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Kinetics of interaction of partially folded proteins with a hydrophobic dye: Evidence that molten globule character is maximal in early folding intermediates

MARKUS ENGELHARD AND PHILIP A. EVANS

Department of Biochemistry and Cambridge Centre for Molecular Recognition, University of Cambridge, Cambridge CB2 1QW, United Kingdom

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Abstract

Interaction with 8-anilino-1-naphthalenesulfonate (ANS) is widely used to detect molten globule states of proteins. We have found that even with stable partially folded states, the development of the fluorescence enhancements resulting from such interactions can be relatively slow and kinetically complex. This is probably because initial binding of the dye can induce subsequent changes in the protein structure, so that the ultimate resulting fluorescence enhancement is not necessarily a good, nonperturbing probe of the preexisting state of the protein. When ANS is used to study folding mechanisms the problem is compounded by the difficulty of distinguishing effects due to the development of dye interactions from those due to the changing populations of folding intermediates.

Many of these complications can be avoided by experiments where the ANS is introduced only after folding has been allowed to proceed for a variable time. The initial fluorescence intensity after mixing, resulting only from rapid and therefore hopefully relatively nonperturbing interactions with the protein, can be monitored at different refolding times to provide a better reflection of the progress of the reaction, uncomplicated by dye interaction effects. Such studies of the folding of carbonic anhydrase and α-lactalbumin have been compared with conventional single-mix experiments and large discrepancies observed. When ANS was present throughout refolding, time-dependent changes attributed to the formation or reorganization of protein–ANS complexes were clearly superimposed on those associated with the actual progress of refolding, and the folding kinetics and population of intermediates were also substantially perturbed by the dye. Thus, it is clear that the pulse method, though cumbersome, should be used where refolding reactions are to be probed by dye binding.

The results emphasize that fluorescence enhancement tends to be greatest in early intermediates, in contrast to what, for carbonic anhydrase at least, might appear to be the case from the more conventional experiments. Later intermediates in the folding of both of these proteins actually induce little fluorescence enhancement and therefore may be quite different in nature from equilibrium molten globule states.

Keywords: α-lactalbumin; carbonic anhydrase; 8-anilino-1-naphthalenesulfonate; folding intermediate; molten globule; protein folding; stopped flow

A wide variety of methods have been used to investigate various aspects of protein folding pathways (Matthews, 1993; Evans & Radford, 1994). Techniques such as CD facilitate relatively straightforward estimation of the variation in secondary structure content during refolding (Labhardt, 1984; Kuwajima et al., 1987), but it is much less obvious how best the development of side-chain interactions should be monitored. Commonly used probes such as intrinsic tryptophan fluorescence are clearly sensitive to side-chain clustering, but they are not necessarily very representative of global structure and the total fluorescence intensity, which is the parameter most easily followed, is difficult to interpret in detail. Combinations of such simple methods with protein engineering offer a more general approach (Matthews, 1993; Serrano, 1994), but here too there are potential problems if the clustering is rather tenuous or nonspecific. It is clear neither whether such behavior would necessarily be detected by simple spectroscopic methods nor how mutations ought to be analyzed in the case of nonspecific interactions. One technique that appears to go some way to addressing the problem uses 8-anilino-1-naphthalene-sulfonate (ANS) as an extrinsic probe. This dye tends to bind to clustered hydrophobic residues and its

Reprint requests to: Philip A. Evans, Department of Biochemistry and Cambridge Centre for Molecular Recognition, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, United Kingdom; e-mail: pe@mole.bio.cam.ac.uk.

fluorescence quantum yield increases markedly as a consequence. This probe has now become almost standard for diagnosing the population of compact, partially folded states, usually referred to as molten globules (Semisotnov et al., 1991).

In an aqueous environment the fluorescence of ANS is very effectively quenched, but a strong intensity enhancement occurs upon interaction of the dye with hydrophobic surfaces, including nonpolar binding sites in the folded states of certain proteins (Stryer, 1965; Mulqueen & Kronman, 1982). If partially folded states of proteins have clusters of hydrophobic side chains that are not yet fully occluded in the native core structure, these might also provide binding sites for the dye and, indeed, molten globule states of several proteins, generated under mild denaturing conditions and shown by viscometric and scattering measurements to have collapsed structures interact very effectively, leading to large fluorescence intensity increases (Tandon & Horowitz, 1989; Semisotnov et al., 1991).

Further studies sought to exploit this interaction with ANS to assess whether comparable molten globule-like states were populated as transient intermediates during the refolding of globular proteins. This was investigated by inclusion of the dye in the refolding buffer in stopped-flow experiments (Semisotnov et al., 1987, 1991; Goldberg et al., 1990; Ptitsyn et al., 1990; Matthews, 1993; Itzhaki et al., 1994). Studies on a wide range of different proteins revealed time courses that were qualitatively remarkably similar in each case: a transient rise in ANS-fluorescence with a maximum reached typically within about 200 ms after the initiation of refolding, followed by a slower return to the low level of fluorescence obtained in the presence of the native states. The kinetics of the slower phase appeared to be similar qualitatively to those of formation of the overall native structure as detected, for example, by recovery of enzymatic activity (Semisotnov et al., 1987).

It was also apparent from these studies that in many cases a proportion of the intensity enhancement had already developed in a "burst phase" that was complete within the dead time of the experiment, typically a few milliseconds (Semisotnov et al., 1991; Mann & Matthews, 1993; Jones et al., 1994, 1995). This suggested a degree of hydrophobic collapse even at this early stage of folding, although in general a subsequent slower rise in intensity was taken to indicate that more extensive clustering followed as later intermediates became populated. A significant point of comparison is that kinetic studies of the refolding of several proteins using stopped-flow CD have revealed that the greater part of the secondary structure is typically formed within the dead time of the experiment (Kuwajima et al., 1987; Chaffotte et al., 1992), more than an order of magnitude faster than the development of the maximal ANS fluorescence. This might suggest that secondary structure formation precedes at least the most extensive phase of hydrophobic collapse of the molecule during folding, thus lending support to the "framework" model for folding pathways (Kim & Baldwin, 1990). Indeed studies of the interaction of ANS with model peptides suggested that the dead time fluorescence enhancement might be attributable simply to interaction with this rapidly formed secondary structure, without the need to invoke any wider hydrophobic interactions (Semisotnov et al., 1991).

