Solvent isotope effects on the refolding kinetics of hen egg-white lysozyme

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(RECEIVED August 9, 1995; ACCEPTED October 23, 1995)

Abstract

Solvent isotope effects have been observed on the *in vitro* refolding kinetics of a protein, hen lysozyme. The rates of two distinct phases of refolding resolved by intrinsic fluorescence have been found to be altered, to differing extents, in D₂O compared with H₂O, and experiments have been conducted aimed at assessing the contributions to these effects from various possible sources. The rates were found to be essentially independent of whether backbone amide nitrogens were protiated or deuterated, indicating that making and breaking of their hydrogen bonding interactions is not associated with a substantial isotope effect. Neither were the rates significantly affected by adding moderate concentrations of sucrose or glycerol to the refolding buffer, suggesting that viscosity differences between H₂O and D₂O are also unlikely to explain the isotope effects. The data suggest that different factors, acting in opposing directions, may be dominant under different conditions. Thus, the isotope effect on the rate-determining step was found to be qualitatively reversed on going to low pH, suggesting that one component is probably associated with changes in the environments of carboxylate groups in forming the folding transition state. This term disappears at low pH as these groups are protonated and an opposing effect then dominates. It was not possible to identify this other effect on the basis of the present data, but a dependence of the hydrophobic interaction on solvent isotopic composition is a likely candidate.

Keywords: folding kinetics; isotope effect; lysozyme; protein folding

Replacement of solvent water by D₂O is well known to affect the thermodynamics of folding of many proteins (Henderson et al., 1970; Gomez-Puyou et al., 1978; Masson & Laurentie, 1988; Antonio et al., 1991; Goto et al., 1993), so that the stability of the folded state is generally enhanced in the deuterated solvent. This presumably reflects differences in the strengths of hydrogen bonds involving NH and OH compared to ND and OD. However, the process of folding involves changes in protein–protein, protein–solvent, and solvent–solvent hydrogen bonding, and it is very difficult to assess the relative contributions of these terms to the overall isotope effects observed. The problem is further complicated in that the size and even the sign of isotope effects are not straightforwardly predicted in complex systems. Some studies on model compounds have been carried out to try to shed light on this problem, but the implications have not been clear cut: for example, opposing isotope effects on the magnitude of the hydrophobic effect were obtained in

comparing different sets of model compounds (Ben-Naim, 1980). Despite these complications, it is clear that isotope effects can potentially provide important insights into the energetics of protein folding. Because these various hydrogen bonding interactions will also be changed to various degrees in intermediates and transition states along the folding pathway, it is to be expected that related isotope effects would be observed on their free energies too, leading to differences in the kinetics of folding and unfolding. This has been investigated in at least one case where, surprisingly, no significant kinetic isotope effect was apparent (Blond-Elguindi et al., 1988). However, no systematic investigation of the possibility appears to have been undertaken in other systems.

In this study, we have investigated kinetic isotope effects on the refolding of hen lysozyme. Refolding kinetics of this protein have been studied using a great variety of methods (Dobson et al., 1994). Recently, in particular, pulsed hydrogen exchange labeling experiments combined with stopped-flow spectroscopic measurements using various detection methods, including CD and a range of fluorescent probes, have provided a wealth of mechanistic information (Radford et al., 1992; Miranker et al., 1993; Itzhaki et al., 1994). Intrinsic tryptophan fluorescence is a particularly convenient probe and, under the conditions studied, this revealed at least three phases of fold-

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ing. Within the first few milliseconds, at a rate too fast to measure, there is significant intensity loss, suggestive of some type of side-chain interactions having developed. Other events occurring on this time scale are indicative of "molten globule" character, including substantial secondary structure and enhancement of the fluorescence of an extrinsic hydrophobic probe, 8-anilino-naphthalenesulphonate. Further developments occur on an intermediate timescale, so that by around 80–100 ms, under the conditions studied, both intrinsic fluorescence and far-UV ellipticity pass through minima. The first substantial protection from hydrogen exchange is also detected by pulse labeling during this period, developing with complex kinetics such that at least two different intermediates must be populated: one having persistent structure in both domains, whereas in the other only the α -domain is sufficiently stable to afford significant protection. The final, slowest phase involves stabilization of the β -domain in most molecules, along with rearrangement of residues into the specific configurations characteristic of the native, functional enzyme.

