Rapid mixing methods for exploring the kinetics of protein folding

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Abstract

Information on the time-dependence of molecular species is critical for elucidating reaction mechanisms in chemistry and biology. Rapid flow experiments involving turbulent mixing of two or more solutions continue to be the main source of kinetic information on protein folding and other biochemical processes, such as ligand binding and enzymatic reactions. Recent advances in mixer design and detection methods have opened a new window for exploring conformational changes in proteins on the microsecond time scale. These developments have been especially important for exploring early stages of protein folding.

Keywords: Ultrafast mixing; Stopped-flow; Continuous-flow; Fluorescence; NMR

1. Introduction

To elucidate the mechanism of any reaction, be it chemical or biochemical, a binding process or a macromolecular conformational change, we need to determine the time-dependent evolution of the molecular species involved. Kinetic techniques fall into two broad categories, equilibrium and relaxation methods. Equilibrium methods extract rate-information without physically or chemically perturbing a system, for example, by measuring dynamic effects on spectral line shape (NMR, EPR or optical), or by observing molecular fluctuations. Relaxation techniques generally rely on a rapid change of an extrinsic variable (e.g., temperature, pressure or solvent composition) to perturb the system and follow its response as it evolves toward a new equilibrium position. The development of laser-based photochemical or thermal triggers has opened new time windows for exploring the dynamics of biological molecules on the micro- and nano-second time scale [1–5]. However, the most common approach for triggering chemical and biological processes relies on turbulent mixing to achieve a rapid change in solvent composition. Historically, some of the earliest rapid kinetic measurements with millisecond time resolution used a continuous-flow arrangement combined with absorbance measurements of the reaction progress at different points downstream [6]. However, continuous-flow experiments were later replaced by the more versatile and economic stopped-flow experiment, which can be coupled with a wide range of spectroscopic probes to monitor reactions with millisecond time resolution [7,8].

Continuous-flow techniques have experienced a renaissance in recent years due to advances in mixer design and detection methods, which made it possible to push the time resolution into the microsecond time range [9–12]. Other techniques make use of two or more consecutive mixing steps to prepare the system in a particular initial state (double-jump stopped-flow), or to execute multiple reaction steps in sequence (quenched-flow). If a reaction can be quenched by manipulating solution conditions (e.g., pH) or lowering temperature, quenched-flow or freeze-quench protocols can be used in combination with slower analytical techniques, such as NMR, EPR or mass spectrometry [13]. To achieve efficient turbulent mixing conditions requires high flow
rates and relatively large channel dimensions, which can consume substantial amounts of material. A more economic technique that uses hydrodynamic focusing to mix solutions under laminar flow conditions has recently been introduced [14,15]. Rapid mixing techniques play a particularly prominent role in kinetic studies of protein folding [16–21]. As with any complex process, time-resolved data are essential for elucidating the mechanism. Even in cases where the whole process of folding occurs in a single step, which is the case for many small proteins, the kinetics of folding and unfolding provide valuable information on the rate-limiting barrier. The effects of temperature and denaturant concentration give insight into activation energies and solvent-accessibility of the transition state ensemble, and by measuring the kinetic effects of mutations, one can gain more detailed structural insight [22–24]. If the protein folding process occurs in stages, i.e., if partially structured intermediate states accumulate, kinetic studies can potentially offer much additional insight into the structural and thermodynamic properties of intermediate states and intervening barriers [18–20,25,26]. The combination of quenched-flow techniques with hydrogen exchange labeling and NMR has proven to be particularly fruitful for the structural characterization of transient folding intermediates [27–29].

