Calcium Binding Peptides from α-Lactalbumin: Implications for Protein Folding and Stability †

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Calcium Binding Peptides from α-Lactalbumin: Implications for Protein Folding and Stability†

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ABSTRACT: The calcium binding protein α-lactalbumin folds via a molten globule intermediate. Calcium does not bind strongly to the unfolded protein or the molten globule, but does bind to the transition state between the molten globule and the native protein. Of interest are the structures formed in the transition state that promote calcium binding. To study the importance of local secondary structure on calcium binding, we have synthesized two peptides corresponding to the calcium binding site that include the flanking C-helix and 3_{10}-helix. The first peptide, elbow-A, consists of residues 72−100 from bovine α-lactalbumin, but with Cys 73, Cys 77, and Cys 91 replaced by alanines. In the second peptide, denoted elbow, the cysteines at position 73 and 91 are included and the nativelike disulfide bond is formed. Both peptides are monomeric and unstructured in aqueous solution and bind calcium weakly with apparent K_d's on the order of 10^{-2} M. In 50% trifluoroethanol (v/v), the peptides are 45% helical as judged by CD. NMR studies performed on elbow and elbow-A in TFE indicate that the helical structure is confined to the C-helix. In this solvent system elbow binds calcium one-to-one with a K_d of 50 μM. Removing the disulfide bond reduces, but does not eliminate calcium binding (K_d = 170 μM in 50% TFE). These results suggest that formation of the C-helix promotes calcium binding and may be a key determinant of calcium binding in the transition state.

The equilibrium molten globule state has characteristics similar to those of kinetic intermediates observed in protein folding experiments. For this reason it has been considered by many to be a good model for these intermediates (Kuwajima, 1989; Christensen & Pain, 1991; Jennings and Wright, 1993; Dobson, 1994; Baldwin, 1994; Ptitsyn, 1995), but one should also see the work of Privalov (1996). Like native proteins, molten globules contain significant secondary structure, and in some cases they have been shown to have nativelike backbone topology. Unlike native proteins, molten globules do not contain all the fixed side-chain interactions needed for the formation of a unique structure. Of interest are the events accompanying the transition from the molten globule state to the native state.

The calcium binding protein α-lactalbumin (α-LA)[1] forms a molten globule under a variety of conditions. The best studied is the acid-denatured state (A-state); however, a molten globule is also formed at neutral pH by removing bound calcium or adding denaturant (Kuwajima, 1976; Dolgikh et al., 1981; Kuwajima et al., 1996). A molten globule is also induced by removing three of the four disulfide bonds via site-directed mutagenesis. This variant which contains the 28−111 disulfide bond forms a molten globule at pH 8.5 (Peng et al., 1995).

α-LA can be divided into two subdomains: an α subdomain containing the four α-helices A−D, and a β subdomain composed of a small β sheet and several loops (Acharya et al., 1989; Acharya et al., 1991; Pike et al., 1996). The structure contains four disulfide bonds. Two, 28−111 and 6−120, are in the α subdomain, and one, 61−77, is in the β subdomain. The fourth, 73−91, connects the C-helix of the α subdomain with a 3_{10}-helix in the β subdomain. These two helices are a part of the helix−loop−helix structure responsible for calcium binding (Figure 1).

Hydrogen−deuterium exchange studies, proline scanning mutagenesis, and disulfide exchange experiments have all been used to probe the structure of the α-LA molten globule (Baum et al., 1989; Chyan et al., 1993; Ewbank & Creighton, 1991; Creighton & Ewbank, 1994; Peng et al, 1995; Schulman et al., 1995; Wu et al., 1995; Schulman & Kim, 1996). The picture that emerges is that there is nativelike backbone topology and significant secondary structure in the

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[1] Abbreviations: α-LA, α-lactalbumin; CD, circular dichroism; CSI, chemical shift index ; DQF-COSY, double quantum filtered correlated spectroscopy; EDTA, ethylenediaminetetraacetic acid; Fmoc, [[(9-flourenylmethyl)oxy]carbonyl]; NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; PAL, 5-(4′-[(Fmoc-amino)methyl]-3′,5′-dimethoxyphenoxy)valeric acid; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TOCSY, total correlated spectroscopy; TSP, 3-(trimethylsilyl)propionate; v/v, volume to volume.

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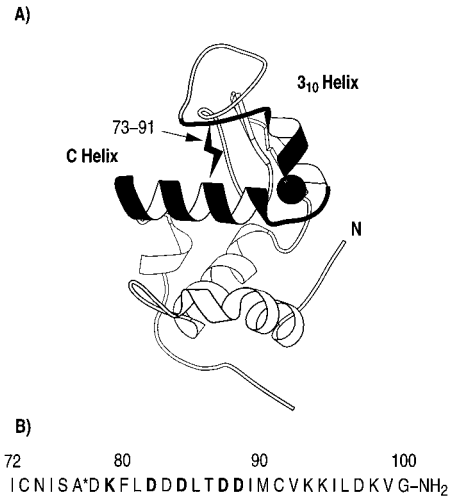

FIGURE 1: (A) Ribbon diagram of α-LA. (Drawing made using the program MOLSCRIPT (Kraulis, 1991).) Residues 72−100 are shaded. Calcium is shown in CPK format. The 3_{10}-helix extends through residues 76−82, and the C-helix is through residues 86−99. The disulfide between Cys 73 and Cys 91 is labeled. (B) Amino acid sequence for the peptide elbow. Sequence and residue numbers taken from bovine α-LA with the exception that an alanine, A*, replaces Cys 77. Residues shown in bold chelate calcium in α-LA. Elbow-A has the same sequence except the cysteines are changed to alanines. The loop peptide consists of residues 79−88.

α subdomain of the molten globule, while the β subdomain is more disordered. It has been shown that a peptide model containing only the α subdomain forms a molten globule-like state (Peng & Kim, 1994). Within the α subdomain, the A-, B-, and D-helices appear to be at least partially formed, while the C-helix is less stable. Studies have also been performed on the structurally homologous protein, equine lysozyme, which is known to form a molten globule state, and similar conclusions were reached (Dael et al., 1993; Griko et al., 1995; Morozova et al., 1995).

The interface between the two subdomains does not show nativelike properties in the molten globule. In contact with both subdomains is the calcium binding loop and the C-helix. The molten globule of α-LA does not bind calcium strongly (Kuwajima et al., 1989), and the C-helix is not intact (Morozova et al., 1995; Schulman et al., 1995; Schulman & Kim, 1996). When calcium is added to the molten globule, it induces formation of the native state (Dolgikh et al., 1981; Ikeguchi et al., 1986; Rao & Brew, 1989; Kuwajima et al., 1990; Ewbank & Creighton, 1993a; Ewbank & Creighton, 1993b; Wu et al., 1996). Kinetic folding studies have also shown that calcium stabilizes the transition state between the molten globule state and the native state (Kuwajima et al., 1989). The transition state between the molten globule and the native protein binds calcium with an estimated K_d of 3 μM, and the molten globule binds calcium with a K_d on the order of 1 mM (Kuwajima et al., 1989). For comparison, native α-LA binds calcium with a K_d of 2−10 nM, depending on conditions (Permyakov et al., 1981; Segewa & Sugai, 1983; Eberhard & Erne, 1991). Presumably, calcium binding helps lock the two subdomains together and therefore promotes the folding of the β subdomain. The details of this step are not known. Certainly crucial to the process are the formation of the specific structures that promote calcium binding. In the crystal structure of α-LA, two of the aspartic acids that chelate calcium come from the first turn in the C-helix. Therefore, formation of the C-helix may increase calcium binding by bringing these two important residues into place, and calcium binding may in turn stabilize the C-helix.