Two crucial assumptions need to be made in drawing these conclusions from stopped-flow experiments with ANS included in the refolding buffer. The first is that the time-dependent changes observed are entirely due to the progressive folding of the protein rather than, for example, relatively slow binding of the ANS to the intermediates populated. The implicit assumption is that once a partially folded state is formed it will interact virtually instantaneously with the ANS to give the characteristic fluorescence enhancement. This would effectively be the case, given the relatively slow time scales of the observed kinetics, if interaction were diffusion limited but this would depend on the nature of the binding event. A study was therefore made of the time development of the fluorescence enhancement resulting from binding of ANS to a preexisting equilibrium molten globule state, that of bovine carbonic anhydrase in 1.9 M guanidinium chloride solution at high pH (Semisotnov et al., 1991). The change was indeed complete within the deadtime of a stopped-flow experiment, lending support to the assumption of instantaneous binding in interpreting refolding data. However, studies of the interaction of this dye with the native state of bovine serum albumin did reveal a rather complex time course, slow enough to follow in stopped-flow experiments, whose interpretation has been the cause of some controversy (Nakatani et al., 1974; Regenfuss & Clegg, 1987).

The second important assumption in analyzing the kinetics is that the ANS simply binds transiently to partially folded intermediates without measurably altering their conformations or stabilities and thus perturbs significantly neither the folding pathway nor its kinetics. In the case of carbonic anhydrase, evidence in support of this came from similar kinetics observed using other probes aimed at monitoring hydrophobic collapse, involving changes in the mobility of EPR probes and in the efficiency of energy transfer between fluorophores (Ptitsyn et al., 1990). However, recent studies of lysozyme refolding suggested that this may not always be the case. Here the presence of the dye appeared to prolong measurably the lifetimes of partially folded states (Itzhaki et al., 1994). This was demonstrated by comparing the time course observed with ANS present throughout refolding with one reconstructed by introduction of a pulse of the dye at various stages into the reaction. In the work described here we have sought to investigate in more detail possible events that might complicate use of the ANS probe for detection of molten globule-like intermediates in folding and to develop further the ANS-pulse method for reconstructing time courses while avoiding many of these problems. The results alter significantly our perception of the kinetic role of molten globule-like intermediates.

Results

Interaction with equilibrium molten globules

As discussed above, binding of ANS to the equilibrium molten globule state of bovine carbonic anhydrase B populated at high pH in the presence of intermediate concentrations of guanidinium chloride has been shown to cause an increase in its fluorescence that is complete within the dead time of a stopped-flow experiment (Semisotnov et al., 1991). We obtained the same result under these conditions (data not shown) but found quite different behavior for another partially folded state of this same protein, formed in this case at low pH, as shown in Figure 1. In a manual mixing experiment at least three phases of time development can be distinguished, starting with a relatively rapid rise in fluorescence intensity that is already complete by the time the first data points are collected, a few seconds after mixing.

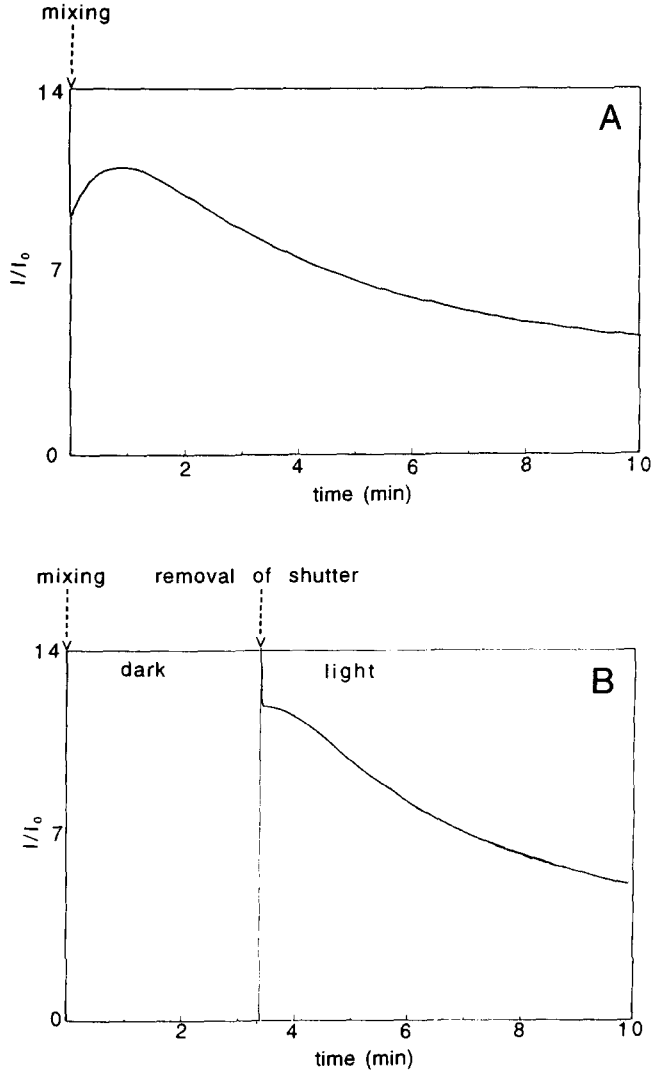

Fig. 1. A: Time dependence of fluorescence of ANS (250 μM final concentration) at 480 nm upon addition to a solution of bovine carbonic anhydrase B (1 μM final concentration) in 0.1 M glycine-HCl buffer at pH 3.6, 20 °C. In this and subsequent figures the intensity (I) is plotted relative to that of free ANS at the same concentration but in the absence of the protein (I_0). Estimated dead time between mixing the solutions and recording the first data point was 15 s. **B:** Experiment performed as in A, but with the sample excluded from the excitation beam during the first part of the time development, as indicated.

