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Review

Chimeras of human lysozyme and α-lactalbumin: an interesting tool for studying partially folded states during protein folding

H. Van Dael

Interdisciplinary Research Centre, K. U. Leuven Campus Kortrijk, B-8500 Kortrijk (Belgium), Fax +32 56 246997, e-mail: Herman.Vandael@kulak.ac.be

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Abstract. Protein folding is an extremely active field of research where biology, chemistry, computer science and physics meet. Although the study of protein-folding intermediates in general and equilibrium intermediates in particular has grown considerably in recent years, many questions regarding the conformational state and the structural features of the various partially folded intermediate states remain unanswered. Performing kinetic measurements on proteins that have had their structures modified by site-directed mutagenesis, the so-called protein-engineering method, is an obvious way to gain fine structural information. In the present review, this method has been applied to a variety of proteins belonging to the lysozyme/α-lactalbumin family. Besides recombinants obtained by point mutations of individual critical residues, chimeric proteins in which whole structural elements (10–25 residues) from α-lactalbumin were inserted into a human lysozyme matrix are examined. The conformational properties of the equilibrium intermediate states are discussed together with the structural characterization of the partially unfolded states encountered in the kinetic folding pathway.

Key words. Lysozyme; molten globule; protein folding; α-lactalbumin; folding intermediates; protein engineering; chimera; stability.

Introduction

Since its discovery in 1921 by Sir Alexander Fleming, lysozyme has become one of the most studied and best characterized of all proteins. The lysozyme variant isolated from hen egg-white (HEWL) was the first enzyme which was submitted to complete X-ray crystallographic analysis and for which a detailed mechanism of action was proposed [1, 2]. It is extensively used as a model in protein chemistry, enzymology, protein crystallography, molecular biology and genetics, immunology and evolution. The progress made by the study of all these different aspects has been reviewed recently [3]. We shall therefore confine this contribution to the folding of different variants of lysozyme and of the related and structurally homologous protein, α-lactalbumin. Especially, we will concentrate on the specific effects induced by inserting isolated residues or whole structural elements from α-lactalbumin in a lysozyme matrix.

Lysozyme and α-lactalbumin are small monomeric proteins of, depending on the species, 123 to 130 residues, and they show the same three-dimensional (3D) structure and fold (fig. 1). They both contain two structural domains stabilized by four intramolecular

disulphide bonds [1, 4–6]. The first domain, the α domain, consists of four α helices along with a 3_{10} helix, encompassing the N- and C-terminal segments of the protein. The other domain, the β domain, contains a large triple-stranded antiparallel β sheet, a small double-stranded antiparallel β sheet, a 3_{10} helix and an irregular loop.

The folding of lysozyme

Ever since the elucidation of the complete tertiary structure, the problem of how a protein folds and acquires its functional properties has been debated. As a result of the pioneering experiments of Anfinsen [7] it is now generally accepted that the information required for an unfolded protein spontaneously to reach its unique 3D structure is all contained in its amino acid sequence. The observed rate of folding and the yield of the correctly folded conformation, however, rule out the possibility that folding is a simple stochastic process [8]. This raises the idea that the folding process proceeds through transient intermediate states of generally increasing order. Initially, these partially folded states could not be isolated for characterization due to their short lifetime.

The unfolding of lysozyme from the native to the denatured state conforms to a reversible two-state transition mechanism [9, 10]. This one-step process has been observed under a wide variety of conditions and shows a high degree of cooperativity. Although extremes of pH, temperature or the presence of various denaturants can generate different types of unfolded states, the thermodynamics and the folding behaviour of lysozymes under equilibrium conditions have always been found to accord well with a two-state model [11–13]. In recent years, many of the biophysical techniques developed to characterize native proteins at equilibrium have been adapted to the structural and thermodynamic identification of these transient intermediate populations during protein folding [14]. By combining different complementary techniques, it has been possible to piece together a detailed tentative model for the folding pathway of HEWL [15]. Recent progress made by following protein folding in real time using nuclear magnetic resonance (NMR) spectroscopy [16], and by monitoring formation of structure during protein folding directly at the level of individual residues [17], provides us with hope that in the future more complete structural information about transient partially folded intermediate states will become available. Both these techniques, which at present can be applied only for slowly folding proteins, need to be refined in order also to cover proteins which fold in a faster way.

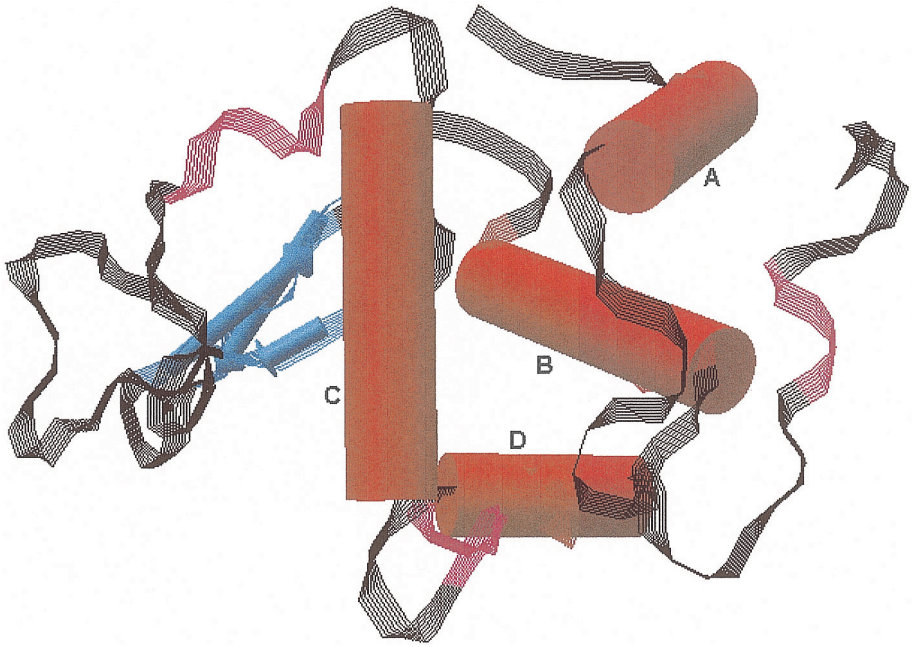

Figure 1. Three-dimensional structure of human lysozyme. The α domain with the four helices (A, B, C, D) and the N and C terminus is on the right side; the β domain with the β sheet and the long loop is on the left side. The red-coloured parts in the ribbon diagram represent the 3_{10} helices.

