X-ray solution scattering studies of protein folding

Mikio Kataoka[1] and Yuji Goto[2]

Protein folding is a reaction in which an extended polypeptide chain acquires maximal packing through formation of secondary and tertiary structures. Compactness and shape are, therefore, critical properties characterizing the process of protein folding. Because the stability of the native state is determined by the subtle free energy balance between the native and denatured states, the characterization of the denatured state is also essential to understand the conformational stability of the native state. We show that solution X-ray scattering is the best technique available today to address these problems. Although the structural resolution of the unfolded or compact denatured states elucidated from solution X-ray scattering is low, it provides a variety of information complementary to that obtained by NMR or X-ray crystallography.

Addresses: [1]Department of Earth and Space Science and [2]Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka 560, Japan. e-mail: [1]kataoka@ess.sci.osaka-u.ac.jp; [2]ygoto@bio.sci.osaka-u.ac.jp

Electronic identifier: 1359-0278-001-R0107

Folding & Design 01 Oct 1996, 1:R107–R114

Introduction

In order to understand protein folding and stability, structural characterization of non-native conformations is as important as determining the high-resolution three-dimensional structure of the native state [1]. We can reveal the high-resolution structure of the native state by either X-ray crystal structure analysis or nuclear magnetic resonance (NMR) solution structure analysis. On the other hand, neither X-ray crystallography nor NMR solution structure analysis is likely to provide a complete description of non-native states such as denatured states and folding intermediates. Since these states are ensembles of various conformations, such states are not expected to yield suitable crystals. Furthermore, such states would not contain definite conformation, although some residual structures have been clarified by NMR [2]. The characterization of the equilibrium and kinetic intermediates by hydrogen/deuterium exchange of amide protons in conjunction with two-dimensional NMR measurements indicates that some regions remain intact in both the secondary and tertiary interactions whereas others are largely unfolded [3]. Structural models of the folding intermediates for several proteins have been proposed on the basis of such results [4].

Since protein folding is a process in which an extended polypeptide chain acquires maximal packing through formation of the secondary and tertiary structures, the change in size and shape during the process is critical to understanding the mechanism of protein folding [5]. Further, the size and the shape are utilized as parameters describing the extent of folding in some theories and models of folding. In order to assess the validities of these theories or models and to compare them with the experimental data, the quantitative description of the size and shape is essential [6,7]. Solution X-ray scattering is probably the best available technique for such a measurement. In fact, solution X-ray scattering and its neutron version have been successfully applied to describe the characteristics of various non-native conformations [8–18].

Recent advances in the techniques of small-angle X-ray solution scattering using synchrotron radiation have now made it possible to obtain accurate data in a relatively short time, thus permitting time-resolved studies, solution scattering measurements under low-contrast solvent conditions and assessment of scattering curves extending well beyond the Guinier region. These developments have led to a breakthrough in the application of this technique to the study of protein folding, enabling equilibrium [8,9,19] and kinetic [20–22] experiments to follow folding directly by size and shape.

Because the application of solution scattering to the study of protein folding is only now becoming popular, there are only a few specific review articles (e.g. [6]), although there are many excellent articles on the classical application of solution X-ray scattering to the structural study of proteins [23,24]. In this review, using recent results (many of them our own), we show how solution X-ray scattering has been applied to the characterization of the intermediate and unfolded states of proteins.

Structural information obtainable by small-angle X-ray scattering studies

Excellent textbooks on small-angle X-ray scattering are available for readers who are interested in the technique [23,24]. The structural information obtainable and useful for the study of protein folding is summarized in Table 1.

The structural information in the scattering experiment is contained in the scattering intensity, $I(Q)$, at momentum transfer, Q, which is defined as $Q = 4\pi\sin\theta/\lambda$, where 2θ and λ are scattering angle and the wavelength of the incident X-rays, respectively. Since there are no preferable orientations in the solute particle, the obtained scattering

Table 1

Analytical methods of solution X-ray scattering, the obtainable structural parameters, and the properties useful for the study of protein folding.