The role of interactions of the solvent in defining and stabilizing the folded states of proteins is clearly very important, but it is not straightforward to monitor changes in protein hydration or water–water hydrogen bonding directly in a refolding experiment. Consequently, most folding experiments focus essentially on the progressive formation of intraprotein interactions and the changing interactions with solvent generally must be deduced indirectly. In the work reported here, we have considered the possibility of using kinetic isotope effects to obtain more direct information about solvent interactions during folding.

Results

The kinetics of folding of lysozyme (20 °C, pH 5.3) as reflected in the intrinsic fluorescence of its tryptophan residues are shown in Figure 1. As has been described in detail previously, this complex profile is characterized by at least three kinetic phases (Itzhaki et al., 1994). In the dead time of the experiment, a substantial decrease in intensity occurs, so that, at the earliest measurable time, its magnitude is reduced by a factor of about 1.9 relative to that of the denatured protein in 6 M guanidine hydrochloride. This seems to be attributable in part to a direct solvation effect on the fluorophores, but, after accounting for this, there remains a substantial component of the amplitude of this change that must be attributed to a genuine structural transition (Itzhaki et al., 1994). The subsequent further fall in intensity, followed by a slow recovery to the limiting native state value, can be fitted well to the sum of two exponentials.

When the refolding buffer is made up in D₂O rather than H₂O (20 °C, $pD_{app} = 5.3$), the fluorescence changes occur qualitatively in the same three kinetic phases, but it is clear that there are significant differences in the actual rates (Fig. 1). Most noticeably, the final slow phase is retarded in the deuterated solvent. This result was very reproducible and, from a series of repeated experiments, a value of $k(H_2O)/k(D_2O)$ of 1.5 ± 0.15 was determined. In contrast, the faster resolved phase has a similar rate in both solvents under these conditions, although this result is rather less certain because this rate is fairly fast relative to the time resolution of the technique. The kinetics of both phases are sensitive to pH, so that one question is whether the differences observed in D₂O might be due simply to errors in pH measurement resulting from isotope effects at the glass elec-

Fig. 1. Time course of refolding of lysozyme in 0.54 M guanidinium chloride at 20 °C, pH 5.3, in 20 mM acetate buffer made up in H₂O (dashed line) and D₂O (full line). The reaction was monitored by intrinsic fluorescence excited at 280 nm and monitored as total intensity above 324 nm. Curves were each fitted to a sum of two exponentials, with time constants of 29 ms and 31 ms for the faster resolved phase in H₂O and D₂O, respectively. For the slower phases, the best-fitting time constants were 380 ms in H₂O and 580 ms in D₂O. Intensities are normalized relative to that of the native protein in H₂O ($I_{N(H)}$).

trode (Glasoe & Long, 1960). A full analysis of the pH dependence was conducted in both solvents (see below), and this showed that the isotope effects are substantially larger than could be accounted for reasonably by such uncertainties. It was concluded, therefore, that the differences in rate observed are bona fide kinetic isotope effects.

It is also apparent in Figure 1 that, throughout most of the time course, the fluorescence intensities are intrinsically smaller in D₂O, although the difference diminishes substantially as equilibrium is approached. This might, at least in part, reflect differences in the population of intermediates, but there can also be an isotope effect on the efficiency of quenching of tryptophan fluorescence by solvent (Itzhaki, 1993). In addition, the fluorescence intensities are sensitive to the ionization state of the protein and the difficulty of comparing pH values in the different solvents is therefore also likely to be a factor here. Although these intensity perturbations could, in principle, provide a probe of fluorophore accessibility through the reaction, it thus seems likely that there are too many other complicating factors to permit straightforward interpretation (Itzhaki, 1993). Another notable difference between the results obtained in the alternative buffers was that the traces obtained in D₂O were markedly more reproducible and free from mixing artefacts than those obtained in H₂O. A similar effect was noted in earlier studies of the refolding of the tryptophan synthase α -subunit and attributed to better matching of the density of D₂O to that of the denatured protein solution, which apparently aids facile mixing (Blond-Elguindi et al., 1988).

