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Unfolding kinetics of dimeric creatine kinase measured by stopped-flow small angle X-ray scattering

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Abstract

The unfolding kinetics of creatine kinase (CK) in various concentrations of urea or guanidine hydrochloride (GuHCl) was investigated by small angle X-ray scattering (SAXS) using synchrotron radiation, and compared with the results obtained by stopped-flow circular dichroism and stopped-flow fluorescence. Using the three methods, the unfolding kinetics of CK fits well to a single exponential function with similar apparent rate constants, and the amplitude of the monophasic kinetics covers the entire range of the equilibrium values. The results suggest that the unfolding time-course measured by integrated SAXS intensity corresponds to the intramolecular loss of globular structure. The refolding kinetics of 8 M urea-denatured CK was monitored in a stopped-flow apparatus by following the spectroscopic changes, and the final state of folding was investigated by SAXS. A substantial part of the ellipticity is recovered within a burst phase, indicating that the secondary structure forms at an early stage in refolding. The R_g value of the final folded state was 33.6 Å when the folding buffer contained 20% glycerol, which is characteristic of native-like compactness and globularity.

Keywords: Protein folding; Monophasic kinetics; Guanidine hydrochloride; Urea; SAXS

1. Introduction

Small angle X-ray scattering (SAXS) is a powerful method to measure the changes in the overall dimensions of a protein molecule [1–3]. It is the best available method for probing changes in compactness during protein folding and unfolding [4,5]. Recent advances in the combined use of the stopped-flow SAXS technique with synchrotron radiation have allowed researchers to apply SAXS to time-resolved measurements of intramolecular protein folding [5].

In this study we investigate the unfolding kinetics of a dimeric protein, creatine kinase (CK). Cytoplasmic CK (EC 2.7.3.2) from rabbit muscle is composed of two identical 43-kDa polypeptide chains of known sequence [6]. It has been demonstrated that the integrated SAXS intensity is particularly sensitive to the globule to coil transition of a protein, while the zero angle intensity, $I(0)$, is more sensitive to intermolecular dissociation/association [5]. In addition, SAXS can describe the shape and the globularity of intermediate conformational states in terms of the size of a molecule estimated from the radius of gyration, R_g. A stopped-flow apparatus adapted for use with the synchrotron SAXS technique [7] was used to measure the time-resolved unfolding curves of CK monitored by the integrated SAXS intensity, and the results were compared with those obtained from stopped-flow fluorescence and stopped-flow CD. The observed results demonstrate the power of the stopped-flow synchrotron SAXS technique for multimeric protein folding studies.

2. Materials and methods

2.1. Materials

CK was prepared and further purified as described previously [8]. The absorbance (1%, 1 cm) value of 8.8 at 280 nm

Abbreviations: CK, creatine kinase; GuHCl, guanidine hydrochloride; β-ME, β-mercaptoethanol; SAXS, small angle X-ray scattering.

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[9] was used for protein concentration measurements. The final preparations used typically had a specific activity of 130–160 μmol min^{-1} mg^{-1} [10] and showed only one band by polyacrylamide gel electrophoresis. Ultrapure urea and guanidine hydrochloride (GuHCl) were obtained from ICN Biochemicals. All other reagents were local products of analytical grade. Twice-deionized water was used throughout.

2.2. Synchrotron SAXS measurements

X-ray solution scattering measurements were performed on the beam line 15A small angle installation (BL-15A) of the Photon Factory of the High Energy Accelerator Research Organization (KEK), Tsukuba, Japan, where a stable beam of photons with a wavelength of 1.5 Å was provided by a bent-crystal horizontally focusing monochromator and a vertically focusing mirror [11]. The detector was a linear position-sensitive proportional counter capable of collecting 256 scattering intensity points along the angular region. Camera length, i.e. sample-to-detector distance, was 1.6 m. For the time-resolved measurements, a total of 94 kinetic points (per frame) were collected, with a time constant (or integration time) chosen between 0.01 and 1 s, depending on the rate of the observed process. The background data for the buffer solution at different concentrations of urea or GuHCl were collected before or after data collection for the protein solutions. The correction of the SAXS data for the difference in the electron density (i.e. contrast) between the protein and the solvent molecules as well as for X-ray absorption by the solution was made according to the standard data provided by Semisotnov (unpublished data). The R_g value was estimated by the Guinier approximation, $I(h) = I(0)\exp(-R_g^2h^2/3)$, where h is the scattering vector given by $h = (4\pi \sin \theta)/\lambda$ (2θ is the scattering angle and λ is the wavelength of X-ray) and $I(0)$ is the scattering intensity at zero angle. It has been demonstrated that the $I(0)$ value is sensitive to intermolecular association and dissociation of protein complexes [5]. The globularity of the protein molecule was examined using a Kratky plot, i.e. $I(h)h^2$ versus h.