One approach used to study the role of local interactions in protein folding or in metal ion binding is to study the conformational preferences of protein fragments (Brown & Klee, 1971; Reid et al., 1981; Bierzynski et al., 1982; Rico et al., 1983, 1984; Kim & Baldwin, 1984, 1990; Oas & Kim, 1988; Blanco & Serrano, 1989; Fontana & Jaenicke, 1989; Montelione & Scheraga, 1989; Marsden et al., 1990; Shaw et al., 1990, 1991; Dyson & Wright, 1991; Shaw et al., 1991; Dyson et al., 1992a,b; Raleigh et al., 1992; Tasayco & Carey, 1992; Cox et al., 1993; Kemmink & Creighton, 1993; Shin et al., 1993a,b; Alexandrescu et al., 1994; Procyshyn & Reid, 1994; Itzhaki et al., 1995; Baldwin, 1995; Yang et al., 1995; Åkerfeldt et al., 1996; Bolin et al., 1996; Smith et al., 1996; Viguera et al., 1996). In this paper we report the results of a study of peptide models of the calcium binding site in α-LA. To study the interplay between helix formation and calcium binding, we have prepared two peptides corresponding to residues 72−100 from bovine α-LA (Figure 1). One peptide, denoted as elbow, contains the C-helix, the calcium binding loop, and the 3_{10}-helix as well as the Cys 73−Cys 91 disulfide bridge which connects the two helices. In the second peptide, elbow-A, Cys 73 and Cys 91 were changed to alanines. A control peptide, corresponding to only the ten residues (K-F-L-D-D-D-L-T-D-D) of the calcium binding loop, the loop peptide, was also prepared.

MATERIALS AND METHODS

Peptide Synthesis and Purification. Peptides were synthesized on a 0.16 mmol scale by solid phase methods using Fmoc-protected amino acids and TBTU-mediated amide coupling on a Millipore 9050 plus automated peptide synthesizer with standard reaction cycles. All β-branched amino acids and all residues which were coupled to β-branched residues were double coupled. Amino acid side chains were protected as follows: Asn and Cys, trityl group; Asp, *tert*-butyl ester; Lys, *tert*-butoxycarbonyl group; Ser and Thr, *tert*-butyl ether. Use of a resin with a PAL linker generated carboxyl terminal primary amides following cleavage from the resin with 91% TFA/3% anisole/3% thioanisole/3% ethanedithiol. Disulfide bonds were formed by air oxidation at pH 8.0 for 12 h. An Elman's test (Stewart & Young, 1984) confirmed the absence of free thiols. The peptides were purified using reverse phase HPLC (C18). An AB gradient was used where the A eluent was 0.1% aqueous TFA and the B eluent was 0.1% TFA/90% CH_3CN/10% H_2O. Fast atomic bombardment (FAB) mass spectrometry and amino acid analysis confirmed the identity of the pure products. Quantitative amino acid analysis (Commonwealth Biolabs, Richmond, VA) was used in triplicate to determine peptide concentrations.

Sedimentation Equilibrium. Samples were dialyzed against 20 mM Tris, 100 mM KCl at pH 7.4 with and without $CaCl_2$ present. Sedimentation equilibrium experiments were performed for two different concentrations of the peptides at 25 °C with a Beckman XL-A analytical ultracentrifuge, using rotor speeds of 30 000, 40 000, and 50 000 rpm. Experiments were carried out using 12 mm pathlength, six-channel, charcoal-filled Epon cells with quartz windows. Data was collected using continuous radial scanning with wavelengths

between 227 and 249 nm. Ten scans were averaged. Partial specific volumes were calculated from the weighted average of the partial specific volumes of the individual amino acids (Cohn & Edsall, 1943). The data were fit globally using either a single species model allowing the molecular weight to vary or monomer−*n*mer equilibrium models holding the molecular weight of the monomer fixed. The HID program from the Analytical Ultracentrifugation Facility at the University of Connecticut was used.

Circular Dichroism (CD) Spectroscopy. CD spectroscopy was performed using an Aviv 62A DS spectrometer at 25 °C. Wavelength spectra are the average of five scans with an averaging time of 3 s per data point and a bandwidth of 1 nm. Solutions contained 20 mM Tris, 100 mM KCl, pH 7.4 with either 100 mM $CaCl_2$ or 1 mM EDTA. Spectra were also taken in 8 M urea, 20 mM Tris, 100 mM KCl, 1 mM EDTA, pH 7.4, and in the presence of 20 mM $DyCl_3$, pH 7.4. For spectra in 50% TFE (v/v), TFE was combined 1:1 by volume with an aqueous solution containing the peptide in 40 mM Tris, 200 mM KCl, pH 7.4, with either 2 mM EDTA or 6 mM $CaCl_2$ (30 mM $CaCl_2$ for the loop peptide). Peptide concentrations were 150 μM for elbow in aqueous solution, 30 μM for elbow in TFE, 160 μM for elbow-A in aqueous solution, 30 μM for elbow-A in TFE, and 670 μM for the loop peptide in TFE.

Concentration Dependent CD. The mean residue ellipticity at 222 nm was measured for different concentrations of peptide in aqueous solution (20 mM Tris, 100 mM KCl, pH 7.4 with either 100 mM $CaCl_2$ or 1 mM EDTA) and in 50% (v/v) TFE. TFE was combined 1:1 by volume with an aqueous solution containing the peptide in 40 mM Tris, 200 mM KCl, pH 7.4, with either 2 mM EDTA or 10 mM $CaCl_2$.

TFE Titrations. TFE titrations of elbow and elbow-A were followed by monitoring the CD signal at 222 nm at 25 °C. Solutions contained 20 mM Tris, 100 mM KCl, pH 7.4, with 5 mM EDTA or 100 mM $CaCl_2$. No corrections were made for nonideal volume effects, but they are known to be small (Rochester & Symonds, 1974).

pH Titrations. Samples were prepared in 2 mM phosphate, 2 mM citrate, 2 mM borate, 10 mM NaCl, and 1 mM EDTA, and the pH was adjusted by the addition of small amounts of concentrated HCl.

Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR experiments were performed on a Bruker Instruments AMX 600 spectrometer and Varian Instruments Inova 500 and 600 MHz spectrometers. Spectra were internally referenced to TSP at 0.0 ppm. 2D spectra were taken of 2.5 mM elbow in 50% H_2O/50% TFE-d_3 (v/v), 100 mM KCl at pH 5.2 (uncorrected), 27 °C, with either 10 mM $CaCl_2$ or 400 μM EDTA. 2D spectra were also taken of elbow-A in 50% H_2O/50% TFE-d_3 (v/v), 100 mM KCl, 500 μM EDTA at pH 7.3 (uncorrected), 27 °C. Assignments were made using standard methods (Wuthrich, 1986).

