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Hydrogen-Exchange Kinetics of Reduced α-Lactalbumin Bound to the Chaperonin GroEL[1]

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α-Lactalbumin in which all the disulfide bonds are fully reduced (RLA) is known to bind strongly to the chaperonin GroEL. Although RLA is more unfolded than the native state and the molten globule state of α-lactalbumin, the CD spectrum of RLA in the far-UV region shows that RLA is not fully unfolded but has an appreciable amount of secondary structure. To investigate whether the secondary structure elements present in RLA are responsible for the recognition of RLA by GroEL or not, we have examined the hydrogen-exchange kinetics of RLA in the presence and absence of GroEL. Our results show that the hydrogen-exchange kinetics of RLA bound to GroEL is identical to that of free RLA. This implies that the secondary structure elements in RLA are not important for the recognition by GroEL, but the unstructured parts of RLA that are not relevant to the stability of the secondary structure provide strong recognition sites of RLA.

Key words: chaperonin, GroEL, hydrogen exchange, α-lactalbumin, molecular chaperone.

Protein folding *in vivo* is an assisted process which is mediated by folding catalysts and by molecular chaperones (*1, 2*). The *Escherichia coli* chaperonin GroEL is the best-studied molecular chaperone so far. GroEL is a homo-oligomeric complex composed of two rings, each containing seven 57-kDa subunits (*3, 4*). GroEL suppresses irreversible aggregation of proteins and promotes productive folding of a protein to the native state with the aid of the co-chaperonin GroES and ATP hydrolysis. Recent studies have demonstrated that the non-native protein accommodated in the GroEL central cavity underneath GroES can fold into the native state in this location (*5-7*).

Although the mechanism by which GroEL mediates protein folding is still obscure, it is clear that the formation of a complex between GroEL and a target protein in its non-native state is an initial step in the process of GroEL action. Hence, to understand the mechanism by which GroEL mediates folding, it is important to know the structural elements and features that are recognized by GroEL. Despite many studies, this remains unclear. Hydrophobic surfaces exposed by a target protein are important for the interaction (*8-11*). It has been reported that GroEL binds a polypeptide in an α-helical conformation (*12, 13*), although it also interacts with an all-β-protein (*14*). It has also been suggested that the molten globule state of a protein is recognized by GroEL (*15-17*). We have, however, shown that disulfide bond-reduced α-lactalbumin is about 100 times more strongly recognized by GroEL than the disulfide-intact protein in the molten globule state (*18, 19*), suggesting that the features recognized by GroEL are not necessarily characteristics of the molten globule state. Electrostatic properties of a target protein have an important influence on the interaction with GroEL (*9, 18*). Since GroEL is highly negatively charged at neutral pH, it has higher affinity for a positively charged polypeptide than for a negatively charged one, and electrostatic screening by ions affects the interaction between GroEL and a target protein.

Analysis of the hydrogen-exchange reaction of a target protein bound to GroEL should be useful for elucidating the structural elements and features important for the recognition by GroEL. In their study of GroEL-promoted hydrogen exchange examined by NMR spectroscopy, Zahn *et al.* have shown that the complete secondary structure and tertiary structure of cyclophilin are globally destabilized when the molecule is complexed with GroEL (*20*). It has also been shown that GroEL binds native barnase and deeply buried amide protons of barnase can exchange with solvent protons when the protein is bound to GroEL, although they are protected from exchange in the native state (*21, 22*). By hydrogen-exchange experiments in combination with mass spectroscopy, Robinson *et al.* have shown that the GroEL-bound state of α-lactalbumin is similar to or more unfolded than the uncomplexed molten globule state of the protein (*16*). These studies have provided a basis for the "annealing" model in which the association of a target protein with GroEL results in the unfolding of an unproductive, or

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Abbreviations: holo-LA, bovine holo-α-lactalbumin; apo-LA, derivative of bovine α-lactalbumin in which the bound calcium ion is removed; CAM-3SS-LA, derivative of bovine α-lactalbumin in which the disulfide bond between Cys6 and Cys120 is selectively reduced and *S*-carboxyamidomethylated; RLA, derivative of bovine α-lactalbumin in which all the disulfide bonds are reduced; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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trapped, folding intermediate, giving the protein a chance to resume folding (*22-24*). However, in the studies of cyclophilin and barnase, the hydrogen-exchange reactions of the GroEL-bound states were compared with those of their native states, and Robinson *et al.* compared the hydrogen-exchange reaction of the GroEL-bound state of one α-lactalbumin derivative with the reaction of another α-lactalbumin derivative in the absence of GroEL. Thus, these three studies do not refer to the hydrogen-exchange characteristics of the non-native, free states that should be recognized by GroEL.

