Further Evidence Suggesting That the Slow Phase in Protein Unfolding and Refolding Is Due to Proline Isomerization: A Kinetic Study of Carp Parvalbumins†

Lung-Nan Lin and John F. Brandts*

ABSTRACT: It was suggested earlier that the slowest step normally observed in protein folding kinetics might be due to cis-trans isomerization of the peptide bonds which are N terminal to proline residues. As a means of further testing this hypothesis, the kinetics of three forms of carp parvalbumins have been examined. Two of these (bands 3 and 5) have no proline residues, while the other (band 2) has a single proline. Other than this, the amino acid sequences show a large degree of homology. The unfolding and refolding of each form have been examined under conditions where circular dichroism and fluorescence measurements suggest that the structure of the three native forms are exceedingly similar, as are the structures of the three denatured forms. In spite of the large similarity in the sequence and structure of these proteins, the kinetic studies show that band 2 invariably exhibits a slow phase which is never seen for band 3 or 5. This becomes several hundred fold slower than the next fastest phase for refolding under some conditions. The rate (ca. 10 s at 25 °C) of the slow phase and its activation energy (18 kcal/mol) are consistent with the proline isomerism hypothesis, and the observed limiting amplitude (ca. 12%) is what would be predicted if this proline were trans in the native state. Also consistent with the isomerism hypothesis is the lack of sensitivity of the rate and activation energy to the concentration of guanidine hydrochloride or calcium in the final refolding mixture. This study provides strong evidence in favor of the idea that proline isomerism is responsible for the slowest phase seen in the folding kinetics of proline-containing proteins.

In spite of the large number of studies of protein folding and unfolding reactions, there is little direct evidence which helps to delineate the sequence of molecular events which occur. Early thermodynamic studies (Brandts, 1964, 1969; Lumry et al., 1966; Tanford, 1970) show that a simple two-state model can explain protein folding satisfactorily for many small proteins. Calorimetric studies in particular show that the ratio of calorimetric enthalpy change to van't Hoff enthalpy change is nearly unity when small proteins undergo a major conformational transition (Jackson & Brandts, 1970; Privalov & Khechinashvili, 1974). However, recent kinetic studies (Tsong et al., 1972; Tsong & Baldwin, 1972; Tsong, 1973) have shown that protein folding is a complex process. Slow and fast kinetic phases, with relaxation times separated by a factor of 100 or more in the baseline region, are usually seen. In order to fit the kinetic data, various protein folding mechanisms have been proposed for different globular proteins (Tsong et al., 1972; Ikai et al., 1973; Tanford et al., 1973). These mechanisms frequently assume that "structural intermediates", present in substantial amounts only in the transition region, are involved in the folding process.

More recently, Hagerman & Baldwin (1976) and Brandts et al. (1975) have shown that, for ribonuclease, the existence of two kinetic phases is not due to the presence of structural intermediates, but rather due to two forms of denatured protein present both inside and outside the transition region. The two forms have nearly identical optical properties. After an extensive study on RNase folding, Hagerman & Baldwin (1976) suggested the folding mechanism

$$U_1 \overset{\text{slow}}{\rightleftarrows} U_2 \overset{\text{fast}}{\rightleftarrows} N$$

to explain their kinetic data, in which U_1 and U_2 are two forms of denatured protein, called the fast (U_2) and slow (U_1) refolding species. Under some conditions, a more complicated mechanism

$$U_1 \overset{\text{slow}}{\rightleftarrows} U_2 \overset{\text{fast}}{\rightleftarrows} I \overset{\text{faster}}{\rightleftarrows} N$$

was used. It was suggested that I may also be an unfolded species populated at equilibrium. However, they were unable to interpret differences between U_1, U_2, and I explicitly in terms of molecular structure. More recently, Hagerman (1977) has analyzed existing literature data for the folding transitions of cytochrome *c* (Ikai et al., 1973) and lysozyme (Tanford et al., 1973) and concluded that the mechanism $U_1 \rightleftarrows U_2 \rightleftarrows N$ may be used to describe their folding and unfolding reactions as well. He suggested that this three species model may provide a fairly general description of the folding kinetics of small proteins.

One specific suggestion (Brandts et al., 1975) has been made to account for the presence of both slow and fast phases in protein folding. This attributes the slowest phase to cis-trans isomerism of the peptide bonds which are N terminal to prolyl residues. That is, before a denatured protein can refold, each of the prolyl residues must be in the same isomeric state as required for the native structure. This isomerism process is slow, as shown in kinetic studies on proline-containing dipeptides. The purpose of this communication is to describe results of an experimental study designed to critically test the validity of the proline isomerism hypothesis. Carp parvalbumins serve as ideal proteins for this study. Of the three major parvalbumin components, bands 3 and 5 contain no proline, while band 2 contains one proline. If the slow phase of protein folding is due to proline isomerization, we should see no slow kinetic phase for bands 3 and 5 while, for band 2 with one proline, we should expect to see a slow kinetic phase with a relative amplitude of 10–20% (assuming the proline is trans in the native form). Another advantage in using carp parvalbumins is that their physicochemical properties and structures are well known. They are highly soluble and of relatively low molecular weight

† From the Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003. *Received March 7, 1978.* This work was supported by Grant GM-11071 from the National Institutes of Health.

0006-2960/78/0417-4102$01.00/0

(11 000–12 000) (Pechere et al., 1971a). In the native form, they strongly bind two calcium ions per molecule (Kretsinger & Nockolds, 1973; Donato & Martin, 1974). The amino acid composition and sequence of all three forms have been determined (Konosu et al., 1965; Kretsinger, 1972; Coffee & Bradshaw, 1973). The crystal structure of band 3 has also been published at a nominal 1.9-Å resolution (Kretsinger & Nockolds, 1973). Besides the differences in proline content, there are only small differences in amino acid composition. These three proteins are also very similar in terms of size, circular dichroism spectra, and high-resolution NMR spectra (Nelson et al., 1976), suggesting that they may be nearly identical in their three-dimensional structure.

A preliminary kinetic study on the proline-free band 5 by the pH-jump method has been reported from this laboratory (Brandts et al., 1977). The results showed no slow kinetic phase and thus favor the argument that proline isomerization is responsible for the slow kinetic phase for proline-containing proteins. However, considering the structural similarity of these three forms of parvalbumins, it is of critical importance to demonstrate the existence of a slow phase for band 2, under conditions where there is no slow phase for bands 5 and 3. In this communication, an extensive kinetic study is reported for all three forms of parvalbumin, using the pH-jump and guanidine-dilution methods.

Materials and Methods

Materials. UltraPure guanidine hydrochloride (Gdn·HCl)[1] was purchased from Schwarz/Mann Co. Sodium acetate, sodium chloride, and calcium chloride were obtained from Fischer Scientific Co. (ACS certified grades). Piperazine, which was used in the eluting solution for purifying parvalbumins, was obtained from Sigma Co. All chemicals were directly used without further purification. Sephadex G-75 and DEAE-Sephadex A-25 were purchased from Pharmacia. Two kinds of carp were used in isolating parvalbumins; one was an Israeli carp purchased from the Arkansas Department of Game and Fish, while the other was an unclassified carp obtained from a fish market in New York City.