Table 1. Amino acid sequence of the Ca^{2+}-binding region in some α-lactalbumins and in natural and recombinant Ca^{2+}-binding lysozymes, compared with the sequence of non-Ca^{2+}-binding human lysozyme.

	Residue number α-lactalbumin													
Species	77	78	79*	80	81	82*	83	84*	85	86	87*	88*	89	90
Human	C	D	K	F	L	D	D	D	I	T	D	D	I	M
Bovine	C	D	K	F	L	D	D	D	L	T	D	D	I	M
Goat	C	D	K	F	L	D	D	D	L	T	D	D	I	V
	Residue number lysozyme†													
	81	82	83	84	85	86	87	88	89	90	91	92	93	94
Equine	C	S	K	L	L	D	E	N	I	D	D	D	I	S
Pigeon	C	S	K	L	R	D	D	N	I	A	D	D	I	Q
M4	C	S	K	L	L	D	D	D	I	A	D	D	V	A
LYLA1	C	D	K	F	L	D	D	D	L	T	D	D	I	M
Human	C	S	A	L	L	Q	D	N	I	A	D	A	V	A

*Residues involved in Ca^{2+}-binding. †Residue numbers are those of human lysozyme and take into account a deletion at position 48 for pigeon lysozyme and at position 70 for equine lysozyme.

The molten globule state of α-lactalbumin

The molten globule state is a well-defined equilibrium intermediate state between the completely folded native and the completely unfolded or fully denatured state. It is characterized by a substantial content of secondary structure which is presumed to be largely nativelike, by a compactness with a radius that is only 10–20% larger than that of the native molecule and by substantially disordered tertiary interactions [18–22]. Additionally, the molten globule state shows a significant binding capacity for hydrophobic probes like 1-anilino-naphthalene-8-sulfonate (ANS) or bis-ANS, as observed by the strongly enhanced fluorescence intensity of the probe upon binding to the partially unfolded protein [18, 23, 24]. Undoubtedly, the most extensively characterized molten globule is that of α-lactalbumin, which is obtained by acid-induced unfolding at low pH, by unfolding in moderate concentrations of a strong denaturant, by reduction of disulphide bonds or by removing a bound Ca^{2+} at neutral pH and low salt concentration [18, 22–28].

One of the best-known functions of α-lactalbumins is their ability to bind Ca^{2+} strongly. This property resides in a typical binding loop extending from residue 72 to residue 88 and thus located just before helix C. In this binding loop, two peptide carbonyls and three carboxylate groups (Asp82, Asp87 and Asp88) act as ligands (table 1). Binding of Ca^{2+} or other metal ions to α-lactalbumin results in the formation of a very stable metalloprotein. The structural and functional properties of α-lactalbumin and its interrelationships with lysozyme are described in excellent reviews [28–32].

The molten globule state in α-lactalbumin is identified as a bipartite structure with a disordered β sheet domain and with an α domain containing substantial secondary structure and a native tertiary fold even though it lacks extensive fixed tertiary interactions [25–27, 33–35]. In the molten globule state most of the amide hydrogens of helix B are protected from exchange, and helix A also shows a high degree of protection. Strikingly, however, helix C, the most highly protected in the native state, is only marginally protected in the molten globule state [34]. Complete folding to the native state is not accomplished until the specificity of side-chain packing has developed. This final folding stage requires additional interactions to lock in the unique tertiary contacts of the native structure. The binding of Ca^{2+} at the domain interface in α-lactalbumin can promote the formation of these contacts and stabilize them [33, 34]. Recent NMR experiments, which probe unfolding at the level of individual residues, demonstrate that human α-lactalbumin molten globule unfolds highly noncooperatively and that simple two-state thermodynamic equations are not sufficient to describe its unfolding transition [36]. It is possible to speculate as to the significance of these results for the assembly of the native protein fold. Some regions of the polypeptide chain are conformationally restricted even at extreme concentrations of denaturants, suggesting that these regions form a particularly stable structural core, whereas the rest of the polypeptide chain is highly denatured. These findings support the idea that the generation of the 3D fold of a protein could occur by the progressive addition of elements of structure to an initially formed core which acts as a template for the folding process [36].

Equilibrium intermediate states in lysozyme

The first equilibrium intermediate state to be found in lysozymes was that which occurs in the apo form of equine lysozyme [37, 38]. Equine lysozyme undergoes a two-step unfolding transition upon heating and in the presence of guanidine hydrochloride (GdnHCl) that is highly dependent on the state of Ca^{2+} binding. Fluorescence emission and ultraviolet (UV) difference absorption spectroscopy suggest that the first transition generates an intermediate state in which sequestration of aromatic side chains from solvent has occurred, whereas the second represents denaturation to a highly unfolded state. Thermodynamic analysis of the effects of excess heat observed upon heating equine lysozyme shows that both these stages represent highly cooperative two-state transitions [39]. The heat capacity increases at both stages, but mainly at the first one. Since the heat capacity increment indicates the exposure of nonpolar groups to water, we can readily assume that both these stages are indeed connected with unfolding of some compact structures in equine lysozyme. Moreover, at the end of the second stage the heat capacity reaches the value specific for the completely unfolded protein in 6 M GdnHCl at that temperature. Circular dichroism (CD) and NMR results indicate that the intermediate state possesses extensive secondary and tertiary structure, although the latter is substantially disordered [38]. The magnitude of the near-UV CD signal and the large enthalpy change associated with the second transition prove that the equilibrium partially folded structure in equine lysozyme is significantly more organized than the classical molten globule identified for α-lactalbumins. Hydrogen-exchange experiments on equine lysozyme show that amides in three of the four major helices of the native protein are significantly protected in the ordered molten globule state at pH 2 [40, 41]. The pattern of protection within the different helices, however, varies significantly. Examination of the pattern in the light of the native structure indicates that the side chains of the protected residues form a compact cluster within the core of the protein. Such an ordered core is already present in the partially unfolded state of equine lysozyme, indicating the existence of substantial nativelike interactions between hydrophobic residues in the intermediate state [41].

