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Structural Features of the Cytochrome *c* Molten Globule Revealed by Fluorescence Energy Transfer Kinetics

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Under mild denaturing conditions, many proteins adopt a nonnative state characterized by compactness, a nativelike secondary structure, but the absence of rigid tertiary structure.[1,2] It has been proposed that this molten globule state is a common intermediate that occurs early in the folding pathways of all globular proteins.[3] Recently, it has been suggested that in some cases molten globules correspond to late-folding intermediates.[2,4] More detailed characterization of molten globules and protein folding intermediates is necessary to clarify the relationship between the two species.

Cytochrome *c* was the first protein in which a globular state induced by salt at low pH was detected and named a "molten globule".[5] Goto et al. later demonstrated that the conformational transition from acid-unfolded protein to this globular state is mediated by anion binding.[6] The structure of the cytochrome *c* molten globule depends strongly on the size of the added anion.[7] In addition to salts, polyols[8] and some alcohols[9,10] have been shown to stabilize the molten globule state of cytochrome *c* at low pH.

We have investigated the conformation of *Saccaromyces cerevisiae* iso-1 cytochrome *c* (cyt *c*) in its molten globule state using fluorescence energy transfer (FET) kinetics. For our experiments, we labeled the thiolate sulfur of C102 in the yeast protein with a dansyl fluorophore (DNS(C102)-cyt *c*); the DNS fluoresces intensely when the protein is unfolded, but it is significantly quenched by energy transfer to the heme in compact conformations. Analysis of the fluorescence decay profiles has provided insights into the distributions of distances between donor (***D***) and acceptor (***A***) labeled residues.[11] In the Förster model, the rate of energy transfer is equal to the decay rate of the unquenched fluorophore (k_0) when the ***D***−***A*** distance is equal to the critical length (r_0). Under typical conditions, FET rates can be measured for ***D***−***A*** distances in the range $0.3r_0 \leq r \leq 1.5r_0$. The critical length ($r_0$) of the DNS-heme FET pair is 40 Å, meaning that a 12−60 Å ***D***−***A*** distance range can be probed in the modified protein. Unlike other probes that report on the average properties of the ensemble (fluorescence intensity, CD, X-ray scattering, absorbance), FET kinetics reveal the conformational heterogeneity of the polypeptide.[12,13]

As expected, the molten globule has very similar helix content to the native state, whereas the acid-denatured protein exhibits no secondary structure (Supporting Information, Figure S1). The CD spectrum in the aromatic region (260−300 nm) shows two sharp peaks (282 and 290 nm) from tryptophan and/or tyrosine[14] for the native state; it is featureless for both the acid-denatured (pH 2) protein and the molten globule ($[SO_4^{2-}] > 0.7$ M), indicating the absence of tertiary structure.[15]

Far-UV CD spectra show that the thermal unfolding transition of the DNS(C102)-cyt *c* molten globule at pH 2 is cooperative (data not shown). The 309 K midpoint temperature is in excellent agreement with that previously observed for the thermal unfolding transition of the cyt *c* (C102T mutant) molten globule.[16] The transition in DNS(C102)-cyt *c* is more than 90% reversible.

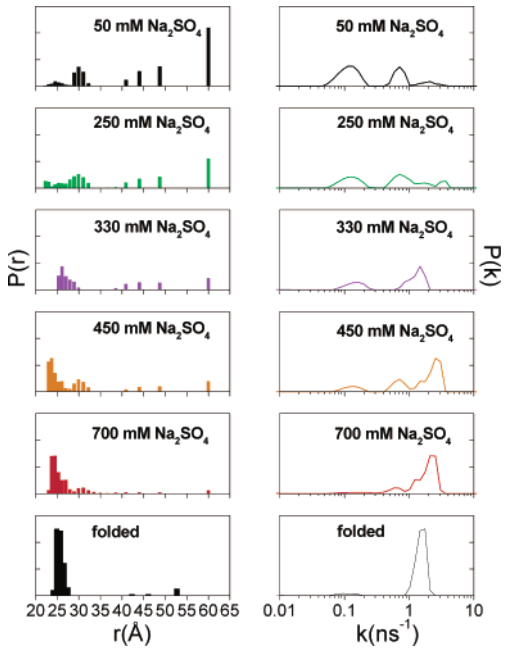

Figure 1. Na_2SO_4-induced changes in the distribution of the luminescence decay rates ($P(k)$, right) and ***D***−***A*** distances ($P(r)$, left) in DNS(C102)-cyt *c* (pH ≈ 2, 22 °C).