Analysis	Description	Obtainable structural parameters	Structural properties
Guinier plot	ln I(Q) versus Q^2	Rg I(0)	Molecular size, compactness Molecular weight, aggregation state
Kratky plot	Q^2*I(Q) versus Q	Peak intensity Persistent length	Compactness and globularity Statistics of chain conformation Molecular shape
P(r)	$(1/2\pi^2)\int I(Q)(Qr)\sin(Qr)dQ$	d_{max}	Molecular size Molecular shape
Scattering profile at high Q region			Tertiary fold

function loses the azimuthal dependence and is circular symmetric about the incident beam. The scattering function is expressed as a time- and space-averaged Fourier transform of the electron density distribution of a solute particle.

In the small Q region, regardless of the detailed particle structure, the scattering function is approximated as $I(Q) = I(0)\exp(-Q^2Rg^2/3)$, where I(0) and Rg are the intensity at 0 angle and the radius of gyration [23,24]. These two quantities are the most fundamental structural parameters obtained by the small-angle X-ray scattering experiment. I(0) is a measure of molecular weight, while Rg is a measure of a size or a molecular compactness. These two parameters are obtained from a Guinier plot, ln I(Q) versus Q^2.

We have shown that the expression of the intensity function in the form of a Kratky plot, Q^2*I(Q) versus Q, is quite useful in protein folding, because the plot has a unique qualitative dependence on molecular shape and conformation [6,8,9]. The scattering profile for globular proteins follows Porod's law — $I(Q) \propto Q^{-4}$ — at large Q values, while the intensity function from a chain molecule, the expected form of a denatured conformation, is proportional to Q^{-2} in the moderate Q region and then varies as Q^{-1} in the high Q region. Thus, the Kratky plot for the native conformation has a distinct peak, the position of which is dependent on Rg, while the plot for a chain molecule gives a plateau and then rises monotonically. Using the integral intensity around a peak, we can follow the folding/unfolding transition in terms of compactness [8,9].

Since the intensity function is an expression in reciprocal space, it is rather difficult to deduce a direct structural image. A distance pair-distribution function, P(r), is given by a sine Fourier transform of the intensity function. P(r) is an expression in real space and is useful to examine the size and shape of a molecule. The size is given as d_{max}, the longest distance in a molecule, where P(r) approaches zero. Since P(r) can be calculated easily from the crystallographic atomic coordinates, it is also useful for comparing the solution structure and the crystal structure or for examining the validity of a model.

The scattering curve in the high Q region (usually 0.2–0.3 < Q) reflects the fluctuations of electron density distribution within a protein molecule, the inner structure, rather than global shape. The protein inner structure appearing in the region (0.2 < Q < 1.0) largely reflects interactions between secondary structural elements, chiefly α-helical segments, since the corresponding distance is from 30 Å to 6 Å. This interpretation is supported by the fact that the low-resolution (6 Å) crystal structure of myoglobin provided the mode of the packing of α-helices [25]. Based on the crystal structure, we can calculate the scattering profile in the high Q region [26,27]. Generally, the scattering profile in the high Q region from a native protein shows a distinct fine structure which is consistent with its crystal structure.

Structural characterization of various conformational states

Various denatured states

An ideal model for an unfolded protein is the 'random coil'. Since the classic experiments of Tanford [28] demonstrated that proteins denatured by guanidine hydrochloride (GuHCl) are random coils, many researchers have assumed that the denatured state is a random coil under all conditions. However, there is evidence that proteins can have significant amounts of residual structure even in 4 M GuHCl [1]. Solution X-ray scattering data can give us information on the extent of the randomness [23,24]. Using neutron solution scattering, Calmettes *et al.* [11,12] showed that GuHCl-denatured phosphoglycerate kinase (PGK) can be explained by a