Backbone amide effects

One consequence of changing the solvent from H₂O to D₂O is that exchangeable hydrogens within the protein will be replaced

at varying rates by deuterium. Of particular significance are likely to be the backbone amide NHs that form the hydrogen bonds that define the secondary structure of the protein. Because these interactions do not persist in the denatured state, the folding process must involve breaking of a set of protein-solvent hydrogen bonds and their replacement by the intramolecular bonds in the folded structure. It might reasonably be expected that the kinetics of this effect would be sensitive to deuteration of these sites, reflecting the differing properties of hydrogen bonds formed by the heavier isotope.

In order to test this possibility, we have measured the rates of refolding in two samples where the solvent conditions were virtually identical and only the isotopic composition of the protein itself was changed. This was achieved by preparing two identical protein samples and denaturing them in 6 M guanidinium chloride solutions, one protiated and the other deuterated. These samples were then incubated for 30 min, which has been shown to be long enough to ensure complete unfolding and isotope exchange in the deuterated solution (Radford et al., 1992), before diluting with a 10-fold excess of protiated 6 M guanidinium chloride at pH 2.5. This resulted in denatured protein solutions differing only marginally in solvent composition, but in which amide exchange has a half life of the order of 15 min (Loftus et al., 1986), so that the isotopic labeling at these sites was very largely conserved during the 2 min or less taken to load the stopped-flow system and perform a refolding experiment. The dilution buffer for refolding was the same for both samples, and the difference in isotopic composition of the final mixtures was less than 1%. The design of these experiments also ensured that the solvent conditions immediately before initiation of refolding were almost the same in the two samples, thus eliminating any possible differences arising from isotope effects on the properties of the unfolded state.

It was found that the refolding kinetics of the two samples were, within the limits of experimental error, indistinguishable. Thus, it appears that the isotopic nature of the backbone amide hydrogens does not have a significant effect on the kinetics of the resolved phases of folding of this protein.

Viscosity effects

The absolute viscosity of 91% D₂O is around 1.2 times that of H₂O (Kestin et al., 1985). In order to test whether the isotope effects on refolding kinetics might be due to this viscosity difference, refolding was monitored in H₂O solutions with co-solvents added to increase the viscosity. Solutions of glycerol and sucrose in water were used at concentrations such that their viscosities were significantly greater than that of D₂O (Weast, 1988). In both 20% (w/v) glycerol (relative viscosity 1.54) and 10% (w/v) sucrose (relative viscosity 1.31), any perturbations to the kinetics were found to be too small to be accurately reproducible and were certainly much smaller, however, than the retardation of the slow phase observed in D₂O. It follows that the solvent viscosity difference can be ruled out as a major contributor to the kinetic isotope effect.

pH-dependence

Because of the problem of differential ionization effects in H₂O and D₂O, it seemed important to measure the isotope effect on refolding rates over a wide pH range in both solvents. The struc-

ture of native lysozyme is known to be virtually unchanged over the pH range used (Imoto et al., 1972), but there is a marked change in the variation of the intrinsic fluorescence during folding at different pH values (Fig. 2A). The substantial fast phase observed at pH 5.3 decreases in amplitude below pH 4 and ultimately vanishes. Below pH 2.5, only one phase is detectable, in which the fluorescence signal now decreases to its limiting value. The variation in amplitudes of the fast and slow phases with pH is summarized graphically in Figure 2B. The rates of both phases also decrease with decreasing pH over this range. The same qualitative behavior was observed in the H₂O and D₂O buffers, although the pH values where the changes in the curves occur do differ.

Fig. 2. A: Time course of refolding of lysozyme in 0.54 M GuHCl, H₂O, 20 °C, at the following final refolding pHs: □, pH 2.6; ○, pH 5.6; and ■, pH 6.9. B: pH dependences of the amplitudes of the resolved phases of intrinsic fluorescence intensity change in H₂O (○ and ●) and D₂O (△ and ▲) during the refolding of lysozyme in 0.54 M GuHCl, H₂O, 20 °C. In both cases, the filled symbols relate to the fast phase of intensity decrease and the open symbols to the subsequent, slower recovery. The amplitudes, in arbitrary units, reflect the total detected intensity change associated with each phase, calculated from a fit of two exponentials to the kinetic data where two phases are measurable, or a single exponential at low pH, where the fast phase vanishes.