In this study, we have investigated the hydrogen-exchange reactions of disulfide bond-reduced α-lactalbumin both in the free state and in the GroEL-bound state. The α-lactalbumin in which the four disulfide bonds are fully reduced (reduced α-lactalbumin; RLA) assumes a more relaxed and expanded structure than the molten globule state of the disulfide-intact protein and exposes hydrophobic surfaces on the protein. RLA is, however, not fully unfolded but has an appreciable amount of secondary structure as revealed by the far-UV CD spectrum. It has been reported that RLA is bound to GroEL so strongly that the complex between them can be detected by gel-filtration chromatography (*15, 19*). Here, we have compared the hydrogen-exchange reactions of RLA in the free state and the GroEL-bound state, and addressed the question of whether the stability of hydrogen bonds in the secondary structure elements present in RLA is changed by GroEL or not. Our results show that the hydrogen-exchange reaction of RLA bound to GroEL is identical to that of free RLA, indicating that the secondary structure elements in RLA are not important for the binding to GroEL. Unstructured and hydrophobic parts of RLA may be more important for the strong recognition of RLA by GroEL. Putative binding sites in RLA recognized by GroEL will be discussed.

MATERIALS AND METHODS

Materials—GroEL was prepared from *E. coli* cells TG1 bearing the expression plasmid pKY206, which was a kind gift from Dr. K. Ito (*25*). The purification method of GroEL was as reported (*12*), except that we used Sephacryl S-300 and DEAE-Sephacel for gel-filtration and ion-exchange chromatography. GroEL was stored in 15% glycerol solution. An extinction coefficient at 280 nm of 0.285 liter·g^{-1}·cm^{-1} was used to determine the concentration of GroEL. We used bovine α-lactalbumin (holo-LA) that had been prepared as described (*26, 27*). Apo-α-lactalbumin (apo-LA) was prepared from holo-LA as described (*19*). Reduced α-lactalbumin (RLA) was prepared as described (*28*). CAM-3SS-LA, a derivative of α-lactalbumin in which the disulfide bond between Cys6 and Cys120 is selectively reduced and *S*-carboxyamidomethylated, was prepared as described (*29*). The concentrations of holo-LA, apo-LA, CAM-3SS-LA, and RLA were determined using molar extinction coefficients at 280 nm of 28,500, 27,700, 27,700, and 27,200 M^{-1}·cm^{-1}, respectively (*28*).

Preparation of Isotope-Labeled Proteins—Holo-LA (125 nmol) was mixed with ^{14}C-labeled apo-LA prepared by the method of Jentoft and Dearborn (*30*); the molar ratio of ^{14}C-labeled apo-LA to holo-LA was 0.003. Reduction of holo-LA containing ^{14}C-labeled apo-LA and tritium-labeling of the amide protons were performed simultaneously in buffer A (100 mM Tris, 100 mM DTT, 1 mM EDTA, 7.2 M urea, pH 8.6; total volume was 44 μl and the concentration of tritium was 2.1 GBq/ml) by incubating the reaction mixture for 10 min at 25°C. The reactions were stopped by adding 1.45 ml of ice-cold buffer B (100 mM HCOOH-HCOONH$_4$, pH 3.0). Chemical reagents and tritium water were separated from the labeled protein by a HiTrap™ desalting column (Pharmacia, Sephadex-G25) equilibrated with buffer B at 4°C. Tritium-labeled RLA was lyophilized and stored until the hydrogen-exchange experiments. The preparation methods of isotope-labeled apo-LA and CAM-3SS-LA were essentially the same as that of RLA except that cold apo-LA and cold CAM-3SS-LA were used instead of holo-LA, and buffer A did not contain DTT.

Hydrogen-Exchange Experiments—The hydrogen-exchange reaction of RLA was initiated by dissolving tritium-labeled RLA in buffer C [20 mM MOPS-KOH (pH 7.0), 1 mM EGTA, 50 mM KCl, 1 mM DTT]. Buffer C contained GroEL when we examined the hydrogen exchange of RLA in the presence of GroEL. Hydrogen-exchange experiments of apo-LA and CAM-3SS-LA were performed in essentially the same manner, except that DTT was not contained in buffer C for the experiment with CAM-3SS-LA and that DTT and KCl were not contained for apo-LA. CAM-3SS-LA was in the apo state during the reaction because the buffer contained EGTA. An aliquot (200 μl) of the reaction mixture was withdrawn at an indicated time and mixed with 300 μl of ice-cold buffer B to stop the exchange reaction. Then tritium in water was removed with a NAP™-5 column (Pharmacia, Sephadex G-25) equilibrated with ice-cold buffer B. The ^{3}H cpm and ^{14}C cpm of the protein eluted from the NAP™-5 column were determined as described using a liquid scintillation counter (*19*). Since the amount of the protein eluted from the NAP™-5 column was different each time, the ratio of ^{3}H cpm to ^{14}C cpm was calculated for normalization. The ratio of ^{3}H cpm to ^{14}C cpm of the labeled protein at zero time was determined by an independent hydrogen-exchange measurement of the same sample at 0°C and pH 3, where hydrogen exchange of the protein hardly occurred on a time scale of minutes.

