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"Partly Folded" State, a New Equilibrium State of Protein Molecules: Four-State Guanidinium Chloride-Induced Unfolding of β-Lactamase at Low Temperature†

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ABSTRACT: Guanidinium chloride- (GdmCl-) induced unfolding of β-lactamase has been investigated by a combination of size-exclusion chromatography (SEC-FPLC) and usual optical methods. It has been shown that at low temperatures this protein unfolds through *two* equilibrium intermediates. The first of these intermediates is the molten globule state, while the other (which we have called a "partly folded" state) is less compact than the molten globule but much more compact than the unfolded state. It also preserves a substantial part of secondary structure of the native or molten globule state. We suggest that this new "partly folded" state of a protein molecule can be the equilibrium counterpart of the first kinetic intermediate of protein folding, formed within a few milliseconds, i.e., after the "burst" stage of folding.

It is well-known that protein folding goes through some kinetic intermediates which can accumulate in the folding process (Kim & Baldwin, 1982, 1990; Ptitsyn, 1987, 1992; Kuwajima, 1989). Early intermediates have small lifetimes, which hinders their structural studies by many standard physical methods. Therefore, it is worthwhile to study *equilibrium* intermediates in the hope that they will to some extent mimic the kinetic ones. The best way to do this is to study protein unfolding by strong denaturants such as urea or GdmCl[1] since these denaturants (unlike, e.g., high temperatures or low pH) can really transform proteins in a more or less completely unfolded state (Tanford, 1968). This opens a possibility to follow all stages of equilibrium unfolding or refolding of a protein and to study equilibrium intermediates, which may be well populated at moderate denaturant concentrations.

It has been shown experimentally that in many cases urea- or GdmCl-induced transitions in small globular proteins can be described in terms of a *two-state* model (Tanford, 1968; Privalov, 1979), which has led to an assumption of the absence of any equilibrium states of protein molecules intermediate between native and unfolded ones (Privalov, 1979, 1992).

On the other hand, it has been established that carbonic anhydrase B (Wong & Tanford, 1973), β-lactamase (Robson & Pain, 1976a,b), bovine (Kuwajima *et al.*, 1976) and human (Nozaka *et al.*, 1978) α-lactalbumins, β-lactoglobulin (Anantanarayanan *et al.*, 1977), and many other proteins [see Ptitsyn (1992) and Bychkova and Ptitsyn (1993) for recent reviews] are unfolded by urea or GdmCl through an *intermediate state* which has a pronounced secondary structure but virtually no pronounced CD spectrum in the near UV region and no enzymatic activity (i.e., no rigid tertiary structure). This state later has been identified (Dolgikh *et al.*, 1981, 1983, 1985) as a specific state of protein molecules which is compact, has a loosely packed protein core and nativelike content of secondary structure, but has no (or has only traces of) rigid tertiary structure, and fluctuates much more strongly than the native state [for reviews see Ptitsyn (1987, 1992), Kuwajima (1989), Christensen and Pain (1991), Baldwin (1991), and Dobson (1992) and references therein]. It is now widely known as the "molten globule" state [the term was proposed by Ohgushi and Wada (1983)].

The molten globule state has been initially predicted (Ptitsyn, 1973) as a *specific* intermediate with a nativelike protein fold, in which α-helices and β-strands are more or less fixed in their approximately native positions in the sequence and 3D space. When this state was first described experimentally (Dolgikh *et al.*, 1981), no evidence on the presence or absence of this fold was available. This made disputable the exact meaning of the "molten globule" term (Kim & Baldwin, 1990; Baldwin, 1991), which may be applied to both specific and nonspecific folding intermediates.

Now evidence is accumulating that (at least in some cases) the pH-induced compact denatured state preserves many important features of the native fold (Baum *et al.*, 1989; Jeng *et al.*, 1990; Hughson *et al.*, 1990; Dobson *et al.*, 1991; Baldwin & Roder, 1991; Harding *et al.*, 1991; Peng & Kim, 1994). This, of course, does not exclude the possibility that a compact state with a pronounced secondary structure but without any well-defined fold may exist at some other conditions.

This paper is probably the first to provide evidence for the existence of *two* equilibrium intermediates between native and unfolded states. It shows that GdmCl-induced equilibrium unfolding of staphylococcal β-lactamase at low temperatures goes through two intermediates, one of which has all features typical of the molten globule state, while the other state is less compact and less strucutred than the first one but still much more compact and more structured than a fully unfolded state.

MATERIALS AND METHODS

Materials. β-Lactamase (EC 3.5.2.6, molecular mass 30.0 kDa) was purified from *Staphylococcus aureus* (strain PC1) by T. Picard (University of Newcastle-upon-Tyne). The homogeneity of the protein was checked by electrophoresis and chromatography in native and denaturing conditions.

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[1] Abbreviations: GdmCl, guanidinium chloride (guanidine hydrochloride); UV, ultraviolet; CD, circular dichroism; NMR, nuclear magnetic resonance; DTT, dithiothreitol; SEC, size-exclusion chromatography; R_s, Stokes radius; V^{el}, elutionvolume; FPLC, fast protein liquid chromatography; N, native state; MG, molten globule state; PF, partly folded state; U, unfolded state of protein molecule.

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Impurities did not exceed 2%. The protein concentrations were determined spectrophotometrically. Lysozyme (from hen egg white), bovine ribonuclease A, and sperm whale myoglobin were from Boehringer Mannheim GmbH (Combithek, Vienna, Austria), Sigma, and Serva, respectively, and were used without additional purification.

DTT was from Serva and was also used without purification. GdmCl was purified by recrystallization from water and from ethanol. The denaturant concentration in solution was measured by the refractive index. All solutions were prepared in double-distilled water and were passed through a 0.2-μm nylon 66 filter (Rainin Instrument Co.).

Preparation of Protein Solutions. The proteins were incubated at the desired temperature for 40 h in 100 mM sodium phosphate buffer, pH 6.8, containing the desired GdmCl concentration. Ribonuclease A was incubated in the same buffer with an additional of 100 mM DTT for 40 h to achieve a complete reduction of disulfide bonds. Protein concentrations for FPLC measurements were about 0.001 mg/mL, for activity measurements 0.1 mg/mL, and for CD measurements 0.8 mg/mL.

Experimental Procedures: (*A*) *Size-Exclusion Chromatography.* The stock solution of protein (containing GdmCl in the desired concentrations) was loaded on the column, equilibrated by the same buffer with the same GdmCl concentration. The flow rate was 20 mL/h. The elution profiles were obtained using a 2158 Uvicord SD instrument (LKB) equipped with a 226-nm filter. This permitted us to use very low protein concentrations. All measurements at 4 °C were made in a cold room.

(*B*) *Molecular Stokes radii* (R_s) were estimated from the elution volumes (V^{el}) measured by a calibrated SEC column (Uversky, 1993) according to

$$R_s = \frac{(1000/V^{el}) - 52.1}{0.725}$$

The SEC column was calibrated both in native conditions (100 mM sodium phosphate buffer, pH 6.8) and in the presence of 6 M GdmCl according to the known procedure (Ackers, 1967, 1970; Davies, 1983; Corbett & Roche, 1984). Stokes radii of proteins used for calibration were determined from the viscosity, sedimentation, and diffusion data [see Uversky (1993)]. The accuracy of determination of R_s values for the studied proteins was no less than 7%.

