A model of dynamic side-chain–side-chain interactions in the α -lactalbumin molten globule

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Abstract

Proteins in the molten globule state contain high levels of secondary structure, as well as a rudimentary, natively like tertiary topology. Thus, the structural similarity between the molten globule and native proteins may have a significant bearing in understanding the protein-folding problem. To explore the nature of side-chain–side-chain interactions in the α -lactalbumin (α -LA) molten globule, we determined the effective concentration for formation of the 28–111 disulfide bond in 14 double-mutant proteins, each containing two hydrophobic core residues replaced by alanine. We compared our results with those of single-alanine substitutions using the framework of double-mutant cycle analysis and found that, in the majority of cases, the effects of two alanine substitutions are additive. Based on these results, we propose a model of side-chain–side-chain interactions in the α -LA molten globule, which takes into consideration the dynamic nature of this partially folded species.

Keywords: α -Lactalbumin; molten globule; effective concentration; side-chain interaction; double-mutant cycle analysis; protein folding

Proteins with a partially folded structure as exemplified by the molten globule state have been considered to be intermediates in protein folding as well as precursors for membrane translocation, ligand binding, and protein–protein or protein–DNA interactions (for reviews, see Ptitsyn 1996; Arai and Kuwajima 2000; see also van der Goot et al. 1991; Gursky and Atkinson 1996; Seeley et al. 1996; Carroll et al. 1997; Lo et al. 1998; Zhang and Matthews 1998). Most partially folded proteins have high levels of secondary structure and a rudimentary, natively like tertiary topology, but

the amino acid side chains in these species remain largely disordered. Despite intensive studies, the mechanism by which a protein folds into the molten globule state is still relatively unclear. Because a random amino acid polymer does not form any specific structure, the formation of the natively like tertiary topology is likely driven by favorable side-chain–side-chain interactions, specified by the particular arrangement of different types of amino acid residues along the primary sequence. This notion is supported by site-directed mutagenesis studies, showing that the alanine substitutions of hydrophobic residues, in particular those that are buried and participate in multiple packing interactions, destabilize the molten globule of α -lactalbumin (α -LA) and apomyoglobin (apoMb; Kay and Baldwin 1996; Song et al. 1998; Wu and Kim 1998; Kay et al. 1999). On the other hand, various biophysical studies have failed to detect specific side-chain–side-chain interactions in the classic molten globules. For example, the loss of the near-UV circular dichroism (CD) signal in the α -LA molten globule suggests that the aromatic side chains are located in a symmetrical environment (Kuwajima et al. 1976; Kuwajima et al. 1985). The NMR spectra acquired in various molten globule-like proteins often display a narrow range

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Abbreviations: α -LA, α -lactalbumin; α -LA-[28–111], a single disulfide variant of human α -LA in which all cysteines except for Cys28 and Cys111 were replaced by alanine; C_{eff} , effective concentration; CD, circular dichroism; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography.

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of chemical shift dispersion that is characteristic of an unfolded protein (e.g., Baum et al. 1989; Alexandrescu et al. 1993). Limited proteolysis, NMR lineshape, and NMR relaxation studies also indicate that the amino acid side chains in the α -LA molten globule undergo significant conformational fluctuations (Baum et al. 1989; Polverino de Lauro et al. 1995; Kim et al. 1999; Bai et al. 2000). Thus, the emergence of a unified view of side-chain–side-chain interactions in the molten globule state of proteins may be necessary to understand the nature of these partially folded species.

