Direct observation of fast protein folding: The initial collapse of apomyoglobin

(molten globule/temperature jump/fluorescence/circular dichroism)

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ABSTRACT The rapid refolding dynamics of apomyoglobin are followed by a new temperature-jump fluorescence technique on a 15-ns to 0.5-ms time scale in vitro. The apparatus measures the protein-folding history in a single sweep in standard aqueous buffers. The earliest steps during folding to a compact state are observed and are complete in under 20 μs. Experiments on mutants and consideration of steady-state CD and fluorescence spectra indicate that the observed microsecond phase requires assembly of an A-(H-G) helix subunit. Measurements at different viscosities indicate diffusive behavior even at low viscosities, in agreement with motions of a solvent-exposed protein during the initial collapse.

A compact molten globule state has been proposed as an early protein-folding intermediate in many cases (1), but its initial formation from the unfolded state during refolding has not been directly observed. Slower (>1-ms) kinetic phases of protein folding, leading from the molten globule state to the native state (2) or from the unfolded state to the fully native state directly (3, 4), have been resolved by stopped-flow techniques. The nanosecond to sub-millisecond time scale, in which the earliest global protein-folding motions are expected to occur, is not easily accessible by these techniques.

Recently, optically induced ligand unbinding in cytochrome c (5) and rapid mixing techniques (6) have been used to monitor folding dynamics on a sub-millisecond time scale. The helix-coil transition in peptides and unfolding of myoglobin (Mb) (7), and the unfolding of RNase A (8) have been monitored by infrared absorption after a temperature jump (T-jump). This very early regime has also been accessed by NMR line shape measurements (9) and can be reached by electron-transfer-induced folding (10). Finally, the nanosecond T-jump technique developed by Eigen and coworkers (for review, see ref. 11) has been applied to folding experiments starting with cold-denatured barstar (12).

Theoretical models currently under discussion address various issues of early folding. A general principle that has emerged is that of "minimal frustration": a free-energy funnel to the native state provides enough (perhaps just enough) smoothness to balance the natural roughness of the folding free-energy surface expected for a heteropolymer, thus allowing fairly efficient folding (13). Depending on the relative location of the transition state, the glass-like transition, and the roughness and sloping of the free-energy surface, this scenario has been played out analytically and in simulations in many limits, including direct two-state folding, collapse to folding intermediates with varying amounts of secondary and tertiary structure, and kinetic traps (14–17). One certainty is that, at least transiently, the protein must pass through a compact ensemble of states while folding.

To investigate early folding events in globular proteins, we have developed an apparatus that allows us to monitor the T-jump-induced refolding dynamics of small proteins. It extends time resolution to the nanosecond time scale and temperature differentials to 30 K, allowing easier observation of larger population changes at shorter times. Some of the requirements in developing our approach were as follows: (i) nanosecond to millisecond time coverage with nanosecond resolution and dead time to follow the earliest large-scale backbone motions up to the stopped-flow time regime; (ii) single-shot acquisition of a sample's history without pump-probe signal averaging or sample flow to allow for small sample quantities (e.g., genetically engineered ones); (iii) experiments in simple aqueous buffers, obviating the need for extraneous dyes or other molecules that could affect early folding dynamics; and (iv) the possibility of fluorescence, CD, or infrared monitoring in different viscosity, temperature, or denaturant concentration ranges.

EXPERIMENTAL METHODS

Outline. The heart of the experiment is shown in Fig. 1. A protein sample is cold denatured in a short path length cell by supercooling the aqueous buffer. CD is used to verify unfolding under the conditions used. The aqueous buffer is directly and rapidly heated (up to 5·10⁹ K/s) by a nanosecond infrared Raman pulse. The unfolded protein is now located in a warmed buffer at a temperature thermodynamically conducive to folding. The folding process is followed by focusing a train of UV laser pulses onto the sample. Here, we report on fluorescence experiments using Trp excitation at 280 nm. Every 15 ns, the UV pulse train induces a protein fluorescence transient. The transients are collected continuously by a photomultiplier and transient digitizer. Finally, the changes in protein fluorescence in 15-ns or larger (averaged) steps are analyzed and correlated to structural changes.

