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# Unfolding and refolding occur much faster for a proline-free protein than for most proline-containing proteins

(proline *cis-trans* isomerization/parvalbumin/protein folding/rate-limiting step)

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**ABSTRACT** **The kinetics for unfolding and refolding of a parvalbumin (band 5) have been examined as a function of pH near the transition region, using stopped-flow techniques. This protein is rather unusual in that it has no proline residues, and therefore serves as a good example to test the hypothesis that the rate-limiting step seen in denaturation reactions is due to the *cis-trans* isomerization of proline peptide bonds in the denatured state.**

**The kinetics for parvalbumin unfolding and refolding are complex, with the data being resolvable into two fast phases at 25°. The slower of the two phases seen for the parvalbumin is about 100 to 500 times faster than the slow phase seen for proline-containing proteins under the same conditions! These results argue strongly in support of the proline isomerization hypothesis. It is also suggested that the slower phase seen for parvalbumin and the second-slowest phase seen for proline-containing proteins might be due to the *cis-trans* isomerization of peptide bonds of non-proline residues.**

A number of small proteins such as ribonuclease, chymotrypsinogen, myoglobin, and cytochrome *c*, have been shown to exhibit a slow kinetic phase in their unfolding and refolding reactions (reviewed in ref. 1). This becomes the dominant phase in refolding reactions and is also evident in unfolding reactions if the final conditions are not too far into the denatured baseline region. One or more fast phases can also be seen under most conditions. These complexities might appear, at first glance, to be due to the presence of structural intermediates that occur in significant concentrations only in the transition region. However, it was shown independently in two laboratories (2, 3) that the existence of two kinetic phases is due directly to the presence of two distinct forms of the denatured protein, at least in the case of ribonuclease. These two denatured forms are almost identical in terms of their physical properties, but one form can refold very fast to the native state while the other form is capable only of slowly refolding to the same native state.

It was suggested in an earlier paper from this laboratory (3) that slow refolding occurred because some of the denatured molecules existed, at any instant in time, with the peptide bonds of their proline residues in the "wrong" state of isomerism. That is, for a native protein in which all of the prolines have to be in the *trans* configuration, any denatured molecules with one or more prolines in the *cis* state would refold slowly, while any denatured molecules with all prolines in the *trans* state would refold rapidly. In terms of this model, the rate-limiting step in denaturation and renaturation would be due to a trivial isomerization of peptide bonds in the denatured state rather than being due directly to any step in the unfolding process itself. This hypothesis would explain why many of the proteins that exhibit complex kinetics also exhibit two-state behavior in terms of rigorous thermodynamic criteria. It was also pointed out that the slow phase in protein folding, with a time constant near 1 min at 25° and near 10 sec at 40°, is of approximately the same velocity as proline isomerization in small peptides.

Even though the above ideas are entirely consistent with all that is known about both protein folding and proline isomerism, direct evidence necessary to prove the hypothesis is still lacking. It is a tough problem to resolve, largely because changes in proline isomerism are very difficult to "see" in the presence of protein unfolding, which process completely dominates most observables.

One way of providing supportive evidence of a more direct nature is to examine the denaturation reactions of proteins that do not contain any proline residues. The slowest phase seen for other proteins should then be absent. This is one approach that we are following and it is the topic of this communication. An examination of the literature quickly reveals that there are not many globular proteins that do not contain proline. Fortunately, some forms of the calcium-binding parvalbumins from fish white muscle do lack proline, and these have been chosen for this investigation.