Calcium Titrations. Calcium titrations were performed using two different methods. For low peptide concentrations, the CD signal at 222 nm was monitored, and for high peptide concentrations, 1D NMR was used to monitor the chemical shifts of resolved proton peaks. Titrations were done both in aqueous solution and in 50% TFE (v/v). All solutions contained 20 mM Tris and 100 mM KCl. Before analysis, 10 mg of the chelating agent Chelex-100 (Bio-Rad) was mixed with the sample for 15 min and then removed by filtration. $CaCl_2$ was added from stock solutions calibrated by EDTA (0.099 mM, Fisher Scientific) titration using murexide as an indicator. For experiments in 50% TFE (v/v), the concentration of elbow was 38 μM for CD and 970 μM for NMR. The concentration of the loop peptide was 200 μM for CD and 930 μM for NMR. Elbow-A aggregates at high concentrations in TFE/$CaCl_2$, and it could only be examined by CD at a concentration of 36 μM. For NMR experiments in aqueous solution the concentrations of peptide were 104 μM for elbow, 1.1 mM for elbow-A, and 2.2 mM for the loop peptide.

Plots of mean residue ellipticity or chemical shift versus $CaCl_2$ concentration were fit to determine apparent K_d's. The data were fit with a nonlinear least squares fitting routine (KaleidaGraph, Abelbeck Software) assuming one binding site.

RESULTS

Three peptides, elbow, elbow-A, and the loop peptide, were synthesized. Elbow consists of residues 72−100 from bovine α-LA, including the disulfide between residues 73 and 91 (Figure 1). The cysteine at position 77, which normally forms a disulfide with residue 61, is replaced with an alanine. In the native protein the C-helix extends from residue 86 to 99, and the groups chelating calcium are the carboxyls of Asp 82, Asp 87, and Asp 88 and the carbonyls of Lys 79 and Asp 84. Residues 76−82 form a small 3_{10}-helix in the native protein. Elbow-A has the same sequence as elbow, except that the two cysteines are replaced with alanines, and the loop peptide contains the calcium binding residues, 79−88.

Analytical ultracentrifugation shows elbow-A to be monomeric at 50 μM in 20 mM Tris, 100 mM KCl, pH 7.4, in the presence and absence of 100 mM $CaCl_2$. Elbow is also monomeric at the same concentration but shows a small amount of aggregation (∼10%) in the presence of 10 and 100 mM $CaCl_2$. Concentration dependent CD experiments also indicate that elbow and elbow-A are monomeric. The CD signal for elbow-A shows no concentration dependence in the presence and absence of $CaCl_2$, and for elbow the CD signal is independent of concentration up to 200 μM peptide in the presence and absence of $CaCl_2$. Above 200 μM the mean residue ellipticity of elbow is concentration dependent with and without $CaCl_2$ present. All further studies on elbow in aqueous solution were done with concentrations below 200 μM. The peptides are also monomeric, as judged by CD, in 50% TFE (v/v). The CD signal at 222 nm recorded in 50% TFE (v/v) is independent of peptide concentration in the presence and absence of $CaCl_2$. Concentration dependent behavior is observed for the elbow-A peptide at peptide concentrations above 500 μM in the presence of $CaCl_2$.

The far-UV CD spectra of the three peptides show them to be largely unstructured in aqueous solution at 25 °C, pH 7.4 (Figure 2). Similar results have been reported for the isolated C-helix from bovine α-lactalbumin (Shimizu et al., 1996) and hen lysozyme (Bolin et al., 1996). The mean residue ellipticity at 222 nm is −4200 for elbow and −4600 for elbow-A. This indicates that formation of the interhelical disulfide bond does not induce additional helical structure. The mean residue ellipticity expected for a 29-residue peptide in a 100% helical conformation is −34 000 (Chen et al.,

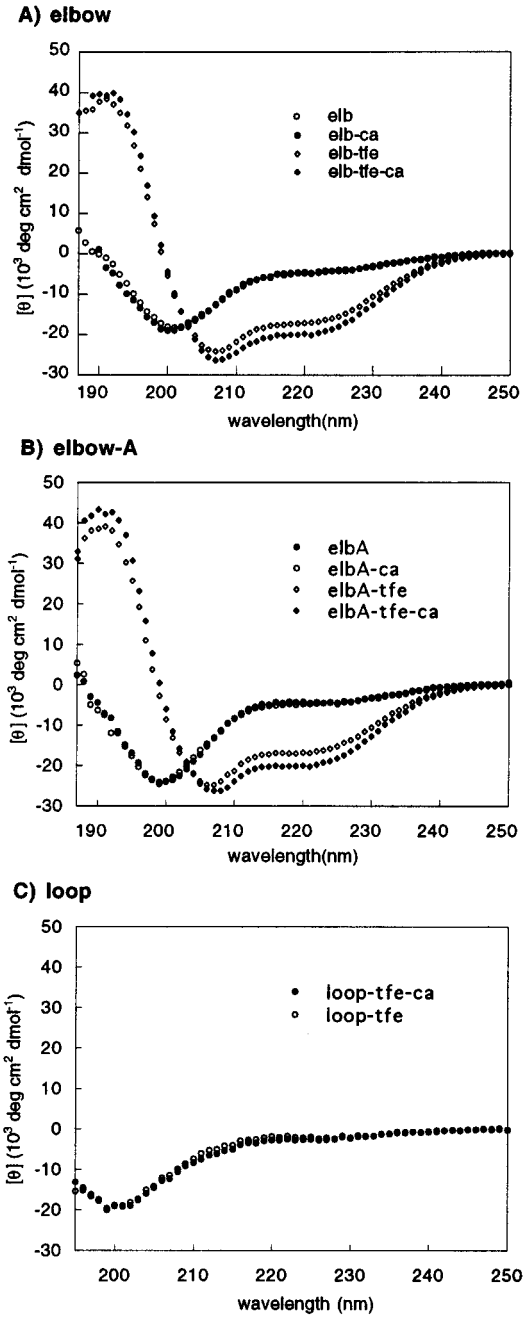

FIGURE 2: Far-UV CD spectra of the peptides in the presence and absence of $CaCl_2$ and 50% TFE (v/v) at 25 °C. Solutions contained 20 mM Tris, 100 mM KCl at pH 7.4. Aqueous solutions contained 100 mM $CaCl_2$ or 1 mM EDTA. In TFE, solutions contained 3 mM $CaCl_2$ (15 mM $CaCl_2$ for the loop peptide) or 1 mM EDTA.

1974; Gans et al., 1991). In 8 M urea the mean residue ellipticity is −2500 for both peptides. Assuming −2500 corresponds to 0% helix and −34 000 to 100% helix, we calculate that elbow is 5% helical and elbow-A 7% helical. The CD signal of both elbow and elbow-A at 222 nm shows a small dependence on pH (Figure 3). The strongest signal is seen at lower pHs. At pH 2.8 the mean residue ellipticity for both peptides is −6000 corresponding to an estimation of 11% helix. Concentration dependent studies at pH 2.5 indicate that the increase in signal at lower pHs is not due to peptide association. Lowering the temperature has no significant effect on the spectra. Also, the addition of $CaCl_2$ up to 100 mM does not change the spectra. These results indicate that either the peptides do not bind calcium or, if

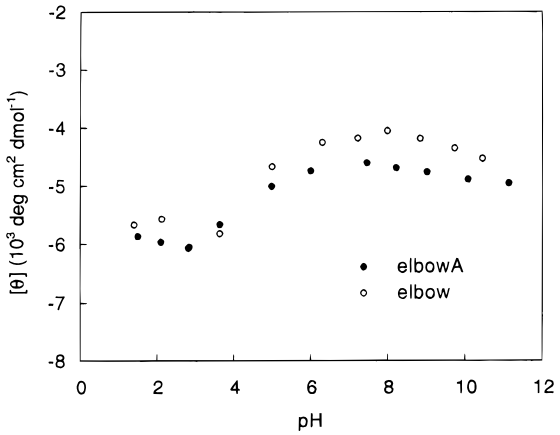

FIGURE 3: Mean residue ellipticity of elbow and elbow-A measured at 222 nm as a function of pH. The buffer contained 2 mM phosphate, 2 mM citrate, 2 mM borate, 10 mM NaCl, and 1 mM EDTA. The data were collected at 25 °C with a peptide concentration of 20 μM.

they do, calcium binding does not induce any additional structure.