Heating and Sample Cell. The sample is held in a custom 0.4-mm path length-fused silica cell (Fig. 1) cooled by two thermoelectric devices. The temperature is held constant to <0.2°C by a thermostat feedback loop. Two 120-mJ, 1.54-μm infrared beams are generated by Raman shifting a 700-mJ neodymium:yttrium/aluminum-garnet laser in a mode-optimized high-efficiency methane cell, resulting in uniform, near-gaussian heating profiles of 2-mm diameter. The counterpropagating beams are delayed by 8 ns from one another to avoid transient grating formation, and heating (by OH overtone relaxation in water) is completed within the pulse duration due to picosecond vibrational equilibration. The two mirror-image exponential absorption profiles add up to a longitudinal temperature uniformity of ±3% over the length of the cell, and the large uniform pump profile minimizes thermal lensing and diffusion effects. The T-jump is measured by transmission of a 1.5-μm diode laser focused to <400 μm.

Abbreviations: T-jump, temperature jump; Mb, myoglobin; apoMb, apomyoglobin; h-apoMb, horse apoMb.

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and calibrated at known temperatures or with free Trp lifetimes. The custom cell design reduces diffusional and shockwave effects in the sample. The latter are also minimized by the 4°C density maximum of water. Any residual shockwave effects and weak anti-Stokes Raman/supercontinuum flashes from the infrared optics do not affect lifetime and folding measurements, as discussed in Fast Kinetic Results (see also Fig. 6).

Kinetic Probing. Probe pulses for Trp fluorescence are generated by tripling a self-mode-locked titanium:sapphire laser in BBO nonlinear crystals. The focused pulse train (~2 mW, 7-nm excitation bandwidth at 280 nm, and 200-μm diameter) probes only the center of the large pump profile. Fluorescence is imaged onto a filtered (U340) 600-ps rise-time microphotomultiplier tube connected to a 2-GS/s digitizer (0.75-GHz bandwidth, 1 million channels). All infrared and UV probe beams are electromechanically shuttered during the experiment to avoid optical sample denaturation.

Protein Samples. Horse skeletal muscle Mb was obtained from Sigma. The heme was removed by the 2-butanol method (18). Purity of the apomyoglobin (apoMb) was verified by UV-visible spectroscopy to exceed 99%. Concentrations of apoMb were determined as described (19). All samples were buffered in 10 mM sodium acetate at pH values shown in Table 1. A Trp-7→Phe-7 sperm whale Mb mutant was provided by S. Sligar (20) and converted to apoMb as described above.

### Table 1. Summary of observed lifetimes for the kinetic phase due to collapse

<table>
<thead>
<tr>
<th>Glycerol (M)</th>
<th>( T_1 ) (°C)</th>
<th>( T_2 ) (°C)</th>
<th>( \eta ) (cP)*</th>
<th>Nominal pH</th>
<th>( \tau_{collapse} ) (μs)</th>
<th>( \tau/ \eta )†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 (2)</td>
<td>22 (3)</td>
<td>1.0</td>
<td>5.9</td>
<td>7 (5)</td>
<td>1.4 (9)</td>
</tr>
<tr>
<td>0</td>
<td>−7 (1)</td>
<td>10 (3)</td>
<td>1.3</td>
<td>5.2</td>
<td>5 (1)</td>
<td>0.8 (2)</td>
</tr>
<tr>
<td>0.1</td>
<td>−8 (1)</td>
<td>10 (3)</td>
<td>1.3</td>
<td>5.2</td>
<td>5 (3)</td>
<td>0.8 (4)</td>
</tr>
<tr>
<td>3</td>
<td>−12 (1)</td>
<td>5 (2)</td>
<td>3.5</td>
<td>5.2</td>
<td>17 (6)</td>
<td>1.0 (4)</td>
</tr>
</tbody>
</table>

*Estimated errors are based on the reproducibility and variation found in several data sets taken under nominally identical conditions.

†Viscosity at final temperature \( T_C \) see ref. 33.