Electrostatic interactions have long been known to influence the stability of a protein. Therefore, at extreme pH many proteins denature because of the presence of destabilizing repulsive interactions between like charges in the native protein [42, 43]. In some cases, however, extremes of pH do not completely denature the proteins but result in denatured states that are only partially unfolded [44, 45]. This is due to the fact that the repulsive forces between ionized residues in the protein may fail to overcome interactions such as hydrophobic forces, salt bridges or other factors that favour folding. This complex interplay between stabilizing and destabilizing forces is a possible explanation for the equilibrium partially folded state found in human lysozyme at low pH [46]. As stated in a previous paragraph, generally the thermal denaturation of c-type lysozymes proceeds without any thermodynamically stable intermediate form [47, 48]. In accordance with this general statement, the unfolding of human lysozyme appears to conform with a two-state unfolding process at least at pH values above 3. This is, however, no longer the case at lower pH values (fig. 2). At pH 1.2, for example, unfolding of the tertiary structure occurs at a temperature approximately 10 °C lower than that of the secondary structure. At this pH and at 60 °C, there is no detectable native tertiary structure remaining, although far-UV CD results show preservation of some 40% of the signal attributable to α-helical elements in the protein. Both these observations point to the existence of a partially folded state of human lysozyme that has at least some characteristic properties of the molten globule state [46]. A similar partially folded state, characterized by a sub-

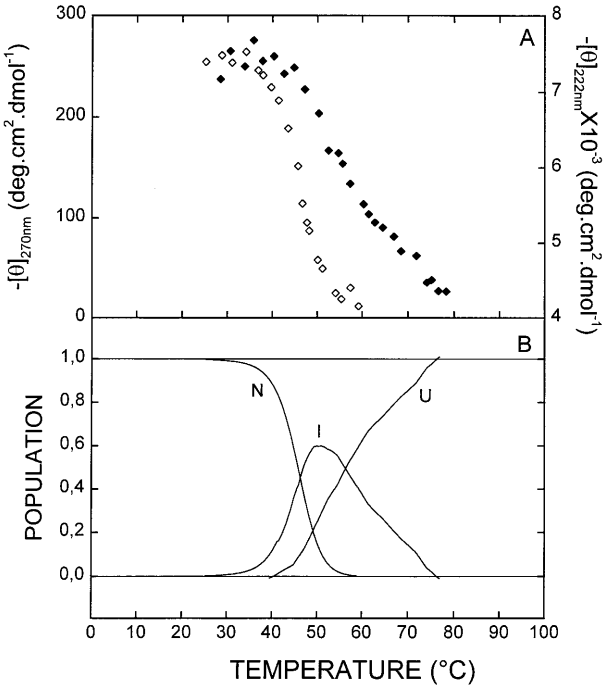

Figure 2. Molten globule behaviour of human lysozyme at pH 1.2 (*A*) Temperature dependence of the ellipticity of HLY in the near--UV (◇, left axis) and the far-UV region (◆, right axis). (*B*) Population of the native (N), intermediate (I) and unfolded (U) states of HLY at pH 1.2 [46].

stantial secondary structure, a large solvent exposure of nonpolar clusters and significantly disrupted tertiary structure, is also found in HEWL at low pH [49]. This acid-unfolded state is obviously only partially unfolded, as substantial enthalpy is associated with further thermal unfolding at this low pH [49].

Conformational studies of peptides in a solution with short-chain alcohols, in particular 2,2,2-trifluoroethanol (TFE) have shown that these agents stabilize regions of the peptide that are helical in the native state [50–52]. Other structural elements, on the contrary, are transformed to nonnative structures. The combination of these effects induced by TFE was observed in HEWL by an ellipticity in the far-UV CD greater than that of the native state and by a near-UV CD intensity that is dramatically reduced compared with that of the native state [53]. This TFE partially unfolded state contains helical structures of great flexibility both as extensions to the nativelike helices and as a nonnative structure in the region of the molecule which forms the C-terminal part of the β sheet in the native state [54]. In contrast, no specific structural preferences are detected in regions corresponding to the long loop and the N-terminal part of the β sheet of native lysozyme. Despite the absence of extensive tertiary interactions, preferential stabilization of nativelike secondary structure by TFE results in a pattern of main-chain dynamics which is similar to that of the native state [55].

A partially folded state of HEWL has also been characterized in dimethylsulphoxide (DMSO). The denaturant property of DMSO is attributed to the strong H-bond-accepting property of the sulphoxide, whereas the two methyl groups presumably interact with the hydrophobic residues of the protein [56, 57]. In 100% DMSO a totally unfolded state of HEWL has been reported [58], but the presence of 10 to 50% DMSO results in a highly ordered partially unfolded state as shown by CD, fluorescence, NMR and hydrogen/deuterium (H/D) exchange measurements [59]. This intermediate resembles the early kinetic intermediate observed during the refolding of HEWL as well as the molten globule state of equine lysozyme at low pH. Like the latter, the intermediate state in HEWL contains an ordered hydrophobic core that comprises helix A, helix B and helix D. Aromatic residues show a tertiary organization as demonstrated by enhanced near-UV CD and by limited exposure of tryptophans to solvent as monitored by iodide quenching of fluorescence [59]. Furthermore, the structural transition from this state to a largely unfolded state is cooperative.

Finally a lysozyme-folding intermediate was revealed by small-angle X-ray scattering experiments [60]. Upon unfolding as a function of urea concentration at pH 2.9, differences in the unfolding transition were observed as monitored by the radius of gyration and by far- and near-UV CD at 222 nm and 298 nm, respectively. These observations of an equilibrium intermediate state in HEWL at pH 2.9, however, have recently been questioned [61]. The latter authors analysed the urea-induced unfolding at the same pH on the basis of steady-state fluorescence measurements, characterization of the folding-unfolding kinetics, double-jump unfolding assays for the amount of native protein and double-jump refolding assays for the amount of unfolded protein. From their analysis they concluded that their results do not provide support for the presence of an equilibrium intermediate state under these conditions, and they warn of possible pitfalls in the experimental detection of equilibrium folding intermediates.

Mutants of human lysozyme

Protein-engineering experiments mostly involve the introduction of point mutations at specific sites into well-characterized enzymes in order to investigate the role of the corresponding amino acid residues either in binding and catalysis or in molecular structure and stability. Also, in human lysozyme genetically engineered mutants have significantly contributed to a better understanding of the binding of various substrates and to the catalytic action of the protein. These results were recently reviewed by Imoto [62]. In the present contribution we will therefore confine our discussion to specific mutations, whereby one or more amino acid residues from the homologous α-lactalbumin are substituted for their equivalent in human lysozyme. Since the stability of a protein can be promoted considerably by the introduction of metal ion-binding sites, several attempts have been made to introduce the Ca^{2+}-binding function of α-lactalbumin in lysozyme by site-directed mutagenesis. Kuroki et al. have shown that introducing two of the necessary Asp residues at the corresponding sites in the human lysozyme molecule (Asp86 and Asp92) results in a functional Ca^{2+}-binding site [63]. This mutant is more stable at 85 °C than the wild-type by 1.9 kcal/mol, and it was found that the enthalpic contribution to the Ca^{2+}-binding reaction was small, whereas entropy release upon binding of Ca^{2+} was essential [64, 65]. Addressing the question whether the simultaneous replacement of both Gln86 and Ala92 by Asp is required for Ca^{2+} binding, we were able to show that a single Asp at position 92 suffices for Ca^{2+} binding [66]. The thermostability of this mutant, A92D, in the apo as well as in the Ca^{2+} form was decreased compared with that of wild-type human lysozyme. Another mutant, M4, in which Ala83,Gln86, Asn88 and Ala92 were replaced by Lys, Asp, Asp and Asp, respectivily (table 1), contains all the residues that are involved in Ca^{2+} binding in α-lactalbumin and shows a marked increase of thermostability [66]. The calcium-binding constants, mea-