## METHODS AND RESULTS

Parvalbumins have been prepared from the white muscle of carp in direct accordance with the procedure described by Pechere *et al.* (4). The final preparative chromatogram on DEAE-Sephadex is shown in Fig. 1. The numbering indicates the probable equivalence of these bands to the bands identified in Pechere's laboratory (4, 5). These workers started with the white muscle from mirror carp and their final chromatogram was on DEAE-cellulose. Our pattern differed somewhat from theirs both in terms of the number of peaks and the relative area under each peak. We obtained essentially the same pattern for three specimens (one an Israeli carp from the Arkansas Department of Game and Fish, one a wild fish from the Connecticut River, and the third an unclassified carp from a fish market in New York City). Identity between our bands and those of Pechere was finally established by amino acid analysis, which was in excellent agreement with theirs. Analysis showed clearly that band 5 contains no proline, so it was used for this study. It also has no tyrosine or tryptophan, but an inordinately large amount of phenylalanine. After purification, this parvalbumin was found to contain 2.2 mol of $Ca^{2+}$ per mol, as measured by atomic absorption. This ratio agrees closely with that measured by Donato and Martin (6).

Because of the lack of tyrosine and tryptophan and because of the low extinction coefficient for phenylalanine, the denaturation reaction of band 5 could not be suitably monitored by UV absorption as has been done with other proteins. We unsuccessfully tried several other methods before finally settling on phenylalanine fluorescence for detection. Shown in Fig. 2A

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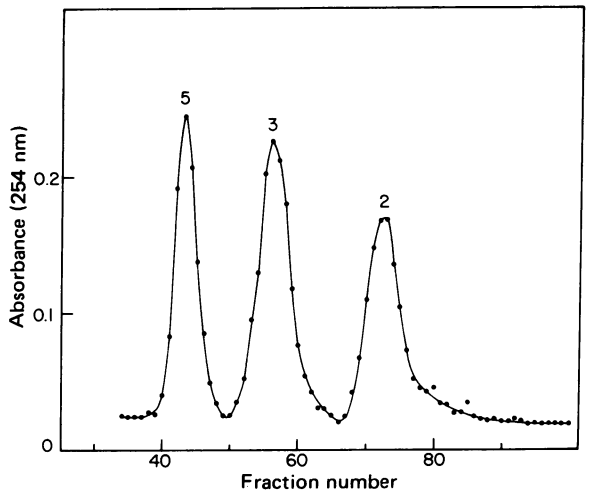



FIG. 1. Final separation of the parvalbumins of carp myogen on DEAE-Sephadex. A preparation containing 150 mg of protein was loaded onto the column. The eluting buffer was identical to that in ref. 4. Elution was accomplished at a flow rate of 25 ml/hr and the volume of fractions was 4 ml. The column was 3 cm × 40 cm.

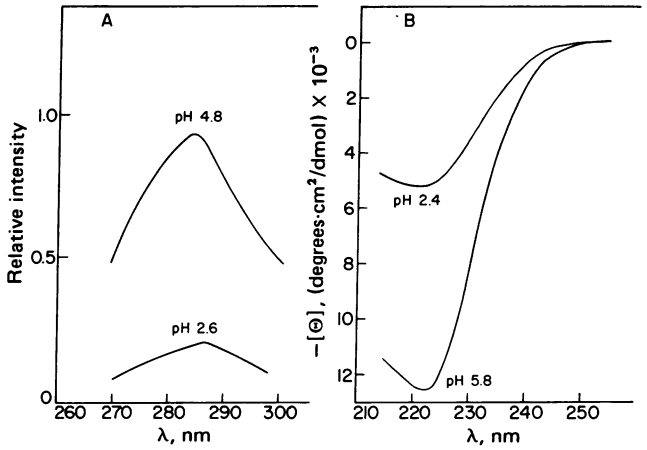
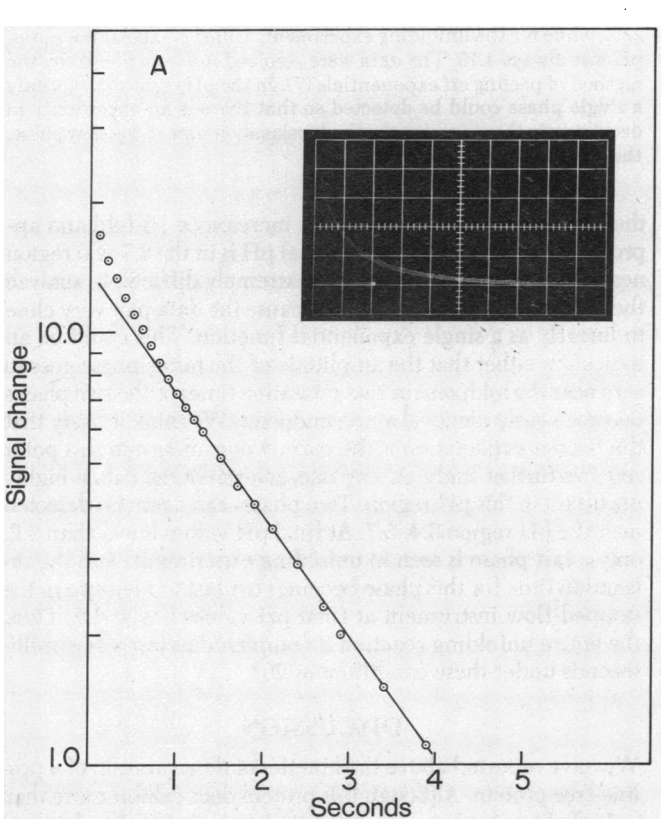



