FOR THE RECORD

Reassessing the folding of the KIX domain: Evidence for a two-state mechanism

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Abstract: The debate about the presence and role of intermediates in the folding of proteins has been a critical issue, especially for fast folders. One of the classical methodologies to identify such metastable species is the "burst-phase analysis," whereby the observed signal amplitude from stopped-flow traces is determined as a function of denaturant concentration. However, a complication may arise when folding is sufficiently fast to jeopardize the reliability of the stopped-flow technique. In this study, we reassessed the folding of the KIX domain from cAMP Response Element-Binding (CREB)-binding protein, which has been proposed to involve the formation of an intermediate that accumulates in the dead time of the stopped flow. By using an in-house-built capillary continuous flow with a 50- μ s dead time, we demonstrate that this intermediate is not present; the problem arose because of the instrumental limitation of the standard stopped flow to assess very fast refolding rate constants (e.g., $\geq 500 \text{ s}^{-1}$).

Keywords: protein folding; kinetics; intermediates; continuous flow

Introduction

One of the most important tasks in studying the folding of a protein is to identify the simplest kinetic scheme describing this complex reaction. Some proteins, such as Chymotrypsin inhibitor 2 (CI2) or the Lysin Motif (LysM) domain, tend to fold in a highly cooperative manner, such that only the fully denatured and native states are stable enough to be populated.^{1–4} Some other proteins fold extremely fast and the characterization of their folding pathways may demand the

use of ultrarapid techniques, such as the laser T-jump.^{5,6} When the stability of individual elements of secondary structure or local nuclei is more marked,⁷ proteins form intermediates, whose structure must be elucidated to describe their role in folding.⁸

A classical signature of folding intermediates is represented by multiphasic kinetics. In some cases, however, intermediates are more elusive and their role must be inferred indirectly.⁹ A classical test to pin down a folding intermediate is based on the dependence on denaturant of the folding and unfolding rate constants and on burst-phase analysis of stopped-flow experiments.¹⁰ By using the latter methodology, the denaturant dependence of the apparent fluorescence amplitude coupled to refolding yields a quantitative assessment for an intermediate. In the absence of ultrafast events (lost in the dead time of the stopped flow), the fluorescence amplitude of refolding experiments is expected to depend linearly on denaturant

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concentration;¹¹ sudden increase or decrease of the fluorescence burst-phase is then interpreted as an experimental evidence for a folding intermediate. In these cases, because the intermediate is obviously populated, the observed folding rate constant is slowed down by a factor equal to $1 + K_{D-I}$ and the dependence of the logarithm of the folding rate constant on denaturant concentration is not linear, the chevron plot displaying the so-called rollover effect.¹² Given that small globular proteins often fold very rapidly,³ folding rates may easily approach the limits of standard stopped-flow instruments; for example, with a folding rate constant of 400 s^{-1} , more than 50% of the total signal change may be lost in a 2-ms dead-time instrument. In these cases, experience suggests that a burst-phase in the refolding amplitudes and a rollover effect in the chevron plot might be vitiated by the limitation of the machine;¹³ and therefore, additional experiments may be necessary to validate the presence of intermediates.

The KIX domain is part of a large multidomain protein, the coactivator CREB-binding protein (CBP), which has recently attracted considerable attention. In fact, KIX is capable of recognizing several intrinsically unstructured proteins, such as the phosphorylated kinase inducible domain (pKID) domain from CREB, the transactivation domain of p53, and the activation domain of the hematopoietic transcription factor c-Myb, which all fold on binding.^{14–17} The folding and unfolding reactions of KIX have been studied both by equilibrium and by kinetic experiments.^{18,19} The analysis of the amplitude and rate constants obtained by refolding experiments suggested the KIX domain to fold via a low-energy intermediate, which accumulates in the dead time of the stopped flow. Because of the low stability of such an intermediate, it is unclear whether this species is the same as that detectable by equilibrium experiments. Since at low urea concentrations, the observed folding rate constants of KIX approach the experimental limitation of the stopped-flow methodology (ca. 400 s^{-1} at $2M$ urea), the rollover effect/burst-phase analysis of this protein demand additional validation. In this work, we have reassessed the folding kinetics of KIX using an in-house-built continuous-flow apparatus with a dead time of about $50 \mu\text{s}$. Data reveal that the rollover effect is absent when refolding was monitored by continuous flow, casting some doubts on the existence of the low-energy burst-phase intermediate, which may result from an intrinsic limitation of the stopped-flow instrument employed.