α -LA is a small, two-domain protein whose molten globule state has been studied extensively. During refolding of denatured α -LA, the protein first folds into a transient intermediate, which is subsequently converted into the native state with a much slower rate constant (Kuwajima et al. 1985; Balbach et al. 1995; Arai and Kuwajima 1996; Forge et al. 1999). This kinetic folding intermediate of α -LA has similar spectroscopic properties to those of the α -LA molten globule, which has been observed under a variety of equilibrium conditions (Ikeguchi et al. 1986; Arai and Kuwajima 1996). These conditions include low pH, the presence of low concentrations of denaturant, removal of the bound calcium in a low-ionic strength buffer, and partial or complete reduction of the disulfide bonds (for reviews, see Ptitsyn 1996; Arai and Kuwajima 2000). Previously, we have used the effective concentration (C_{eff}) for formation of the 28–111 disulfide bond as a probe for the nativelike tertiary topology in the α -LA molten globule (Peng et al. 1995; Song et al. 1998). α -LA-[28–111] is a single disulfide variant of human α -LA, in which all cysteines except Cys28 and Cys111 have been replaced by alanine. Due to the loss of the other three disulfide bonds, α -LA-[28–111] does not fold into the native state. Instead, it remains in a molten globule state even at neutral pH. This is important because the disulfide C_{eff} experiments can only be performed under neutral to slightly basic pH conditions. In addition, the simple disulfide bond configuration in α -LA-[28–111] allows a straightforward separation and quantitative assessment of the amount of oxidized and reduced proteins. Using this system, we have identified a number of hydrophobic residues for which alanine substitutions significantly reduce the C_{eff} for formation of the 28–111 disulfide bond (Song et al. 1998). In the current study, we have extended our work to include proteins with double-alanine substitutions. We compared our current results to those from single-alanine substitution studies using the framework of double-mutant cycle analysis (Horovitz and Fersht 1990). Interestingly, in the majority of the double-mutant proteins, the effects of two alanine substitutions are additive, implying that the two mutations can be considered as independent. To explain these results, as well as the results for single-alanine substitution studies, we proposed a model of dynamic side-chain–side-chain interactions in the α -LA molten globule,

which can account for all the experimental data that are so far available.

Results and discussion

Double-mutant cycle analysis as applied to the C_{eff} measurement

In the original form of double-mutant cycle analysis, the difference in the interaction energy (ΔG_I) between the folded (F) and unfolded (U) states is given by (see equation 6 in Horovitz and Fersht 1990)

$$\Delta G_I(ij)_F - \Delta G_I(ij)_U = \{(\Delta G_{\text{unf}}^0(P_{ij}) - \Delta G_{\text{unf}}^0(P_{i0})) - (\Delta G_{\text{unf}}^0(P_{0j}) - \Delta G_{\text{unf}}^0(P_{00}))\} \quad (1)$$

where $\Delta G_{\text{unf}}^0(P_{ij})$, $\Delta G_{\text{unf}}^0(P_{i0})$, $\Delta G_{\text{unf}}^0(P_{0j})$, and $\Delta G_{\text{unf}}^0(P_{00})$ are the free energy of unfolding for the wild-type protein, the protein with mutation in the j th residue, the protein with mutation in the i th residue, and the double-mutant protein, respectively. If the effects of two mutations are independent or if the interaction energies in both the folded and unfolded states are the same, equation 1 is reduced to

$$\Delta G_{\text{unf}}^0(P_{i0}) + \Delta G_{\text{unf}}^0(P_{0j}) = \Delta G_{\text{unf}}^0(P_{ij}) + \Delta G_{\text{unf}}^0(P_{00}) \quad (2)$$

Similarly, the difference in the interaction energy between the oxidized and reduced proteins can be written as

$$\Delta G_I(ij)_{\text{ox}} - \Delta G_I(ij)_{\text{red}} = \{(\Delta G_{\text{redox}}^0(P_{ij}) - \Delta G_{\text{redox}}^0(P_{i0})) - (\Delta G_{\text{redox}}^0(P_{0j}) - \Delta G_{\text{redox}}^0(P_{00}))\} \quad (3)$$

where $\Delta G_{\text{redox}}^0(P_{ij})$, $\Delta G_{\text{redox}}^0(P_{i0})$, $\Delta G_{\text{redox}}^0(P_{0j})$, and $\Delta G_{\text{redox}}^0(P_{00})$ are the redox free energy for the wild-type protein, the protein with mutation in the j th residue, the protein with mutation in the i th residue, and the double-mutant protein, respectively. In analogy to equation 2, if the effects of two mutations are independent or if the interaction energies in both the oxidized and reduced proteins are the same, equation 3 can be reduced to

$$\Delta G_{\text{redox}}^0(P_{i0}) + \Delta G_{\text{redox}}^0(P_{0j}) = \Delta G_{\text{redox}}^0(P_{ij}) + \Delta G_{\text{redox}}^0(P_{00}) \quad (4)$$