sured at pH 7.5 and 25 °C, are 8×10^3 M^{-1} for A92D, 1×10^7 M^{-1} for M4 and only 2×10^2 M^{-1} for lysozyme from human milk. All these results clearly show that it is perfectly feasible to transfer the Ca^{2+}-binding capacity of α-lactalbumin to the corresponding site in human lysozyme, either in a partial or in a more complete way, depending on the specific place and on the importance of the mutation. Stability of these mutants increases accordingly to their Ca^{2+}-binding capacity. The folding behaviour of these mutants, however, has not changed to the same extent. Only the apo form of mutant M4 shows a slightly pronounced tendency to adopt a molten globule conformation at normal pH. This behaviour is more explicit at pH 1.2, where upon a temperature-induced denaturation the population of the intermediate state amounts to 60% compared with 25% for natural human lysozyme [46]. With this perspective we turned to the construction of chimeras of human lysozyme and bovine α-lactalbumin, in which more substantial parts of the latter protein are inserted into the former. The study of the thermodynamics and folding kinetics of these constructs will probably shed more light on the determinants of molten globule behaviour in the lysozyme/α-lactalbumin family in particular and in globular proteins in general.

Construction of LYLA1

LYLA1 (fig. 3, table 1) was created by exchanging the amino acid sequence 76–102 of HLY for the corresponding region of BLA (72–97). This implanted part is composed of a small two-turn 3_{10} helix, the complete Ca^{2+}-binding loop and the entire central helix C. Compared with the homologous HLY sequence, the transplanted BLA segment shows a deletion at the penultimate position. Consequently the adjacent Pro103 is moved one step in the direction of helix C, and this could cause the last turn of the transplanted helix to unwind. Modelling of this chimera, however, predicted a good fit in the contact zone, resulting in reasonable stability of the engineered protein. The hybrid gene was transferred to the shuttle vector pAB24, resulting in the pABAGLYLA1 expression plasmid [67]. Then the *Sac*-

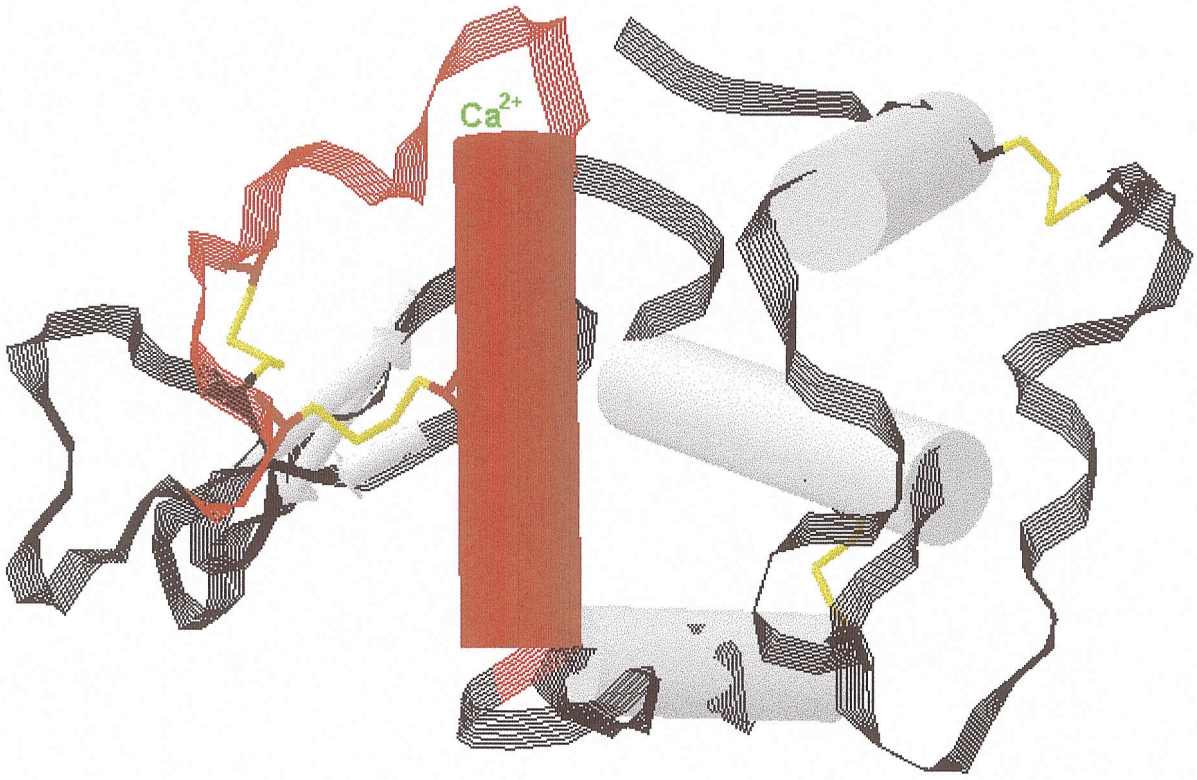

Figure 3. Three-dimensional structure of LYLA1 from the same viewpoint as in figure 1. The fragment inserted from α-lactalbumin is coloured in red, the four S–S bridges in yellow. The location of the bound Ca^{2+} ion (green) is also indicated.

charomyces cerevisiae strain GRF 182 was tranformed with this plasmid. After selection in uracil- and leucine-selective medium, yeast cells were grown for 5 to 7 days in a 10 litre fermentor. The chimeric protein was purified from the culture medium by cycles of ion exchange and gel filtration. The average yield was about 8 mg/l of culture, and the final product was homogeneous on SDS-polyacrylamide gel electrophoresis (PAGE). N-terminal sequence analysis indicated that the secreted protein had been correctly processed by the heterologous host cells. Further details of this expression procedure together with immunochemical analysis and proof of the enzymatic activity of the chimera were described by Pardon et al. [67]. Ca^{2+}-binding experiments showed a single strong binding site with $K = 2.5 \times 10^8\ M^{-1}$. One-dimensional NMR spectra of the chimera, performed in collaboration with C. Dobson and co-workers, were well dispersed with many resolved resonances in the methyl and aromatic regions, indicating a completely folded protein with ordered tertiary structure. In order to analyse the secondary structure of LYLA1 in more detail, two-dimensional NMR methods were employed (fig. 4). Clear evidence for four α helices was found, corresponding closely to helices A–D in the structure of the parent human lysozyme. Importantly, the existence of helix C transplanted from α-lactalbumin is clearly evident. These data showed overall marked similarity between the secondary structure of LYLA1 and human lysozyme, even in the inserted region [67]. For the helix C region specifically, a comparison was made between the differences in residual chemical shifts between LYLA1 and HLY on the one hand and between LYLA1 and BLA on the other. This analysis shows that in the second half of the helix, there is a much better correlation with the chemical shifts of BLA than with those of HLY, indicating that in this region the structure of LYLA1 is more likely to be akin to that of BLA.

