Equilibrium Folding Studies of Cellular Retinoic Acid Binding Protein, a Predominantly β-Sheet Protein†

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ABSTRACT: We have examined the conformational behavior under various unfolding conditions of a predominantly β-sheet protein, cellular retinoic acid binding protein (CRABP). Urea unfolding–refolding of CRABP is a highly cooperative process that can be approximated by a two-state model. Acid denaturation is also cooperative and reversible and leads to a state containing nonnative residual structure: Below pH 2.6, CRABP contains a substantially larger amount of α-helix than under native conditions. CRABP adopts up to 75% α-helix in solutions containing a high percentage of 2,2,2-trifluoroethanol. The acid-denatured state of CRABP undergoes a conformational change to a state containing predominantly β-sheet structure upon the addition of small amounts of Na_2SO_4. This conformational malleability may be important for the folding mechanism of CRABP. The possible implication of nonnative α-helical structure in the folding of CRABP is discussed.

There is growing evidence that the refolding of small monomeric proteins in vitro occurs along pathways that can be described in terms of intermediate partially folded states (Kim & Baldwin, 1990; Creighton, 1990; Matthews, 1993; Baldwin, 1993a) and that the secondary structure present in these intermediate states is generally nativelike (Kuwajima, 1989). A picture of a general folding mechanism has emerged in recent years for proteins containing a large percentage of α-helix or mixed α-helix and β-sheet in their native states. There appear to be at least two types of discrete folding intermediates between the completely unfolded and the native states in the folding pathways of such proteins: (1) A compact, loosely packed molten globule-like intermediate (Christensen & Pain, 1991; Ptitsyn, 1992; Kuwajima, 1992; Baldwin, 1993a); the average content and the location of the secondary structure in the compact intermediate are similar to those of the folded state. Kinetic studies of protein folding suggest that the compact intermediate results from hydrophobic collapse of the unfolded state, or of an early folding intermediate, and later undergoes local rearrangements that lead to a tight packing of the atoms inside the core and thus to the native state (Ptitsyn & Semisotnov, 1991; Baldwin, 1993b). (2) An early folding intermediate that contains a substantial fraction of secondary structure formed within the first 10 ms of refolding (Ptitsyn, 1991; Jeng & Englander, 1991; Kuwajima, 1992). This intermediate is different from the compact molten globule form of proteins and has an expanded nonglobular (or collapsed unfolded) form (Baldwin, 1991). It was proposed that this structural intermediate is the precursor form that precedes the formation of the compact molten globule of such proteins (Ptitsyn, 1991; Chaffotte et al., 1991, 1992).

Very little is known about how a β-sheet protein folds (Baldwin, 1991, 1993a). Some examples of the folding of β-sheet proteins have been reported (Kuwajima et al., 1987; Büchner et al., 1991; Ropson et al., 1992; Varley et al., 1993). It is likely that folding of β-sheet proteins follows the sequential mechanism with kinetically identifiable intermediates, as suggested in the folding study of interleukin-1β (Varley et al., 1993) and β-lactoglobulin (Kuwajima et al., 1987). However, little is known about the nature of the intermediates. Do β-sheet proteins form molten globule or pre-molten globule intermediates? If so, will these intermediates contain only native secondary structure (β-sheets), or is there any nonnative secondary structure, for example, α-helix, involved in the folding pathway? It would not be surprising if nonnative secondary structure is involved in the folding of β-sheet proteins. A β-sheet represents a two-dimensional arrangement of a polypeptide that usually involves more than one strand, while an α-helix is formed by a single chain. In studies of synthetic polypeptides, it has been shown that α-helices fold within microseconds (Gruenewald et al., 1979). On the other hand, the formation of β-sheets in model peptides may take from tens of milliseconds to hours, depending on the sequence and the conditions (Finkelstein, 1991). The time scales of folding of β-sheet proteins are within seconds to minutes, which are similar to those of α and/or α–β proteins. It is likely that, in general, unfolded polypeptide chains have a much higher tendency to form α-helices than β-sheets, and the formation of helices may provide the most efficient way to nucleate an initial hydrophobic collapse in any type of protein.

Characterization of the equilibrium states of proteins in diverse unfolding conditions can shed light on their intrinsic conformational properties and, hence, give insight into factors that guide protein folding mechanisms. Of particular interest is acid denaturation, as it has been shown that low pH induces partially folded states in several proteins [for reference, see Kuwajima (1989) and Goto et al. (1990)]. These equilibrium, partially folded states resemble molten globule states and are much like early kinetic intermediates in the folding process (Kuwajima, 1992; Baldwin, 1993a,b). In this article, we describe equilibrium folding–unfolding studies of a predominantly β-sheet protein, cellular retinoic acid binding protein (CRABP,[1] MW 15 000), paying particular attention to acid-induced unfolding. CRABP belongs to a family of proteins that binds small hydrophobic ligands, the so-called β-clam protein family (Gordon et al., 1991). We have built a three-dimensional model of CRABP (Zhang et al., 1992) based on the crystal structure of myelin P2 protein (Jones et al., 1988).

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This structure consists of two nearly orthogonal β-sheets, formed by five antiparallel β-strands each, and a short region containing an α-helix–turn–helix near the N-terminal end (Zhang et al., 1992). Preliminary NMR characterization of the solution structure of CRABP is fully consistent with this model (J. Rizo, Z.-P. Liu, and L. M. Gierasch, unpublished results). The β-clam protein fold may occur more widely, as it was recently reported that the sequence of the *N*-methyl-D-aspartate (NMDA) receptor has a region homologous to the β-clam proteins (Petrou et al., 1993). The activity of the NMDA receptor is modulated by direct interaction with arachidonic acid and certain other types of fatty acids (Miller et al., 1992), implying that β-clam modules may have important functions in the central nerve system. Study of the structure and folding mechanism of this class of proteins should aid in understanding their functions.

Our results indicate that, in acidic conditions (pH below 2.6), CRABP is partially folded. The acid-unfolded protein contains substantially more α-helix than the native protein as judged by its CD spectrum. Addition of Na_2SO_4 to the acid-denatured protein causes a conformational change to a state containing predominantly β-sheet structure, but still with a significantly larger α-helix content than native CRABP. We also studied the tendency of CRABP to adopt α-helical structure in the absence of native tertiary interactions by looking at the structure of CRABP in aqueous TFE solution. In solutions containing a high percentage of TFE, CRABP adopts almost 75% α-helix and can reversibly renature from this state by dilution into aqueous buffer. These results imply that a good part of the CRABP sequence has the potential to adopt an α-helical conformation. Hence, it is tempting to speculate that nonnative α-helical structure may be involved in the folding mechanism of CRABP.