2. Burst-phase signals in stopped-flow experiments

In many cases, stopped-flow and quenched-flow measurements of protein folding reactions show indications of unresolved rapid processes occurring within the dead time (e.g. [30–35]). This is illustrated by Fig. 1, which shows the kinetics of refolding of cytochrome c measured by stopped-flow fluorescence (panel A) along with equilibrium fluorescence data vs. denaturant concentration (panel B). The protein was unfolded by addition of 4.5 M guanidine hydrochloride (GuHCl), which lies in the baseline region above the cooperative unfolding transition (Fig. 1B). The refolding reaction was triggered by 6-fold dilution with buffer (0.1 M sodium acetate, pH 5), resulting in a final GuHCl concentration well within the folded baseline region. The data points in Fig. 1A were recorded by sampling the fluorescence emission above 325 nm (using a glass cutoff filter) at logarithmically spaced time intervals. The first time point corresponds to the instrumental dead time of 2.5 ms, which was calibrated using a standard test reaction ([36]; see below). The observed decay fits to a series of two exponential phases (solid line), a major one with a time constant of about 8 ms and a minor one with a time constant in the 100 ms range. Extrapolation of the observed kinetics back to t = 0 yields the initial signal, S(0), which is compared in Fig. 1B (arrow) with the equilibrium unfolding transition plotted on the same fluorescence scale (relative to unfolded cytochrome c at 4.5 M GuHCl). The initial signal observed in this and a series of additional stopped-flow experiments at different final GuHCl concentrations are consistently below the relative fluorescence of the unfolded state, S_{pred}(U), predicted by linear extrapolation of the unfolded-state baseline (see dashed line in (B)). (B) Effect of the denaturant concentration on the initial (squares) and final (circles) fluorescence signals, S(0) and S(∞), measured in a series of stopped-flow refolding experiments at different final GuHCl concentrations.

![Fig. 1. Stopped-flow fluorescence evidence for an unresolved rapid process (burst phase) during folding of cytochrome c (pH 5, 10°C).](image-url)
techniques, and provided a strong incentive for the development of faster methods for triggering and observing structural changes during the first millisecond of refolding.

3. Turbulent mixing

Most rapid mixing schemes rely on turbulent mixing to achieve complete mixing of two (or more) solutions. Mixers of various design are in use ranging from a simple T-arrangement to more elaborate geometries, such as the Berger ball mixer \[37\]. The goal is to achieve highly turbulent flow conditions in a small volume. The turbulent eddies thus generated can intersperse the two components down to the micrometer scale. However, the ultimate step in any mixing process relies on diffusion to achieve a homogeneous mixture at the molecular level. Given that the diffusion time, \( t = r^2 / D \), over which molecules have to diffuse, it takes a molecule with a diffusion constant \( D = 10^{-5} \text{ cm}^2/\text{s} \) about 1 ms to diffuse over a distance of 1 \( \mu \text{m} \). Thus, the mechanical mixing step has to intersperse the two components on a length scale of less than 1 \( \mu \text{m} \) to achieve sub-millisecond mixing times. The onset of turbulence is governed by the Reynolds number, \( Re \), defined as

\[
Re = \frac{\rho v d}{\eta},
\]

where \( \rho \) is the density (g/cm\(^3\)), \( v \) is the flow velocity (cm/s), \( d \) describes the characteristic dimensions of the channel (cm), and \( \eta \) is the viscosity of the fluid (e.g., 0.01 poise for water at 20°C). To maintain turbulent flow conditions in a cylindrical tube, \( Re \) has to exceed values of about 2000.

Turbulence is important not only for achieving efficient mixing, but also for maintaining favorable flow conditions during observation. In stopped-flow and quenched-flow experiments, turbulent flow insures efficient purging of the flow lines. In continuous-flow measurements, turbulent flow conditions in the observation channel lead to an approximate "plug flow" profile, which greatly simplifies data analysis compared to the parabolic profile obtained under laminar flow conditions. The time resolution of a rapid mixing experiment is ultimately limited by the stability of the mixture in the flow cell, which is limited by convective flow or diffusion of reagents in and out of the observation volume. For slow reactions with time constants longer than a few minutes, manual mixing experiments are generally more reliable. Stopped-flow mixing is usually coupled with real-time optical observation using absorbance (UV through IR), fluorescence emission or circular dichroism spectroscopy, but other biophysical techniques, including fluorescence lifetime measurements \[39,40\], NMR \[41,42\], and small-angle X-ray scattering (SAXS \[43\]), have also been implemented.

The interpretation of stopped-flow data requires a careful calibration of the instrumental dead time by measuring a pseudo-first-order reaction tuned to the time scale of interest (i.e., a single-exponential process with a rate-constant approaching the expected dead time) and an optical signal matching the application.
Common test reactions for absorbance measurements include the reduction of 2,6-dichlorophenolindophenol (DCIP) or ferricyanide by ascorbic acid [44]. A convenient test reaction for tryptophan fluorescence measurements is the irreversible quenching of N-acetyltryptophanamide (NATA) by N-bromosuccinimide (NBS). For fluorescence studies in or near the visible range, one can follow the pH-dependent association of the Mg$^{2+}$ ion with 8-hydroxyquinoline, which results in a fluorescent chelate [45], or the binding of the hydrophobic dye 1-anilino-8-naphthalene-sulfonic acid (ANS) to bovine serum albumin (BSA), which is associated with a large increase in fluorescence yield [46].