Lanthanide ions have often been used to study calcium-binding proteins and peptides partly because the increased charge on the lanthanide should result in stronger binding. Addition of $DyCl_3$ to aqueous solutions of elbow and elbow-A at pH 7.4 resulted in only small changes to the CD spectra. The addition of 20 mM $DyCl_3$ changed the signal at 222 nm from −4200 to −5000 for elbow and from −4600 to −5500 for elbow-A.

$CaCl_2$ titrations followed with 1D NMR show that calcium binds weakly to the peptides in aqueous solution. For all three peptides there are small but reproducible chemical shift changes (0.01−0.05 ppm) associated with the addition of millimolar amounts of $CaCl_2$. For some peaks a plot of chemical shift versus $CaCl_2$ concentration could be fit to yield an apparent K_d assuming a single calcium binding site in the peptide. It is important to emphasize that for all three peptides different K_d's ranging between 5 and 80 mM are obtained depending on which peak is followed. This indicates that all three peptides most likely contain multiple weak binding sites for calcium. It is not surprising that these peptides have multiple binding sites given that they are largely unstructured, with five aspartic acids in the loop peptide and seven in elbow and elbow-A. Short peptides derived from the loop region of EF-hand proteins have also been shown to bind calcium nonspecifically with multiple binding sites (Marsden et al., 1988).

Both elbow and elbow-A become more helical with the addition of TFE, and their CD spectra are sensitive to the presence of calcium while in TFE (Figure 2). Peptides with an intrinsic tendency to form helical conformations often show a dramatic increase in helical content upon the addition of TFE, and TFE is widely used to induce and stabilize helical structures in peptides (Nelson & Kellenbach, 1986; Cammers-Goodwin et al., 1996). Previously TFE was thought to only induce significant helical structure in peptides with an intrinsic propensity to adopt a helical conformation. This has led to the description of the effect of TFE as helix-enhancing rather than helix-inducing. There are, however, a number of examples of peptides that form helices in TFE but adopt a different conformation in the native state (Zhong & Johnson, 1992; Fan et al., 1993; Waterhous et al., 1994;

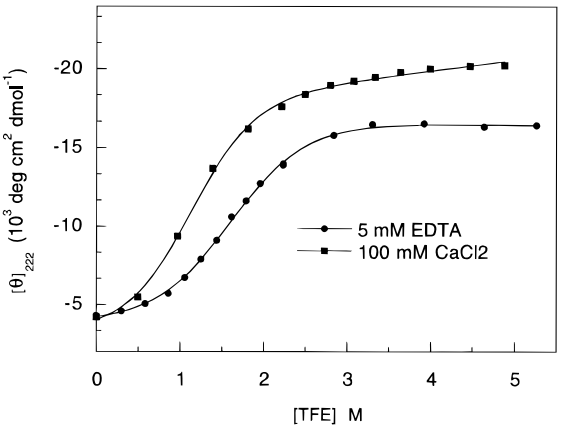

FIGURE 4: Mean residue ellipticity of elbow at 222 nm as a function of TFE concentration (M) in the presence and absence of calcium. Solutions contained 16 μM elbow in 20 mM Tris, 100 mM KCl at pH 7.4 in 100 mM $CaCl_2$ (squares) or 5 mM EDTA (circles).

Hamada et al., 1995). The exact mechanism by which TFE stabilizes the helical conformation is still controversial. Early work on the S-peptide argues against simple dielectric effects, at least in this system (Nelson & Kallenbach 1986). Several studies support the notion that TFE acts by weakening hydrogen bonds between backbone amides and the solvent, thus destabilizing the unfolded state (Conio et al., 1970; Llinas & Klein, 1975; Storrs et al., 1992; Cammers-Goodwin et al., 1996), while other work suggests a model in which TFE promotes helix formation by preferentially interacting with the helical state (Jasanoff & Fersht, 1994; Rajan and Balaram, 1996).

As TFE is added to elbow and elbow-A the mean residue ellipticity at 222 nm becomes more negative until the signal levels off at 20%−30% TFE. The midpoint of the transition occurs at lower concentrations of TFE with calcium present, and the endpoint of the titration corresponds to a more helical state (Figure 4). CD spectra of elbow in 30%, 50%, and 80% TFE are nearly identical. In 50% TFE (v/v) the mean residue ellipticity of elbow measured at 222 nm is −16 800 without calcium and −19 500 with calcium. These values correspond to 45% helix without calcium and 54% helix with calcium. In the native protein 45% of the sequence is in an α-helix and 20% is in a 3_{10}-helix. For elbow-A the mean residue ellipticity at 222 nm is −16 700 without calcium and −19 800 with calcium, corresponding to 45% and 55% helix, respectively. It is important to point out that structures other than helices can contribute to the ellipticity at 222 nm and that the rotational strength of the transitions that give rise to the helix signal at 222 nm depends upon the local backbone conformation (Manning et al., 1988; Manning & Woody, 1991).

2D NMR of elbow shows that the C-helix is intact in 50% TFE (v/v) in the absence of calcium. For residues 86−99, which make up the C-helix in the native protein, there are numerous i to $i + 3$ NOEs from the α-protons to the NH and β-protons. This is strong evidence that these residues are helical in elbow (Wuthrich, 1986). In addition, as expected for a helix, there are strong NH_i to NH_{i+1} crosspeaks for these residues. The chemical shift indices (CSI) also indicate a helix between residues 86 and 99 (Wishart et al., 1992). In the native protein there is a 3_{10}-helix between residues 76 and 82. There is no definitive evidence that this helix exists in elbow. There are some strong NH_i to NH_{i+1}