Preparation and Amino Acid Analysis of Carp Parvalbumins. Parvalbumins were prepared from the white muscle of carp. The procedures described by Pechere et al. (1971a) have been employed with one significant modification: DEAE-Sephadex A-25 instead of DEAE-cellulose was used in the final stage of purification. Both the size of the column and the elution rate were also somewhat different. All chromatography was performed at room temperature. The pure parvalbumins obtained from the DEAE column were dialyzed against a large volume of 0.01 M NaCl solution. The sample was used immediately or frozen at −20 °C for later use. Protein concentrations were estimated spectrophotometrically on a Cary 14 spectrophotometer. An extinction coefficient $E_{1cm}^{1\%}$ (at 259 nm) of 1.80 was used for bands 3 and 5, and a value of 2.00 was used for band 2. The calcium content of parvalbumins was determined using a Perkin-Elmer 303 A atomic absorption spectrophotometer.

Since most literature studies (Pechere et al., 1971a; Kretsinger & Nockolds, 1973) were carried out on parvalbumins isolated from mirror carp, it was necessary to establish that the proteins used in this study correspond to those described in the literature. The amino acid compositions were therefore determined with a Spinco Model 120 B automatic amino acid analyzer. The proteins were hydrolyzed in 6 M HCl at 110 °C for 22 h in accordance with the procedures of Moore & Stein (1951). The amount of each amino acid was calculated using the height–width method of analysis.

Circular Dichroism (CD) Measurements. CD measurements were made on a Cary 60 spectropolarimeter with a 6002 CD accessory. The samples were scanned slowly at room temperature from 350 to 210 nm using a cell of 1-mm pathlength. It was not possible to obtain satisfactory CD data below 210 nm because of low transmitted light.

Fluorescence Measurements. Equilibrium fluorescence measurements were performed on a Perkin-Elmer MPF-44 fluorescence spectrophotometer. The instrument was run in the energy mode with both slits set at 1.5 mm. The measurements were made using a 1-cm quartz cell thermostated at 22 °C. Corrections were made for the background fluorescence. For bands 3 and 5, with no tryptophan or tyrosine but with high content of phenylalanine, fluorescence was determined with the excitation wavelength at 259 nm. The maximum fluorescence was observed at 283 nm. For band 2, with one tyrosine and also a high content of phenylalanine, the excitation wavelength was either 259 nm or 275 nm. Due to energy transfer, strong tyrosine fluorescence and no phenylalanine fluorescence was always observed for band 2, with an emission peak at 303 nm.

Stopped-Flow Measurements. The kinetic measurements were carried out on a Durrum stopped-flow spectrophotometer, using fluorescence detection. The Durrum fluorescence accessory, used in conjunction with the Xenon lamp accessory, gave adequate sensitivity. Two separate thermostating baths were used; one to thermostat the two driving syringes and another to thermostat the observation chamber. Such an arrangement permits one to minimize the thermal mixing artifact. A Tektronix 549 storage oscilloscope with a 1A7A differential amplifier was used to record the signal output. The voltage on the photomultiplier tube was controlled in the range of 0.60–0.65 kV with slit width settings at 5–2 mm, depending on protein concentration. The excitation wavelength was set at 259 nm and an optical filter (no. 26-4630 or 26-4622), purchased from Corning Glass Co., was placed before the photomultiplier tube to cut off the excitation light. Volume ratio syringes, purchased from Durrum, were used for guanidine dilution studies. The dead time was found to be in the range of 5–10 ms when high guanidine dilution was involved.

We found that the conventional water–water or guanidine–water mixing control was not adequate for eliminating temperature artifacts in fluorescence detection experiments. The following precautions were therefore taken before each kinetic experiment. A thermocouple, connected to a Honeywell Electronic 19 recorder, was used to eliminate the temperature difference between the syringe bath and observation chamber. One-tenth degree difference in temperature was detectable. Then, a water–guanidine mixing control was carried out. Any baseline aberrations were corrected by adjusting the thermostating water bath for the observation chamber. Finally, a fluorescence control run, which involved mixing Gdn·HCl and acetyl-L-phenylalanine, was performed to be sure that no large baseline artifacts were present. A detailed discussion of thermal artifacts due to dilution of Gdn·HCl has been given by Ikai (1971).

Results

Amino Acid Composition. The three major components of carp parvalbumins (bands 2, 3, and 5) were prepared from both the Israeli carp and the unclassified carp. The final DEAE-Sephadex chromatogram was shown in an earlier paper

[1] Abbreviation used: Gdn·HCl (GuHCl in figures), guanidine hydrochloride.

TABLE I: Amino Acid Composition of Carp Parvalbumins as Number of Residues per Molecule.

amino acid	band 2[a]			band 3[a]			band 5[a]		
Gly	9.52	9.40	9	8.39	7.44	8	8.24	8.72	9
Ala	20.49	21.53	20	19.44	18.70	20	20.18	18.24	17
Leu	8.09	9.61	9	8.14	8.59	9	7.95	8.83	9
Ile	4.80	6.15	5	3.39	3.41	5	3.63	4.90	6
Ser	4.76	6.44	7	4.41	4.59	5	4.20	5.14	6
Thr	3.86	4.40	4	5.40	5.07	5	4.62	6.88	7
Met	0	0	0	0	0	0	0	0	0
½-cystine	0.41	0	1	trace	0.64	1	trace	0	1
Pro	0.88	0.95	1	0	0	0	0	0	0
Phe	10.40	10.50	9	10.27	9.16	10	9.24	9.72	10
Tyr	0.86	1.01	1	0	0	0	0	0	0
Trp			0			0			0
Asp	16.04	16.54	13	15.50	15.53	14	17.12	16.18	13
Asn			3			3			3
Glu	9.21	10.11	8	7.71	7.75	6	7.88	8.25	6
Gln			2			2			2
NH_3	5.53	7.06		5.15	6.88		5.99	5.76	
Lys	10.66	11.50	13	9.50	10.55	13	9.58	13.33	11
His	0	0	0	1.11	1.04	1	0.80	1.03	1
Arg	0.86	1.04	1	1.00	0.97	1	0.95	0.97	1

[a] Column 1, this work; column 2, data of Konosu et al. (1965); column 3, data of Coffee & Bradshaw (1973) and Kretsinger (1972).

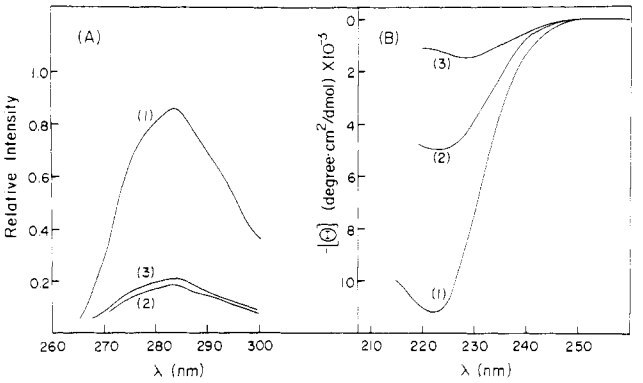

FIGURE 1: The CD and fluorescence properties of band 3 at 23 °C. (A) Fluorescence spectra: (1) pH 5.8, no Gdn·HCl; (2) pH 2.18, no Gdn·HCl; (3) pH 5.8, 3.5 M Gdn·HCl. The excitation wavelength was 259 nm and protein concentration was 0.058%. (B) CD spectra: (1) pH 5.8, no Gdn·HCl; (2) pH 2.18, no Gdn·HCl; (3) pH 5.8, 3.5 M Gdn·HCl. The protein concentration was 0.058%. All solutions were buffered in 0.035 M sodium acetate which was 0.01 M NaCl.

