Demonstration by burst-phase analysis of a robust folding intermediate in the FF domain

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The role of intermediates in the folding reaction of single-domain proteins is a controversial issue. It was previously shown by different methods that an on-pathway intermediate is populated in the presence of sodium sulphate during the folding of the FF domain from HYPA/FBP11. Here we demonstrate using analysis of the amplitudes of kinetic traces that this burst-phase folding intermediate is present at different salt concentration and at various pH, and is also found in roughly 30 site-directed mutants. The intermediate appears robust to changing conditions and thus fulfils an important criterion for a productive molecular species on the folding reaction pathway.

Keywords: FF domain/stopped-flow spectrometry/burst-phase analysis/kinetics/protein folding

Introduction

We wish to understand pathways and mechanisms of folding of small single-domain proteins to learn fundamental principles and to benchmark simulation (Fersht and Daggett, 2002). Some single-domain proteins fold by simple two-state kinetics (Jackson and Fersht, 1991; Jackson, 1998), whereas intermediates appear to be transiently populated during the folding reaction of other proteins (Wildegger and Kiefhaber, 1997; Shastry and Roder, 1998; Bai, 1999; Ferguson et al., 1999; Capaldi et al., 2001; Teilum et al., 2002; Mayor et al., 2003b; Sánchez and Kiefhaber, 2003; Travaglini-Allocatelli et al., 2003; Chi et al., 2007; Gianni et al., 2007). The FF domain from HYPA/FBP11 folds via an on-pathway intermediate (Jemth et al., 2004). The best evidence for the existence of a folding intermediate is provided by direct measurement of the kinetic phase associated with the transition between the intermediate and the denatured state (Fersht, 1999). Unfortunately, the faster phase often occurs on a time-scale not amenable to stopped-flow measurements, and even if other techniques such as temperature-jump and continuous-flow spectrometry are employed, the fast phase may be difficult to monitor. An indirect way of detecting intermediates is analysis of the amplitudes of the kinetic traces obtained by stopped-flow spectrometry. If the sum of the kinetic amplitudes do not match up with the equilibrium endpoints, a burst phase is said to be present. There are several examples in the literature of burst-phase intermediates in fast folding protein domains (Qi et al., 1998; Shastry and Roder, 1998; Ferguson et al., 1999; Capaldi et al., 2001; Teilum et al., 2002; Jemth et al., 2004; Magg and Schmid, 2004). These burst-phase species are sometimes referred to as collapsed states, rather than folding intermediates, as their degree of native structure or relevance to productive folding has been questioned (Sosnick et al., 1997; Qi et al., 1998; Krantz et al., 2002; Magg and Schmid, 2004; Crespo et al., 2006; Li et al., 2007). Whether a molecular species is an intermediate or an off-pathway collapsed state may be partly semantic, but there is the underlying issue if the intermediate/collapsed state is mechanistically ‘important’ for the folding reaction, i.e. if it is obligatory, in the strictest sense, for the protein to reach its native conformation. Is the intermediate always populated en route to the native state, or is it non-productive and redundant for correct folding? Whereas the question if an intermediate is always obligatory is next to impossible to answer, it is possible to show that the intermediate is present under widely varied conditions. We showed previously that a fast phase in the folding of the FF domain (detected by continuous-flow fluorimetry) satisfied all classical criteria for an on-pathway intermediate (Jemth et al., 2004) and this phase correlated with the burst phase observed by stopped-flow spectrometry. These data were recently corroborated by NMR relaxation dispersion experiments (Korzhev et al., 2007). In the present paper, we complement the previous studies by using burst-phase amplitude analysis to show that the intermediate is robust in the sense that it is present under varying conditions (salt, pH, and during refolding from the acid-denatured state, and on mutation) and thus fulfils one criterion for a mechanistically important intermediate.