2.3. Kinetic measurement of unfolding using a stopped-flow apparatus

All the experiments were performed in 10 mM Tris–HCl buffer, pH 8.3, containing 5 mM β-mercaptoethanol (β-ME), at 25 °C. The unfolding reaction, induced by concentration jumps of GuHCl or urea, was followed by the SAXS intensity, far-ultra violet CD or intrinsic fluorescence using a stopped-flow apparatus. The stopped-flow attachment for SAXS measurements [7] used a 1:6 volume ratio of the two solutions, driven pneumatically with nitrogen gas at a pressure of 7–8 kg cm^{-2}. The dead time of the experiment was less than 3 s. Fluorescence experiments were carried out with a Unisoku stopped-flow spectrophotometer equipped with a mixing device using a 1:6 volume ratio of the two solutions, driven pneumatically with nitrogen gas at a pressure of 4–5 kg cm^{-2}. The emission intensity was monitored at wavelengths above 330 nm using a 330 nm cut-off filter, after excitation at 294 nm with a slit width of 1 mm. The pathlength of the cell for fluorescence was 2 mm and the dead time was less than 10 ms. CD stopped-flow experiments were performed using the same apparatus as in the SAXS measurements, with a cell of 1 mm light pathlength and a reduced dead time of less than 10 ms.

The kinetic data were analyzed by non-linear least-squares fitting to the equation:

$$A(t) = \sum_i A_i \exp(-k_i t) + A_\infty$$

where $A(t)$ is the value of integrated SAXS intensity, fluorescence intensity or CD signal at a given time t, A_i is the amplitude corresponding to each individual phase (i), k_i is the associated rate constant, and A_∞ is the amplitude at infinite time.

3. Results

3.1. Time-resolved protein unfolding monitored by the integrated SAXS intensity

As the integrated SAXS intensity is sensitive to the transition between the random coil state and the globular state [5], the time course of protein unfolding can be monitored by direct measurement of the difference in the integrated SAXS intensity. Fig. 1 shows the time-resolved change of the integrated SAXS intensity during unfolding of CK initiated by a concentration jump to 1.5 M GuHCl. It can be seen that the time-resolved decrease in the integrated SAXS intensity reflects the transition of the protein from the globular to the non-globular state. The unfolding kinetics at various concentrations of urea or GuHCl fit well to a single exponential equation and the rate constants obtained are shown in Table 1. Kratky and Guinier plots for CK at the beginning and end of the protein unfolding reaction are shown in Fig. 2. The four kinetic frames at around 4 and 46 s were averaged in order to obtain a satisfactory signal/noise ratio and to obtain the R_g and the $I(0)$ values at the beginning and end of the reaction. The time-resolved changes in R_g and $I(0)$ values during the unfolding reaction were calculated from the same data set (Fig. 3), and they also fit well to a single exponential equation. The rate constants obtained were 0.21 ± 0.01 and 0.076 ± 0.005 s^{-1} calculated from the $I(0)$ and R_g values, respectively. Compared with the value derived from the integrated SAXS intensity, the rate constant obtained from $I(0)$ is identical, while the value from R_g is around 2.75-fold lower. This small difference is probably a reflection of the fact that CK unfolding is a complex process [12–14]. The different structural parameters derived from the SAXS data may vary in their sensitivity to the different intermediates populated during unfolding. From detailed analysis of the equilibrium data, the denatured state ensemble under the conditions of the