The complexity of this pH dependence of the absolute rates clearly complicates interpretation of kinetic isotope effects. Double jump experiments have shown that the fast phase reflects the accumulation of an intermediate (S.T. Gladwin & P.A. Evans, unpubl. results) and therefore its disappearance at low pH could, in principle, indicate a significant difference in the folding pathway. For this reason, care must be used in comparing data obtained at widely differing pH values. For convenience, however, we show in Figure 3 the pH dependence of the rate of the slow refolding phase in H₂O and D₂O over the whole range. The curves are qualitatively similar, but there is a measurable difference in rate constants over most of this pH range and, remarkably, the isotope effect is actually reversed below pH 3, so that the rate is now faster in D₂O than in H₂O. It is clear that the curves are not just shifted relative to one another along the pH axis, as would be expected if the apparent isotope effect arose only as a result of the difficulties of measuring pD values accurately and accounting for pK_a differences of titratable groups in the alternative solvents. Thus, whereas these effects may play a significant role, there must be other components to the isotope effects observed.

Ionic strength dependence

The ionic strength dependence of the rates of the two resolved folding phases is shown in Figure 4. As we reported previously, the rate of the slower phase is significantly diminished in concentrated salt solutions, whereas that of the faster phase is much less affected (Itzhaki et al., 1994). The behavior of the slow phase is a kinetic salt effect, suggesting that the formation of specific favorable ionic interactions within the protein structure occurs in this final, slow phase of folding. Remarkably, in contrast to this marked variation of the actual rate of the slow phase, it is clear from Figure 4 that the magnitude of the isotope effect, $k(\text{H}_2\text{O})/k(\text{D}_2\text{O})$ is virtually independent of the

Fig. 3. pH dependence in H₂O (○ and ●) and D₂O (△ and ▲) of the rates of the slow phase of refolding of lysozyme in 0.54 M GuHCl, H₂O, 20 °C, observed through intrinsic fluorescence. Filled symbols indicate an increase in intensity, open symbols a decrease. Error bars reflect estimated uncertainties in individual rates, based on reproducibility of the values in repeated experiments; apparent error limits on individual least-squares fits were negligible in comparison.

Fig. 4. Ionic-strength dependence in H₂O (○ and ●) and D₂O (△ and ▲) of the rates of the changes in intrinsic fluorescence during the refolding of lysozyme at pH 5.3. In both cases, the filled symbols represent the rates of the fast intensity decrease and the open symbols the rates of the subsequent, slower intensity recovery. Error bars reflect estimated uncertainties in individual rates, based on reproducibility of the values in repeated experiments. Amplitudes of the fluorescence changes were found to be independent of the ionic strength, within the limits of experimental error, in these experiments. Ionic strengths indicated were calculated from the concentrations of all salts present under the final refolding conditions, including guanidine hydrochloride, acetate buffer, and variable amounts of NaCl.

ionic strength. In the case of the faster phase, the rates appear to diverge somewhat as the salt concentration is increased, though the isotope effect remains relatively small compared to that on the slow phase.

Discussion

When a globular protein folds in aqueous solution, the complexity of interactions involving the water and other hydrogen bonding groups in protein folding means that deuterium isotope effects cannot be interpreted easily. We have sought, in this paper, to assess the significance of various potential contributory effects and have been successful at least to the extent of eliminating some likely candidates.

One potential contributor to the isotope effect would be the making and breaking of hydrogen bonds involving backbone amide hydrogens as the protein folds. This was quite clearly ruled out, however, by the absence of any significant differences when the folding kinetics of samples differing in isotopic composition only at these sites were compared. This does not necessarily mean that such interactions are not being made and broken during the kinetic phases observed: it could simply be that any associated isotope effect is too small to measure. On the other hand, it is also possible that the isotope effect is absent because formation of intramolecular hydrogen bonds is already essentially complete within the dead-time of the experiment. Stopped-flow far-UV CD experiments indicate that a substantial amount of backbone folding does indeed occur within the dead time and deconvolution of a reconstructed spectrum of the protein at this stage has suggested that this might be close to a native complement of secondary structure (Chaffotte et al.,

1992). Pulsed hydrogen exchange labeling studies did not pick up this very rapidly formed structure, but these experiments were designed to detect only very stable elements of structure and, because this stability may depend on longer-range interactions developed later in the pathway, this result does not conflict with the notion of relatively labile native-like secondary structure being formed at a very early stage (Radford et al., 1992). Recent studies using a variant of the exchange labeling experiment that focuses on the structure present during the dead time have indeed now detected exchange protection in the α -helical segments of the sequence but not, intriguingly, in the β -sheet region (S.T. Gladwin & P.A. Evans, in prep.). The interpretation of the lack of an isotope effect attributable to reorganization of amide hydrogen bonding thus remains open to question.