Materials and methods

Buffers were prepared in 18 MΩ H2O by mixing the compounds of the respective buffer, to obtain the required pH value. For pH 3.3 and 3.7, sodium formate buffer was used, for pH 4.7 and 5.7 sodium acetate and for pH 7.0 sodium 3-[N-morpholino]propanesulphonate. Sodium chloride or sodium sulphate was added to obtain the appropriate conditions. Wild-type FF domain and mutants were expressed and purified as described previously (Jemth et al., 2005).

Dead-time calibration

The zero time-point of a kinetic trace as presented by the stopped-flow software is arbitrary and could differ from the true mixing time with several milliseconds. Calibrations of the zero time-point and dead-time (the time from the zero time to the first good data point) were done by mixing N-acetyltryptophanamide (NATA) with different concentrations of excess N-bromosuccinimide (NBS) (under pseudo-first order reaction conditions). The zero time-point and dead-time may change with urea concentration, and the
The NATA-NBS reaction usually used for the calibration will not allow all conditions to be monitored, because pre-mixing NBS and urea affects the reaction by an unknown mechanism. All amplitude analyses were based on kinetic traces that were adjusted to actual time points of mixing determined by NATA-NBS experiments at different [urea]. This correction was fairly large for both stopped-flow machines used. The experimental data were fitted to single exponential equations. All the initial data points were omitted from the traces until satisfactory fits to single exponentials were obtained, and a common point of intersection was achieved for a range of NBS concentrations (Fig. 1). The point of intersection is the effective zero time to use for calculating the amplitudes of the traces in the refolding experiments, and the 'dead-time' points should be omitted from those experiments. As the dead time and zero time points vary with the concentration of denaturant used, it is crucial to calibrate the stopped-flow spectrometers under identical conditions to those used in the folding experiments, with NATA taking the place of the protein and NBS in the dilution buffer (Fig. 1a and b). Note that the calculated first-order rate constant will be independent of the time-point for mixing, but the amplitude of the trace will vary; an early time zero (e.g. at ~2 ms) will yield an artificially low amplitude if not corrected for, and vice versa. The true mixing time-point should thus be subtracted from all the experimental ones (e.g. 0-(-2) ms, 0.1-(-2) ms etc.) (Fig. 2a).

**Stopped-flow kinetic experiments**

Unfolding of the FF domain was performed by 11-fold dilution of protein in buffer into urea solutions in a stopped-flow spectrometer [π*—180 spectrometer (Applied Photophysics)].

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**Fig. 1.** Determination of mixing time-point in a stopped-flow experiment. (a) KinetAsyst SF-61DX2 stopped-flow (Hi-Tech); 40 μM NATA, 7 M urea in 50 mM sodium acetate, pH 5.7, 100 mM NaCl was diluted 11-fold into solutions containing different concentrations of excess N-bromosuccinimide (NBS) (2.0–2.8 mM) in buffer. (b) π*—180 stopped-flow (Applied Photophysics); 15 μM NATA, 8.2 M urea in 50 mM sodium acetate, pH 5.7, 100 mM NaCl was diluted 11-fold into solutions containing different concentrations of excess NBS and 1.16 M urea in buffer. Single exponentials were fitted to the kinetic traces obtained and data points were omitted until the fitted lines extrapolated to a common point of intersection, which is the zero time-point. Here the point of intersection was found to be around 2.2 ms (a) and ~3.5 ms (b). (Inset) measured mixing time points plotted against [urea]; the fitted polynomial was used to correct the data in Figs. 3b–d, 4 and 6. (e) Interrupted unfolding double jump experiments were used to estimate the contribution of cis-trans Pro isomerisation to the total refolding amplitude. FF domain in buffer was mixed 1:1 with 8 M urea in buffer, and incubated for different delay times at the resulting 4 M urea. In the second mixing, the protein was refolded in 2 M urea. The measured rate constant at 2 M urea concentration was ~70 s⁻¹. The amplitudes of the refolding traces were plotted versus the delay time and a double exponential was fitted to data. The amplitude of the slower phase, 15% of the total, results from the cis-trans isomerisation (k_{obs} = 0.003 ± 0.002 s⁻¹).

