Unfolding of dimeric creatine kinase in urea and guanidine hydrochloride as measured using small angle X-ray scattering with synchrotron radiation

Jun-Mei Zhou[a,*], Ying-Xin Fan[a], Hiroshi Kihara[b], Kazumoto Kimura[c], Yoshiyuki Amemiya[d]

[a] *National Laboratory of Biomacromolecules, Institute of Biophysics, Academia Sinica, Beijing 100101, China*
[b] *Physics Laboratory, Kansai Medical University, Uyamahigashi, Hirakata, Osaka 573, Japan*
[c] *Division of Medical Electronics, Dokkyo University School of Medicine, Mibu, Tochigi 321-02, Japan*
[d] *Faculty of Engineering, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan*

Received 30 May 1997; revised version received 19 August 1997

Abstract The denaturation of dimeric creatine kinase (CK) induced by urea and guanidine hydrochloride (GuHCl) has been studied by small angle X-ray scattering (SAXS), which is a direct way to measure the changes in the overall dimensions of a protein molecule. The radii of gyration (R_g) of CK are 29 ± 0.4 Å in the native state and 46 ± 1.5 Å in the unfolded state in either 8 M urea or 3 M GuHCl. The transition curves of urea denaturation derived from the R_g values and the zero angle intensity ($I(0)$) are similar to that from intrinsic fluorescence, indicating that the changes in the molecular shape, the tertiary structure and the dissociation of the subunits proceed simultaneously. In the case of GuHCl-induced denaturation, the dramatic increases both in R_g and in $I(0)$ in 0.3–0.5 M GuHCl suggest clearly that soluble aggregates form at low GuHCl concentrations. The aggregates dissociate and the molecule unfolds at higher GuHCl concentrations. The results suggest that the mechanisms of CK denaturation in urea and in GuHCl are somewhat different and the intermediate in GuHCl denaturation can much more easily form soluble aggregates.

Key words: Creatine kinase; Protein unfolding; Small angle X-ray scattering; Dissociation; Association

1. Introduction

The changes in the secondary and tertiary structure of proteins during folding and unfolding can be monitored by various methods such as circular dichroism (CD), intrinsic fluorescence, Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR). Direct measuring of changes in the compactness during protein folding is, however, very difficult. Small angle X-ray scattering (SAXS) is a powerful technique for such measurements [1–4]. It is well known that the SAXS pattern is sensitive to the size and shape of a scattering molecule [1]. The size of a molecule can be estimated using the radius of gyration (R_g), obtained by analysis of the SAXS patterns using Guinier plots, while the shape and globularity of proteins can be described in terms of a Kratky plot and the pair distribution function (P(r) function). SAXS has been used for monitoring the conformational changes of globular proteins [2–5]. The properties of the folding intermediates characterized by the method are of importance for understanding protein folding. In addition, SAXS is also a useful tool for studying protein aggregation, which often accompanies the folding process [5].

Cytoplasmic creatine kinase (CK; EC 2.7.3.2) from rabbit muscle is a dimer which is made up of two identical 43-kDa polypeptide chains of known sequence [6]. Although the three-dimensional structure of the isoenzyme has not been obtained yet, it has been postulated by biochemical and biophysical methods that the protomer of the enzyme is composed of two flexibly linked domains and the subunit is assembled asymmetrically. The unfolding and refolding of the enzyme have been studied extensively by intrinsic fluorescence, CD and other biophysical methods [7–10]. The folding mechanisms of CK induced by urea and guanidine hydrochloride (GuHCl) have been investigated. In the present study, the changes in the size and shape of the enzyme molecule during unfolding in urea and GuHCl were studied with the SAXS technique.

2. Materials and methods

2.1. Materials

Creatine kinase was prepared and further purified as described previously [11]. The $A^{1\%}_{1cm}$ value of 8.8 [12] was used for protein concentration measurements. The final preparations usually had a specific activity of 130–160 μmol/min/mg. Ultrapure urea and GuHCl were obtained from Nacalai Tesque, Inc., Kyoto, Japan. Dithiothreitol (DTT) was a Sigma product. All other reagents were local products of analytical grade.

2.2. Synchrotron small angle X-ray scattering measurement

Solution scattering measurements were performed at the beam line 15 small angle installation (BL-15A) of the Photo Factory, National Laboratory for High Energy Physics, at 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 [13]. Samples in a quartz cell with 1 mm pathlength were irradiated for 300 s at 25°C. The protein concentration was 7.5 mg/ml. Background data for the buffer solution at different concentrations of urea and GuHCl were collected before or after data collection for the protein solutions. The correction of the SAXS data for the difference in electron density (contrast) between the protein and 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 data were processed at the Division of Medical Electronics, Dokkyo University School of Medicine [5]. The R_g was estimated by the Guniner 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 the 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 associ-

*Corresponding author. Fax: (86) (10) 62022026.
E-mail: zhoujm@sun5.ibp.ac.cn

Abbreviations: SAXS, small angle X-ray scattering; CK, creatine kinase; GuHCl, guanidine hydrochloride; MG, molten globule

PII S0014-5793(97)01120-4

ation and dissociation of protein complexes [5]. The globularity of protein molecule was examined with a Kratky plot, i.e. $I(h)h^2$ versus h.