Gel-Filtration Chromatography—Lyophilized RLA was dissolved in buffer C with or without GroEL. The concentration of RLA was 6.5 μM. The concentration of GroEL, if present, was 13 μM. The solution was incubated for 10 min at 25°C, and an aliquot (1 ml) was applied to a Sephadex G-100 column (1 × 40 cm) equilibrated with buffer C. The eluted proteins were detected by measurement of the fluorescence intensity excited at 295 nm. The chromatography and detection of the proteins were performed with a Waters 626 LC system and a Waters 474 scanning fluorescence detector.

CD Measurements—The CD spectra of RLA, apo-LA, and CAM-3SS-LA at pH 7.0 were measured in the same buffers as used in the hydrogen-exchange experiments. The spectrum of holo-LA was measured in buffer D [20 mM MOPS-KOH (pH 7.0), 50 mM KCl]. The spectra of RLA at pH 8.5 and 2 were measured in buffer E [20 mM Tris-HCl (pH 8.5), 1 mM EGTA, 50 mM KCl, 1 mM DTT] and 10 mM HCl, respectively. All the CD measurements were performed in a Jasco J-720 spectropolarimeter at 25°C.

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RESULTS

CD Spectra—Figure 1A shows the CD spectrum of RLA compared with the spectra of other forms of α-lactalbumin, holo-LA, apo-LA, and CAM-3SS-LA, in the far-UV region between 190 and 240 nm. These spectra were measured using a cell with 0.1-mm path length. The spectrum of RLA was taken at pH 8.5 where no aggregation of RLA was observed (see below), and the spectra of the other forms of α-lactalbumin were taken at pH 7.0. As can be seen from Fig. 1A, the breakage of the disulfide bond between Cys6 and Cys120 in CAM-3SS-LA makes the molecule more unfolded than disulfide-intact apo-LA and holo-LA, and the breakage of all the other disulfide bonds in RLA makes the RLA molecule even more unfolded. However, the CD spectrum in the far-UV region apparently indicates that RLA is not fully unfolded but still has an appreciable amount of secondary structure. The amounts of secondary structure in RLA and in the other forms of α-lactalbumin were thus calculated by three methods (*31-33*) from the CD spectra shown in Fig. 1A, and the results are summarized in Table I. It is clear that RLA contains quite large amounts of secondary structure. Thus, if these elements of the secondary structure, most probably α-helices, are recognized by GroEL, the stability of the secondary structure would be changed by GroEL.

Since the hydrogen-exchange experiments on RLA and GroEL-bound RLA were carried out at pH 7.0, the CD spectrum of RLA was first measured at pH 7.0. At pH 7.0, however, RLA gradually aggregated at 30 μM (the minimum protein concentration required for the CD measurement using a cell with 0.1-mm path length). This aggregation was observed as a change of CD spectra (Fig. 1B) and as an increase in optical density at 350 nm (data not shown). The increase of CD intensity of RLA between 210 and 235 nm at pH 7.0 may be due to formation of intermolecular β-structure by aggregation (Fig. 1B). The RLA sample that was prepared for the hydrogen-exchange experiments was subjected to CD measurement at the protein concentration required for hydrogen-exchange experiments (6.5 μM) to examine the presence or absence of intermolecular aggregation (see below).

Gel-Filtration Chromatography—The gel-filtration experiments on RLA in the presence and absence of GroEL were performed under the same conditions as used in the following hydrogen-exchange experiments, *i.e.*, at pH 7.0 at two different temperatures, 0 and 25°C, to confirm the binding between GroEL and RLA under those conditions. RLA in buffer C was applied to a Sephadex G-100 column.