The effective concentration for formation of an intramolecular disulfide bond is defined by (Creighton 1983)

$$C_{\text{eff}} = K_{\text{intra}}/K_{\text{inter}} \quad (5)$$

where K_{intra} is the intramolecular equilibrium constant for formation of the disulfide bond, which is linked to the redox free energy by the Boltzmann relationship

$$K_{\text{intra}} = \exp(-\Delta G_{\text{redox}}^0/RT) \quad (6)$$

K_{inter} is the intermolecular equilibrium constant for the same reaction, which serves as a reference state. In practice, K_{inter} can be substituted by the redox potential of a small molecule reference reagent, such as glutathione, and can be considered as a constant. The additivity relationship (equation 4) can be rewritten using C_{eff} , except that the addition sign must be replaced by a multiplication sign

$$C_{\text{eff}}(P_{i0}) \times C_{\text{eff}}(P_{0j}) = C_{\text{eff}}(P_{ij}) \times C_{\text{eff}}(P_{00}) \quad (7)$$

Experimentally, because the ratio of C_{eff} for a mutant protein versus the wild type can often be measured more accurately, equation 7 can be transformed to

$$C_{\text{eff}}(P_{00})/C_{\text{eff}}(P_{ij}) = [C_{\text{eff}}(P_{i0})/C_{\text{eff}}(P_{ij})] \times [C_{\text{eff}}(P_{0j})/C_{\text{eff}}(P_{ij})] \quad (8)$$

where $C_{\text{eff}}(P_{ij})$, $C_{\text{eff}}(P_{i0})$, $C_{\text{eff}}(P_{0j})$, and $C_{\text{eff}}(P_{00})$ are the effective concentration for the wild-type protein, the protein with mutation in the j th residue, the protein with mutation in the i th residue, and the double-mutant protein, respectively.

Additivity of double-alanine substitutions in the α -LA molten globule

In this study, we examined the C_{eff} for formation of the 28–111 disulfide bond in 14 double-mutant proteins. These proteins were constructed based on α -LA-[28–111], each containing two hydrophobic residues replaced by alanine. Together, nine residues have been selected for mutagenesis, all of them are buried and located in the α -helical domain. The location and side-chain orientation of these residues are shown in Figure 1A. These residues were selected based on two criteria. First, at least one residue was selected from each secondary structure element. Second, none of the residues are less than three amino acids apart from each other. Six of the selected residues (L8 and L12 on the A-helix, L23 and I27 on the B-helix, Y36 on the loop between the α -helical and β -sheet domain, and W118 on the C-terminal 3_{10} helix) are critical residues that have been identified earlier (Song et al. 1998). Alanine substitutions of these residues reduce the C_{eff} for formation of the 28–111 disulfide bond by more than 40%. Figure 1B shows the distances and the number of van der Waals interactions between these residues in native α -LA. Double-alanine substitutions of these six residues were investigated systematically. We have constructed 11 double-mutant proteins in which mutations in