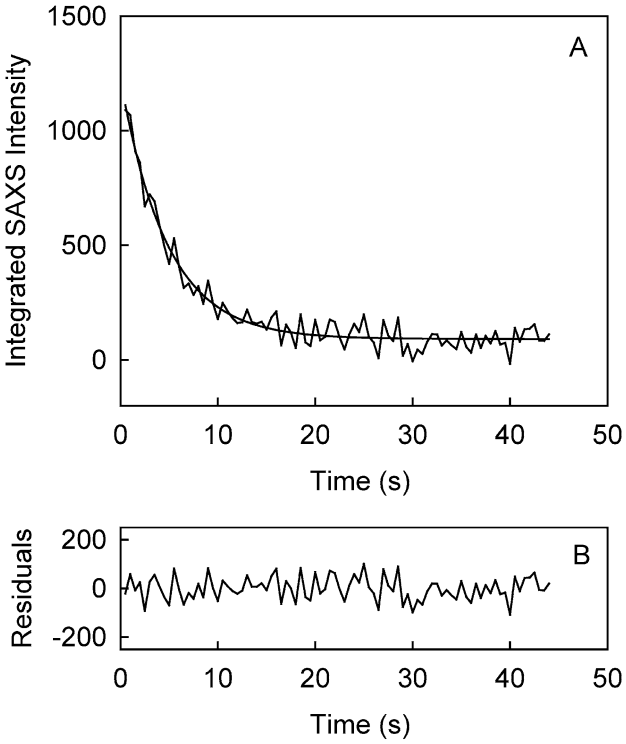

Fig. 1. Kinetics of CK unfolding as monitored by the integrated SAXS intensity at a final GuHCl concentration of 1.5 M. The protein concentration was 3 mg ml^{-1}. The number of accumulations was 32. The continuous line represents the theoretical curve obtained by fitting to a single exponential function, giving a rate constant of $k = 0.21 \pm 0.01$ s^{-1}. The lower panel shows the residual between the experimental points and the theoretical curve.

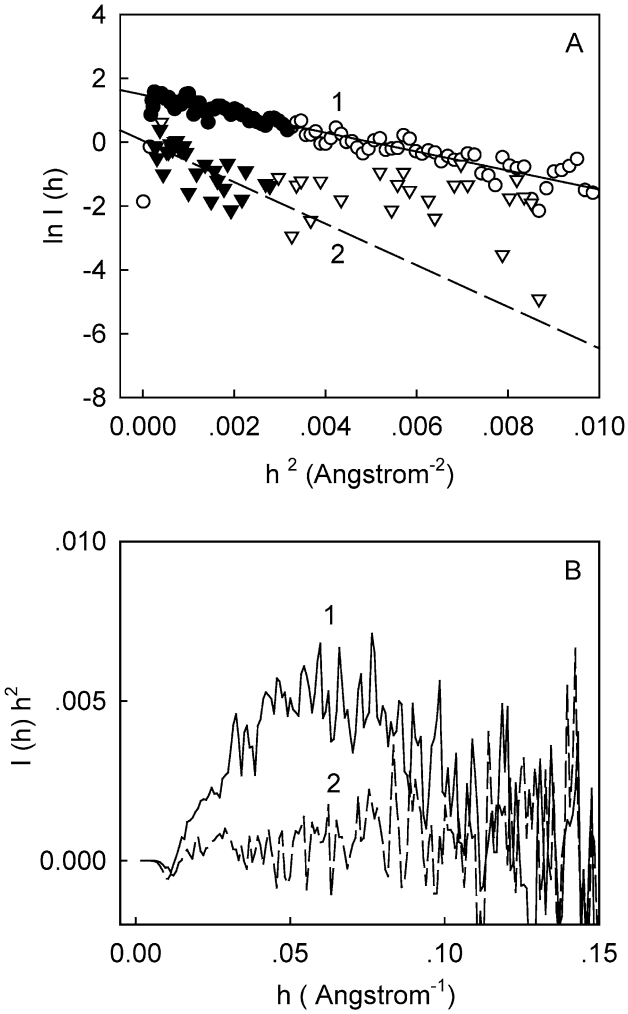

Fig. 2. Guinier plots (A) and Kratky plots (B) for CK at the beginning (around 4 s; curve 1) and end (around 46 s; curve 2) of the unfolding reaction, which were derived from analysis of the unfolding kinetics shown in Fig. 1. In each case, four kinetic frames were averaged in order to improve the signal/noise ratio. (A) The symbols are the data for Guinier plots and the filled symbols represent the fitting region to obtain the R_g value. The fitted results are shown as straight lines. The corresponding R_g values are 29.8 Å at the beginning and 43 Å at the end of unfolding.

experiment (1.5 M GuHCl) consists predominantly of a pre-molten globule state (75%), with most of the remainder in a molten-globule state [14]. Consistent with this, the data presented here indicate that the properties of the final state observed in the kinetic experiments is slightly more compact than the fully denatured state (Fig. 3A), and has around 30% of native secondary structure (Fig. 4A), but lacks distinct globular structure (Fig. 2B). The amplitude of the monophasic unfolding kinetics determined by integrated intensity and R_g values covers the entire range of the equilibrium values at the different final urea and GuHCl concentrations (see [15]). Therefore, the part of the unfolding curve measured by the integrated SAXS intensity corresponds to the loss of compact, globular structure for CK.