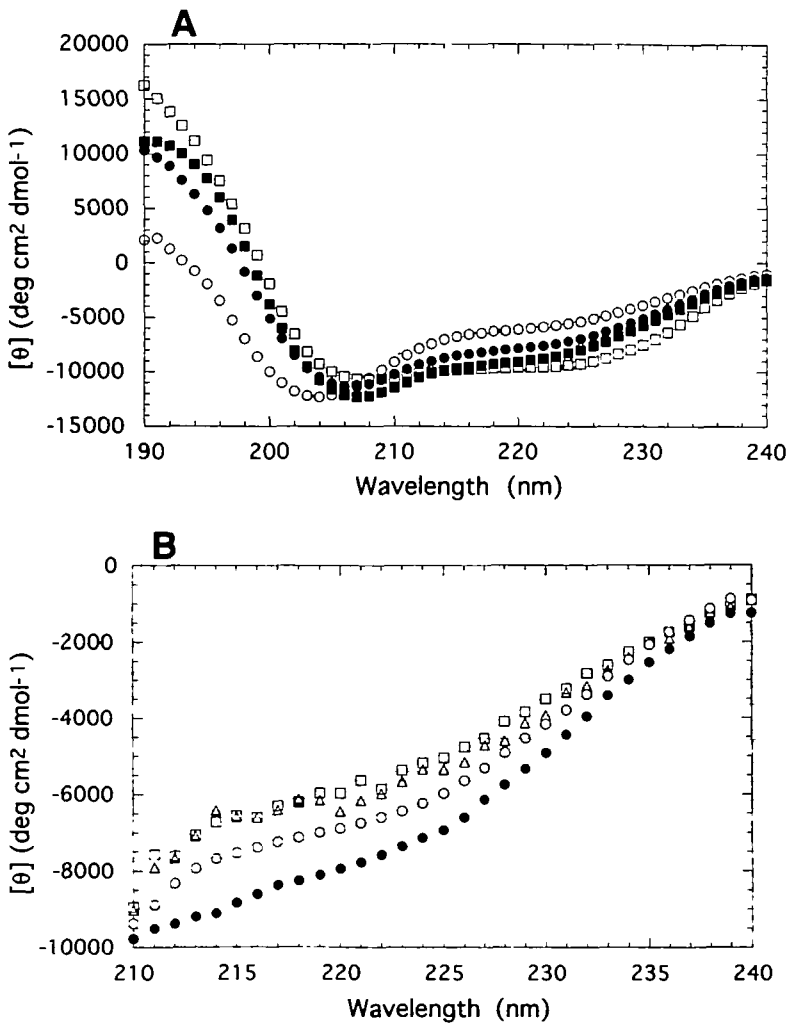

Fig. 1. **(A) CD spectra of α-lactalbumin variants measured with a cell with 0.1-mm path length.** The protein concentrations were between 90 and 270 μM for all measurements. Open squares (□), holo-LA; filled squares (■), apo-LA; filled circles (●), CAM-3SS-LA; open circles (○), RLA. The spectra of holo-LA, apo-LA, and CAM-3SS-LA were measured at pH 7.0. The spectrum of RLA was measured at pH 8.5. **(B) CD spectra of RLA.** Open triangles (△) represent the spectrum measured just after dissolving the lyophilized protein prepared for the hydrogen-exchange experiments in buffer C at pH 7.0 (protein concentration was 12.9 μM). The lyophilized protein was also dissolved in buffer C at pH 7.0 at a protein concentration of 30.1 μM, and the CD spectrum was measured after 15 min (○) and 65 min (●). Open squares (□) represent the spectrum at pH 2 (protein concentration was 37.5 μM). The spectra of RLA at 15 and 65 min after dissolving the lyophilized protein were measured using a cell with 0.1-mm path length. The other spectra were measured using a cell with 1-mm path length.

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Buffer C contained a twice molar excess of 14-mer GroEL when the chromatography of RLA was performed in the presence of GroEL. Figure 2 shows that the peak of RLA disappeared at both temperatures when GroEL was present. Thus, RLA is bound to GroEL under the conditions used in the hydrogen-exchange experiments.

Hydrogen-Exchange Kinetics of RLA—For the hydrogen-exchange experiments on RLA in the presence and absence of GroEL, all the exchangeable protons in RLA were first exchanged for tritium in a solution containing tritium water. Since RLA is susceptible to aggregation, the labeling of RLA with tritium was done in a solution containing 7.2 M urea at 25°C to circumvent the aggregation. The labeled protein was separated from urea and solvent tritium on a Sephadex G-25 column and then lyophilized for the following hydrogen-exchange experiments. We measured the CD spectrum of the labeled RLA at a protein concentration of 13 μM in the same buffer as used in the hydrogen-exchange experiments and confirmed that there was no aggregation, *i.e.*, the spectrum just after dissolving the protein in the buffer was identical to the spectrum of non-aggregated RLA at pH 2 (Fig. 1B). When RLA had been labeled in a solution without urea at 25°C, however, the light scattering of the RLA solution measured in terms of the optical density at 350 nm increased and the CD spectrum of RLA changed from the spectrum of non-aggregated RLA at pH 2 (data not shown). We also confirmed that all four disulfide bonds were fully reduced in labeled RLA by quantitative analysis of the amount of thiol groups in the protein (*34, 35*). The thiol analysis by the Ellman method detected 7.6 thiol groups per molecule of α-lactalbumin.