(*C*) *Relative areas of chromatographic peaks* were estimated from the elution profiles by their deconvolution using a curves synthezator (SC-2, Biopribor, Pushchino, Russia). The deconvolution accuracy was ~10%.

(*D*) *Enzymatic activity of β-lactamase* was measured by the rate of increase of absorption at 235 nm, which is the measure of benzylpenicillin cleavage (Jansson, 1965). The reaction was initiated by addition of 20 μL of the protein stock solution to 2000 μL of the reactive mixture containing benzylpenicillin (~0.1 mg/mL).

Equipment. Size-exclusion chromatography experiments were performed using a Superose-12 column and the FPLC equipment (Pharmacia, Uppsala, Sweden). Circular dichroism measurements were made with a Jasco-600 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan). Enzymatic activity measurements were performed using a Specord M40 spectrophotometer (Carl Zeiss, Jena, Germany). Fluorescent measurements were made with an Aminco (SPF-1000CS) corrected spectrofluorometer (American Instrument Corp., Silver Spring, MD). All these spectral instruments were equipped with a temperature-controlled holder.

RESULTS

Size-Exclusion Chromatography. The study of protein unfolding was performed by a combination of traditional methods (like near and far UV CD) and size-exclusion chromatography. SEC permits one to evaluate dimensions of protein molecules in different conformers (Ackers, 1967, 1970; Davies, 1983; Corbett & Roche, 1984; Uversky, 1993) with high accuracy at low concentrations (~0.01–0.001 mg/mL). It was also shown (Corbett & Roche, 1984; Uversky *et al.*, 1992, 1993; Uversky, 1993) that a chromatographic (FPLC or HPLC) column does not shift the equilibrium between the native, molten globule, and unfolded states and therefore can be safely applied to studying the transitions between these states.

Application of size-exclusion chromatography is most promising for studying "all-or-none" transitions between two states of different compactness, like native–coil (Corbett & Roche, 1984; Uversky, 1993) and molten globule–coil (Uversky *et al.*, 1992, 1993) transitions. If the time of exchange between "compact" and "less compact" states is larger than the characteristic time of chromatography (10–20 min), a *bimodal* distribution of molecules on their sizes is observed. In this case, size-exclusion chromatography opens a quite new possibility as it permits *separate* measurement of the dimensions of "compact" and "less compact" molecules (see, e.g., Figures 2B and 4B). Moreover, SEC in principle provides a possibility for *a separate study of other properties of "compact" and "less compact" molecules* by collecting a corresponding fraction from the column (Uversky *et al.*, 1992, 1993) or by combining SEC with structural methods, e.g, with fluorescence. This is a new kind of information since traditional structural methods (e.g., circular dichroism, viscosity, etc.) provide us with the data *averaged over all conformations which exist within the transition region.*

In the case of slow exchange it is possible to determine fractions of molecules in "compact" (f_C) and "less compact" (f_{LC}) states from the elution profile. These fractions are merely equal to relative areas of the corresponding elution peaks:

$$f_C = \frac{S_C}{S_C + S_{LC}} \quad f_{LC} = 1 - f_C = \frac{S_{LC}}{S_C + S_{LC}} \qquad (1)$$

where S_C and S_{LC} are the areas under the elution peaks corresponding to "compact" and "less compact" molecules, which can be compared with fractions of native (rigid and active) and denatured (nonrigid and nonactive) molecules. It is well-known that some parameters (e.g., biochemical activity or near UV CD) dramatically change upon denaturation, while they are almost the same for all denatured states. As denaturation of a small protein is an "all-or-none" process (Privalov, 1979), each of these parameters can be presented as superpositions of contributions of native (N) and denatured (D) molecules:

$$X = f_N X_N + f_D X_D = f_N X_N + (1 - f_N) X_D \qquad (2)$$

where X is the measured parameter and X_N and X_D are its values for native and denatured molecules, respectively, while f_N and f_D are fractions of native and denatured molecules. Equation 2 leads to the well-known equations:

$$f_D = \frac{X - X_N}{X_D - X_N} \quad f_N = 1 - f_D = \frac{X_D - X}{X_D - X_N} \qquad (3)$$

which are valid in all cases when protein denaturation as such is an "all-or-none" process, irrespective of other transitions which may follow denaturation.

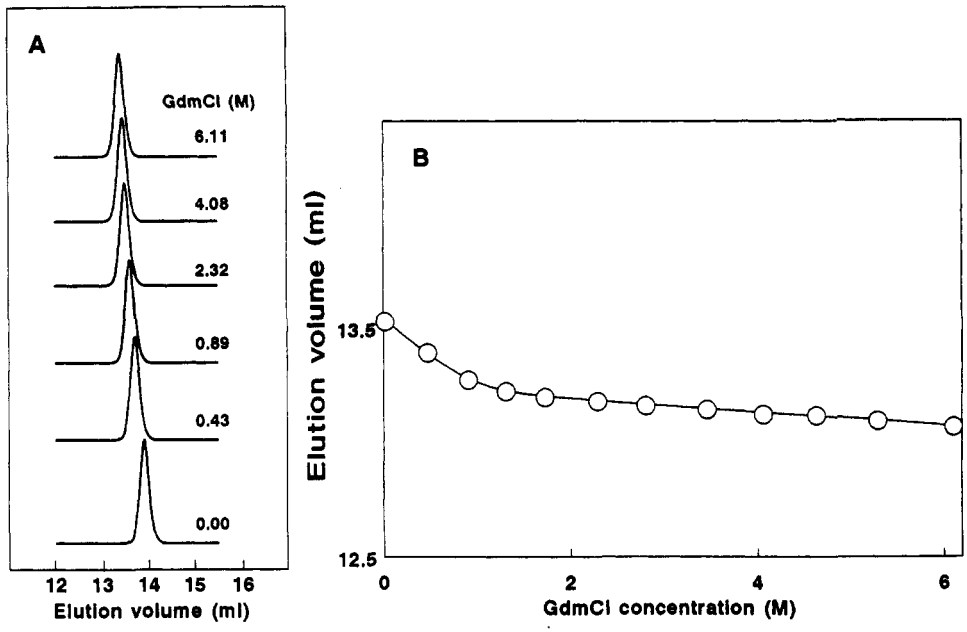

FIGURE 1: (A) Elution profiles of reduced ribonuclease A at 4 °C in 0.1 M sodium phosphate buffer, pH 6.8, for different GdmCl concentrations. (B) Dependence of elution volume V_{el} on GdmCl concentration.

In the case of a two-state N ⇄ U transition between native and unfolded states, eqs 1 and 3 give identical information, as in this case $f_C = f_N$ and $f_{LC} = f_D = f_U$ (f_U is a fraction of unfolded molecules; see below, Figure 8A). However, in the case of three-state transition through a compact intermediate (molten globule) state, denaturation and unfolding become decoupled and eqs 1 and 3 permit us to determine the fraction of molecules in all three states, as $f_U = f_{LC}$ and

$$f_{MG} = f_D - f_{LC} = f_C - f_N \tag{4}$$

(see below, Figure 8B).