3. Results

3.1. Changes in the R_g value and $I(0)$ with the changes in concentrations of urea or GuHCl

As shown in Fig. 1 (curve 1), the R_g value of CK in the native state is 29 ± 0.4 Å and changes little at urea concentrations below 3 M. The R_g value increases sharply at urea concentrations between 3.0 and 4.2 M, reaching a value of 46 ± 1.5 Å. Further increases of the urea concentration result in no further changes in the R_g value. The good agreement between the curves plotted from the R_g values and that from intrinsic fluorescence indicates that the swell of the molecular size occurs simultaneously with the changes in the tertiary structure of the molecule. The value of $I(0)$ decreases to a value of about 50% of that in the native state between 3.0 and 4.5 M urea (Fig. 1, curve 2), indicating clearly that the dissociation of the dimer takes place cooperatively with the conformational transitions.

Previous studies by intrinsic fluorescence and CD spectra ([9,10], Fan et al., submitted for publication) suggested that the unfolding transition of CK in GuHCl is a two-stage process involving an equilibrium intermediate which resembles the definition of the 'molten globule' (MG) and is most stable at 0.8 M GuHCl. The CK unfolding curve monitored by R_g value shows a different behavior. The R_g value increases dramatically, from about 29 to 55 Å, within the native-MG transition region (0.3–0.8 M GuHCl), then it decreases to a value of about 46 Å with a further increase of the GuHCl concentration.

The value of $I(0)$ increases sharply at GuHCl concentrations higher than 0.2 M and reaches a value about 2.7-fold of that of the native state at 0.8 M GuHCl. The value of $I(0)$ decreases gradually with a further increase in the GuHCl concentration (Fig. 2, curve 2). The $I(0)$ value in 3 M GuHCl is the same as in 8 M urea. The increases in the values of the R_g and $I(0)$ show clearly that, at the protein concentration used in the present study, an intermolecular association occurs at GuHCl concentrations of 0.3–0.8 M. With further increases of the GuHCl concentration, the intermolecular aggregates dissociate, accompanied by the unfolding of the molecule.

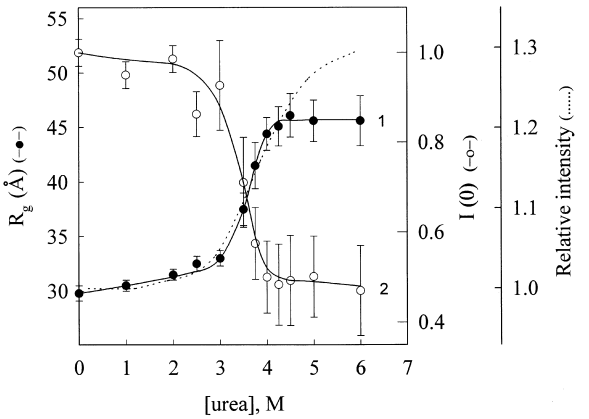

Fig. 1. Urea-induced equilibrium unfolding transition of CK monitored by the apparent value of R_g (1) and by the apparent zero-angle intensity (2). Prior to the measurement, the enzyme was incubated with the desired concentrations of urea in 50 mM Tris-HCl buffer, pH 8.3 at 25°C for 12 h. The protein concentration was 7.5 mg/ml. The transition curve monitored by intrinsic fluorescence is also shown as a dotted line for comparison [7].

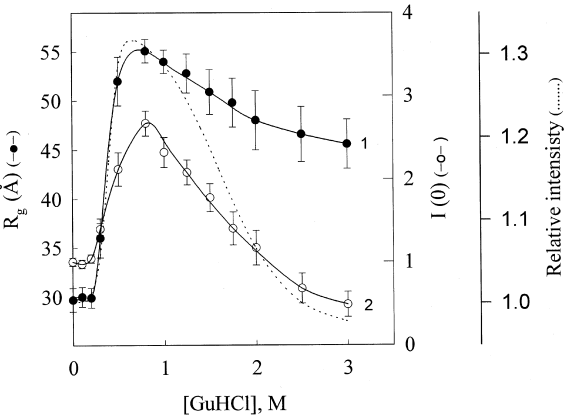

Fig. 2. GuHCl-induced equilibrium unfolding transition of CK monitored by the apparent value of R_g (1) and by the apparent zero-angle intensity (2). Prior to the measurement, the enzyme was incubated with the desired concentrations of GuHCl in 50 mM Tris-HCl buffer, pH 8.3 at 25°C for 12 h. The protein concentration is 7.5 mg/ml. The transition curve monitored by intrinsic fluorescence was also shown as a dotted line for comparison (Fan et al., unpublished data).