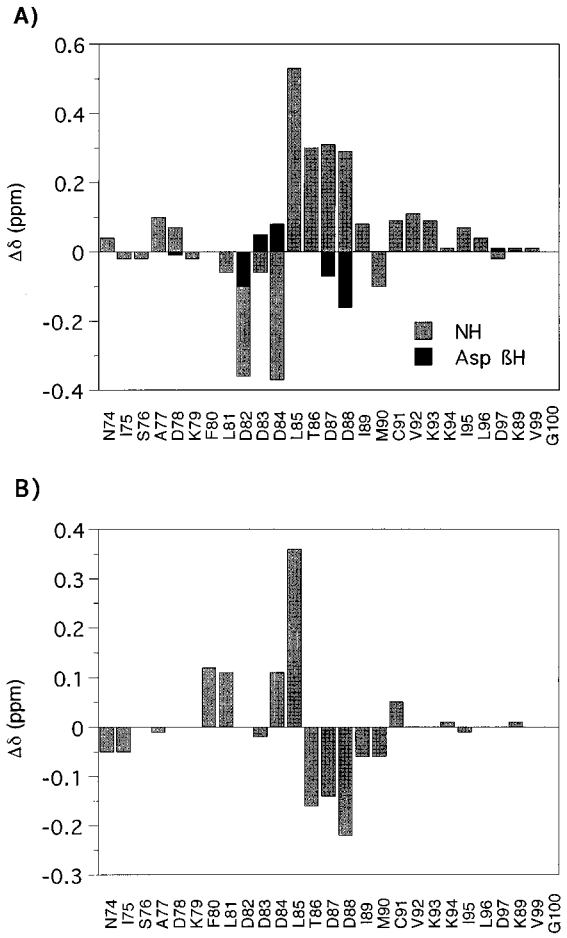

FIGURE 5: Differences in chemical shifts for the calcium bound and the calcium free form of the peptide elbow in 50% TFE. A positive bar indicates that the chemical shift of the holo-form is larger. (A) Values are shown for the NH-protons and for the β-protons on aspartic acids. For each aspartic acid, the β-proton which has the largest chemical shift difference was graphed. (B) Values are shown for the α-protons.

NOEs between these residues, but there are no i to $i + 2$ or i to $i + 3$ connectivities. Also, there are no NOEs indicative of tertiary structure. In the crystal structure, Phe 80 packs between the 3_{10}-helix and the C-helix, placing the ring protons within 3.2 Å of the β-protons on Asp 87, Asp 88, and Cys 91 and the methyl protons of Ile 75. No NOEs are seen between these protons in elbow.

When $CaCl_2$ is added to elbow, there are large changes in chemical shifts for most of the residues which form the binding loop in the native protein, 79−88 (Figure 5). The only exception is Lys 79, which shows very small changes in the chemical shift of its α- and NH-protons. In the crystal structure Lys 79 chelates calcium with its carbonyl and is in the 3_{10}-helix. There are seven aspartic acids in elbow, and only the ones in the binding loop show significant changes in their β-proton chemical shifts. These results provide strong evidence that calcium binds to the same region of the peptide as in the native state. Also noticeable is that the chemical shifts for the α-protons on Thr 86, Asp 87, and Asp 88 all shift upfield by over 0.1 ppm with the addition of $CaCl_2$. A shift to this direction often correlates with a more helical conformation (Wishart et al., 1992). NOE data from both apo- and holo-elbow indicate that these residues comprise the first turn of the C-helix. With calcium though, the i to $i + 3$ NOEs from the α-protons to the β-protons in

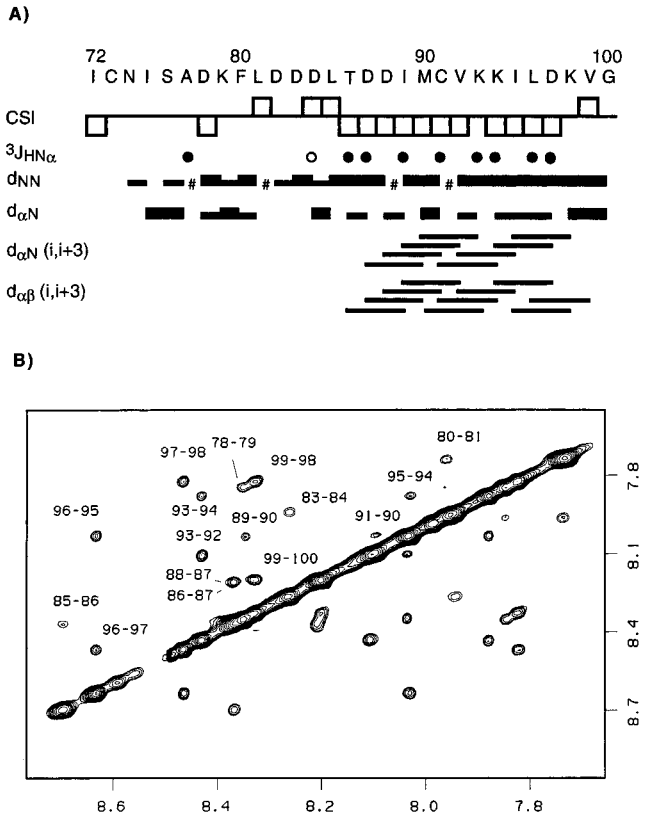

FIGURE 6: (A) Sequential NOEs for elbow in 50% TFE (v/v), 10 mM $CaCl_2$ show that the C-helix is formed. Similar results are seen without calcium. The sample was at 27 °C, pH 5.2 (uncorrected). For i to $i + 1$ NOEs, the thickness of the line indicates strong (1.8 Å−2.7 Å) or medium−weak (1.8 Å−5.0 Å) NOEs. # indicates that a possible peak is under the diagonal. CSI refers to chemical shift index and is based on α-CH chemical shifts (Wishart et al., 1992). Below the horizontal indicates a CSI of −1 and above the horizontal indicates +1. Random coil chemical shifts in 50% TFE are assumed to be the same as those in water (Merutka et al., 1995). $^3J_{HN\alpha}$ couplings are shown as a filled circle if they are below 6 Hz, and as an open circle if they are above 8 Hz. (B) The NH−NH region of a NOESY of elbow in 50% TFE, 10 mM $CaCl_2$. Peaks are labeled by residue number.

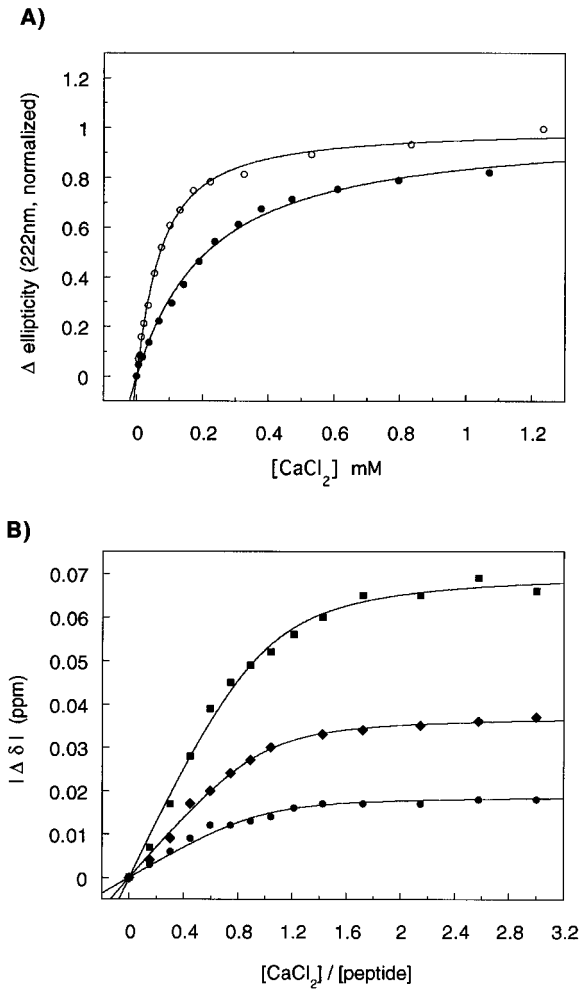

FIGURE 7: (A) Calcium titrations of elbow (filled circle) and elbow-A (open circle) in 50% TFE (v/v), monitored by the CD signal at 222 nm. The change in ellipticity is normalized from 0 to 1. For elbow the mean residue ellipticity increased from −16 700 to −19 800, and for elbow-A it increased from −16 800 to −19 500. The peptide concentrations were 37 μM for elbow and 34 μM for elbow-A. The curve fits give a K_d of 50 μM for elbow and 170 μM for elbow-A. (B) Calcium titration of elbow in 50% TFE (v/v) at high peptide concentration demonstrates one-to-one binding. 1D NMR was used to follow chemical shifts as a function of [$CaCl_2$]. Changes in chemical shifts are plotted for three protons: β Ala 77 (filled circles), α Cys 91 (filled diamonds), and α Asp 84 (filled squares). The peptide concentration was 970 μM.