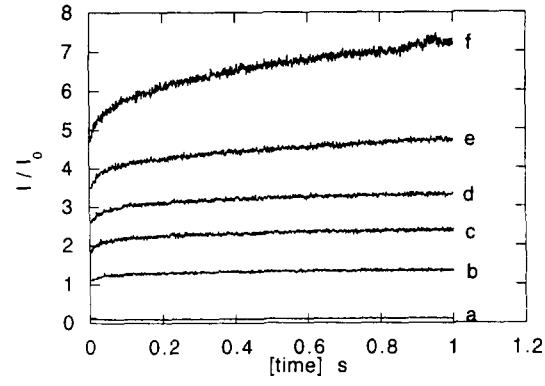

Fig. 2. Time dependence of the ANS fluorescence enhancement observed in stopped-flow experiments where the dye was mixed at various concentrations with 12.5 μM carbonic anhydrase B in 0.1 M glycine HCl buffer at pH 3.6, 20 °C. ANS concentrations were: a = 0 μM; b = 50 μM; c = 100 μM; d = 150 μM; e = 200 μM; f = 250 μM.

A further, slower increase follows, so that the intensity peaks after about 30 s, after which a much slower decay ensues, leading toward a very small limiting fluorescence.

The final decay in fluorescence in this experiment seemed at first sight especially surprising because it appears to imply that the enhancement is not an equilibrium property of the system. It was found, however, that this phase was an artifact due to photobleaching in the fluorimeter beam. This was demonstrated by keeping the sample in the dark for some minutes before starting to monitor its fluorescence, as shown in Figure 1B. Far from having decayed during this incubation time in the dark, the first measured fluorescence signal in this experiment has a slightly greater intensity than the maximal value attained in the continuously illuminated sample. Experiments were performed with dark exposure times between 2 and 10 min and it was found that approximately the same initial intensity was observed in each case. Even after these prolonged preincubations in the dark, the kinetics of the intensity decay are clearly complex, suggesting that more than just a first order photolysis reaction is involved. Nonetheless, all of these observations are consistent with the decay phase occurring only on exposure to the light beam.

In order to focus, in particular, on the initial time development of the fluorescence enhancement, stopped-flow experiments were performed to allow much more rapid events to be resolved. Kinetic traces observed over a range of ANS concentrations all showed essentially the same qualitative features, as exemplified in Figure 2. Even with a dead time now reduced to about 3 ms, a significant proportion of the intensity increase observed (65–85% of total fluorescence enhancement) was complete before the first data points were acquired. The subsequent further rise in intensity does not occur in a single exponential phase, adding further to the evident complexity of the overall kinetics.

The time development over 10 s in these experiments could be modeled adequately by a sum of two exponentials over the entire range of ANS:protein concentration ratios studied. The rate constants obtained from the fitting procedure are essentially invariant, within the limits of experimental error, suggesting that the kinetics are not limited by a simple bimolecular binding event (Fig. 3). The variation in amplitude of the different phases of the time course was also investigated. Figure 4 shows that neither the dead time change nor the limiting intensity obtained after prolonged incubation in the dark show clearcut saturation behavior, even when the ANS is in 20-fold excess. The binding curves in both cases appear to deviate from simple hyperbolae, suggesting that there may be multiple, interacting binding sites with significantly different dissociation constants.

The possibility that this complex behavior might be unique to the low pH molten globule state of carbonic anhydrase was checked by studying the interaction of ANS with partially folded states of two other proteins. Bovine α-lactalbumin and an acyl carrier protein from *Saccharopolyspora erythraea* form molten globule states at acidic pH and the interaction of each with ANS

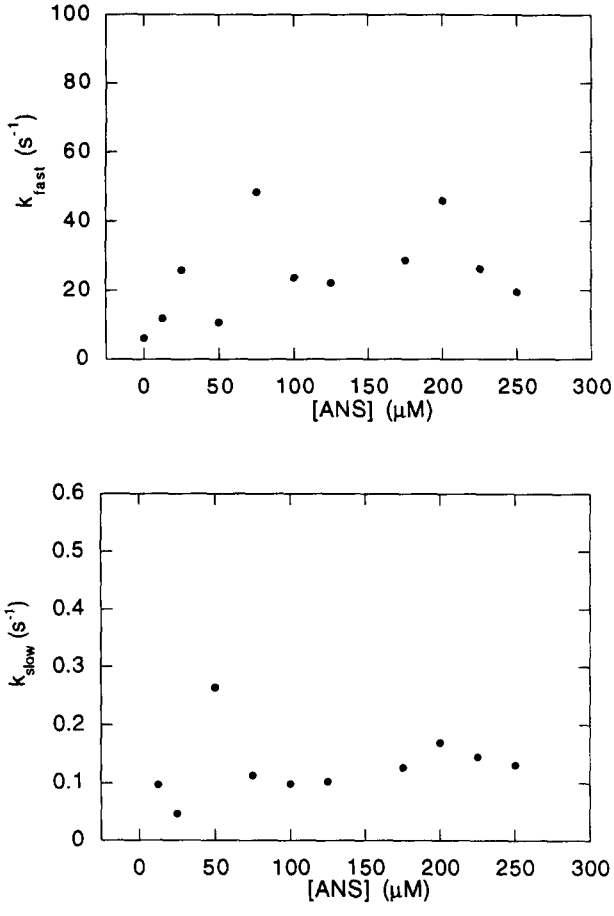

Fig. 3. Dependence on ANS concentration of the kinetics of fluorescence enhancement observed in the stopped-flow studies of its interaction with carbonic anhydrase B. Raw data, as exemplified in Figure 2, were fitted to a sum of two exponentials with rate constants k_{fast} and k_{slow}, and the values of these parameters are plotted here.

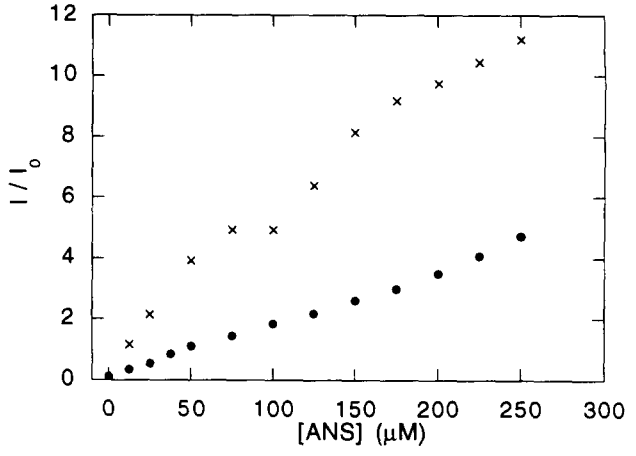

Fig. 4. Dependence on ANS concentration of the fluorescence intensity enhancement observed upon addition of 12.5 μM carbonic anhydrase B at pH 3.6, 20 °C: ●, initial enhancement observed in a stopped-flow experiment with a dead time of 3 ms; ×, enhancement observed after manual mixing and preincubation in the dark for 10 min before measuring the fluorescence. Note that the data from the manual mixing experiments are based on emission intensities at a fixed wavelength (480 nm), whereas in the stopped-flow experiments the emission was effectively integrated over all wavelengths in excess of the filter cutoff. The data sets are therefore not directly comparable, but this does not affect any of the conclusions drawn.

