the globular acidic form while the loop region of the native structure is flexible and partly disordered.

Structural information on partially folded proteins is essential for understanding the mechanism of folding and the principles of structure stabilization. Despite extensive efforts, attempts to detect and characterize equilibrium folding intermediates of small globular proteins have been unsuccessful due to the cooperative nature of folding–unfolding transitions. Consequently, it is now widely accepted that the equilibrium between native and denatured states can be described by a two-state model [for reviews, see Tanford (1968) and Privalov (1979, 1982)]. However, folding intermediates have been shown to accumulate transiently in some cases (Kim & Baldwin, 1982, 1990), but the structural description of these short-lived states requires application of indirect methods such as trapping of disulfide intermediates (Creighton, 1978) or hydrogen-exchange labeling and 2D NMR[1] (Udgaonkar & Baldwin, 1988; Roder et al., 1988). The possibility of a more direct physical characterization of folding intermediates is suggested by recent reports that some proteins, when placed under mildly denaturing conditions, assume compact nonnative equilibrium states with high secondary structure content and fluctuating tertiary structure [reviewed by Ptitsyn (1987) and Kuwajima (1989)].

A well-studied example of this so-called molten globule state is the acid-denatured form (A state) of α-lactalbumin (α-LA). The far-UV CD spectrum of the A state is similar to that of the native protein, indicating the presence of native-like secondary structure, but the aromatic CD band is absent, suggesting disordered or fluctuating tertiary structure (Kuwajima et al., 1976). Hydrodynamic measurements show that the A

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[1] Abbreviations: 2D NMR, two-dimensional nuclear magnetic resonance; A state, compact acidic protein state; α-LA, α-lactalbumin; CD, circular dichroism; cyt *c*, horse heart cytochrome *c*; pD, pH-electrode reading in D_2O solution; COSY, *J*-correlated spectroscopy.

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state of α-LA is nearly as compact as the native state (Dolgikh et al., 1981). The transition from the native state to the A state is cooperative while thermal unfolding of the A state is noncooperative by calorimetric criteria (Dolgikh et al., 1985; Pfeil et al., 1986). The relevance of these results is stressed by recent reports of kinetic folding intermediates with properties resembling those of the molten globule state (Ikeguchi et al., 1986; Ptitsyn et al., 1990).

Cytochrome *c* (cyt *c*) is known to exist in several pH- and salt-dependent conformational states with altered heme ligation (Theorell & Akeson, 1941; Babul & Stellwagen, 1972; Dyson & Beattie, 1982; Goto et al., 1990). At low ionic strength, acid denaturation occurs in a single cooperative transition centered around pH 2.5 resulting in a fully unfolded state, probably lacking axial heme ligands (Babul & Stellwagen, 1972). However, acidification at high ionic strength or high concentration of HCl leads to a compact high-spin state lacking the native methionine ligand (Stellwagen & Babul, 1975; Robinson et al., 1983; Goto et al., 1990). This globular acidic form of oxidized cyt *c* (A state) resembles the native protein (N state) in terms of intrinsic viscosity and the CD spectrum in the far-UV region, but its stability toward thermal unfolding is drastically reduced (Robinson et al., 1983; Potekhin & Pfeil, 1989). On the basis of these properties and the absence of native conformational shifts in the proton NMR spectrum, Ohgushi and Wada (1983) proposed that the A state of cyt *c* represents a molten globule state.

It is difficult to obtain direct structural information on molten globule states because of their low stability and dynamic nature. The most detailed characterization of a nonnative protein resulted from recent NMR studies of α-LA by Baum et al. (1989). Using magnetization transfer and hydrogen-exchange methods, they identified some conformationally shifted side-chain resonances at acid pH and assigned several amide protons in helical segments of the native protein that were protected from exchange in the A state, suggesting the presence of partly folded structure.

For cyt *c*, the direct application of conventional NMR approaches is complicated by the presence of a paramagnetic high-spin iron at acid pH. This limitation can be overcome by use of hydrogen-exchange methods which can provide structural and dynamic information at a site-resolved level even under conditions that are unsuitable for direct NMR investigation. Previous studies on the folding pathway of cyt *c* (Roder et al., 1988) and the complex of cyt *c* with a monoclonal antibody (Paterson et al., 1990) made use of a similar strategy. In this study, hydrogen–deuterium exchange was carried out under conditions that favor the globular A state (pD 2.2, 1.5 M NaCl), and the partially exchanged samples were analyzed by 2D NMR under native conditions. This approach makes it possible to measure NH exchange rates in the nonnative form for all those amide protons that can be trapped in the refolded protein (44 H-bonded peptide groups), and thus provides detailed structural information on the acidic form of cyt *c*.