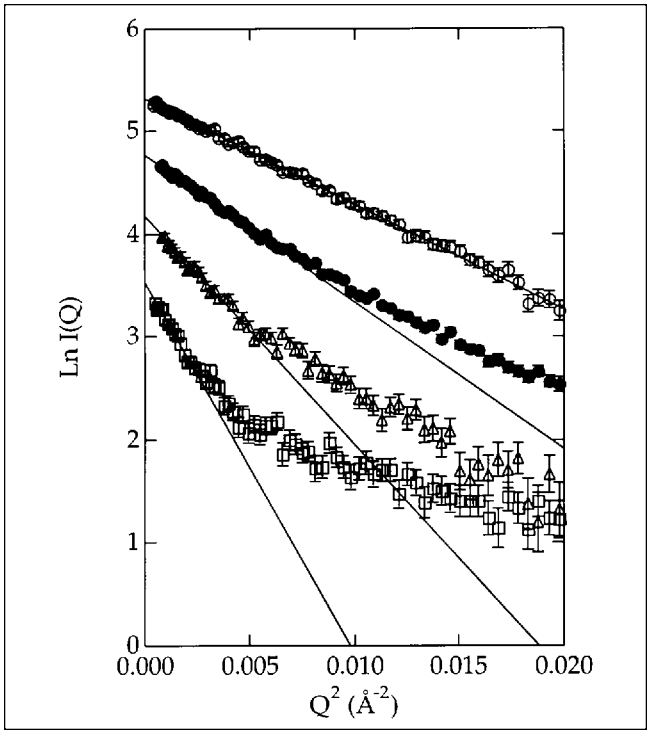
random flight chain to some extent. They also created a molecular model of the fully unfolded state of PGK using a freely jointed chain of spheres [11]. As their analysis of the scattering data combined with statistical mechanics of polymer molecules is successfully applied to PGK, this kind of analysis is expected to apply to the other conformational states of various proteins. Further, there is still room for improvement. For example, Calmettes *et al.* [11,12] did not include any excluded volume effect, which is significant in a real chain molecule in solvent. This means that the random flight chain is not necessarily a good expression even for the fully unfolded state by GuHCl.

Structural variations in denatured states are shown for the *Streptomyces* subtilisin inhibitor (SSI). Konno *et al.* [18] have shown by X-ray solution scattering that heat-denatured (D), cold-denatured (D′) and urea-denatured (D_{urea}) states of SSI are distinct conformational states. The radii of gyration are 25.8, 20.7 and 32–35 Å for the D, D′ and D_{urea} states, respectively [18]. The native SSI has an Rg of 20.1 Å, which is close to that of the D′ state. The native SSI is a homo-dimeric protein, while every denatured state is monomeric. Thus, even for the D′ state, the molecule is quite expanded relative to the native state. The molecular shape of the D′ state is globular, while the D_{urea} state is an expanded chain conformation with a persistent length. The D state appears to have some residual structure, but the amount of the structure is less than that of the D′ state. Upon addition of urea to the D or D′ states, a conformational transition to the D_{urea} state is observed [18]. This example demonstrates that the denatured state is not a random coil. The compact and globular cold-denatured state of SSI is in contrast to the cold-denatured state of PGK. The radii of gyration of PGK are 24.4 and 71–78 Å for the native and cold-denatured states, respectively [14]. The scattering profile from the cold-denatured PGK is well explained by a chain conformation with a persistent length. Since cold denaturation is a thermodynamically important phenomenon, further detailed structural studies on the cold-denatured state are essential for a complete understanding of protein folding.

Molten globule state

Much attention has been paid to characterizing pathways of protein folding and the discrete intermediate states. The most popular equilibrium intermediate state is the molten globule state. According to the original definition, the molten globule state has native-like secondary structure and compactness but disordered tertiary structure [29–31]. Conformational states with properties similar to the molten globule state were detected in various proteins under mild-denaturing conditions, such as low concentrations of denaturant or at acid pH, and subsequently proposed to be similar to kinetic intermediates accumulated during the refolding of several proteins [4,29,30]. On the other hand, recent theoretical and kinetic studies suggest that folding of small globular proteins may occur very rapidly without the stable intermediate states [32,33]. This new view also suggests that folding intermediates observed so far, in particular the molten globule state, may in fact be kinetically trapped and misfolded species. Therefore, the exact role of the molten globule states in protein folding is controversial at present.

Figure 1



Guinier plots of scattering curves from the various conformational states of myoglobin: (○) holomyoglobin and (●) apomyoglobin native state, (△) trichloroacetate-stabilized molten globule state, and (□) acid-unfolded state. The scattering curves at infinite dilution obtained by extrapolation of the scattering data within the concentration range 1–20 mg ml^{-1} are presented. For clarity, each plot is shifted along the ln I(Q) axis. Reproduced from [10].