Even in the case of a four-state transition through a "compact" and "less compact" ("partly folded"; see below) intermediates when the "less compact" intermediate exchanges slowly with the "compact" intermediate but quickly with unfolded molecules, SEC permits us to evaluate separately fractions of molecules in both "less compact" states. In fact, elution volume averaged over the both "less compact" states (see below, Figure 4B) is equal to

$$\langle V_{LC}^{el} \rangle = \frac{f_{PF} V_{PF}^{el} + f_U V_U^{el}}{f_{PF} + f_U} = V_{PF}^{el} + \frac{f_U}{f_{LC}} (V_U^{el} - V_{PF}^{el}) \tag{5}$$

where f_{PF} is a fraction of the "less compact" ("partly folded") intermediate and V_{PF}^{el} is its elution volume. Therefore

$$f_U = f_{LC} \frac{\langle V_{LC}^{el} \rangle - V_{PF}^{el}}{V_U^{el} - V_{PF}^{el}} \tag{6}$$

and f_U (as well as $f_{PF} = f_{LC} - f_U$) can be calculated from f_{LC} and $\langle V_{LC}^{el} \rangle$ if the values of V_U^{el} and V_{PF}^{el} can be estimated (see below, Figure 8C).

If "compact" and "less compact" states are in a fast exchange, SEC can give only the value of elution volume averaged over all states of protein molecules (see below, Figure 3B):

$$\langle V^{el} \rangle = f_C \langle V_C^{el} \rangle + f_{LC} \langle V_{LC}^{el} \rangle \tag{7}$$

where $\langle V_C^{el} \rangle$ is the elution volume averaged over all "compact" states. It is very much similar to the usual solution methods which average protein parameters in a similar way:

$$X = f_N X_N + f_I X_I + f_U X_U \tag{8}$$

where f_I is the fraction of molecules in intermediate state(s) and X_I is the value of parameter X in this state (or its value averaged over all intermediates).

However, even in this case SEC has an important advantage, as eq 8 can be used to estimate values of f only if X_I is close either to X_U or to X_N. In the first case eq 8 is reduced to eq 2, while in the second case

$$X = f_N X_N + f_U X_U = (1 - f_U) X_N + f_U X_U \tag{9}$$

and

$$f_U = \frac{X - X_N}{X_U - X_N} \tag{10}$$

As elution volumes of the native and molten globule states are relatively close to each other, f_U can be evaluated from eq 10 as

$$f_U = \frac{\langle V^{el} \rangle - V_C}{V_U^{el} - V_C^{el}} \tag{11}$$

where elution volumes of "compact" (V_C^{el}) and unfolded (V_U^{el}) states can be obtained for any given denaturant concentration by extrapolation of baselines before and after transition.

This is an advantage of the elution volume as compared with, say, far UV CD spectra. In fact, far UV ellipticities are in general different in the native, intermediate, and unfolded states (Wong & Hamilin, 1974; Jagannadham & Balasubramanian, 1985; Bolotina, 1987; Rodionova *et al.*, 1989). Therefore, say, $\langle f_{220} \rangle$ is described by full equation 8 which limits the use of relative changes of $[\Theta]_{220}$

$$f_{220} = \frac{[\Theta]_{220} - [\Theta]_{220}^{N}}{[\Theta]_{220}^{U} - [\Theta]_{220}^{N}} \tag{12}$$

for a real estimate of protein unfolding. As for hydrodynamic and scattering properties of protein, they are also relatively close in the native and molten globule states, but it is very

Table 1: Molecular Dimensions of Proteins in Different Conformational States

	conformational states			
	compact (minimum)	compact (maximum)	less compact (minimum)	less compact (maximum)
Reduced Bovine Ribonuclease A (M = 13.7 kDa) (4 °C)				
GdmCl (M)	0.00		0.00	6.00
V^{el} (mL)	15.13[a]		13.55	13.07
R_s (Å)	19.3[a] (1.00)[b]		29.9 (1.55)	33.7 (1.75)
Hen Egg White Lysozyme (M = 14.3 kDa) (4 °C)				
GdmCl (M)	0.00	4.71	3.73	6.00
V^{el} (mL)	15.10	14.89	14.37	13.88
R_s (Å)	19.5 (1.00)	20.8 (1.07)	24.1 (1.24)	27.5 (1.41)
Sperm Whale Myoglobin[c] (M = 16.9 kDa) (4 °C)				
GdmCl (M)	0.00	1.96	1.14	6.00
V^{el} (mL)	14.97	14.62	13.07	12.50
R_s (Å)	21.4 (1.00)	22.5 (1.05)	33.7 (1.57)	38.5 (1.80)
S. aureus β-Lactamase (M = 30.0 kDa) (23 °C)				
GdmCl (M)	0.00			6.00
V^{el} (mL)	14.49			11.17
R_s (Å)	23.5 (1.00)			51.6 (2.20)
S. aureus β-Lactamase (M = 30.0 kDa) (4 °C)				
GdmCl (M)	0.00	0.92	0.28	6.00
V^{el} (mL)	14.46	13.88	13.36	11.05
R_s (Å)	23.5 (1.00)	27.2 (1.16)	31.4 (1.34)	53.0 (2.25)

[a] The values obtained for native bovine ribonuclease A with intact S–S bonds. [b] Figures in parentheses represent the increase of the R_s value as compared with the native state. [c] Data taken from Uversky (1993).

difficult to measure them in the transition region due to the tendency of an intermediate state to aggregation.

Unfolded Protein. To obtain reference data for size-exclusion chromatography of an unfolded protein, we have used reduced bovine ribonuclease A, which is widely considered as a model of unfolded protein in water solutions. Figure 1A presents FPLC data for this protein at different GdmCl concentrations. It shows a single elution peak which slightly shifts to smaller elution volumes with an increase in GdmCl concentration (see also Figure 1B). This shift reflects "normal" swelling of unfolded protein chains in good solvents (see below). It corresponds to a rather small increase of the Stokes radius—only by 13% between 0 and 6 M GdmCl (calculated from the data of Table 1). This behavior of the protein which does not undergo GdmCl-induced cooperative transition can be used as a reference point for other cases.

Two-State ($N \rightleftarrows U$) Transitions. Figure 2 presents FPLC data for hen egg white lysozyme, one of the proteins whose unfolding by a strong denaturant was successfully described by one sigmoidal curve, consistent with the "all-or-none" transition (Tanford *et al.*, 1966; Tanford, 1968). In accordance with this suggestion, Figure 2A demonstrates a *bimodal* distribution of lysozyme elution volumes in the transition region. This strictly shows that lysozyme undergoes an "all-or-none" transition between "compact" and "less compact" states, slowly exchanging with each other. The small (7%) swelling of the native state (see the top curve in Figure 2B) will be discussed below [see also Corbet and Roche (1984)]. It is important to emphasize that the swelling of a "less compact" state (see the lower curve in Figure 2B) is also relatively small—only by 14% between 3.7 and 6.0 M GdmCl (calculated from the data of Table 1). This complies with the idea that this state is really unfolded (cf. the data for unfolded ribonuclease presented above).

