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ARTICLE

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Conformational stability of LYLA1, a synthetic chimera of human lysozyme and bovine α-lactalbumin

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Abstract LYLA1 is a chimeric protein mainly consisting of residues originating from human lysozyme but in which the central part (Ca^{2+}-binding site and helix C) of bovine α-lactalbumin has been inserted. The equilibrium unfolding of this hybrid protein has been examined by circular dichroism and tryptophan fluorescence techniques. The reversible denaturation process induced by temperature or by addition of chemical denaturant is three-state in the case of apo-LYLA1 and two-state in the presence of Ca^{2+}. The Ca^{2+}-bound form of the chimera exhibits higher stability than both wild-type lysozyme and α-lactalbumin. The stability of the apo-form, however, is intermediate between that of the parent molecules. Unfolding of apo-LYLA1 involves an intermediate state that becomes populated to a different extent under various experimental conditions. Combination of circular dichroism with bis-ANS fluorescence experiments has permitted us to characterize the acid state of LYLA1 as a molten globule. Furthermore our results strongly suggest the presence of multiple denatured states depending on external conditions.

Key words Lysozyme · α-lactalbumin · Chimera · Circular dichroism · Protein stability · Molten globule

Abbreviations HLY human lysozyme · BLA bovine α-lactalbumin · LYLA1 chimeric protein derived from human lysozyme and bovine α-lactalbumin as described in the text · GdnHCl guanidinium hydrochloride · CD circular dichroism · bis-ANS 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonate

Introduction

Many proteins undergo reversible transitions between a highly ordered native state and a disordered unfolded state in a two-state process. Lysozymes are typical examples of this category. In some patients, however, during unfolding one or more partially folded states become populated. One of the best-known examples that conforms to the latter model is that of α-lactalbumin which unfolds in a three-state process via a molten globular intermediate (Dolgikh et al. 1981; Kuwajima 1989; Ptitsyn et al. 1990; Griko et al. 1994). Nevertheless, α-lactalbumin is homologous to lysozyme with regard to sequence and structure. Both these proteins contain four α-helices, which make up one domain encompassing the amino- and carboxy-terminal segments, and a triple-stranded antiparallel β-sheet that together with a long loop makes up much of a second domain. The structure is stabilized by four disulfide bridges. All the α-lactalbumins have been shown to possess a strong Ca^{2+}-binding site (Stuart et al. 1986) and binding of Ca^{2+} or other metal ions to this site results in a drastic improvement of the thermostability of these proteins (Segawa and Sugai 1983; Desmet et al. 1987). In a few lysozymes, the residues responsible for Ca^{2+} binding in α-lactalbumin are conserved and Ca^{2+} binding is now known to be a property of lysozymes of different origin: horse milk (Nitta et al. 1987; Desmet et al. 1989), pigeon egg white (Nitta et al. 1988), dog milk (Zeng et al. 1990) and echidna milk (Teahan et al. 1991). This Ca^{2+}-binding property, however, is totally absent in the other c-type lysozymes.

These intriguing differences in unfolding behavior, metal binding capacity and resulting conformational stability have prompted us to construct chimeric proteins in which well-considered structural elements are exchanged between lysozyme and lactalbumin. As a result we were able recently to report on LYLA1, a chimera derived from human lysozyme in which the Ca^{2+}-binding loop and the adjacent central helix C of bovine α-lactalbumin were inserted (Fig. 1). This chimera was proven to be a stable, well-structured protein which has conserved the lytic activity of lysozyme and which has acquired the Ca^{2+}-binding capacity (Pardon et al. 1995). An interesting feature of this hybrid is that the transplanted part (residues 76-102: HLY numbering) is situated in the contact zone between the two domains.

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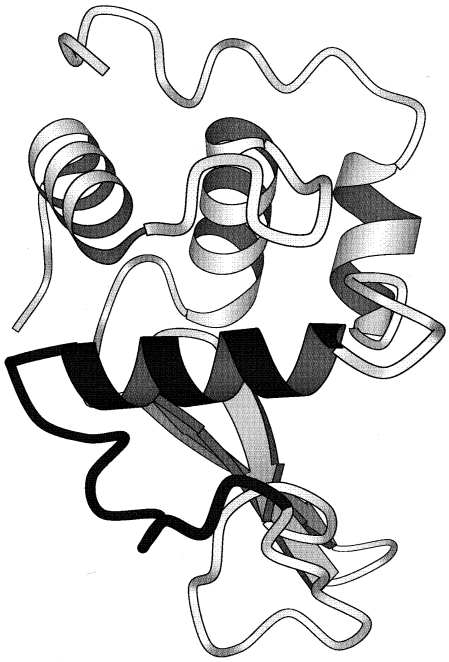

Fig. 1 Schematic representation of the three-dimensional structure of LYLA1. Starting from the structure of HLY (Brookhaven Data bank code 1 LZ1), the insertion of the BLA part (in dark) was accomplished by the BRUGEL modelling package (Delhaise et al. 1984) on a Silicon Graphics Iris Indigo Workstation. The diagram was generated using the program MOLSCRIPT (Kraulis 1991)

Extensive kinetic studies with optical spectroscopic and NMR techniques have shown that the two structural domains in lysozymes are also distinct folding domains with specific kinetic behaviour (Miranker et al. 1991; Radford et al. 1992). In α-lactalbumin the molten globule state 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 (Baum et al. 1989; Alexandrescu et al. 1993; Schulman et al. 1995). Complete folding to the native state, however, is not accomplished until the specificity of sidechain packing has developed. This final folding stage requires additional interactions to lock in the unique tertiary contacts of the native structure. The binding of calcium at the domain interface in α-lactalbumin can promote the formation of these contacts and stabilize them (Schulman et al. 1995, Wu et al. 1996).