The hydrogen-exchange reaction of RLA was initiated by dissolving the labeled RLA in buffer C. In Fig. 3A, the exchange reaction of free RLA in the absence of GroEL is compared with the exchange reaction of fully unfolded RLA in 5 M guanidine hydrochloride. The exchange reaction of the fully unfolded protein was too fast to measure under the present conditions, and all the tritium in the protein was exchanged within the first 20 s. The exchange reaction of free RLA in the absence of the denaturant, however, shows significant protection against exchange, and the fraction of tritium remaining was 0.17 at 10 s and 0.07 at 20 s, suggesting that these amide protons may be protected by hydrogen bonds formed in the secondary structure in RLA. Next, we investigated the exchange reaction of RLA in the presence of GroEL, and this was compared with the exchange reaction of free RLA. When the hydrogen-exchange kinetics was measured in the presence of GroEL, buffer C contained a twice molar excess of 14-mer GroEL. The results show that the hydrogen-exchange kinetics are the same whether GroEL is present or not (Fig. 3A). We also investigated the hydrogen-exchange reaction of apo-LA in the molten globule state. The results show that the exchange rate of apo-LA in the absence of GroEL is slower than that of RLA (Fig. 3A), indicating that some amide protons in apo-LA are more protected than those in RLA. We also investigated the hydrogen-exchange reaction of CAM-3SS-LA. The results show that the exchange rate of CAM-3SS-LA is intermediate between those of RLA and apo-LA (Fig. 3A).

At pH 7.0 and 25°C, the hydrogen-exchange rate is so fast that about 80% of tritium in RLA is exchanged within 10 s (Fig. 3A). Thus, we next examined the hydrogen-exchange kinetics of RLA at pH 7.0 and 0°C. The results (Fig. 3B) show that the hydrogen-exchange kinetics of RLA at 0°C remains the same whether GroEL is present or not. Even when the concentrations of both proteins were about three times higher, no more change in the hydrogen-exchange kinetics of GroEL-bound RLA was observed. These results

TABLE I. **Secondary structure fractions of α-lactalbumin variants calculated from three sets of reference data.** The contents of secondary structure were calculated from the far-UV region spectra (190-240 nm) shown in Fig. 1A according to the methods of Greenfield and Fasman (*31*), Saxena and Wetlaufer (*32*), and Chen *et al.* (*33*). f_α, f_β, and f_c are the fractions of α-helix, β-sheet, and random coil, respectively.

	Greenfield and Fasman (1969)			Saxena and Wetlaufer (1971)			Chen *et al.* (1974)		
	f_α (%)	f_β (%)	f_c (%)	f_α (%)	f_β (%)	f_c (%)	f_α (%)	f_β (%)	f_c (%)
Holo-LA at pH 7.0	15	44	41	35	9	56	37	11	52
Apo-LA at pH 7.0	14	41	45	31	11	58	33	15	52
CAM-3SS-LA at pH 7.0	12	40	48	31	8	61	30	16	54
RLA at pH 8.5	10	34	56	26	5	69	24	14	62

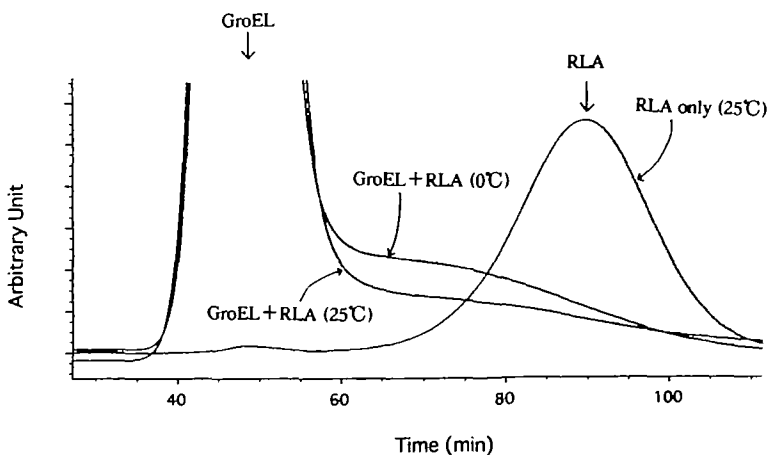

Fig. 2. **Gel-filtration chromatography of RLA in the presence and absence of GroEL at pH 7.0 at 25 and 0°C.** The complex between GroEL and RLA eluted at the void volume (elution time of around 48 min) and RLA alone eluted at around 90 min on the column. The eluted proteins were monitored by measuring the fluorescence intensity excited at 295 nm.