**STEADY-STATE RESULTS**

We have chosen horse apoMb (h-apoMb) for our first experiment because a wealth of information on its cold denaturation (21), CD (22), millisecond folding dynamics (2), structure (23), molten globule states (24), and fluorescence properties (25) is available. Our cold denaturation data on h-apoMb are similar to the results obtained by Nishii and coworkers (22). Assuming a two-state model with folded/unfolded states, the population of the native state as a function of temperature is plotted in Fig. 2. The cold-denatured state probably contains some residual helix, although the heat- and cold-denatured baseline in Fig. 2 is well-fitted by a single line. Cold and pressure denaturation experiments on RNase by Nash and coworkers (26) indicate protection factors for a few residues in the pressure/cold-denatured state that are substantially higher (≈100) than those in the heat-denatured state (≈1–5) but substantially lower than those in the native protein (10^−3−10^−4), and the higher cold denaturation transition temperature of apoMb implies even smaller protection factors for the latter. Calorimetric measurements also suggest a state at least close to a random coil (21), and a number of steady-state and kinetic fluorescence experiments described below also indicate a loose initial state. However, until isotopic two-dimensional NMR or similar data become available, a partially assembled A-G-H complex cannot be ruled out entirely in the cold-denatured initial state. It is worth reiterating that our experiment does not take place under cold denaturation conditions; the conditions simply provide an initial unfolded structure from which the protein starts refolding in the warmed buffer.

Fluorescence/mutagenesis experiments have shown that specific amino acid residues can dominate Trp quenching (27, 28). In certain cases, this is the case for observed nonexponential behavior in the presence of only one Trp residue (28). Using known bimolecular quenching rates of Trp (27), our kinetic modeling using the rate model of Van Gilst and coworkers (28) indicates that the major contribution to fluorescence changes in h-apoMb comes from Trp-14 (A helix) and that its lifetime and quenching are most substantially affected by Met-131 (H helix), whose side chain is in van der Waals contact with the Trp ring (Fig. 3).

The kinetic modeling of Trp quenching includes rates for fluorescence, reversible bimolecular quenching by specific residues, and a background quenching rate (solvent and remainder of protein) (27, 28) using structural parameters for holo-Mb. In h-apoMb, Trp-7 is predicted to be heavily quenched (short lifetime) by neighboring Glu-6 and Gln-8 residues and shows no specific quenching outside the A helix; Trp-14 is weakly quenched by Ala-15 and Val-13 residues but

**Summary.** The folding history of the protein in an aqueous buffer is thus obtained in a single shot in real time, requiring no dyes for energy transfer, extensive pump-probe signal averaging, or large samples for sample flow. The dependence of the early folding kinetics on the protein environment can be studied by changing solvent viscosity (e.g., glycerol solutions) or by adding denaturants.
shows strong specific quenching by Met-131 in the H helix (Met Cterminus methyl to Trp Cz5 contact). The following control experiments (Fig. 4) support the modeling. Wild-type sperm whale apoMb has fluorescence properties that are very similar to those of h-apoMb (no differences in the critical residues described above). The Trp-7→Phe-7 sperm whale mutant in Fig. 4 has a slightly larger slow component (locally quenched Trp-7 absent); guanidine-HCl-denatured h-apoMb has a large slow component with a smooth temperature dependence (no Trp-14-Met-131 quenching at any temperature, leaving only background quenching of the Trp); cold-denatured h-apoMb also has a large slow component, which rapidly decreases at higher temperature (Trp-14-Met-131 contact upon folding). Kinetic data (see below) on an Met-131-free mutant indicates that Met is indeed critical to Trp-14 quenching. A more detailed analysis with other residues is not warranted until uncomplexed apoMb structures become available, and it is not likely to affect our general conclusions.

**FAST KINETIC RESULTS**

Fig. 5 summarizes a fast folding experiment with h-apoMb, and conditions of several other experiments are listed in Table 1. The raw fluorescence data are analyzed by singular value decomposition (30) to eliminate noise components and by least-squares fitting. They show a clear progression from the cold-denatured value to compact-state/native values (as obtained on the same apparatus or a phase fluorimeter in a control after 0.5 ms or under steady-state conditions).

