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Folding of an all-β protein: Independent domain folding in γII-crystallin from calf eye lens

(eye lens proteins/domain structure/folding intermediate/protein folding/protein stability)

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ABSTRACT **γII-crystallin from calf eye lens consists of two homologous domains, each composed of two similar "Greek key" motifs. As a consequence of the bilobal structure, a biphasic transition is seen upon unfolding by urea at low pH (monitored by circular dichroism, fluorescence emission, and ultracentrifugal analysis). In 3.3 ± 0.5 M urea, a stable intermediate is formed at equilibrium, whereas 5.5 M urea causes maximum denaturation. Unfolding/folding kinetics display a complex pattern characterized by two kinetic phases. Both reactions exhibit strong dependence on the urea concentration; in the range of the respective transition, their rates are extremely slow ($k \approx 1 \times 10^{-4}\ s^{-1}$). The kinetic mechanism of unfolding and refolding may be described by a three-state model: native ⇌ intermediate ⇌ denatured. The rate-determining steps are domain folding rather than domain pairing or proline isomerization. Kinetic analysis of the unfolding/folding of the intermediate populated in 3.0 M urea, pH 2.0, reveals that the kinetic and the equilibrium intermediates have similar structures. Limited proteolysis of γII-crystallin by pepsin in 3 M urea, pH 2, allows the NH_2-terminal domain of the protein to be isolated. Unfolding/refolding of the fragment parallels the second transition in the above scheme, thus proving that the intermediate contains the COOH-terminal domain in its random state, whereas the NH_2-terminal domain is still in its native conformation. In conclusion, folding of γII-crystallin proceeds through the independent sequential structuring of the domains.**

Eye lens proteins are not subject to the general protein turnover; they remain in their native conformation during the whole life-span of the organism. The molecular mechanism underlying the extreme long-term stability is presently unknown.

γ-crystallins, the main components of the core region of the eye lens, have been characterized in detail: the amino acid sequences of most proteins from calf eye lens are known, and for γII-crystallin, the crystal structure has been solved at high resolution (1). The protein is a monomer of 20 kDa that does not contain disulfide bonds; it consists of two homologous domains, each composed of two "Greek key" motifs forming a sandwich of two four-stranded antiparallel β-pleated sheets (2, 3). Because γII-crystallin is almost entirely composed of β-sheets, this protein should be a good model for the analysis of β-structure formation.

γII-crystallin is a highly stable protein that preserves its native state at pH 1–10. In 0.1 M phosphate buffer, pH 7, the protein is stable to 75°C; at higher temperature, irreversible aggregation occurs. In 7 M urea, denaturation is only seen at elevated temperature or low pH; under these conditions, a two-step equilibrium transition suggests independent domain folding (4).

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We report here equilibrium and kinetic studies on the domain folding of γII-crystallin. Sedimentation analysis, intrinsic fluorescence, circular dichroism, and thermal analysis prove that the overall reaction at pH 2 can be quantitatively described by a three-state model: $N \rightleftharpoons I \rightleftharpoons D$, where N, I, and D stand for the native, intermediate, and denatured states of the protein, respectively. I is an intermediate state common to both the equilibrium transition *and* the kinetics of denaturation/renaturation. As shown by equilibrium and kinetic folding experiments with the isolated NH_2-terminal domain (obtained by limited proteolysis), I represents the intact protein with its COOH-terminal domain unfolded.

MATERIALS AND METHODS

Preparation of γII-Crystallin and Its NH_2-Terminal Domain. γII-crystallin from calf eye lenses was prepared according to the method of Björk (5), modified by van Dam (6) and Nesslaüer (7). Purity of the protein was ascertained by SDS/PAGE, isoelectric focusing, and spectroscopic techniques: Phast-system (Pharmacia), fluorescence spectrophotometer MPF 44A (Hitachi/Perkin–Elmer), spectropolarimeter J-500/DP-500N (Jasco, Easton, MD). Fast protein liquid chromatographic separations (Pharmacia) made use of the gradient programmer GP-250 with UV-1 monitor.