each of these residues were paired with mutations in all other residues except those on the same secondary structure element (for this purpose, the B-helix and the interdomain loop were considered to be the same secondary structure element). The remaining three residues (I85, I101, and W104) are located on the C- and D-helices. Substitutions of these residues by alanine have a relatively small effect on the C_{eff} for formation of the 28–111 disulfide bond. Thus, only one double-mutant protein involving each of these three residues was investigated. Altogether, the 14 double-mutant proteins can be divided into two groups. In the first group, the two residues substituted by alanine are separated by less than 5 Å in the native α -LA structure. These proteins are L8A/I27A, L8A/Y36A, L12A/L23A, L12A/I27A, L12/I85A, I27/W118A, and Y36/W118A. In the second group, the two residues substituted by alanine are separated by greater than 7 Å in the native α -LA structure. These proteins are L8A/L23A, L8A/W118A, L12A/Y36A, L12A/W118A, L23A/W118A, I27/I101A, and I27/W104A. Proteins in the second group can be considered as internal controls, because if the α -LA molten globule has a nativelike structure and the residues interact with each other strongly, we would expect that the results obtained for the first group will be different from that obtained for the second group.

Figure 2 shows the results of C_{eff} measurement for all 14 double-mutant proteins expressed as the ratio of $C_{\text{eff}}(\text{mutant})$ versus $C_{\text{eff}}(\text{wild type})$. These results are compared with the products of the same ratio as obtained for two single-alanine substituted proteins using equation 8. (These single-substitution results were taken from Song et al. [1998].) In general, the experimental results and the calculation agree reasonably well, with an average difference <15%. In addition, there is no significant difference between proteins in the first group and that in the second group. Thus, to a first-order approximation, the effects of two alanine substitutions in α -LA molten globule are additive or the mutations can be considered as independent. We can offer three possible explanations for this result. First, the α -LA molten globule may not have a nativelike structure. This is unlikely because there is a large amount of experimental evidence suggesting that in the α -LA molten globule, the structure of the α -helical domain is compact and nativelike (for reviews, see Ptitsyn 1996; Arai and Kuwajima 2000). Second, it is possible that the interaction energies in the oxidized and reduced proteins have the same magnitude, such that they cancel each other out. This is also unlikely because such a coincidence should not occur in all 14 double-mutant proteins. In addition, because the oxidized α -LA-[28–111] is significantly more stable than the reduced protein, if the two mutated residues normally interact, the interaction strength will unlikely be the same in both the oxidized and reduced proteins. Finally, it is possible that the side-chain–side-chain interactions in the α -LA molten globule are averaged out by fluctuations. Although this idea seems to be contra-

Fig. 1. (A) The structure of human α -LA in the native state (PDB code 1HML). The backbone topology and the disulfide bonds are shown in gray. The side chains of nine hydrophobic residues that were selected for mutagenesis studies are shown in black. The names of secondary structural elements and disulfide bonds are labeled. (B) The minimum distances (upper triangle) and the number of van der Waals interactions (lower triangle) between six selected residues. Alanine substitutions of these residues significantly lower the C_{eff} for formation of the 28–111 disulfide bond. Distances less than 5 Å are underlined.

Fig. 2. Double-mutant cycle analysis of double-alanine substituted α -LA-[28–111]. The ratios of the C_{eff} measured in the double-mutant proteins versus that of the wild type are shown in black. For comparison, the products of the same ratio obtained for two single-alanine substituted proteins are shown in gray. An asterisk indicates that the two mutated residues are separated by less than 5 Å in the native protein. The y-axis is plotted in a logarithmic scale such that the length of the column is proportional to the free energy.

dictory to the observation that specific residues are required at certain positions of the α -LA molten globule (e.g., see Wu and Kim 1998), a more careful analysis suggests that this idea is actually consistent with all the experimental data. In the following section, we will present a model of side-chain–side-chain interactions in the α -LA molten globule that takes into consideration the dynamic nature of this partially folded species.