was studied at pH 2.0. In both cases time dependences qualitatively similar to that observed for carbonic anhydrase B were observed (data not shown). There was a significant intensity rise during the dead time of manual mixing, followed by a further increase to a maximum at around 240 s for α-lactalbumin and 15 s for the acyl carrier protein. In both cases, as with carbonic anhydrase, there ensued finally a slow photolytic decay.

Interactions during protein refolding

If the interaction of ANS with transient folding intermediates were as kinetically complex as it can evidently be with equilibrium molten globules, interpretation of the time-dependent fluorescence intensity could clearly be problematic. We have investigated this possibility for two proteins whose refolding has been studied in some detail, using interaction with ANS as one probe of intermediate formation: bovine carbonic anhydrase B and human α-lactalbumin.

The time course of ANS fluorescence during the refolding of carbonic anhydrase is shown in Figure 5A. Using the conventional approach, where ANS is present throughout the time course, there appears to be only a limited intensity increase during the dead time of the stopped-flow experiment, the greater part of the enhancement developing in an apparently biphasic manner, such that at least 90% of the maximal enhancement is achieved within 1 s, with the remainder developing over several seconds. These results are in accord with those reported previously under the same conditions (Ptitsyn et al., 1990). In that case a much slower ultimate decay of the fluorescence over several minutes was also observed, attributed to completion of folding to yield the native state; our present experiments did not extend to monitoring events on this time scale because of the problems we experienced with photolysis, as discussed above.

A second strategy was adopted for constructing the refolding curve under the same conditions, as illustrated in Figure 5B. In this case, the ANS was introduced in a second mixing event, after the reaction has been allowed to proceed for some time in the absence of the dye. The initial intensities observed after mixing in the ANS were extracted from these data and plotted for comparison with the conventional experiment in Figure 5A. What is being measured here is effectively a reflection of the interactions that develop within 2 ms of the addition of a pulse of dye at various stages of folding. The refolding conditions before the introduction of the ANS pulse are otherwise identical to those in the original experiment, except that the protein concentration is twofold higher, so that when the dye solution is added, the final protein and dye concentrations in the observation cell are exactly reproduced.

The differences between the two curves are quite remarkable. In the curve derived from the ANS pulse data, the maximal fluorescence enhancement is now observed at the shortest measurable time, around 20 ms into refolding in this experiment. Most of this enhancement is then rapidly lost so that during the first 1 s, when the intensity is rising in the conventional experiment,

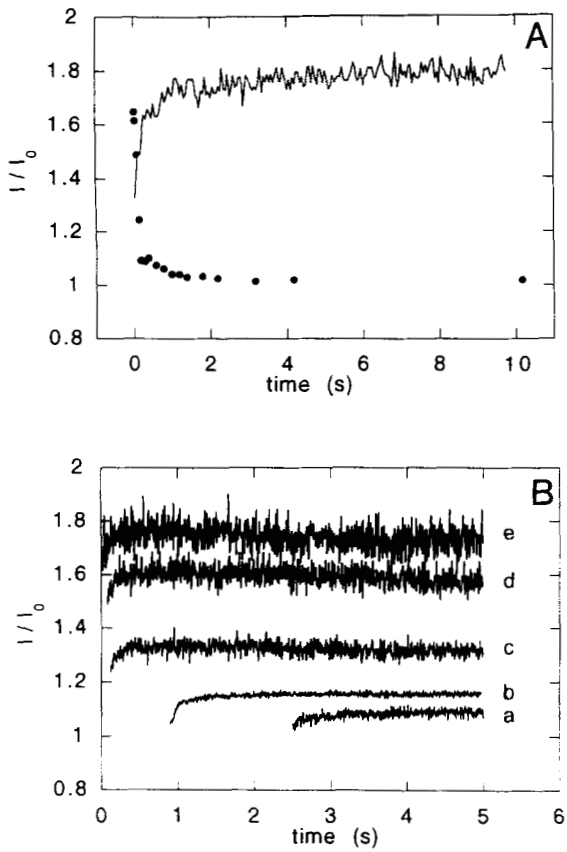

Fig. 5. A: Time course of refolding of bovine carbonic anhydrase B as reflected in ANS fluorescence enhancement. The full line is the result obtained in a conventional, one-mix experiment, where a solution of the protein in 8 M urea was diluted two-fold to initiate refolding. The final refolding buffer composition was 3 μM protein in 4 M urea, 50 mM Tris HCl, pH 8.0, 23 °C; 240 μM ANS was present throughout refolding. The points (●) represent initial intensity enhancements determined by ANS pulse experiments in which the dye was introduced in a second mixing stage after refolding had proceeded in its absence for the time indicated. The refolding conditions were identical to those of the one-mix experiment except that the protein concentration was 6 μM and the dye was absent; after addition of the ANS the conditions were identical to those in the one-mix experiment. **B:** Representative data from the ANS pulse experiments, with variable periods of refolding before introduction of the dye (a = 2,496 ms; b = 896 ms; c = 120 ms; d = 80 ms; e = 20 ms). Data plotted in A correspond, in effect, to the first data point of each trace, although in practice these initial intensities were averaged over a period of several milliseconds of continuous flow to improve accuracy.