3.2. Time-dependent protein unfolding monitored by spectroscopic methods

The unfolding kinetics of CK was monitored in a stopped-flow apparatus by following changes in ellipticity at 222 nm

Table 1
Unfolding rate constants for CK under different conditions

Unfolding condition	Rate constants (s^{-1}) [a]			
	Stopped-flow SAXS [b]	Stopped-flow fluorescence	Far-UV CD at 222 nm	
			Stopped-flow mixing	Manual mixing
0.8 M GuHCl	0.010 ± 0.004	0.0118 ± 0.0002	0.013 ± 0.003	0.0100 ± 0.0002
1.5 M GuHCl	0.21 ± 0.01	0.380 ± 0.007	0.26 ± 0.04	0.16 ± 0.02
3.0 M GuHCl	2.0 ± 0.6	7 ± 1	7 ± 4	– [c]
8.0 M urea	0.11 ± 0.04	0.119 ± 0.007	0.10 ± 0.04	0.0367 ± 0.0003

[a] The error shown is the standard error obtained from the fit.
[b] Determined from the time-resolved change in the integrated SAXS intensity.
[c] Too fast to be determined.

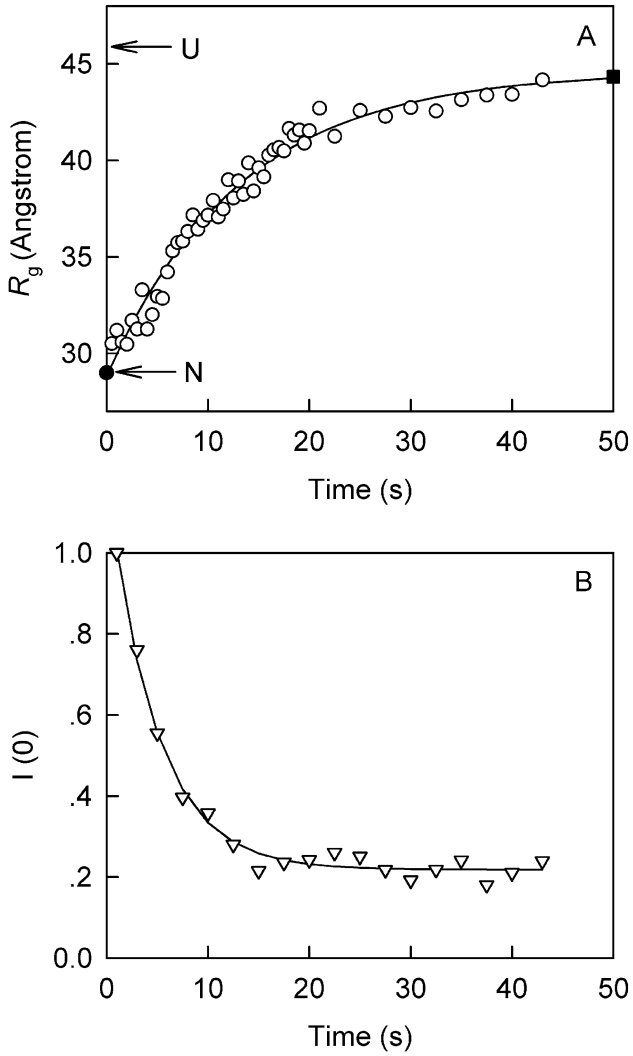

Fig. 3. Time-resolved changes in the values of R_g (A) and $I(0)$ (B) during unfolding of CK in 1.5 M GuHCl. The initial and the final values of R_g are nearly indistinguishable from those of the equilibrium native (●) and unfolded (■) states, respectively. Unfolding from the native state fits well to a single exponential function with a rate constant of 0.076 ± 0.005 s^{-1} derived from R_g and 0.21 ± 0.01 s^{-1} derived from $I(0)$. *N* and *U* represent the equilibrium values for the native and fully unfolded states, respectively.