Figure 2C gives a comparison of a fraction of *denatured* molecules ($f_D = 1 - f_N$) obtained from near UV molar ellipticity with a fraction of "*less compact*" molecules, f_{LC}, obtained from the relative area under the corresponding elution peak

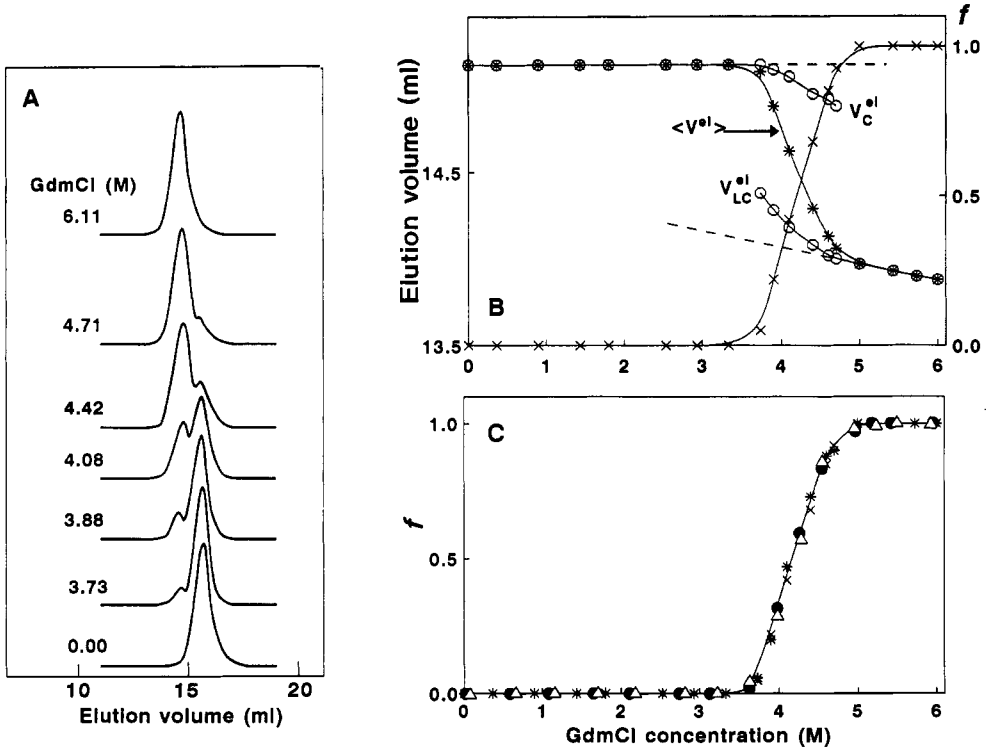

FIGURE 2: (A) Elution profiles of hen egg white lysozyme at 4 °C in 0.1 M sodium phosphate buffer, pH 6.8, for different GdmCl concentrations. (B) GdmCl dependence of fractions of "less compact" molecules (×), calculated by eq 1, and of elution volumes of "compact" ($\langle V_C^{el} \rangle$) and "less compact" ($\langle V_{LC}^{el} \rangle$) molecules (○). (*) GdmCl dependence of the average elution volume $\langle V^{el} \rangle$ calculated from $\langle V_C^{el} \rangle$ and $\langle V_{LC}^{el} \rangle$ by eq 7. Baselines are shown by dotted curves. (C) Comparison of the fraction of *denatured* molecules obtained from molar ellipticity $[\Theta]_{277}$ (●), the fraction of "less compact" molecules obtained from the relative area of a corresponding FPLC peak (×), and the fraction of *unfolded* molecules evaluated from averaged elution volume $\langle V^{el} \rangle$ (*) and the relative change of molar ellipticity $[\Theta]_{220}$ (△).

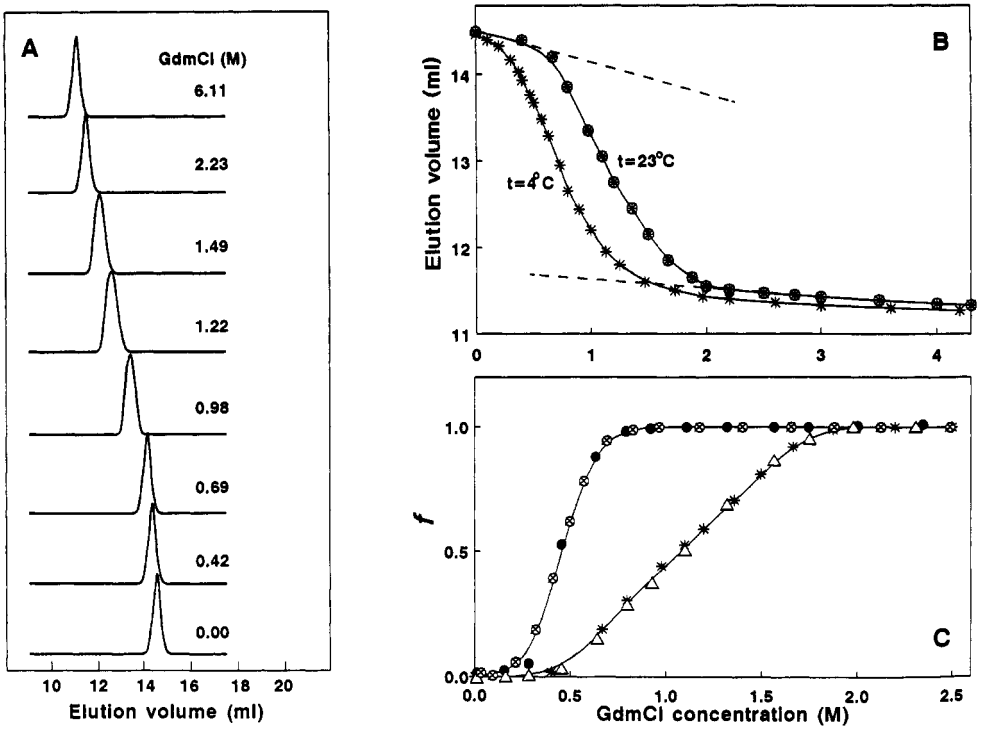

FIGURE 3: (A) Elution profiles of staphylococcal β-lactamase at 23 °C in 0.1 M sodium phosphate buffer, pH 6.8, for different GdmCl concentrations. (B) Dependence of average elution volumes $\langle V^{el} \rangle$ at 23 °C on the GdmCl concentration (*). Baselines are shown by dotted curves. The analogous dependence at 4 °C (see Figure 4B) is shown for comparison (*). (C) Comparison of the fraction of *denatured* molecules, obtained from esterase activity (⊗) and molar ellipticity $[\Theta]_{277}$ (●), with the fraction of *unfolded* molecules evaluated from the average elution volume $\langle V^{el} \rangle$ (*) and the relative change of molar ellipticity $[\Theta]_{220}$ (△).

and with the "unfolding degree" evaluated by eq 11 from the average elution volume. All three curves practically coincide, which shows that in this case the denaturation coincides with unfolding. In addition, the relative changes of far UV ellipticity also shown in Figure 2C—see eq 12—demonstrate that the secondary structure is destroyed in this case simultaneously with denaturation (i.e., the loss of tertiary structure) and unfolding (i.e., the increase of molecular dimensions). Similar results have been also obtained for sperm whale myoglobin unfolding by urea at 25 °C (Corbet & Roche, 1984) and by GdmCl at 4 °C (Uversky, 1993).

Three-State ($N \rightleftarrows U$) *Transition.* Figure 3 presents data for the GdmCl-induced unfolding of staphylococcal β-lactamase at 23 °C. It has been shown long ago that this protein (Robson & Pain, 1976a,b) is unfolded by GdmCl through a stable intermediate which later has been suggested (Christensen & Pain, 1991) to be the molten globule state.