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**Fig. 2.** (a) Kinetic trace for refolding of wild-type FF in 1.6 M urea, 50 mM sodium acetate, pH 5.7, 100 mM NaCl. The effect of correction for zero time-point (+3.4 ms, see Fig. 1b) is large for the fitted amplitude (2.1 and 1.4 for corrected and uncorrected, respectively), but negligible for the rate constant (126 and 127 s⁻¹). (b) Examples of chevron plots of wild-type FF, showing the magnitude of k_{obs} values measured in the present study (usually <200 s⁻¹). The amplitude analyses of these two experiments are shown in Fig. 4.
Photophysics, Leatherhead, UK), and a KinetAsyst SF-61DX2 from Hi-Tech (Salisbury, UK). Kinetic experiments monitored tryptophan fluorescence on excitation at 280 nm and detection of emitted light after passage through either a 330 ± 25 nm bandpass or a 360 nm cut-off filter. The kinetics of helix formation was also followed by far-UV circular dichroism (CD) at 222 nm on the pi-180 spectrometer. All data for a burst-phase analysis were collected on the same day, as the intensity of the lamp may vary from day-to-day and affect the measured fluorescence. A drift in the lamp intensity would affect the result: it is crucial that the endpoints of refolding and unfolding traces are similar in the region where they overlap. After correction of folding experiments with the FF domain, there is still a loss of amplitude when using the 330 nm bandpass emission filter. If the 360 nm cut-off filter is used the hyperfluorescent burst phase will be enhanced by the correction (Figs 3c and 4d; Jemth et al., 2004). Thus, for the FF domain, since different emission filters yield either hypo- or hyperfluorescent burst phases, the effect of an erroneous dead-time determination can never account for the burst phase: either it is present using one of the filters, or both.

**Temperature-jump fluorimetry**

Fluorescence kinetics were measured using a conventional pump-probe, laser-based temperature-jump instrument. Heating was achieved using the 1574 nm output from a BigSky CFR400 NdYAG laser incorporating an OPO device. Pulse width was 10 ns at FWHM. The transmission of water at this wavelength is ~70% for the 0.5 mm pathlength cell used, thus minimising temperature gradients across the sample as with the IR setup. Temperature jumps of 4°C were initiated at 1 Hz frequency and the average of 500 acquisitions was sufficient to give good signal to noise. Heating was complete within ~20 ns as judged from the fluorescence change of NATA in control measurements. The size of temperature jumps was determined prior to each experiment from the amplitude of the decrease in NATA fluorescence compared with a standard curve determined in separate equilibrium

![Fig. 3. Burst-phase analysis of refolding and unfolding traces measured by stopped-flow spectrofluorimetry and far-UV circular dichroism (CD). Filled circles, endpoints of refolding (corrected for Pro phase) and unfolding traces. The endpoints were fitted to the standard two-state solvent denaturation equation, and the fitted solid line is referred to as the denaturation curve. Diamonds, observed starting points for refolding [i.e. refolding amplitudes, corrected for both zero time-point and the Pro phase, subtracted from (a and b) or added to (c and d) the denaturation curves, yielding the observed starting points for unfolding]. Plus signs, endpoints from mixing NATA with urea in the stopped-flow apparatus. The solid lines in a, c, and d are the extrapolated denatured baselines obtained from the fit of the solvent denaturation equation.](image-url)
measurements on the temperature-jump setup or on a conventional fluorimeter. Degassed and filtered samples of FF domain at ~100 μM were slowly pumped through the 0.5 × 2 mm quartz flow cell at 5 μl min⁻¹ during data acquisition. Fluorescence excitation used the CW 284 nm output from a LEXEL SHG 95, a frequency doubled krypton gas laser source. The probing beam was focused within the heated volume and centred using pinhole targets. Fluorescence emission was detected through a 320–450 nm bandpass filter using a Hamamatsu R7400U photomultiplier placed in close contact with the sample cell. The photomultiplier used a custom voltage divider and pre-amp and the output was digitised by input to a Tektronix TDS 5032 oscilloscope. The initial fluorescence intensity was subtracted from the data automatically during each acquisition with custom-built electronics to allow maximum use of dynamic range during the D/A conversion. We estimate the overall bandwidth of the detection electronics to be around 20 MHz.