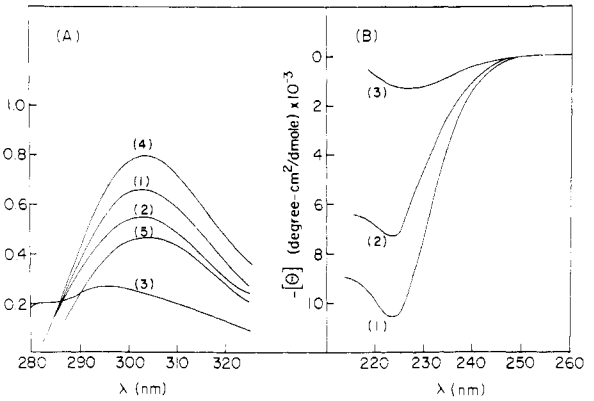

FIGURE 2: The CD and fluorescence properties of band 2 at 23 °C. (A) Fluorescence spectra: (1) pH 5.8, no Gdn·HCl (excitation at 259 nm); (2) pH 2.00, no Gdn·HCl (excitation at 259 nm); (3) pH 5.8, 3.5 M Gdn·HCl (excitation at 275 nm); (4) pH 5.8, no Gdn·HCl (excitation at 275 nm); (5) pH 5.8, 3.5 M Gdn·HCl (excitation at 275 nm). Protein concentration was 0.011%. (B) CD spectra: (1) pH 5.8, no Gdn·HCl; (2) pH 1.9, no Gdn·HCl; (3) pH 5.8, 3.5 M Gdn·HCl. The protein concentration was 0.044%. All solutions were buffered in 0.035 M sodium acetate which was 0.01 M NaCl.

(Brandts et al., 1977) and was essentially identical for the two specimens. It differs somewhat from that of Pechere et al. (1971a) in terms of the number of peaks and the relative area under each peak. Pechere et al. (1971a) were able to isolate an additional minor component (band 1), and their band 5 was split into two subcomponents. The integrated areas for bands 2 and 5 were larger than for band 3, whereas we find band 3 to have the largest area. We found no splitting for band 5 and no evidence of band 1. However, they found that splitting of band 5 and resolution of band 1 were not observed by disc electrophoresis.

Despite these differences in the chromatogram, amino acid compositions were found to be in good agreement with those reported from Pechere's laboratory (Konosu et al., 1965). A comparison is given in Table I. It shows that the three forms of parvalbumin have abnormally high content of phenylalanine, but have no tryptophan. The most notable difference in amino acid composition is that band 2 has one proline, one tyrosine, and no histidine, while bands 3 and 5 have one histidine, no proline, and no tyrosine. Our amino acid analysis detected about 0.5 half-cystine residue in band 2, and only a trace amount of cysteine residues in bands 3 and 5, while Konosu et al. (1965) found that band 3 contains a small amount of half-cystine and that bands 2 and 5 have none. Also listed in Table I are the results from a recent sequence analysis and X-ray structure determination on parvalbumins from Mirror carp by Kretsinger (1972), which are also in good agreement with ours. The atomic absorption measurements show that all three forms of parvalbumins contain 2.1–2.2 calcium ions per protein molecule, which is consistent with analysis reported by other investigators (Pechere et al., 1971b; Donato & Martin, 1974).

CD and Fluorescence Studies. The fluorescence and CD spectra of bands 2 and 3 in water and in 3.5 M Gdn·HCl are shown in Figures 1 and 2. In Figure 1, the decrease from the native fluorescence intensity for band 3 is seen to be very large when the protein was denatured by either guanidine or by exposure to low pH. It is also seen in Figure 1 that the ellipticity of band 3 in guanidine solutions is sharply decreased to −1200 from −11 000 in the native form. In pH 2.18 solution, the θ_{223}

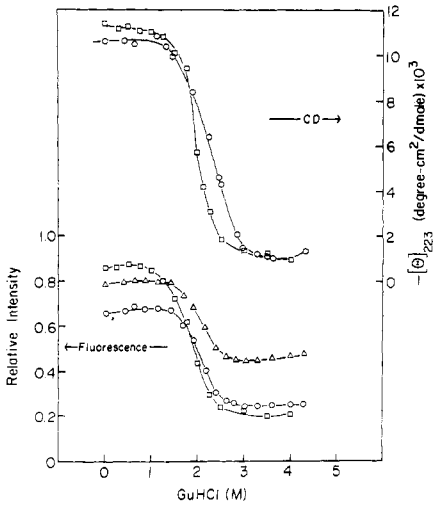

FIGURE 3: The CD and fluorescence transition curves for Gdn·HCl unfolding of bands 2 and 3 at 23 °C and at pH 5.8. For band 2 (circles), the ellipticity value at 223 nm and fluorescence intensity at 303 nm (excitation at 259 nm) are plotted. Also shown (triangles) is fluorescence intensity at 303 nm for excitation at 275 nm. For band 3, the squares show ellipticity data at 223 nm and the fluorescence intensity at 283 nm (excitation at 259 nm). The protein concentration and solvent were the same as those of Figures 1 (band 3) and 2 (band 2).

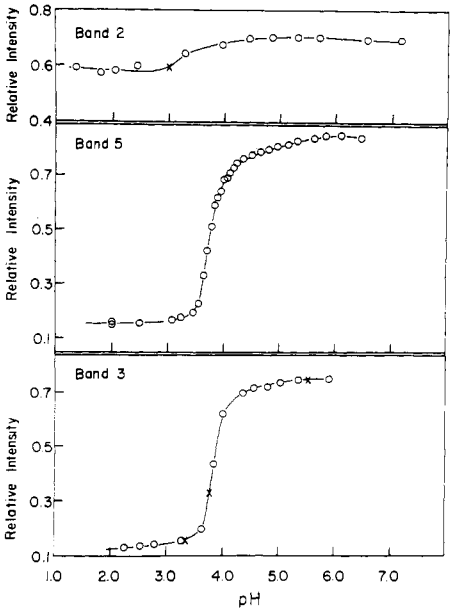

FIGURE 4: The pH-induced transition curves for bands 2, 3, and 5 at 25 °C (0.04 M sodium acetate, 0.01 M NaCl). Fluorescence intensity at 283 nm is shown for bands 3 and 5, and fluorescence intensity at 303 nm for band 2. The excitation wavelength was 259 nm. Protein concentrations were 0.028%, 0.013%, and 0.029% for bands 2, 3, and 5, respectively. (X shows values for samples previously denatured at pH 2.5.)

value is only reduced to about −5000 from −11 000 at pH 5.8. From the fluorescence and CD data, we could suggest that, in 3.5 M guanidine solution, parvalbumin band 3 has undergone a major conformational change and lost nearly all of its ordered structure. At pH 2.18 in water, the protein also has undergone a major conformational change, but still retains some of its helicity. Fluorescence and CD studies on band 5 in 3.5 M Gdn·HCl solution and at acid pH in water gave very similar results as for band 3, some of which have been published (Brandts et al., 1977).

