Optical Triggers of Protein Folding

Sparked by recent developments in the theory of protein folding (*1*), experimentalists have been developing new methods for studies on the submillisecond time scale (*2*, *3*). Optical techniques have great potential in this area because of the many different ways that laser pulses can be employed to trigger folding. Torbjörn Pascher *et al.* (*4*) describe a method that uses a photoinduced electron transfer reaction to initiate the folding of reduced cytochrome c. The idea takes advantage of the difference in stability of the oxidized and reduced proteins. Optical excitation of an Ru^{2+} complex [Ru(2,2′-bipyridine)$_3^{2+}$] produces a long-lived excited state that reduces the oxidized heme of cytochrome c in less than 1 μs. Reduction of the unfolded oxidized protein initiates the folding of the more stable reduced protein.

This method is conceptually similar to the triggering technique used by Jones *et al.* (*2*), in which folding of reduced cytochrome c was initiated by photodissociation of the heme–carbon monoxide complex (Fig. 1). Because there is a larger difference in stability between oxidized and reduced cytochrome c than between reduced cytochrome c and its CO complex, the electron transfer method permits observation of folding under lower denaturant concentrations (Fig. 2) (*5*). The power of these triggering techniques is that they can be used to study events in the largely unexplored nanosecond-microsecond time regime.

Pascher *et al.* observed an ~40 μs process by optical absorption, which they pointed out is consistent with the kinetics of intramolecular methionine and histidine binding described in detail by Jones *et al.* (*2*, *6*). They further suggested that the ~40-μs phase could also correspond to the collapse of the unfolded polypeptide to compact denatured structures (*2*, *4*). Because of the similarity of the two optical triggering methods, we can provide a test of this hypothesis by measuring tryptophan fluorescence following photodissociation of the CO complex under almost identical solvent conditions. Cytochrome c contains a single tryptophan at position 59, about 40 residues distant along the chain from the heme. The tryptophan fluorescence is weakly quenched by excitation energy transfer to the heme in the unfolded state. It is almost completely quenched in the native conformation and at heme-tryptophan distances less than about 2.0 nm, as would occur in compact states of cytochrome c (*7*). Because the tryptophan fluorescence should decrease by at least 10-fold upon forming compact structures, it provides a very sensitive probe of polypeptide collapse.

We searched for fluorescence changes following nanosecond photodissociation of the CO complex over a range of guanidine hydrochloride concentrations (3.5 M to 5.0 M). We obtained the simple result that there is no observable fluorescence change, even at the lowest denaturant concentration, which strongly favors folding in the absence of CO (over 99.99% native at equilibrium) (Fig. 3). Thus, using this probe, there is no evidence for collapse to compact structures at any time between 10 ns and the completion of CO rebinding at 10 ms.

This result might be unexpected. Removing a ligand by photodissociation or reducing the net charge on the heme from +1 to 0 by electron transfer makes the heme more hydrophobic. Both optical triggers are therefore expected to increase the interaction of the large hydrophobic heme with noncoordinating amino acid residues, as well as alter the binding of ligands from the polypeptide chain (Fig. 1). Although these changes stabilize the native structure relative to unfolded denatured structures at equilibrium (Fig. 2), it is not clear that they could also cause collapse of the unfolded polypeptide before formation of the native structure [which begins at tens of milliseconds (*4*)]. In contrast, changing the solvent from "good" to "poor" by diluting a chemical denaturant strengthens the hydrophobic interaction among many amino acid residues. If these interactions are sufficiently strong, they can drive rapid collapse to compact denatured states (for example, "molten globule") (*1*, *8*). Our results indi-

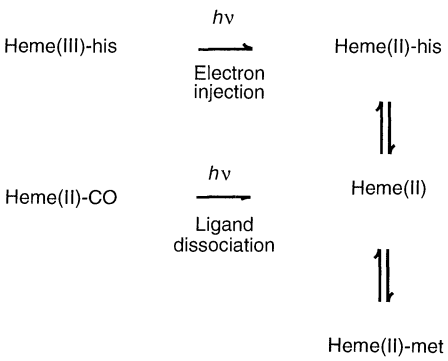

Fig. 1. Comparison of optical triggers for the folding of reduced cytochrome c. In the experiment of Pascher *et al.* (*4*), the heme iron in the initial denatured state of cytochrome c is oxidized. Axial ligands are histidine 18 and histidine 33 (or possibly histidine 26), as shown by nuclear magnetic resonance measurements (*10*). Electron injection reduces the heme iron. In the experiment of Jones *et al.* (*2*), dissociation of CO from the reduced heme produces a high-spin, five-coordinate complex. Jones *et al.* observed optical absorption changes beginning at hundreds of nanoseconds and modeled the changes in the time-resolved spectra as arising from complex formation with the methionines 65 and 80 (rate constants for binding and dissociation of 2.5×10^4 s^{-1} and 2.5×10^5 s^{-1}), and with histidines 26 and 33 (rate constants for binding and dissociation of 2.5×10^3 s^{-1} and 6×10^3 s^{-1}) (*2*).

