Hydrogen Exchange of the Tryptophan Residues in Bovine, Goat, Guinea Pig, and Human α-Lactalbumin†

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ABSTRACT: Hydrogen exchange of the individual tryptophan residues of bovine, goat, guinea pig, and human α-lactalbumin has been studied by both ultraviolet and NMR spectra. The assignment of the slowly exchanging imino proton resonances to the tryptophan residues (Trp26 and Trp60) was obtained by comparison of the nuclear Overhauser effect difference spectra of bovine, guinea pig, and human α-lactalbumin. Taking account of the thermal unfolding of each α-lactalbumin, the hydrogen exchange rates of the individual tryptophan residues are analyzed. The temperature dependence of the exchange rates classified their exchange mechanisms into two exchange processes: the "low activation energy process" and the "high activation energy process" which is associated directly with the global thermal unfolding of the protein. Trp26 of α-lactalbumin exchanges through the high activation energy process. The exchange behavior of Trp26 of guinea pig α-lactalbumin suggests a difference of the globally unfolded state of the protein from the other species. The exchange mechanism of Trp60 of human α-lactalbumin is the low activation energy process in contrast with those of the bovine and goat proteins, although their global thermodynamic properties are similar to each other. Trp104 and Trp118 of α-lactalbumin exchange through the low activation energy process, and the reaction rates are affected by the local structural differences around the tryptophan residues among these proteins. The results presented in this paper indicate that the hydrogen exchange rate through the low activation energy process provides the information only about the local nature of a protein while that through the high activation energy process provides the information about the global nature of a protein.

α-Lactalbumin is a small globular protein found in mammalian milk and a component of lactose synthase (Hill & Brew, 1975). This protein and chicken-type lysozyme are homologous proteins evolved from a common ancestor (Hill & Brew, 1975; Hall et al., 1982; Qasba & Safaya, 1984; Shewale et al., 1984), and so the homology between such functionally distinct proteins has stimulated numerous comparative studies on their structure and stability.

The labile hydrogen in a protein exchanges with aqueous solvent hydrogen, and the exchange rate for a given hydrogen of the protein depends critically on the structure and dynamic properties of the protein [reviewed in Hvidt and Nielsen (1966), Barksdale and Rosenberg (1982), Woodward et al. (1982), Wagner (1983), and Englander and Kallenbach (1984)]. The hydrogen exchange method is thus useful for getting information about the structures and the dynamics of proteins.

Since the tryptophan imino proton is labile, this can be used to investigate the hydrogen exchange kinetics of proteins. Six tryptophan residues of hen egg white lysozyme are known to be in widely different environments in the native structure (Blake et al., 1978), and the individual exchange rates of these imino protons measured by nuclear magnetic resonance (NMR)[1] spectroscopy have been determined over wide ranges of temperatures and pHs (Wedin et al., 1982; Delepierre et al., 1983, 1987; Endo et al., 1988).

Recently, we have studied the hydrogen exchange of tryptophan residues in bovine α-lactalbumin by UV spectroscopy (Harushima et al., 1988). The two imino protons exchange faster with lower activation energies, and their exchange rates are not affected by the protein stability. The exchange rates of the other two imino protons are very slow, and the reaction is mediated by the transient global unfolding under the strongly native condition. The exchange mechanism of the slowly exchanging imino protons in bovine α-lactalbumin is markedly different from the exchange mechanism of imino protons in lysozyme. Wedin et al. (1982) reported that all tryptophan residues in lysozyme exchange with low activation energies under the native condition.

In order to investigate the difference in the exchange mechanism between the two proteins, it is necessary to assign each imino proton to the specific tryptophan residue in α-lactalbumin. Some researchers observed tentatively assigned the exchanging tryptophan residues in bovine α-lactalbumin on the basis of the results of the Raman spectra (Takesada et al., 1976; Miura et al., 1988). Takesada et al. assigned the two slowly exchanging residues to Trp26 and Trp104, considering a putative three-dimensional model structure which has been constructed on the basis of the lysozyme structure (Browne et al., 1969). However, Miura et al. assigned one of the fast-exchanging imino protons to Trp60 and one of the slowly exchanging imino protons to Trp118 by the analogy of the corresponding tryptophan residues in lysozyme.

The primary structures of more than 10 α-lactalbumin species have been determined [Hall et al., 1982; Shewale et al., 1984; and see references in Gaye et al. (1987) and Godovac-Zimmermann et al. (1987)]. The sequence homologies between the different species are high (for example, see Table I), suggesting close similarities of the backbone structure, and the solvent accessibility and the packing of their conserved residues (Miller et al., 1987). It will be expected that the conserved tryptophan residues show similar exchange behavior

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[1] Abbreviations: CD, circular dichroism; CIDNP, chemically induced dynamic nuclear polarization; COSY, two-dimensional *J*-correlated spectroscopy; Ac-Trp-OEt, *N*-acetyl-L-tryptophan ethyl ester; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; UV, ultraviolet.

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and similar chemical shifts in the NMR spectra. The substitution of the tryptophan residues from one species to another may provide the exact assignment of the tryptophan protons. Such substitutions from bovine α-lactalbumin are reported at different positions; one is in guinea pig α-lactalbumin, and another is in human α-lactalbumin (see Table I). Therefore, we have investigated the exchange behavior and the NMR spectral characteristics of the tryptophan imino protons in bovine, guinea pig, and human α-lactalbumin and also in goat α-lactalbumin which has the same tryptophan residues with the bovine protein as a control.

In this study, taking account of the thermal unfolding of each α-lactalbumin, the hydrogen exchange mechanism of the tryptophan residues is classified into the two exchange processes according to each activation energy. We elucidate that the exchange rates through the "low activation energy process" are affected only by the local environment of an observed proton; on the other hand, the exchange rates through the "high activation energy process" are affected by the global nature of a protein. As a result, it is concluded the apparent difference in the exchange mechanism between bovine α-lactalbumin and lysozyme under the native condition is due to the difference in the exchange rates through the low activation energy process caused by the local environmental difference around the imino protons between the two proteins.