In our previous work (Pardon et al. 1995) we established the three-state thermal unfolding behaviour of apo-LYLA1. This means that exchanging residues 76-102 of human lysozyme for the corresponding ones of α-lactalbumin, induces a molten globule behaviour in the whole chimera.

In the present study it is our aim to describe in detail the occurrence of the intermediate state in this chimera as a function of a set of external parameters such as temperature, Ca^{2+} content, denaturant concentration and pH. Special attention is focused on the acid state which has been fairly well characterized by circular dichroism and fluorescence spectroscopy. In view of further characterization of the structural basis of this molten globule by NMR spectroscopy, this study is essential for determining the conditions in which the interplay of various denaturing agents leads to a maximal population of the intermediate form.

Materials and methods

Production of LYLA1

LYLA1 (Fig. 1) was created by exchanging the amino acid sequence 76-102 of HLY for the corresponding part of BLA (72-97), which is constituted by the Ca^{2+}-binding loop and the big central α-helix (helix C) (Pardon et al. 1995). This hybrid gene was transferred to the shuttle vector pAB24, resulting in the pABAGLYLA1 expression plasmid. Then the *S. cerevisiae* strain GRF182 was transformed with this plasmid. After selection in uracil and leucine-selective medium, yeast cells were grown in YPD medium, buffered with 50 mM MES to pH around 6.0, at 28 °C for 5–7 days in 10 l batches in a fermentor and were supplemented with 4% ethanol after exhaustion of the carbon source. The chimeric protein was purified from the culture medium by cycles of ion exchange (Streamline-SP and Fractogel-SO_3 EMD 600) and gel filtration (Sephacryl HR100). The average yield was 8–9 mg/l of culture, and the final product was homogeneous on SDS-PAGE. From isoelectric focusing a pI of about 9.3 was calculated. N-terminal analysis, using a gas phase sequenator, indicated that the secreted protein had been processed correctly by the heterologous host cells.

Immunochemical analysis, muramidase activity measurements and Ca^{2+}-binding properties of this chimera were described previously (Pardon et al. 1995).

Acid and chemical denaturation

Protein concentration was determined by UV absorption spectroscopy at 280 nm on a Beckman DU-70 spectrophotometer. Based on the calculated molecular weight and the Tyr and Trp content the following values were adopted for $\varepsilon_{1\%}$: LYLA1: 25.0; HLY (from U.S. Biochemicals): 25.5 and BLA (from Sigma): 20.1.

In the acid denaturation experiments a small aliquot of 2 N HCl was added to 3 ml of a 0.4 mg/ml protein solution in 20 mM KH_2PO_4, pH = 4.0. Subsequently the acquired pH value was determined, the temperature of the sample was re-equilibrated at 25 °C and the ellipticities at 270 nm and 222 nm were recorded. This cycle of addition of aliquots of concentrated HCl was repeated with the same sample from pH 4 down to pH 0. The CD signals were corrected for the consecutive dilution effects.

In GdnHCl-induced unfolding experiments, a separate solution was made up for each point of the denaturation curve. These solutions with constant protein (0.3 mg/ml)

but increasing denaturant concentrations were prepared volumetrically starting from a protein stock solution of 5 mg/ml and a denaturant stock solution of 7.5 M. Extremely pure GdnHCl was purchased from U.S. Biochemicals. As GdnHCl is hygroscopic the exact concentration was determined from refractive index measurements (Nozaki 1972). In order to allow the solution to reach an equilibrium state, experiments were only started one hour after mixing the denaturant with the protein.

Reversibility of the temperature- or GdnHCl-induced transitions was checked by lowering temperature or denaturant concentration after the unfolding experiment. Lytic activity of LYLA1, measured using *M. luteus* as substrate at pH = 6.2 (Pardon et al. 1995), was conserved after the denaturation run.

Circular dichroism spectroscopy

Circular dichroism spectra were obtained on a Jasco J-600A spectropolarimeter. In the near-UV spectral region (250–320 nm), the ellipticity monitored at 270 nm or at 292 nm is mainly due to aromatic residues and reflects the extent of specific tertiary interactions in the protein. In the far-UV region (190–250 nm), the ellipticity at 222 nm was taken as a measure for the α-helical content of the protein. The spectrometer was equipped with a system for temperature control in 10 mm or 1 mm cells and was calibrated with 10-camphorsulfonic acid at 290.5 nm. The data were expressed as residual ellipticity $[\theta]$ ($deg \cdot cm^2 \cdot dmol^{-1}$) using 115.1, 113.1 and 115.2 as the mean residue weight for LYLA1, HLY and BLA, respectively. Protein concentration was normally about 0.3 mg/ml.

The apparent fraction of protein present in the native, intermediate or unfolded state can be determined by monitoring the ellipticity at 222 nm and 270 nm as a function of pH, temperature and denaturant concentration (Ikeguchi et al. 1986). Population curves of the various states can be generated by fitting the data points at 270 nm by a sigmoidal curve typical for two-state transitions and the data points at 222 nm by a sigmoidal curve if the transition is highly cooperative or by a five-term polynomial in the case of a broad unfolding process. The population of the I-state is obtained by subtracting the sum of the N- and U-populations from 1.