Figure 2 shows the change in the CD and fluoresence spectra for band 2 under similar conditions. In 3.5 M guanidine solution, the small CD and fluorescence for band 2 also indicate that nearly total unfolding has taken place. The θ_{223} value for band 2 is decreased to −1100 in high concentrations of guanidine. However, in water at pH 2, band 2 undergoes a relative small CD and fluorescence change; it only loses about one-third of its apparent helical content and 30% of its fluorescence intensity. It seems reasonable to assume that band 2 is more stable toward acid denaturation and is probably only partially unfolded relative to bands 3 and 5 under the same conditions.

The major conformational change for band 2 also can be observed from its excitation spectra. In the native form, the excitation spectra for band 2 has two comparable peaks at 276 nm and 267 nm for emission at 303 nm, suggesting energy transfer from phenylalanine to the single tyrosine. However, in 3.5 M guanidine, the peak at 267 nm has decreased and become a small shoulder, as suggested by the data shown in Figure 2. Energy transfer apparently becomes less efficient after unfolding.

Equilibrium Transition Curves. Further studies on the equilibrium unfolding for bands 2 and 3 are shown in Figure 3, where θ_{223} and fluorescence intensity (at 283 nm for band 3 and 303 nm for band 2) are plotted as functions of guanidine concentration. The CD and fluorescence transition curves are almost coincident for each protein. The transition midpoint for band 3 is located at 1.80 M guanidine. For band 2, the transition midpoint is at 2.2 M guanidine, again reflecting higher stability for band 2. The relative fluorescence change for band 2 is seen to be larger with the excitation wavelength at 259 nm than at 275 nm. The reversibility for unfolding was tested by appropriate dilutions. Nearly 100% reversibility was observed when such reversals were carried out within 1 h after exposure to denaturing conditions.

The CD and fluorescence transition curves for band 5 are almost identical with those of band 3. A transition midpoint was observed at 1.80 M guanidine for both CD and fluorescence.

pH-induced transition curves for bands 2, 3, and 5 were also measured in guanidine-free solutions by fluorescence, with results shown in Figure 4. A transition midpoint at about pH 3.75 was observed for both bands 3 and 5. Because of its higher stability, a well-defined transition is not seen for band 2. An earlier fluorescence study (Burstein et al., 1975) on bands 3 and 2 at various pH values led to similar observations.

Although these results suggest some differences in the stabilities of bands 2, 3, and 5, there is no evidence of structural differences. The CD spectra for the native forms of each protein are probably identical within error. This agrees with a recent NMR study (Nelson et al., 1976), where a striking degree of coincidence in peak positions led to the conclusion that the different forms of carp parvalbumin are extremely similar in native structure. Also, the guanidine-denatured forms of bands 2, 3, and 5 display virtually identical CD spectra and all have very low fluorescent intensity for phenylalanine excitation. We conclude then that the native $\rightleftharpoons$ denatured equilibria probably involve nearly the same initial and final states for all three parvalbumins. This is an important point since it means that the large differences in kinetic behavior (discussed below) are probably not caused by conformational differences between bands 2, 3, and 5 in either the native or denatured states.

Kinetic Studies in Guanidine Solutions. Extensive kinetic studies were carried out for bands 2, 3, and 5, using guanidine dilution at 25 °C. All kinetic data were obtained with excitation wavelength at 259 nm. The peel-off method was used for data analysis in cases where two or more kinetic phases were

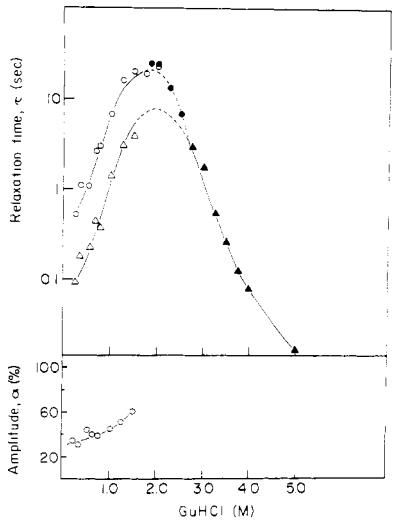

FIGURE 5: The relaxation times and the relative amplitudes for band 3 at 25 °C and pH 5.8 as a function of the final Gdn·HCl concentration. For refolding experiments (open symbols), protein was initially in 3.5 M Gdn·HCl solution, while, for the unfolding experiments (filled symbols), no Gdn·HCl was present in the initial protein solution. All final solutions were buffered in the solvent used for data of Figure 1. Triangles and circles represent the fast and slow phases, respectively. In the transition region and denatured baseline region, only one phase was resolved. The relative amplitude is plotted only where two phases could be resolved. See text for details.

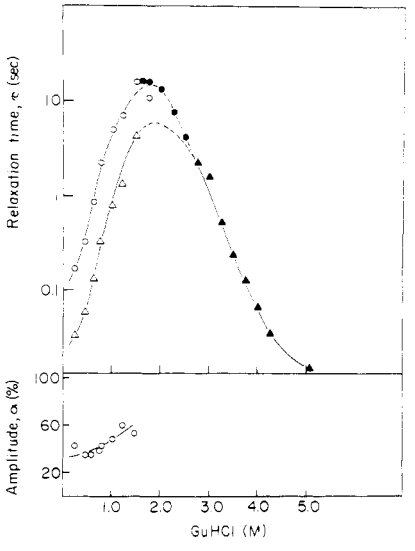

FIGURE 6: The relaxation times and the relative amplitudes for band 5 at 25 °C and pH 5.8 as a function of final Gdn·HCl concentration. For details, see Figure 5 legend.

indicated. The parvalbumins were in 3.5 M Gdn·HCl when refolding was initiated. For unfolding experiments, initial conditions were no Gdn·HCl, 0.01 M NaCl, pH 5.8. As mentioned above, all three forms of parvalbumins exist in nearly the same conformation in both 3.5 M Gdn·HCl and in H_2O solutions. Even so, rather large differences in refolding kinetics are evident between band 2 on the one hand, and band 3 or 5 on the other hand. All three forms of parvalbumins show a large fast phase in refolding, which can be separated into two phases. However, band 2 alone shows an additional slow phase which accounts for about 12% of the total amplitude and has a time constant close to 10 s. No comparable slow phase has ever been seen for band 3 or 5 under any refolding conditions.