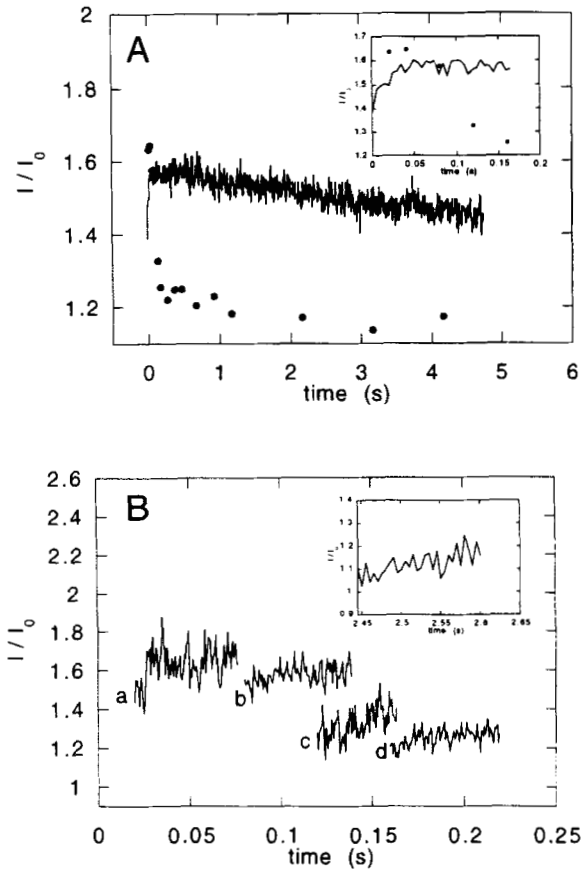

Fig. 6. A: Time course of refolding of human α-lactalbumin as reflected in ANS fluorescence enhancement. The full line is the result obtained in a conventional, one-mix experiment, where a solution of the protein in 9 M urea was diluted six-fold to initiate refolding. The final refolding mixture comprised 10 μM protein in 1.5 M urea, 50 mM Tris-HCl, pH 8.0, 8 °C; 240 μM ANS was present throughout refolding. The points (●) represent initial intensity enhancements determined by ANS pulse experiments, analogous to those described in Figure 5. The insert shows an expansion of the first 160 ms of the time course. **B:** Representative data from the ANS pulse experiments, with variable periods of refolding before introduction of the dye (a = 20 ms; b = 80 ms; c = 120 ms; d = 160 ms). Data plotted in A correspond, in effect, to the first data point of each trace. The insert shows the first 160 ms of a trace obtained with a refolding period of 2,440 ms before introducing the dye.

it actually dwindles to around 10% of its initial value when the ANS pulse method is used. A small proportion of the enhancement decays rather more slowly but by around 2 s the fluorescence observed in the pulse experiment is almost unperturbed by the protein; in contrast, the intensity obtained in the conventional experiment is still rising at this time.

Once the ANS has been introduced in the pulse experiment, the subsequent time development of the intensity can provide further insights, as illustrated in Figure 5B. The initial intensity enhancement is invariably followed by a further, slower increase, qualitatively similar to the faster resolved phase of the time course in the conventional refolding experiment (Fig. 5A). Even when more than 2 s is allowed to elapse before the dye is introduced, by which time the protein has reached a state that causes negligible initial intensity enhancement, this subsequent rise remains apparent, though diminished in amplitude. Clearly, this element of the time-dependent behavior is caused by the dye and does not reflect an independent refolding event.

The alternative experimental approaches were also applied to the refolding of human α-lactalbumin (Fig. 6A). The curve obtained with ANS present throughout is similar to that previously reported (Ptitsyn et al., 1990). In this case the greater part of the maximal fluorescence enhancement has already developed during the dead time and there is then a further rise, so that the intensity peaks at around 40 ms before beginning to decline slowly toward its limiting value. It should be noted that in the case of this protein there seems to be a significant enhancement (around 20%) of the fluorescence even by the native protein.

In this case, the early stages of the time development are qualitatively similar in the two curves, so that maximal enhancements occur at around the same time in the two curves. In the later stages, however, the curves differ sharply, with a very much more rapid loss of intensity characterizing the ANS pulse data. By 1 s virtually all of the enhancement over and above that of the native protein appears to be lost in this experiment, whereas this takes at least 20 s in the conventional experiment.

Figure 6B shows data from pulse experiments with α-lactalbumin. The initial intensity enhancement is invariably followed by a slower subsequent rise over some 200 ms or so, even when there have been delays much longer than this before introduction of the dye. Significantly, however, this time development is substantially slower than that observed during the first 40 ms or so in the conventional one-mix experiment and the amplitude is diminished at very early times. These observations, together with the coincidence with the time course reconstructed from pulse experiments, suggest that in this case the faster resolved phase in the conventional experiment may actually be faithfully reporting the progress of folding, with events driven by dye binding being significant only on a longer time scale.

As a comparison, the refolding kinetics of human α-lactalbumin were also followed by stopped-flow CD, as shown in Figure 7. In this case most of the increase in negative ellipticity at 225 nm is complete within the dead time of the reaction but there is an additional smaller component that develops over approximately 2 s during which time a CD signal in the near-UV region (289 nm) also develops. The low amplitude slow phase in the reconstructed ANS pulse fluorescence curve is qualitatively similar in decay rate (Fig. 6A), but it is clear that the decay in the single-mix ANS fluorescence experiment is slower than any event detected by CD, consistent with the notion that the presence of the dye retards the transition to the native state. Neither are there any features in the CD comparable to the intensity rise over the first 40 ms or so, or to the subsequent major phase of intensity decline in the fluorescence time development derived from the ANS pulse data.