As a practical example, Fig. 2 shows a series of DCIP absorbance measurements at several ascorbic acid concentrations used to estimate the dead time of our Biologic SFM-4 stopped-flow instrument equipped with a FC-08 microcuvette accessory (Molecular Kinetics, Indianapolis, IN). The reaction was started by mixing equal parts of DCIP (0.75 mM in water at neutral pH, where the dye is stable) with L-ascorbic acid at pH 2 at final concentrations ranging from 6 to 25 mM. Absorbance changes measured at 525 nm, near the isosbestic point between the acidic and basic forms of DCIP, are plotted relative to the absorbance of the reactant in water, measured in a separate control (Fig. 2, inset).

After the flow comes to a full stop, the absorbance decays exponentially with rate constants of about 290, 780, and 1400 s$^{-1}$ at ascorbate concentrations of 6, 14, and 25 mM, respectively, which is consistent with a pseudo-first-order reduction process with a second-order rate constant $k'' \approx 5.7 \times 10^5$ s$^{-1}$ M$^{-1}$. At shorter times, the exponential fits intersect at an absorbance change $\Delta A = 0$, which corresponds to the level of the oxidized DCIP measured in the control. The delay between this intercept and the first data point that joins the fitted exponential provides an estimate of the instrumental dead time,

$$t_d = 0.55/C_{0.5} \text{ ms}.$$  

Alternatively, the dead time can be estimated from the signal level of the continuous-flow regime prior to closure of the stop valve, $I_{cf}$, using the following equation:

$$t_{cf} = -\ln([I_{cf} - I_{\infty}]/[I_0 - I_{\infty}])/k,$$  

where $I_{\infty}$ is the baseline at long times, $I_0$ is the signal of the reactant (in this case, DCIP mixed with an equal volume of water), and $k$ is the first-order rate constant obtained by exponential fitting. Note that $t_{cf}$ does not account for the finite stop time and other stop-related artifacts, and is thus always shorter than $t_d$, which explains why some manufacturers of stopped-flow instruments prefer to cite $t_{cf}$ over the more realistic operational dead time, $t_d$. In the present example, Eq. (2) will be applied to determine the dead time of the instrument.

Fig. 2. Estimation of the dead time of stopped-flow absorbance measurement on a Biologic SFM-4 instrument with FC-08 micro-cuvette accessory, using the reduction of DCIP by ascorbic acid as a test reaction. Equal parts of DCIP in water (pH 7) and sodium ascorbate (pH 2) at final concentrations of 6 (circles), 14 (squares), and 25 mM (triangles) were mixed at a total flow rate of 12 ml/s. The inset shows an expanded plot at early times with dashed lines indicating the dead time (~0.5 ms). Absorbance changes at 525 nm are plotted relative to the absorbance of a control (diamonds) measured by mixing DCIP with 10 mM HCl.
yields $t_d$ in the range of 0.25–0.4 ms, compared to $t_d \approx 0.55$ ms obtained by the extrapolation (Fig. 2).

4.2. Continuous-flow

In a continuous-flow experiment, the reaction is again triggered by turbulent mixing, but, in contrast to stopped-flow, the progress of the reaction is sampled under steady-state flow conditions as a function of the distance downstream from the mixer [47,48]. This avoids artifacts related to arresting the flow and makes it possible to use relatively insensitive detection methods. Thus, continuous-flow measurements can achieve shorter dead times compared to stopped-flow, but this comes at the expense of sample economy. Most earlier versions of this experiment involved point-by-point sampling of the reaction profile while maintaining constant flow at high rates (several ml/s for a conventional mixer). The prohibitive amounts of sample consumed limited the impact of continuous-flow techniques until advances in mixer design made it possible to achieve highly efficient mixing at lower flow rates [9,11,49], and an improved detection scheme allowed simultaneous recoding of a complete reaction profile in a few seconds [12]. These developments lowered both the dead time and sample consumption by at least an order of magnitude, and made routine measurements on precious samples with dead times as short as 50 $\mu$s possible.