MATERIALS AND METHODS

Expression and Purification of CRABP. The plasmid pT7-7/CRABP (Zhang et al., 1992) was used to transform the *Escherichia coli* strain BL21(DE3) (Studier et al., 1990). An overnight culture in 5 mL of Luria–Bertani (LB) medium containing 25 μg/mL carbenicillin (CB) inoculated from a freshly transformed plate was diluted to 500 mL of LB–CB. The cells were incubated at 37 °C until the cell density reached an absorbance of 0.6–1.0 at 600 nm. At this time, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM to induce the synthesis of T7 RNA polymerase (Studier et al., 1990). The culture was incubated for another 3 h at 37 °C before its harvest by centrifugation. To obtain uniformly ^{15}N-enriched protein, the overnight culture grown in rich LB–CB medium was diluted 20-fold to M9 minimal medium with $^{15}NH_4Cl$ (Cambridge Isotope Laboratory) as the sole nitrogen source. The cell culture was harvested after 4 h of IPTG induction.

Cell pellets from 500 mL of culture were resuspended in 50 mL of buffer containing 10 mM Tris-HCl at pH 8.0, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM β-mercaptoethanol (β-ME), and 15% sucrose. The cells were lysed by addition of 5 mg of lysozyme and 5 mL of 1% Triton X-100 and incubation at 30 °C for 30 min. Two minutes of sonication (15-s bursts with 30-s intervals between the bursts) were used to shear the DNA. The majority of the CRABP was expressed as cell inclusion bodies. Since from previous studies we knew that WT CRABP could be folded properly from the urea-denatured state, we decided to purify the protein from inclusion bodies, which greatly simplified the purification procedure compared with the original one (Zhang et al., 1992). The cell suspension was pelleted by centrifugation at 13000*g* for 15 min, and the pellets (containing the inclusion bodies) were washed two times, first with 10 mM Tris-HCl at pH 8.0, which included 0.5% Triton X-100, and second with the buffer alone. Finally, the washed inclusion bodies were suspended in 20 mL of 8 M urea/10 mM Tris-HCl (pH 8.0)/0.1 M β-ME/1 mM EDTA. After a 2-h incubation at room temperature, solubilized protein from the inclusion bodies was in the supernatant.

Refolding of CRABP was accomplished by 20-fold dilution of the solubilized inclusion bodies to refolding buffer/10 mM Tris-HCl at pH 8.0 and 5 °C. The addition of denatured protein to refolding buffer was carried out slowly to keep the local concentrations of the folding intermediates low. The solution was kept cold (5 °C) overnight. The folded protein was purified by DEAE ion-exchange chromatography. The folded protein elutes at a ca. 0.1 M NaCl concentration from a 40-mL DEAE Sepharose CL-6B (Sigma, St. Louis, MO) column using 10 mM Tris-HCl (pH 8.0) as eluant and a linear gradient of the salt concentration from 0 to 0.2 M. The typical yield of protein is ca. 15 mg/L cell culture. The biochemical and biophysical properties of CRABP purified from the cell inclusion bodies are the same as those purified from the cytosol fraction of the bacteria cells, as judged by their ligand binding activity and CD, fluorescence, and NMR spectra (Liu, 1993; Zhang et al., 1992).

For some NMR studies (see Results), we used lyophilized CRABP. The lyophilized form of CRABP was prepared by first dialyzing the protein extensively against Milli-Q water (Milligen, Bedford, MA) and then concentrating to 3 mg/mL with an Amicon filtration device (Beverly, MA). The concentrated protein solution was then quickly frozen with liquid nitrogen and lyophilized overnight. Two-dimensional NMR spectroscopy of lyophilized CRABP samples shows that the protein conformation is not affected by the lyophilization process (unpublished results).

Transverse Urea Gradient Gel Electrophoresis. Gels (8 × 7 × 0.15 cm) were prepared with a linear 0–8 M urea gradient and a compensating 15–11% (w/v) gradient of acrylamide, as described in Goldenberg (1989). The gel solutions were buffered with 0.05 M Tris–acetate (pH 7.5) (0.05 M Tris base adjusted with acetic acid). The electrode buffer was made by dissolving 0.6 g of Tris base and 2.88 g of glycine in 1 L of water. Typically, the sample of native protein was prepared by diluting the stock protein solution with 4× loading buffer (44% glycerol/0.44 M β-ME/5 mM phosphate buffer (pH 7.0)/0.16% bromophenol blue). Usually, a sample of 20–30 μg of protein in 100–200 μL was loaded. The solution was incubated at room temperature for 20 min prior to loading. The unfolded protein sample was prepared by adding solid urea to a final concentration of 8 M and β-ME to 0.1 M. The sample was incubated at room temperature for 1 h, and a drop of 0.2% bromophenol blue was added 20 min prior to loading. The native and unfolded protein samples were applied to

[1] Abbreviations: 1D, one-dimensional; 2D, two-dimensional; ANS, 1-anilinonaphthalene-8-sulfonic acid; CB, carbenicillin; CD, circular dichroism; CRABP, cellular retinoic acid binding protein; DEAE, (diethylamino)ethyl; EDTA, ethylenediaminetetraacetic acid; FID, free induction decay; FTIR, Fourier transform infrared; GdnHCl, guanidinium hydrochloride; HSQC, heteronuclear single quantum coherence; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria–Bertani; NMDA, *N*-methyl-D-aspartate; NMR, nuclear magnetic resonance; PMSF, phenylmethanesulfonyl fluoride; RA, retinoic acid; RMS, root-mean-square; TFE, 2,2,2-trifluoroethanol; UV, ultraviolet; β-ME, 2-mercaptoethanol; $[\theta]_m$, mean residue ellipticity; WT, wild type.

identical gels prepared at the same time and electrophoresed in parallel at room temperature. The gels were usually electrophoresed for 10 h at a constant voltage of 30 V.