Fluorescence spectroscopy

Fluorescence measurements were performed on an Aminco SPF-500 spectrofluorimeter connected to a personal computer for data handling. In the titration experiments of 1 μM bis-ANS with protein, the excitation wavelength was fixed at 385 nm. The emitted light, integrated between 420 nm and 600 nm was used as indicator for the fluorescence intensity. Wavelength shifts of the fluorescence signal were reflected in the intensity ratio I_{460}/I_{510}. Intrinsic tryptophan fluorescence spectra were recorded on the same spectrofluorimeter with excitation at 280 nm.

Size-exclusion chromatography

In size-exclusion chromatography experiments we used the Superose-12 column and fast protein liquid chromatography (FPLC) equipment of Pharmacia. To study very dilute protein solutions, the Spectra 200 UV-VIS Detector (Spectra-Physics) was fixed at 226 nm. All experiments were performed at 4 °C and in the presence of 0.1 M Na_2SO_4 in order to eliminate weak hydrophobic interactions between the protein and the gel matrix (Golovchenko et al. 1992). As, even in these conditions, LYLA1 was somewhat retarded on the column, we report elution volumes rather than Stokes radii.

Results

Thermal unfolding at pH 4.5

As previously mentioned (Pardon et al. 1995), thermal unfolding studies by CD and by tryptophan fluorescence at pH 4.5 show that apo-LYLA1 does not unfold in a simple two-state process. An intermediate state is observed in a restricted temperature range. Indeed the tertiary structure of this chimera denatures with a $T_m = 72.6$ °C whereas unfolding of the secondary structure has a considerably higher $T_m = 80.9$ °C. The same authors have also proven the stabilization of the chimera by Ca^{2+}-binding. In the presence of 10 mM Ca^{2+}, the unfolding curves obtained in the far- and near-UV region coincide completely with a T_m value of 90 °C, indicating that in this case unfolding involves only native and totally unfolded states without population of an intermediate state.

Chemical unfolding at pH 4.5

Analysis of unfolding transitions in the presence of denaturants provides important information on the basis of conformational stability of proteins. Besides urea, GdnHCl is probably the most commonly used protein denaturant. As the concentration of GdnHCl needed to obtain a certain degree of denaturation is lower than the corresponding concentration of urea (Pace 1990) and as use of urea at low pH causes a problem arising from its titratable group (Hagihara et al. 1994), in all our experiments GdnHCl was preferred as denaturant. Morever, comparable studies of the unfolding equilibria of the parent proteins, lysozyme and α-lactalbumin, have been performed by treatment with GdnHCl (Ikeguchi et al. 1986).

Figure 2 A shows the effects of GdnHCl on the CD spectra of LYLA 1 at pH 4.5, monitored at 222 nm and 270 nm. The tertiary structure of apo-LYLA1 unfolds at a distinctly lower denaturant concentration ($C_{1/2} = 3.1$ M) than the secondary structure ($C_{1/2} = 3.7$ M). Calculation of the population of the various states, as described previously (Haezebrouck et al. 1995), indicates that a fraction of apo-LYLA1 is present in the intermediate state (Fig. 2B). Apo-BLA (at

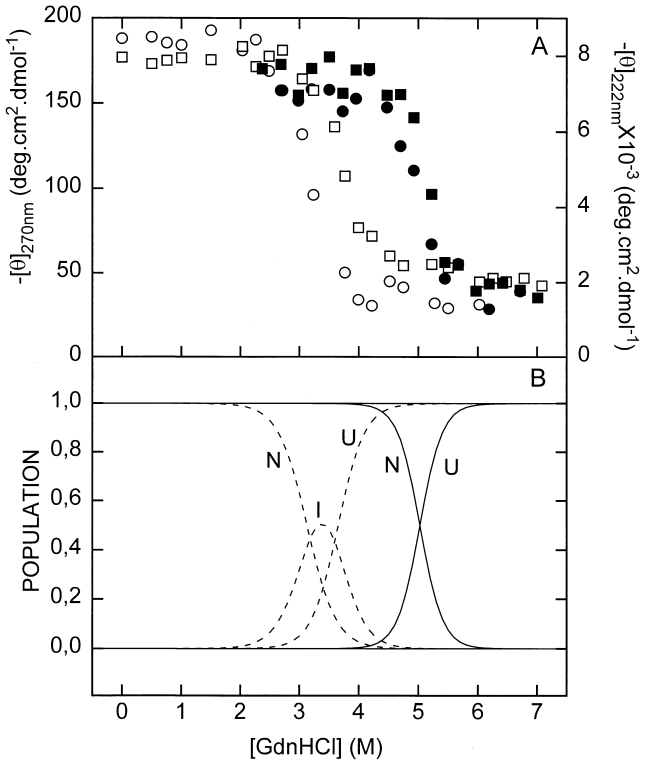

Fig. 2A, B Chemical denaturation of LYLA1 at pH 4.5. **A** shows the residual ellipticities at 270 nm (○, *left scale*) and 222 nm (□, *right scale*) for apo-LYLA1 in 10 mM NaAc, 90 mM NaCl, and the same parameters (*filled symbols*) for Ca-LYLA1 in 10 mM NaAc, 90 mM NaCl, 10 mM Ca^{2+}. **B** shows the relative population of the N, I and U state for the apo-form (– – –) and for the Ca^{2+}-form (–)