![Fig. 2](image-url)  
**Fig. 2.** (A) Mean residue ellipticity at 222 nm (disks) for h-apoMb in the -8-95°C range (pH 5.9). Data collected after the sample was held at -7.2°C for 1 h (fully reversible cold denaturation); the CD of mostly denatured protein (sample between 70-95°C for 1 h, then cooled to 20°C); solid line, a fit to a thermodynamic two-state model (21, 22), including fitted temperature-dependent baselines (dashed line). The unfolding thermodynamic parameters are ΔH = 195(17) kJ/mole, ΔS = 0.575(49) kJ/mole-deg, and ΔCp = 5.1(5) kJ/mole-deg. (B) The fraction of folded protein (the hatched zone represents the uncertainty due to thermodynamic parameters errors). The disks are the folded fraction in 3 M glycerol, which still shows substantial cold denaturation. CD data for 0 and 3 M glycerol buffers at pH 5.2 are similar to those in A and not shown here; the uncertainty in the 3 M glycerol folded fraction is similar to that shown for 0 M, pH 5.9 conditions. T-jumps in the -10 to 10°C range can produce 25-40% population changes.

![Fig. 3](image-url)  
**Fig. 3.** A model of folded h-apoMb, based on ref. 23 (holo-protein), showing the Trp-14 and Met-131 residues involved in fluorescence quenching upon A-H helix contact. The color coding corresponds to theoretically derived folding units (29). (The folding units in this figure were derived from an energy landscape analysis by picking protein segments and then identifying segments with the largest ratio of stability gap to the spread of their molten globule energies.) G-H form such a unit (red) and the A helix is part of another unit (green), in which it shows the least structural fluctuations during folding simulations (15). This is highly suggestive of the postulated A-G-H folding intermediate (2) as well as the very fast collapse phase observed in our work.

We have employed a number of different methods to analyze the progression of individual fluorescence transients, including exponential fits with different floating parameters. Fig. 5E shows a typical result of fitting deconvoluted decays using a bi-exponential function. However, in this experiment, we are interested not in the details of individual fluorescence transients but in the progressive change of fluorescence as the protein folds. The most robust method of analysis thus bypasses the need for fluorescence lifetimes and instrument response functions entirely (Fig. 5C). An "initial" or "unfolded" fluorescence profile, f1, and a "final" or "compact" profile, f2, are generated from data before and long after the T-jump; a linear least-squares fit then reconstructs the data at intermediate times from the f1 by fitting their two amplitudes, A1 and A2. The fraction x2 = A2/(A1 + A2) is plotted as a function of time delay. x2 is not very sensitive to residual amplitude fluctuations and mostly correlates with the shape (i.e., lifetime) of the fluorescence decays as they progress from cold-denatured to compact/native-like.

The kinetic behavior is typically as follows. Before the T-jump, x2 = 0; within the next probe pulse (<15 ns), x2 rises instantaneously to 0.8-0.9. This is followed by a rise of a few microseconds to x2 = 1. The instantaneous change is nearly identical to a control experiment with free Trp or acetyl-Trp (Fig. 6). We assign it purely to the temperature dependence of the emission (local motion and population effects) rather than any <15-ns global folding motion. This is also verified by fluorescence lifetime measurements of h-apoMb at -8°C and 10°C in the presence and absence of denaturants (Fig. 4; Steady-State Results).

Fig. 6 illustrates another refolding transient obtained in 3 M glycerol solution at higher viscosity. The refolding amplitude is a larger fraction of the total amplitude in this case because
the initial temperature (−12°C vs. 0°C in Fig. 5) resulted in a larger initial fraction of unfolded protein (see CD in Fig. 2). These data will be discussed in detail below. Fig. 6B shows a control experiment performed with an aqueous Trp solution jumped by 22°C. Only the instantaneous phase due to the temperature dependence of the emission is evident in this test and similar tests, confirming that the microsecond phase is inherent to the protein-folding process. Furthermore, a kinetic trace (data not shown) obtained for a Met-131 → Ala-131 sperm whale apoMb mutant (purified sample kindly provided by R. Baldwin and M. Kay, Stanford University) refolding to the acid-globule state at pH 5.2 shows no microsecond phase at our signal-to-noise level, indicating that Met-131 is indeed the major quencher and that the A-G-H complex is likely not preformed in the cold-denatured state.