Sample Preparation and Circular Dichroism Measurement. Acid- and salt-induced conformational transitions of CRABP were carried out with protein (3 μM) that had been dialyzed extensively against Milli-Q water (Milligen, Bedford, MA). For the TFE titration, the protein solutions contained 4 mM phosphate buffer (pH 7.0) and the appropriate TFE concentration (ranging from 5% to 70% by volume). Refolding of CRABP was initiated by diluting a concentrated solution of protein in 60% TFE/10 mM phosphate buffer (pH 7.0) into phosphate buffer (pH 7.0) to a final protein concentration of 2.56 μM. All of the samples were spun in a microcentrifuge for 15 min before the CD measurements, as partial aggregation sometimes occurred. All CD spectra were recorded from 190 to 260 nm every 1 nm on an Aviv Model 60DS spectropolarimeter at room temperature using a 2-mm path length cell. Spectra were base-line-corrected and smoothed with programs provided by Aviv Associates. Protein concentrations were determined spectrophotometrically, using an extinction coefficient of $\epsilon = 2.8 \times 10^4\ M^{-1}\ cm^{-1}$, and by quantitative amino acid analysis. For urea titration of the acid-unfolded state of CRABP, CRABP (100 μM) was first unfolded in 14 mM HCl. To this HCl-unfolded protein sample was added the appropriate amount of a 10 M solution of urea to give a final protein concentration of 50 μM, 14 mM HCl, and various concentrations of urea ranging from 0 to 5 M. The CD spectrum of the sample was recorded with a 0.1-mm path length cell.

pH-Induced Folding–Unfolding of CRABP. pH-induced unfolding of CRABP was performed by 30-fold dilution of a 78 μM solution of CRABP (2 mM Tris-HCl, pH 8.0) into 10 mM citric acid, with the pH ranging from 2.1 to 4.3. The refolding of CRABP was carried out by 30-fold dilution of HCl-unfolded CRABP (78 μM in 14 mM HCl, pH 2.6) into 10 mM citric acid, with the pH ranging from 3.1 to 4.0. Refolding of CRABP at pH 7.0 was performed with 10 mM phosphate buffer. The samples were prepared at 5 °C and equilibrated for 15 min at room temperature prior to the measurement. The folding–unfolding of CRABP was monitored by CD at 25 °C.

Quantitation of Secondary Structure from CD Spectra. The secondary structure of CRABP was estimated from the CD spectra of the protein from 190 to 240 nm with the program CONTIN (Provencher & Glöckner, 1981). The concentration of the protein in the native state was determined by amino acid analysis and used for calculation of the mean residue ellipticities for all of the CD spectra, including those of nonnative states. For the spectra of nonnative states, we found it necessary to reduce the parameter RUSER14, which is proportional to the protein concentration, by a fraction <10% in order to obtain the best fit. Decreased effective concentration may be due to partial aggregation of the protein.

NMR Studies of the Native and Acid-Unfolded States of WT CRABP. All NMR spectra were recorded on a Varian VXR500 spectrometer operating at a proton frequency of 500 MHz. For one-dimensional spectra, lyophilized protein samples were dissolved in 0.7 mL of the corresponding solvent (protein concentrations were 100 μM). The spectra were the result of 256–1000 scans and were acquired with a 6800-Hz spectral width. Exponential multiplication with 1-Hz line broadening was used before Fourier transformation. Heteronuclear single quantum coherence (HSQC) spectra (Bodenhausen & Ruben, 1980) were acquired on uniformly ^{15}N-labeled protein samples. The HSQC spectrum of native CRABP was obtained on a 3 mM protein solution in 12 mM Tris-d_{11} buffer at pH 7.5. The acid- and urea-unfolded samples were obtained by dissolving the lyophilized protein in 14 mM HCl (0.1 mM protein concentration) or in 7 M urea/14 mM HCl (1 mM protein concentration), respectively. In all cases, the solutions contained 10% D_2O for frequency lock. The water resonance was suppressed by very low power presaturation. Quadrature detection in the t_1 dimension was achieved by the hypercomplex method (States et al., 1982). Each HSQC spectrum consisted of 2 × 100 FIDs containing 1024 complex points each, with 16 (native and urea states) or 96 (acid state) scans per FID; the total acquisition times were ca. 2 and 10 h, respectively. Proton and ^{15}N spectral widths were 6800 and 1800 Hz, respectively. The data sets were processed using the program FELIX (Biosym Technologies Inc., San Diego). Gaussian and 60° shifted sine-bell apodizations were used in the t_2 and t_1 dimensions, respectively. Zero-filling in the latter dimension yielded matrices of 1024 × 1024 real data points.

To measure deuterium exchange rates in the acid-denatured state, lyophilized protein was dissolved in 20 mM DCl (0.3 mM protein concentration, pD 2.5 at 5 °C), and a series of HSQC spectra was recorded with total acquisition times between 45 min (2 × 70 FIDs, 16 scans per FID) and 2 h (2 × 100 FIDs, 32 scans per FID).

RESULTS

Reversibility of Folding–Unfolding of CRABP Assayed by Transverse Urea Gradient Gel Electrophoresis. The far-UV CD spectra of CRABP under various solvent conditions are shown in Figure 1. The CD spectrum of native CRABP is typical of a predominantly β-sheet protein, with a characteristic minimum ellipticity at 218 nm (Figure 1a). The CD spectrum of CRABP also shows a shoulder at 230 nm, which is probably due to aromatic residues (Manning & Woody, 1989). This is consistent with the observation of a CD signal in the near-UV region (data not shown). The protein is unfolded in aqueous solution with concentrations of urea higher than 4 M, as indicated by the loss of the CD signal at wavelengths above 215 nm (see Figure 1b). To analyze the folding–unfolding transition of CRABP in urea, we used polyacrylamide gel electrophoresis, where protein samples were applied across the top of a slab gel containing a urea concentration gradient perpendicular to the direction of electrophoresis (Goldenberg, 1989). Figure 2 shows the urea gradient gels starting with native CRABP (Figure 2a) and with CRABP that had been denatured in 8 M urea prior to loading (Figure 2b). The electrophoretic patterns can be fit, at first approximation, to a two-state folding–unfolding model. At low urea concentration (<3 M), the protein is folded and migrates rapidly. At high urea concentration (>4 M), the protein is unfolded and migrates more slowly. At intermediate urea concentrations (3–4 M urea), only folded and unfolded conformations are present in a significant amount, and the mobility of the mixture is determined by the weighted average of the mobilities of the folded and unfolded forms.

The appearance of the electrophoretic pattern in the transition region depends on the ratio between the half-time ($t_{1/2}$) of the conversion between the folded and unfolded forms and the electrophoresis time (T). If $t_{1/2}$ is about 10 times T or larger, then two separate bands with mobilities corresponding to folded and unfolded conformers will be seen. If $t_{1/2}$ is close to T or smaller, then a smeared band will be seen in the transition region (Goldenberg, 1989). The appearance