We have been investigating the structure of molten globule or compact denatured states by solution X-ray scattering [8–10,18,19,34,35]. Figure 1 shows the Guinier plots of the scattering curves of various conformational states of myoglobin: the native state of holomyoglobin (M), the native state (N), the molten globule state stabilized by sodium trichloroacetate (MG(TCA)), and the acid-unfolded state (U_A) of apomyoglobin [10]. The scattering curves are at infinite dilution extrapolated from measurements at concentrations of 2–20 mg ml^{-1}. Because the scattering curve generally shows a dependence on protein concentration, consideration of the concentration dependence is critical to obtaining reliable data. It should

Table 2

Comparison of the radius of gyration (Rg) for the native and molten globule states and its increment in the molten globule for various proteins.

Protein	Rg for N state (Å)	Rg for MG state (Å)	ΔRg/Rg(N) (%)
Cytochrome *c* [8]	13.5	17.4†	28.8
		17.0‡	25.9
Holomyoglobin [10]	17.5		12.6‡‡
Apomyoglobin [10]	19.7	23.1§	17.3§§
			32.0##
Staphylococcal nuclease [17]	16.2	21.2#	30.9
α-Lactalbumin*	15.7	17.2**	9.6
		17.2††	9.6

*M Kataoka, Y Goto, unpublished data. †The molten globule is stabilized at pH 2.0 with 100 mM NaCl. ‡The molten globule is stabilized at pH 2.0 with the acetylation of lysine residues. §The molten globule is stabilized at pH 2.0 with 50 mM Na trichloroacetate. #The Rg value for the fragment which lacks 13 amino acid residues from the C terminus. **The Rg value for apo-α–lactalbumin at pH 8.0. ††The molten globule is stabilized at pH 2.0. ‡‡Increment from holomyoglobin native state to apomyoglobin native state. §§Increment from apomyoglobin native state to molten globule state. ##Increment from holomyoglobin native state to molten globule state.

be noted that, although the Guinier approximation holds for the region $QRg \leq 1.3$, the linear region of the Guinier plot for a globular protein sometimes extends up to $QRg = 3$, as was the case of holomyoglobin. (A more detailed discussion of the appropriate region for analysis of the Guinier plot can be found in [10].)

In the Guinier plot, a steeper slope indicates a larger Rg. Including the results of the unfolded states in urea (U_{urea}) or GuHCl (U_{GuHCl}), Rg for myoglobin increases in the order:

$$M < N < MG(TCA) < U_A < U_{urea} = U_{GuHCl}$$

The molecular size of the molten globule is compact compared to the unfolded state, but is expanded relative to the native state. Similar results have been obtained for cytochrome *c* [8], staphylococcal nuclease (SNase) [9,16] and α-lactalbumin [30,36]. The Rg values and their increment at the molten globule for various proteins are summarized in Table 2. It is clear that the molten globule is not as compact as the native state. Ptitsyn and co-workers [30,36] claimed that the increment in Rg at the molten globule should be less than 10% of the Rg value for the native state, based on their observation for α-lactalbumin. However, the increment of Rg, ΔRg/Rg(native), is up to 30%. The increment is about 10% only for α-lactalbumin. Among these proteins, α-lactalbumin is the only one that possesses intramolecular disulfide bonds. Therefore, we conclude that the molten globule has a tendency to expand from the native state but that intramolecular disulfide bonds restrict the expansion [10].