Materials and Methods

Bovine, goat, guinea pig, and human α-lactalbumins were prepared from fresh milk of the respective species by the methods described previously (Kuwajima et al., 1976; Hiraoka & Sugai, 1984). The concentrations of α-lactalbumin were determined by absorbance spectrophotometry at 280 nm, using extinction coefficients, $E^{1\%}_{1cm}$, of 20.1 for bovine and goat (Kuwajima et al., 1980), 18.4 for human (Nozaka et al., 1978), and 17.2 for guinea pig α-lactalbumin (Takase et al., 1978). For Ac-Trp-OEt, a molar extinction coefficient at 280 nm of 5600 M^{-1} (Fasman, 1976) was used. All chemicals used were guaranteed reagent grade. All pH values reported are direct pH meter readings without isotope corrections.

The thermal unfolding curves of the proteins were obtained by CD measurements at 270 nm in a Jasco J-500A spectropolarimeter or a Union CD-1000 spectropolarimeter, and these were analyzed as described previously (Kuwajima et al., 1986; Mitani et al., 1986). The reversibility of the thermal transition was checked by cooling in each experiment. Protein concentrations used were (2.4–3.8) × 10^{-5} M.

The methods of observation and analysis of the hydrogen exchange of the tryptophan residues by UV spectroscopy were essentially the same as described previously (Harushima et al., 1988). In order to measure the hydrogen exchange curve with a small volume of the solution, a special stopped-flow apparatus was constructed in Unisoku Inc., Osaka. The new one was compactly composed of two driving syringes, a double two-jet mixer, and two observation cell compartments. The driving syringes worked pneumatically. The mixing volume ratio of the two solutions in the syringes was 1:9. Either of the two observation flow cells on the same flow line was used properly. The monochromatic light was introduced to either of the observation cells by an optical quartz fiber glass, and a photomultiplier was attached to the cell compartment on the light path. One of the observation cell compartments immediately adjoined the mixing compartment to get the short dead time. In order to observe the reaction stably for a long time period, the other observation flow cell was distant from the mixing compartment, and small ball-check valves (ruby balls of 3-mm diameter) were placed before and after the second cell on the flow line. When measurements were done in the second cell, the first cell was replaced by a block with a thin zig-zag flow line. The dead time of the first observation cell was 5 ms, and that of the second was 83 ms at 5 kg/cm^2 driving pressure, and these were determined by the method of Paul et al. (1980). For measurements over 1000 s, the exchange reaction was initiated by manual mixing and monitored in a Shimadzu UV-3000 spectrophotometer in the double-wavelength mode. The reference wavelength used was 320 nm where the absorption of the solution was almost zero. The change in the protein concentration of the solution was suppressed by sealing up the surface of the solution with liquid paraffin in the optical cuvette. The optical path of the observation cell was 10 mm for both spectrophotometric measurements. The output signals from the stopped-flow spectrophotometer were stored in an NEC 9801 VM2 microcomputer through a 12-bit A/D converter, and the signals from the Shimadzu spectrophotometer were stored in an NEC 9801 VM21 microcomputer through a GP-IB interface for subsequent data analysis.

Proton NMR spectra were taken on a Jeol GX-500 spectrometer in the Fourier-transform mode at the High Resolution NMR Laboratory, Faculty of Science, Hokkaido University. Data sets were 16K with a sweep width of 8000 Hz. NOE difference spectra were obtained by alternately collecting eight acquisitions with on-resonance irradiation for 0.3 s and eight acquisitions with off-resonance irradiation. All chemical shifts were measured from an internal standard, 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionic acid sodium salt (TSP). When the hydrogen exchange took place in the NMR probe at a given temperature, accumulated free induction decays were taken successively and stored on a magnetic disk. The hydrogen exchange rates of the individual proton resonances were obtained by plotting the resonance area against a recorded time which corresponds to the middle time from the start and to the end of the accumulation of the free induction decay of the spectrum.

Results

Thermodynamic Properties of Goat, Guinea Pig, and Human α-Lactalbumin. The four α-lactalbumins have negative aromatic CD bands around 270 nm (Cowburn et al., 1972; Kuwajima et al., 1976; Nozaka et al., 1978). The aromatic CD bands disappear with the melting of the tertiary structure of the protein. The exchange of some protons is correlated with the stability of a protein (Wagner & Wüthrich, 1979), and bovine α-lactalbumin contains two such imino protons while the two other imino protons of the tryptophan residues are not correlated with the thermal stability (Harushima et al., 1988). Thermal unfolding curves of human, goat, and guinea pig α-lactalbumin have been studied in the presence and the absence of 1 mM Ca^{2+} at pH 7.0 and 0.1 M NaCl. Since bovine α-lactalbumin is a Ca^{2+} binding protein, the stability of the protein is remarkably enhanced by the presence of free Ca^{2+} ion in the solution. Similar Ca^{2+}-induced stabilization is also found in the thermal transition curves of goat, guinea pig, and human α-lactalbumin. In the absence of 1 mM Ca^{2+}, the thermal transitions of these apoproteins begin below 25 °C and are complete at about 60 °C. In the presence of 1 mM Ca^{2+}, these holoproteins are in the native state below 50 °C and are in the thermally unfolded state above 80 °C (data not shown).