In 1985, Regenfuss et al. [9] described a capillary jet mixer consisting of two coaxial glass capillaries with a platinum sphere placed at their junction. The reaction progress was monitored in a free-flowing jet, using conventional photography to measure fluorescence vs. distance from the mixer. Measurements of the binding kinetics of ANS to bovine serum albumin indicated that dead times less than 100 $\mu$s can be achieved with this mixer design. More recently, several laboratories reported continuous-flow resonance Raman and fluorescence studies of enzyme and protein folding reactions on the sub-millisecond time scale, using machined mixers with dead-times of about 100 $\mu$s [10,11,49]. More widespread use of these methods has been hampered by a number of technical and experimental difficulties. Continuous-flow experiments involving a free-flowing jet [9,11,49,50] are fraught with difficulties due to instability and scattering artifacts. The use of a conventional camera with high-speed monochrome film for fluorescence detection is inadequate due to the low sensitivity of the film in the UV region, limited dynamic range, and the non-linearity of the film response. Finally, prohibitive sample consumption makes continuous-flow experiments that record a kinetic trace one point at a time feasibly only for highly abundant proteins [10,51–53].

We were able to overcome many of these limitations by combining a highly efficient quartz capillary mixer, based on the design of Regenfuss et al. [9], with a flow cell and an improved detection system involving a digital camera system with a UV-sensitized CCD detector [12]. A diagram of the experimental arrangement is shown in Fig. 3. Two Hamilton syringes driven by an Update (Madison, WI) quenched-flow apparatus deliver the reagents to be studied at moderate pressure (<10 atm) into each of the two coaxial capillaries. The outer capillary consists of a thick-walled (6 mm o.d. and 2 mm i.d.) quartz tube, which is pulled to a fine tip (~200 $\mu$m i.d. at the end), using a glassblowing lathe or a simple gravity method. The inner capillary (360 $\mu$m o.d., 150–180 $\mu$m i.d., purchased from Polymicro Technologies, Phoenix, AZ) with a ~250 $\mu$m platinum sphere suspended at the end is positioned inside the tapered end of the outer capillary. The sphere is formed by melting the end of 50 $\mu$m diameter platinum wire. Thin glass rods fused to the inner wall of the outer capillary (tapering down to diameter of ~20 $\mu$m) prevent the sphere from plugging...
The reagents are forced through the narrow gap between the sphere and the outer wall where mixing occurs under highly turbulent flow conditions. The fully homogeneous mixture emerging from the mixer is injected into the 250 μm × 250 μm flow channel of a fused-silica observation cell (Hellma, Plainview, NY) joined to the outer capillary by means of a hemispherical ground-glass joint. Typical flow rates are 0.6–1.0 ml/s resulting in a linear flow velocities of 10–16 m/s through the 0.25 × 0.25 mm² channel of the observation cell.

The reaction progress in a continuous-flow mixing experiment is measured by recording the fluorescence profile vs. distance downstream from the mixer. A conventional light source consisting of an arc lamp (we currently use a 350 W Hg arc lamp in a lamp housing from Oriel, Stratford, CT), collimating optics and monochromator, is used for fluorescence excitation. Relatively uniform illumination of the flow channel over a length of 10–15 mm is achieved by means of a cylindrical lens. A complete fluorescence vs. distance profile is obtained by imaging the fluorescent light emitted at a 90° angle onto the CCD detector of a digital camera system (Micromax, Roper Scientific, Princeton NJ) containing a UV-coated Kodak CCD chip with an array of 1317 × 1035 pixels. The camera is equipped with a fused silica magnifying lens and a high-pass glass filter or a band-pass interference filter to suppress scattered incident light.

Fig. 4 illustrates a typical continuous-flow experiment, using the quenching of NATA by NBS as a test reaction. The upper panels show raw data obtained by averaging the intensity across the flow channel vs. the distance d downstream from the mixer. Panel (A) shows the intensity profile for the quenching reaction, Ie(d), at final NATA and NBS concentrations of 40 μM and 4 mM, respectively (mixing 1 part of 440 μM NATA in water with 10 parts of 4.4 mM NBS in water). Panel (B) shows the distribution of incident light intensity, Ic(d), measured by mixing the same NATA solution with water. Panel (C) shows the scattering background, Ib(d), measured by passing water through both capillaries. Panel (D) shows the corrected kinetic trace, fIrel(t), obtained according to

\[ fI_{rel} = \frac{I_e - I_b}{I_c - I_b}. \]

Distance was converted into time on the basis of the known flow rate (0.8 ml/s in this example), the cross-sectional area of the flow channel (0.0625 mm²), and the length of the channel being imaged (12.5 mm, or 9.5 μm per pixel). The signal measured at points below the entrance to the flow channel is well fitted by a single-exponential decay. The trace at fIrel = 1 in Fig. 4D represents a control for the mixing efficiency measured as follows. The 440 μM NATA stock solution used in the experiment above was diluted 11-fold with water and filled into both syringes. The continuous-flow trace recorded at the same flow rate was then background-corrected and normalized according to Eq. (3), using the Ie trace recorded previously in which the same NATA solution was diluted 11-fold in the capillary mixer.