Figure 3A shows that β-lactamase has only one elution peak which is substantially shifted to smaller elution volumes with the increase in GdmCl concentration (see also Figure 3B). This shift corresponds to a 2.2–2.3-fold increase in the β-lactamase Stokes radius, which is much larger than the GdmCl-dependent increase in dimensions obtained for the unfolded molecules: 1.13–1.14 for unfolded lysozyme, ribonuclease, and myoglobin (see above). A larger GdmCl-induced increase of protein dimenisons in the first case reflects the *shift of the equilibrium* between compact and unfolded states much more remarkably than the swelling of any of these states. This is additionally confirmed by the fact that the FPLC peaks of β-lactamase are much broader in the middle of transitions (where both compact and unfolded molecules are present) than outside the transition region (see Figure 3A).

Figure 3C presents a comparison of fractions of *denatured* molecules, obtained from the enzymatic activity and near UV ellipticity by eq 3, with a fraction of "less compact" (in this case unfolded) molecules, obtained from the average elution volume by eq 11. The great difference between denaturation and unfolding curves demonstrates the existence of a denatured but compact intermediate, whose population as a function of GdmCl concentration is shown in Figure 8B (see below). Figure 3C shows also relative changes of molar ellipticity in the far UV region f_{220}, which practically coincides with the f_U evaluated from $\langle V^{el} \rangle$. This means that the unfolding of molecules (i.e., an increase in their dimensions) in these cases is accompanied by disruption of their secondary structure. Thus, the equilibrium intermediate has no activity and no rigid tertiary structure but has nativelike compactness and nativelike secondary structure; i.e., it is the molten globule state.

Four-State ($N \rightleftarrows MG \rightleftarrows PF \rightleftarrows U$) *Transition.* The results on two- and three-state transitions provide a background for the data which we obtained for *Staphylococcus aureus* β-lactamase at low temperature.

Figure 4 shows our FPLC data (elution profiles and GdmCl dependence of elution volumes) for β-lactamase at 4 °C. The existence of two well-resolved elution peaks in the transition regions in Figure 4A [see also Uversky *et al.* (1992, 1993)] clearly shows the *bimodal* distribution of molecular dimensions. This means the existence of an "all-or-none" transition between "compact" and "less compact" states.

Unlike two-state transitions observed for myoglobin and lysozyme (see above), these transitions occur at larger GdmCl concentrations than protein denaturation. This is demonstrated in Figure 5, in which the fraction of molecules in a "*less compact*" *state* f_{LC} (obtained by eq 1 from the relative area of "left" peaks in Figure 3) is compared with the fraction of *denatured* molecules, obtained from near UV ellipticity and activity (see Figure 5B) by eq 3; see also Figure 8B.

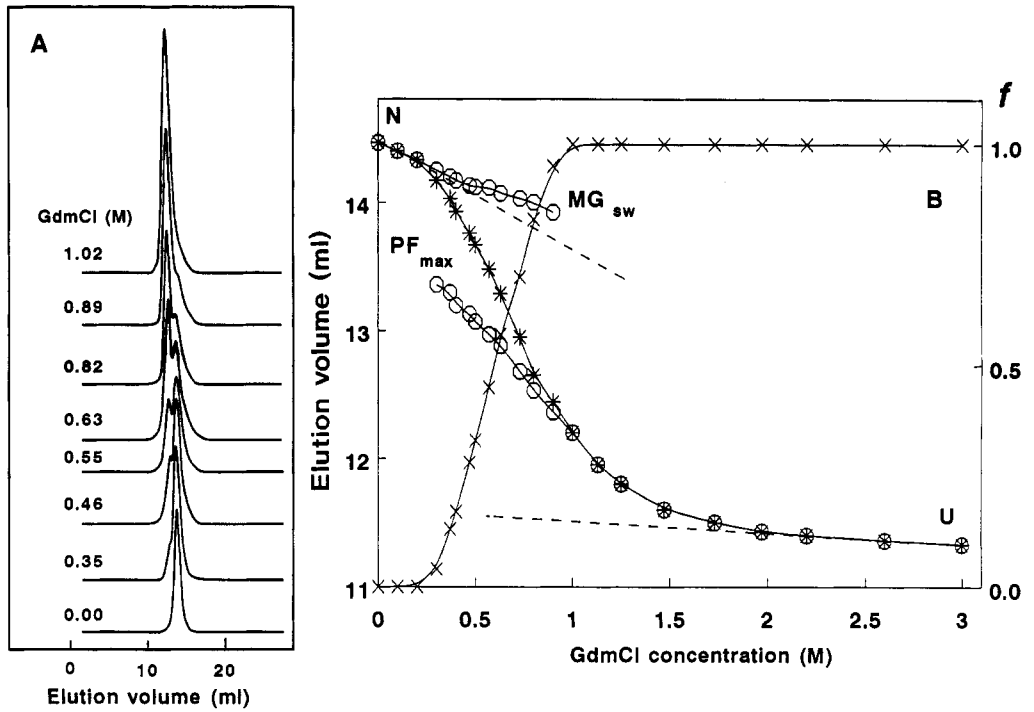

FIGURE 4: (A) Elution profiles of staphylococcal β-lactamase at 4 °C in 0.1 M sodium phosphate buffer, pH 6.8, for different GdmCl concentrations. (B) Dependence of the relative number of "less compact" molecules (×) and elution volumes (○) of "compact" $\langle V_C^{el} \rangle$ (top curve) and "less compact" $\langle V_{LC}^{el} \rangle$ (lower curve) molecules from GdmCl concentrations. (*) GdmCl dependence of the average elution volume $\langle V^{el} \rangle$ calculated from $\langle V_C^{el} \rangle$ and $\langle V_{LC}^{el} \rangle$. Baselines are shown by dotted curves.

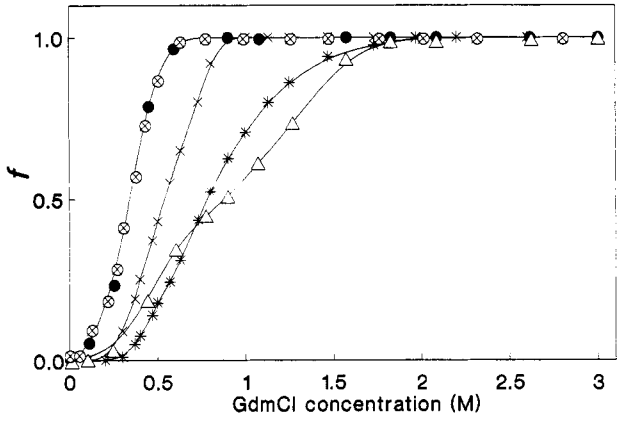

FIGURE 5: Stages of unfolding of staphylococcal β-lactamase at 4 °C: (⊗ and ●) fraction of *denatured* molecules obtained from the enzymatic activity and near UV ellipticity $[\Theta]_{277}$, respectively, by eq 3; (×) fraction of "less compact" molecules obtained from relative areas of corresponding FPLC peaks by eq 1; (*) fraction of *unfolded* molecules evaluated from the average elution volume $\langle V^{el} \rangle$ by eq 11; (△) relative change of far UV ellipticity $[\Theta]_{220}$ obtained by eq 12.