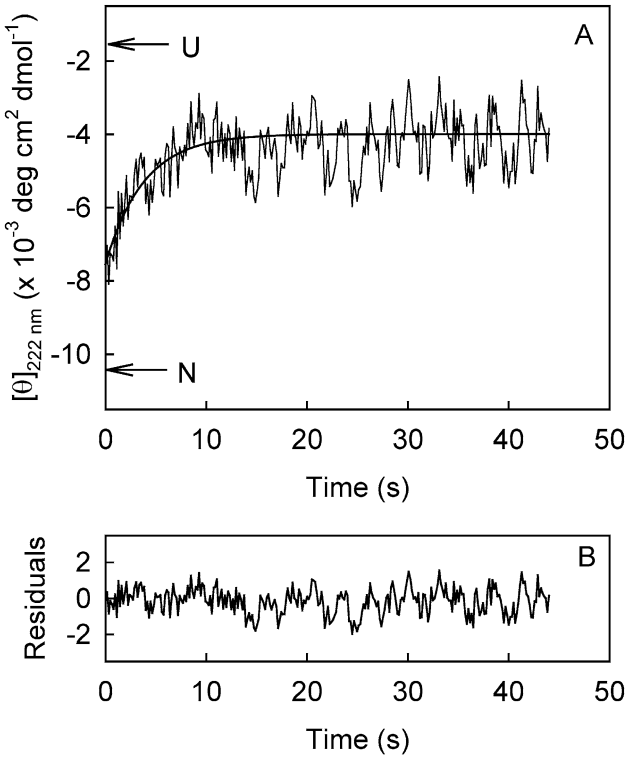

Fig. 4. Kinetics of CK unfolding as monitored by ellipticity changes at 222 nm with a final GuHCl concentration of 1.5 M. Details are as in Fig. 1, except that the final protein concentration was 0.3 mg ml^{-1}, and the number of accumulations was 5. Unfolding from the native state fits well to a single exponential function with a rate constant of k = 0.26 ± 0.04 s^{-1}. *N* and *U* represent the equilibrium values for the native and fully unfolded states, respectively.

or by intrinsic fluorescence above 330 nm with excitation at 294 nm. Figs. 4 and 5 show typical kinetic traces for unfolding of CK at a final GuHCl concentration of 1.5 M, monitored by CD and fluorescence, respectively. The unfolding kinetics at various concentrations of GuHCl or urea fit well to a single exponential equation (Figs. 4B and 5B) and the same apparent rate constants are obtained by the two methods (Table 1). As can be seen from the above results, the same time course of unfolding is observed when monitoring by the integrated SAXS intensity or by spectroscopic methods (Table 1). The unfolding rate constants obtained from the CD signal by manual mixing agree with those measured by stopped-flow mixing (Table 1). Therefore, the unfolding/folding kinetics measured by the stopped-flow apparatus accurately reflects the complete structural change that occurs during unfolding. This demonstrates the applicability of the SAXS stopped-flow method to protein folding studies.

3.3. Time-dependent refolding from 8 M urea-denatured CK monitored by spectroscopic methods

The refolding of CK was initiated by a concentration jump from 8 M urea, where the protein is completely unfolded, to 1.14 M urea with and without 20% glycerol. The refolding curves of CK were monitored by following changes in CD ellipticity at 222 nm in a stopped-flow apparatus or by manual mixing, and by changes of intrinsic fluorescence above 330 nm with excitation at 294 nm in a stopped-flow apparatus. It is clear that a substantial part of the ellipticity change from the unfolded to the native state occurs within the dead time of the stopped-flow apparatus (10 ms) in a burst phase, indicating the formation of pronounced secondary structure at an early stage in refolding. The kinetic traces for refolding after the burst phase fit well to a single exponential equation and the same apparent rate constant is obtained by the two methods (Table 2).

The occurrence of aggregation at higher protein concentrations affected the quality of refolding data that could be obtained from stopped-flow SAXS, especially in the refolding buffer without glycerol. Previous investigation suggests

Table 2

Refolding conditions	Rate constants (s^{-1}) [a]		
	Stopped-flow fluorescence	Far-UV CD at 222 nm	
		Stopped-flow mixing	Manual mixing
10 mM Tris pH 8.3	0.0103 ± 0.0005	0.013 ± 0.002	0.0077 ± 0.0001
20% glycerol with 10 mM Tris pH 8.3	0.0111 ± 0.0004	0.015 ± 0.002	0.0105 ± 0.0002

[a] The error shown is the standard error obtained from the fit.

that addition of glycerol can slow down the rate of subsequent folding after the burst phase and prevent the formation of the off-pathway intermediate [16]. Under these conditions (at lower protein concentrations), the refolded state populated at equilibrium is spectroscopically indistinguishable from the native state and exhibits greater than 50% native activity, suggesting a native-like structure. When the refolding reaction was carried out in buffer with 20% glycerol, the R_g value of the final refolded state monitored by static state measurement of SAXS was 33.6 Å, which shows a 15% increase compared to the native state value of 29.0 Å [14], but is significantly less than 45.4 Å which is obtained when refolding is carried out in buffer without glycerol. The slightly increased size of the refolded state obtained in glycerol compared to the native R_g value could reflect a small degree of aggregation when refolding at the high protein concentrations required for SAXS experiments, even in the presence of glycerol. Alternatively, the native-like state populated in the presence of 1.14 M urea and 20% glycerol may have a slightly expanded structure.