Kinetic data were fitted using the Kaleidagraph package (Synergy Software, Reading, USA).

**Results**

Intermediates in protein folding reactions are often ephemeral species that elude the experimentalist. Direct detection of the intermediate is preferable, but burst-phase analysis is an indirect method. Any intermediate detected with burst-phase analysis should therefore be established with other techniques, and transient aggregation or bimolecular events must also be ruled out.

**Bimolecular events**

Burst phases could arise from transient association of monomers (Oliveberg, 1998), and such bimolecular events can be tricky to disprove (Ferguson *et al*., 2004). However, by varying the final protein concentration it is possible to detect transient association/aggregation (Went *et al*., 2004). Observed rate constants as well as the relative magnitude of the burst phase was independent of protein concentration for the FF domain. Amplitude analyses at 0.36–9 mM final protein concentration yielded similar chevron plots and hyperfluorescent burst phases (with the 360 nm cut-off filter, not shown), suggesting that a protein–protein association is not responsible for the burst phase. Furthermore, a bimolecular event at 0.4 mM final protein concentration would have an observed rate constant well within the accessible range of the stopped-flow (<500 s⁻¹) unless the association rate constant is unrealistically high, but only a single exponential phase was observed in the experiments.

**Temperature-jump fluorimetry**

The fast phase previously identified by continuous-flow fluorimetry and associated with formation of the on-pathway intermediate (Jemth *et al*., 2004) was detected by temperature-jump fluorimetry on the double mutant L25A/D46N (Fig. 5). In the presence of 50 mM sodium acetate, 0.5 M Na₂SO₄, pH 5.7, this mutant, which is a model for the intermediate (Jemth *et al*., 2004), populated both the native state and the intermediate, as shown by the two phases recorded using the bandpass filter. The amplitude of the fast phase was very small, but careful control experiments with
NATA showed that it was not an artefact. The phase disappeared at low temperature where fully denatured protein was no longer populated. For wild-type FF, only the slow phase was visible due to the low population of the intermediate at equilibrium (not shown).

Dead-time calibration
Burst-phase analysis of protein folding is dependent on accurate dead-time and zero time-point calibrations at several urea concentrations, which were done as described in the Materials and methods section (Fig. 1a and b). The dead-time calibration curve shown as inset to Fig. 1b was used to correct the refolding amplitudes for the experiments shown in Figs 3b–d, 4 and 6.

Slow proline phases
There are two proline residues in the structured part of the FF domain. To estimate the fraction of refolding amplitude loss resulting from slow cis-trans Pro isomerisation, we performed double jump unfolding-refolding experiments (Brandts et al., 1975) (Fig. 1c). The Pro phase amplitude was determined as 15% of the total refolding amplitude and all refolding amplitudes were thus multiplied by 1.15.

Folding kinetics and burst-phase analysis
The observed rate constants for refolding and unfolding of wild-type FF were determined as a function of urea concentration using stopped-flow fluorimetry. At emission wavelengths of 310–350 nm the fluorescence decreased upon unfolding and at higher wavelengths it increased. Two emission filters could thus be used, one 330 ± 25 nm interference filter and one 360 nm cut-off filter. There are two Trp residues in the FF domain, Trp11 and Trp36. As shown by the fluorescence spectra of the Trp11→Phe and Trp36→Phe mutants, Trp36 is the main contributor to the decrease in fluorescence around 330 nm, and Trp11 for the increase at wavelengths >360 nm, upon unfolding (Fig. 7). Apart from a slow Pro isomerisation phase (Fig. 1c), kinetic traces followed first-order kinetics, and amplitudes and endpoints of the reactions were determined by fitting either single exponentials or single exponential plus a linear slope to data (Fig. 2a). Examples of chevron plots ($k_{obs}$ versus [urea]) showing the magnitude of the rate constants in the experiments are shown in Fig. 2b. After acquisition of kinetic traces, burst-phase analysis was performed as follows. The basic idea is that the amplitudes of refolding and unfolding traces must add up to the total change at equilibrium for the reaction, as monitored by, in this case, fluorescence. The endpoints from refolding and unfolding kinetic traces were plotted versus [urea] and fitted to the standard two-state denaturation curve. Then, the unfolding and refolding amplitudes were either added to or subtracted from this curve, depending on the fluorescence emission filter used. Figure 3 shows amplitude analyses in 50 mM sodium acetate, 100 mM NaCl (283 K) on a (a) KinetAsyst SF-61DX2 (Hi-Tech) and (b–d) π=180 stopped-flow instrument (Applied photophysics). In all other amplitude analyses, the π=180 apparatus was used in the experiments.