A summary of the relaxation times obtained for both unfolding and refolding is given in Figures 5, 6, and 7 for bands 3, 5, and 2, respectively. The relaxations of bands 3 and 5 are very similar and can be resolved mathematically into two phases in the refolding baseline region. The relaxation times of these two phases differ by only a factor of 4 to 7. In the far transition region and denatured baseline region, only one phase was observed for these two forms of parvalbumin. Refolding and unfolding reactions were found to give identical relaxation times when jumping to the same final conditions near the transition midpoints. The slowest relaxation times for both parvalbumins are obtained at 1.8 M guanidine, which is the midpoint of the equilibrium transition curves. The rates for band 3 are consistently slower than those of band 5. At the transition midpoint, for example, the relaxation time for band 5 is about 16 s, compared with about 25 s for band 3.

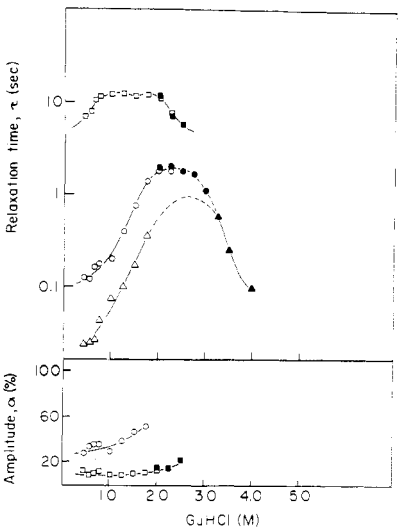

FIGURE 7: The relaxation times and the relative amplitudes for band 2 at 25 °C and pH 5.8 as a function of final Gdn·HCl concentration. For refolding experiments (open symbols), the initial Gdn·HCl concentration was 3.5 M, while for the unfolding experiments (filled symbols) no Gdn·HCl was present in the initial protein solution. Triangles, circles, and squares represent the fast, intermediate, and slow phases, respectively. In the transition region, the intermediate and fast phases converged into one phase. In the unfolding baseline region, also only one phase was seen. Whether these single phases represent the extension of the intermediate or fast phase cannot be ascertained. The relative amplitude was plotted only where at least two phases can be resolved. See text for details.

The amplitudes for the slower phase for bands 3 and 5 are shown also in Figures 5 and 6. A limiting value of ca. 30% is reached in the native baseline region for both proteins. This slowly increases to about 60% in the transition region at the point where the data can no longer be resolved as two phases. Beyond this point, it is not possible to know for certain if the single phase which is seen is the continuation of the fast or slow phase, or if it corresponds to a mixture of the two phases having very similar relaxation times. On the basis of pH-jump kinetics (discussed below) where the two phases are better resolved, it appears likely that the amplitude of the slow phase approaches 100% near the transition midpoint and then sharply decreases to zero in the denatured baseline region. Although this is a fairly complicated pattern of behavior, it will be shown later that this agrees well with the behavior expected on the basis of calculations.

The relaxation times for the proline-containing parvalbumin, band 2, are shown in Figure 7. Compared with bands 3 and 5, a striking difference in kinetic pattern is apparent. An additional slow phase, observable from the native baseline region to the transition midpoint, is seen. The amplitude of this phase varies from about 8% to 15% of the total amplitude, while the

relaxation time is close to 10 s at 25 °C. The two faster phases (i.e., the intermediate phase and the fast phase) for band 2 show a kinetic pattern very similar to the two phases seen for bands 3 and 5. Although the relaxation times are significantly shorter for band 2, the ratio of relaxation times is almost identical (ca. five times) for each of the three parvalbumins. On the other hand, the ratio of the relaxation times for the intermediate and slow phase for band 2 is nearly 50 in the refolding baseline region. It seems fairly clear from this that it is the slowest phase for band 2 which has no analogue in the kinetics for bands 3 and 5.

The greater stability of band 2, mentioned earlier in connection with the equilibrium studies, is also apparent in the kinetics since the maximum in relaxation times occurs at 2.2 M guanidine, compared with 1.8 M for bands 3 and 5. The amplitude for the intermediate phase is also shown in Figure 7, and it behaves quite similarly to the amplitude for the slow phase of bands 3 and 5.

pH-Jump Studies. The pH-induced unfolding and refolding reactions were also studied in detail for bands 3 and 5 in the absence of guanidine hydrochloride. Less extensive studies were carried out for band 2. The relaxation times for band 3 are shown in Figure 8. A comparable study for band 5 was reported in an earlier paper (Brandts et al., 1977) and is similar in all respects. The basic kinetic pattern is not unlike that seen in Gdn·HCl solutions. Only two phases are seen, with relaxation times exhibiting a maximum at the transition midpoint. The amplitude of the slowest of the two phases is about 30% in the native baseline and increases to near 100% close to the midpoint. However, two phases are again resolved over a limited pH region on the denatured side of the midpoint. In the denatured baseline region, only the fastest phase is seen.

The major difference between these results and those obtained in guanidine solutions is the magnitude of the relaxation times. Whereas the rates of the slower phase are close to 25 s at the transition midpoint in guanidine solutions, they are only about 4 s in water.

Because band 2 is more stable, it is possible to only partially denature it by pH variations in the absence of guanidine, as shown earlier. However, some refolding studies were carried out from an initial pH of 1.9. Even though amplitudes were small due to the limited unfolding, suitable data were obtained in the native state baseline region. Relaxation times for the fast and intermediate phases were 0.003 and 0.023 s at pH 4.9 and 0.005 and 0.026 s at pH 4.4. The slow phase, constituting 10–15% of the total amplitude, had a relaxation time of about 5 s or nearly the same as in solutions with guanidine hydrochloride. Thus, the large acceleration in rates for the fast and intermediate phases, seen in the absence of guanidine, is not observed for the slow phase.

Activation Enthalpies of the Refolding Reactions. Previously, it was shown that the activation energy for the slow phase, obtained from refolding experiments into the native state baseline region, should be that which is characteristic of the elementary process which determines the rate for the slow phase (Brandts et al., 1975). Thus, if the proline isomerism hypothesis is correct, one would expect an activation energy for the slow phase which is close to that for model compounds exhibiting proline isomerism (ca. 16–20 kcal/mol) and the value should be nearly independent of guanidine concentration.

The activation energy for the slow phase for band 2 has been measured over the temperature range from 18 to 37 °C at final guanidine concentrations of 0.43, 0.76, and 1.25 M and a pH of 5.8. The initial guanidine concentration was 3.5 M in all cases. The activation energy was determined from plots of the logarithm of the reciprocal relaxation time vs. reciprocal temperature. Activation energies were estimated as 17, 16, and 18 kcal/mol at guanidine concentrations of 0.43, 0.76, and 1.25 M, respectively, with errors of approximately ±2 kcal/mol. These are in excellent agreement with expectations based on the proline isomerism model.