To isolate the NH_2-terminal domain fragment, partially unfolded γII-crystallin (obtained by 24-hr incubation of the protein in 3 M urea/0.1 M NaCl/HCl, pH 2.0, at 20°C) was subjected to limited proteolysis by pepsin [pepsin/γII-crystallin ratio 1:10 (wt/wt)]. Purification by gradient elution chromatography on S-Sepharose (0–0.3 M NaCl in 50 mM sodium acetate buffer, pH 5.0/3 M urea/1 mM EDTA/1 mM dithioerythritol, led to electrophoretically pure material (8). NH_2-terminal and COOH-terminal amino acid sequence analysis of the fragment revealed that the NH_2-terminal domain remains intact; Phe-88 becomes the COOH terminus. The COOH-terminal domain is digested to small fragments (9). To further characterize the fragment, analytical ultracentrifugation (Beckman, Spinco model E) was used in addition to the previously mentioned spectroscopic techniques.

Ultrapure urea was supplied by Schwarz/Mann; all other chemicals were of analytical grade. Bi-distilled water was used throughout.

Equilibrium and Kinetics of Unfolding/Folding. The urea-induced unfolding transitions of γII-crystallin at equilibrium were determined by incubating the samples at various urea concentrations for 24 hr in 0.1 M NaCl/HCl, pH 2.0 (20°C). Denaturation was followed by dichroic absorption at 222 nm, fluorescence emission (λ_{exc} = 280 nm), and sedimentation analysis. To monitor the kinetics of unfolding, the change in

Abbreviations: N, native; I, intermediate; D, denatured (these abbreviations are restricted to equations).

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fluorescence emission at 320 and 360 nm was measured at various urea concentrations. For the refolding kinetics, the protein was first denatured completely (incubation in 8 M urea, pH 2.0, for 5 hr, 20°C) or transformed into the stable intermediate (3 M urea, pH 2.0, for 24 hr, 20°C); subsequently refolding was initiated by adding renaturation buffer in 20-fold excess (final protein concentration was 20 μg/ml for 0.5–2.0 M urea and 46 μg/ml for concentrations of urea > 2.0 M).

The kinetics of the $N \rightleftharpoons I$ transition was also studied by gel-filtration (fast protein liquid chromatography on Superose 12, HR 10/30) measuring heights of the corresponding peaks at 280 nm over time. The approach is based on the fact that retention times for N and I significantly differ (32.6 and 35.6 min, respectively), whereas no distinction between retention times for I and D can be made. About 10 μg of protein, withdrawn at different time intervals, was applied to the column. The elution was accomplished with 0.1 M glycine/HCl buffer, pH 2.0, at a flow rate of 0.5 ml/min.

Analysis of the kinetic data made use of the algorithm developed by Hagerman and Baldwin (10).

RESULTS AND DISCUSSION

γII-Crystallin Shows a Biphasic Unfolding Equilibrium Transition. The urea-induced equilibrium unfolding transitions of γII-crystallin at acidic pH are biphasic (Fig. 1). As monitored by circular dichroism, fluorescence emission, and sedimentation analysis a stable intermediate (I) dominates at a urea concentration of ≈3 M. Beyond 4 M urea, I is transformed to the denatured state D. The fact that the amplitudes of the two transitions vary at different wavelengths of fluorescence emission may be attributed (*i*) to differences in the environments of the fluorophores and (*ii*) to domain interactions involved in the $N \rightleftharpoons I$ transition. For the change in sedimentation velocity one would expect that I would show a higher anisotropy compared with N. This is clearly reflected by the decrease in $s_{20,w}$. Up to ≈1 M urea, no change in the native conformation of γII-crystallin is detectable, even at a pH as low as 2; on the other hand, beyond 5.5 M urea, all structural parameters reach a plateau value, suggesting that the protein is in its randomized state. The fact that under this condition the sedimentation coefficient exhibits an exceedingly low value proves that the coil must be extended due to the high positive net charge of the polypeptide chain at pH 2. At 2.8–3.8 M urea, pH 2.0, a stable equilibrium intermediate I is formed so that the overall reaction may be described by a three-state model according to $N \rightleftharpoons I \rightleftharpoons D$. The denaturation and renaturation profiles coincide, proving that both steps in the sequential reaction are reversible, with midpoints of transition at 2.0 and 4.5 M urea, respectively.

Limited proteolysis by pepsin in 3 M urea, pH 2.0, allows γII-crystallin to be cleaved into the intact NH_2-terminal domain (residues 1–88) and smaller fragments originating from the COOH-terminal part of the polypeptide chain. As shown below, the intermediate I and the NH_2-terminal domain exhibit similar properties (cf. Fig. 5).

Because the yield of renaturation from the unfolded to the native state ($D \rightarrow N$) is close to 100%, kinetics of the various reactions can be studied in both the forward and the reverse directions. The corresponding analyses were done under conditions defined by the equilibrium experiments, covering the whole range of urea concentrations.