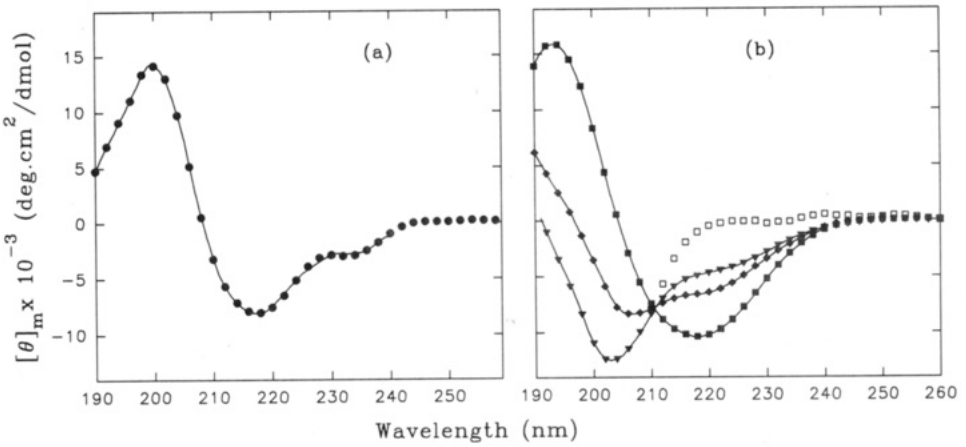

FIGURE 1: CD spectra of CRABP (ca. 3 μM): (a) in H_2O at pH 7.0 (●); (b) in H_2O with 5 mM HCl at pH 2.6 (▼), 5 mM $HClO_4$ at pH 2.6 (♦), 5 mM HCl/2 mM Na_2SO_4 (■), and 5 M urea (□). The symbols represent experimental data and are plotted every 2 nm. The lines are best fits from the CONTIN program. No CD data could be obtained in 5 M urea below 210 nm due to the large absorption of the solvent; no attempt was made to curve-fit these data.

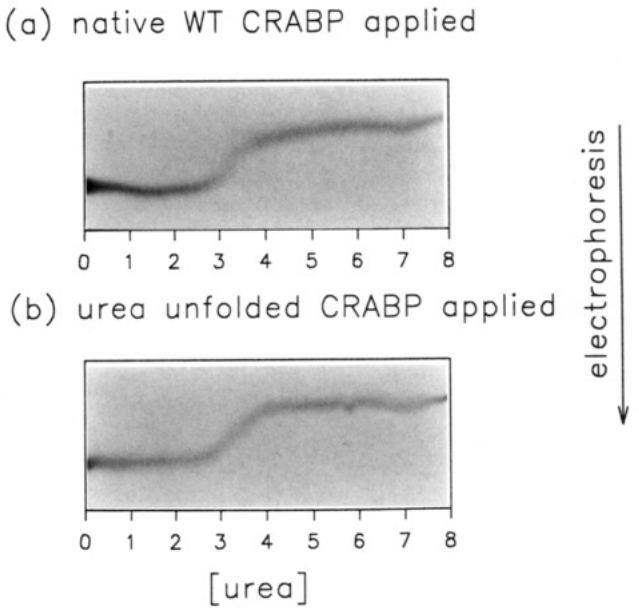

FIGURE 2: Transverse urea gradient gel electrophoresis of CRABP. The gels contained a linear gradient of 0–8 M urea, from left to right, perpendicular to the direction of electrophoresis. Twenty-five micrograms of protein in 150 μL of 10 mM Tris-HCl (pH 8.0) were loaded. (a) The folded and (b) urea-unfolded proteins were applied across the top of the gel and electrophoresed toward the cathode (indicated by the arrow) at room temperature, pH 7.5, for about 10 h.

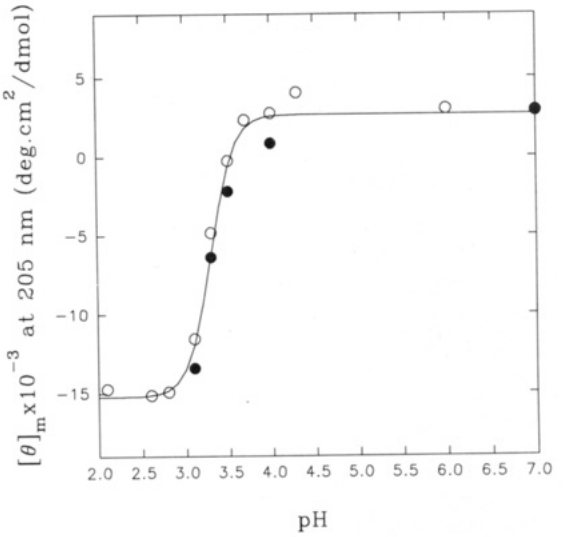

FIGURE 3: Acid unfolding (○) and refolding (●) transition of CRABP monitored by CD at 205 nm as a function of pH at 25 °C. For the unfolding part of the curve, native CRABP was diluted into 10 mM citric acid solutions with pH's ranging from 2.1 to 4.3. For the refolding transition, HCl-unfolded CRABP was diluted into 10 mM citric acid buffered solutions with pH's ranging from 3.1 to 4.0. For comparison, points corresponding to CRABP in 10 mM phosphate buffer at a pH close to neutral before and after acid unfolding have also been included.

of the transition in CRABP is characteristic of a fast interconversion relative to *T*. Figure 2 also suggests that the folding–unfolding of CRABP is reversible: The electrophoretic patterns are almost superimposable, and both display a continuous transition between the folded and unfolded bands, with a single inflection point at 3.2 M urea concentration. CRABP regains its native conformation after urea denaturation and refolding into aqueous buffer, as indicated by its CD and fluorescence spectra and its retinoic acid binding activity (data not shown).

Acid-Induced Unfolding of CRABP. Acid-induced unfolding of proteins is often incomplete, and the residual structure can usually be unfolded further upon the addition of urea or GdnHCl (Tanford, 1968). Unfolded proteins are frequently observed to adopt a "molten globule" type of conformation under refolding conditions (Kuwajima, 1992; Ptitsyn, 1992). Fink and co-workers (Goto et al., 1990) showed that for some proteins, these molten globule-like conformations can be obtained at acidic pH by variation of pH, temperature, and anion concentration.

The CD spectrum of CRABP remains unaltered by changes in pH between 3.7 and 10. The CD spectrum of CRABP in 5 mM HCl at pH 2.6 (Figure 1b) indicates that the protein loses its native structure and becomes largely unfolded under these conditions. As acid unfolding of CRABP with 10 mM citric acid at pH's below 2.6 yields a CD spectrum very similar to that observed in 5 mM HCl (data not shown), we studied the acid-induced folding–unfolding transition of CRABP using protein solutions buffered with 10 mM citric acid and the pH adjusted from 2.1 to 4.3. The unfolding–refolding transition, as monitored by CD at 205 nm, is illustrated in Figure 3. The acid-induced unfolding of CRABP occurs in a quite narrow pH range, between pH 2.8 and 3.5, and appears to be a reversible, two-state cooperative process. As can be observed in Figure 1b, significant negative ellipticity is still observed near 220 nm in the CD spectrum of HCl-unfolded CRABP, which indicates the presence of residual structure. Under the