As the thermal unfolding of α-lactalbumin is known to be represented by a two-state transition, we can calculate the enthalpy change of the thermal unfolding at the transition temperature (T_m), $\Delta H_u(T_m)$ (Hiraoka & Sugai, 1984; Pfeil

Table I: Positions of Tryptophan Residues, Homologies (Percent), and Number of Substitutions in α-Lactalbumin Species

					bovine	goat	guinea pig	human
bovine	Trp26	Trp60	Trp104	Trp118		93%	67%	75%
goat	Trp26	Trp60	Trp104	Trp118	9		65%	78%
guinea pig	Trp26		Trp104	Trp118	41	43		70%
human		Trp60	Trp104	Trp118	31	28	36	

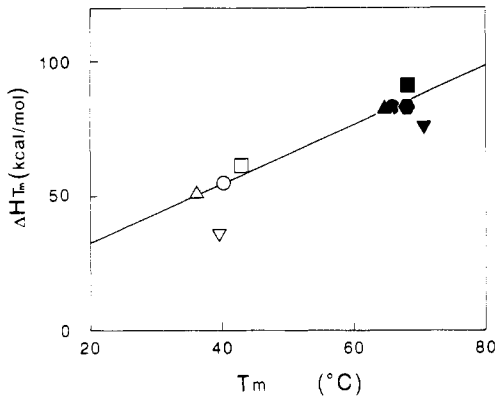

FIGURE 1: Plot of the enthalpy change of thermal unfolding versus the corresponding unfolding temperature of various α-lactalbumins in 50 mM sodium cacodylate and 50 mM NaCl at pH 7.0 in the presence (closed symbols) and the absence (open symbols) of 1 mM Ca^{2+}. The species are bovine (○), goat (□), human (△), and guinea pig (▽). The hexagon (●) denotes the value of bovine α-lactalbumin in D_2O solution. The solid line which shows $\Delta H_u(T)$ of bovine α-lactalbumin and the values of $\Delta H_u(T_m)$ of the bovine protein in H_2O are quoted from a previous study (Kuwajima et al., 1986). In the case of guinea pig α-lactalbumin, 2 mM EDTA was added to remove the free Ca^{2+} ion in the solution.

& Sadowski, 1985; Kuwajima et al., 1986; Mitani et al., 1986). The observed values are plotted against T_m in Figure 1. Generally, the enthalpy change of the thermal unfolding of a protein linearly increases with temperature with a slope of the heat capacity change of the unfolding, ΔC_p. The temperature dependence of ΔH_u of bovine α-lactalbumin and the values of $\Delta H_u(T_m)$ under the same condition obtained previously were also included in Figure 1 for comparison (Kuwajima et al., 1986). Because the enthalpy change upon Ca^{2+} binding to the native structure may be negligible as observed in bovine α-lactalbumin (Kuwajima et al., 1986), the difference in $\Delta H_u(T_m)$ between the holoprotein at 1 mM Ca^{2+} and the apoprotein in the absence Ca^{2+} may arise from the temperature dependence of ΔH_u for each α-lactalbumin. The ΔH_u at a temperature T, $\Delta H_u(T)$, is almost the same among bovine, goat, and human α-lactalbumin, while the $\Delta H_u(T)$ of guinea pig α-lactalbumin is a little smaller than those of the others. The ΔC_ps are the same among these species in the experimental error.

Using the following equation, we can calculate the Gibbs free energy change of the unfolding:

$$\Delta G(T) = T\Delta C_p \ln (T_m/T) + [\Delta H_u(T_m) - T_m\Delta C_p](T_m - T)/T_m = -RT \ln K_u \quad (1)$$

where T is the absolute temperature, R the gas constant, and K_u the equilibrium constant of the thermal unfolding (Privalov, 1979). The value, 1.1 kcal mol^{-1} $°C^{-1}$, of ΔC_p which has previously been obtained for bovine α-lactalbumin also satisfies the present results (Kuwajima et al., 1986). The calculated stability at 25 °C is in the order goat α-lactalbumin > bovine α-lactalbumin > human α-lactalbumin > guinea pig α-lactalbumin both in the presence and in the absence of 1 mM Ca^{2+}. The difference in the stability among the former three α-lactalbumins which have the same $\Delta H_u(T)$ values arises from the difference in the entropy change of the thermal unfolding.

Using the difference in ΔG (at 25 °C) between 0 and 1 mM Ca^{2+}, we can calculate the apparent binding constant of Ca^{2+}, K_b^{app}, for each species at 0.1 M Na^+ as described previously (Kuwajima et al., 1986; Mitani et al., 1986). The values of K_b^{app} are 9.3×10^6 M^{-1} for goat α-lactalbumin, 8.7×10^6 M^{-1} for human α-lactalbumin, and 7.3×10^6 M^{-1} for guinea pig α-lactalbumin. These values are similar to that obtained previously for bovine α-lactalbumin under the same condition (Kuwajima et al., 1986; Mitani et al., 1986).

D–H Exchange of the Tryptophan Residues Obtained by UV Spectroscopy. The tryptophan residue contents and the positions in the primary sequences are listed for the four α-lactalbumins in Table I. The number of substitutions and the percent degrees of homology in the primary sequence between these species are also listed in Table I. As shown previously, the four tryptophan residues in bovine α-lactalbumin show the biphasic hydrogen exchange kinetics. The two residues are involved in each of the kinetic phases (Harushima et al., 1988).

Exchange curves of goat, guinea pig, and human α-lactalbumin were measured by the absorption increase in the native state. The exchange curve of goat α-lactalbumin is biphasic like that of bovine α-lactalbumin. However, both exchange curves of guinea pig and human α-lactalbumins are triphasic. In order to evaluate the number of residues involved in each phase, we examined the wavelength dependence of the kinetic amplitude of each phase in these α-lactalbumins. The observed kinetic difference spectra thus obtained are shown in Figure 2. These kinetic difference spectra are characteristic of hydrogen exchange of the tryptophan residues in the native state. Considering the composition of the tryptophan residues in each α-lactalbumin and the intensities of the spectra, it is concluded that two tryptophan residues are involved in each phase of goat α-lactalbumin, and each of the three phases of both guinea pig and human α-lactalbumin involves one tryptophan residue. The above result of goat α-lactalbumin is well expected from the high sequence homology to bovine α-lactalbumin. The exchange behavior of both human and guinea pig α-lactalbumin shows differences in the environments of the conserved tryptophan residues from those in bovine α-lactalbumin.