LYLA1A is another chimera produced by an analogous procedure, but in this case the amino acid sequence 76–92 of HLY was exchanged for the corresponding one of BLA (72–88). This means that the entire Ca^{2+}-binding loop of BLA was inserted into HLY together with the first turn of the C helix. In this construct, however, the remaining part of the C helix remains lysozyme-like.

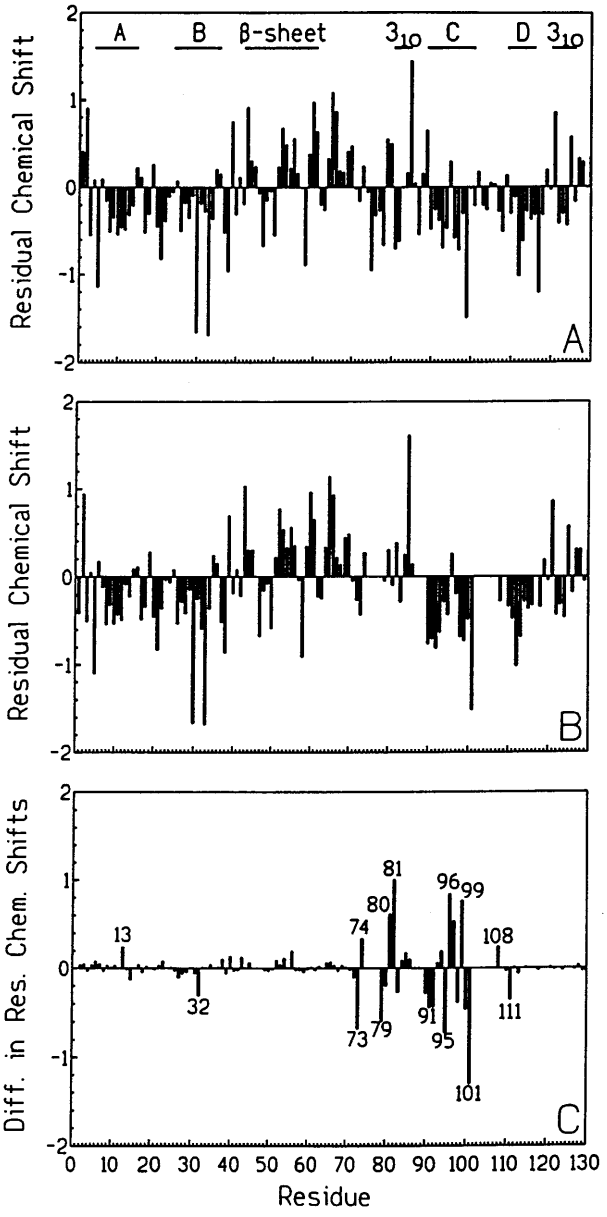

Figure 4. Residual Cα-H shift comparison. The residual chemical shift is calculated by subtracting the random coil shift from the measured shift for human lysozyme (*A*) and for LYLA1 (*B*). The elements of secondary structure are indicated. Residues 75–78 and 87–89 have not been assigned for LYLA1. In panel *C*, the difference in residual chemical shift between LYLA1 and HLY is indicated. The 15 most perturbed resonances are labelled [67].

Conformational stability of LYLA1

Thermal unfolding curves derived from tryptophan fluorescence and CD measurements at 222 nm and 270 nm show that apo-LYLA1 does not unfold in a simple two-state process. The midpoint temperature T_m for the unfolding of the tertiary structure of this chimera is 72.6 °C, whereas the secondary structure unfolding has a considerably higher T_m (80.9 °C). In the presence of 10 mM Ca^{2+}, however, the unfolding curves obtained in the far- and near-UV region coincide completely, indicating that only the native and the totally unfolded state are involved in this process without interference of

a partially unfolded intermediate state. Furthermore, as T_m increases to 90 °C, the binding of Ca^{2+} provokes a significant stabilization of the protein. The occurrence of an intermediate state in apo-LYLA1 and its disappearance together with the concomitant stabilization upon Ca^{2+} binding were also demonstrated in GdnHCl-induced unfolding studies at pH 4.5 [68].