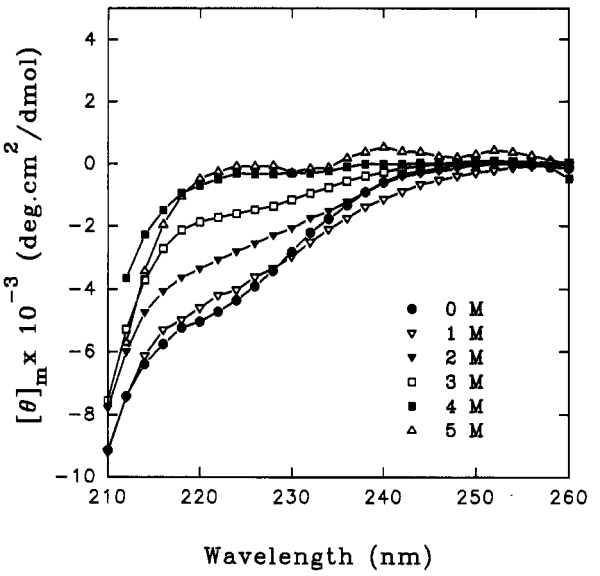

FIGURE 4: Urea titration of the acid-unfolded state of CRABP monitored by CD. CRABP (100 μM) was unfolded in 14 mM HCl. To this solution was added the appropriate amount of urea in 10 M solution to give a final protein concentration of 50 μM, 14 mM HCl (pH 2.6), and urea concentrations ranging from 0 to 5 M. The CD spectra were recorded with a 0.1-mm path length cell at room temperature. Experimental data points are plotted every 2 nm.

Table 1: Estimated Secondary Structure Contents of Various Conformational States of CRABP (3 μM) Using the Program CONTIN[a]

conditions	% α-helix	% β-sheet	remainder
H_2O, pH 7	6 (1.3)	54 (2.6)	40 (2.7)
5 M urea[b]	mostly random		
5 mM HCl	17 (1.0)	29 (1.3)	54 (1.9)
5 mM $HClO_4$	22 (1.1)	26 (1.4)	52 (2.1)
5 mM HCl and 2 mM Na_2SO_4	18 (0.5)	51 (1.0)	31 (1.0)

[a] In parentheses are indicated the standard errors as calculated by CONTIN [see van Stokkum et al. (1990)]. The RMS deviations between the calculated and experimental curves were 146 deg cm^2/dmol for the data in H_2O at pH 7 and between 50 and 63 deg cm^2/dmol for the other curves (see also Figure 1). [b] No CD data were obtained for these conditions below 210 nm due to the large absorption of the solvent. Thus, no attempt was made to curve-fit these data.

same conditions, the near-UV CD of the native state has disappeared (data not shown), arguing that the remaining signal in the far-UV region does not arise from aromatic residues. The residual structure remaining in the acid-denatured state of CRABP can be unfolded further by the addition of urea (Figure 4), which yields a CD signature characteristic of a random polypeptide. The transition between the acid- and urea-denatured states occurs at urea concentrations between 2 and 4 M.

Although the CD spectra of CRABP unfolded with HCl or citric acid are very similar, a somewhat different spectrum is obtained with $HClO_4$-induced unfolding (Figure 1b). This effect is probably due to differences in the binding ability of the corresponding anions to the denatured protein (Goto et al., 1990). Despite the noted differences, the shapes of the CD spectra of HCl- and $HClO_4$-unfolded CRABP both suggest the presence of a substantial amount of α-helical conformation (Figure 1b). To estimate the secondary structure contents from the CD data shown in Figure 1, we used the program CONTIN (Provencher & Glöckner, 1981). The calculated structure contents and standard errors are given in Table 1. The calculated curves are superimposed with the experimental

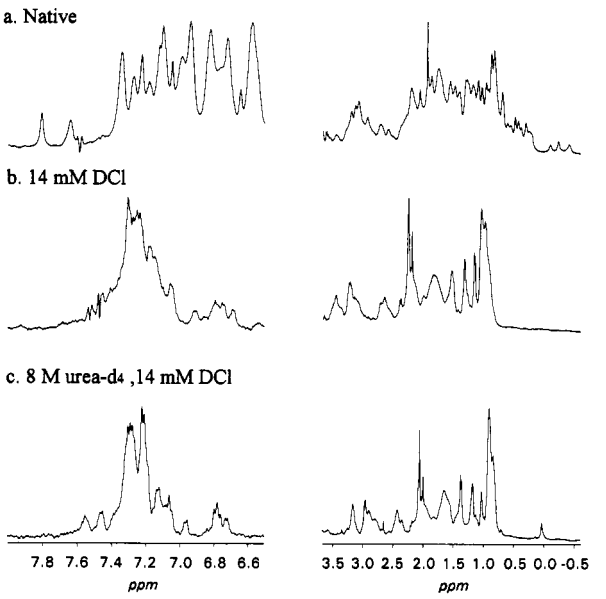

FIGURE 5: Aromatic and aliphatic regions of one-dimensional 500-MHz ^{1}H NMR spectra of CRABP in (a) D_2O, (b) 14 mM DCl in D_2O, and (c) 8 M urea-d_4/D_2O containing 14 mM DCl.

data in Figure 1. The fits obtained with CONTIN were very good, with root-mean-square (RMS) deviations between experimental and calculated curves of 146 deg cm^2/dmol for the native CD, and between 50 and 63 deg cm^2/dmol for the other curves.[2] The estimation of secondary structure by CONTIN indicates that about 17% and 22% of α-helical conformation exists in HCl- or $HClO_4$-denatured protein, respectively, while the β-sheet content estimation is 29% and 26% in the two solutions, respectively. The estimation of α-helix in native CRABP is ca. 6%. Therefore, the α-helix content in acid-unfolded CRABP is significantly higher than that in native CRABP.

To determine whether intermolecular interactions stabilize the helical conformation in acid-unfolded CRABP, we recorded a series of spectra in 14 mM HCl at room temperature where the protein concentration was varied. No changes in the CD spectrum were observed for a protein concentration range of 3–120 μM (data not shown), arguing strongly for monomeric behavior under these conditions. This conclusion was also supported by sedimentation velocity analysis of acid-denatured CRABP at 10 μM protein concentration (M. A. L. Atkinson, personal communication). At room temperature and concentrations of CRABP above 150 μM, the protein begins to aggregate as judged by the appearance of the CD spectrum. At 5 °C, the acid-unfolded state is stable for several hours at 300 μM protein concentration.

NMR Studies on the Urea- and Acid-Unfolded States. In order to obtain additional information on the unfolded states of CRABP, we have performed some preliminary studies by nuclear magnetic resonance (NMR). The aliphatic and aromatic regions of the one-dimensional (1D) NMR spectrum of 100 μM CRABP in 14 mM DCl/D_2O (pD 2.6) are shown in Figure 5, where they are compared with the 1D NMR spectra of the native protein in D_2O and of CRABP in 8 M urea-d_4/D_2O. The dispersion of signals in the aromatic and aliphatic regions of the NMR spectrum of acid-unfolded CRABP is much more similar to that of the urea-unfolded

[2] We chose CONTIN after comparing several curve-fitting methods because it was far more successful in yielding high-quality fits to CRABP CD data. The slightly larger RMS deviation for the native CD is due to the difficulty introduced in the fitting by the shoulder at 230 nm.