Although the exchange kinetics are triphasic for both human and guinea pig α-lactalbumin, the rates are different between them. The exchange rate of the second phase in human α-lactalbumin equals that of the bovine fast phase. On the other hand, the reaction rate of the fastest phase in guinea pig α-lactalbumin equals that of the bovine fast phase. The three phases are thus termed "very fast, fast, and slow" for human α-lactalbumin and "fast, middle, and slow" for guinea pig α-lactalbumin.

In order to investigate the exchange mechanism of the tryptophan residues, we examined the temperature dependence of the exchange rate of each phase in goat, guinea pig, and human α-lactalbumin at pH 7.0 in the presence of 1 mM Ca^{2+}. The Arrhenius plot of each phase is shown in Figure 3, and the results of bovine α-lactalbumin and Ac-Trp-OEt obtained previously are also shown in the same figure (Harushima et al., 1988). The values of the activation energies of the hydrogen exchange reactions of these tryptophan residues are

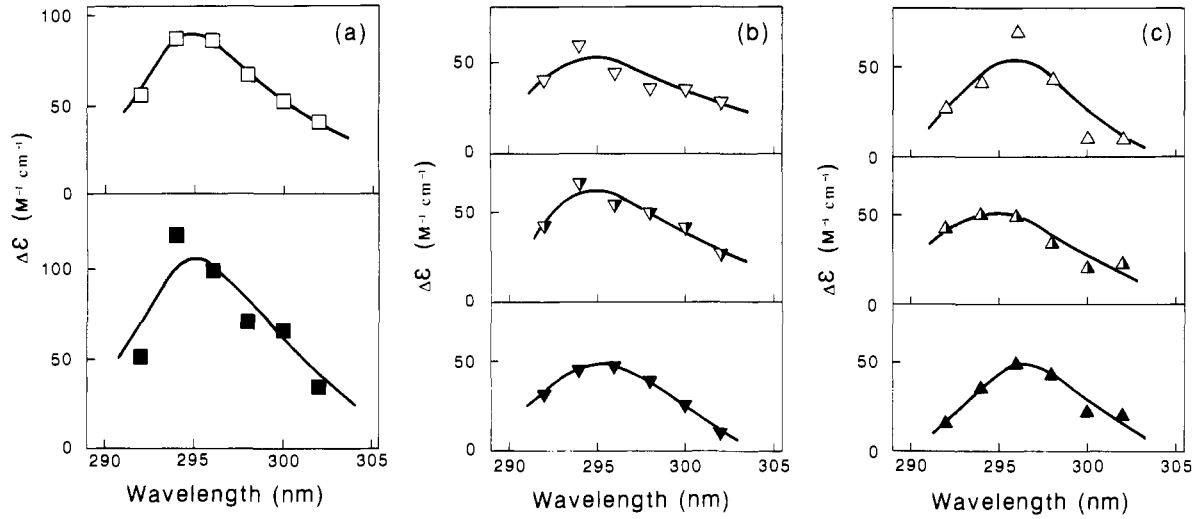

FIGURE 2: Kinetic difference spectra due to hydrogen exchange of tryptophan residues in native α-lactalbumins at pH 7.0. The observed exchange rates of each kinetic phase are 0.28 (□) and 0.0015 s^{-1} (■) at 41.1 °C in goat α-lactalbumin (a), 0.52 (▽), 0.018 (▽), and 0.00088 s^{-1} (▼) at 48.4 °C in guinea pig α-lactalbumin (b), and 1.7 (△), 0.10 (△), and 0.0015 s^{-1} (▲) at 36.42 °C in human α-lactalbumin (c). The solution contained 50 mM sodium cacodylate, 50 mM NaCl, and 1 mM $CaCl_2$. The protein concentrations used are from 45 to 87 μM.

listed in Table II. Some interesting features are found in Figure 3 and Table II. The activation energy of the human very fast phase is smaller than that of Ac-Trp-OEt which is a model substance for the completely exposed residue. The fast phases in the four α-lactalbumins are the same in the absolute rate and in the activation energy with each other. The activation energy of the guinea pig middle phase is similar to those of the fast phases of the four α-lactalbumins. For the human slow phase, the activation energy is significantly small compared with those in the other species, and the exchange rate is similar to that of the guinea pig middle phase.

Comparison of the Proton Stability and the Hydrogen Exchange Rate in D_2O with Those in H_2O. Generally, in the NMR method the exchange reactions of protons to deuterons are traced in D_2O solution, while in our UV method the reaction of deuterons to protons has been studied in H_2O solution. Therefore, in order to compare the hydrogen exchange results by the UV measurements with those by the NMR ones, we investigated the effects of isotopes on the protein stability and on the exchange rate.

In some cases, a difference in the stability of a protein is found between H_2O and D_2O solutions (Hvidt & Nielsen, 1966). The increment of the thermal stability of bovine α-lactalbumin in D_2O was reported before finding the strong Ca^{2+} binding ability of the protein (Takesada et al., 1973). We have reinvestigated the effects of isotopic solvent in the presence of 1 mM Ca^{2+}. As shown in Figure 1, bovine α-lactalbumin is slightly more stable in D_2O than in H_2O at the same Ca^{2+} concentration. The difference is not serious, because the stability change between in H_2O and in D_2O is smaller than that between bovine and goat α-lactalbumin in H_2O, and there is no significant change in the tryptophan hydrogen exchange between the two proteins (see Figures 1 and 3).

The kinetic isotope effects on the hydrogen exchange of the tryptophan residue were also investigated by observation of reverse reaction in D_2O by the UV method. As shown in Figure 3, the exchange rates of the imino proton of Ac-Trp-OEt in D_2O (diamonds in panel a) are the same as in H_2O (dotted line) at pH 7.0. The similar results for the amide proton exchange were previously reported (Barksdale & Rosenberg, 1982).