FIG. 2. Change in the physical properties of band 5 upon denaturation at 25°. (*A*) Fluorescence spectra of parvalbumin in 0.04 M sodium acetate/0.01 M sodium chloride under nondenaturing conditions (pH 4.8) and under denaturing conditions (pH 2.6). The excitation wavelength was 259 nm and the protein concentration was 0.022%. Spectra were obtained on a Perkin–Elmer MPF-44. (*B*) Circular dichroism spectra of parvalbumin under nondenaturing conditions (pH 5.8) and under denaturing conditions (pH 2.4). The protein concentration was 0.03%. The spectra were recorded on a Cary 60 instrument.

is the change in fluorescence that occurs when band 5 is denatured by changing the pH from 4.8 to 2.6 at 25°. This indicates that there is an enormous loss in fluorescence intensity at 285 nm when the protein is denatured. This is a completely reversible change if the sample is not left under denaturing conditions for more than an hour.

In order to be certain that the changes in fluorescence are actually the result of protein unfolding, the circular dichroism was also examined over nearly the same range of conditions. The corresponding changes in ellipticity are shown in Fig. 2*B*. It is seen that there are large changes in circular dichroism that accompany the changes in fluorescence, suggesting that the fluorescence changes do result from unfolding of the molecule.



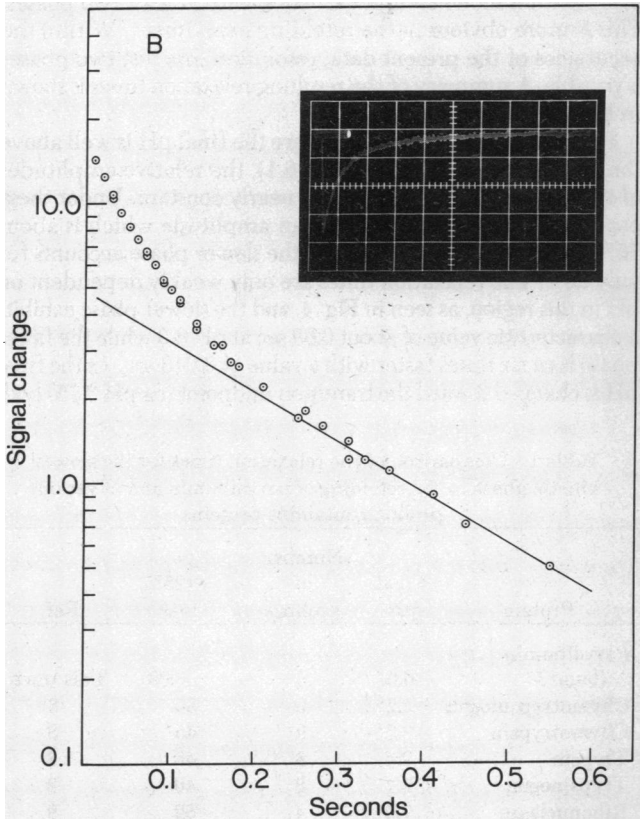




FIG. 3. The unfolding (*A*) and refolding (*B*) kinetics of parvalbumin band 5 at 25° and at a protein concentration of 0.04%. The trace in *A* shows fluorescence changes after the jump pH 4.76–3.62, using a sweep speed of 1 sec/cm. The trace in *B* shows the changes resulting from the pH jump 2.67 to 4.77, using a sweep speed of 0.1 sec/cm. In each case, the second partial sweep was started approximately one minute after the initial jump and this was used to determine the infinite time value. The semilogarithmic plots of each set of data are also shown.

More detailed studies indicate that both fluorescence and ellipticity change in a sigmoidal fashion as a function of pH under the conditions of Fig. 2, with the midpoint of the transition being near pH 3.75 for both observables. The circular dichroism change induced by changing the pH is very substantial. The mean residue ellipticity at 223 nm changes from −12,500 degrees·cm²/dmol for the high-pH form to −5,300 degrees·cm²/dmol for the low-pH form. This, along with the large loss in fluorescence, suggests that the process that is being observed is a major unfolding reaction and not a trivial rearrangement of structure. For comparison, the pH-induced denaturation of ribonuclease was examined by circular dichroism. The mean residue ellipticity at 215 nm was found to be −10,200 degrees·cm²/dmol for the native form (pH 4, 40°) and −6,000 degrees·cm²/dmol for the denatured form (pH 2.1, 40°). Thus, the circular dichroism change for denaturation of parvalbumin is substantially larger than that for ribonuclease.