Fig. 4. A typical continuous-flow mixing experiment. The upper panels show raw intensity profiles vs. distance from the mixing region. (A) Raw kinetic trace (NATA mixed with NBS). (B) Fluorescence control (NATA mixed with water). (C) Scattering background (water delivered from both syringes). (D) Corrected kinetic trace calculated according to Eq. (3) (lower trace), and a control designed to determine mixing efficiency (upper trace; see text).
Immediately after entering the flow channel, the ratio approaches unity, indicating that NATA is completely mixed with water. Alternatively, mixing efficiency could be assessed using a very fast reaction completed within the dead time, such as the quenching of tryptophan (or NATA) by sodium iodide.

To estimate the dead time of the continuous-flow experiment, we measured the pseudo-first-order NATA-NBS quenching reaction at a final NATA concentration of 40 μM and several NBS concentrations in the range 2–32 mM. Fig. 5A shows a semilogarithmic plot of the relative fluorescence, $f_{rel}$, calculated according to Eq. (2), along with exponential fits (solid lines). Since $f_{rel} = 1$ corresponds to the unquenched NATA signal expected at $t = 0$, the intercept of the fits with $f_{rel} = 1$ indicates the time point $t = 0$ where the mixing reaction begins. The delay between this point and the first data point that falls onto the exponential fit corresponds to the dead time of the experiment, $\Delta t_d$, which in this example is 45 ± 5 μs. In Fig. 5B, the rate constants obtained by exponential fitting are plotted as a function of NBS concentration. The slope of a linear fit (solid line) yields a second-order rate constant for the NBS-induced chemical quenching of NATA of $7.9 \times 10^5$ M$^{-1}$ s$^{-1}$, which is consistent with data obtained by stopped-flow measurements at lower NBS concentration [36]. This agreement, together with the linearity of the second-order rate plot (Fig. 5B), demonstrates the accuracy of the kinetic data. Continuous-flow measurements on our instrument and others built according to our design have provided much new insight into the kinetics of protein folding and enzyme reactions on the submillisecond time scale [54–63].

5. Detection methods

5.1. Tryptophan fluorescence

Table 1 lists common detection methods used in rapid mixing studies of the kinetics of folding and other conformational changes in proteins. The fluorescence emission properties of tryptophan and tyrosine side chains provide information on the local environment of these intrinsic chromophores. For example, a fully solvent-exposed tryptophan in the denatured state of a protein typically shows a broad emission spectrum with a maximum near 350 nm and quantum yield of ~0.14, similar to that of free tryptophan or its derivative, NATA. Burial of the tryptophan side chain in an apolar environment within the native state or a compact folding intermediate can result in a substantial blue-shift of the emission maximum (by as much as 25 nm) and enhanced fluorescence yield. These changes are a consequence of the decrease in local dielectric constant and shielding from quenchers, such as water and polar side chains. In other cases, close contact with a polar side-chain or backbone moiety gives rise to a decrease in fluorescence yield upon folding. Most polar amino acid side chains (as well as main chain carbonyl and the terminal amino and carboxyl groups) are known to quench tryptophan fluorescence, probably via excited-state electron or proton transfer [64,65]. Thus, the straightforward measurement of fluorescence intensity vs. folding or unfolding time can provide useful information on solvent accessibility and proximity to quenchers of an individual fluorescence probe. Complications due to the presence of multiple fluorophores can be avoided by using mutagenesis to replace any additional tryptophans (e.g. [66,67]). Because Trp is a relatively rare amino acid, proteins with only one tryptophan are not uncommon, or in the case of Trp-free proteins, a unique fluorophore can be introduced by using site-directed mutagenesis (e.g. [68,69]).