Folding and unfolding kinetics of wild-type FF domain was also monitored by far-UV CD, to measure the extent of formation of helices. The amplitude analysis from this experiment is shown in Fig. 3d. The advantage with CD over fluorescence is that the assumption of linear baselines is more valid and that the property measured, helix formation, is better structurally defined than that from fluorescence.
The CD amplitude loss was close to the experimental error. But, data may have been slightly overcorrected for the zero time-point; the comparison between different stopped-flow machines (Fig. 3) and the lack of amplitude loss at high urea in the fluorescence experiments (even at pH 3.3) where the observed rate constants are high (Figs 2b and 4a) suggest that the correction using the standard curve in Fig. 1b (inset) may be too large. Indeed, NMR relaxation dispersion data suggest that two of the helices are partially formed in the intermediate (Korzhnev et al., 2007).

The dependence of the observed folding/unfolding rate constants on [urea] was measured at different salt concentration at constant pH (5.7) and temperature (283 K). Amplitude analyses for data collected at (a) 10 mM sodium acetate, and (b) 50 mM sodium acetate, and 400 mM sodium sulfate are shown in Fig. 6. Data were also collected from pH 3.3 to 7.0 at a constant ionic strength (0.147 M) and temperature (283 K) (Fig. 4).

Thirty-three of the mutants from a Φ value analysis (Jemth et al., 2005) were subjected to amplitude analysis. Out of these only A39G displayed perfect apparent two-state behaviour, i.e. no missing or extra amplitude. Some mutants displayed a slightly larger and some a smaller burst phase. Due to the errors involved in the analysis it was difficult to quantify the change in burst phase upon mutation. Qualitatively it was clear that the observed burst phase was modulated by mutation but still present in most cases. Note that the different mutations can affect the fluorescence yield of the denatured, intermediate and native states, respectively, and although A39G does not display a burst phase it could still fold via the intermediate state.

**Discussion**

We show here that the intermediate in the folding reaction of the FF domain is present at low and high salt, at different pH values and upon refolding from the acid-denatured state, as judged by amplitude analysis. It is also present in at least 32 out of 33 mutants of the FF domain (produced for a Φ-value analysis; Jemth et al., 2005). Thus, independently of the conditions an intermediate state is populated upon refolding of the FF domain, and this would argue that the overall folding mechanism is robust to change in conditions. Importantly, the FF burst-phase species is not generated by certain solvent conditions such as high sodium sulphate concentration, as was proposed for the S6 protein (Otzen and Oliveberg, 1999).

Burst-phase analysis is a classical but indirect way of detecting intermediates in protein folding and it must therefore be exercised with caution. The identification of an intermediate should not be solely dependent on a burst-phase analysis. The use of amplitude analysis as a probe for the intermediate in the FF domain is justified by the following experiments: (i) the use of different filters giving hypo- and hyperfluorescent burst phases, respectively, unequivocally demonstrate the presence of a burst-phase species in the folding of the FF domain; (ii) the detection of a fast phase with both continuous flow (Jemth et al., 2004), and temperature-jump fluorimetry (Fig. 5) and (iii) evidence from NMR (Korzhnev et al., 2007). In addition, molecular dynamics simulation of unfolding demonstrated the presence of an on-pathway intermediate (Jemth et al., 2004).