Kinetic studies were done on band 5, using fluorescence optics on a Durrum–Gibson stopped-flow apparatus. Excitation was carried out at 259 nm with a slit width of 5 mm while the fluorescent light was observed at 90° through a glass filter that had a sharp cutoff at about 280 nm. The kinetics were observed for pH jumps that resulted in either unfolding or refolding. The initial pH of unfolding jumps was 4.76 in all cases, whereas the initial pH for refolding experiments was always 2.67. The final pH was varied systematically in both the unfolding and refolding experiments.

Two oscilloscope traces are shown in Fig. 3. Shown in 3*A* is a trace resulting from unfolding to a final pH of 3.62, while 3*B* shows the trace for refolding to pH 4.77. The corresponding semilogarithmic plots of these data are also shown in Fig. 3. It is evident from these data that the denaturation reaction of this parvalbumin shows complex kinetics, with at least two phases. This is more obvious in the refolding experiment. Within the accuracies of the present data, resolution into just two phases is possible. A summary of the resulting relaxation times is shown in Fig. 4.

For refolding experiments where the final pH is well above the transition region (*ca* pH 4.6–6.1), the relative amplitudes of the fast and slow phase remain nearly constant. Under these conditions, the faster phase has an amplitude which is about 75% of the total amplitude while the slower phase accounts for only 25%. The relaxation times are only weakly dependent on pH in this region, as seen in Fig. 4, and the slower phase exhibits a characteristic value of about 0.08 sec at pH 6.0 while the faster phase is *ca* six times faster with a value of 0.013 sec. As the final pH is changed toward the transition midpoint (*ca* pH 3.75) both the fast and slow relaxation times increase *ca* 15-fold and approach a maximum. When the final pH is in the 3.7–4.0 region near the transition midpoint, it is extremely difficult to analyze the data in terms of two phases because the data plot very close to linearly as a single exponential function. This could be an indication either that the amplitude of the faster phase goes to zero near the midpoint or that relaxation times of the two phases become nearly identical at the midpoint. We think it likely that the former explanation is the correct one, although this point requires further study. At any rate, analysis of the data is highly uncertain in this pH region. Two phases can again be detected over the pH region 3.4–3.7. At final pH values lower than 3.2, only a fast phase is seen in unfolding experiments and the relaxation time for this phase becomes too fast to measure in the stopped-flow instrument at final pH values below 2.5. Thus, the entire unfolding reaction is completed in just a few milliseconds under these conditions at 25°.