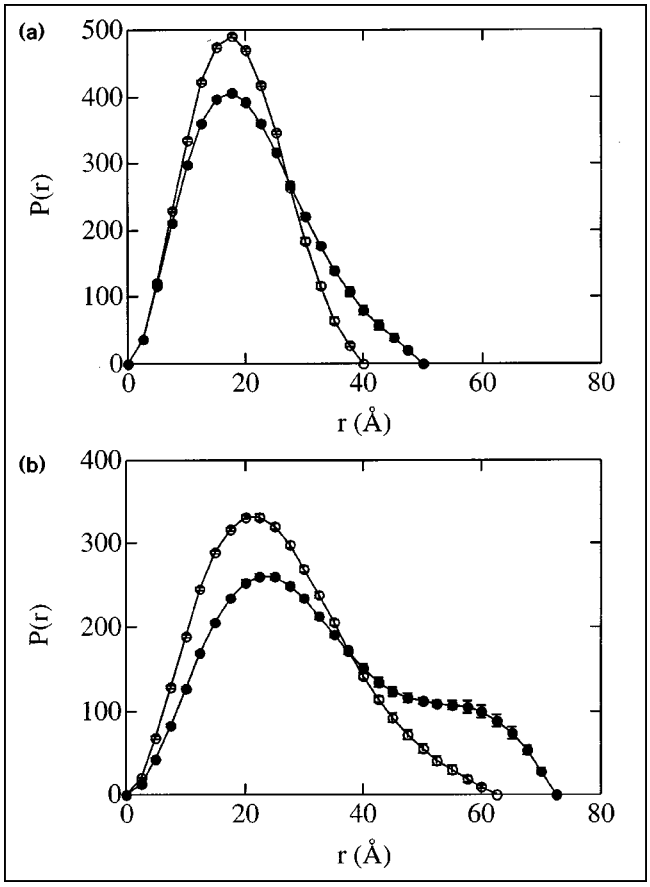
The distance distribution functions, P(r), for the native and molten globule states are compared for cytochrome *c* and apomyoglobin in Figure 2 [10]. The value of d_{max}, the longest dimension of the molecule, can be estimated from the point where the function approaches zero. Thus, d_{max} changes from 40 Å to 50 Å for cytochrome *c* and from 63 Å to 73 Å for apomyoglobin in going from the native state to the molten globule state, consistent with the results obtained by Guinier plot. Both the native states show a monophasic peak, indicating globular structure. The interesting and important properties of P(r) for molten globules are that the apomyoglobin molten globule shows a peak around 20 Å and a shoulder, i.e. a biphasic P(r), while the cytochrome *c* molten globule gives a single peak, a monophasic P(r), like the native state. Examples of the biphasic P(r) are shown in the truncated form of staphylococcal nuclease [9,16,17] and thermally denatured ribonuclease A [37], and the monophasic P(r) is shown in the molten globule of α-lactalbumin [38]. A monophasic P(r) is characteristic of a spherical structure like the native state. A typical example of a biphasic P(r) is shown in calmodulin, which takes a dumbbell-like shape [38]. However, there is no evidence that the molten globule or compact denatured states of these proteins are composed of two distinct domains connected with a linker. We also demonstrated that molten globules having a monophasic P(r) show distinct fine structures in the scattering profile in the high Q region, suggesting a significant amount of interaction among secondary structural elements, i.e. a significant amount of tertiary fold. Molten globules having a biphasic P(r) show small and broad diffraction maxima in the high Q scattering profile, suggesting a small amount of tertiary fold [10]. Taking into account these observations, we interpret the biphasic P(r) of the molten globule as resulting from a hydrophobic core with flaring tail(s). Theoretical calculation of the intermediate states with a lattice model reproduces the bimodal P(r) function, which may

be one of the common structural properties of the early folding intermediates [7]. Thus, P(r) function provides a direct image of the folding intermediates, although neither the atomic details nor a unique structural image are derived. It is also useful for examining the validity of models or theories of protein folding.

As described above, solution X-ray scattering has revealed structural variations in the molten globule. The formation and the stability of the globular and highly compact molten globule of α-lactalbumin would be mainly attributed to the intramolecular disulfide bonds (M Kataoka, Y Goto, unpublished data). Those of the globular and relatively compact molten globule for cytochrome *c* would be mainly attributed to the hydrophobic interaction between the heme and polypeptide, as well as different segments of the polypeptide [35]. The molten globule of apomyoglobin is composed of a hydrophobic core formed by helices A, B, G and H [39] with a fluctuating connection loop. It is important to note that the structural image obtained from solution X-ray scattering for α-lactalbumin is somewhat different from that obtained by NMR and studies of disulfide bond formation [3]. Probably, whereas the β-domain is substantially disordered, it collapses because of the presence of the disulfide bonds.

The original model of the molten globule state, the uniform melting of the sidechain packing and the total maintenance of the secondary structure, is already contradicted by various methods, including NMR [3] and calorimetry [40]. The results of the solution X-ray scattering also support the inappropriateness of the original model. Moreover, the solution X-ray scattering indicates that there are a variety of intermediate states. Although the subdomain model of the intermediate state is important [40], the results of solution X-ray scattering suggest significant loosening of the tertiary fold in the remaining structure.