Results and Discussion

The dependence of the folding and unfolding rate constants of KIX versus denaturant concentration is reported in Figure 1, compared with data previously published.¹⁸ While above $2M$ urea the data obtained in this laboratory agree with those previously

Figure 1. Chevron plot of KIX obtained by stopped flow. Semilogarithmic plot of the observed rate constants for folding and unfolding of KIX versus [urea] at pH 7.5 at 25°C in 10 mM NaPi and 150 mM NaCl . The chevron plots of KIX protein with His-tag (gray circles) and without His-tag (black open circles) obtained by stopped flow are superposed with that previously published by Horng et al.¹⁸ (black filled circles).

published, a clear difference was observed at low denaturant concentrations (i.e. at $[\text{urea}] < 1.5M$). Prompted by such a discrepancy, we resorted to complement the standard stopped-flow data with experiments carried out with an ultrarapid in-house-built continuous-flow instrument. Because of the high amount of protein required for continuous-flow experiments, in analogy to previous work carried out on the protein Im7,²⁰ we carried out these experiments with the His-tagged version of KIX. Importantly, the presence of the tag has no significant effect on both the stability and folding kinetics of KIX; Figures 1 and 2 show a comparison between the His-tagged and non-His-tagged proteins, the latter being the same construct used in Ref. 18. A typical refolding trace of KIX measured at 25°C in the presence of 10 mM phosphate and 150 mM NaCl pH 7.5 and $0.6M$ urea is reported in Figure 2(A). The complete chevron plot from stopped- and continuous-flow experiments, reported in Figure 2(B), shows that the rollover previously seen at low urea concentrations is not visible when the refolding rate constant is measured with an instrument characterized by a much smaller dead time. Thus, it is likely that the rollover effect reflects the intrinsic limitation of the stopped-flow instrument. Furthermore, an underestimate of the refolding rate constant would correspond to an artifactual burst phase in the amplitude analysis.

In an effort to investigate further the presence/absence of a rollover effect in the folding of KIX, we carried out kinetic experiments also in the presence of a stabilizing salt, namely $0.4M$ sodium sulfate

Figure 2. Folding kinetics of KIX from μ s to ms by stopped flow and continuous flow. (A) Refolding traces of KIX measured by the continuous-flow apparatus. Both traces were recorded in 10 mM NaPi pH 7.5 and 150 mM NaCl and 25°C, in the absence (gray, 0.6M urea) and in the presence (black, 0.54M urea) of 0.4M Na_2SO_4 . Lines are the best fit to a single exponential decay. (B) Semilogarithmic plot of the observed rate constants for folding and unfolding of KIX versus [urea] at pH 7.5 in 10 mM NaPi and 150 mM NaCl, obtained in the presence (squares) and in the absence (circles) of 0.4M Na_2SO_4 . The data obtained after His-tag cleavage in the presence and absence of 0.4M Na_2SO_4 are reported as gray triangles. Lines are the best fit to a two-state chevron plot. Folding rate constants higher than 500 s^{-1} as obtained by stopped flow (Fig. 1) have been omitted from this analysis. The folding rate constants in the absence of denaturant extrapolated to zero urea are 1900 s^{-1} in the absence and 4600 s^{-1} in the presence of 0.4M Na_2SO_4 .