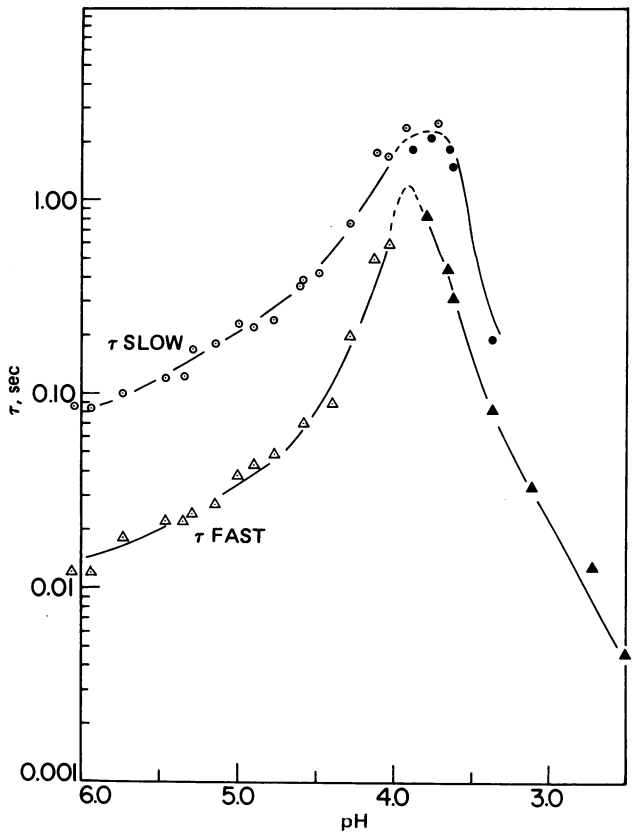



FIG. 4. The pH dependence of the relaxation times for the fast and slow phase for band 5. The abscissa gives the final pH of the jump in each case for both the slow phase (circles) and the fast phase (triangles). For refolding experiments (open symbols), the initial pH was 2.67, while for the unfolding experiments (filled symbols) the initial pH was always 4.76. The data were resolved into two phases by the method of peeling off exponentials (7). In the pH region 3.7–4.0 only a single phase could be detected so that there is an uncertainty in deciding whether to assign the single relaxation time to the slow phase, the fast phase, or to both.

Table 1. Comparison of the relaxation times for the slowest kinetic phase in the refolding of parvalbumin and of various proline-containing proteins

| Protein | Final pH | Number of prolines | τ (25°), sec | Ref. |
|---|---|---|---|---|
| Parvalbumin (band 5) | 6.0 | 0 | 0.08 | This work |
| Chymotrypsinogen | 2.2 | 9 | 55 | 8 |
| Chymotrypsin | 2.7 | 9 | 45 | 8 |
| Trypsin | 2.4 | 8 | 45 | 8 |
| Trypsinogen | 2.2 | 8 | 40 | 8 |
| Ribonuclease | 7.0 | 4 | 52 | 9 |
| Cytochrome *c* | 4.0 | 4 | 8 | 10 |
| Lysozyme | 6.3 | 2 | 17* | 11 |

* 32°.