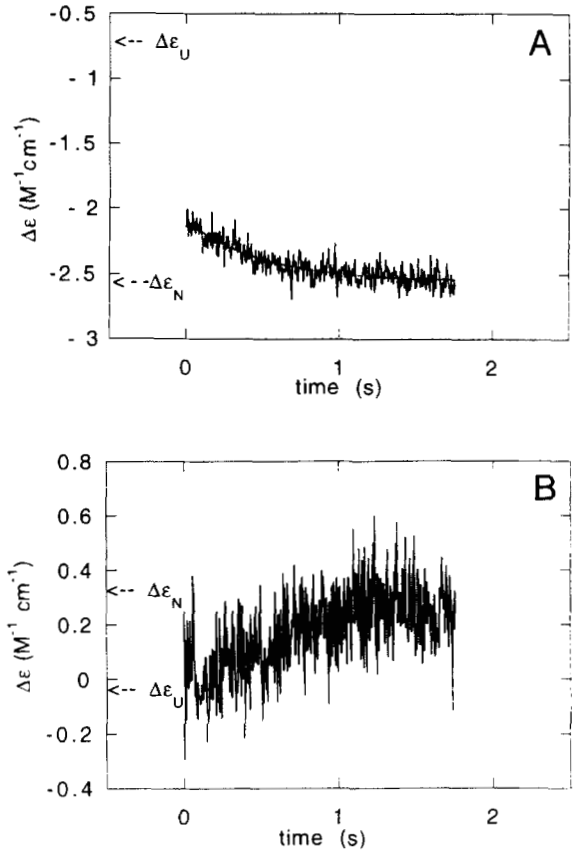

Fig. 7. Time course of refolding of human α-lactalbumin as monitored by CD at **(A)** 225 nm and **(B)** 289 nm. Refolding conditions were the same as those used in the ANS fluorescence studies (Fig. 6), except that the dye was omitted and the protein concentration was 160 μM. Values of the dichroism in the unfolded state ($\Delta\epsilon_U$) and the native state ($\Delta\epsilon_N$), determined from equilibrium measurements, are indicated in each case.

Discussion

Interaction with equilibrium molten globules

The complex time-dependent changes in fluorescence intensity as ANS interacts with the acidic molten globule state of carbonic anhydrase B are in stark contrast to the very simple behavior observed for the same protein at high pH in the presence of denaturant, when the entire intensity enhancement was too fast to measure (Semisotnov et al., 1991). The immediate conclusion to be drawn from this is that these species, although both may meet the criteria defining molten globule states, may actually have significantly different structural properties. One factor that may be significant is that the low pH form has a much greater tendency to associate and the much greater magnitude of the ANS fluorescence enhancement in the presence of this state has been linked to this phenomenon (Semisotnov et al., 1991).

The qualitatively similar results obtained for two other proteins, although these were not investigated in great detail, suggest that this kind of complex time dependence may be rather common when ANS interacts with partially folded states. The nature of the events involved cannot be discerned in detail but some conclusions can be drawn, especially from the concentration dependence studies. The shape of the binding curves (Fig. 4) suggests that there are several, nonequivalent ANS binding sites on the carbonic anhydrase acidic molten globule molecule and a simple model for the kinetics might propose that although binding to some of these is facile and complete within the dead time, others are kinetically less accessible, so that binding occurs in the slower observed kinetic phases. The rates of such simple bimolecular binding events, however, would be expected to depend on the product of the protein and ligand concentrations and this is not the case, as is evident in Figure 3. The lack of concentration dependence of the rates of either of the two resolved phases of intensity increase suggests that they must be associated with changes whose rates are limited by essentially unimolecular events.

Interestingly, a similar issue arose in kinetic studies of the interaction of ANS with the native state of bovine serum albumin. Here too, the rates were found to be independent of the concentrations of both dye and protein and in the original study the authors concluded that initial binding occurred during the dead time, with subsequent intensity changes reflecting conformational changes within the complex (Nakatani et al., 1974). In a later study, however, which extended the observations down to much shorter times, an alternative model was developed which

did not require the postulate of conformational changes. The apparent first order kinetics were explained by a very fast initial binding of ANS in a nonequilibrium distribution among several possible binding sites, followed by a slower redistribution according to the thermodynamic affinities of the sites. The rate of the latter process being limited by the unimolecular dissociation of the initial complex, it appeared to have first order kinetics. This situation could arise only because the protein was in excess, so that there was competition among potential binding sites for a limited amount of ANS (Regenfuss & Clegg, 1987). In the present study, however, concentration independence of the rate constants was observed over a range of concentration ratios up to a 20-fold excess of ANS. In addition the binding curves (Fig. 4) show that the protein is not saturated even at this dye concentration and equilibrium dialysis studies have suggested that only about 2 dye molecules bind in the limiting case (Semisotnov et al., 1991). There could, therefore, not have been such a competitive binding situation under our conditions, which effectively rules out such an explanation in the case of the carbonic anhydrase molten globule.

Another potential complication arises from the exposure of hydrophobic surfaces in partially folded proteins, which renders them liable to aggregation in solution. Interaction of ANS with these same nonpolar groups might be associated with the time-dependent events observed. However, the lack of a significant effect of protein concentration on the kinetics suggests that the fluorescence changes do not arise from an increase in association, for which a kinetic reaction order of at least two would be expected. An alternative possibility might be the disruption by ANS of preexisting aggregates, which could be a first order process. However, although the low pH form of carbonic anhydrase does indeed seem to be associated (Semisotnov et al., 1991), that of bovine α-lactalbumin, which shows qualitatively similar time-dependent behavior with ANS, has been shown to be monomeric (Dolgikh et al., 1985). It therefore seems unlikely that changes in the association state of the protein can provide a general explanation of the time-dependent behavior observed.

These considerations leave us with the conclusion that the time dependence observed is most likely to be associated with conformational changes within the initially formed ANS–molten globule complex. This does not seem unreasonable, given that the lack of a fixed tertiary structure would be expected to make such states relatively plastic. Interestingly, conformational fluctuations with rates in a similar range have been detected by NMR in the case of a molten globule state of guinea pig α-lactalbumin (Baum et al., 1989). Changes in the stability of partly folded states as a result of dye binding have been noted previously (Teschke & King, 1993; Shi et al., 1994) and NMR has revealed perturbations to the structure of a relatively native-like state of apomyoglobin when ANS binds (Cocco & Lecomte, 1994), but in general the extent of structural perturbations to molten globule states is difficult to gauge because the dye interferes with many spectroscopic methods.

Interaction with kinetic intermediates

The time scale of the events observed when ANS interacts with these equilibrium molten globules, whatever their precise explanation, is immediately of concern in relation to the use of this probe in kinetic studies of protein refolding. The time dependence of fluorescence could reflect changes actually brought about by binding of the dye, as well as the progress of refolding, and it seems unlikely that these factors could easily be distinguished in a simple experiment where the dye is present throughout. The idea behind the ANS pulse method is that the initial fluorescence enhancement when the dye is introduced might, in general, provide a better reflection of the preexisting state of hydrophobic collapse of the protein at any instant. Although there could be some structural reorganization as a result of binding even within this time, it would clearly be expected to be less extensive than could occur through subsequent slower transitions. In addition, the pulse method avoids the risk of changes in the pathway or kinetics of refolding due to dye binding.