Figure 2



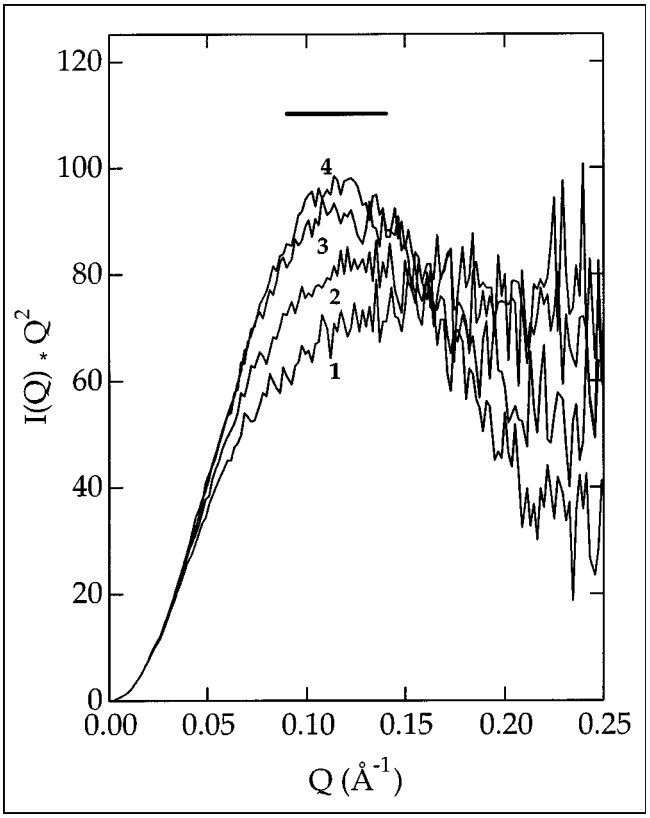
P(r) functions of the native and molten globule states for (a) cytochrome *c* and (b) apomyoglobin. Open symbols indicate the native state and solid symbols indicate the salt-induced molten globule state. The results for apomyoglobin are reproduced from [10].

Folding transition obtained by small-angle X-ray scattering studies

Solution X-ray scattering can also be utilized to follow a folding/unfolding reaction in terms of the molecular compactness or the molecular shape [8,9]. Figure 3 shows the titration of acidic cytochrome *c* with NaCl in the form of a Kratky plot [8]. While cytochrome *c* is in an acid-unfolded state at pH 2.0, addition of NaCl induces the formation of a molten globule. Figure 3 shows that the transition from the unfolded state to the molten globule state is a structural transition from a chain-like conformation to a globular conformation, which is clear from the appearance of a peak at Q = 0.12 [8]. A remarkable feature of the figure is the clear isoscattering point around Q = 0.15. Similar results are obtained for the formation of the molten globule from the acid-unfolded state by acetylation. The existence of the isoscattering point strongly suggests that the transition approaches a two-state mechanism. This represents one of the first characterizations of a transition involving the non-native states of a protein.

Integral intensity around the peak is a measure of compactness or globularity. The transition curve measured by the integral intensity for the NaCl-induced transition was compared with the transition curves obtained spectroscopically [8]. The folding transitions from the acid-unfolded state to the molten globule measured by various techniques give a common transition curve with a midpoint at 0.05 M NaCl, indicating a two-state transition. In contrast, a difference between the transition curves obtained by solution X-ray scattering and that obtained by fluorescence is seen in the case of urea-induced unfolding of SNase [9], suggesting a gradual structural transition. Note that Rg is not necessarily a good parameter by which to follow structural transitions, because the Rg for intermediate states cannot be described by a linear combination of the Rg values of the native state and the unfolded state.

Figure 3



Kratky plots of cytochrome *c* as a function of the NaCl concentration. NaCl concentrations of curves 1–4 are 0.0, 0.02, 0.05 and 0.3 M, respectively. The horizontal bar indicates the integral region for the evaluation of integral intensity. Reproduced from [8].