The lack of comparable effects when sucrose or glycerol cosolvents were added to the refolding medium suggests that the viscosity difference between D₂O and H₂O cannot account for the observed isotope effect either. Such an effect might have been anticipated if the rate of either resolved phase were limited by a "diffusion-collision" process of structural development (Karplus & Weaver, 1976). However, fluorescence quenching studies have shown that, within the first 100 ms, the fluorescing tryptophans become significantly shielded from quencher, suggesting that the structure is already highly condensed by this stage (Itzhaki et al., 1994). It would be surprising if the rate of the subsequent slow phase were limited by diffusional processes and the lack of viscosity dependence on this phase, at least, is therefore not unexpected.

The pH dependence of the isotope effect could be thoroughly investigated only for the slow phase because the fast phase actually disappears under acidic conditions. A number of potential pH-dependent contributions to the overall isotope effect can be identified, including effects due to differences in pK_a values of titratable groups in the two solvents. The situation is a little more straightforward in pH ranges where the folding of lysozyme is essentially independent of ionization equilibria: this is the case at very low pH values (below about 1.5), where the titratable groups of lysozyme are fully protonated in both folded and unfolded states. Above about pH 5, all acidic side chains are ionized in both states, and only His-15 titrates in the region between pH 5 and neutral (Donovan et al., 1960; Sophianopoulos & Weiss, 1964). As might therefore be expected, the rates measured show only limited pH dependence in these regions, but there is a substantial kinetic isotope effect in both cases, showing clearly that the effect is not just a consequence of errors due to the use of uncorrected readings from a glass electrode. Furthermore, the opposite sign of the isotope effect in these two pH ranges shows that there must be more than one contributor to the overall effect, at least one of which depends on the ionization of titratable groups.

In the intermediate pH range, there is the added complexity that several groups will be protonated in the unfolded but not the folded state, so that apparent kinetic isotope effects could result from differences in these ionization equilibria. To the extent that changes in the rate constants can be correlated with titration of such groups, the pK_a values actually appear quite similar (Fig. 3), but the true pD value in D₂O is probably around 0.4 units higher than it appears because we have not corrected for isotope effects on the glass electrode; this would mean that pK_a values in the protein are significantly higher in D₂O, as expected (Wehry & Rogers, 1966). In any case, it is clear that

the isotope effect in this region is likely to be uninterpretable because there are too many possible contributions.

It seems plausible that the change in environment of some carboxylate groups on forming the transition state in the slow phase of folding, presumably involving their partial desolvation, may carry an additional free energy penalty in D₂O because of an isotope effect on the ion solvation interactions. There are no wholly buried ionic groups in the native lysozyme structure, but several of them do form specific interactions at the protein surface (Blake et al., 1967), and formation of these would undoubtedly entail stripping off some of the water of hydration. At pH values above 4, this isotope effect might be large enough that, overall, the folding itself would be retarded in D₂O, as observed. At the low pH extreme, this effect would disappear because of protonation of acidic groups, so that a different contribution to the overall isotope effect could become predominant, thus accounting for the observed reversal in the direction of the effect.

The question would then remain of the origin of this second contribution to the isotope effect. Although carboxylate groups become uncharged at low pH, there remain a large number of cationic groups, and at least some of these must become partially desolvated during folding, which might likewise be expected to be associated with an attendant isotope effect. Because the molecular details of the hydration of cationic and anionic groups clearly differ, we cannot exclude the possibility that an isotope effect of opposite sign to that associated with anionic groups results from changes in the solvation of positively charged groups on folding of the protein. Another factor that is likely to be associated with an isotope effect is the hydrophobic effect, as discussed below. A further possible complication that needs to be borne in mind is that the pathway itself may be altered as a result of the titration of acidic groups. If the extent of hydrophobic burial in the transition state, for example, were greatly altered as a result, there might be an indirect pH dependence of the contribution to the isotope effect even from the hydrophobic effect. The loss of the fast phase in the fluorescence-detected kinetics indeed suggests some alteration in the distribution of intermediates populated over this pH range, so this possibility cannot be excluded readily.