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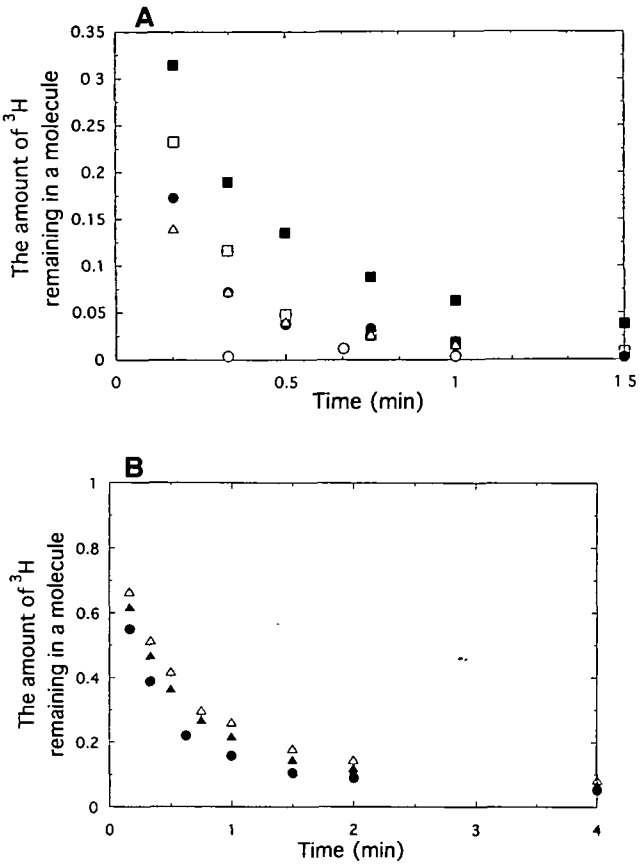

Fig. 3. **(A) Hydrogen-exchange reactions of RLA in the presence of GroEL (△) and in the absence of GroEL (●) at pH 7.0 and 25°C.** Hydrogen-exchange of apo-LA (■) and CAM-3SS-LA (□) is also shown in the figure. The concentrations of apo-LA, CAM- 3SS-LA, and RLA were 6.5 μM and the concentration of GroEL was 13.0 μM. The hydrogen-exchange reaction of RLA in the presence of 5 M guanidine hydrochloride (○) is also shown in the figure for reference. **(B) Hydrogen-exchange reactions of RLA in the presence and absence of GroEL at 0°C.** Open triangles (△) and filled circles (●) represent the reactions of 6.5 μM RLA at pH 7.0 in the presence and absence of 11.6 μM GroEL, respectively. Filled triangles (▲) represent the reaction of 19.6 μM RLA at pH 7.0 in the presence of 31.7 μM GroEL. The amount of tritium remaining in the molecule at zero time is 1 in the ordinates of both figures.

in Fig. 3, A and B, thus demonstrate that the interaction between GroEL and RLA leads to no further protection against or promotion of the amide-proton exchange in RLA. Thus, the hydrogen bonds in the secondary structure elements in RLA are neither stabilized nor destabilized when RLA is bound to GroEL, although RLA has quite large amounts of secondary structure, as described above. What are recognized in RLA by GroEL are thus not the secondary structure elements, but unstructured parts, which have no influence on the stability of hydrogen bonds in the secondary structure, in the RLA molecule (see "DISCUSSION").

DISCUSSION

In the local-unfolding model of hydrogen exchange, the proton transfer reaction with solvent catalysts is interrupted by stable hydrogen bonding in a protein, and the exchange of the protected hydrogens occurs *via* transient open states in which hydrogen bonds are broken (*36, 37*). Therefore, the hydrogen-exchange reaction is sensitive to the stability of the hydrogen bonds, which are mainly formed in secondary structure in a protein. The hydrogen exchange is thus a useful technique to investigate the stability of secondary structure of a target protein in the complex with GroEL and to understand the role of secondary structure in the formation of the complex between the proteins. Several hydrogen-exchange studies, including the studies of cyclophilin and barnase bound to GroEL, have already been reported (*20-22*). Robinson *et al.* have also shown by means of hydrogen-exchange experiment that the GroEL-bound state of α-lactalbumin is similar to or more unfolded than the uncomplexed molten globule state of the protein (*16*). However, in the studies of cyclophilin and barnase, the hydrogen-exchange reactions of the GroEL-bound states were compared with those of their native states. Robinson *et al.* compared the hydrogen-exchange kinetics of the GroEL-bound state of an α-lactalbumin derivative (a disulfide bond-rearranged species) with the kinetics of CAM-3SS-LA, which was not bound by GroEL, because the former derivative aggregated when GroEL was absent. Thus, in all of these studies, the hydrogen-exchange reactions of the GroEL-bound states were compared with those of the states that were not strongly recognized by GroEL, thus providing little information about the structural features in the proteins that were important for the recognition by GroEL. It is important to investigate the hydrogen-exchange characteristics of a non-native, free state that is recognized by GroEL and to compare these characteristics with those of the GroEL-bound state. RLA exists in a non-native state that must contain the structural features recognized by GroEL even when GroEL is absent. RLA is thus a pertinent model protein to investigate the structural determinants in a target protein required for binding to GroEL.