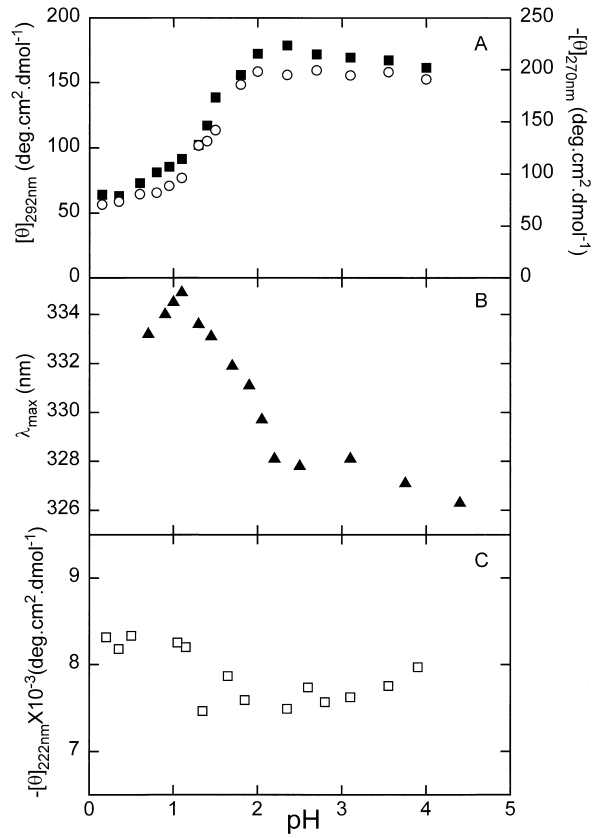

Fig. 3A–C Acid denaturation of apo-LYLA1. HCl titration was done at 25 °C starting from 20 mM KH_2PO_4. Tertiary structure organization is followed by CD at 270 nm and 292 nm (**A**) and secondary structure at 222 nm (**C**). **B** shows the wavelength at which the Trp fluorescence intensity reaches its maximum

pH 7, 25 °C, $[Na^+] = 0.1$ M) was found to unfold with $C_{1/2} = 1.3$ M for the tertiary structure and $C_{1/2} = 2.3$ M for the secondary structure (Ikeguchi et al. 1986). Both values are markedly lower than the corresponding ones for apo-LYLA1 but their difference is greater than in the case of apo-LYLA1. As a consequence, the intermediate state in apo-BLA occurs in a broader concentration range and to a greater extent than in apo-LYLA1.

In the presence of 10 mM Ca^{2+} the unfolding curves for LYLA1, monitored at both wavelengths, coincide completely and conform to a pure two-state transition with a midpoint $C_{1/2} = 5.0$ M (Fig. 2). These results strongly suggest, firstly, that Ca-LYLA1 unfolds without adopting an intermediate state and, secondly, that the binding of Ca^{2+} ions to apo-LYLA1 results in a significant increase in stability. As one could expect, Ca-LYLA1 is more resistant towards chemical unfolding than Ca-BLA for which the transition midpoint was determined at 3.5 M in the presence of 12 mM Ca^{2+} (Ikeguchi et al. 1986). The GdnHCl-induced transition of lysozyme on the other hand is not influenced by the presence of 12 mM Ca^{2+} and conforms to a two-state process with midpoint at 4 M GdnHCl (Ikeguchi et al. 1986). This indicates that Ca-LYLA1 has a greater stability than Ca-BLA due to its predominant lysozyme character, and also than lysozyme itself due to the extra stabilization provoked by the Ca^{2+}-binding.

Acid denaturation

The denaturation process, induced by acid titration at 25 °C, was followed by CD measurements in the far-UV (222 nm) and in the near-UV (270 and 292 nm) range (Fig. 3). In the pH range from 2 to 1, most of the tertiary interactions become disrupted but even at pH 0 a certain amount of tertiary structure is retained. Similar observations were made using tryptophan fluorescence. The wavelength at which the tryptophan fluorescence intensity shows its maximum, shifts from 328 nm at pH 2 to 334 nm at pH 1. For comparison, the native to acid-state transition shifts the λ_{max} of BLA from 327 nm to 340 nm while in HLY it remains fixed at 331 nm. The ellipticity at 222 nm remains nearly constant down to pH 1.5 and becomes more negative at lower pH.

The behaviour of this chimera in acidic conditions is obviously different from that of BLA and HLY. In the whole pH range concerned (1–4.5), apo-BLA adopts a molten globule state in agreement with earlier observations (Segawa & Sugai 1983) and with recent data (Fink et al. 1994). By contrast, the acid titration of lysozyme by the latter authors showed no significant change in either near- or far-UV signals down to pH 1. Our previous study of lyso-

Table 1 Thermal unfolding of apo-LYLA1 at various pH values. The temperature region in which the intermediate state occurs is indicated together with the temperature at which the maximum population is obtained. The total population of the intermediate state is calculated from the surface under the population curve as a function of temperature

pH	$T_{m,270}$ (°C)	$T_{m,222}$ (°C)	Characteristics of intermediate state			
			Temperature region (°C)	T_{max} (°C)	Maximum population (procent)	Total population (arbitrary units)
4.35	72.6	80.9	57–95	76.8	65	8.8
2.85	60.4	70.3	51–85	65.5	61	9.8
2.55	54.7	64.5	42–84	59.9	57	10.6
1.85	34.8	57.6	17–89	43.4	80	25.5
1.10	25.3	57.6	11–97	36.4	97	35.4
4.50 1 M GdnHCl	60.2	70.6	45–90	66.3	53	12.7
4.50 2 M GdnHCl	48.1	52.3	40–70	52.0	31	4.4

zyme at low pH also confirms that, at least at room temperature, the conformational state of human as well as chicken lysozyme, is completely native (Haezebrouck et al. 1995).