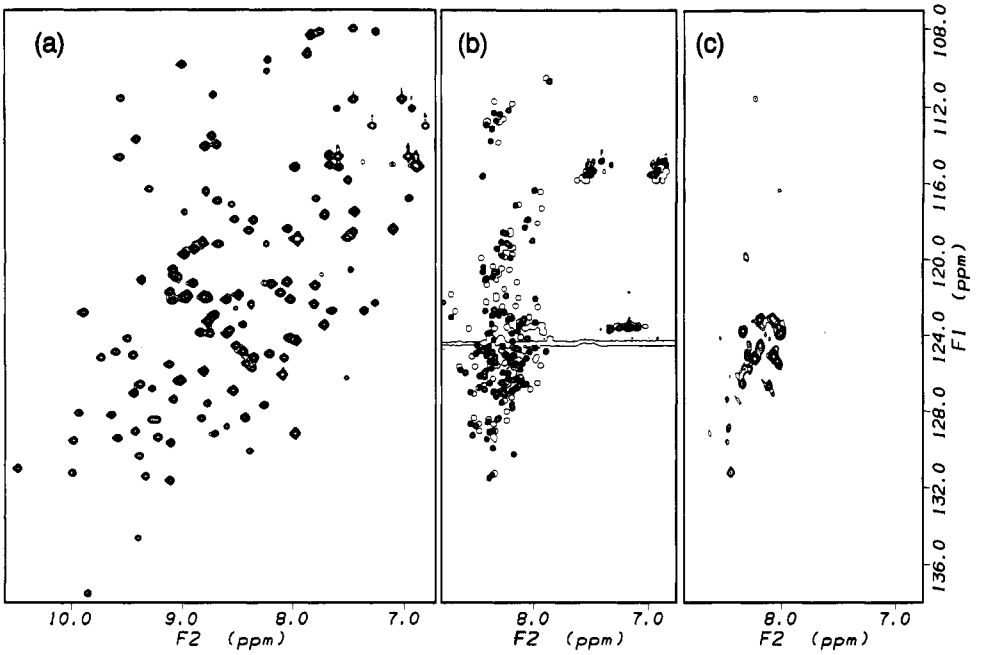

FIGURE 6: (a) ^1H–^{15}N HSQC spectrum of CRABP in 12 mM Tris-d_{11} at pH 7.5. (b) Superposition of the ^1H–^{15}N HSQC spectra of CRABP in 14 mM HCl and in 8 M urea with 14 mM HCl; only one contour has been plotted for the former, while 12 contours are plotted for the latter. (c) HSQC spectrum of CRABP (0.3 mM) in 20 mM DCl (pD 2.5) at 5 °C after 210 min of NH/D exchange.

protein than to that observed for native CRABP, which is consistent with the CD data. In particular, the absence of signals above 0.7 ppm, which in folded proteins usually corresponds to buried methyl groups near aromatic π-systems, reflects the lack of a compact, specifically folded core in acid-unfolded CRABP. Some differences in the aromatic region can be observed between the spectra of acid- and urea-unfolded CRABP, suggesting that aromatic residues are involved in the residual structure present in the acid-unfolded form. However, it is difficult to draw more specific conclusions from the 1D NMR spectra due to overlapping of the large number of resonances present.

The application of homonuclear two-dimensional NMR methods to obtain more detailed conformational information on the acid-unfolded state of CRABP is hindered by the necessity to use a very low concentration of protein to avoid aggregation. However, heteronuclear correlation experiments have been shown to be very sensitive due to the high efficiency of the magnetization transfer between directly bonded nuclei. Hence, we recorded a ^1H–^{15}N heteronuclear single quantum coherence (HSQC) spectrum (Bodenhausen & Ruben, 1980) on homogeneously ^{15}N-labeled samples of CRABP in native and in urea and acid unfolding conditions. In ^1H–^{15}N HSQC spectra, a cross-peak is observed for every hydrogen directly bonded to a nitrogen atom. The HSQC spectrum of native ^{15}N-labeled CRABP is shown in Figure 6a. A superposition of the HSQC spectra of urea- and acid-unfolded CRABP is shown in Figure 6b. The distribution of cross-peaks for the acid-unfolded protein is again much more similar to that of urea-unfolded CRABP than to the distribution observed for the native form. The larger dispersion of chemical shifts in native CRABP is a consequence of the well-defined secondary structure and a compact, specific tertiary structure. Despite the overall similarity in the distribution of cross-peaks for the acid- and urea-unfolded proteins, there appears to be a somewhat larger dispersion of chemical shifts in the HSQC spectrum of acid-unfolded CRABP, consistent with the presence of residual structure indicated by CD. The observation that very few cross-peaks of the acid- and urea-unfolded forms coincide shows that conformational differences between the two states exist throughout the whole sequence. Note that the intrinsic effect of urea on the random coil chemical shifts of amide protons is very small (<0.03 ppm; Jiménez et al., 1986). There is a clear tendency for both the proton and the ^{15}N resonances of the acid-unfolded state to be shifted upfield with respect to the urea-unfolded state, which is consistent with the formation of α-helical structure (Wishart et al., 1991). The upfield shifts are also consistent with enhanced protection from the solvent (a strong hydrogen-bond acceptor) without the formation of strong, specific intramolecular hydrogen bonds. Our results suggest that much of the CRABP sequence may be involved in structure formation in the acid-denatured state.

To test further for the presence of residual structure in acid-unfolded CRABP, we performed hydrogen-exchange experiments. Amide protons are labile to exchange with solvent, and the exchange rate can be slowed by many orders of magnitude when an NH is involved in intramolecular hydrogen bonding. A protection factor, P, which is defined as the ratio between the exchange rate in poly-D,L-alanine (Molday et al., 1972; Englander et al., 1979; Robertson & Baldwin, 1991) and the observed exchange rate, is usually used as an indicator of the degree of protection from the solvent. A series of HSQC spectra of CRABP (0.3 mM) in 20 mM DCl (pD 2.5) at 5 °C were recorded at different times after sample preparation. The HSQC spectrum obtained after 210 min of exchange is shown in Figure 6c. These experiments indicate that 17% of the backbone amide protons have a protection factor P greater than 10, and another 8% are modestly protected from exchange (P is between 2 and 10). These results are consistent with the presence of residual structure in the acid-unfolded state of CRABP. The magnitude of these protection factors is very similar to those of the folding intermediates of oxidized horse cytochrome c and sperm whale apomyoglobin (Baldwin & Roder, 1991) and those of the alcohol forms of ubiquitin (Pan & Briggs, 1992). However, these protection factors are still several orders of magnitude smaller than those commonly found in native proteins. Thus, the residual structure in the acid-unfolded CRABP is not very stable.