## DISCUSSION

We have reported above the kinetics of denaturation of a proline-free protein. Although this protein does exhibit more than a single kinetic phase, we nevertheless feel that the observed results do support the idea that proline isomerization is responsible for the slow phase for those proteins that contain proline. To illustrate this, Table 1 contains a comparison of our

results with those found for proline-containing proteins. In each case, the relaxation times that are listed pertain to refolding experiments into the native state baseline region at 25° and thereby should be suitable for direct comparison. It is seen from this comparison that the slower kinetic phase for this parvalbumin is *ca* 100 to 500 times faster than the slowest kinetic phase seen for each of the proteins that contain proline. Therefore, it seems certain that the rate-limiting step for the parvalbumin is not the same molecular process as for these other proteins. Furthermore, it seems very possible that it is the absence of proline that is directly accountable for these tremendous differences in the rates of refolding. Even so, it should be reemphasized that the parvalbumin is also unusual in that it contains no tyrosine or tryptophan and somewhat unusual in that it contains no disulfide bonds. All of the other proteins listed in Table 1 contain either tyrosine or tryptophan, or both. All of the proteins in Table 1 contain disulfide bonds except parvalbumin and cytochrome *c*.

The particular case of staphylococcal nuclease has not been included in Table 1. The refolding of this protein, which has six prolines, was studied by Epstein *et al.* (12) using pH perturbations in a stopped-flow instrument. They characterized two fast phases (relaxation times of *ca* 0.5 and 0.05 sec. at pH 3.8 and 15°). No slow phase was reported, although later unpublished studies (H. F. Epstein, personal communication) did reveal a much slower phase in unfolding. Also, S. Hawley (personal communication) carried out extensive kinetic studies on this protein, using pressure perturbation and a conventional spectrophotometer. His results show a large slow phase with a relaxation time (pH 4.5, 15°) near 100 sec extrapolated to 1 atmosphere. Thus, whether or not this protein behaves similarly to the other proline-containing proteins in Table 1 cannot be deduced with certainty at this time, but it seems at least possible that it exhibits a comparable slow phase.

If the hypothesis about proline isomerization is correct, one might then wonder why two distinct kinetic phases are still seen for the parvalbumin. Because the amplitude of the slower phase goes to zero for unfolding experiments with jumps ending in the denatured baseline region, it seems very likely that the complications are due to the existence of two or more species of denatured protein that have very similar physical properties but which refold with different rates to the native state. That is, the following scheme can be suggested:

$$N \rightleftharpoons D_f \rightleftharpoons D_s$$

In this model, the denatured species $D_f$ and $D_s$ have essentially the same fluorescent properties but $D_f$ refolds to native $N$ in a fast conformational step while the equilibration of $D_s$ to $D_f$ is slower. We feel that the two relaxations seen for parvalbumin might then be equivalent to the $\tau_2$ and $\tau_3$ relaxations that Baldwin and colleagues have found for ribonuclease (7), with the $\tau_1$ phase missing for parvalbumin because of the absence of proline. It is not yet known what the $D_f \rightleftharpoons D_s$ equilibration might be. One possibility is that it involves equilibration of the *cis–trans* isomers of peptide bonds for non-proline residues. Preliminary nuclear magnetic resonance data suggests that non-proline residues might exhibit relaxation times for isomerism that are much faster than for proline residues if the corresponding rates (unpublished observations from this laboratory) for *N*-methyl acetamide and *N,N*-dimethyl acetamide can be used as a guide.

In summary, the absence of a very slow phase in the denaturation and renaturation kinetics for band 5 is consistent with the idea that the process responsible for the slow phase exhibited by other proteins is *cis–trans* isomerization of proline residues. The present study further suggests that the actual process of folding or unfolding a protein might occur considerably faster than thought heretofore. Apparently, the only phase in the parvalbumin kinetics that involves significant conformational changes is the fastest phase. For unfolding or refolding experiments ending in either baseline region, this process has a time constant of only a few milliseconds at 25°, although it slows down somewhat near the transition point. Thus, proteins might be able to find their way into the correct structure exceedingly quickly once the peptide bonds are in their proper isomeric state.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