Materials and Methods

A 6 mM solution of oxidized horse heart cytochrome *c* (type VI from Sigma Chemical Co.) was prepared by dissolving the lyophilized protein in 1.5 M NaCl solution in H_2O adjusted to pH 2.2 by addition of HCl. Hydrogen–deuterium (H–D) exchange was initiated by transferring the protein into an unbuffered D_2O solution of 1.5 M NaCl adjusted to pD 2.2 (uncorrected electrode reading), spinning Sephadex G-25 columns being used (3-mL bed volume, 30-s centrifugation at ~400*g*). The solution was incubated at 20 °C, and 0.5-mL aliquots were collected after H–D exchange times ranging from 2 min to 500 h. The exchange reaction was quenched in a second spinning column equilibrated at 4 °C with a D_2O solution of 50 mM sodium phosphate and 50 mM ascorbate at pD 5.3. Under these conditions, cyt *c* refolds completely within seconds. Ascorbate was included in the quench buffer because cyt *c* is substantially more stable in the reduced form and the exchange for most amide protons is slower (Wand et al., 1986). In order to minimize solvent artifacts in the NMR spectra, it was necessary to lower the ascorbate concentration to 12 mM, by use of a third spinning column. The samples were stored at 4 °C prior to NMR analysis.

Proton NMR spectra were recorded at 30 °C on a Bruker AM 500 spectrometer operating at 500 MHz. COSY spectra (Nagayama et al., 1980) were recorded in the magnitude mode by collecting 96 transients of 1024 complex data points covering a spectral width of 9090.9 Hz for 400 t_1 increments (5 μs to 44 ms). Total acquisition time was 15 h. The data were processed on a MicroVax II computer with the program FTNMR (courtesy of Dr. D. Hare, Hare Research, Woodinville, WA).

The pH dependence of cytochrome *c* in 1.5 M NaCl was studied by monitoring the Soret absorption band at 395 nm. Half of a cyt *c* stock solution in 1.5 M NaCl (unbuffered) was titrated to pH 1 with 1.5 M HCl. To the other half, kept at pH 7, an equal volume of 1.5 M NaCl solution was added, resulting in a final cyt *c* concentration of 12 μM for both stock solutions. The two solutions were mixed in various ratios to yield a range of pH values. Absorption spectra were recorded at room temperature on a Cary 118 spectrophotometer. The isosbestic point was determined to be at 405 nm.

Results and Discussion

Experimental Design. To measure the kinetics of hydrogen exchange in the cyt *c* A state, 12 separate samples of oxidized cyt *c* were exchanged for different time periods (2 min to 500 h) at pD 2.2 (D_2O, 1.5 M NaCl) and quenched at pD 5.3, as described above. The extent of exchange at individual NH sites was quantified by recording COSY spectra on each sample under identical conditions (see Materials and Methods). Cross-peak volumes were measured for 44 resolved NH–C_αH cross peaks [assigned by Wand et al. (1989)], and exchange rates were determined from their exponential decay as a function of the H–D exchange time. A COSY spectrum recorded on a freshly prepared sample of reduced cyt *c* in D_2O at pD 5.3 was used to set cross-peak intensities at $t = 0$.

In order to determine the structural contribution to H exchange, we used the measured exchange rates, k_{ex}, to calculate protection factors $P = k_c/k_{ex}$. For each amide proton, intrinsic exchange rates, k_c, were calculated from the previously calibrated pH and temperature dependence for peptide NH exchange (Englander & Poulsen, 1969; Englander et al., 1979) and the sequence dependence according to the rules of Molday et al. (1972). The results are shown in Figure 1 and listed in Table I together with the protection factors measured in an earlier exchange study on native oxidized cyt *c* at neutral pH (Wand et al., 1986; Roder, Wand, Milne, and Englander, unpublished data).