Thermal denaturation at low pH

In order to elucidate in more detail the conditions under which secondary and tertiary interactions are lost, and to what extent an intermediate state occurs, the thermal unfolding of apo-LYLA1 was examined at various acidic pH values ranging from pH 4.5 to 1 (Table 1).

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 whereby all tertiary interactions are lost in a restricted temperature range (Fig. 4A). The evolution of the corresponding midpoints of transition ($T_{m,270}$) is presented in Fig. 4C. For comparison, the unfolding data of human lysozyme are included (Haezebrouck et al. 1995). They indicate that the tertiary structure of the latter protein is more stable than that of 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.

Combination of the ellipticity data at 270 nm (Fig. 4A) and at 222 nm (Fig. 4B), shows that from pH 4.5 down to pH 2.5 an intermediate state is present at a nearly constant population. At lower pH values this population increases drastically.

From Fig. 4B it is also clear that, even at 95 °C, unfolding is not yet completed. The remaining ellipticity at this temperature depends on pH ranging from about 5.0×10^3 (pH 4.5) to 3.5×10^3 deg · cm^2 dmol^{-1} (pH 1.1). At low pH the unfolding process also shows an important loss of cooperativity.

Bis-ANS fluorescence spectroscopy

The binding of proteins to the hydrophobic probes ANS or bis-ANS is widely used as an indicator for molten globule behaviour and for the occurrence of intermediate states. As 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 a titration with HLY (Fig. 5A). Whereas an acid environment (pH 1.3) does not influence the fluorescence of the probe in the case of HLY, titration with LYLA1 at pH 1, 25 °C shows a strongly enhanced fluorescence comparable with that obtained in the molten globule state of BLA. These data clearly indicate that, upon acidification, LYLA1 exposes some hydrophobic regions within the collapsed structure. Monitoring the temperature-dependent behaviour of the fluorescence at pH 1.1 provides supplemental information about the conformational changes in the acid state (Fig. 5B). A first conformational transition (10 °C–30 °C) indicates that λ_{max} shifts to lower wavelengths (from 486 to 482 nm) referring to a bis-ANS probe able to penetrate into the protein and to bind to a hydrophobic region. This transition is concurrent with the ellipticity changes observed at 270 nm (Fig. 4A). Around 30 °C the intensity ratio reaches a maximum corresponding with a maximum population of the intermediate state determined at 35 °C by our CD experiments (Table 1). Above this temperature, the pattern of hydrophobic residues that are able to bind bis-ANS desintegrates, leading to a considerably lower fluorescence with maximum shifted to higher wavelengths.

Combination of denaturing agents

From the previous data it is clear that temperature, GdnHCl and low pH, each on their own induces three-state unfolding behaviour showing an intermediate unfolded state to be present in a restricted temperature, denaturant or pH range. In order to get insight into how changing these parameters possibly shifts the equilibria between the different states and affects the amount of intermediate state, the combined effects of two of these agents were examined.