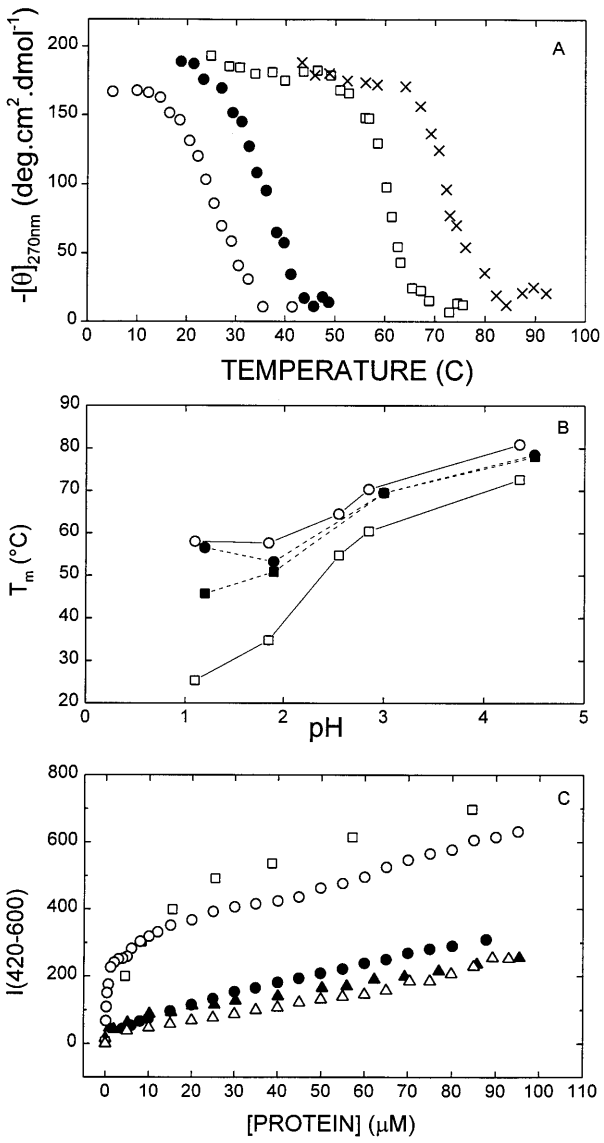

Figure 5. The partially folded state of LYLA1 at low pH. (*A*) Thermal unfolding of the tertiary structure deduced from ellipticity data at 270 nm and at pH = 4.35 (×), 2.85 (□), 1.85 (●) and 1.1 (○). (*B*) pH dependency of the midpoint of thermal transition at 270 nm (squares) and at 222 nm (circles) for apo-LYLA1 (open symbols) and for HLY (filled symbols). (*C*) Bis-ANS binding to LYLA1 compared with its binding to HLY and BLA. The integrated fluorescence intensity between 420 and 600 nm of bis-ANS is depicted as a function of protein concentration at 25 °C: HLY at pH 7.5 (▲) and pH 1.3 (△); apo-LYLA1 at pH 7.5 (●) and at pH 1.1 (○); and apo-BLA at pH 7.5 (□) [68].

The acid denaturation of apo-LYLA1 at 25 °C takes place between pH 2 and 1, but at the lowest pH a certain amount of tertiary structure is still retained. A more detailed picture of the behaviour of LYLA1 in acidic conditions has been obtained by analysing the thermal unfolding curves at various pH values (fig. 5A). The native protein has nearly the same residual ellipticity at 270 nm at any of these pH values, and in each case unfolds in a cooperative process. From the evolution of the $T_{m,270}$ values presented in figure 5B, it is clear that wild-type human lysozyme has a more stable tertiary structure than the chimera over the whole pH range. Especially at low pH this difference in stability increases and amounts to 20 °C at pH 1.1 [68]. Calculation of the population of the different states at low pH shows the presence of an intermediate state in the temperature range 57–95 °C at pH 4.5 with a maximum population of 65% at 76.8 °C. At pH 1.1, the intermediate occurs in a broader temperature range going from 11 to 97 °C and obtains a maximum population of 97% at 36.4 °C. Both temperature and pH are parameters that can be used to shift the equilibria between the different states and hence to maximize the population of the equilibrium partially unfolded state.

The binding of the hydrophobic probes ANS or bis-ANS to proteins is widely used as an indicator for molten globule behaviour and for the occurrence of intermediate states [18, 23, 24]. Because in an expanded state hydrophobic domains become more accessible to the probe, a large enhancement of fluorescence and a blue shift of the maximum is observed. When bis-ANS is titrated with apo-LYLA1 at pH 7.5 and 25 °C, fluorescence evolves as in titration with HLY (fig. 5C). The results for the experiments at low pH, however, are totally different. Whereas an acid environment does not influence the fluorescence of the probe in the case of HLY, titration with LYLA1 at pH 1 shows a strongly enhanced fluorescence comparable with that observed in the molten globule state of BLA [68]. These data clearly indicate that, upon acidification, LYLA1 exposes some hydrophobic regions within the collapsed structure.

Biological relevance of folding intermediates

The folding process of a protein in vitro is likely to differ in many details from that inside the cell. Apart from obvious differences in folding such as temperature, pH, ionic strength and protein concentration, the presence of disulphide bonds at the onset of folding is artificial and is likely to affect the stability of the various intermediate states [12, 69]. In the molten glob-

ule conformation of α-lactalbumin, for example, the nonnative disulphide bonds predominate in contrast to the popular idea that molten globules generally have a nativelike topology [70, 71]. The latter view was reinforced by studies on two variants of human α-lactalbumin in which, in the one case, only the disulphide bonds in the α domain, and in the other, only those in the β domain are kept intact [33, 35, 72]. A further complication in the comparison between the folding process in vitro and in vivo arises as folding in the cell is assisted by other proteins acting as catalysts, such as protein disulphide isomerase and prolyl isomerase [73, 74]. In addition, it is now firmly established that in vivo a significant fraction of newly synthesized polypeptides requires the assistance of molecular chaperones for their folding [75–77]. Chaperones generally recognize the nonnative states of many different polypeptides, primarily by binding to solvent-exposed hydrophobic amino acid residues or to surfaces that are buried in the native state. These interactions serve to prevent or reverse off-pathway folding reactions that lead to aggregation. One of the best-studied chaperones is GroEL, composed of 14 identical 57-kDa subunits, which are arranged in two stacked heptameric rings [78, 79]. As the molten globule state is one of the structural features that can be recognized by GroEL, α-lactalbumin is a favourite substrate. Binding to GroEL in a three-disulphide derivative of bovine α-lactalbumin appears to involve relatively disordered partly folded states resembling intermediates formed in the very early stages of kinetic folding [80]. Furthermore, it was shown that the refolding of apo-α-lactalbumin is retarded by GroEL, indicating that it recognizes the molten globule state of the protein [81]. The binding was found to be entropy-driven at room temperature, and the heat capacity change for the binding was found to be largely negative, indicating that GroEL binds to α-lactalbumin through hydrophobic interactions [82].

During recent years evidence has mounted that nonnative states of proteins in a cell could be responsible for amyloid fibril formation [83–86]. In the environment of the cell, the high density of proteins results in a high probability of intermolecular interactions, particularly in the case of incompletely folded proteins that are likely to have hydrophobic groups exposed to the solvent. A number of diseases are thought to be associated with the inability of mutant proteins to avoid aggregation during folding. Other diseases are associated with proteins that are expressed normally but which subsequently undergo a transition to a different conformational state that has a strong tendency to aggregate. A schematic representation of the competitive denaturation and fibrillogenesis under partially denaturing conditions is given in figure 6 [86]. The core structure of the amyloid fibrils consists of β sheets with the strands perpendicular to the long axis of the fibre. Recently, two natural variants of human lysozyme, Ile56Thr and Asp67His, have been reported to form amyloid deposits in the viscera, apparently causing hereditary nonneuropathic systemic amyloidosis, resulting in death by the age of 40 [83]. Given the conservative nature of both these mutations, it is likely that X-ray crystallography of the variant proteins shows structures that are very similar to those of the wild-type human lysozyme and that leave the four disulphide bonds intact [87–88]. Closer inspection of the two diffraction patterns, however, demonstrates that subtle but structurally significant changes at the interface region between the α and β domains occur in both these variants, indicating that in the variants the interface region is less constrained than in the wild-type protein [88]. This common feature of both structures implies that the organization of the domain interface could be an important factor in their amyloidogenic properties. Both variants are also less thermostable, with midpoints of denaturation approximately 12 °C lower than that of the wild-type protein at pH 5. More important, the unfolding transition of the two amyloidogenic variants, although reversible under conditions in which fibril formation did not occur, is not cooperative [88]. This results in a partly folded state being significantly populated near the midpoint of unfolding. Such behaviour is quite different from the cooperative unfolding displayed by the wild-type protein under these conditions but is similar to the thermal unfolding of HLY at extremely low pH, where