A model of side-chain interaction in the molten globule state

Our model is based on the following observations. (1) Single alanine substitutions in α -LA molten globule have a significant effect on the C_{eff} for formation of the 28–111 disulfide bond. (2) The effects of two alanine substitutions appear to be independent. (3) The stability of the α -LA molten globule is lower than that of the native protein and the amino acid side chains undergo significant conformational fluctuations. The schematics of the model are shown in Figure 3. In the native state, each residue in the protein interacts with a small number of other residues defined by the native three-dimensional structure and these interactions

are present 100% of the time. However, in the molten globule state, because of the higher side-chain mobility, each residue now can interact with a large number of other residues. For each given pair, the specific interaction is only present a small fraction of the time. In order to understand the energetic consequence of mutations, one must take an average of all possible conformations. Figure 3B shows a hypothetical example. If one truncates the side chain of a particular residue, in the native state the protein will lose all side-chain interactions mediated by that residue. The same is true for the molten globule state, except that the total interaction strength now is lower, because the molten globule is less stable. The biggest difference between the native and the molten globule state is in how the truncation of one residue affects another. In the native state, if one first truncates residue II or III in Figure 3B, then the subsequent truncation of residue 2 will have 50% of the effect as truncation of residue 2 in the wild-type protein. This is the origin of the interaction energy. However, in the molten globule state, because of the dynamic averaging, the truncation of residue II or III will only have 30% of the effect as truncation of residue 2 in the wild-type protein. (This is a two-dimensional model. In three-dimensional space, it is likely that the effect of dynamic averaging will be more

Fig. 3. A model of dynamic side-chain-side-chain interactions in the α -LA molten globule. (A) A schematic illustration. In the native protein, the side-chain interactions are rigid and specific. In the molten globule, the amino acid side chains are flexible and the interactions are averaged by conformational fluctuations. (B) The strength of side-chain-side-chain interactions between residue 2 and residues I, II, III, and IV in the native and molten globule states of proteins. The values are hypothetical. Note that the sum of all interactions in the molten globule state is ~50% of that in the native state, because the molten globule is less stable. The specific interaction between a given pair of residues is even lower due to dynamic averaging.

significant.) In other words, the two residues will behave more independently.

Discussion and future prospects

Our data suggest that in the α -LA molten globule, specific pairwise interactions between amino acid side chains are significantly attenuated. This is consistent with the disrupted side-chain packing and a noncooperative thermal unfolding transition. However, one should not conclude that favorable side-chain-side-chain interactions are not important for maintaining the nativelike structure in the α -LA molten globule. Instead of relying on specific pairwise interactions, each residue in the molten globule can be considered as interacting with a dynamic ensemble or a mean field created by many other residues. The specificity of side chain-side chain interactions in the molten globule state becomes less important, because it is averaged out by fluctuation. However, the strength of such interactions may still be responsible for maintaining the stability of the molten globule. Thus, eliminating all interactions mediated by a particular side chain will have a significant effect. Although this is a general model, each individual residue in the α -LA molten globule is expected to behave somewhat differently.

For example, the discrepancy between the measured and predicted values for residue pairs 8–27, 8–36, 12–36, and 36–118 are somewhat larger than the experimental error. Whether this is an indication of residual-specific interactions remains to be investigated.

Although the idea of dynamic side-chain averaging has been implicit in the discussion of molten globules for some time, this is the first time that double-mutant cycle analysis has been used to directly measure the strength of specific side-chain-side-chain interactions in a molten globule. Thus, it is still unclear to what extent this model is applicable to other systems. The molten globule states of equine and canine milk lysozyme both exhibit a cooperative thermal denaturation and partially ordered side chains as indicated by calorimetry and near-UV CD spectroscopy (Van Dael et al. 1993; Griko et al. 1995; Morozova-Roche et al. 1997; Koshiba et al. 1999; Kobashigawa et al. 2000). It is possible that specific side-chain-side-chain interactions are more pronounced in these systems. From a different point of view, it would be nice if double-mutant cycle analysis can be used to detect the differences in conformational specificity in different states of proteins. In order to achieve this, one needs to choose a system that can switch between the molten globule and the native state, such that the interaction

energy can be measured and compared under two different conditions. The results of single-residue substitutions in the pH 4 intermediate of apoMb exhibit a similar pattern as in the α -LA molten globule (Kay and Baldwin 1996; Kay et al. 1999). In this regard, apoMb could be a good model system for future study.