γII-Crystallin Shows Biphasic Folding Kinetics. The kinetics of *unfolding* are monophasic below 3 M urea and biphasic above 3 M urea. Fig. 2 illustrates the time course at 3.0 and 4.5 M urea, using the increase in fluorescence at 360 nm to monitor unfolding. At 4.5 M urea, the unfolding kinetics display a complex pattern. "Pealing off" (10) allows a fast kinetic phase (relaxation time τ = 200 s), which accounts for ≈75% of the total amplitude to be separated from a slow phase (τ = 7200 s), which accounts for ≈25% of the total amplitude. Relaxation times of the two reactions strongly depend on urea concentration (cf. Fig. 4). With regard to the kinetic mechanism, the two phases can be attributed to the (fast) $N \rightarrow I$ and the (slow) $I \rightarrow D$ transitions. In the range of 4–6 M urea, the relative proportion of the amplitudes remains constant. The sum of both amplitudes accounts for the complete change in fluorescence, as expected from the equilibrium transition. Under strongly denaturing conditions (>6 M urea), part of the fluorescence change escapes detection.

To follow the kinetics of *refolding*, urea concentration was shifted from 8.0 M (complete denaturation after >5-hr incubation) to various final concentrations between 5.0 and 0.5 M. Fig. 3 depicts the refolding kinetics at 1.0 and 3.0 M urea, monitored by the fluorescence emission at 360 and 320 nm, respectively. The kinetics at 1 M urea represent the sum of two phases (Fig. 3*A*). The faster one (τ = 57 s) may be correlated to the $D \rightarrow I$ step, whereas the slower one (τ = 2200 s) corresponds to $I \rightarrow N$. The slow phase emerges only under

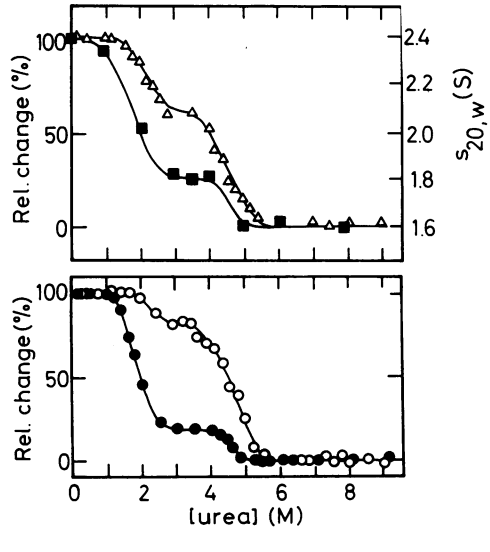

FIG. 1. Urea-induced unfolding of γII-crystallin in 0.1 M NaCl/HCl, pH 2.0, at 20°C. (*Upper*) Circular dichroism (△) (protein concentration, 100 μg/ml); sedimentation coefficient $s_{20,w}$ (■) (protein concentration, 200 μg/ml). S, Svedberg units. (*Lower*) Relative (Rel.) change in fluorescence emission at 360 nm (●) and 320 nm (○) (protein concentration, 40 μg/ml).

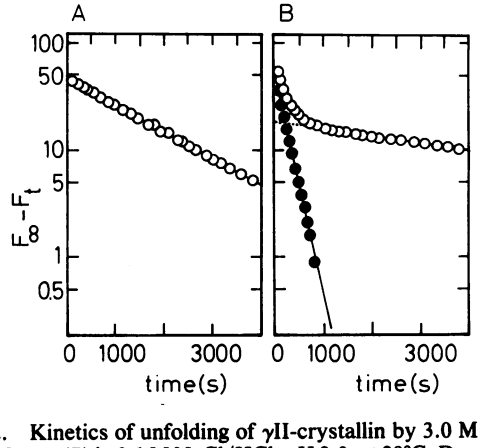

FIG. 2. Kinetics of unfolding of γII-crystallin by 3.0 M urea (*A*) and 4.5 M urea (*B*) in 0.1 M NaCl/HCl, pH 2.0, at 20°C. Denaturation was initiated by 50-fold dilution of native protein, pH 2.0, in the corresponding urea solution. Fluorescence emission at 360 nm, protein concentration 37 μg/ml. F_t and F_∞, fluorescence signals at time t and in the end of the experiment, respectively.