[Fig. 2(B)]. A typical refolding trace of KIX recorded in presence of 0.4 M sodium sulfate is reported in Figure 2(A). As expected, the salt shifts the denaturation midpoint to higher urea concentrations (from 4M in the absence to 5M in the presence of 0.4 M sodium sulfate) and increases the folding rate constant by a factor of 3. In analogy to what observed in the absence of sodium sulfate, when the data are recorded using the stopped-flow and the continuous-flow instruments,

time course is always monoexponential, and there is no indication of refolding rollover effect. Thus, the chevron plot of KIX appears to satisfy a simple two-state folding behavior without accumulation of intermediates.

Concluding Remarks

The KIX domain of CBP has been reported to fold through an early intermediate based on two classical observations: significant burst-phase amplitudes and rollover in the folding limb of the chevron plot.¹⁸ In carrying out a detailed chevron plot analysis by combining stopped-flow and continuous-flow experiments, both in the presence and in the absence of the stabilizing agent sodium sulfate 0.4M, we did not detect the previously observed signatures for such an intermediate. Even if there are several examples in the literature of burst-phase intermediates in fast folding protein domains,^{10,21–23} this effect must be interpreted with some caution. In fact in some cases, a rollover may arise from protein aggregation,²⁴ shift of the transition state along the reaction coordinate²⁵ or inadequate pH control on buffer dilution. In this study, we exemplify that the saturation of the apparent rate constants at low urea may induce apparent rollover effect. In the latter case, ultrafast mixing experiments, classically introduced to monitor the accumulation of short-lived intermediates,^{26–30} allow to infer unambiguously the existence of an intermediate, if any. By integrating stopped-flow and continuous-flow mixing experiments, in fact, we obtained for the folding kinetics of KIX a completely linear chevron plot typically diagnostic of a simple two-state model.

Material and Methods

Expression and purification of KIX domain

KIX from *Mus musculus* CBP (residues 587–672) cloned into pRSET vector (Invitrogen) and resulting in a clone expressing His-tagged KIX, was generously provided by Prof. Per Jemth (Uppsala University, Sweden). The plasmid was used to transform *Escherichia coli* BL-21 (DE3), and protein expression was initiated by inducing 1-L bacterial cultures with 1-mM isopropyl- β -D-thiogalactopyranoside at an A600 of ~ 0.5 . The cultures were grown overnight at 25°C, and the bacteria were harvested by centrifugation. KIX was purified by using a nickel(II)-charged chelating Sepharose FF (Amersham Biosciences) column equilibrated with 40 mM Tris-HCl and 400 mM NaCl, pH 8.5. After washing with the same buffer, the His-tagged Kix was eluted with 250 mM imidazole. The sample was then diluted fourfold in 40 mM Tris and pH 8.5, and all minor impurities were removed by the purification step on a Q-sepharose column equilibrated with 40 mM Tris and pH 8.5. The flow-through containing the protein was collected and concentrated. When necessary, His-tag cleavage was carried out

with thrombin (Sigma Aldrich) using 2.0 U for mg of protein at 4°C for 15 h. KIX was further purified from thrombin using para-aminobenzoic acid (PABA) column (GE Healthcare). The purity of the protein was confirmed by sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE).

Stopped-flow measurements

Single mixing kinetic folding experiments were carried out on an SX-18 stopped-flow instrument (Applied Photophysics, Leatherhead, UK); the excitation wavelength was 280 nm, and the fluorescence emission was measured using a 320-nm cutoff glass filter. In all experiments, refolding and unfolding were initiated by a 11-fold dilution of the denatured or the native protein with the appropriate buffer. The buffer used was 10 mM sodium phosphate, 150 mM NaCl, and pH 7.5. Final protein concentration was typically 1 μ M. The observed kinetics were always independent of protein concentration (from 0.5 to 5 μ M after mixing), as expected from monomolecular reactions without effects due to transient aggregation.²⁴

Continuous-flow experiment

Continuous-flow measurements were performed using a homemade instrument of design and methodology similar to that published by Shastry and Roder,³⁰ as described in Refs. 31 and 32.

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