Effect of Salts on Acid-Unfolded CRABP. The addition of salts to acid-denatured proteins often results in a collapsed conformation with properties similar to those of a molten globule state (Goto et al., 1990). In studies of the effects of various anions on the acid-unfolded states of cytochrome *c* and apomyoglobin, Fink and co-workers proposed that the conformational transitions of these two proteins from the acid-induced unfolded state to a molten globule state are promoted by electrostatic binding of the anions to the positively charged sites of the protein (Goto et al., 1990). We performed similar experiments with CRABP. Addition of sodium sulfate (final concentration 2 mM) to HCl-denatured CRABP (3 μM) induces a conformational change to a state which is referred to here as the A state (Figure 1b). The CD spectrum of CRABP in the A state displays a minimum at 218 nm characteristic of β-structure, as is observed in native CRABP. However, the minimum near 218 nm in the A state is broadened and the maximum at 200 nm is shifted to 195 nm. Estimation of the secondary structure of the A state by the program CONTIN reveals that the β-sheet content of CRABP in the A state is close to that in native CRABP (51%, Table 1) and the α-helix content is ca. 18%. The A state is very prone to aggregation and hence difficult to characterize in detail. Our attempts to obtain reproducible NMR data on the A state have so far been unsuccessful.

The concentration of Na_2SO_4 required to cause this change in acid-unfolded CRABP is comparable to that in acid-unfolded cytochrome *c* and apomyoglobin, suggesting that the effect is not specific to CRABP. We also examined the effect of the addition of KCl on the acid-unfolded CRABP. It produces a similar conformational change as sodium sulfate, but at much higher salt concentration (>0.2 M). These results are similar to those reported by Goto et al. (1990) for cytochrome *c* and apomyoglobin. It is likely that the effect of the addition of Na_2SO_4 on the conformation of the acid-induced unfolded state of CRABP is due to the binding of SO_4^{2-} to the positive charges on CRABP, as in the case of cytochrome *c* and apomyoglobin. Neutralization of charges in CRABP causes the collapse of the protein. Several attempts to determine the aggregation state of CRABP in the A state by sedimentation equilibrium analysis suggest that addition of Na_2SO_4 causes a range of aggregated states.

Conformation of CRABP Induced by 2,2,2-Trifluoroethanol (TFE). The presence of a substantial amount of α-helix in acid-denatured CRABP prompted us to investigate the intrinsic tendency of the CRABP sequence to adopt α-helix in the absence of native tertiary interactions. TFE is often used as a structure-promoting solvent in peptide and protein conformational studies (Lehrman et al., 1990; Sönnichsen et al., 1992; Buck et al., 1993), and the propensity of peptides to adopt α-helical conformation in TFE appears to correlate well with the secondary structure of the corresponding segments in the native proteins (Lehrman et al., 1990; Dyson et al., 1992). The nature of the interaction between TFE and polypeptides is not well understood, but it has been proposed that TFE disrupts hydrophobic interactions and strengthens intramolecular hydrogen bonding (Sönnichsen et al., 1992). TFE-induced conformational changes in CRABP are shown in Figure 7. The native β-structure of CRABP is stable up to 10% TFE (v/v). With increasing concentrations of TFE, CRABP undergoes a conformational change. At 45% TFE, CRABP starts taking up α-helical structure, and at 60% TFE the α-helical content of CRABP reaches a plateau estimated at 75% by the program CONTIN. Aggregation and concomitant disappearance of the CD signal occur within 5 h at

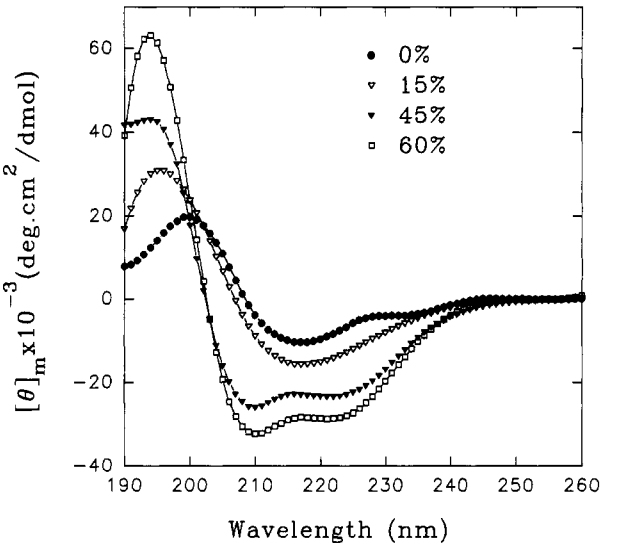

FIGURE 7: TFE titration of CRABP (3 μM in 10 mM phosphate buffer, pH 7.0) as monitored by CD. TFE concentrations are % (v/v).

30–40% TFE. In solutions containing greater than 45% TFE or less than 20% TFE, no aggregates and no loss of CD signal were observed for a period of 2 days.

The reversibility of the TFE-induced conformational change was assayed by diluting a concentrated solution of CRABP in 60% TFE into 10 mM phosphate buffer at pH 7.0 to a final 5% TFE concentration. The CD spectrum of refolded CRABP in 5% TFE and the spectrum of native CRABP in 5% TFE are superimposable (not shown), indicating that the native CRABP conformation can be regained after unfolding with 60% TFE.

DISCUSSION

Unfolding of native CRABP by either urea or acid is reversible and highly cooperative. In both cases, the transition between the folded and unfolded states follows a two-state mechanism to a first approximation. However, the unfolded states in urea and in acid are different. The acid-induced unfolded state (U_A state) retains a significant amount of residual structure relative to the urea-unfolded state (U state). This is not surprising since both theoretical and experimental evidence have shown that acid induces partial unfolding of many proteins, depending on the experimental conditions (Tanford, 1968; Alonso et al., 1991; Goto et al., 1990; Kuwajima, 1992; Shortle, 1993). However, what is of special interest is the nature of the residual structure in the U_A state and the possible relationship of the U_A state to the folding mechanism of CRABP. The acid-unfolded form of CRABP appears to contain a significantly larger amount of α-helix than does native CRABP, which contains predominantly β-structure. Indeed, a largely β-sheet protein such as CRABP can be almost all α-helical in 60% TFE, and these two conformations can be reversibly interconverted depending on the content of TFE in the solution.