Figure 5 shows that in this case the protein molecules denature without a substantial increase in their molecular dimensions. The denaturation is followed by another "all-or-none" transition, which transforms the already denatured molecules in a "less compact" state. This means that protein unfolding involves at least two "all-or-none" transitions – the first one is protein *denaturation* and the second one is protein *expansion* to a "less compact" state (Uversky *et al.*, 1992, 1993). Usually only the second transition can be observed by size-exclusion chromatography, as only this transition is accompanied by a substantial increase of molecular dimensions.

Figure 4B shows also the GdmCl dependence of the elution volume averaged over "compact" and "less compact" states by eq 7. It is just the elution volume which we could observe if "compact" and "less compact" states would exchange rapidly even at a low temperature. Its comparison with the same elution volumes observed at room temperature (where two states really interconvert rapidly) is shown in Figure 3B. GdmCl dependences of average elution volumes are similar at room and low temperatures; the only difference is that at a low temperature protein molecules are less stable, as is usually the case for globular proteins (Privalov, 1992).

The upper and lower curves in Figure 4B present elution volumes of "compact" and "less compact" states of β-lactamase. One can see that the elution volume of a compact state slightly decreases with the increase in GdmCl concentration. Similar behavior has also been observed for myoglobin (Corbett & Roche, 1984; Uversky, 1993) and lysozyme (see Figure 2B) and has been attributed to the small swelling of native molecules (Corbett & Roche, 1984). However, this decrease can also reflect the transition of molecules from the native state to the slightly less compact molten globule, or it may be the result of a superposition of these processes.

As for the behavior of "less compact" molecules (see the lower curve in Figure 4B), we can see two well-defined stages in the GdmCl dependence of their elution volumes. From the very appearance of "less compact" molecules, their elution volume drastically decreases with an increase in GdmCl concentration. However, at larger (more than 2 M) GdmCl concentrations, the dependence of the elution volume on the denaturant concentration becomes much less pronounced and very similar to those obtained for unfolded proteins [see Figures 1 and 2 as well as Corbett and Roche (1984) and Uversky (1993)]. The small dependence almost certainly reflects the "normal" swelling of unfolded protein molecules in good solvents [see, e.g., Damaschun *et al.* (1991)], well described by the classical Flory's theory (Flory, 1953).

A much stronger GdmCl dependence of V_{LC}^{el} at a lower GdmCl concentration cannot be explained by "normal" expansion of random coil and can be attributed to the presence

(within the frames of the elution peak corresponding to "less compact" protein molecules) of intermediate(s) rapidly interconvertible with the unfolded state.

Figure 4B shows also that the elution volume V_{LC}^{el} of "less compact" molecules (and consequently the average elution volume $\langle V^{el} \rangle$) progressively decreases long after the "all-or-none" transition between "compact" and "less compact" states (monitored by the relative area of the corresponding FPLC peaks) is over, i.e., at GdmCl concentrations at which all protein molecules are already in a "less compact" state. This differs from such transitions as denaturation of lysozyme (Figure 2B) or myoglobin (Uversky, 1993), in which only a very small decrease of V_{LC}^{el} (and correspondingly of $\langle V^{el} \rangle$) is observed outside the transition region.

Figure 5 compares GdmCl dependence of the activity (or near UV ellipticity) and of the fraction of "less compact" molecules (f_{LC}) with GdmCl dependence of an average elution volume $\langle V^{el} \rangle$. All data are given in relative units—see eqs 1, 3, and 11. The figure shows the existence of at least *three* stages of protein unfolding: (1) protein *denaturation* (measured by enzymatic activity or near UV circular dichroism), (2) two-state *transition into a "less compact" state* (measured by a bimodal distribution of elution volumes), and (3) *real protein unfolding* (measured by a decrease of the average elution volume). For comparison, Figure 5 also shows relative changes f_{220} in the far UV molar ellipticity $[\Theta]_{220}$, obtained from Figure 6B (see below) by eq 12 (this curve will be discussed below).

The existence of three well-resolved phases of unfolding suggests the presence of at least two stable intermediates between the native and unfolded states. These data show that one of these intermediates is compact and exchanges very slowly with "less compact" state(s) while the other intermediate (or intermediates) is less compact and rapidly exchanges with the unfolded state.

Additional information on the existence of less compact intermediate(s) at rapid interconversion with the unfolded state is suggested by the study of the shape of the FPLC profiles near the middle of transition between a compact and a less compact state. It looks like a superposition of a narrow and symmetrical peak of compact molecules on a broader and asymmetrical peak of less compact molecules, which suggests the existence of molecules with different elution volumes within the latter. Analogous broadening of FPLC peaks has been observed for β-lactamase and carbonic anhydrase as well as human and bovine α-lactalbumins (Uversky, 1993) in the region of coexistence of rapidly exchanging compact and unfolded molecules (see, for example, Figure 3A).

The above results show that the GdmCl-induced unfolding of β-lactamase at low temperature goes through at least two equilibrium intermediates: the first one with compactness similar to that of the native state and the other one(s) with compactness intermediate between the native and the unfolded states. The first intermediate has all the features of a typical molten globule [see also Uversky *et al.* (1992, 1993)]—it is compact and its far UV CD spectrum suggests the existence of a very pronounced secondary structure (see Figure 6), while its near UV CD spectrum is almost absent, which is evidence for the absence of rigid tertiary structure (see Figure 7).

Thus there is little doubt that the first intermediate in low-temperature unfolding is the molten globule. What are the properties of the second intermediate which we call a "*partly folded*" *state* of protein molecules?

Properties of a "Partly Folded" State: *(A) Compactness.* A deeper insight into the properties of the second intermediate

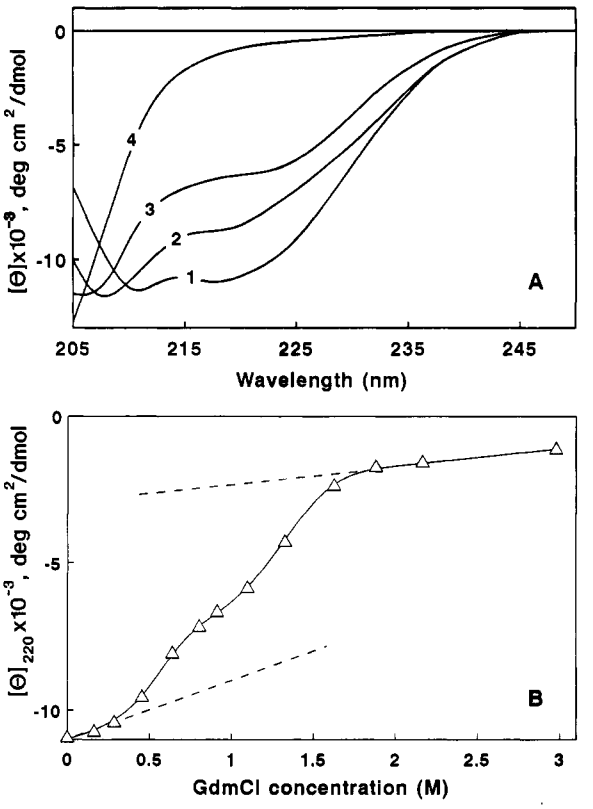

FIGURE 6: (A) Far UV circular dichroism spectra of staphylococcal β-lactamase at 4 °C in 0.1 M sodium phosphate buffer (pH 6.8) at different GdmCl concentrations: (1) 0 M (N-state), (2) 0.64 M (mixture of MG-, PF-, and U-states), (3) 0.91 M (mixture of PF- and U-states), and (4) 4.84 M GdmCl (U-state). (B) GdmCl dependence of $[\Theta]_{220}$ (Δ). Baseline for the unfolded state is shown by a dotted curve.

can be obtained if we use the unique capability of size-exclusion chromatography to study separately "compact" and "less compact" states when these states interconvert slowly enough to be resolved by this technique.