The above observations guarantee direct comparison between the results of UV measurements and those of NMR.

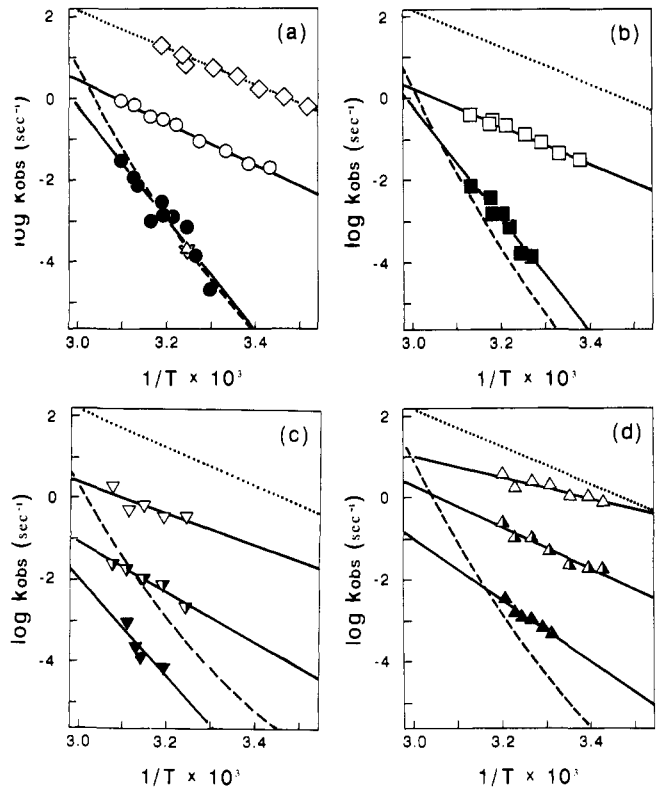

FIGURE 3: Arrhenius plots for hydrogen exchange reactions of Trp104, -118 (○) and Trp26, -60 (●) in bovine α-lactalbumin (a), Trp104, -118 (□) and Trp26, -60 (■) in goat α-lactalbumin (b), Trp104 (or -118) (▽), Trp118 (or -104) (▼), and Trp26 (▼) in guinea pig α-lactalbumin (c), and Trp118 (or -104) (△), Trp104 (or -118) (▲), and Trp60 (▲) in human α-lactalbumin (d) at pH 7.0. The solution conditions are the same as in Figure 2. The dotted line represents the exchange rate of Ac-Trp-OEt in H_2O solution. The diamonds (◇) in panel a indicate the exchange rate of Ac-Trp-OEt in D_2O solution. The dashed line in each panel represents the calculated value of the exchange rate in each species with the assumption that the exchange reaction is induced by the global unfolding in the EX_2 kinetics (see Discussion). The assignments of each kinetic phase are discussed in the text. The exchange rates in bovine protein and Ac-Trp-OEt in H_2O solution are quoted from a previous study. The observed exchange rates of Trp26 (△) and Trp60 (▽) from the NMR measurements are also shown in panel a.

Assignment of the Residues Involved in the Slowly Exchanging Tryptophan Residues. The assignment of the

Table II: Activation Energies[a] and Tryptophan Residue Assignments (in Parentheses) of Hydrogen Exchange Reactions in α-Lactalbumins[b]

	bovine	goat	guinea pig	human
very fast phase				11.6 ± 2.4 [Trp104 (or -118)]
fast phase	23.3 ± 0.6[c] (Trp104, Trp118)	21.3 ± 1.3 (Trp104, Trp118)	17.1 ± 7 [Trp104 (or -118)]	23.2 ± 2.7 [Trp118 (or -104)]
middle phase			27.9 ± 3.4 [Trp118 (or -104)]	
slow phase	62.5 ± 7.3[c] (Trp26, Trp60)	63.6 ± 7.3 (Trp26, Trp60)	54.7 ± 18.8 (Trp26)	34.4 ± 4.4 (Trp60)
Ac-Trp-OEt	20.5 ± 1.0[c] (free)			

[a] Values are expressed in kilocalories per mole. [b] The tryptophan residues assigned to each kinetic phase are discussed in the text. [c] The values are quoted from our previous paper (Harushima et al., 1988).

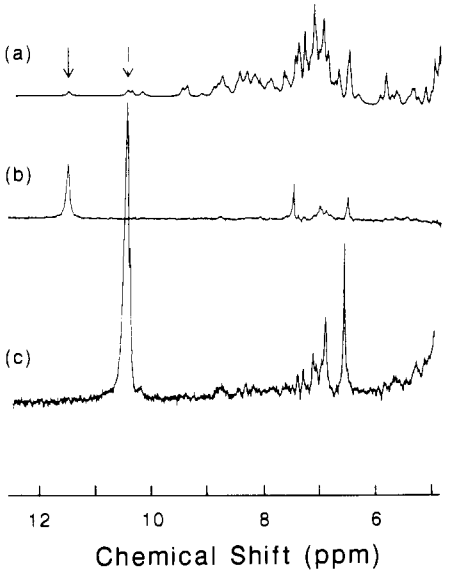

FIGURE 4: (a) 500-MHz ^1H NMR spectrum in the low-field region of bovine α-lactalbumin in D_2O solution (100 mM NaCl and 1 mM $CaCl_2$) at pH 6.6 and 25 °C. (b) Selective NOE difference spectrum with on-irradiation for 0.3 s at 11.52 ppm; (c) that at 10.46 ppm. The arrows in the normal spectrum represent the irradiated peaks.

tryptophan residues with the slowly exchanging imino protons was made as follows: the NMR spectra were observed after the disappearance of the fast exchanging labile proton resonances, and the slowly exchanging imino proton resonances were distinguished from the remaining amide protons in the low-field region by use of the selective NOE difference spectra. The NOE is strongly correlated with the internuclear distance. Irradiation on an amide resonance brings the most pronounced effect on the C_α-proton resonance. The selective NOE difference spectrum of the imino proton indicates the chemical shifts of the C-2 and the C-7 protons of the tryptophan residue, since the nearest proton from the imino proton is the C-2 proton and the next is the C-7 proton in the same residue. Comparison of the sets of the chemical shifts of the slowly exchanging tryptophan residues among bovine, human, and guinea pig α-lactalbumin led to the assignment of the tryptophan residues in the primary structures.