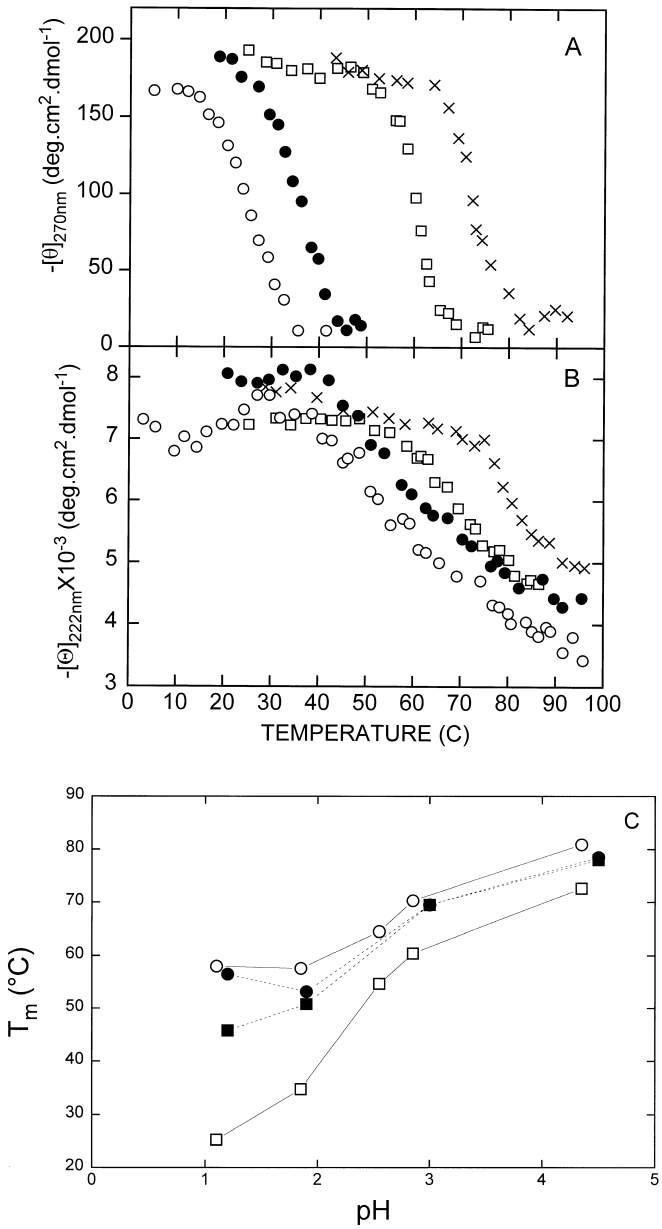

Fig. 4A–C Thermal unfolding of apo-LYLA1 at different low pH values. **A** Thermal unfolding of the tertiary structure deduced from ellipticity data at 270 nm and at pH 4.35 (X), 2.85 (□), 1.85 (●), 1.1 (○). **B** Thermal unfolding of the secondary structure deduced from ellipticity data at 222 nm at variable pH. Same symbols as in **A**. **C** 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*)

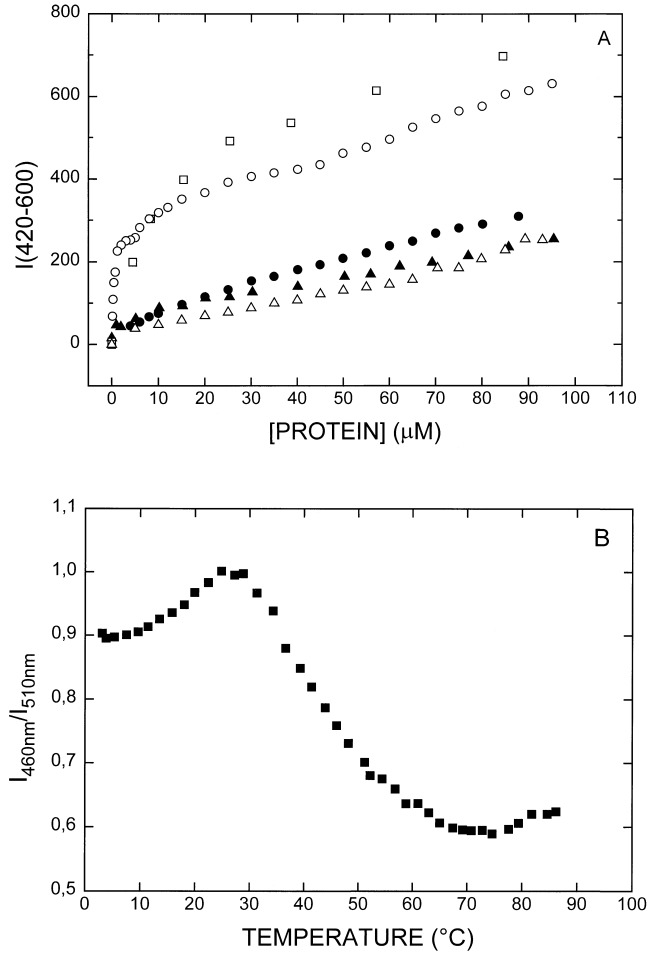

Fig. 5A, B Bis-ANS binding to LYLA1 compared with its binding to HLY and BLA. **A** Integrated fluorescence intensity between 420 and 600 nm of bis-ANS as a function of increasing protein concentration at 25 °C: HLY at pH 7.5 (▲) and pH 1.3 (△); apo-LYLA1 at pH 7.5 (●) and at pH 1.1 (○) and apo-BLA at pH 7.5 (□). **B** Temperature dependency of the fluorescence intensity ratio I_{460}/I_{510} for LYLA1: 50 μM apo-LYLA1 mixed with 1 μM bis-ANS at pH 1.1 in 20 mM KH_2PO_4

Addition of 1 M GdnHCl to apo-LYLA1 at pH 4.5 shifts both $T_{m,270}$ and $T_{m,222}$ to lower values (Table 1) and broadens the temperature range in which an intermediate state is observed with a slight decrease of its maximum population. The unfolding curve measured at 270 nm shows a steep and cooperative transition with $T_m = 60.2$ °C (not shown). Monitored at 222 nm, the transition starts at higher temperature and extends over a broader range (Fig. 6). Upon the addition of 2 M GdnHCl at the same pH (Table 1), the temperature at which the intermediate starts to appear is further shifted to a lower value and as the totally unfolded state is fully populated at 70 °C, under these conditions, secondary structure interactions are also strongly labilized.

In the same perspective the GdnHCl-induced unfolding was followed at pH = 1.85 (Fig. 7A). At this pH and at 25 °C, LYLA1 is almost native in the absence of denaturant (see also Fig. 3), but small amounts of GdnHCl are able to break tertiary interactions ($C_{1/2} = 0.6$ M) as measured from θ_{270nm}. At a concentration of 1.5 M GdnHCl almost the whole CD signal at 270 nm is lost and this remains so up to 6 M GdnHCl. By contrast, the residual ellipticity at 222 nm is constant up to 1.5 M and decreases in a broad unfolding process to a level of 2.10^3 deg · cm^2 dmol^{-1} at 6 M GdnHCl. The corresponding population curves indicate that at pH 1.85, 25 °C and 1.6 M GdnHCl, LYLA1 is nearly 100% in the intermediate state (Fig. 7B).