The use of tryptophan fluorescence to explore early stages of protein folding is illustrated in Fig. 6, which shows results on staphylococcal nuclease (SNase).
recently obtained in our laboratory [70]. A variant with a unique tryptophan fluorophore in the N-terminal \( \beta \)-barrel domain (Trp76 SNase) was obtained by replacing the single typtophan in wild-type SNase, Trp140, with His in combination with Trp substitution of Phe76. The fluorescence of Trp76 is strongly enhanced and blue-shifted under native conditions relative to the denatured state in the presence of urea (Fig. 6A), indicating that upon folding the indole ring of Trp76 moves from a solvent-exposed location to an apolar environment within the native structure. An intermediate state with a fluorescence emission spectrum similar to, but clearly distinct from, the native state was detected in equilibrium unfolding experiments (dashed line in Fig. 6A). In contrast to WT* SNase (P47G, P117G, and H124L background), which shows no changes in tryptophan fluorescence prior to the rate-limiting folding step (\( \tau_2 \approx 100 \text{ ms} \)), the F76W/W140H variant shows additional changes (enhancement) during an early folding phase with a time constant of about 80 \( \mu \text{s} \) (Fig. 6B). The fact that both variants exhibit the same number of kinetic phases with very similar rates confirms that the folding mechanism is not perturbed by the F76W/W140H mutations. However, the Trp at position 76 reports on the rapid formation of a hydrophobic cluster in the N-terminal \( \beta \)-sheet region while the wild-type Trp140 is silent during this early stage of folding.

5.2. ANS fluorescence

Valuable complementary information on the formation of hydrophobic clusters at early stages of folding can be obtained by using ANS as extrinsic fluorescence probe [71,72]. Fig. 7 illustrates this with recent results on the Trp76 variant of SNase introduced above. Panel (A) shows continuous-flow measurements of the enhancement in ANS fluorescence that accompanies early stages of SNase folding under native conditions (U \( \rightarrow \) N), and during formation of the A-state, the compact acid-denatured state of SNase (U \( \rightarrow \) A). Also shown is the kinetics of ANS binding to the pre-formed A-state. While the rate of ANS binding to the A-state in the presence of 1 M KCl shows the linear dependence on ANS concentration characteristic of a second-order binding process (panel (B)), the rates observed under refolding conditions (both to the native state and compact A-state) level off at \( \sim 120 \mu \text{M} \) ANS. The limiting ANS-independent rate at higher concentrations thus is due to an

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Table 1
Common detection methods used in kinetic studies of protein folding

Fig. 6. Folding mechanism of SNase probed by tryptophan fluorescence. (A) Fluorescence emission spectra of the Trp76 variant of SNase under native and denaturing conditions (solid) and a folding intermediate populated at equilibrium (dashed). The spectrum of the intermediate was determined by global analysis of the fluorescence spectra as a function of urea concentration (pH 5.2, 15 \( ^\circ \text{C} \)). (B) Time-course of folding (triggered by a pH-jump from 2 to 5.2) for wild-type SNase (Trp140) and a single-tryptophan variant (Trp76) measured by continuous-flow (<\(10^{-3}\) s) and stopped-flow (>\(10^{-3}\) s) fluorescence.
intramolecular conformational event that precedes ANS binding. The rate of this process closely matches that of the earliest phase detected by intrinsic fluorescence of Trp76 (Fig. 6B), confirming that both processes reflect a common early folding step. The results are consistent with the rapid accumulation of an ensemble of states containing a loosely packed hydrophobic core involving primarily the \( \beta \)-barrel domain. In contrast, the specific interactions in the \( \alpha \)-helical domain involving Trp140 are formed only during the final stages of folding.

### 5.3. FRET

Fluorescence resonant energy transfer (FRET) can potentially give more specific information on the changes in average distance between fluorescence donors and acceptors. For example, cytochrome \( c \) contains an intrinsic fluorescence donor–acceptor pair, Trp59, and the covalently attached heme group, which quenches tryptophan fluorescence via excited-state energy transfer \([73]\). We have made extensive use of this property to characterize the folding mechanism of cytochrome \( c \) \([28,74,75]\), including the initial collapse of the chain on the microsecond time scale \([54,55]\).