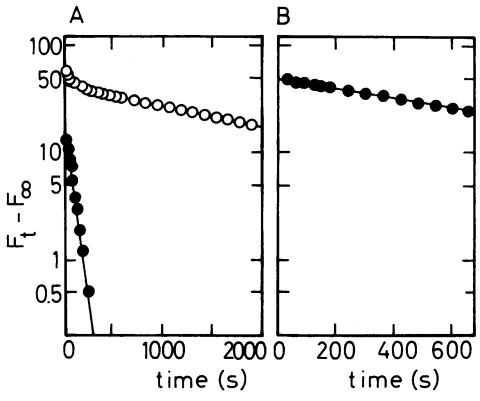

FIG. 3. Kinetics of refolding of γII-crystallin after denaturation in 8.0 M urea, pH 2.0, 20°C. (*A*) Refolding in 1.0 M urea monitored by fluorescence emission at 360 nm. (*B*) Refolding in 3.0 M urea monitored by fluorescence emission at 320 nm. F_t and F_∞, fluorescence signals at time *t* and in the end of the experiment, respectively.

native-like conditions (≤2.0 M urea); during refolding under conditions where mainly *I* is populated at equilibrium (2.5–3.0 M urea), one single (fast) relaxation is detectable (Fig. 3*B*). As for denaturation kinetics, the relaxation times depend strongly on urea concentration: the reactions become >20-fold faster by decreasing urea concentration from 2.0 to 0.5 M (Fig. 4). The sum of both amplitudes accounts for the whole change in fluorescence expected from the equilibrium data.

Summarizing the dependence of relaxation times on the urea concentration, two separate V-shaped profiles are obtained (Fig. 4). The respective partial reactions correspond to the $N \rightarrow I$, $I \rightarrow N$, $D \rightarrow I$, and $I \rightarrow D$ transitions, in agreement with the three-state model with one single intermediate on the pathway of denaturation/renaturation.

Folding/Unfolding of the Equilibrium Intermediate Are Single-Phase Reactions. To compare the characteristics of the kinetic intermediate with the folding behavior of the equilibrium intermediate, denaturation/renaturation kinetics were determined starting with the intermediate equilibrated at 3.0 M urea, pH 2.0. Both denaturation and renaturation of the equilibrium intermediate confirm the above mentioned ki-

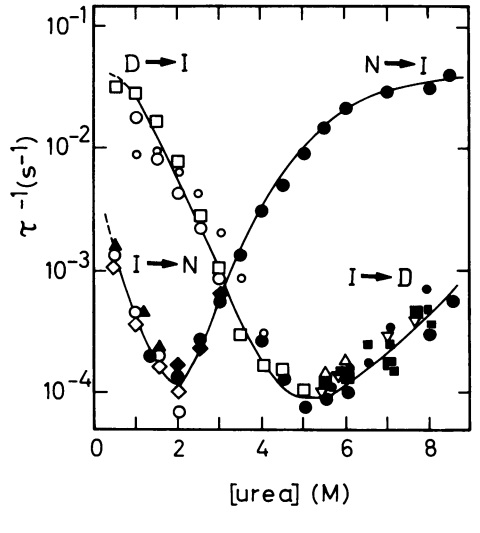

FIG. 4. Dependence of rate constants of denaturation/renaturation of γII-crystallin on urea concentration: 0.1 M NaCl/HCl, pH 2.0, at 20°C. Fluorescence emission of native γII-crystallin at 360 nm (unfolding ●, folding ○), 320 nm (unfolding ■, folding □), fast protein liquid chromatography gel filtration (unfolding ◆, folding ◇). Fluorescence emission of intermediate (*I*) at 360 nm (unfolding ▲, folding △) and 320 nm (unfolding ▽). Fluorescence emission of NH_2-terminal fragment at 360 nm (unfolding ●, folding ○) and 320 nm (unfolding ■).

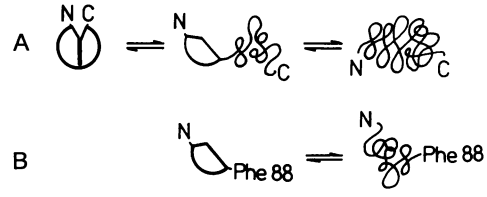

FIG. 5. Schematic representation of the unfolding/folding of γII-crystallin (*A*) and its NH_2-terminal domain fragment (*B*). N, NH_2 terminal; C, COOH terminal.

netic analysis (Fig. 4). Unfolding of the intermediate exhibits only the slow phase with relaxation times indistinguishable from the slow unfolding of the native protein (previously assigned to $I \rightarrow D$). On the other hand, refolding of the intermediate reflects the slow renaturation step of the completely unfolded molecule ($I \rightarrow N$). The results prove that the kinetic and equilibrium intermediates are identical.