The NMR spectrum of bovine α-lactalbumin in the low-field region is shown in Figure 4a. It is expected from the hydrogen exchange results of the UV measurements that there are two imino proton resonances in this region. Spectra b and c in Figure 4 are the NOE difference spectra with irradiations of the peaks at 11.52 and 10.46 ppm, respectively. These NOE difference spectra are typical ones with irradiation of the imino proton of a tryptophan residue. The difference spectrum indicates that the chemical shifts of one of the slowly exchanging tryptophan residues are 11.52 (N-1), 7.50 (C-2), and 6.54 ppm (C-7). The chemical shifts of another residue in bovine α-lactalbumin are 10.46 (N-1), 6.56 (C-2), and 6.90 ppm (C-7).

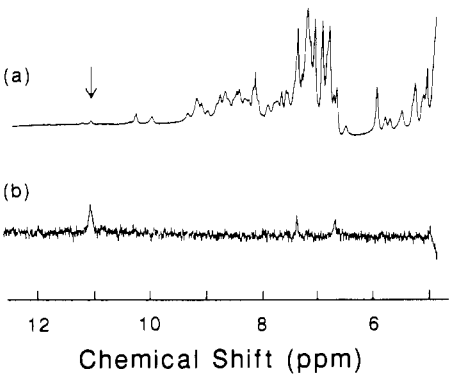

FIGURE 5: (a) 500-MHz ^1H NMR spectrum in the low-field region of human α-lactalbumin in D_2O solution (100 mM NaCl and 1 mM $CaCl_2$) at pH 6.6 and 25 °C. (b) NOE difference spectrum with on-irradiation at 11.06 ppm for 0.3 s in D_2O solution (100 mM NaCl and 15 mM $CaCl_2$) at pH 6.5 and 25 °C.

The exchange rates of the slowly exchanging imino protons in bovine α-lactalbumin were measured by observing the time-dependent disappearance of the two resonances in the NMR spectra. The observed exchange rates show fair agreements with the results from the UV method (Figure 3a).

The NMR spectrum in the low-field region which contained one residual N-1 signal of the slowly exchanging tryptophan in human α-lactalbumin is shown in Figure 5a. Spectrum b in Figure 5 shows the NOE difference spectrum with irradiation of the peak at 11.06 ppm. This difference spectrum is very similar to spectrum b in Figure 4. The chemical shifts of the N-1, C-2, and C-7 protons of the slowly exchanging tryptophan residue in human α-lactalbumin are 11.06, 7.37, and 6.68 ppm, respectively. There is a resonance with a similar chemical shift to another N-1 resonance of bovine α-lactalbumin in the human spectrum (Figure 5a). When the resonance at 10.25 ppm was irradiated, the most pronounced NOE appeared at the C_α-proton region (data not shown). The above observation confirms the results of the UV experiments that only one imino proton remains under this condition.

The low-field region NMR spectrum of guinea pig α-lactalbumin is shown in Figure 6a. This spectrum was recorded immediately after the protein was dissolved in the D_2O buffer solution at pH 7.0, 1 mM Ca^{2+}, and 25 °C. Under this condition, we can expect to observe two imino proton resonances with full intensity and half-intensity remaining from the UV hydrogen exchange results (see Figure 3c). There is no residual resonance above 11 ppm in Figure 6a in contrast to bovine and human α-lactalbumin. The difference NOE spectrum on irradiation at 10.56 ppm is shown in Figure 6b. This spectrum is very similar to spectrum c in Figure 4. The chemical shifts of the N-1, C-2, and C-7 protons of the slowly exchanging tryptophan residue in guinea pig α-lactalbumin are 10.56, 6.58, and 7.02 ppm, respectively.

Considering the positions of the tryptophan residues in the primary sequences of bovine, human, and guinea pig α-lactalbumin shown in Table I, it is concluded that the slowly

Table III: Chemical Shifts of Proton Resonances in Slowly Exchanging Tryptophan Residues[a]

	Trp26						Trp60		
	N-1	C-2	C-4	C-5	C-6	C-7	N-1	C-2	C-7
bovine	10.46	6.56	7.29	5.68	5.27	6.90	11.52	7.50	6.54
guinea pig	10.56	6.58				7.02			
human							11.06	7.37	6.68

[a] Chemical shifts are in ppm referenced to TSP.

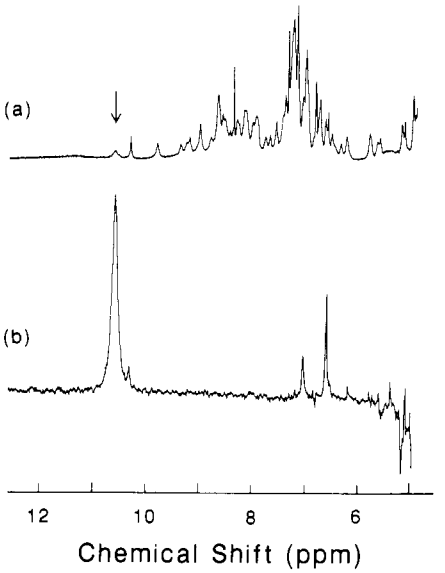

FIGURE 6: (a) 500-MHz ^1H NMR spectrum in the low-field region of guinea pig α-lactalbumin in D_2O solution (50 mM sodium cacodylate, 50 mM NaCl, and 1 mM $CaCl_2$) at pH 7.0 and 25 °C. The spectrum was recorded within 25 min after the protein was dissolved in the buffer. (b) NOE difference spectrum with on-irradiation at 10.50 ppm for 0.3 s in D_2O solution (100 mM NaCl and 1 mM $CaCl_2$) at pH 7.0 and 25 °C.