3.2. Changes in the globularity of CK during denaturation by urea and GuHCl

Figs. 3 and 4 show the Kratky plots of CK at different concentrations of urea and GuHCl. The Kratky plot of native CK shows a clear peak at an h value of 0.062 (curve 1 in both Figs. 3 and 4). The peak diminishes gradually at urea concentrations higher than 3 M and becomes a plateau at urea concentrations above 4.25 M (Fig. 3). The result shows that the CK molecule is globular in the native state but is chain-like in the unfolded state. The globule-coil transition coincides with that monitored by intrinsic fluorescence and CD.

As shown in Fig. 4, at low GuHCl concentrations, the peak of the Kratky plots shifts to small values of h, from 0.062 to about 0.03 $Å^{-1}$. The result gives further strong evidence for intermolecular association at low GuHCl concentrations. Size exclusion chromatography (Zhang et al., unpublished data) and sedimentation velocity [10] studies have shown that the dimeric CK dissociates into monomers at GuHCl concentrations of 0.4–1 M at the protein concentrations used in the investigations, usually lower than 1 mg/ml. Thus it has been concluded that the monomeric GuHCl-induced intermediate is unstable and forms aggregates at higher protein concentrations. As the GuHCl concentration continues to increase, the protein associates gradually disappear, as indicated by the decreases in the distinct peak in the Kratky plots. The Kratky plot becomes a plateau at 3 M GuHCl showing that the enzyme is in an unfolded state which is similar to the case of urea denaturation.

4. Discussion

The changes in the size and shape of the CK molecule during urea- and GuHCl-induced unfolding transitions were monitored directly with the SAXS technique. SAXS gives unique and essential information for describing the protein

unfolding process. The value of R_g reflects the changes in the size of the protein molecule during unfolding while the Kratky plot directly shows the shape of the molecule. If there is no intermolecular association and dissociation, $I(0)$ is a constant during protein unfolding; therefore, it is useful for determining dissociation and association accompanying the unfolding process [5]. Although there was a more than 50-fold difference in the protein concentrations employed, the urea-induced unfolding transition curves measured by SAXS coincide with those obtained by intrinsic fluorescence and far-UV CD spectra, indicating that the swelling of the molecule, the changes in the tertiary and secondary structure as well as the dissociation of the subunits occur cooperatively during urea denaturation. The GuHCl-induced unfolding, however, shows a much more complex behavior. It can be clearly seen from changes in the R_g and $I(0)$ values and the Kratky plot that there is intermolecular association at a GuHCl concentration of 0.4–1.0 M. This strongly suggests that the equilibrium intermediate in the GuHCl-induced unfolding process is unstable at the concentration used. However, it is interesting to note that the associates are soluble and can refold to the native state after removal of the denaturant.

Urea and GuHCl are two of the most commonly used denaturants for protein denaturation, and they are believed to have a similar mode of action [14,15]. However, urea is generally known to be about half as effective in protein unfolding and dissociation as GuHCl [16]. Comparison of the unfolding and inactivation of CK by urea and GuHCl suggested that although these two denaturants are similar in some aspects, there are some significant differences [10,17]: (1) GuHCl is much more effective in the unfolding and dissociation of the enzyme. (2) Previous studies indicated that GuHCl-induced unfolding transition monitored by intrinsic fluorescence, far-UV CD, and enhancement of ANS fluorescence takes place in two stages, suggesting the existence of an equilibrium intermediate, which is not stable and forms associates at higher protein concentrations as indicated by the present SAXS study. However, in the case of urea denaturation, there is no such clearly observed intermediate. (3) The unfolding of CK in 3 M GuHCl differs from that in 8 M urea as indicated by the intrinsic fluorescence spectra [7], but the molecular size and shape are similar as shown in the present study.