A limitation of the method used is that only amide protons with sufficiently slow exchange rates in the native state can be observed, namely, those with exchange times longer than about 12 h at pD 5.3 ($P > 10^3$). According to the recently redetermined X-ray structure of horse cyt *c* (Bushnell et al., 1990), most of these strongly protected amide protons are involved in intramolecular hydrogen bonds. The only exceptions are Thr19 and Phe36 which form H-bonded interactions

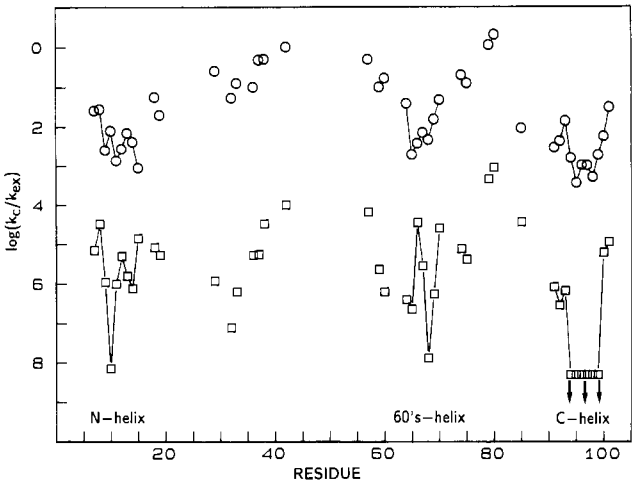

FIGURE 1: Profiles of H-exchange protection factors for oxidized cyt *c* in the A state (○) and the native state (□). The logarithm of protection factors $P = k_c/k_{ex}$ is plotted against the corresponding residue number for 44 amide protons for which the exchange reaction can be quenched by refolding to the native reduced form. A lower limit of *P* is given for the most slowly exchanging amide protons (residues 94–98) in the native state.

with crystallographically defined internal water molecules. These observations confirm the previously noted correlation between H-exchange slowing and hydrogen bonding (Englander & Kallenbach, 1984; Wand et al., 1986) and thus support the conclusion that amide protons with large protection factors in the A state are similarly involved in hydrogen bonds (though additional contributions from factors such as solvent accessibility and electrostatic effects may well occur). Although H-bond acceptors cannot be identified directly, the identity of H-bond acceptors can often be inferred by comparison of the patterns of protection observed under native and nonnative conditions.

Helices. Figure 1 shows that the most slowly exchanging amide protons in the A state are located in the three major helical segments of cyt *c*, just as in the native form. All of the amide protons that exhibit slow H exchange due to helical H-bonds in native cyt *c* (residues 7–15 in the N-helix, 64–70 in the 60's helix, and 91–101 in the C helix) are also protected from exchange at acid pH. The profiles of protection factors along the helices (Figure 1) are qualitatively similar in the two forms. For example, in both forms the first three and the last two H-bonded amide protons in the C-helix exchange more rapidly than the protons in the center of the helix. Evidently, the three helical segments of the native cyt *c* structure are preserved in the A state.

In the structure of native cyt *c* (Bushnell et al., 1990), the three long α-helices are grouped around one edge of the heme group. Each helix provides several apolar side chains that together constitute a major part of the hydrophobic core of cyt *c*. Most of the highly protected amide protons in the native state are clustered around the hydrophobic core, and the fact that these protons are still strongly protected in the acidic form suggests that the core is at least partially present in the A state, although probably not as tightly packed as in the native structure.

A more detailed comparison of the protection factors for the N-helix and the 60's helix (Figure 1; Table I) reveals some interesting differences. The most striking case is the NH of Phe10 which is by far the slowest proton in the N-helix under native conditions ($P = 10^8$); in the A state it is one of the faster protons. These differences in exchange patterns may reflect differences in tertiary interactions among the helices and with other parts of the structure.