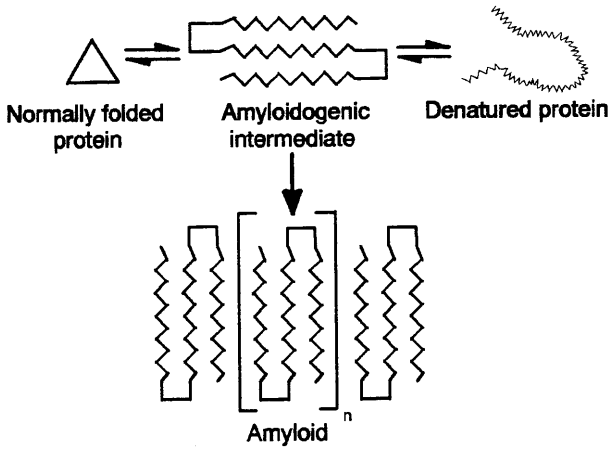

Figure 6. Schematic representation of the competitive denaturation and fibrillogenesis pathways in amyloidogenic proteins under partially denaturing conditions. Ile56Thr and Asp56 His mutations in HLY appear to function by shifting the equilibrium between the normally folded protein and the amyloidogenic intermediate under mildly denaturing conditions where the wild-type protein would be stable [86].

the protein unfolds through a partially structured equilibrium intermediate [46]. Therefore, the cause of this amyloid disease has to be sought in the fact that the mutations alter the folding pathway, making the amyloidogenic intermediate more accessible. Based on these results, a model for lysozyme fibrillogenesis in which association of the partly folded forms of the variants occurs through the unstable β domain has been proposed [88]. The development of stable β structure through such intermolecular association could then act as a template for the progressive recruitment of polypeptide chains into the nascent fibril.

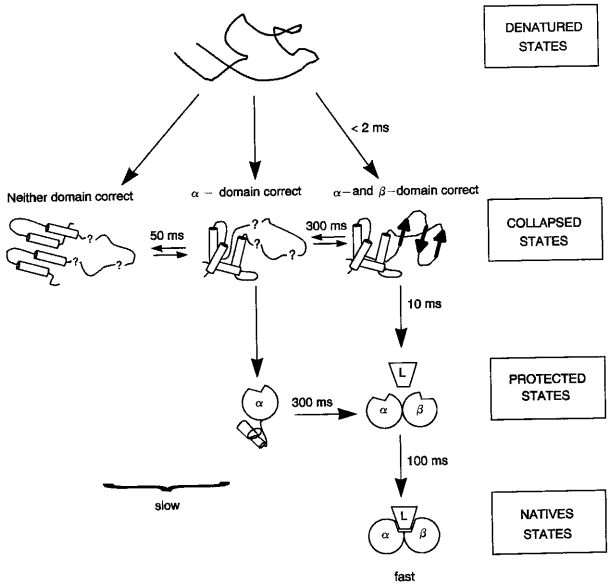

Figure 7. Schematic representation of a possible folding pathway of HEWL (pH 5.5, 20 °C). Both the fast and slow tracks of folding are illustrated. Domains with nativelike hydrogen-exchange protection are represented by spheres, and the enzyme with a competent active site, situated at the interface between the two folding domains and with ligand (L) bound, can be seen as the outcome of both tracks [89, 92].

The folding kinetics of lysozyme and α-lactalbumin

As mentioned before, a number of biophysical techniques has been employed to determine the folding kinetics of lysozyme [14]. The acquisition of this assembly of structural information has resulted in the scheme of a possible folding pathway, depicted in figure 7 [15, 89]. Within a few milliseconds a compact state of the protein is formed which has a nativelike content of secondary structure [11, 90]. However, this collapsed state has been shown to be heterogeneous. By using interrupted refolding experiments, Kiefhaber has demonstrated that refolding of lysozyme occurs on two parallel pathways [91]. Most of the molecules refold along a slow kinetic pathway with well-populated partially folded states, but a fraction of them folds faster. This fast-folding population is seen to form persistent structure in both domains on a 10-ms time-scale. The rate-determining step on this fast track involves docking of the two constituent domains of the protein. Binding studies with the fluorescent substrate inhibitor 4-methylumbelliferyl-N,N′-diacetyl-β-chitobiose (MeU-diNAG) reveal that the completely native state with a competent active site is obtained on a 100-ms time-scale [92]. In this fast-folding alternative, the data are consistent with a model in which each domain forms a persistent structure prior to their docking in a locally cooperative manner on a time-scale comparable to that of the folding of small single-domain proteins [92].

On the slow folding track, the structure formed at the early stage of folding ($<$ ms) is not persistent, indicating that these states are highly dynamic and rapidly fluctuating. In this case, persistent structure in the α domain is formed on a 50-ms time-scale, whereas the formation of a stably structured β domain is not completed until at least 300 ms. During this folding process, therefore, kinetically distinct populations of molecules can be distinguished, and partially folded states can be detected. Recent double-jump experiments [93, 94] showed that the two folding pathways are not caused by slow equilibration reactions in the unfolded state. Rather, kinetic partitioning occurs very early in lysozyme refolding, giving the molecule the chance to enter the direct folding pathway or the slow-folding alternative.

Compared with HEWL, the human protein shows marked differences in its folding behaviour [95]. First, the overall rate of refolding is fourfold faster than that of the hen protein. Second, although refolding of the α domain takes place before refolding of the β domain, formation of the former domain is not fully cooperative, and amides in helices A and B, and in the C-terminal 3_{10} helix, become protected before those in helices C and D. The observation of these differences in the folding characteristics of the hen and human variants provides evidence that the cooperative but local assembly of regions of secondary structure is a key aspect of protein folding. Our own measurements of fluorescence intensity in a stopped-flow experiment (fig. 8) show that 75% of the total fluorescence change between the unfolded and the native states of human lysozyme has already occurred within the dead time (<2 ms) of the instrument, indicating that a burst-phase intermediate state is formed [96]. The remaining decrease in fluorescence can be fitted to a double exponential function with $k_1 = 41$ s^{-1} and $k_2 = 7.7$ s^{-1}.