The formation of ionic interactions in the transition state associated with the slow phase is clearly manifest in the ionic-strength dependence of the rate, as discussed above. It is remarkable, however, that, although this effect is very pronounced at pH 5.3, the magnitude of the attendant isotope effect is virtually insensitive to ionic strength (fig. 4). Given the evidence from the pH dependence that interactions of ionic groups make a significant contribution to the isotope effect, this might seem rather surprising. However, the salt and isotope effects can be seen to be linked principally with quite distinct components of the free energy of activation associated with changing the environment of ionic groups in folding: a positive term due to weakening of ion-solvent interactions, which will be subject to an isotope effect but not strongly dependent on ionic strength, and a negative term due to improvement of intraprotein charge-charge interactions which, conversely, will be ionic-strength dependent, but largely independent of the isotopic composition of the solvent, because this has little effect on the dielectric constant.

In the case of the fast phase, there is much less sensitivity to ionic strength, but, interestingly, such effect as there is evidently is different in H₂O and D₂O, so that the magnitude of the isotope effect on this phase is enhanced at high salt. This suggests

that the lack of a significant isotope effect under the conditions of Figure 1 may actually reflect the cancellation of opposing contributions, the balance between which is slightly altered by an increase in ionic strength.

The other major component of the energetics of protein folding that could be linked to a kinetic isotope effect is the hydrophobic interaction. Because this is a consequence of the disruption and reorganization of water-water hydrogen bonds when nonpolar groups are exposed, it might clearly be expected to be liable to a solvent-isotope effect. This is borne out by comparative measurements of the solubility of nonpolar groups in the two solvents, although the size and even the sign of the effect seem to depend on the nature of the solutes involved, so that it is hard to predict the expected effect in protein folding (Ben Naim, 1980). Because the resolved phases of folding apparently involve changes in the environments of relatively hydrophobic side chains, as exemplified by the tryptophan fluorophores, it seems likely that they both involve desolvation of nonpolar surfaces and there is also evidence from estimates of the heat capacity change in forming the transition state, albeit measured under very different conditions, to suggest that burial of hydrophobic side chains is an important aspect of the rate-limiting step in refolding (Segawa & Sugihara, 1984). Thus, a kinetic isotope effect associated with the hydrophobic effect might well be expected in refolding. Our simple interpretation of the pH dependence would suggest that this might make a roughly constant contribution to the overall isotope effect, favoring refolding in D_2O , over the whole pH range. There is, however, no direct evidence to confirm this.

It is clear from these considerations that understanding isotope effects on protein-folding kinetics is far from straightforward. We have shown that changes in the environment of anionic groups on folding are probably linked to a major contribution to the isotope effect, but that there must also be other factors with a different pH dependence. We have also managed to show that some potential contributions are actually negligible in this system. Nonetheless, at present there is too much ambiguity to draw many firm conclusions, and we cannot with any certainty add a great deal to our picture of the folding pathway of lysozyme on the basis of the data presented here. On the other hand, there is clearly a lot of information here that may be of value in assessing other experimental data that are likely to be forthcoming in the future. It will also be interesting in future studies to see to what degree isotope effects similar to those described here for hen lysozyme are a general feature of protein folding. In contrast to lysozyme, the refolding kinetics of the tryptophan synthase α -subunit (Blond-Elguindi et al., 1988) and of barnase (A.R. Fersht, pers. comm.) are relatively insensitive to changing the solvent from H_2O to D_2O . This could be due to cancellation of opposing contributions, as evidently occurs partially for lysozyme, at least at some pH values, or it could be that the transition states associated with the resolved phases of lysozyme refolding have features that are absent from those of the other proteins, thus conferring on lysozyme an unusual sensitivity to isotopic contribution.

Materials and methods

Hen egg-white lysozyme (grade I) was purchased from Sigma and used without further purification. Deuterium oxide (99.9% D atom) was purchased from Goss Scientific and all other reagents

were of analytical grade. Guanidium chloride was perdeuterated by dissolution in D_2O and freeze drying after a brief incubation at room temperature. The process was repeated three times to ensure a high level of deuteration.