Table I: H-Exchange Protection Factors, *P*, for the Globular Acidic Form of Cyt *c* (A State) and Comparison with Native Oxidized Cyt *c*[a]

residue	*P*(A state) (pD 2.2)[b]	*P*(native) (pD 7.0)[c]	log [*P*(native)/ *P*(A state)]
K7	39	1.5×10^5	3.6
K8	36	3.0×10^4	2.9
I9	389	9.0×10^5	3.4
F10	126	1.4×10^8	6.0
V11	726	1.0×10^6	3.1
Q12	362	2.0×10^5	2.7
K13	143	6.3×10^5	3.6
C14	240	1.3×10^6	3.7
A15	1120	7.0×10^4	1.8
H18	18	1.2×10^5	3.8
T19	51	1.9×10^5	3.6
G29	4	8.4×10^5	5.3
L32	19	1.3×10^7	5.8
H33	8	1.6×10^6	5.3
F36	10	1.9×10^5	4.3
G37	2	1.8×10^5	4.9
R38	2	3.0×10^4	4.2
Q42	0.8	1.0×10^4	4.1
I57	2	1.5×10^4	3.9
W59	10	4.3×10^5	4.6
K60	6	1.6×10^6	5.4
L64	26	2.5×10^6	5.0
M65	507	4.4×10^6	3.9
E66	260	2.8×10^4	2.0
Y67	140	3.5×10^5	3.4
L68	211	7.4×10^7	5.5
E69	65	1.8×10^6	4.4
N70	21	3.9×10^4	3.3
Y74	5	1.3×10^5	4.4
I75	8	2.4×10^5	4.5
K79	0.9	2.2×10^3	3.4
M80	0.5	1.1×10^3	3.4
I85	109	2.7×10^4	2.4
R91	334	1.2×10^6	3.6
E92	227	3.5×10^6	4.2
D93	71	1.5×10^6	4.3
L94	619	$>2.0 \times 10^8$	>5.5
I95	2743	$>2.0 \times 10^8$	>4.9
A96	952	$>2.0 \times 10^8$	>5.3
Y97	959	$>2.0 \times 10^8$	>5.3
L98	1935	$>2.0 \times 10^8$	>5.0
K99	519	$>2.0 \times 10^8$	>5.6
K100	171	1.6×10^5	3.0
A101	32	8.4×10^4	3.4

[a] Protection factors $P = k_c/k_{ex}$ determined from measured H–D exchange rates, k_{ex}, and the corresponding intrinsic exchange rate, k_c, calculated according to Molday et al. (1972). The statistical errors in k_{ex} (determined by least-squares analysis) vary between 2% and 15%, depending on experimental factors such as COSY cross-peak intensities, degree of exchange covered, and pH variation between samples. The error in *P* is expected to be substantially larger due to possible systematic errors in the calculation of k_c. [b] H–D exchange rates for oxidized horse cyt *c* at 20 °C in D_2O, pD 2.2, and 1.5 M NaCl. [c] H–D exchange rates for oxidized horse cyt *c* at 20 °C, in D_2O and mM sodium phosphate, pD 7.0 [from Wand et al. (1986) and Roder, Wand, Milne, and Englander (unpublished data)].

Nonhelical H-Bonds. In native oxidized cyt *c*, we find 14 slowly exchanging amide protons outside the three helical regions. Two of these (His18 and Arg38) form H-bonds in reverse turns; the rest are involved in irregular tertiary H-bonded interactions between nonsequential chain segments (Bushnell et al., 1990). In the A state, the protection factors for nonhelical protons cover a range from 2 to 110 with the majority falling under 10, whereas the same protons exhibit protection factors from 2×10^4 to 6×10^6 in the native protein (Table I). On average, the protection factors from nonhelical protons are about 200-fold smaller than for helical protons in the A state. The amide protons of Gly37, Arg38, Gln42, Ile57, Lys79, and Met80 exhibit slowing factors from 0.5 to 2; within

the uncertainty of the measurements and the calculation of intrinsic exchange rates, these protons are essentially unprotected in the A state. Thus, it appears that several of the tertiary H-bonds and reverse turns of the native structure are only marginally stable or even completely absent in the A state. These observations are consistent with a highly dynamic structure in the nonhelical regions of the A state, particularly in the bottom part of the structure [viewed in standard orientation; cf Figure 1 in Bushnell et al. (1990)], which consists of several irregular loops (Ω-loops; Leszczynski & Rose, 1986) joined by H-bonded interactions. The smallest protection factors outside the loop region are observed for the amide protons of Lys79 ($P = 0.9$) and Met80 ($P = 0.5$), which are H-bonded to a propionate side chain of the heme and the O_γ of Thr78, respectively (Bushnell et al., 1990). The fact that these protons are unprotected in the A state is consistent with earlier spectroscopic evidence (Stellwagen & Babul, 1975) that the native Met80 ligand bond to the heme iron is severed in the acid form.