The refolding kinetics of α-lactalbumin were investigated using kinetic CD [97] and stopped-flow absorption measurements [98], with varying concentrations of denaturant and free Ca^{2+} [99] and recently using a combination of the former techniques, completed by acrylamide quenching and pulsed hydrogen-exchange NMR [100]. A burst-phase intermediate was observed to form within the first few milliseconds of the refolding process. This intermediate was characterized by pronounced, hydrogen-bonded secondary structure, exposure of hydrophobic surfaces and the absence of tertiary structure. All these properties indicate that the burst-phase intermediate in the refolding of α-lactalbumin is identical with the molten globule state, observed under equilibrium conditions. Nativelike helices in the α domain may be formed in the burst-phase intermediate, whereas a disordered β domain persists. Further folding to the fully native state happens in a monoexponential process, with $k = 4.2 \times 10^{-2}$ s^{-1} [100]. In the presence of 10 mM Ca^{2+} ions, refolding proceeds in a biexponential and significantly faster process with rate constants of 26 s^{-1} and 3.5 s^{-1}, respectively [99; Van Dael et al., unpublished]. The binding of $Ca^{2+}$to α-lactalbumin obviously promotes rapid folding. Because the rate-determining folding step of the apo protein is associated with the organization of the β domain and the subsequent docking of both domains in a fully native structure, Ca^{2+} binding seems to affect the interfacial region of the protein to a considerable extent. This is in accordance with the observation that the Ca^{2+}-binding site does not belong exclusively to the α domain but extends largely to the β domain [101, 102].

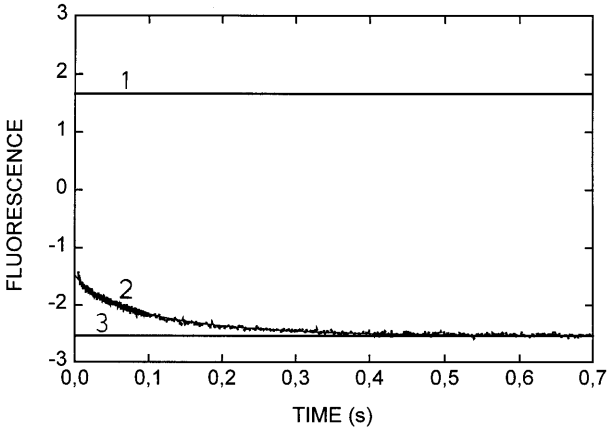

Figure 8. Time course of the refolding of HLY in 20 mM Tris, 80 mM NaCl, 10 mM EDTA, pH 7.5 at 25 °C. Refolding is induced by 11-fold dilution by the buffer solution obtaining a final concentration of 0.54 M GdnHCl. Curve 1 indicates the equilibrium fluorescence of the unfolded state in 6 M GdnHCl. Curve 2 gives the time-dependent evolution of the signal that can be fitted to a biexponential function (full line). Curve 3 gives the fluorescence of the folded protein.

Refolding of chimeric constructs

Human lysozyme and apo α-lactalbumin differ markedly in stability. Whereas wild-type human lysozyme unfolds highly cooperatively at pH 4.5 with $T_m = 78$ °C [12, 66], carefully demetalized apo α-lactalbumin at pH 7.5 is already completely unfolded at 30 °C [103]. As can be expected from these results, the stability of human lysozyme will decrease when structural elements of lysozyme are replaced for the corresponding ones of α-lactalbumin. Insertion of the Ca^{2+}-binding site of α-lactalbumin into human lysozyme (LYLA1A) causes a rather small reduction of T_m to 76.6 °C, and the subsequent implantation of helix C of α-lactalbumin (LYLA1) further reduces T_m to 72.6 °C. In both cases, the binding of Ca^{2+} to the chimeras provokes considerable stabilization, resulting in $T_m =$ 90 °C. In general, stable domains in a protein are formed first, and folding is completed by the formation of the less stable parts, so it is of great interest to compare these stability results with the rate constants observed during refolding. Specifically, for bovine α-lactalbumin and human lysozyme it has been shown that their rate constants differ by more than a factor of 100 and that nativelike helices in the α domain may be formed in the burst-phase intermediate, with the β domain still disrupted [11, 97, 100]. Refolding measurements on mutants and chimers, therefore, can inform us about the influence of mutations on secondary structure formation, on the establishment of the various domains and on their joining together into a fully active native structure.

Our results, which will be published in detail elsewhere, can be summarized as follows. A burst-phase intermediate is formed in M4, LYLA1A and LYLA1. As proved by far-UV CD stopped-flow experiments, this burst-phase intermediate shows extensive nativelike secondary structure. Just as in wild-type HLY, 80–90% of the ellipticity change occurring during the unfolded to native state transition is accomplished in the first refolding step, which leads to the burst-phase intermediate state. Arrangement of the tertiary structure, however, happens to a markedly lower extent (60%) in this intermediate state and is completed to form a competent native state in a biexponential process. The concomitant rate constants for all these constructs have decreased compared with the rate constants observed for HLY, indicating that the presence of these inserted residues is not favourable for folding. The decrease in stability is accompanied by a decrease in folding speed. Upon Ca^{2+} binding to these constructs, the folding speed increases

significantly, indicating that the conformational rearrangment induced by Ca^{2+} binding favours folding as well as stability. Matching of the two domains in chimers of HLY is clearly enhanced by the presence of Ca^{2+} at the binding site. The acceleration of the folding speed upon binding of Ca^{2+}, however, is not a general phenomenon. In pigeon lysozyme, which also shows Ca^{2+}-binding capacity and concomitant stabilization, the rate constants are exactly the same in the apo- and the Ca^{2+}-bound forms [104].

Conclusion

Although structural elucidation of the mechanism of protein folding has progressed in a considerable way during the last few years, it is clear that many crucial aspects of the fundamental features of the folding process are not yet completely understood. In the present contribution we have shown that the application of different spectroscopic techniques to the same protein combined with the study of the effects of modifications introduced by protein engineering of the system can provide essential information. It is evident, however, that results from many systems will be needed to establish more fully the fundamental rules describing folding. Improvement of experimental strategies trying to reveal the extremely rapid burst-phase events in folding and novel theoretical methods designed to predict which structure a given sequence will adopt will certainly contribute greatly to the further understanding of protein folding.

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