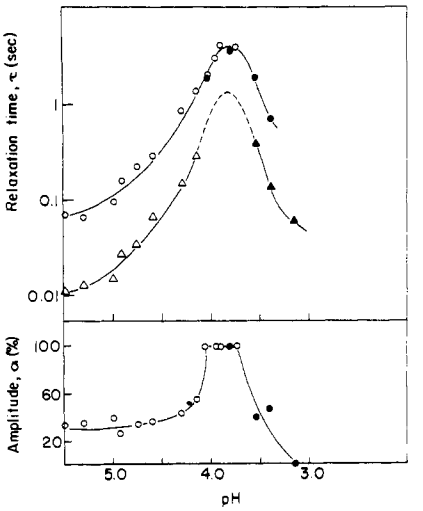

FIGURE 8: The pH dependence of the relaxation times and the relative amplitudes for band 3 at 25 °C. The abscissa gives the final pH in each case for both the slow phase (circles) and the fast phase (triangles). For refolding experiments (open symbols), the initial pH was always 2.45, while for the unfolding experiments (filled symbols), the initial pH was 6.2. All final solutions were buffered in 0.035 M sodium acetate, 0.01 M NaCl. In the pH region 3.75–4.0, only a single phase was resolved. We assign it as the slow phase.

An attempt was made to determine activation energies for the intermediate and fast phase for band 2. However, considerable scatter was introduced by errors encountered in peeling off the slow phase from the intermediate phase. The data were consistent with a value of ca. 10 kcal for both phases. Much better results were obtained on band 3 since complications from the slow phase are absent. Activation energies of 9.0 ± 2 kcal/mol were obtained for both phases for band 3. Because the two phases are not extremely well separated on the time axis, these values contain moderately large errors.

The Dependence of Relaxation Times on Ca^{2+} Concentration. Since parvalbumins have a high affinity for Ca^{2+} and since the ion appears to be absolutely required for the stability of the native state, it would be anticipated that those kinetic phases involved *directly* in conformational changes might show a large dependence on Ca^{2+} concentration. All three forms of parvalbumin contain two tightly bound ions per molecule, and the X-ray structure (Kretsinger & Nockolds, 1973) shows that the two binding centers encompass six helical sections. Efforts in our laboratory to remove these two calcium ions invariably caused denaturation. On the other hand, low concentration of Ca^{2+} (0.1 M and below) caused no detectable change in the rate of isomerism of proline dipeptides such as Gly-Pro (unpublished results from this laboratory). Thus, the dependence of relaxation times on Ca^{2+} concentration might provide some discriminating evidence as to which phases, if any, involve isomerism.

To study the effect of Ca^{2+}, refolding from the guanidine-denatured (3.5 M) species was examined as a function of the final calcium concentration. The results are given in Figure 9 for bands 5 and 2, respectively. (Band 3 was also studied and behaved nearly identically with band 5.) For band 5, it is seen that both the fast and slow phases are markedly accelerated by the addition of Ca^{2+} to the refolding solution. For band 2,

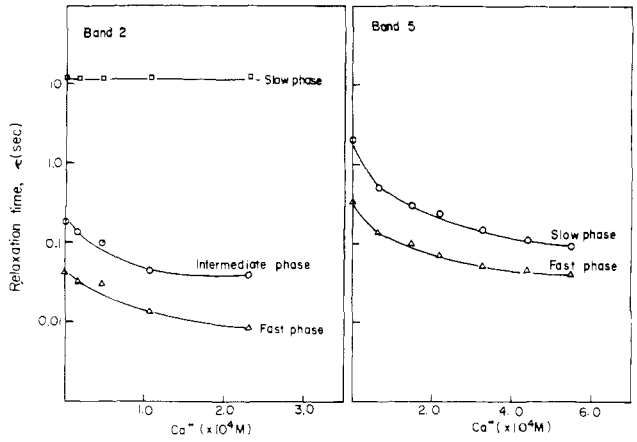

FIGURE 9: The dependence of relaxation times on Ca^{2+} concentration for bands 2 and 5 refolding at 25 °C. The initial Gdn·HCl concentration was 3.5 M and the final Gdn·HCl concentration was 0.76 M. Calcium ion was added as $CaCl_2$. The final protein concentrations were 0.09% for band 2 and 0.1% for band 5. The solvent was that of Figure 1.

the fast and intermediate phases are accelerated in much the same way as the fast and slow phases for bands 3 and 5. It is quite striking, however, that the slowest phase for band 2 exhibits no sensitivity whatsoever to Ca^{2+} concentration, since both the relaxation times and the relative amplitude were invariant up to the highest concentration examined. This situation prevailed at final concentrations of guanidine hydrochloride of 1.75 and 0.38 M as well as the concentration (0.76 M) used for the data of Figure 9. Thus the differences in relaxation times become considerably greater in moderate concentrations of Ca^{2+}, with the slow phase being about 300 times slower than the intermediate phase and about 1500 times slower than the fast phase in the presence of 0.2 mM Ca^{2+}.

These results are important for at least two reasons. First, the Ca^{2+} dependence observed for bands 2 and 5 helps to establish the idea that the two kinetic phases seen for bands 3 and 5 are analogous to the fast and intermediate phases seen for band 2 since all exhibit high calcium sensitivity. Second, the complete lack of sensitivity of the slow phase of band 2 to calcium is in agreement with the idea that this phase involves proline isomerism.

Discussion

The three forms of parvalbumin are very closely related both chemically and structurally. According to the data of Kretsinger (1972), bands 3 and 5 differ in sequence in only four of 108 amino acids while bands 2 and 5 differ in only fifteen amino acids. Perhaps most striking is the fact that band 2 contains one proline, one tyrosine, and no histidine while bands 3 and 5 have no proline, no tyrosine, and one histidine. In view of the high degree of similarity in sequence, it is not surprising to find evidence suggesting a strong structural analogy. Our CD spectra show the three native forms to be identical within error. Recent ^{13}C NMR studies (Nelson et al., 1976) also suggest that bands 2 and 3 have remarkably similar structure in the native form. Also, CD and fluorescence data indicate that the guanidine-denatured forms are also nearly identical and probably very close to a randomly coiled structure. It then seems that each form of parvalbumin equilibrates between the same initial and final state in folding and unfolding reactions, so any major differences in the number of kinetic phases would appear not to be due to differences in the folding pathways for these similar proteins. Thus, one must look elsewhere for a reasonable explanation for the extra phase.

Even for bands 3 and 5, at least two phases are present in the refolding kinetics in the native baseline region and in part of the transition region. Since only one phase is seen for unfolding experiments with jumps ending in the denatured baseline region, it seems reasonable to assume that there exist two or more unfolded species which have identical spectroscopic properties but which refold with different rates to the native state. It can also be convincingly argued that this is the case from results obtained at various initial conditions. The simplest mechanism which is generally consistent with our results is

$$D \overset{\text{slow}}{\rightleftarrows} X \overset{\text{fast}}{\rightleftarrows} N \qquad (1)$$

If X were a structural intermediate present only in the transition region, then we should expect to see two phases in refolding jumps from the transition region to the native baseline and only one phase for baseline-to-baseline refolding jumps. However, we see two phases for both of the above jumps with nearly constant relative amplitudes, which means that X and D exist in nearly the same proportions in the transition region and in the denatured baseline region. Since parvalbumins were shown to be virtually complete random coils in the denatured baseline region (i.e., in high concentrations of guanidine), it follows that X must be a denatured species and not a structural intermediate. Therefore, the minimal mechanism for bands 3 and 5 is

$$D_2 \overset{\text{slow}}{\rightleftarrows} D_3 \overset{\text{fast}}{\rightleftarrows} N \qquad (2)$$

where D_3 is a denatured form which can refold to the native state somewhat faster than can the denatured form D_2. Because the relative amplitudes of the fast and slow phase are nearly independent of initial conditions, it follows that the $D_2 \rightleftharpoons D_3$ equilibrium is not altered very much by changing the concentraion of guanidine hydrochloride. This puts some serious restrictions on the type of structural processes which might be involved in this step. This analysis also argues for the idea that *large conformational changes occur only in the fastest phase which is seen for parvalbumins.*