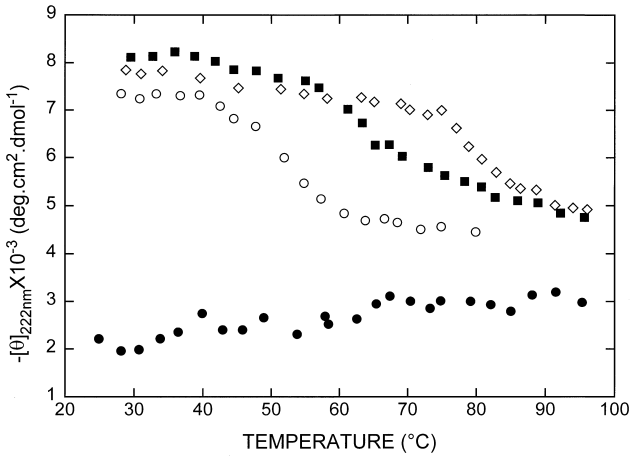

Fig. 6 Thermal denaturation of apo-LYLA1 at pH 4.5, 10 mM NaAc, 90 mM NaCl, followed by CD at 222 nm in the presence of GdnHCl 0 M (◇), 1 M (■), 2 M (○) and 6 M (●)

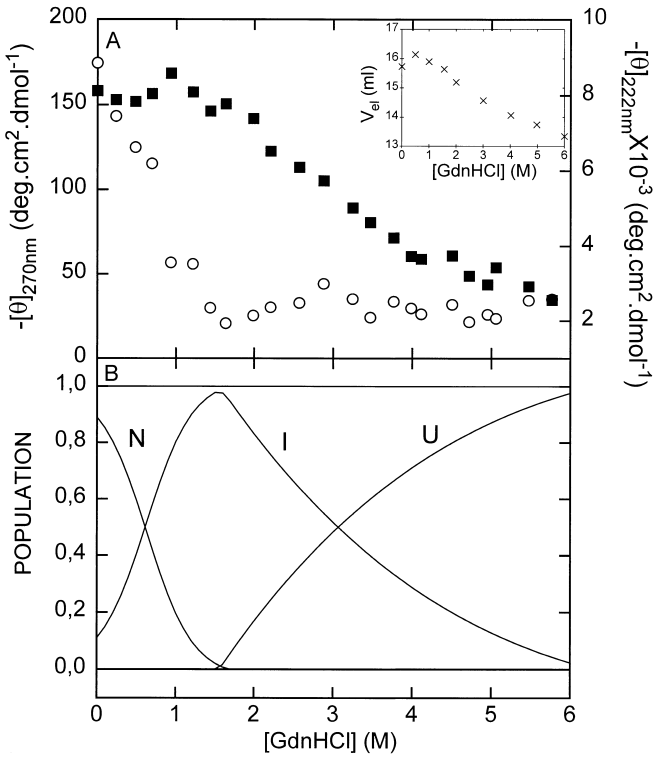

Fig. 7 Chemical denaturation of LYLA1 at pH 1.85. In the *upper part* the residual ellipticity at 270 nm (○) and 222 nm (■) is presented. Conditions: 20 mM Tris, 80 mM NaCl, 10 mM Ca^{2+} and 25 °C. The insert shows the elution volume obtained in size-exclusion chromatography experiments as a function of denaturant concentration. The *lower part* depicts the population in the native, intermediate and unfolded states, respectively

Size-exclusion chromatography

Uversky (1993) demonstrated that size-exclusion FPLC is a suitable method to estimate molecular dimensions of proteins in the molten globule state. Since the column does not affect the equilibrium between the different conformations (N, I and U), this technique can be applied for monitoring possible changes of molecular dimensions upon protein unfolding. Therefore, we followed the GdnHCl-induced denaturation of LYLA1 at pH 1.85 with gel filtration. In the whole concentration range only a single elution peak appears. With increasing GdnHCl concentration it shifts to smaller volumes in a way comparable with the ellipticity decrease at 222 nm (Fig. 7 insert).

In 0 M GdnHCl, LYLA1 elutes in a single sharp peak with an elution volume (V_{el}) of 15.7 ml. In the conditions where the chimera is in a nearly pure I-state (1.6 M GdnHCl, pH = 1.85) a single peak with V_{el} = 15.6 was observed.

Two important conclusions can be drawn from these measurements. Firstly, the single sharp peak at 1.6 M GdnHCl indicates that the I-state of LYLA1 is monomeric and we can rule out the possibility that aggregation phenomena complicate our data. Secondly, the I-state of the chimera is nearly as compact as the N-state.

Discussion

Our results show that in the case of apo-LYLA1 an equilibrium intermediate state can be generated by chemical or acid denaturation as well as by temperature. By modifying these external parameters the equilibria between the N-, I- and U-states shift and as a consequence the corresponding population of the partially folded state changes. In this way a nearly pure I-state can be generated for example in 1.6 M GdnHCl at pH 1.85 and at room temperature.