Another important aspect of folding derives from equilibrium measurement of various conformational states of myoglobin. Myoglobin consists of eight helices designated A–H. It has been reported that helices A, B, G and H are formed in the molten globule induced by sodium trichloroacetate [39]. Helical content and the contribution of hydrophobic interactions in stabilizing these conformational states were examined and compared with the change in Rg and d_{max} [10,34]. The results indicate that the protein becomes more compact with formation of the secondary structure and that the tight packing of the sidechains occurs at the late stage of protein folding, i.e. the transition between the molten globule and native states. Similar results were obtained with cytochrome *c* [34].

Time-resolved studies

Important issues that cannot be accessed through the equilibrium measurements described above include whether kinetic folding intermediates are identical to equilibrium intermediates and whether the compaction is preceded by secondary structure formation. Kinetic measurements using various spectroscopic techniques lead to the conclusion that some of the structural properties of the kinetic intermediate are close to those of the equilibrium intermediate [41]. However, few direct measurements of size and shape have been reported so far. Time-resolved solution X-ray scattering is expected to give critical information concerning the above issues. Both stopped-flow [42] and temperature-jump systems [43] have been developed for kinetic solution scattering experiments using synchrotron radiation. Although the first feasibility study on protein folding was reported in 1988 [43], no concrete conclusion about the above-mentioned issues was reached. Although the recent kinetic X-ray scattering studies clearly indicate that molecular association or aggregation is a major problem, they also suggest that the compaction occurs concomitantly with the secondary structure formation [20,22], and that the kinetic intermediate is as compact as the equilibrium intermediate [21]. It is apparent that the incident intensity of the present synchrotron radiation is not sufficient to record the scattering intensity with high qualities. The use of the new generation synchrotrons, such as ESRF (France), APS (USA) and SPring-8 (Japan), will benefit the kinetic study of protein folding.

Future perspectives

The solution scattering technique has been successfully applied to the protein folding problem. Solution scattering can give information on size and shape and is, thus, useful to describe structural characteristics of non-native states of proteins. We have shown the structural diversity in molten globule states as well as in the highly denatured states. The important issue that should now be addressed is how these structures are different. Reliable structural models for non-native structures are required to describe this structural variation. The development of new and effective analytical methods for solution scattering data is also required

In this regard, Doniach *et al.* [45] have indicated that the scattering curve from the molten globule of cytochrome *c* reported by Kataoka *et al.* [8] may be explained by a partially folded and partially unfolded model. However, the calculated scattering curves for their model are slightly different from the experimentally observed scattering curve. The difference is best observed at the region QRg = 3–4. According to the theoretical treatment of scattering, the structural properties reflected in this region are the surface structure. We noticed that the intensity function of this region is approximated as $I(Q) \propto Q^x$, and the x value is a good index by which to discriminate the native, the compact denatured and the fully unfolded state (M Kataoka, unpublished data). The x values for the native states of various proteins are distributed in the range $-4.5 > x > -6$, while the x values for the molten globule or compact denatured states are in the range $-4 < x < -2.5$. The molten globule states with a unimodal P(r)

function have x values close to –4, while states with a bilobic P(r) function have x values between –2.5 and –3. The unfolded states have x values larger than –2.3. Thus, there are no ambiguities in classifying each conformational state. We have reported that the native state of apomyoglobin has a property intermediate between the holomyoglobin native state and apomyoglobin molten globule state [10]. The x value for apomyoglobin native state is exactly intermediate between these two states. Further theoretical and model-oriented studies are required to reveal the physical meaning of the x value.

Although kinetic X-ray scattering study is now feasible using synchrotron radiation, the data are still insufficient in many respects. Development of a new effective area detector and a triggering system with a short dead time is essential to follow the kinetic studies using new generation synchrotron radiation.

Acknowledgements

We thank Andrew D Robertson (University of Iowa) for critical reading of the manuscript. This work was supported by Grants-in-Aids for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. Our X-ray solution scattering measurements were performed with the SAXES diffractometer installed at BL10C of the Photon Factory, Tsukuba, Japan. The experiments were performed under an approval of the program advisory committee. The authors are grateful to their collaborators Y Hagihara, D Hamada, H Kamikubo, M Hoshino, J Yunoki and N Hirota.

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