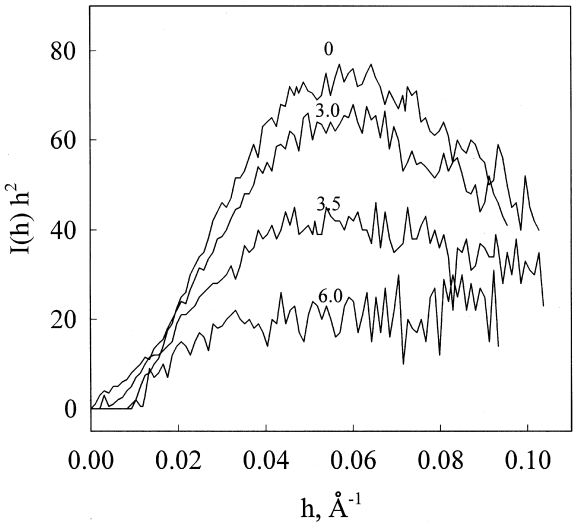

Fig. 3. Kratky plots of CK at various urea concentrations. The experimental conditions were the same as in Fig. 1. The numbers on each curve denote urea molar concentrations.

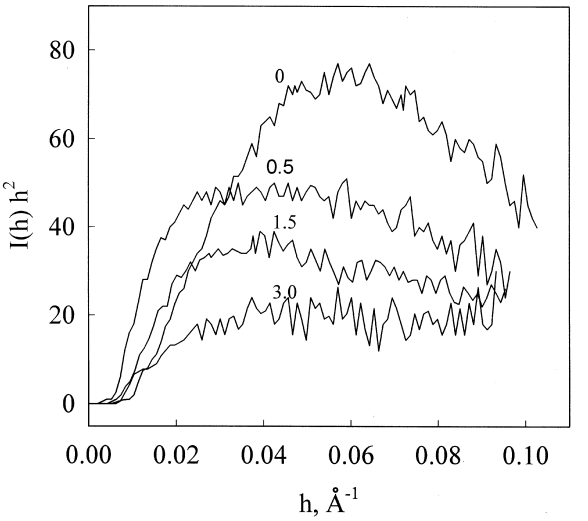

Fig. 4. Kratky plots of CK at various concentrations of GuHCl. The experimental conditions were the same as in Fig. 2. The numbers on each curve denote GuHCl molar concentrations.

Acknowledgements: This work was supported in part by the Pandeng Project of the China National Commission of Science and Technology. One of the authors (J.M.Z.) was supported by the Japanese Society for Fellowships for Foreign Scientists (ID 96275). The experiment at BL-15A of PF was performed under the approval of the Photon Factory Advisory Committee (Proposal No. 95P016).

References

[1] Glatter, O. and Kratky, O. (1982) in: Small Angle X-ray Scattering, pp. 1–515, Academic Press, London.
[2] Lattman, E.E. (1994) Curr. Opin. Struct. Biol. 4, 87–92.
[3] Kataoka, M., Nishii, I., Fujisawa, T., Ueki, T., Tokunaga, F. and Goto, Y. (1995) J. Mol. Biol. 249, 215–248.
[4] Sosnick, T.R. and Trewhella, J. (1992) Biochemistry 31, 8329–8335.
[5] Semisotnov, G.V., Kihara, H., Kotova, N.V., Kimura, K., Amemiya, Y., Wakabayashi, K., Serdyuk, I.N., Timchenko, A.A., Chiba, K., Nikaido, K., Ikura, T. and Kuwajima, K. (1996) J. Mol. Biol. 262, 559–574.
[6] Putney, S., Herlihy, W., Royal, N., Pang, H., Vasken Aposhian, H., Pickering, L., Belagaje, R., Biemann, K., Page, D., Kuby, S. and Schimmel, P. (1984) J. Biol. Chem. 259, 14327–14330.
[7] Yao, Q.Z., Tian, M. and Tsou, C.L. (1984) Biochemistry 23, 2740–2744.
[8] Zhou, H.M. and Tsou, C.L. (1987) Biochim. Biophys. Acta 869, 69–74.
[9] Gross, M., Lusting, A., Wallimann, T. and Furter, R. (1995) Biochemistry 34, 10350–10357.
[10] Couthon, F., Clottes, E., Ebel, C. and Vial, C. (1995) Eur. J. Biochem. 160–170.
[11] Yao, Q.Z., Hou, L.X., Zhou, H.M. and Tsou, C.L. (1982) Sci. Sin. 25B, 1186–1193.
[12] Noda, L., Kuby, S.A. and Lardy, H. (1954) Methods Enzymol. 2, 605–610.
[13] Amemiya, Y., Wakabayashi, K., Hamanaka, T., Wakabayashi, T. and Hashizume, H. (1983) Nucl. Instr. Methods 208, 471–477.
[14] Hibbard, L.S. and Tulinsky, A. (1978) Biochemisty 17, 5460–5468.
[15] Yao, M. and Bolen, D.W. (1995) Biochemistry 34, 3771–3781.
[16] Pace, C.N. (1986) Methods Enzymol. 131, 267–280.
[17] Yao, Q.Z. and Tsou, C.L. (1985) in: Molecule Architecture of Proteins and Enzymes (Bradshaw, R.A. and Tang, J., Eds.), Academic Press, New York.