Relative Destabilization. For a more quantitative analysis of the relative changes in exchange rates between the two forms of cyt *c*, it is useful to consider the ratios of protection factors, listed in Table I in the form $\Delta \log P = \log [P(\text{native})/P(\text{A state})]$. Within the framework of the structural unfolding model for H exchange (Englander & Kallenbach, 1984), this quantity can be interpreted as the change in local structural stability of individual segments in the native form and in the A state. However, in view of the complexity of the system, a rigorous thermodynamic treatment is not warranted at this time.

The average $\Delta \log P$ value for all observed protons is 4.2 (i.e., 16000-fold rate acceleration in the A state). The largest $\Delta \log P$ values (>5) are observed for the most slowly exchanging amide protons in the helical regions of the native protein (residues 10, 68, and 94–99), indicating that these core protons experience the largest destabilization in the N to A transition. Some of the nonhelical residues, including Gly29, Leu32, His33, and Lys60, also exhibit high $\Delta \log P$ values (5.3–5.8). The regions around Leu32 and Lys60 thus appear to be strongly perturbed in the A state. The smallest $\Delta \log P$ values are observed for Ala15, Glu66, and Ile85, indicating that these sites remain relatively well protected in the A state. The low $\Delta \log P$ value for Ile85 (2.4) is particularly interesting. The NH of Ile85 is involved in a tertiary H-bond with the carbonyl oxygen of Leu68, and both side chains are part of the central hydrophobic core of the native structure. The high degree of protection for Ile85 in the A state of cyt *c* thus provides additional evidence for the presence of a hydrophobic core and supports the conclusions drawn from the analysis of helical protons (see above). However, one cannot rule out the possibility that the Ile85 NH finds another, nonnative, H-bond acceptor under acidic conditions.

Comparison with Other Proteins. The proposal that the acidic form of cyt *c* represents a molten globule state (Ohgushi & Wada, 1983) can now be reconsidered in light of the new structural information presented above. The A state of cyt *c* resembles a molten globule state as defined by Ptitsyn (1987) in terms of the following properties:

(*a*) *Compactness.* Hydrodynamic measurements, including viscosity (Stellwagen & Babul, 1975), dynamic light scattering (Ohgushi & Wada, 1983), and gel-exclusion chromatography (Goto et al., 1990), show that the A state is essentially as compact as the native state.

(*b*) *High Secondary Structure Content.* The present NH exchange results show that the three major helices of cyt *c* are present in the A state, which is consistent with the observation that the far-UV CD spectrum is very similar in both forms (Robinson et al., 1983; Goto et al., 1990).

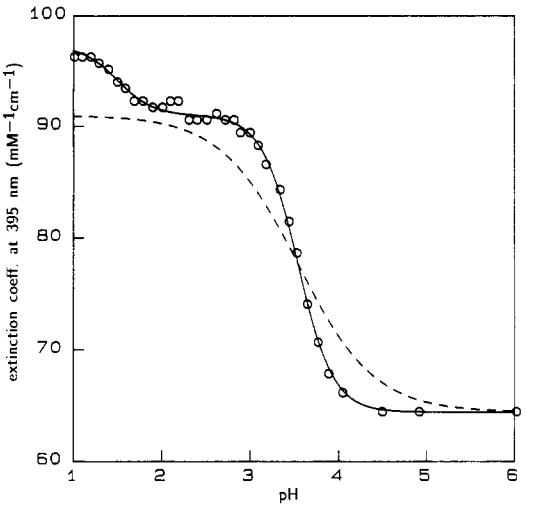

FIGURE 2: Effect of pH on the extinction coefficient ϵ at 395 nm for oxidized horse cyt *c* in 1.5 M NaCl. The solid curve represents a nonlinear least-squares fit of the equation $\epsilon(\text{pH}) = (\epsilon_A - \epsilon_N) \times 10^{n(pK_1-pH)} + (\epsilon_U - \epsilon_A) \times 10^{n(pK_2-pH)}$, with $pK_1 = 3.5$ (N to A transition), $pK_2 = 1.5$ (A to U transition), and $n = 2.2$. For comparison, the dashed curve shows a simple titration curve for a transition with a pK_a of 3.5 and $n = 1$.