Fluorescence measurements underlying the previous data have been supplemented by fast protein liquid chromatography analyses on Superose 12 (HR 10/30). The time-dependent changes in the elution profiles during the $N \rightleftharpoons I$ transition corroborate the fluorescence kinetics within the range of error (Fig. 4).

The NH_2-Terminal Domain of γII-Crystallin Folds Independently. A simple model explaining the equilibrium and kinetic data can be deduced from the domain structure of γII-crystallin. As illustrated in Fig. 5*A*, the three states may be attributed to (*i*) the native protein with its two homologous domains, each forming a sandwich of two four-stranded antiparallel β-pleated sheets, (*ii*) the intermediate with the NH_2-terminal domain intact, and (*iii*) the fully randomized protein. To prove this model, the NH_2-terminal domain (residues 1–88, isolated by limited proteolysis) was investigated with respect to stability and folding properties. Fig. 6 shows that its equilibrium transition occurs at 4.4 M urea, in agreement with the $I \rightarrow D$ transition of the intact protein depicted in Fig. 1. The kinetics of denaturation/renaturation show only one relaxation (as monitored by fluorescence emission at 320 and 360 nm). Except for a slight deviation under strongly native conditions, the corresponding rate constants confirm the $I \rightarrow D$ and $D \rightarrow I$ transitions seen for the intact two-domain protein, thus corroborating the model (Fig. 5*B*).

Further evidence is obtained from calorimetric studies where the enthalpy of unfolding (ΔH_{cal}) of the complete molecule has been found to be twice the apparent enthalpy ($\Delta H_{van't\ Hoff}$) determined from the temperature dependence of the equilibrium constants (9). In this context, we note that calorimetric measurements yield bimodal scans with $T_{m,1}$ =

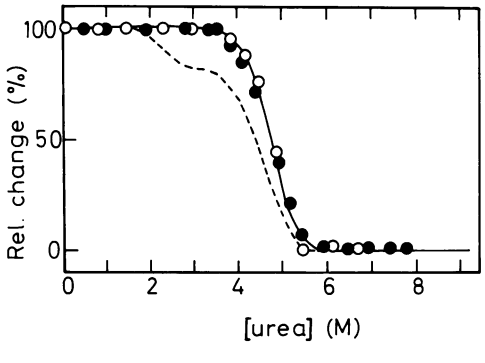

FIG. 6. Urea-induced unfolding (●) and refolding (○) of the NH_2-terminal fragment of γII-crystallin in 0.1 M NaCl/HCl, pH 2.0, at 20°C. Relative (Rel) change in fluorescence emission at 320 nm (protein concentration, 20 μg/ml). ---, Denaturation transition of intact γII-crystallin (cf. Fig. 1 *Lower*).

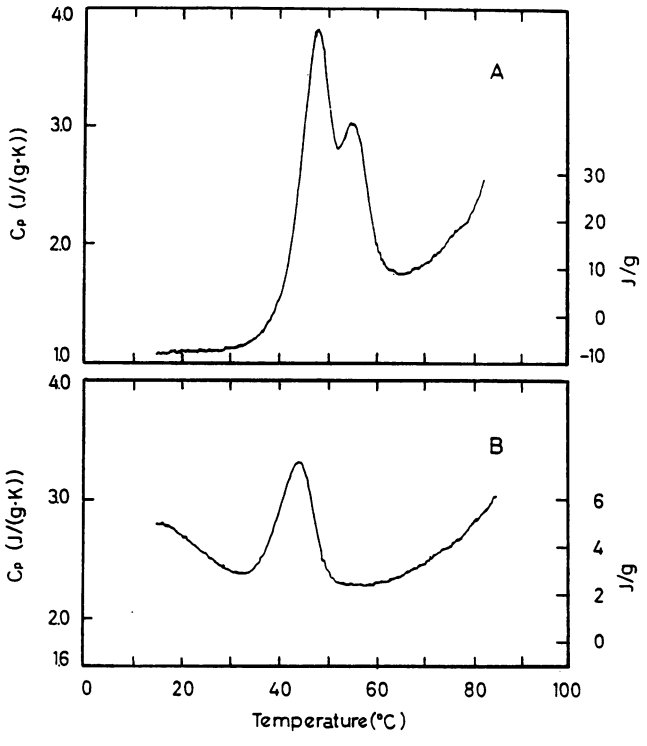

FIG. 7. Temperature dependence of the molar heat capacity (Cp) of γII-crystallin in 0.1 M NaCl/HCl, pH 2.0. Protein concentration, 2.0 mg/ml; heating rate 1°C/min. (*A*) Thermal transition $N \rightarrow I \rightarrow D$ in the absence of urea. (*B*) Thermal transition $I \rightarrow D$ in the presence of 3.0 M urea.