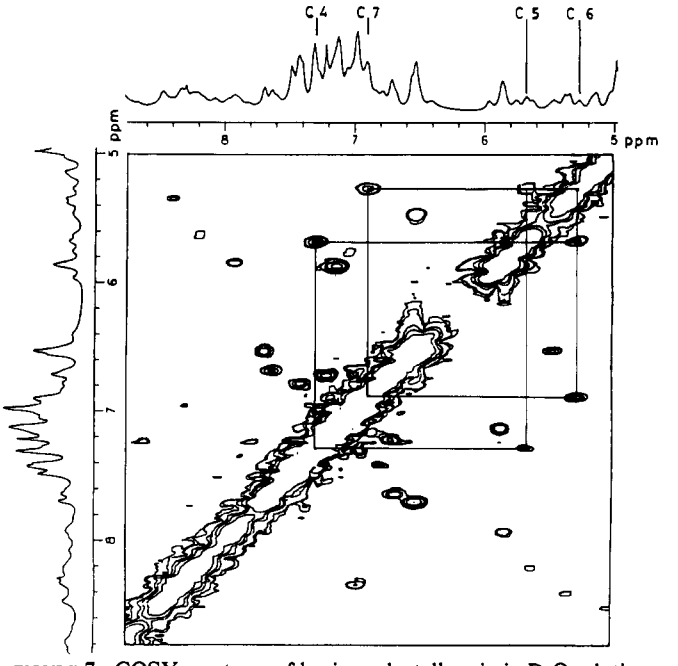

FIGURE 7: COSY spectrum of bovine α-lactalbumin in D_2O solution (100 mM NaCl and 1 mM $CaCl_2$) at pH 6.5 and 25 °C. The region showing the aromatic ring–proton cross-peaks is depicted. The lines indicate the spin systems of Trp26.

exchange tryptophan residues are Trp26 and Trp60. The resonances at 10.46 ppm in Figure 4c (bovine) and at 10.56 ppm in Figure 6b (guinea pig) are due to the N-1 proton of Trp26, since in human α-lactalbumin the correspondent resonance is not found because of the absence of the residue in the primary sequence. In the same way, the resonances at 11.52 ppm in Figure 4b (bovine) and at 11.06 ppm in Figure 5b (human) are due to the N-1 protons of Trp60, because of its absence in guinea pig α-lactalbumin.

To make additional assignment of the proton resonances of the slowly exchanging tryptophan residues in bovine α-lactalbumin, the COSY spectrum (see Figure 7) was obtained under a condition similar to that in Figure 4. From the NOE results, the chemical shift of the C-7 proton of Trp26 is determined to be 6.90 ppm. As shown in Figure 7, the resonance at 6.90 ppm couples with the well-resolved single proton triplet resonance at 5.27 ppm (C-6), which couples with the other single proton triplet resonance at 5.68 ppm (C-5). The resonance at 5.68 ppm also couples to 7.29 ppm (C-4). The assignments of these resonances to Trp26 were confirmed by the observation of the weak NOEs at 5.27 and 5.68 ppm in Figure 4c. For Trp60, we could not estimate the chemical shifts of the C-6, C-5, and C-4 resonances from the 6.54 ppm resonance (C-7) in Figure 7.

The chemical shifts of the resonances of the tryptophan residues in three α-lactalbumins assigned here are listed in Table III.

Discussion

Validity of the Assignments. The results obtained here are the following: the two slowly exchanging tryptophan residues are Trp26 and Trp60 in bovine (and probably also goat) α-lactalbumin. The slow phase of human α-lactalbumin is assigned to Trp60, and that of guinea pig α-lactalbumin is to Trp26. The other phases of α-lactalbumin are thus assigned to Trp104 and Trp118. In hen egg lysozyme, the hydrogen exchange rate of Trp28 which corresponds to Trp26 in α-lactalbumin is the slowest in rate among the six tryptophan residues, and that of Trp63 which corresponds to Trp60 in α-lactalbumin is very fast (Wedin et al., 1982). The difference in the exchange behavior of Trp60 (Trp63) between the two proteins suggests the local conformational differences around the residue. The resolution of the X-ray three-dimensional structure of α-lactalbumin is not enough to show the details in the local conformational differences between the two proteins (Smith et al., 1987; Phillips et al., 1987). However, the conformational difference around Trp60 has been pointed out by the model building studies on α-lactalbumin (Browne et al., 1969; Warme et al., 1974). The comparative study of bovine, human, and guinea pig α-lactalbumin by the solvent perturbation spectroscopic method has suggested that Trp26 and Trp60 are buried (Takase et al., 1978). The photo-CIDNP study on several α-lactalbumins has shown the directly polarized (exposed) tryptophan residue is only Trp104 (Berliner & Kaptein, 1981). The previous studies on environments of the tryptophan residues support the validity of our assignments in this study. These assignments permit us to compare the individual exchange behavior at the same residue among the four species.