Combination of the data acquired from circular dichroism, intrinsic and extrinsic fluorescence measurements also enabled us to construct a preliminary picture of the acid state of LYLA1. It obviously corresponds in many respect to that of a molten globule. Studies on α-lactalbumin in particular have defined the molten globule as a compact denatured state with extensive secondary structure but with few and non-specific tertiary interactions. As shown by the CD spectra, the A-state of LYLA1 certainly conforms to these criteria. In addition, the fluorescence increase observed in bis-ANS binding experiments, suggests a collapsed structure that differs from the native state in that hydrophobic domains of the protein become more accessible to the probe. The red shift of the maximum of the intrinsic tryptophan fluorescence also indicates a higher degree of exposure of the Trp residues. The transition of the native to the intermediate state is in every case reversible and highly cooperative.

The propensity to form an intermediate state is also present in the parent proteins, human lysozyme and bovine α-lactalbumin, but to a totally different extent. At pH 1 and room temperature HLY is completely native. However, above 35 °C the native conformation transforms into an intermediate state, the population of which reaches its maximum at 50 °C (Haezebrouck et al. 1995). Repulsive forces between positively ionized groups have been invoked here

to explain the occurrence of this intermediate state. BLA, however, shows very few tertiary interactions at this pH and no transition is detected over the whole temperature region measured (5 °C–70 °C). When temperature is increased, secondary structure disappears only gradually, indicating that a mixed population of different intermediate states is present, which slowly convert into the totally unfolded state.

The unfolding behaviour of LYLA1 clearly lies in-between that of the parent proteins and is in some aspects reminiscent of the one reported for equine lysozyme. The latter protein, in contrast to most other lysozymes, binds Ca^{2+} as it contains all the residues that act as Ca^{2+}-binding ligands in the lactalbumins. It was demonstrated before that thermal, chemical and acid unfolding of the equine protein progresses via an intermediate state (Van Dael et al. 1993; Morozova et al. 1995). Looking at the unfolding behaviour of LYLA1 and equine lysozyme, one is forced to conclude that the occurrence of a molten globular intermediate state is closely associated with Ca^{2+} binding. It must be noted, however, that the presence of the Ca^{2+}-binding residues as such is not sufficient for generating a three-state unfolding. Pigeon lysozyme, that binds Ca^{2+} just like equine lysozyme, unfolds in a very cooperative two-state process without involving intermediate forms upon thermal (Haezebrouck 1992) or GdnHCl denaturation at neural pH (Nitta et al. 1993). Rather than through their intrinsic ability to bind Ca^{2+}, the binding residues seem to exert their influence through their integration in the network of tertiary interactions that hold together both domains of the protein. This view is in agreement with two recent reports on the heat denaturation and the stability, respectively, of equine lysozyme. In the first one, the analysis of the unfolding enthalpy functions of various lysozymes leads to the conclusion that the loss of interdomain cooperatively in equine lysozyme is directly related with the atomic packing in the interior of the molecule and specially at the interface of the α- and β-domain (Griko et al. 1995) exactly where the Ca^{2+}-binding site is located. The second one shows that the side chains of residues protected from hydrogen-exchange form a compact cluster within the core of equine lysozyme. That core remains present in the molten globule state and could be crucial in directing polypeptide chains to their native structure in the folding process (Morozova et al. 1995). In the case of LYLA1 more fundamental structural studies will be required in order to reveal the details of packing order and hydrophobic cluster formation in this hybrid protein.

At pH 4.35 and 2.85 the unfolding of the tertiary structure of LYLA1 proceeds very cooperatively as illustrated by the steep thermal unfolding curves (Fig. 4A). At lower pH, although a small broadening of the transition curve appears, the transition retains a high degree of cooperativity. At these low pH values, the unfolding of secondary structure, however, progresses very gradually without showing cooperativity (Fig. 4B). This refers to the consecutive unfolding of distinct structural entities. In terms of population of intermediate states, these observations lead to the following picture. At pH 4.35 the heat denaturation of tertiary and secondary structure occurs at temperatures that are both relatively high and that differ only by 8.3 °C (Table 1). This means that the totally unfolded state is attained after going through partly folded intermediates that are only modestly populated. At decreasing pH both T_m values also decrease but at a markedly different rate. As a result an increasing population of intermediate is observed together with a broadening of the temperature range in which intermediate states exist (Table 1).

Furthermore, our experiments show that at the highest temperatures (>90 °C) unfolded states are generated with ellipticities at 222 nm that depend on the other experimental conditions. Low pH (Fig. 4B) as well as the presence of denaturant (Fig. 6) causes a further reduction of ellipticity and hence of the amount of helix in the polypeptide chain. In contrast to earlier statements (Privalov et al. 1989, Griko et al. 1994), the high temperature limit for the ellipticity at 222 nm is not always the same in the absence and the presence of GdnHCl, nor is it universal for all proteins. Our data also give experimental support to the denatured state model recently predicted from two- and three-dimensional HP (hydrophobic-polar) lattice computer simulations (Dill et al. 1995, Dill & Shortle 1991). As a result of this model the denatured state of a protein is not a unique state but a broad ensemble of conformations that responds to changes in external conditions. In strongly denaturing conditions the most populated denatured species are highly unfolded. In less denaturing conditions the most populated species are rather compact.

As a conclusion we can state that, by inserting the Ca^{2+}-binding site and the helix C of α-lactalbumin into human lysozyme, we have created a chimeric protein, apo-LYLA1, that adopts a molten globule state in a very wide range of conditions. Its Ca^{2+}-binding property provides a supplemental possibility to restore the native conformation. Unlike α-lactalbumin, it is perfectly stable in neutral conditions at room temperature and it seems to be a very suitable protein for further studies on the structural basis of native to molten globule transitions in proteins.

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