48°C and $T_{m,2}$ = 56°C, corresponding to the independent "melting" of the COOH- and NH_2-terminal domains in the $N \rightarrow I$ and $I \rightarrow D$ transitions, respectively (Fig. 7). Thermal unfolding of the intermediate state yields only one single transition, corresponding to the unfolding of the NH_2-terminal domain. In this case, the shift in T_m from 56°C to 43°C can be attributed to 3 M urea.

Domain pairing is not rate-determining in the process of renaturation of γII-crystallin: in contrast to other two-domain proteins, where merging of domains has been found to depend on solvent viscosity (11), refolding of γII-crystallin is not affected by saccharose or glycerol (9).

CONCLUSIONS

There is clear evidence from structural data that γII-crystallin consists of two structurally independent domains. High-resolution x-ray analysis revealed that the high sequence homology between the domains is reflected in a strong similarity at the level of the 3-dimensional structure. Thus, biphasic transition profiles might be attributed to either of three mechanisms: (*i*) independent domain folding/unfolding, (*ii*) sequential domain separation with subsequent cooperative unfolding of the two domains, and (*iii*) formation of a loosely structured intermediate ("molten globule") followed by the "melting" of the whole polypeptide chain. Evidence gained from the present experiments clearly supports the first alternative.

The results of the equilibrium and kinetic studies strongly suggest independent unfolding/folding of the two domains in γII-crystallin. The intermediate state represents the partially unfolded protein with one domain unfolded and the other still in its native state. Because both domains are known to be closely similar in their amino acid sequences as well as their spatial organization (3), investigation of the separate domains may be used to determine which of the two domains exhibits higher stability. As taken from the accessibility toward proteolysis, the NH_2-terminal fragment seems more stable than the COOH-terminal domain. However, thermal analysis clearly shows that the ΔH_{cal} values for the two transitions are closely similar, suggesting only marginal differences in the thermodynamic stability of the two domains.

Unfolding under strongly denaturing conditions, as well as renaturation under native-like conditions, show complex kinetics, pointing to a stable kinetic intermediate on the pathway of unfolding/folding. Comparing the respective phases of the kinetics of the overall renaturation reaction with the folding kinetics starting from the stable equilibrium intermediate, identical results have been obtained, in accordance with the structural similarity of both the kinetic intermediate and the stable intermediate of the equilibrium transition.

The folding and not the pairing of the individual domains is rate-determining in the self-organization of γII-crystallin. Pairing or merging contributes neither to the kinetic mechanism nor to the higher overall stability of the protein. This fact is indicated by the similarity between the denaturation profiles seen for the entire protein ($I \rightarrow D$) and its NH_2-terminal domain fragment. The present investigation of the NH_2-terminal domain as part of the entire protein and as isolated fragment refers to conditions where the COOH-terminal domain is either denatured or removed. The clear-cut analysis of the contribution of domain interactions to the folding and stability of the individual domains would require experiments involving the isolated COOH-terminal domain that has not been accessible by using limited proteolysis.

Because the rate constants of the slow folding reactions are found to depend strongly on the denaturant concentration (cf. Fig. 4), the cis–trans isomerization reaction of Xaa-Pro peptide bonds cannot be a rate-limiting step in the kinetics of domain folding (12, 13). However, that proline isomerization becomes rate determining under strongly denaturing or strongly native conditions cannot be excluded.

The folding of γII-crystallin proceeds through the sequential structuring of its domains. In the urea-induced unfolding at pH 2.0, the NH_2-terminal and the COOH-terminal domains undergo a sequential unfolding transition. At physiological pH the analysis is hampered by the extreme stability of the protein. What is the physical reason for this stability remains to be solved. Similarly, the findings that the unfolding/folding of γII-crystallin is extremely slow in the transition range and that the kinetics are strongly influenced by the denaturant need explanation. This holds especially because other proteins of comparable size show significantly faster folding, even in the transition range. Whether the observed folding pattern is a general feature of β-structure formation would be important to elucidate.

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