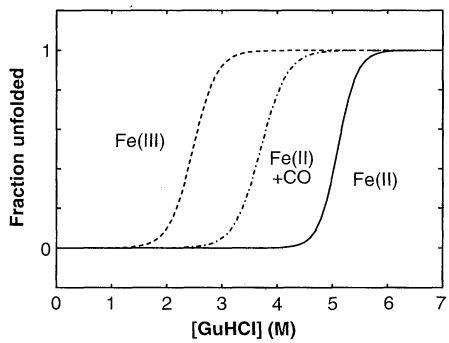

Fig. 2. Fluorescence-detected guanidine hydrochloride unfolding curves for oxidized cytochrome c (dashed), reduced cytochrome c (continuous), and reduced cytochrome c under one atmosphere of CO (dash/dot) at 40°C pH 6.5 to 7.0. Curves are taken from (*2*) and (*4*).

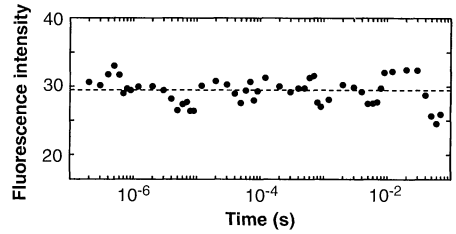

Fig. 3. Fluorescence intensity following photodissociation of CO from unfolded cytochrome c (3.75 M GuHCl, 0.1 M potassium phosphate, pH 7.0, 20°C). Under these conditions, 55% of the molecules are unfolded before photodissociation, while 0.05% of the molecules are unfolded in the absence of CO at equilibrium (Fig. 2). Fluorescence intensity at 360 nm, using 266 nm excitation with a single (~5 ns) pulse from a Nd:YAG laser, was measured as a function of the time delay after ≥75% photodissociation with the 532-nm harmonic of another Nd:YAG laser. Integrated intensity was measured with a photomultiplier tube and digitizing scope. Time between photodissociation pulses was 400 seconds, in order to permit complete recovery of the equilibrium distribution of unfolded states with CO bound, native states, and compact states with CO bound. Native-like states with CO bound rapidly convert to the native state after CO photolysis, but recover slowly as a result of the slow replacement of methionine with CO. This process does not contribute to the fluorescence signal because the tryptophan fluorescence is quenched in both states. Each point is the result of smoothing data from single excitation and probe pulses for each time delay.

cate that the changes in heme-protein interactions produced by the optical triggers are insufficient to do this. These optical triggers do, however, provide a unique opportunity to investigate the nanosecond-microsecond dynamics of the unfolded protein prior to surmounting the free energy barrier that separates the unfolded structures from the native state (9).

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5. As with many different types of single-laser-pulse, initiated processes, reversibility of the reaction puts limits on the *longest* times that can be studied. For cytochrome c, reversal of the photochemistry aborts folding at a few milliseconds. In the electron transfer experiment, reoxidation of the reduced cytochrome by the Ru^{3+} complex occurs, while in the photodissociation experiment CO rebinds. Both of these problems could be overcome by using a continuous wave laser to maintain reduction or ligand dissociation after the initial excitation pulse.
6. The relaxation times corresponding to the rate constants obtained by Jones *et al.* (*2*) for the kinetic scheme in Fig. 1 are ~4 μs and ~120 μs. After reduction in the electron transfer experiment, the heme is mainly six-coordinate, and the same two relaxation times should be observed as in the CO photodissociation experiment. At most wavelengths, however, the amplitude will be dominated by the slower relaxation time corresponding mainly to histidine dissociation and rebinding. Considering the small difference in solution conditions (for example, the slightly higher pH in the experiments of Pascher *et al.*, which would result in less protonation of the histidines and faster binding rates) and the report of a single relaxation time in the electron-transfer experiment, we assume that the two techniques are giving consistent results, as also concluded by Pascher *et al.* (*4*). Thus, although the initial conditions are different in the two experiments, after ~100 μs they should produce the same distribution of denatured structures.
7. The quantum yield of fluorescence relative to the quantum yield in the absence of heme quenching is given by Forster theory as $[1 + (r_o/r)^6]^{-1}$, where r is the heme-tryptophan distance and r_o, which depends on the "overlap integral" of the normalized fluorescence spectrum of the tryptophan and the absorption spectrum of the heme, is ~3.5 nm. In a random coil distribution of heme-tryptophan distances, where the mean distance is ~7 nm the average relative quantum yield is 0.8, while at a distance of 2.0 nm the relative quantum yield is only 0.03. In the native conformation this distance is 1.0 nm.
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11. We thank A. Szabo and E. Henry for helpful discussions.