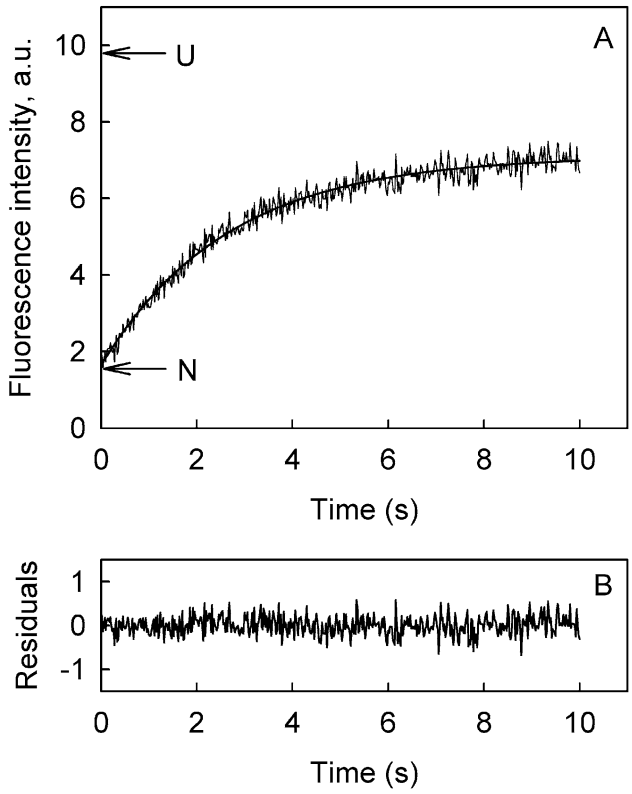

Fig. 5. Kinetics of CK unfolding as monitored by changes in intrinsic fluorescence at a final GuHCl concentration of 1.5 M. Details are as in Fig. 1, except that the final protein concentration was 0.5 mg ml^{-1}, and the number of accumulations was 6. Unfolding from the native state fits well to a single exponential function with a rate constant of $k = 0.380 \pm 0.007$ s^{-1}. *N* and *U* represent the equilibrium values for the native and fully unfolded states, respectively.

4. Discussion

The urea- and GuHCl-induced unfolding of CK has been measured by SAXS previously under steady-state conditions [15]. Good agreement was observed between the equilibrium transition curves measured by the R_g values and by intrinsic fluorescence. However, it was evident from a change in the maximum position of the Kratky plots, that at the protein concentration used (7.5 mg ml^{-1}), intermolecular association occurs within the GuHCl concentration range 0.3–1.5 M. This leads to a 2.5-fold increase in the $I(0)$ value and a 1.8-fold increase in R_g. In contrast, no intermolecular association is observed for urea-induced unfolding.

In this study, we focus on the kinetic unfolding reaction of CK. It is shown here that the integrated SAXS intensity can be successfully applied as a direct method to monitor the intramolecular loss of globular structure during protein unfolding. Synchrotron SAXS combined with the stopped-flow technique can be used to measure the kinetic course of protein unfolding from globular to random coil state, provided unfolding occurs on the second to minute time-scale or longer. In many cases, the backbone secondary structure and the rigid tertiary structure are lost simultaneously during protein unfolding [12]. The SAXS method used in combination with other physicochemical techniques [5] is able to throw light on the interrelation between secondary structure formation and globularization during protein folding. Due to the visible aggregation during GuHCl- or urea-induced refolding of CK when the protein concentration is higher than 2 mg ml^{-1}, only the unfolding kinetics can be investigated by stopped-flow SAXS. The results indicate that the loss of globular structure occurs together with the disruption of secondary and tertiary structures. Previous stopped-flow SAXS studies have focused on monomeric proteins [4,17]. The present study demonstrates the applicability of this technique to investigate the folding/unfolding of multimeric proteins.

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