**DISCUSSION**
We assign the ~7-μs kinetic phase in Fig. 5 to the time required for the unfolded ensemble (U) to form a compact (but still strongly hydrated) state. Using CD experiments with 5-ms time resolution, Jennings and Wright (2) have recently reported evidence for a molten globule (MG) state as a first step in apoMb folding. Their evidence is based on different (from native, N) urea titration curves and CD spectra of a <5-ms burst phase, which acquires 75% of the full apoMb optical rotation within the experimental dead time. While our fluorescence measurements cannot conclusively distinguish between a U → N or U → MG transition, it is very unlikely, based on diffusion and contact lifetimes for a 153-link polypeptide chain at ~1°C viscosity, that we are observing a very fast U → N phase. Rather, our 7-μs phase reflects the formation of a more compact state in which the Trp-14 and Met-131 residues have approached from a value closer to ~40 Å (random coil value) to a ~9 Å native Cα-Cα separation. This is in agreement with the expectation that the A-G-H complex of apoMb would be the earliest structure to form in the folding process, based on amide proton NMR protection data (2).

This collapse rate is slightly faster than expected from recent experiments by Eaton and coworkers (5, 6), which yielded diffusional collision times of residues in cytochrome c near 40 μs in the presence of denaturants. There are two points of view when comparing our results with theirs. The expected smaller roughness of the free-energy surface under their strongly denaturing conditions would point toward 40 μs being a lower limit on the collapse time; on the other hand, the shallower funnel under their denaturing conditions would point toward 40 μs being an upper limit. In any case, both numbers are close to the expected purely diffusional time constant, which should be of the same order as the collapse time for formation of a compact state. Our measured smaller time constant indicates that the A-H “collision” in apoMb occurs somewhat faster than expected from simple diffusion of a chain in a solvent, perhaps due to “boosting” of the polypeptide chain by non-native hydrophobic contacts.

If the microsecond phase in Figs. 5 and 6 indeed leads from a largely unfolded coil to a more compact state with substantial native-like contacts in the A-G-H complex, one would expect a significant Kramers (i.e., diffusional) dependence of the rate on viscosity even at low viscosities (unless a convective motion “burrowing” through the solvent were involved). Ansari and coworkers (31) have measured the viscosity dependence of the structural rearrangement rate of folded Mb after photolytic detachment of CO. Their observed rearrangements are relatively small in scale compared with those during refolding, but they are globally distributed over the protein, particularly near the heme pocket. In the 0.7- to 3-cP viscosity range, their rate for conformational rearrangement in folded Mb is viscosity independent, whereas it decreases rapidly at higher viscosities. Their data are well-fitted by a modified Kramers expression that includes an internal protein friction term σ, such that the rate is proportional to (σ + η)−1, where η is the solvent viscosity (31, 32).

We have measured the folding rate at 3.5-cP viscosity using 3 M glycerol/water solutions (33) of h- apoMb (Table 1). The resulting fit to a 17-μs kinetic phase is shown in Fig. 6. The refolding transient is considerably slower in this experiment than in 0 or 0.1 M glycerol experiments. This result agrees better with a strict Kramers-like decrease of the rate as a function of viscosity than with a constant rate, if solvent-induced changes in the activation energy can be neglected. We have reason to believe that the 10°C and 22°C measurements in Table 1 indicate that the activation energy must be small to begin with; “stabilizing” solvents such as glycerol are known to be excluded from the immediate vicinity of the protein (34) and are likely to solvate an unfolded chain and hydrated transition state in a similar way, creating only a small change in activation barrier.

The process in Figs. 5 and 6 is, therefore, due to solvent-exposed—most likely large-scale—motions of the protein backbone as it collapses to a compact state and not due to small-scale changes dominated by self-friction of a protein that has already largely excluded its aqueous environment. This is also supported by the CD data in Fig. 3; the data are well-fitted by a two-state model with the same baseline for the heat- and cold-denatured states. Therefore, our initial state should have largely coil-like characteristics with some residual helicity, unlike the native state or the structured A-G-H bundle inferred by Jennings and Wright for the molten globule (2).