Table 1 presents Stokes radii calculated for β-lactamase from its elution volumes at different GdmCl concentrations, which correspond to the maximal and minimal values of the elution volumes of "compact" (top curves) and "less compact" molecules. Maximal and minimal elution volumes of "compact" molecules correspond to the native state and the swollen molten globule state, respectively (see Figure 4B, N and MG_{sw}). Maximal and minimal elution volumes of "less compact" molecules correspond to the partly folded (upper estimate; see below) and unfolded states, respectively (see Figure 4B, PF_{max} and U). We can estimate only the top value of the elution volume of a "partly folded" state as the maximal elution volume of "less compact" molecules. These volumes should be equal if all less compact molecules at the point of their appearance were "partly folded".

It is seen that the swollen molten globule state of β-lactamase has a Stokes radius ~16% larger than in the native state. This exactly coincides with the analogous estimates (14% ± 2%) for acid- and temperature-induced molten globule states for bovine and human α-lactalbumins (Ptitsyn, 1992) obtained from sedimentation (Gilmanshin *et al.*, 1982), dynamic light scattering (Gast *et al.*, 1986), and diffusion (Bychkova *et al.*, 1990) data. The upper estimate of Stokes radius of "partly folded" molecules is 34% larger than its native value, while the Stokes radius of unfolded molecules is 2.2 times larger than the native one.

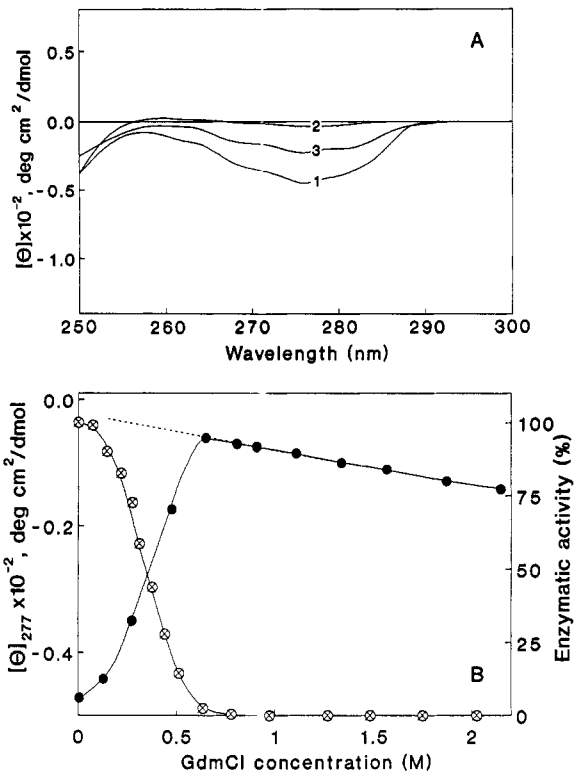

FIGURE 7: (A) Near UV circular dichroism spectra of staphylococcal β-lactamase at 4 °C in 0.1 M sodium phosphate buffer (pH 6.8) at different GdmCl concentrations: (1) 0 M, (2) 0.64 M, and (3) 4.84 M GdmCl. Curves 1 and 3 refer to N- and U-states, respectively, while curve 2 corresponds to the GdmCl concentration at which β-lactamase loses its enzymatic activity. Spectra are weakly pronounced as β-lactamase does not contain tryptophan residues. (B) GdmCl dependence of enzymatic activity (⊗) and $[\Theta]_{277}$ (●). Baselines are shown by dotted curves.

Stokes volumes V_S[3] calculated from these R_S values in the native (~54 000 Å^3), molten globule (~84 000 Å^3), partly folded (~120 000 Å^3) and unfolded (~620 000 Å^3) states show that the "partly folded" state belongs to the class of reasonably compact states. In fact, its hydrodynamic volume is only 1.5 times larger than in the molten globule state and 2.2 times larger than in the native state (as compared with the 11-fold increase of the volume in the unfolded state).

(B) Secondary Structure. Figure 6A shows far UV circular dichroism spectra of β-lactamase at 4 °C at different GdmCl concentrations, which correspond to their native state, the point of vanishing of native (active) molecules (0.64 M), the point of vanishing of compact molecules (0.91 M), and the point at which all molecules are completely unfolded (4.84 M). These spectra at intermediate GdmCl concentrations refer to the *mixture* of different states: curve 2 corresponds to *denatured* molecules (i.e., to the mixture of MG-, PF-, and U-states), while curve 3 corresponds to *less compact* molecules (i.e., to the mixture of PF- and U-states).

Figure 6B shows GdmCl dependence of $[\Theta]_{220}$ (see also Figure 5) which consists of two phases with a bend at ~1 M GdmCl, i.e., near the point of disappearance of compact molecules. This suggests that GdmCl dependence of $[\Theta]_{220}$ is a superposition of at least two curves; the first curve may reflect the transition of compact molecules into the parly folded state, while the second curve is referred to further unfolding of protein molecules.

(C) Populations. The main results of our studies of two- and multiple-state protein unfolding are presented in Figure 8. Figure 8A shows the results for *two-state transition*, in