A critical issue for the significance of our results is the accuracy in the estimation of secondary structure content from CD data by the program CONTIN. The fits yielded by CONTIN for the CD data in Figure 1 are very good, with standard errors between 1% and 3% for the individual contents of each type of secondary structure and small RMS deviations between calculated and experimental curves (Table 1 and Figure 1). Still, the most important source of uncertainty in any method used to estimate secondary structure from CD

data is to what extent the CD of the protein of interest can be described as a function of the reference spectra. The contribution from the inadequacy of the reference spectra to the error in the secondary structure determination is very difficult to quantitate. In addition, structure prediction by CONTIN is highly sensitive to errors in protein concentrations.

However, there are several arguments supporting our conclusion that the difference between the helical content of the native and the U_A states of CRABP is significant. We are currently solving the native structure of CRABP by NMR, and our preliminary data indicate that the protein contains approximately 10% α-helix, which is also the helical content in the modeled structure we constructed on the basis of the crystal structure of the homologous myelin P2 protein (Zhang et al., 1992). The difference between this α-helix content and that calculated by CONTIN (6%, Table 1) may be due to the contribution of aromatic residues to the far-UV CD. The fit of the CD spectra of the acid-denatured states of CRABP is less likely to be affected by the contribution from the aromatic residues, as no near-UV CD signal is observed for these states, and hence is expected to be more reliable than the fit of the native CD. In addition, a detailed analysis of the quality of the secondary structure prediction by CONTIN has shown that this method is particularly accurate in estimating α-helical content and has a higher uncertainty in the estimation of β-sheet and random coil (Provencher & Glöckner, 1981; Johnson, 1988). Furthermore, comparison of the secondary structure estimates of different states of a protein is more likely to be reliable than comparison of different proteins. Finally, the protein concentrations obtained from UV absorption and quantitative amino acid analysis are very reproducible (±5%), and hence we feel confident that this variable contributes very little to the error in secondary structure determination.

The results obtained in TFE/water mixtures give further insight into the tendency of the CRABP sequence to adopt α-helical conformation. It has long been noted that alcohols, in particular halogenated alcohols such as trifluoroethanol, can induce helical structure in several proteins (Tanford, 1968). The structures of several predominantly β-sheet proteins have been studied in alcohols. β-Lactoglobulin (8% α-helix, 44% β-sheet in its native state) adopts ca. 50% α-helix in 50% ethanol (v/v) (Dufour & Haertlé, 1990). However, the helical content of ubiquitin (15% α-helix and 38% β-sheet) in 60% ethanol is decreased relative to the native state, while a significant amount of β-sheet is retained (Harding et al., 1991). Concanavalin A (2% helix and 51% β-sheet in native conditions) appears to adopt a predominantly α-helical structure in 30% TFE, as determined by FT-IR (Jackson & Mantsch, 1992), but no quantitation of helical content was described. Oxidized hen egg white lysozyme contains ca. 34% α-helix in native conditions whereas it is 61% helical in 50% TFE, as judged by CD (Buck et al., 1992). Our results show that CRABP adopts about 75% α-helix in 60% TFE. From the data described above, it appears that TFE can induce α-helical structure, even in predominantly β-sheet proteins. However, the amount of TFE needed to induce α-helical structure and the extent of such structure vary from one protein to another.

A question follows: Is the high α-helicity of CRABP in 60% TFE meaningful? In peptide systems, TFE does not induce secondary structure indiscriminately. Isolated segments of proteins have higher helical content in the regions that are helical in the native state (Lehrman et al., 1990; Sönnichsen et al., 1992; Dyson et al., 1992). Therefore, TFE is often used as a test for the intrinsic ability of peptides to adopt a helical structure in the native state. Although segments that are helical in the native state have high helix tendency when isolated, the reverse may not necessarily be true. Our results and those described for other β-sheet proteins (see above) argue that not all segments that have high helical tendency are helical in their native states. This is not surprising. In proteins, the secondary structure is part of a compact globular structure and is stabilized by many long-range interactions. Thus, even if a peptide segment has a high helical propensity, it will only be helical in a protein if it can contribute productively to form tertiary structure.

The results presented in this article argue that a good part of the CRABP sequence has an intrinsic tendency to adopt α-helical conformation in the absence of native tertiary interactions. The question that immediately arises is whether nonnative α-helix is involved in the folding mechanism of CRABP. It is possible that, in the early folding steps of CRABP, some regions of the protein form α-helix, as in the U_A state, and provide initiation sites for a hydrophobic collapse, which could facilitate the formation of β-structure in other regions of the molecule. The formation of a collapsed intermediate state in the folding pathway may parallel the formation of the A state of CRABP, which has a substantially higher β-sheet content than the U_A state, but a similar amount of α-helix. Although these ideas on the folding mechanism of CRABP are at this point highly speculative and need to be tested with kinetic methods of analysis, they are supported by a number of indirect arguments. For example, the presence of residual structure in acid-denatured CRABP is a manifestation of the intrinsic conformational tendencies of the CRABP sequence and the need to sequester hydrophobic surface, factors that should also govern the initial stages of its folding mechanism. Also, local secondary structure and hydrophobic clusters have been found in the unfolded forms of many proteins (Montelione & Scheraga, 1989; Shortle, 1993), and partially unfolded states of several monomeric proteins obtained under mild denaturing conditions have been found to resemble kinetic intermediates observed during refolding of such proteins (Baldwin, 1993b). Studies on several proteins, including the β-sheet protein β-lactoglobulin, show that substantial amounts of secondary structure are formed during the first 10 ms of folding (Kuwajima et al., 1987; Semisotnov et al., 1991; Chaffotte et al., 1992; Serrano et al., 1992). Since the time scale of β-sheet formation in model peptides ranges from tens of milliseconds to hours (Auer & Miller-Auer, 1986; Snell & Fasman, 1973; Finkelstein, 1991), early folding events may exploit α-helices [which can form in microseconds (Zana, 1975)] or turns to nucleate β-sheet formation.

Whether nonnative structure is involved in protein folding is a subject of much debate (Darby et al., 1992; Weissman & Kim, 1991; Matthews, 1993) and is central to protein folding mechanisms. Because of its simple topology, CRABP is an ideal target to explore this issue and to gain further insights into folding pathways for β-sheet proteins. We are currently solving the structure of CRABP using multidimensional NMR techniques. Although the NMR data are fully consistent with the overall fold of the modeled structure of CRABP, the solution structure obtained by NMR will provide a more reliable basis to interpret and analyze in detail the folding studies. In the future, we will obtain additional information on the partially folded states of CRABP using NMR

techniques, in particular to study what regions of the U_A state may adopt nonnative α-helix. Such information will be compared with kinetic data obtained from stopped-flow CD measurements, from deuterium exchange/folding competition experiments (Miranker et al., 1991), and from pulse-labeling experiments (Baldwin, 1993b) to explore whether nonnative α-helical structure is involved in the folding mechanism of CRABP.

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