Singlet value decomposition data near τ = 0 (average of several folding transients taken under nominally identical conditions, data not shown) with <20-ns dead time show a small kinetic phase leading to a longer lifetime immediately after τ = 0, before the shortening due to Met-131 quenching. The lifetime of several hundred nanoseconds of this component may be due to incipient protection of Trp-14 from the
solvent by a newly formed A helix before its “collision” with the G-H complex, but better signal-to-noise and solvent quenching studies will be required to verify this.

Recent experiments by Nölling and coworkers (12) report a very small folding phase of ~300 μs in T-jumped barstar. As shown in Fig. 5F, some of our runs show indications of a 100- to 500-μs phase that cannot be resolved with the present signal-to-noise ratio. If it is indeed present, it could be analogous to the early kinetics observed by Nölling and coworkers. One may speculate that this phase is due to smaller-scale rearrangements of the protein after initial collapse (with rearrangements of the hydration shell), as these would have a much less severe effect on the quenching of Trp fluorescence. Perhaps it is even a very fast phase leading to the native state (although data in ref. 12 indicate that it may still be solvent-exposed). In that case, our prediction is that this phase should show a minimal viscosity dependence over the 1- to 3-cP range considered here. Our faster phase would have been difficult to observe with their apparatus, if one is indeed present in barstar.

In conclusion, our study with <20-ns dead time and time resolution shows that collapse of the unfolded state to a compact state involving at least the A- and H-helix backbone is complete in a few microseconds and involves significant motions of the backbone through the solvent. It remains to be seen from appropriate fluorescence/mutagenesis experiments whether other parts of partially folded apoMb (CD-E-F) collapse more slowly or incompletely on this time scale. Future nanosecond time-resolution CD and infrared experiments will show how the timing of any postulated secondary structure nuclei compares to the collapse rate.

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Fig. 5. Experimental data and kinetic fit. (A) Slow diffusional relaxation of cell temperature following the T-jump (see Table 1). On a logarithmic scale (B) covering six orders of magnitude in time, the baseline-subtracted fluorescence transients are shown from -10 μs to +490 μs (with respect to the T-jump as marked; pH 5.9, 3·10^{-4} M protein). The T-jump is followed by an “instantaneous” phase and a 5- to 7-μs phase reaching the steady-state fluorescence. Individual transients (15-ns length) mark one point in the folding evolution. Depending on the time scale, data are averaged in 1-600 transient blocks to extract maximal signal-to-noise at long times. (C) To analyze the data, two fluorescence decay functions, f_1 and f_2, are generated by averaging the initial and final 8 μs of the interval. The data and f_i are singular value decomposed. The circles in C are constructed from two singular value decomposition components. Data can be fitted to a bi-exponential decay convolved with the instrument response function (D), which is precisely known from lifetime measurements using calibrated compounds (p-terphenyl) or 420-nm scattering as an instantaneous response. (E) The extracted decays, with τ_1 = 3.7 ns, A_1 = 60%, τ_1' = 1.2 ns, τ_2 = 3.34 ns, A_2 = 43%, and λ_2' = 1.0 ns. Generally, the lifetimes are not of interest, and a two-parameter linear least-squares fit of the data to the fixed functions f_1 and f_2 is used to determine their amplitudes, A_1 and A_2. (F) The resulting 7(5)-μs kinetic phase in x_2 = A_2/(A_1 + A_2), which accounts for 15% of the signal, the rest being due to the instantaneous temperature effect, x_2 is independent of overall fluorescence intensity and depends only on the shape (i.e., lifetime) of the signal as it progresses from unfolded toward the compact state signature. The right side of F shows the 30- to 490-μs time scale. Small phases of <0.5-μs and >100-μs duration may also be present but cannot be resolved with the present signal-to-noise ratio.

Fig. 6. (A) Kinetic transient for h-apoMb in 3 M buffered glycerol (pH 5.2). The 17(6)-μs phase of this sample [3.5 cP at 5°C (1 P = 0.1 P±)] is significantly slower than observed in pure aqueous buffer (1.3 cP at 10°C). At low viscosity, this indicates that large-scale protein motions during folding dominate over internal protein friction effects. (B) Control experiment using Trp (pH 5.9, T-jump from 0 to 22°C). Only an instantaneous component is found in this and similar controls with acid-denatured h-apoMb and a Met-131 → Ala mutant of sperm whale apoMb.