We recently combined ultrafast mixing experiments with FRET to monitor large-scale structure changes during early stages of folding of acyl-CoA binding protein (ACBP), a small (86 residue) four-helix bundle protein \([61]\). ACBP contains two tryptophan residues on adjacent turns of helix 3, which served as fluorescence donors, and an AEDANS fluorophore covalently attached to a C-terminal cysteine residue (introduced by mutation of Ile86 to Cys) was used as an acceptor (Fig. 8A). Earlier equilibrium and kinetic studies, using
intrinsic tryptophan fluorescence, showed a cooperative unfolding transition and single-exponential (un)folding kinetics consistent with an apparent two-state transition. Even when using continuous-flow mixing to measure intrinsic tryptophan fluorescence changes on the sub-millisecond time scale (Fig. 8B), we found only minor deviations from two-state folding behavior. However, when we monitored the fluorescence of the C-terminal AEDANS group while exciting the tryptophans, we observed a large increase in fluorescence during a fast kinetic phase with a time constant of 80 μs, followed by a decaying phase with a time constant ranging about 10–500 ms, depending on denaturant concentration (Fig. 8C). The large enhancement in FRET efficiency is attributed to a major decrease in the average distance between helix 3 and C-terminus of ACBP. The fact that the early changes are exponential in character suggests that the initial compaction of the polypeptide is limited by an energy barrier rather than chain diffusion. The subsequent decrease in AEDANS fluorescence during the final stages of folding is attributed to a sharp decrease in the intrinsic fluorescence yield of the two tryptophans due to intramolecular quenching. The specific side chain interactions responsible for quenching are established only in the close-packed native structure and are not present during the initial folding event. These observations indicate that the early (80 μs) folding phase marks the formation of a collapsed, but loosely packed and highly dynamic, ensemble of states with overall dimensions (in terms of fluorescence donor–acceptor distance) similar to that of the native state. Accumulation of partially structured states with some native-like features may facilitate the search for the native conformation. Because of their short lifetime and low stability, such intermediates can easily be missed by conventional kinetic techniques, whereas the continuous-flow FRET technique offers the temporal resolution and structural sensitivity to detect even marginally stable intermediates populated during early stages of folding.

5.4. Continuous-flow absorbance

Although fluorescence is inherently more sensitive, our capillary mixing instrument can also be adapted for continuous-flow absorbance measurements on the microsecond time scale. The fully transparent flow cell used for fluorescence measurements is replaced with a custom-made partially opaque absorbance flow cell of the same dimensions (0.25 mm pathlength). Relatively uniform illumination with minimal changes to the optical arrangement (Fig. 3) was achieved by using a 2 mm fluorescence cuvette filled with a highly turbid suspension (non-dairy creamer works well) as scattering cell. As in fluorescence measurements, a complete reaction profile can be recorded in a single 2–3 s continuous-flow run by imaging the flow channel onto the CCD chip. Using the DCIP-ascorbate reaction described above (cf. Fig. 2), we measured dead times as short as 40 μs at the highest flow rate tested (1.1 ml/s).

To validate the technique, we measured the changes in heme absorbance in the Soret region (~360–430 nm) associated with the folding of oxidized horse cytochrome c. The reaction was initiated by a rapid jump from pH 2, where the protein is fully unfolded, to pH 4.7, where folding occurs rapidly with minimal complications due to non-native histidine–heme ligands. A series of kinetic traces covering the time window from 40 μs to 1.2 ms were measured at different wavelengths spanning the Soret region (Fig. 9). A parallel series of stopped-flow experiments (data not shown) was performed under matching conditions to extend the data to longer times (2 ms–10 s). Global fitting of the family of kinetic traces to sums of exponential terms yielded three major kinetic phases with time constants of 65 μs, 500 μs, and 2 ms, respectively, consistent with accumulation of two intermediate species, I₁ and I₂, with absorbance properties distinct from both the initial (U) and final (N) states. In previous continuous-flow fluorescence measurements on cytochrome c [54,55], we also observed three kinetic phases with very similar time constants, indicating that a basic four-state mechanism is sufficient to describe the folding process of cytochrome c in the absence of complications due to non-native heme ligation and other slow events, such as cis–trans isomerization of peptide bonds.

Fig. 9. Initial stages of refolding of acid-denatured oxidized cytochrome c at pH 5 monitored by continuous-flow absorbance measurements at different wavelengths spanning the Soret heme absorbance band. The lines represent a global fit of a four-state folding mechanism to the family of kinetic traces.
5.5. Other detection methods for ultrafast folding studies

Continuous-flow measurements have been coupled with several other biophysical techniques, including resonance Raman spectroscopy [10], CD [52], EPR [76,77], and SAXS [53,78,79]. In their pioneering work, Takahashi et al. [10] used resonance Raman spectroscopy to monitor changes in heme coordination during folding of cytochrome c on the sub-millisecond time scale. Their findings confirmed and extended prior results on the involvement of heme ligation in folding of cytochrome c, based on stopped-flow absorbance and fluorescence measurements [80–82].