We have tried to treat the relaxation data quantitatively in terms of the mechanism of eq 2. For this purpose, we have used the method of Hagerman & Baldwin (1976), where experimental values of relaxation times and equilibrium constants can be used to calculate relative amplitudes for each relaxation. These calculated values can then be compared with the corresponding experimental amplitude estimates as a check for self-consistency. This comparative method serves primarily as a test to be sure all relaxations have been observed and that no artifactual processes are included, rather than as a test for the validity of a detailed mechanism. The application of this procedure to our data obtained from guanidine dilution experiments is rather pointless, since the separate transitions are nonresolvable in the transition region and denatured baseline region. The data obtained from pH-jump experiments in the absence of guanidine can be used, since the different phases are separable over much of the transition zone. The results obtained for band 5 are shown in Figure 10. The calculations were made from the relaxation times shown in Figure 4 of a previous paper (Brandts et al., 1977) and equilibrium measurements of fluorescence as a function of pH. A pH-independent value of 4.0 was assumed for the D_3/D_2 ratio, and D_2 and D_3 were also assumed to have identical optical properties. The calculated curve qualitatively reproduces the large fluctuations seen in the experimental data. The quantitative agreement is not exceedingly good, however, and this can probably be attributed to the difficulties involved in obtaining good separation of two kinetic phases with fairly similar re-

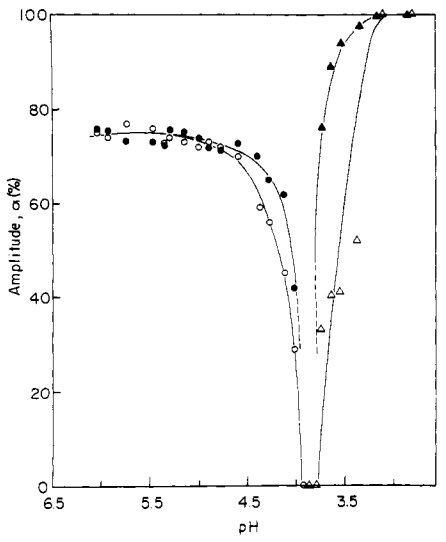

FIGURE 10: Comparison of the fast phase amplitude predicted from the relaxation times with those measured for bands 5 at 25 °C. For refolding experiments (circles), the initial pH was 2.67, while, for the unfolding experiments (triangles), the initial pH was 4.75. Filled symbols represent predicted value; open symbols represent experimental data. Near the transition midpoint, only a single phase was detected experimentally, so that no calculation could be made. See text for details.

laxation times. We do feel the agreement provides some support for the experimental validity of the data as well as showing consistency with the general mode of eq 2.

For band 2, an extra slow phase is seen in addition to the two faster phases. Although somewhat faster, the intermediate and fast phases for band 2 are otherwise similar in their kinetic pattern to the slow and fast phases for bands 3 and 5. They also exhibit the high sensitivity to calcium ion seen for the two phases of bands 3 and 5. The intermediate and fast phases for band 2 are therefore assumed to be analogous to the slow and fast phases for bands 3 and 5. The kinetic scheme for band 2 is modified then only by the inclusion of an additional slow step:

$$D_1 \overset{\text{slow}}{\rightleftharpoons} D_2 \overset{\text{interm}}{\rightleftharpoons} D_3 \overset{\text{fast}}{\rightleftharpoons} N \qquad (3)$$

Because of the disappearance of the slow phase in unfolding jumps to the denatured baseline and because the relative amplitude of the slow phase in refolding jumps does not depend strongly on the initial conditions, it seems certain, by the same arguments given above, that D_1 is also a denatured species whose concentration relative to D_2 and D_3 is insensitive to the amount of guanidine hydrochloride in solution. Because of the high degree of unfolding of the denatured form of band 2, the extra kinetic phase must be due to some guanidine-insensitive topological feature of the random chain present only for band 2. Since band 2 contains one proline and bands 3 and 5 contain none and since the high energy barrier for proline isomerism is well-documented, it seems all but certain that the slow step involves the cis–trans isomerism of the single proline in band 2. There are several other facts which support this conclusion. (1) The time constant for the slow phase (ca. 10 s at 25 °C) is close to that expected for proline isomerism (Brandts et al., 1975). (2) The activation energy of 17 kcal/mol for the slow phase is well in the range normally found for peptide isomerism. (3) Assuming that the single proline of band 2 is confined to the trans state in the native protein, one would predict that a slow phase involving isomerism should account for ca. 10–20% of the total amplitude, in excellent agreement with that observed experimentally (8–15%) for band 2. (4) The rate and amplitude of a slow phase due to isomerism should be independent of the addition of small amounts of Ca^{2+}, on the basis of the behavior of model systems. The slow phase for band 2 was found to be Ca^{2+} independent, even though the two faster phases showed a strong dependence. (5) For model proline-containing systems, the rate and activation energy for isomerism are almost totally independent of the concentration of guanidine hydrochloride up to concentrations of at least 4 M. For band 2, the slow phase exhibited a time constant, activation energy, and amplitude which were independent of the concentration of guanidine, as would be anticipated if the slow phase involves isomerism.

All of these facts taken together form a strong case in support of the idea that the slow phase for band 2 is rate-limited by proline isomerism, which also explains why it is not seen for the very similar parvalbumins, bands 3 and 5. Since other proteins known to contain proline also exhibit a slow phase[2] with many of the same kinetic features (Brandts et al., 1975), it is tempting to generalize this conclusion. In fact, it seems highly likely that proline isomerism will occur for all such proteins and that it will be considerably slower than "real conformational changes" involving alterations in Ramachandron angles. It is indeed difficult to imagine how kinetic complications from proline isomerism could be avoided in conformational reactions. This is not to say that complications from other slow processes could not be present as well. One example where this might happen is with ribonuclease where properties of the slow phase seem not to be easily rationalized if it involves only proline isomerism (Nall et al., 1978). In particular, the large dependence of relaxation time and activation energy on the concentration of guanidine salts is contrary to that observed for proline-containing model compounds. (It is also quite different than what we have observed for the slow phase of band 2.) This has prompted Baldwin and colleagues (Nall et al., 1978) to suggest that the ribonuclease slow phase may involve slow rearrangement of disulfide loops as well as perhaps involving proline isomerism.