If a protein can fold into a natively like tertiary fold without specific pairwise interactions, we speculate that it should be possible to predict the overall structure of a protein by using only single-residue information. This idea is consistent with the observation that proteins with a randomized hydrophobic core can often fold into the same structure as the wild-type protein and even have the same activity (Lim and Sauer 1989; Lim and Sauer 1991; Axe et al. 1996). Similarly, in the α -LA molten globule and T4 lysozyme, it has been shown that the entire hydrophobic core can be replaced by leucine or methionine (Gassner et al. 1996; Wu and Kim 1997). This single-residue approximation resembles the mean field approximation used in statistical physics. Computer algorithms based on this approximation have been developed recently to model biological macromolecules (for review, Koehl and Delarue 1996). Recent structural prediction studies also show that the algorithms based on single-residue information, such as PSI-BLAST, perform as well as algorithms based on specific pairwise interactions (for review, Jones 2000). Thus, it is possible that studies along this line will eventually lead to the design of more efficient structural prediction algorithms.

Materials and methods

Proteins

Two methods were used to construct the genes encoding double-mutant proteins. First, for those proteins that contain one alanine substitution in the N-terminal half of α -LA and another in the C-terminal half, the gene was made by restriction-enzyme digestion of the vectors containing single-alanine substitutions by the enzymes *Xba*I and *Bgl*II and subsequent exchange of the smaller fragment containing the first 56 residues of α -LA (the *Xba*I site is located 39 bp upstream of the synthetic α -LA gene in the cloning vector pAED4 and the *Bgl*II site is located between residues 55 and 57 in the β -sheet domain). Second, for those proteins that contain two alanine substitutions relatively close to each other, the genes were made by site-directed mutagenesis (Kunkel et al. 1987) using the vector containing a single-alanine substitution as a template. All mutations were confirmed by manual or automated DNA sequencing throughout the entire coding region. Proteins were expressed in *Escherichia coli* BL21 and purified as described previously (Peng and Kim 1994; Schulman et al. 1995). Purified proteins were lyophilized and stored at -80°C .

Disulfide C_{eff} measurement

Disulfide C_{eff} measurements were performed as described previously using oxidized and reduced glutathione as reference reagents (Peng et al. 1995; Song et al. 1998). Concentrations of protein-

stock solutions were determined from their absorbance at 280 nm in 6 M guanidine hydrochloride (Edelhoch 1967). As a control, all reactions were started from both the oxidized and reduced proteins. After the reaction had reached equilibrium, the reaction mixtures were separated on a reverse-phase HPLC by using a Vydac C18 analytical column, and the amount of oxidized and reduced proteins was quantified by using a peak integration algorithm (Millennium 3.0, Waters Cooperation). The difference in C_{eff} for reactions started from different initial conditions was typically $<10\%$. For all except three double-mutant proteins, the C_{eff} measurements have been repeated three to four times and the standard deviation is indicated by the error bar in Figure 2. In order to reduce experimental variability, for each set of experiments, a sample of wild-type α -LA-[28-111] was always included as an internal standard. Only the ratio of $C_{\text{eff}}(\text{mutant})/C_{\text{eff}}(\text{wild type})$ was used to calculate the additivity relationship. The errors for the single-alanine substitution studies were estimated based on 5% error for each measurement and the rule of error propagation.

Circular dichroism

CD spectroscopy was used to check the amount of secondary structure in double-mutant proteins. These experiments were performed on a Jasco J-715 CD spectropolarimeter equipped with a thermoelectric temperature controller. The shapes of the CD spectra for all double-mutant proteins are similar to that of the wild type, although those proteins with a low C_{eff} also often exhibit a slightly lower amount of α -helical secondary structure. The difference was typically $<20\%$.

Molecular graphics and computational tools

The distances and the number of van der Waals contacts between different residues in human α -LA were calculated using the program Insight II and the PDB data set 1HML assuming a 5 Å cutoff for van der Waals interactions. Figure 1A was prepared using the program MOLMOL (Koradi et al. 1996) and Pov-front.

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