The gross differences in the curves obtained using the conventional approach and those reconstructed from ANS pulse data fully bear out these considerations. Although the key qualitative conclusion from earlier work, that states capable of binding ANS and therefore probably resembling equilibrium molten globule states are populated transiently during refolding, is fully supported by the present results, it must be concluded that the detailed kinetics observed in studies based on the conventional one-mix experiment do not, in general, provide a true reflection of the progress of folding in the absence of the dye.

Even at the shortest measurable times, substantial differences were observed between the conventional and reconstructed curves obtained with both proteins studied. When a pulse of ANS is administered after 20 ms or so of refolding the dye binds effectively, causing a substantial fluorescence enhancement. However, when the ANS is actually present during these first few milliseconds, the curious result is that there is significantly less enhancement, especially with carbonic anhydrase. This must mean that the presence of the dye significantly affects the progress of folding in these early stages, perturbing the population of intermediates. What is really surprising is that the consequence is actually less rather than more fluorescence enhancement, which, if we were to assume a simple relationship between the extent of binding and the magnitude of fluorescence enhancement, would suggest that the presence of ANS actually inhibits formation of states to which it can bind. This seems nonsensical, and a more likely explanation would be that the dye is binding to at least as great an extent but that as a result of the differences in the partially folded structures, the fluorescence quantum yield is less effectively enhanced. In any case, the remarkable point is that these different early intermediates seem only to be formed because the ANS is present from the very outset of folding. Just a few milliseconds in the absence of the dye are apparently sufficient to ensure that alternative structures are formed instead, which produce the larger fluorescence enhancement when introduced to ANS.

In the case of carbonic anhydrase, the conventional experiment then shows a sharp rise in fluorescence intensity, which, perversely, is almost mirrored by an equally sharp fall in intensity achieved in the ANS pulse experiment. This again suggests that the change observed when ANS is present throughout is an artifact of its presence and does not reflect the change in the population of collapsed intermediates that would be observed otherwise. This is a very important result because it means that for this protein at least, the intermediates most resembling a molten globule state are actually populated maximally very early in folding rather than appearing more gradually, as would be supposed based on the conventional ANS fluorescence experiments.

Thus, it certainly cannot be concluded that secondary structure formation precedes hydrophobic collapse in this case. This question cannot, indeed, be addressed because the events are too fast for stopped-flow methodology.

In the case of α-lactalbumin, the CD experiment showed a large ellipticity change at 225 nm during the stopped-flow dead time which, assuming that the helical structure in the protein makes the largest contribution at this wavelength, suggests that secondary structure formation is already at an advanced stage by this time. Taken together with the concomitant ANS fluorescence enhancement, this observation thus precludes the possibility of defining the nature of initiation of folding from these data.

After the dead time, the behavior of the ANS fluorescence in the α-lactalbumin experiment differs from that in the carbonic anhydrase case. There is a further increase in the intensity, which, in this case, is apparent both in the conventional and reconstructed curves. Furthermore, the rate of this increase is faster than those of increases due to dye binding effects that are observed in the pulse experiment throughout folding and even, indeed, in the native state itself. It seems likely, therefore, that in contrast to the carbonic anhydrase case, the conventional method may be faithfully reporting the progress of folding of α-lactalbumin in these early stages. Thereafter, however, it is clear that the presence of the dye does perturb the kinetics substantially.

A feature common to both of the proteins studied here, and to lysozyme as we reported previously (Itzhaki et al., 1994), is that the final loss of the intensity enhancement is evidently retarded significantly when ANS is present continuously. This seems quite reasonable because if ANS binds preferentially to partially folded states, the need to dissociate from it will tend to raise the activation barrier to their transition into the native state. One consequence of this is that it has tended to obscure the fact that the ability to bind the dye actually diminishes rather quickly, if it is monitored without this perturbation, by the pulse method. Thus, in the case of carbonic anhydrase, for example, although the native state is restored with a half life of around 140 ± 20 s (Ptitsyn et al., 1990), ANS binding capability is virtually lost within the first 2 s of the reaction (Fig. 5A). Similarly for α-lactalbumin, most of the fluorescence enhancement associated with the early intermediate is lost within the first 100 ms of the reaction (Fig. 6A), but the formation of the native structure, as judged by near-UV CD, occurs over 2 s or so (Fig. 7B). This means that the later intermediates in the folding pathways of these proteins actually do not behave like molten globule states, in that their ANS binding capability is not significantly greater than that of the native state itself. To understand the full significance of this we would have to know more about the nature of the binding sites that ANS occupies in these states, but it would seem to suggest that hydrophobic surfaces become effectively excluded at a relatively early stage, later intermediates presumably having higher packing densities and insufficient accessibility or plasticity to admit the dye molecules.

Conclusions

The results presented here demonstrate that the interaction of ANS with molten globule states of proteins can be a kinetically complex process, probably because the rapid initial binding of the dye can induce subsequent, slower conformational changes in the partially folded structure or because binding to some sites is dependent on particular conformational fluctuations, so that the kinetics may be limited by their frequency. Consequently, the ultimate fluorescence enhancement resulting from the interaction may not be a good, nonperturbing probe of the nature and extent of preexisting structure in the partially folded state. We have argued that the initial intensity observed in a stopped-flow mixing experiment may come closer to providing a true reflection of the average conformation of the protein before the dye is added.

These considerations are especially relevant when the use of ANS to detect partially folded compact intermediates during refolding is considered. We have shown that many of the potential complications can be avoided by performing a series of double mix stopped-flow experiments, in which the ANS is introduced after folding has been allowed to proceed for a variable time. This enables the time course of the initial fluorescence intensity, resulting from rapid and therefore hopefully relatively nonperturbing interactions with the protein, to be determined. Comparisons with results obtained using a conventional single mix stopped-flow method have indeed revealed marked discrepancies between the time courses. The differences when the ANS is present throughout refolding can be attributed partly to time-dependent changes associated with formation or reorganization of protein–ANS complexes, superimposed on those genuinely associated with the progress of refolding. There is also clear evidence, however, of perturbation of the distribution of intermediates as a result of interaction with the dye. We therefore recommend strongly that, despite the considerably greater inconvenience and the relatively small number of data points that can be obtained from the pulse experiment, this approach should be used in experiments where dye binding is to be used to probe refolding reactions. In the systems we looked at the kinetic behavior was clearly complex such that a detailed fitting of the curves was not possible; however, in simpler cases, at least, the experiment could be used for quantitative kinetic analysis.