Landry *et al.* have reported that GroEL binds a polypeptide in an α-helical conformation and proposed that GroEL interacts with sequences in an incompletely folded chain that have potential to adopt an amphipathic α-helix structure and that the chaperonin binding promotes the formation of the helix (*12, 13*). However, the binding constant between GroEL and the polypeptide reported (10^3 to 10^5 M^{-1}) is much smaller than that between GroEL and RLA (about 10^7 M^{-1}). It has been suggested that there is a minimum threshold length of 55-65 amino acid residues for a polypeptide to be strongly bound by GroEL (*38*). Therefore, the polypeptide employed by Landry *et al.* may be so short (13 amino acids) that GroEL does not bind it strongly. The results of Landry *et al.* thus do not necessarily mean that α-helical elements are indispensable for the formation of a stable complex between GroEL and the target protein that has a sufficient size for the binding to GroEL. RLA has a size of 123 amino acid residues, and it is strongly bound to GroEL and has an appreciable amount of α-helix (Fig. 1 and Table I). RLA is thus a suitable protein to investigate whether the secondary structure elements present in a target protein are responsible for the formation of a stable complex with GroEL.

Since there must be dynamic equilibrium between the structural (α-helical) and unfolded (open) forms in the regions where the α-helical secondary structure of RLA is formed, GroEL would recognize either α-helical or open

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form of these regions, if they are the regions bound by GroEL. Thus, in the following, we consider how the binding of either form of these regions to GroEL would affect the dynamic equilibrium between the two forms and hence the hydrogen-exchange kinetics of the amide protons. The hydrogen-exchange reaction of the amide protons in the regions where the α-helical structure of RLA is formed is, in general, represented by the local-unfolding model as:

$$H \rightleftharpoons C \xrightarrow{k_{ex}} C^* \quad (1)$$

where H represents an α-helical form in which the hydrogen exchange of the amide protons with solvent is protected by hydrogen bonding; C represents an open form, from which hydrogen exchange of the amide protons takes place with a rate constant of k_{ex}; and C* represents the state in which the amide protons have been exchanged with solvent protons. The rate constants of structural opening and closing between H and C are expected to be close to the helix-coil transition rate of a polypeptide, so that they must be much faster than k_{ex} (EX2 mechanism) in RLA. Therefore, the apparent exchange rate constant of the amide protons in the regions where the α-helical structure of RLA is formed (k_{app}) is given by:

$$k_{app} = \frac{[C]}{[H]+[C]} \times k_{ex} \quad (2)$$

where [H] and [C] are the concentrations of H and C. The concentration [H] or [C] is the sum of the concentrations of the free and the bound species, if the species is bound to GroEL, and it is assumed that the binding of C to GroEL does not change the exchange rate k_{ex}. Then, if H is bound to GroEL, [C] decreases because of the dynamic equilibrium between H and C (see Scheme 1). As a result, k_{app} decreases according to Eq. 2. If C is bound to GroEL, [C] increases, and as a result, k_{app} increases according to Eq. 2. Therefore, if the regions where the α-helical structure of RLA is formed are bound to GroEL, the hydrogen-exchange rate of RLA should increase or decrease when GroEL is present. However, our results show that the hydrogen-exchange kinetics of RLA bound to GroEL is identical to that of free RLA, although RLA has a quite large amount of α-helical secondary structure. Our results thus indicate that the regions where the α-helical structure of RLA is formed are not bound to GroEL. Theoretically, it might be possible that, when C is bound to GroEL, some amide protons of the bound segment are protected from hydrogen exchange because of the shielding of the amide protons by binding to GroEL, and as a result, the apparent hydrogen-exchange rate of RLA does not change. However, it is unlikely that the increase in [C] and the shielding of the amide protons by binding to GroEL would exactly cancel each other out. In any event, it is clear from our results that the α-helical form (H in Scheme 1) of RLA is not recognized by GroEL. Nevertheless, our results do not necessarily mean that the secondary structure elements of a protein are not recognized at all by GroEL. In RLA, the unstructured parts are much more strongly recognized by GroEL than the secondary structure elements, so that the affinity of these elements for GroEL might become irrelevant.