the first turn of the helix are over twice as strong as those without calcium. One possibility is that the addition of calcium reduces fraying at the start of the helix by binding to Asp 87 and Asp 88, and therefore enhances the helical character of Thr 86, Asp 87, and Asp 88. Such behavior would at least partly explain the additional helicity seen in the CD spectra with the addition of calcium.

The NOE pattern for calcium-bound elbow is similar to that of the apo-peptide (Figure 6). The C-helix is formed, but there is no conclusive evidence that the 3_{10}-helix is formed. Eight of the residues in the C-helix have measured $^3J_{HN\alpha}$ coupling constants <6 Hz. This is indicative of a helical structure (Bystrov, 1976). The only other residue with a coupling constant below 6 Hz is Ala 77. There is a weak tertiary NOE between the ring protons of Phe 80 and the α-proton on Asp 88 which was not observed in the apo-peptide. In the native protein the distance between these two protons is 3.2 Å.

2D NMR on elbow-A shows that the C-helix is intact in 50% TFE in the absence of calcium. There are i to $i + 3$ NOEs from the α-protons to the NH and β-protons for residues 85−99. The chemical shift indices for residues 86−99 are all −1 (indicative of a helix) except for Lys 98. Almost all the N-terminal residues, 72−85, have indices equal to 0 (indicative of random coil). The exceptions, Asp 83, Asp 78, and Ile 72 have indices equal to −1. There are only two weak NOEs indicative of any regular secondary structure in residues 72−85. There are i to $i + 3$ NOEs between the α-proton on Lys 79 and the β-protons on Asp 82, and between the α-proton on Asp 78 and the β-protons on Leu 81. These are weak NOEs indicating that there may be a partially stable helix between residues 78 and 82. These residues comprise the last turn of the 3_{10}-helix in the native protein.

Elbow and elbow-A bind calcium more strongly in TFE than in aqueous solution. In 50% TFE (v/v) elbow binds calcium with a K_d of 50 μM (Figure 7a). A calcium titration with a high concentration of elbow showed that binding is one to one (Figure 7b). Elbow-A binds calcium with a K_d of 170 μM in 50% TFE (Figure 7a). Interpreting these results is complicated because TFE may be enhancing

binding in more than one way. One possibility is that forming the C-helix enhances binding. Another possibility is that the tighter binding is simply due to solvent effects separate from changes in peptide structure. The dielectric constant of TFE (26.7) is lower than that of water (78.5), and thus, unfavorable electrostatic repulsion between the negatively charged side chains in the apo-peptide may be enhanced in TFE-containing solutions. Alternatively, hydration of the calcium ion may be different in the mixed solvent systems.

K_d's for calcium binding by elbow vary between 80 μM at 32% TFE (v/v) and 10 μM at 85% TFE (v/v). From CD spectra it appears that there is no change in structure of the peptide over this range of TFE concentrations. Also, the NOE pattern in 80% TFE (v/v) is similar to the one in 50% TFE (v/v). Increasing the percentage of TFE from 32% (v/v) to 85% (v/v) increases calcium affinity by a factor of 8 with no apparent changes in peptide structure. However, going from an unfolded peptide (in H_2O) to a folded peptide (in 32% TFE (v/v)) increases calcium affinity by a factor of 125. We interpret these results to mean that although part of the increased binding observed in TFE may be due to solvent effects, the major cause of enhanced binding is due to the formation of the C-helix. Additional evidence for this conclusion comes from TFE titrations in the presence and absence of calcium and from control experiments with the loop peptide.

If formation of the C-helix results in increased calcium binding, then calcium should stabilize the C-helix and alter the helix−coil transition induced by TFE. With calcium present, the helix−coil transition for elbow occurs at lower concentrations of TFE, indicating that calcium is stabilizing the C-helix (Figure 4). The TFE titration curves have been fit using the formalism developed by Jasanoff and Fersht (1994). In 100 mM calcium the midpoint of the titration is at 1.1 M TFE (8% v/v) and without calcium present the midpoint is at 1.6 M TFE (12% v/v). The calculated $\Delta\Delta G$ (with Ca^{2+} − without Ca^{2+}) for helix formation is −2.0 kcal/mol at 50% TFE (v/v). Also noticable is that the helix−coil transition is steeper in the presence of calcium. The constant of proportionality, the *m*-value, is 1.6 kcal/M with calcium and 1.3 kcal/M without calcium.

Studies with the loop peptide provide further evidence that a stable C-helix is important for calcium binding. The loop peptide does not contain the C-helix, but includes all the calcium binding residues. The CD spectra of the loop peptide in 50% TFE (v/v) show small changes in mean residue ellipticity (on the order of 1000 deg cm^2 dmol^{-1}) at 220 nm with the addition of $CaCl_2$, but a plot of ellipticity versus $CaCl_2$ concentration can not be fit to a single binding site model. A titration followed by 1D NMR gives results similar to those observed for the loop peptide in water. Depending on which peaks are followed, different apparent K_d's are obtained. In this case though, the lowest K_d's are on the order of 1 mM. The α- and β-protons of Asp 84 give apparent K_d's of 1.2 and 1.6 mM, respectively. The α-proton of Leu 85 and the methyl protons of Thr 86 give an apparent K_d of 0.9 mM. In 50% TFE (v/v) the loop peptide binds calcium approximately 20 times more weakly than elbow and 6 times more weakly than elbow-A. This indicates that the extra structure in elbow and elbow-A is important for stronger binding and is important for forming a unique calcium binding site. The 2D NMR experiments performed on elbow and elbow-A indicate that the C-helix is the most significant structure formed in 50% TFE (v/v).

DISCUSSION

In aqueous solution, the peptides elbow and elbow-A are largely unstructured and bind calcium only weakly. Metal binding (Ca^{2+} or Dy^{3+}) does not induce secondary structure formation in elbow and elbow-A, indicating that interactions with the rest of the protein are required to stabilize the C-helix and the 3_{10}-helix. Also, formation of the 73−91 interhelical disulfide is not sufficient to stabilize the C-helix in the absence of interactions with the rest of the protein.