Apart from those caveats already mentioned, there is one more issue that must be carefully considered in an amplitude analysis. It is usually assumed that the fluorescence signal of NaCl or Na₂SO₄ (Fig. 8). There was an increasing loss of amplitude with increasing salt, reflecting the stabilisation of the burst-phase intermediate. Note that the stabilising effect of Na₂SO₄ on the burst phase (i.e. the decrease in refolding amplitude) is due mainly to electrostatic screening and not its ‘Hofmeister properties’ (Baldwin, 1996) as NaCl was essentially as effective. A similar experiment was done with wild-type protein, using Na₂SO₄ as salt, but due to the high observed rate constants in water, 3.2 M urea had to be included and the jump was thus from the urea-denatured state.

**Refolding from the acid-denatured state**

The FF domain can be denatured by lowering the pH, which allowed refolding from the acid-denatured state. The protein is also stabilised by salt, so the refolding rate constants can be measured as a function of salt concentration. Refolding of the mutant E15A/V30A involved only buffer and salt. The mutant is highly destabilised, and in 50 mM sodium acetate, pH 5.7, 100 mM NaCl and 283 K only some 70% of the molecules populate the native conformation. At lower salt concentration and pH, the mutant is unfolded and can be refolded by mixing with sodium acetate buffer and salt. The protein was jumped from water, acidified to pH 4, into sodium acetate buffer containing different concentrations of NaCl or Na₂SO₄ (Fig. 8).

**Fig. 7.** Fluorescence spectra of (a) wild-type FF and (b) Trp11Phe and (c) Trp36Phe mutants, showing the influence of the respective tryptophan residue on the fluorescence. Solid line is the spectrum of native protein and dashed line is the urea denatured protein.
the denatured state is constant, or depends strictly linearly on the concentration of denaturant so its value can be extrapolated into the region where the protein is folded (linear baselines; cf. Figs 3, 4, 6 and 8; Johnson and Fersht, 1995). The slope of NATA versus [urea] is a straight line (Fig. 3b), but that of the starting point of folding, the denatured state under folding conditions, may not be a straight line. The slope of the denatured baseline can be estimated by adding mutations that will cause the protein to unfold under native conditions and use this construct instead of NATA to measure the slope at different [urea]. However, the species created in this fashion may very well be regarded as a model of the folding intermediate as found for the FF domain (Jemth et al., 2004), engrailed homeodomain (Mayor et al., 2003a; Religa et al., 2005, 2007) and Im7 (Spence et al., 2004). Hence, the slope of the baseline for the unfolded species at low denaturant can never be accurately defined, and this is the crux of the matter. All that can be said is that the denatured state of the protein is not a random coil at low [urea]. Whether this species is an ‘obligate’ on-pathway intermediate or a non-specific collapsed state, due to polypeptide chain contractions upon transfer to a different buffer with a change in spectral properties on release of denaturant (Sosnick et al., 1997; Qi et al., 1998), must be assessed by other means. First, the temperature-jump data presented here suggest that a change in solvent conditions is not the cause of the burst-phase species. Secondly, by using the destabilised mutant E15A/V30A, refolding rate constants and amplitudes could be measured in the absence of urea (Fig. 8). The denatured state obtained in this way should be distinct from the urea-denatured one, but still a loss of amplitude was detected as judged both by the dependence of the amplitudes on salt (Fig. 8b), and the observed starting points of refolding compared with the expected one (Fig. 8c). The burst phase in refolding experiments thus seems to be dependent on neither urea as denaturant nor sulphate to stabilise its structure (Cobos and Radford, 2006).

In conclusion, a burst-phase species is present under all conditions investigated, corroborating previous conclusions (Jemth et al., 2004; Korzhnev et al., 2007) of a productive intermediate in the folding pathway of the FF domain.

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