(*c*) *Fluctuating Tertiary Structure.* The NH exchange data reported in Table I show that the majority of the nonhelical H-bonds of native cyt *c* are strongly destabilized or completely absent in the A state, suggesting that some regions of the tertiary structure are highly flexible. On the other hand, the high degree of protection observed for most of the helical amide protons indicates the presence of stabilizing tertiary contacts between the helices. The acid-induced transition of cyt *c* thus appears to be accompanied by local increases in structural flexibility rather than a uniform melting of the tertiary structure.

For other proteins, the dynamic nature of molten globule states has been based on qualitative observations such as the absence of a near-UV CD band and the absence of conformationally shifted resonances in the proton NMR spectrum (Dolgikh et al., 1985; Ptitsyn, 1987), but the application of these tests to the acidic form of cyt *c* is complicated by the presence of the heme group. The CD spectrum of the heme interferes with the observation of the aromatic CD band, and paramagnetic line broadening and shift effects caused by the high-spin iron render a meaningful interpretation of the NMR spectrum difficult [cf. Ohgushi and Wada (1983)].

On the basis of unpublished NMR and calorimetric data, Ptitsyn (1987) questioned whether the A state of cyt *c* can be described as a molten globule state and suggested that it has more native-like character than other acid-denatured proteins. However, the following observations argue against the possibility that the A state of cyt *c* simply represents a destabilized form of the native protein. According to calorimetric results reported by Potekhin and Pfeil (1989), the native form of cyt *c* and the A state are thermodynamically distinct states separated by a phase transition involving large changes in enthalpy and heat capacity. However, in contrast to other proteins (Pfeil et al., 1986), the cyt *c* A state undergoes a second cooperative transition to a more extensively unfolded state. The cooperativity of the N to A transition is also evident in Figure 2, which shows the pH titration of the heme absorbance at 395 nm for oxidized cyt *c* in 1.5 M NaCl. Comparison with a simple one-proton titration curve (dashed line) indicates that

the transition between the N and the A state centered around pH 3.5 involves more than one titrating group. Furthermore, Goto et al. (1990) have shown that when HCl is added to native cyt *c*, the protein first unfolds (near pH 2) and then undergoes a second transition to a compact state at HCl concentrations above 0.2 M. This behavior is very similar to that of other proteins, including apomyoglobin and β-lactamase, which are known to exist as molten globule states under these conditions.

The NH exchange rates measured in this study (Figure 1; Table I) provide further evidence for structural differences between the A state and native cyt *c*. In particular, several tertiary H-bonds of the native structure are lost in the A state, and the profiles of protection factors for helical regions are significantly different in the two states.

It is thus evident that, at low pH and high salt concentration, cyt *c* assumes a globular conformation that is structurally and thermodynamically distinct from both native and fully unfolded states. The question whether it can be properly classified as a molten globule state seems somewhat arbitrary, especially since the conformational properties of the destabilized protein states categorized as molten globules are not yet clearly defined. The present study shows that acid-destabilized cyt *c* exhibits many native-like characteristics while other properties are clearly nonnative. It can be noted that native-like elements of secondary and tertiary structure have recently been reported for the acidic forms of α-lactalbumin (Baum et al., 1989), the classical example of a molten globule state, and also for apomyoglobin (Hughson et al., 1990).

Conclusions

The results presented in this paper demonstrate that hydrogen exchange and 2D NMR methods are well suited for obtaining structural and dynamic information on nonnative protein states in spite of their low stability and dynamic nature. The main advantage of this approach is that the H–D exchange reaction can be carried out under nonnative conditions unsuitable for direct structural investigations (e.g., NMR or X-ray crystallography), while the extent of exchange is monitored by 2D NMR spectroscopy under native conditions where NH exchange is slow and proton resonance assignments are available. This approach makes it possible to measure exchange rates for all those amide protons that exchange slowly in the refolded protein.

The NH exchange results reported here show that the three major α-helices of cyt *c* remain folded in the acid-destabilized state at high salt concentration, but the majority of the amide protons involved in nonhelical H-bonds are only marginally protected, suggesting a drastic destabilization of these tertiary interactions. The transition from the native state to the globular acidic state results in the loss of some specific interactions, including some salt bridges and at least one axial heme ligand (Met80). This leads to increased structural flexibility and partial disruption of the H-bonded structure, primarily in the regions containing irregular loops, while the helices and parts of the hydrophobic core remain in a native-like conformation.

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