Similar studies have been done with EF-hand peptides but with different results. Peptides corresponding to sequences from the helix−loop−helix structure of EF-hands bind calcium and calcium binding induces helix formation (Reid et al., 1981; Marsden et al., 1990; Procyshyn & Reid, 1994; Åkerfeldt et al., 1996). There is, however, a crucial difference between the EF-hand peptides and the peptides derived from α-LA. EF-hands are usually found paired in proteins with significant packing between the helices and loops (Strynadka & James, 1989; Ikura, 1996), while α-LA only has one calcium binding site. EF-hand peptides dimerize and form a nativelike domain upon the addition of calcium (Shaw et al., 1990, 1992). Elbow and elbow-A do not dimerize and are not expected to dimerize.

Stabilization of the C-helix by the addition of TFE leads to moderate to strong calcium binding. The binding site is formed from the same region which binds calcium in the intact protein. Presumably, formation of the C-helix promotes binding by bringing Asp 88 and Asp 89 into place relative to the rest of the binding residues. Removal of the 73−91 disulfide reduces but does not eliminate calcium binding, indicating that formation of the C-helix rather than the disulfide is the major determinant of calcium binding in elbow and elbow-A.

Work on disulfide variants of α-LA has shown that full length α-LA without the disulfides, 73−91 and 61−77, does not bind calcium (Wu et al., 1996). It is likely though, that the C-helix is not formed in this variant of α-LA since it has been shown that the C-helix is not formed in a variant of α-LA containing only the 28−111 disulfide (Schulman & Kim, 1996). Thus, in the full length protein, the 73−91 disulfide may be important for C-helix stability and therefore needed for calcium binding. In the peptides studied here, stabilization of the C-helix by changing solvent conditions leads to moderately strong calcium binding.

An important step in the folding of α-LA from molten globule to native protein is the formation of nativelike contacts between the partly nativelike α-subdomain and the less structured β-domain. The C-helix and the calcium binding loop contact both subdomains and do not have nativelike characteristics in the molten globule. This implies that stabilization of the C-helix and calcium binding are important events in the transition from the molten globule to the native protein. We have provided evidence that a stable C-helix is important for calcium binding and that formation of the 73−91 disulfide is not sufficient to induce strong calcium binding in the absence of a structured C-helix. The formation of the C-helix coupled with calcium binding is likely a crucial step which allows for nativelike contacts between the α- and β-subdomains.

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SUPPORTING INFORMATION AVAILABLE

^{1}H assignments for elbow in 50% TFE with and without calcium and for elbow-A in 50% TFE without calcium (4 pages). Ordering information is given on any current masthead page.

REFERENCES

Acharya, K. R., Ren, J., Stuart, D. I., Phillips, D. C., & Fenna, R. E. (1991) *J. Mol. Biol. 221*, 571−581.
Acharya, K. R., Stuart, D. I., Walker, N. P. C., Lewis, M., & Phillips, D. C. (1989) *J. Mol. Biol. 208*, 99−127.
Åkerfeldt, K. S., Goyne, A. N., Wilk, R. R., Thulin, E., & Linse, S. (1996) *Biochemistry 35*, 3662−3669.
Alexandrescu, A. T., Abeygunawardana, C., & Shortle, D. (1994) *Biochemistry 33*, 1063−1072.
Baldwin, R. L. (1994) *Bio Essays 16*, 207−210.
Baldwin, R. L. (1995) *Biophys. Chem. 55*, 127−135.
Baum, J., Dobson, C. M., Evans, P. A., & Hanley, C. (1989) *Biochemistry 28*, 7−13.
Bierzynski, A., Kim, P. S., & Baldwin, R. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2470−2474.
Blanco, F. J., & Serrano, L. (1995) *Eur. J. Biochem. 230*, 634−649.
Bolin, K. A., Pitkeathly, M., Miranker, A., Smith, L. J., & Dobson, C. M. (1996) *J. Mol. Biol. 261*, 443−453.
Brown, J. E., & Klee, W. A. (1971) *Biochemistry 10*, 470−476.
Bystrov, V. F. (1976) *Progr. Nucl. Magn. Reson. Spectrosc. 10*, 41−82.
Cammers-Goodwin, A., Allen, T. J., Oslick, S. L., McClure, K. F., Lee, J. H., & Kemp, D. S. (1996) *J. Am. Chem. Soc. 118*, 3082−90.
Chen, Y.-H., Yang, J. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350−3359.
Christensen, H., & Pain, R. H. (1991) *Eur. Biophys. J. 19*, 221−229.
Chyan, C.-L., Wormald, C., Dobson, C. M., Evans, P. A., & Baum, J. (1993) *Biochemistry 32*, 5681−5691.
Cohn, E. J., & Edsall, J. T. (1943) *Proteins, amino acids and peptides as ions and dipolar ions*, Reinhold Publishing Corporation, New York.
Conio, G., Patrone, E., & Brighetti, S. (1970) *J. Biol. Chem. 245*, 3335−3340.
Cox, J. P. L., Evans, P. A., Packman, L. C., Williams, D. H., & Woolfson, D. N. (1993) *J. Mol. Biol. 234*, 483−492.
Creighton, T. E., & Ewbank, J. J. (1994) *Biochemistry 33*, 1534−1538.
Dael, H. V., Haezebrouck, P., Morozova, L., Arico-Muendel, C., & Dobson, C. M. (1993) *Biochemistry 32*, 11886−11894.
Dobson, C. M. (1994) *Curr. Biol. 4*, 636−640.
Dolgikh, D. A., Gilmanshin, R. I., Brazhnikov, E. V., Bychkova, V. E., Semisotnov, G. V., Venyaminov, S. Y., & Ptitsyn, O. B. (1981) *FEBS Lett. 136*, 311−315.
Dyson, H. J., & Wright, P. E. (1991) *Annu. Rev. Biophys. Biophys. Chem. 20*, 519−538.
Dyson, H. J., Merutka, G., Waltho, J. P., Lerner, R. A., & Wright, P. E. (1992a) *J. Mol. Biol. 226*, 795−817.
Dyson, H. J., Sayre, J. R., Merutka, G., Shin, H.-C., Lerner, R. A., & Wright, P. E. (1992b) *J. Mol. Biol. 226*, 819−835.
Eberhard, M., & Erne, P. (1991) *Eur. J. Biochem. 202*, 1333−1338.
Ewbank, J. J., & Creighton, T. E. (1991) *Nature 350*, 518−520.
Ewbank, J. J., & Creighton, T. E. (1993a) *Biochemistry 32*, 3677−3693.
Ewbank, J. J., & Creighton, T. E. (1993b) *Biochemistry 32*, 3694−3707.
Fan, P., Bracken, C., & Baum, J. (1993) *Biochemistry 32*, 1573−1582.
Fontana, A., & Jaenicke, R. (1989) *Eur. J. Biochem. 183*, 513−518.
Gans, P. J., Lyu, P. C., Manning, M. C., Woody, R. W., & Kallenbach, N. R. (1991) *Biopolymers 31*, 1605−1614.
Griko, Y. V., Freire, E., Privalov, G., Dael, H. V., & Privalov, P. L. (1995) *J. Mol. Biol. 252*, 447−459.
Hamada, D., Kuroda, Y., Tanaka, T., & Goto, Y. (1995) *J. Mol Biol. 254*, 737−746.
Ikeguchi, M., Kuwajima, K., & Sugai, S. (1986) *J. Biochem. 99*, 1191−1201.
Ikura, M. (1996) *TIBS 21*, 14−17.
Itzkahi, L. S., Nieira, J. L., Ruiz-Sanz, J., De Prat Gay, G., & Fersht, A. R. (1995) *J. Mol. Biol. 254*, 289−304.
Jasanoff, A., & Fersht, A. R. (1994) *Biochemistry 33*, 2129−2135.
Jennings, P. A., & Wright, P. E., (1993) *Science 262*, 892−896.
Kemmink, J., & Creighton, T. E. (1993) *J. Mol. Biol. 234*, 861−878.
Kim, P. S., & Baldwin, R. L. (1984) *Nature 307*, 329−334.
Kim, P. S., & Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631−660.
Kraulis, P. J. (1991) *J. Appl. Cryst. 24*, 946−950.
Kuwajima, K. (1989) *Proteins 6*, 87−103.
Kuwajima, K. (1996) *FASEB J. 10*, 102−108.
Kuwajima, K., Nitta, K., Yoneyama, M., & Sugai, S. (1976) *J. Mol. Biol. 106*, 359−373.
Kuwajima, K., Mitani, M., & Sugai, S. (1989) *J. Mol. Biol. 206*, 547−561.
Kuwajima, K., Ikeguchi, M., Sugawara, T., Hiraoka, Y., & Sugai, S. (1990) *Biochemistry 29*, 8240−8249.
Llinas, M., & Klein, M. P. (1975) *J. Am. Chem. Soc. 97*, 4731−4737.
Manning, M. C., Illangasekare, M., Woody, R. W. (1988) *Biophys. Chem. 31*, 77−86.
Manning, M. C., & Woody, R. W. (1991) *Biopolymers 31*, 569−586.
Marsden, B. J., Hodges R. S., & Sykes, B. D. (1988) *Biochemistry 27*, 4198−4206.
Marsden, B. J., Shaw, G. S., & Sykes, B. D. (1990) *Biochem. Cell Biol. 68*, 587−601.
Merutka, G., Dyson, H. J., & Wright, P. E. (1995) *J. Biomol. NMR 5*, 67−81.
Montelione, G. T., & Scheraga, H. A. (1989) *Acc. Chem. Res. 22*, 70−76.
Morozova, L. A., Haynie, D. T., Arico-Muendel, C., Dael, H. V., & Dobson, C. M. (1995) *Nature Struct. Biol. 2*, 871−875.
Nelson, J. W., & Kallenbach, N. R. (1986) *Proteins: Struct. Funct. Genet. 1*, 211−217.
Oas, T. G., & Kim, P. S. (1988) *Nature 336*, 42−48.
Peng, Z.-y., & Kim, P. S. (1994) *Biochemistry 33*, 2136−2141.
Peng, Z.-y., Wu, L. C., & Kim, P. S. (1995) *Biochemistry 34*, 3248−3252.
Permyakov, E. A., Yarmolenko, V. V., Kalinichenko, L. P., Morozova, L. A., & Burstein, E. A. (1981) *Biochem. Biophy. Res. Commun. 100*, 191−197.
Pike, A. C., Brew, K., & Acharya, K. R. (1996) *Structure 4*, 691−703.
Procyshyn, R. M., & Reid, R. E. (1994) *J. Biol. Chem. 269*, 1641−1647.
Ptitsyn, O. B. (1992) In *Protein Folding* (Creighton, T. E., Ed.) pp 243−300, W. H. Freeman and Co., New York.
Ptitsyn, O. B. (1995) *Curr. Opin. Struct. Biol. 5*, 74−78.
Privalov, P. L. (1996) *J. Mol. Biol. 258*, 707−723.
Raleigh, D. P., Evans, P. A., Pitkeathly, M., & Dobson, C. M. (1992) *J. Mol. Biol. 228*, 335−342.
Rajan, R., & Balaram, P. (1996) *Int. J. Pept. Protein Res. 48*, 328−336.
Rao, K. R., & Brew, K. (1989) *Biochem. Biophys. Res. Commun. 163*, 1390−1396.
Reid, R. E., Gariepy J., Saumd, A. K., & Hodges, R. S. (1981) *J. Biol. Chem. 256*, 2742−2751.
Rico, M., Nieto, J. L., Santoro, J., Bermejo, F. J., Herranz, J., & Gallego, E. (1983) *FEBS Lett. 162*, 314−319.
Rico, M., Gallego, J., Santoro, J., Bermejo, F. J., Nieto, J. L., & Herranz, J. (1984) *Biochem. Biophys. Res. Commun. 123*, 757−763.