The observation of ANS fluorescence enhancements within the first few milliseconds of refolding reactions has been influential in supporting the view that the early intermediates populated at this stage already have some resemblance to equilibrium molten globule states (Mann & Matthews, 1993; Matthews, 1993; Jones et al., 1994). The results of the ANS pulse experiments presented in this paper strongly reinforce this view and emphasize that, contrary to what has in some cases appeared to be the case from the more conventional experiments, dye binding capability tends actually to be maximal at an early stage and to be lost fairly rapidly thereafter. Thus, the major difference in the picture of refolding pathways that emerges from this new way of exploiting the ANS binding phenomenon is that although molten globule-like behavior seems to be a significant aspect of the properties of early intermediates, species formed later in the pathway appear to be inaccessible to the dye, suggesting that they may actually be fundamentally different in their level of structural organization.

Materials and methods

Bovine carbonic anhydrase, bovine and human α-lactalbumins (calcium-bound forms), and ANS were obtained from Sigma and used without further purification. A fatty acid synthase acyl carrier protein from *S. erythraea* was overexpressed in *Esche-*

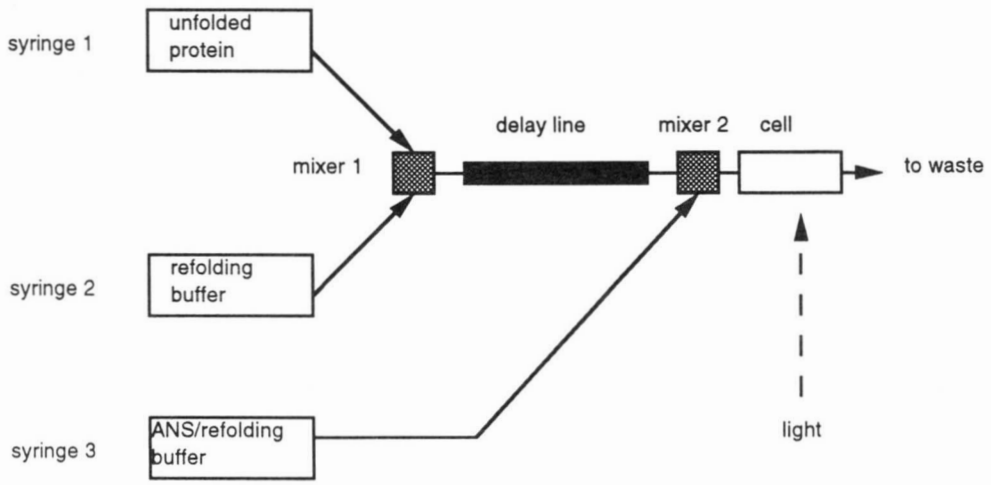

Fig. 8. Schematic illustration of the ANS pulse experiment. Denatured protein solution and refolding buffer are loaded into syringes 1 and 2, respectively, whereas the ANS solution is placed in syringe 3. Refolding is initiated by combining the protein and refolding buffer in mixer 1, after which the mixture travels through a variable volume delay line leading to the second mixer, where the ANS is introduced. The exit from this mixer leads to the observation cuvette with a minimal intervening volume ensuring a dead time of just a few milliseconds. The instrument can be operated in "continuous mode" in which all three syringes are driven simultaneously and the age of the refolding solution at the instant the dye is introduced is determined just by the rate of flow through the delay line and its volume. Alternatively, longer aging times can be obtained by allowing the refolding mixture to rest in the delay line for a measured interval before pushing it through to the second mixer.

richia coli and isolated using the method described by Morris et al. (1993), with minor variations. Each of the proteins used was judged to be at least 95% pure by SDS-gel electrophoresis. All other materials used were of analytical grade.

Equilibrium binding measurements and kinetic experiments with manual mixing were performed on a Jasco FP-777 fluorimeter, in a quartz cell of 1-cm pathlength. Fluorescence was excited at 350 nm and emission spectra were recorded between 420 and 530 nm; excitation and emission bandwidths were 5 nm. Rapid kinetic events were studied using a Biologic SFM3 stopped-flow module coupled to a Jobin-Yvon CD6 circular dichrograph, which served simply as the light source (350-nm excitation wavelength with a 5-nm bandwidth). A fiber optic was used to convey fluorescent emission from the side window of the stopped-flow cell to a photomultiplier tube via a colored glass filter, which passed only light of wavelengths exceeding 435 nm. A Biologic PMS-200 detection system was used to digitize and amplify the signal.

Refolding experiments were performed using the stopped-flow system in two ways. In the conventional experiments the reaction was initiated by dilution of the protein from a concentrated denaturant solution with a suitable buffer containing ANS. Data were recorded by monitoring the fluorescence signal in real time from the end of the stopped-flow push. The dead time in these experiments was on the order of 3 ms. ANS pulse experiments, on the other hand, required two mixing stages, as illustrated in Figure 8. In this case the refolding buffer did not contain ANS, and the protein concentration after the first mix was twice that in the conventional experiments. The refolding time in these experiments was, in effect, the delay between the first and second mixes, which was varied by changing the length of the connecting delay line and, for longer times, by interruption of the flow. The second mixing stage was used to introduce the ANS and generate a composition identical to that of the solution monitored in the conventional experiment. The dead time between mixing in the ANS and collection of the first data point was about 2 ms. Accuracy in these experiments was improved by averaging over several points from samples of the same age by monitoring while the syringes were still moving. In effect, this is then a continuous flow experiment (Chaffotte et al., 1992). In practice, essentially the same results were obtained by taking the first point after the stop in each case. In both types of experiment, a pulse sequence was used that enabled a baseline to be recorded with ANS but no protein in the cell, thus allowing the effects observed during the experiment to be expressed as proportional intensity enhancements (for clarity the baselines are not shown in the figures).

Stopped-flow CD measurements were made using the same Biologic SFM-3 instrument coupled to the Jobin-Yvon CD6 which, in this case, acted as both light source and detection system. The dead time in this case was around 3 ms.

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