We feel that the results of this study are strongly in favor of the hypothesis that proline isomerism is the rate-limiting step for folding of proline-containing proteins. Even so, it should be reemphasized that the slowest phase for bands 3 and 5, which contain no proline, is in the same time range as the slow phase for band 2 under certain conditions near the transition midpoint (cf. Figures 5 and 6 with Figure 7). However, under some other conditions, large inherent differences are seen between the slowest phase of bands 3 and 5 and the slow phase for band 2. For example, in the refolding baseline region the slow phases for bands 3 and 5 become 20 to 50 times faster than for band 2. Also, the slowest phases for bands 3 and 5 are markedly accelerated by addition of small amounts of Ca^{2+} (Figure 9), while the slow phase for band 2 is insensitive to Ca^{2+}. These different patterns of behavior show rather clearly that the slow phase for band 2 is an inherently different type of process than the slowest phases for bands 3 and 5. On the other hand, we have no good explanation for why the two phases for bands 3 and 5 become so slow in the transition region. It might have something to do with the facts that bands 3 and 5 are more easily denatured by Gdn·HCl (Figure 3) and have higher isoelectric points (4.47 and 4.25 for bands 5 and 3 vs. 3.95 for band 2).

[2] Although no slow phase was originally reported to occur in the unfolding and refolding of staphylococcal nuclease (Epstein et al., 1971), it appears now as if there may actually be a large slow phase which is generally similar to that of ribonuclease and other small proteins. See Brandts et al. (1977) for a more complete discussion.

If D_1 in the mechanism of eq 3 is a denatured molecule with the single proline in the "wrong" isomeric state for refolding, it is still difficult to decide what might be the specific structural features of D_2 which prevent it from refolding as fast as D_3. It does seem possible that the "D_2 state" also exists for other proteins, since ribonuclease has been suggested to have three kinetically separable unfolded forms with relative relaxation times somewhat similar to those for band 2 (Hagerman & Baldwin, 1976). Parvalbumins have no disulfide bonds, so slow chain rearrangements associated with cross-linking cannot be present. The $D_2 \rightleftharpoons D_3$ equilibrium is not strongly shifted by guanidine hydrochloride, so it appears not to involve a small amount of folded structure which forms only in D_3. This being the case, one must consider trivial stereochemical rearrangements of the same type involved in the slowest phase. There is no evidence in the literature for slow isomeric equilibration for any amino acid side chains, and the only other obvious possibility would be cis–trans isomerism of peptide bonds for non-proline residues. In view of the fact that monosubstituted amides isomerize much faster than disubstituted amides (the coalescence temperature for *N*-methylformamide is over 20 °C lower than for *N,N*-dimethylformamide), one would anticipate that isomerism of non-proline residues would appear as a separate kinetic phase, distinct from the isomerism of proline residues. Since there are a very large number of non-proline residues normally involved directly in unfolding reactions, very low equilibrium concentrations of the cis form in the denatured state will be effective in preventing rapid refolding. For example, for a protein with 100 residues, an equilibrium fraction of 0.005 cis form for each residue would lead to 40% of the denatured molecules having one or more cis residues. This 40% fraction would, therefore, be incapable of fast refolding. Although NMR studies have never detected the cis form for non-proline residues, fractions below 1% might easily have been overlooked.

On the other hand, the rather complex behavior of the relaxation time of the intermediate phase and the strong dependence on Ca^{2+} argue against the most simple model one might imagine for peptide isomerism. Some of these complications might arise from strong coupling between the intermediate and fast phases, but more critical information is necessary before one can decide for certain what precise structural changes are involved in the intermediate phase.

In summary, the results of this study provide very strong evidence in favor of the idea that the slowest step normally seen in conformational reactions involves the proline peptide bonds getting into the proper isomeric state so that refolding can actually occur. The second slowest step for proline-containing proteins and the slowest step for proline-free proteins also may involve a similar type of trivial configurational change since it apparently occurs with no inherent change in spectroscopic properties and since the equilibrium is guanidine insensitive. Although it is suggested that isomerism of peptide bonds for non-proline residues could be involved in this latter process, no direct evidence now exists to support the idea and certain features in the kinetics seem to require a more complicated model than peptide isomerism in its simplest form. At any rate, only the fastest phase seen for parvalbumin appears to involve true conformational changes as they are normally defined. In refolding and unfolding where the final conditions are far removed from the transition midpoint, this process takes place with a time constant of a few milliseconds at room temperature, although it slows down considerably in the transition region particularly when guanidine hydrochloride is present. It appears then that folding into the native structure can occur quite fast once the stereochemistry of the chain is "correct."

References

Brandts, J. F. (1964) *J. Am. Chem. Soc. 86,* 4302.

Brandts, J. F. (1969) *Biol. Macromol. 2,* 213.

Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14,* 4953.

Brandts, J. F., Brennan, M., & Lin, L.-N. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 4178.

Burstein, E. A., Permyakov, E. A., Emelyaneko, V. I., Bushveva, T. L., & Pechere, J.-F. (1975) *Biochim. Biophys. Acta 400,* 1.

Coffee, C. J., & Bradshaw, R. A. (1973) *J. Biol. Chem. 248,* 3305.

Donato, H., & Martin, R. B. (1974) *Biochemistry 13,* 4575.

Epstein, H. F., Schecter, A. N., Chen, R. F., & Anfinsen, C. B. (1971) *J. Mol. Biol. 60,* 499.

Hagerman, P. J. (1977) *Biopolymers 16,* 731.

Hagerman, P. J., & Baldwin, R. L. (1976) *Biochemistry 15,* 1462.

Ikai, A. (1971) Ph.D. Dissertation, Duke University.

Ikai, A., Fish, W. F., & Tanford, C. (1973) *J. Mol. Biol. 73,* 165.

Jackson, W., & Brandts, J. F. (1970) *Biochemistry 9,* 2294.

Konosu, S., Hamoir, G., & Pechere, J.-F. (1965) *Biochem. J. 96,* 98.

Kretsinger, R. H. (1972) *Nature (London), New Biol. 240,* 85.

Kretsinger, R. H., & Nockolds, C. E. (1973) *J. Biol. Chem. 248,* 3313.

Lumry, R., Biltonen, R., & Brandts, J. F. (1966) *Biopolymers 4,* 917.

Moore, S., & Stein, W. H. (1951) *J. Biol. Chem. 192,* 663.

Nall, B. T., Garel, J.-R., & Baldwin, R. L. (1978) *J. Mol. Biol. 118,* 317.

Nelson, D. T., Opella, S. J., & Jardetzky, O. (1976) *Biochemistry 15,* 5552.

Pechere, J.-F., Demaille, J., & Capony, J.-P. (1971a) *Biochim. Biophys. Acta 236,* 391.

Pechere, J.-F., Capony, J.-P., & Ryden, L. (1971b) *Eur. J. Biochem. 23,* 97.

Privalov, P. L., & Khechinashvili, N. N. (1974) *J. Mol. Biol. 86,* 665.

Tanford, C. (1970) *Adv. Protein Chem. 24,* 1.

Tanford, C., Aune, K. C., & Ikai, A. (1973) *J. Mol. Biol. 73,* 185.

Tsong, T. Y. (1973) *Biochemistry 12,* 2209.

Tsong, T, Y., & Baldwin, R. L. (1972) *J. Mol. Biol. 69,* 145.

Tsong, T. Y., Baldwin, R. L., & Elson, E. L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69,* 1809.