The studies of barnase and cyclophilin (*20-22*) have shown that both proteins bound to GroEL are apparently unfolded and that all the amide protons rapidly exchange with solvent in the GroEL-bound states. However, in these studies, the hydrogen-exchange reactions of the GroEL-bound states were compared with those of the native states. Therefore, it does not follow from these studies that both proteins bound to GroEL are fully unfolded as they are in strong denaturant. Our results indicate that the unstructured parts of RLA are sufficient for the strong interaction of RLA with GroEL and the interaction with GroEL does not bring about further unfolding of the α-helical structure in RLA.

It is known that the hydrophobicity and the electric charge of a target protein are both important for the interaction between GroEL and the target protein; hydrophobic and/or positively charged parts must be more strongly recognized by GroEL than other parts (*8, 9, 18*). Thus, to investigate which parts in RLA are important for the interaction between GroEL and RLA, we prepared a hydropathy plot of α-lactalbumin by the method of Kyte and Doolittle (*39*) and also calculated the average electric charge along the primary structure of the protein (Fig. 4). Studies of the molten globule state of α-lactalbumin have shown that the A, B, and D-helices, and the 3_{10}-helix near the C terminus constitute the core of the molten globule (*40, 41*). These helices that form the core of the molten globule may also form the α-helical parts present in RLA. The other parts that are unfolded in the molten globule state should also be unfolded in RLA, and may provide the strong recognition sites of RLA by GroEL. In Fig. 4, those helices that form the core of the molten globule state are also indicated. As shown in Fig. 4, the part between residues 90 and 100 is positively charged and shows high hydropathic indices. The part between residues 70 and 80 is also hydrophobic, while the average charge is almost neutral. We propose that these parts that do not form the

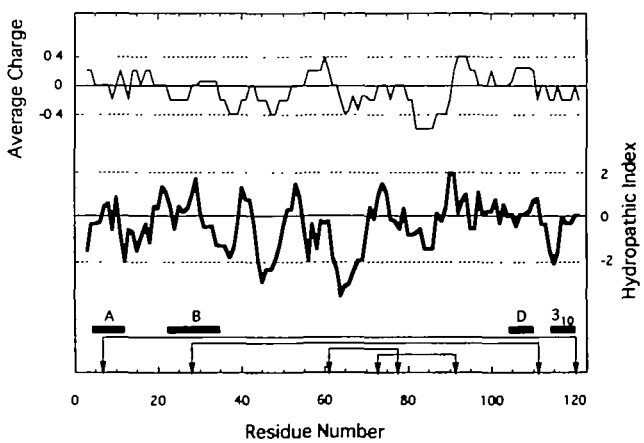

Fig. 4. **Hydropathic indices and average charges along the primary structure of α-lactalbumin.** Hydropathic indices and electric charges of five contiguous residues were averaged and plotted in the figure. Thin and thick line represent the average charge and the hydropathic index, respectively. The hydropathic indices of amino acids according to Kyte and Doolittle (*39*) were used for the hydropathy plot. The average charge was calculated assuming that the electric charges of (Glu and Asp), His, (Lys and Arg), and the other amino acids are −1, +0.25, +1, and 0, respectively. The N and C termini are assumed to have the electric charges of +1 and −1, respectively. At the bottom of the figure, the positions of the helices that form the core of the molten globule state are indicated. The arrowheads on the *X* axis indicate the positions of the eight cysteines of α-lactalbumin. Those cysteines that form a disulfide bond are connected by a line.

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secondary structure in the molten globule and have high hydrophobicity and/or positive charges provide the strong recognition sites of RLA by GroEL. It should also be noted that there are three cysteines located in these parts (residues 73, 77, and 91) that form disulfide bonds in the disulfide-intact native α-lactalbumin (see at the bottom of Fig. 4). It has been shown that CAM-3SS-LA that has three native disulfide bonds excluding that between Cys6 and Cys120 is not bound to GroEL and that GroEL exhibits only weak affinity for CAM-2SS-LA, a derivative of α-lactalbumin in which disulfide bonds 6-120 and 28-111 are selectively reduced and *S*-carboxyamidomethylated (*15*). This suggests that the breakage of disulfide bonds 6-120 and 28-111 does not bring about the structural change of the protein which leads to strong interaction with GroEL. Therefore, it is very likely that the breakage of the two remaining disulfide bonds, 61-77 and 73-91, located in the proposed parts, allows the amino acid residues in these parts to be easily accessible to GroEL and results in the strong recognition of RLA by GroEL.

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