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Response: In our report about the electron-transfer (ET)–initiated folding of ferrocytochrome c (cyt c^{II}) (*1*), we noted rapid changes in the visible absorption spectrum corresponding to a process with a time constant of ~40 μs. We indicated that this observation was consistent with studies of cyt c^{II} folding initiated by CO dissociation where the fast dynamics were attributed to changes in heme ligation (*2*). We also suggested that these dynamics might correspond to the collapse of the protein into a compact denatured state, as has been proposed for apomyoglobin on the basis of laser-temperature-jump measurements (*3*). Our transient absorption data could not distinguish between the two possibilities, and these fast folding dynamics were a relatively minor component of the study described in our report. Chan *et al.*, with the use of tryptophan (Trp) fluorescence as a probe, found no significant collapse of the protein on the submillisecond time scale following dissociation of CO from unfolded cyt c^{II}. Clearly, multiple spectroscopic probes must be employed to study protein folding; accordingly, we are currently developing time-resolved Trp fluorescence as a probe for ET-initiated folding of cyt c^{II}.

A particularly significant difference between our work and that of Chan *et al.* is the nature of the unfolded form of the protein. The initial state in ET-triggered cyt c^{II} folding is guanidine hydrochloride (GuHCl) denatured ferricytochrome c (cyt c^{III}) (*1*, *4*); in contrast, Chan *et al.* start with CO-ligated cyt c^{II} in the presence of GuHCl (*2*). Higher GuHCl concentrations are required to unfold CO-bound cyt c^{II} than cyt c^{III}. Apparently, the positive charge on the heme in cyt c^{III} has a significantly greater destabilizing effect than does CO binding to the ferroheme. Given these differences, it is reasonable to question whether the two unfolded states are the same. Investigations of cyt c^{III} folding using stopped-flow kinetic spectroscopy have been interpreted in terms of a minor collapse during the mixing dead time (~2 ms) (*5*). Nevertheless, the reduction in Trp fluorescence observed in this burst phase is measurably greater than the changes found by Chan *et al.*; this reduction has been attributed to a decrease in the Trp to heme distance of ~5 Å. Given the possible differences between the unfolded forms of the proteins, we must use Trp fluorescence as an additional probe in our studies of ET-initiated cyt c^{II} folding.

The complex process of protein folding is believed to involve dynamics that span more than 12 orders of magnitude in time (picoseconds to minutes). The power of optical triggering methods (such as photo-induced CO dissociation and ET chemistry) is that they lay open a large part of this time regime for direct examination.

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Complementary DNA for 12-Kilodalton B Cell Growth Factor: Misassigned

The 12-kD B cell growth factor (BCGF), also known as low molecular weight–BCGF or BCGF, is a cytokine produced by activated T lymphocytes (*1–4*). Several functions have been implicated for this factor. Most importantly, 12-kD BCGF has been suggested to be a common progression factor for human B lymphocytes and to have an autocrine role in B cell neoplasms (*5*). Twelve-kilodalton BCGF was purified to homogeneity in 1985 (*3*), and cloning of the corresponding cDNA was reported 2 years later (*4*). However, these studies have not been confirmed, and the exact molecular connection between the widely used commercial BCGF (purified from the supernatants of mitogen-activated lymphocytes), natural 12-kD BCGF, and the reported cDNA has remained ambiguous. Recently, a genomic segment that clearly corresponds to the reported cDNA sequence was identified (*6*, *7*). Sequencing of the genomic DNA of 12-kD BCGF by Ziętkiewicz *et al.* (*6*)