CD spectroscopy in the far-UV (peptide) region provides an overall measure of secondary structure content, and is thus an especially valuable technique for protein folding studies (e.g. [30,31]). However, the low inherent sensitivity of the technique, together with various flow artifacts, such as strain-induced birefringence, has limited the resolution of stopped-flow CD measurements to the 10 ms time range [83]. Akiyama et al. [52] were able to extend the time resolution down to the 400 l/s range by coupling an efficient turbulent mixer (T-design) with a commercial CD spectrometer. Their continuous-flow measurements of CD spectral changes in the far-UV region revealed the formation of (helical) secondary structure during the second and third (final) stages of cytochrome c folding. The same group recently designed a mixer/flow-cell assembly with a dead time as short as 160 l/s for continuous-flow SAXS measurements on a synchrotron [53]. They were thus able to follow the changes in size (radius of gyration, \( R_g \)) and shape (pair distribution derived from scattering profiles) associated with refolding of acid-denatured cytochrome c under conditions similar to those used in our absorbance measurements (Fig. 9). The intermediate formed within their dead time, which corresponds to the product of the 65 l/s process in Fig. 9, is substantially more compact (\( R_g \sim 20 \text{Å} \)) than the acid-denatured state (\( R_g = 24 \text{Å} \)). This finding clearly shows that cytochrome c undergoes a partial chain collapse during the initial folding phase, confirming earlier fluorescence data [54,84].

6. A quenched-flow method for H–D exchange labeling studies on the microsecond time scale

H–D exchange labeling experiments coupled with NMR detection [27–29,85,86] are important sources of structural information on protein folding intermediates. These experiments generally rely on commercial quenched-flow equipment to carry out two or three sequential mixing steps, which limits the time resolution to a few milliseconds or longer. To push the dead time of quenched-flow measurements into the microsecond time range, we made use of our highly efficient capillary mixers [12]. The device (illustrated in Fig. 10A) uses a quartz capillary mixer similar to that used for optical measurements, but without observation cell, to generate a homogeneous mixture of solutions A and B. The mixture emerges from the capillary as a fine (200 μm diameter) jet with a linear velocity of up to 40 m/s at the highest flow rate used (1.25 ml/s). A second mixing event can be achieved simply by injecting the jet into a test tube containing a third solution C; the extremely high flow velocity ensures very efficient mixing. To determine the dead time (i.e., the shortest delay between the two mixing events), we carried out a series of H–D exchange experiments on a pentapeptide (YGGFL). Rapid exchange of the backbone amide protons with solvent

Fig. 10. A capillary mixing device for quenched-flow measurements on the microsecond time scale. (A) A capillary mixer similar to that in Fig. 3, but without flow cell, is used to generate a fast free-flow jet. A second mixing event occurs upon impact of the jet with a quench buffer solution. (B) Quenched-flow NMR measurement of the H–D exchange reaction of backbone NH groups of a model peptide (YGLFG). Extrapolation of the exponential decay in normalized NH resonance intensity (solid curves) yields an estimated dead time of 60 μs.
deuterons was achieved by mixing a H$_2$O solution of the peptide with 5-fold excess of D$_2$O buffered at pH 9.7 (uncorrected pHe meter reading). The exchange reaction was quenched by injecting the mixture into ice-cooled acetate buffer at pHe 3. Under these quench conditions, the rate of exchange for some of the peptide NH groups (Gly3, Phe4, and Leu5) is sufficiently slow (10, 45, and 70 min, respectively) to determine their residual NH intensity by recording one-dimensional $^1$H NMR spectra. The measurement corresponds to an effective exchange time over a 1 ms time window, and the Gly2 NH continues to decay under quench conditions. Extrapolation of the fits up to the NH intensity expected at $t=0$ (measured in a separate control) indicates that the first measurement corresponds to an effective exchange time of 60 ± 10 μs, thus defining the time of death of the measurement. We previously used a similar setup to measure the protection of amide protons at early times of refolding of β-lactoglobulin [58].

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References