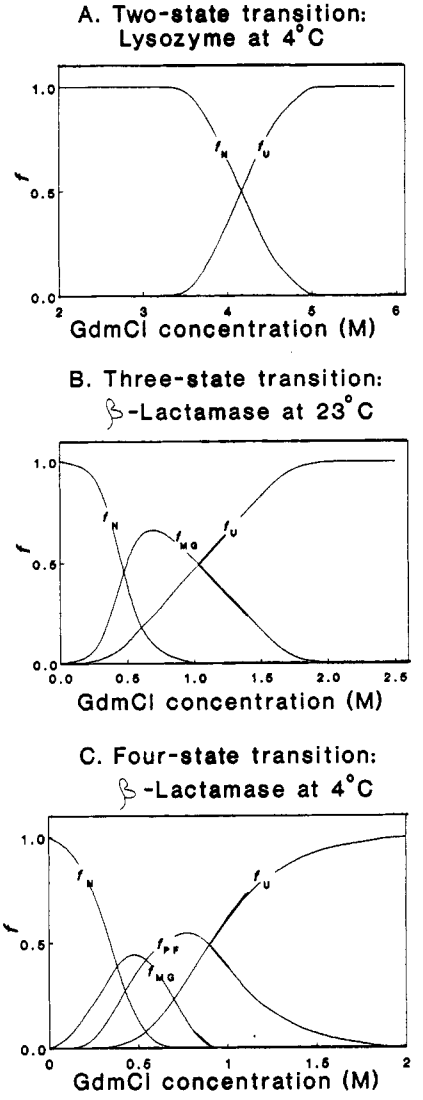

FIGURE 8: (A) Two-state transition in hen egg white lysozyme at 4 °C. The fraction of native molecules (f_N) was calculated from $[\Theta]_{270}$ by eq 3; the fraction of unfolded molecules (f_U) was obtained from the relative area of the corresponding FPLC peaks, the average elution volume $\langle V^{el} \rangle$, and $[\Theta]_{220}$ by eqs 1, 11, and 12, respectively. (B) Three-state transition in staphylococcal β-lactamase at 23 °C. The fraction of native (f_N) molecules was determined from the activity and near UV ellipticity data by eq 3; the fraction of unfolded molecules (f_U) was obtained from the average elution volume $\langle V^{el} \rangle$ and $[\Theta]_{220}$ by eq 11 and 12, respectively. In this case $f_N + f_U < 1$ and the fraction of the intermediate (molten globule) state $f_{MG} = f_{LC} - f_N$. (C) Four-state transitions in staphylococcal β-lactamase at 4 °C. The fraction of native molecules (f_N) was determined from the activity and near UV ellipticity by eq 3; the fraction of "less compact" molecules (f_{LC}) was obtained from the relative area of a corresponding FPLC peak by eq 1; the fraction of unfolded molecules (f_U) was obtained from the average elution volume of "less compact" molecules $\langle V^{el}_{LC} \rangle$ by eq 5. In this case $f_{MG} = f_C - f_N = 1 - f_{LC} - f_N$, but $f_{MG} + f_{LC} + f_N < 1$ and the fraction of a "less compact" intermediate (partaly folded state) $f_{PF} = 1 - f_{MG} - f_U$. In eq 5 V^{el}_U was obtained by extrapolation of the corresponding baseline, while instead of V^{el}_{PF} its upper estimate (see the text) was used.

which all protein molecules can be either in the native or in the completely unfolded state ($f_N + f_U = 1$). Figure 8B shows the results for *three-state transition* through a compact intermediate (the molten globule state), whose population can be obtained as a difference between fractions of unfolded and denatured molecules ($f_{MG} = f_D - f_U = 1 - f_N - f_U$).

Finally, Figure 8C shows the data for *four-state transition* through at least two intermediates, the compact molten globule state and the less compact partly folded state. A relative

number of native (f_N), molten globule (f_{MG}), partly folded (f_{PF}), and unfolded (f_U) molecules of β-lactamase as a function of GdmCl concentration has been calculated from three basic curves: the denaturation ($f_D = 1 - f_N$), monitored by near UV ellipticity and activity (see eq 3); the transition to a "less compact" state (f_{LC}), monitored by a relative area of the corresponding FPLC peak (see eq 1); and the unfolding (f_U), monitored by an elution volume of "less compact" molecules (see eq 5). Then relative populations of two intermediate states have been calculated by obvious equations:

$$f_{MG} = f_D - f_{LC} \quad f_{PF} = f_{LC} - f_U \tag{13}$$

Figure 8C shows how the molten globule state appears upon unfolding and then transforms into the partly folded state, which finally transits into the unfolded state. Though these three transitions partly overlap, it is quite clear that β-lactamase at 4 °C can exist in at least four quite different states: the native state, the molten globule state, the partly folded state(s), and the unfolded state.

DISCUSSION

Twenty years ago one of the authors of this paper (Ptitsyn, 1973) suggested a stage model of protein folding which later has been named (Kim & Baldwin, 1982) a "framework model". The main idea [see also Ptitsyn (1987, 1991, 1992)] was that protein folding consists of three main steps starting with the formation of embryos of fluctuating secondary structure, followed by the collapse of these embryos in a compact intermediate, and terminating by the transition of this compact intermediate into the native protein. An important assumption was that the main results achieved at each stage are not reconstructed very much at subsequent steps. This means that the fluctuating secondary structure is formed at the first stage around the native positions of α- and β-regions and that the rough mutual positions of these regions in a compact intermediate are close to those in the native protein. The discovery of the equilibrium molten globule state of proteins in mild denaturing conditions was the first indirect confirmation of this idea, as this equilibrium intermediate has been shown to have a number of important features of the protein 3D structure [see Baldwin (1991), Ptitsyn (1992), and Dobson (1992) for reviews and especially the recent paper of Peng and Kim (1994)]. It has been also revealed (Dolgikh *et al.*, 1984; Semisotnov *et al.*, 1987; Ptitsyn *et al.*, 1990; Goldberg *et al.*, 1990) that proteins fold through a kinetic intermediate which has all properties of the molten globule state [see Ptitsyn (1991, 1992) and Ptitsyn and Semisotnov (1991) for recent reviews]. This kinetic molten globule state has been shown to have many important features of the native 3D structure, including nativelike locations of many α-helices and β-strands in the protein sequence (Udgaonkar & Baldwin, 1988, 1990; Roder *et al.*, 1988; Bycroft *et al.*, 1990; Matouscheck *et al.*, 1992a,b; Serano *et al.*, 1992; Roder *et al.*, 1988; Elöve *et al.*, 1992; Radford *et al.*, 1992), a nativelike (but less tightly packed) protein core (Bycroft *et al.*, 1990; Matouscheck *et al.*, 1992a,b; Serano *et al.*, 1992), and even some nativelike epitopes (Goldenberg *et al.*, 1990). On the other hand, the existence of an equilibrium counterpart of this compact kinetic intermediate (the equilibrium molten globule state) has strongly contributed to the understanding of this kinetic intermediate.

Much less is known on the nature of the first kinetic intermediate. Many experiments (Kuwajima *et al.*, 1987, 1988; Gilmanshin & Ptitsyn, 1987; Goldberg *et al.*, 1990; Ptitsyn & Semisotnov, 1991; Elöve *et al.*, 1992; Radford *et al.*, 1992) have shown that proteins usually achieve from 50% to 100% of their native far UV circular dichroism within the first few milliseconds of their folding, which suggests a very fast formation of a rather substantial part of their secondary structure. The existence of a very fast stage of ANS binding (Ptitsyn & Semisotnov, 1991) and the very fast partial heme quenching of tryptophan fluorescence in cytochrome *c* (Elöve *et al.*, 1992) suggests that this "burst" stage of protein folding leads to a state which is partly compact; however, many additional experiments are needed to check this assumption.

It is possible that a new intermediate described in this paper can be an equilibrium counterpart of the first stage of the protein folding kinetics. In fact, it has a substantial part of the native secondary structure, is relatively compact, and according to our unpublished data for carbonic anhydrase, even weakly binds ANS.

There is also another analogy between the equilibrium partly folded state and the kinetic intermediate of protein folding. First, the kinetic intermediate accumulates upon protein folding, as it is formed much faster (within 0.01 s) than a subsequent intermediate, the molten globule state, which needs 0.1–1.0 s to be formed [see Ptitsyn (1991, 1992) for recent reviews]. This means that the first kinetic intermediate is separated by a high potential barrier from the kinetic molten globule state. On the other hand, the equilibrium partly folded state is also separated from the equilibrium molten globule state by a large potential barrier as the transition between these states belongs to the "all-or-none" type [see Uversky *et al.* (1992, 1993) and this paper].

Further experiments are required to check the assumption on the analogy between the equilibrium "partly folded" states of protein molecules revealed in this paper and the first kinetic intermediate of their folding. If this assumption is confirmed, it may open new possibilities for studying early kinetic intermediates of protein folding by their equilibrium counterparts.

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