In the native state, the heme in DNS(C102)-cyt *c* is low-spin with Met80/His18 axial coordination. The Soret band is centered at 410 nm, and the Met80 → Fe(III) charge-transfer transition is clearly visible at 695 nm (Supporting Information, Figure S2). In the acid-denatured state, the protein is high-spin; that is, neither Met80 nor His18 is coordinated.[17−19] Both the Q-bands and the Soret absorption blue-shift from their positions in the native state. The Soret is centered at 396 nm, and there is a band at 620 nm attributable to an electronic transition in a high-spin heme.[20] The Soret band is centered at 400 nm in the spectrum of the molten globule, and the Q-bands red-shift relative to their positions in the acid-denatured protein. The heme in the cyt *c* molten globule is believed to be mixed spin, with His18 coordinated to iron in both spin states; Met80 is coordinated in the low-spin species, but not in the high-spin state.[15,17,18,21]

We have obtained ***D***−***A*** distance distributions in DNS(C102)-cyt *c* under conditions favoring the molten globule (Figure 1). At salt concentrations of 100 mM or lower, the polypeptide ensemble is highly heterogeneous: 50% of the polypeptides are in extended conformations with ***D***−***A*** distances >35 Å; the remaining 50% are in compact conformations with ***D***−***A*** distances <35 Å. As the salt concentration is increased further (100−500 mM), the fraction of

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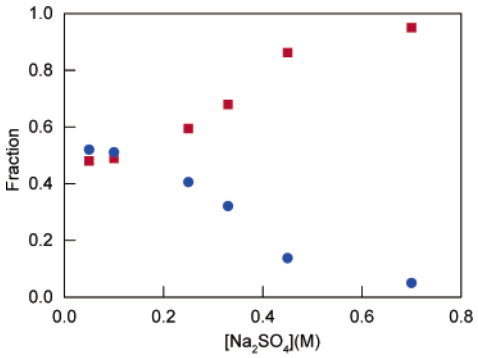

Figure 2. Fraction of compact (r < 35 Å, ■) and extended (r > 35 Å, ●) distributions as a function of Na_2SO_4 concentration.

polypeptides in extended conformations decreases in favor of compact structures, but both populations remain heterogeneous. At high salt concentrations (≥700 mM), all of the polypeptides are compact with ***D***−***A*** distances between 25 and 30 Å. The fractional populations (Figure 2) of compact (r < 35 Å) and extended (r > 35 Å) distributions parallel amplitude changes observed by circular dichroism upon addition of salt, but the FET-probed transition from the acid-denatured state to the molten globule is more prominent. FET kinetics measurements clearly reveal that the populations of compact and extended polypeptides are extremely heterogeneous below 700 mM [Na_2SO_4].

FET kinetics measured during DNS(C102)-cyt *c* folding show that dilution of denaturant to concentrations favoring native protein conformations ([GuHCl] = 0.13 M) does not produce complete collapse of the polypeptide ensemble.[13] Within the deadtime of stopped-flow measurements, we find that only 40% of the protein population has formed compact structures. The compact ensemble has a mean DNS-heme separation of ∼27 Å, which is greater than that of the native protein, indicating that the collapsed molecules are not fully folded. As the population of proteins with the native fold increases, extended and compact polypeptides disappear on comparable time scales. On the basis of these observations as well as folding kinetics measurements on Co-substituted cyt *c*,[22] we conclude that extended and collapsed populations in this folding intermediate are in rapid equilibrium.

The picture is similar in the case of "refolding" the acid-denatured protein with added Na_2SO_4. At relatively low salt concentrations ([Na_2SO_4] = 50 mM), both collapsed and extended structures are present at equilibrium. The compact ensemble has a mean DNS−heme separation of ∼30 Å, and the extended polypeptide population exhibits DNS−heme distances greater than 40 Å. The collapsed structures in the molten globule are reminiscent of the compact ensemble observed during DNS(C102)-cyt *c* refolding.[13] As the salt concentration is increased, the compact ensemble becomes more compact and shifts to shorter distances (25−30 Å) with a concomitant decrease of the population of extended conformations. Thus, at high salt concentrations, the ***D***−***A*** distances in the collapsed ensemble are virtually indistinguishable from those in the native population.

The structural homogeneity of folded proteins can be disrupted by a variety of chemical and physical perturbations. FET kinetics measurements on DNS(C102)-cyt *c* reveal a large degree of such heterogeneity in the acid-denatured protein. High salt concentrations convert the observed complex mixture of conformations into an ensemble of compact (r < 35 Å) polypeptides with a mean ***D***−***A*** separation quite close to that of the native protein (25 Å). This molten globule is somewhat more compact and far more homogeneous than the ensemble of polypeptides present in the burst intermediate formed during cyt *c* folding.[13,23−26] A clear transition between the acid-denatured and molten globule forms of cyt *c* is not apparent from most ensemble-averaged spectroscopic probes (UV−vis absorption, CD, fluorescence intensity). FET kinetics, however, provide definitive evidence for the formation of a uniformly compact molten globule at salt concentrations greater than 700 mM. It remains to be determined if polyols, alcohols, and other molten-globule stabilizing agents[27−29] are as effective as anions in shifting the collapsed/extended equilibrium to a position in which compact states are dominant.

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Supporting Information Available: Far- and near-UV CD spectra and absorption spectra of DNS(C102)-cyt *c*. Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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