Fluorescence detection of the folding kinetics of hen lysozyme was achieved using a Biologic SFM3 stopped-flow module, coupled via a fiber-optic link to an external fluorimeter (Jasco FP777), which served as a light source. An excitation wavelength of 280 nm and band width of 10 nm was employed, and emission was measured above 324 nm by inserting a colored glass long-band filter between the stopped-flow cell and the photomultiplier tube. A Biologic PMS200 detection system was used. The temperature in the stopped-flow unit was maintained at 20 °C using water circulating from a thermostated bath. Under the experimental conditions employed, the dead time of the equipment was 3 ms.

In most experiments, unfolded lysozyme in 6 M guanidine hydrochloride was diluted 11-fold with 20 mM sodium acetate buffer, pH 5.3, to a final protein concentration of 125 μ M. For refolding in D_2O , 20 mM sodium acetate/ d_3 -acetic acid was used as the refolding buffer. The isotope effects quoted (in all experiments except for those that measure the effect of amide exchange, see below) are therefore for H_2O compared with 90.9% D_2O . Between 15 and 20 traces were averaged for each experiment.

For studies of the effect of isotopic substitution of amide hydrogens, deuteration of all exchangeable sites was achieved by dissolving the protein at 50 mg/mL in 6 M deuterated guanidinium chloride in D_2O , followed by an incubation of at least 30 min at room temperature. This solution, with the protein already unfolded, was then diluted 10-fold into 6 M guanidinium chloride (protiated) in H_2O at pH 2.5, under which conditions amide exchange is very slow, and rapidly loaded into the stopped-flow apparatus. The refolding experiment was performed (11-fold dilution with 20 mM acetate, H_2O , pH 5.3, as before) within 2 min of the dilution from deuterated into protonated guanidine hydrochloride, so that exchange back to NH before refolding was minimal. The kinetics were then compared with those for refolding of the usual protiated protein (unfolded at 5 mg/mL in 6 M guanidine hydrochloride, refolding by 11-fold dilution with 20 mM acetate, H_2O , pH 5.3). Thus, the solvent conditions (99% and 100% H_2O , respectively) are very nearly identical for the two experiments, so that any effect on the refolding rate may be assigned exclusively to the effect of deuteration of amide sites.

For studies of viscosity effects, solutions of glycerol and of sucrose were prepared in the refolding buffer (20 mM acetate, pH 5.3), and the pH checked and readjusted to pH 5.3 where necessary, no correction to pH being made for any solvent effects. For the ionic-strength dependence experiments, sodium chloride solutions spanning a range of concentrations from 0 to 2.5 M were made up in 40 mM sodium acetate, pH 5.3, and the pH readjusted to pH 5.3 where necessary, using dilute acetic acid or d_3 -acetic acid.

For the pH-dependence studies, the following refolding buffers were made up in H_2O and D_2O : 20 mM KCl/HCl at pH values up to 2; 20 mM sodium citrate/citric acid at pH 3.0–3.5; 20 mM sodium acetate/acetic acid at pH 4–6; 20 mM Na_2HPO_4/NaH_2PO_4 at pH 6–7. The final refolding pH was measured for each by performing a manual 1:10 mix of 20 mg/mL lysozyme in 6 M guanidine hydrochloride with the buffer. The pH or pD was determined using a glass electrode

at 20 °C. pD values quoted are not adjusted for any isotope effect at the electrode. It has been estimated that the true pD in a D₂O solution is around 0.4 units higher than that measured (Glasoe & Long, 1960), but there are also effects on the pK_a values of groups in a protein that tend to offset this to some extent (Bundi & Wüthrich, 1979), so that it is actually very difficult to obtain with any certainty conditions where the state of ionization of the protein is the same in the two solvents. For this reason, we concluded that the only safe course was to monitor refolding over a range of pH values in each case and compare the overall dependencies.

Acknowledgments

We are grateful to Dek Woolfson and Sharon Gladwin for valuable discussions. This work has been supported by the Biotechnology and Biological Sciences Research Council of the UK. P.A.E. is a Lister Institute Research Fellow.

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