Rochester, C. H., & Symonds, J. R. (1974) *J. Fluorine Chem. 4*, 141−148.
Schulman, B. A., & Kim, P. S. (1996) *Nature Struct. Biol. 3*, 682−687.
Schulman, B. A., Redfield, C., Peng, Z.-y., Dobson, C. M., & Kim, P. S. (1995) *J. Mol. Biol. 253*, 651−657.
Segewa, T., & Sugai, S. (1983) *J. Biochem. 93*, 1321−1328.
Shaw, G. S., Hodges, R. S., & Sykes, B. D. (1990) *Science 249*, 280−283.
Shaw, G. S., Hodges. R. S., & Sykes, B. D. (1991) *Biochemistry 30*, 8339−8347.
Shaw, G. S., Findlay, W. A., Semchuk, P. D., Hodges, R. S., & Sykes, B. D. (1992) *J. Am. Chem. Soc. 114*, 6258−6259.
Shimizu, A., Ikeguchi, M., Kobayashi, T., & Sugai, S. (1996) *J. Biochem. 119*, 947−952.
Shin, H. C., Merutka, G., Waltho, J. P., Wright, P. E., and Dyson, H. J. (1993a) *Biochemistry 32*, 6348−6355.
Shin, H. C., Merutka, G., Waltho, J. P., Tennant, L. L., Wright, P. E., and Dyson, H. J. (1993b) *Biochemistry 32*, 6356−6364.
Smith, L. S., Fiebig, K. M., Schwalbe, H., & Dobson, C. M. (1996) *Folding Design 1*, R95−R106.
Stewart, J. M., & Young, J. D. (1984) *Solid Phase Peptide Synthesis*, W. H. Freeman, New York.
Storrs, R. W., Truckses, D., & Wemmer, D. E. (1992) *Biopolymers 32*, 1695−1702.
Strynadka, N. C. J., & James, M. N. G. (1989) *Annu. Rev. Biochem. 58*, 951−998.
Tasayco, M. L., & Carey, J. (1992) *Science 255*, 594−597.
Viguera, A. R., Jimenez, M. A, Rico, M., & Serrano, L. (1996) *J. Mol. Biol. 255*, 507−521.
Waterhous, D. V., & Johnson, W. C., Jr. (1994) *Biochemistry 33*, 2121−2128.
Wishart, D. S., Sykes, B. D., & Richards, F. M. (1992) *Biochemistry 31*, 1647−1651.
Wu, L. C., Peng, Z.-y., & Kim, P. S. (1995) *Nature Struct. Biol. 2*, 281−285.
Wu, L. C., Schulman, B. A., Peng, Z.-y., & Kim, P. S. (1996) *Biochemistry 35*, 859−863.
Wuthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, John Wiley & Sons, Inc., New York.
Yang, J. J., Buck, M., Pitkeathly, M., Kotik, M., Haynie, D. T., Dobson, C. M., & Radford, S. E. (1995) *J. Mol. Biol. 252*, 483−91.
Zhong, L., & Johnson, W. C., Jr. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4462−4465.

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