Hydrogen Exchange of Individual Tryptophan Residues. The hydrogen exchange mechanism in a native protein is

described by the two-step reaction scheme (Hvidt & Nielsen, 1966):

$$N \underset{k_f}{\overset{k_u}{\rightleftharpoons}} O \overset{k_e}{\rightharpoonup} \text{exchanged} \quad (2)$$

where N represents a native structure where an observed hydrogen is not permitted to exchange and O represents an open structure where the hydrogen exchanges with a solvent isotope by a rate constant k_e. N and O are in dynamic equilibrium with an unfolding rate constant, k_u, and a folding one, k_f. There are two exchange pathways for the hydrogen depending on the structures of O (Woodward et al., 1982). One is the so-called the "low activation energy process", and the overall structure of O is folded. The "solvent penetration model" and the "local unfolding model" were proposed for the mechanism of the low activation energy process [reviewed in Woodward et al. (1982) and Englander and Kallenbach (1984)]. The other is the "high activation energy process", the cooperative global unfolding mediates the exchange reaction. In both exchange pathways, the observed exchange rate, k_{obs}, is (Hvidt & Nielsen, 1966)

$$k_{obs} = k_u k_e/(k_f + k_e) \quad (3)$$

There are two limiting kinetic situations:

(1) If $k_f \ll k_e$ ("EX_1 kinetics")

$$k_{obs} = k_u \quad (4)$$

The apparent activation energy means the activation energy of the unfolding, E_u*.

(2) If $k_f \gg k_e$ ("EX_2 kinetics")

$$k_{obs} = (k_u/k_f)k_e = K_u k_e \quad (5)$$

where K_u is an equilibrium constant of the unfolding. The apparent activation energy is the sum of the enthalpy of the unfolding, ΔH_u, and the activation energy of the intrinsic exchange reaction from O, E_e*. In general, the EX_1 kinetics are rare in a protein, and the reported EX_1 kinetics have been observed only in the high activation energy process in the protein (Nakanishi & Tsuboi, 1974; Ohta et al., 1977; Segawa et al., 1981; Wedin et al., 1982; Roder et al., 1985; Harushima et al., 1988; Kawata et al., 1988); therefore, each exchange process of the tryptophan residues in α-lactalbumin is classified into the low or the high activation process as it follows EX_2 kinetics.

If the exchange reaction is mediated by the high activation energy process in the EX_2 kinetics, the exchange rate will be expected by using eq 1 and 5. In a previous study, we have shown that the exchange reactions of Trp26 and Trp60 in bovine α-lactalbumin are the above cases in the presence of 1 mM Ca^{2+}. The observed exchange rate of these residues shows good agreement with the expected value by eq 5 where the K_u is calculated from the thermal unfolding curve by using eq 1 and k_e is the observed hydrogen exchange rate in the thermally unfolded state; the value has been found to be 3 times smaller than that of Ac-Trp-OEt. In this study, we have also measured the thermal unfolding of goat, guinea pig, and human α-lactalbumin by the CD spectra under the same conditions as used for the hydrogen exchange. We have calculated the exchange rate by eq 1 and 5 on the same assumptions as used for calculation of the exchange rate in the bovine protein. The dashed lines in Figure 3 show the calculated values of the exchange rates.

Hydrogen Exchange of Trp26 and Trp60 in α-Lactalbumin. The observed exchange reactions of Trp26 and Trp60 in goat α-lactalbumin are well explained by the high activation energy process as in bovine α-lactalbumin (Figure 3b). However, the exchange reactions of both Trp26 in guinea pig α-lactalbumin and Trp60 in human α-lactalbumin are not explained in the same way as for the bovine protein. Trp26 in guinea pig α-lactalbumin exchanges slower than the expected value (Figure 3c); on the other hand, Trp60 in human α-lactalbumin exchanges faster than the expected value (Figure 3d). The interpretations of the discrepancy between the observed exchange rate and the expected one in guinea pig α-lactalbumin are different from those in human α-lactalbumin.

Hydrogen Exchange of Trp26 in Guinea Pig α-Lactalbumin. The exchange mechanism of Trp26 in guinea pig α-lactalbumin is the high activation energy process, because the difference in the activation energy between Trp26 in guinea pig α-lactalbumin and a free tryptophan residue is nearly equal to the value of ΔH_u of this protein at the respective temperature (see Table II and Figure 1). The exchange reaction of Trp26 in guinea pig α-lactalbumin follows EX_2 kinetics, since the logarithm of the exchange rate depends linearly on pH with a slope of unity in accord with the specific base catalysis of the intrinsic exchange reaction (data not shown). The difference in the exchange behavior of Trp26 between bovine and guinea pig α-lactalbumin can be explained by the difference in the k_e of eq 5. In other words, the imino proton of guinea pig α-lactalbumin is more protected to exchange than that of bovine in the thermally unfolded structure. This interpretation is supported by other observations. According to Sommers and Kronman (1980), the iodide quenching of the tryptophan fluorescence in the thermally unfolded conformation of guinea pig α-lactalbumin is weaker than that of the other species; the tryptophan residues are structurally protected against solvent under the thermally unfolded state.

Hydrogen exchange through the high activation energy process provides precious informations about the global nature of the protein. The difference in k_e at each site in the high activation energy process gives the information on the residual structure of the transiently globally unfolded state. The protection by the residual structure would be extended to all tryptophan residues in the unfolded structure, since the observed exchange rate of Trp118 (or Trp104) is also smaller than the calculated one in guinea pig α-lactalbumin (see Figure 3c). It is well-known for the other α-lactalbumins that the thermally unfolded state is not completely unfolded but shows an intermediate conformational state (Kuwajima & Sugai, 1978; Sommers & Kronman, 1980; Kuwajima et al., 1985; Dolgikh et al., 1985; Pfeil, 1987; Harushima et al., 1988). The residual structure in the transiently unfolded state is also suggested in the hydrogen exchange measurements of the amide protons in bovine α-lactalbumin by NMR spectroscopy. Several amide protons exchanged about 1 order of magnitude slower than the imino protons of Trp26 and Trp60 (data not shown), although amide protons exchange faster than imino protons at pH 7.0 in a completely unfolded structure of a protein (Molday et al., 1972; Takahashi et al., 1978; Englander et al., 1978; Harushima et al., 1988). The difference in the exchange behavior of Trp26 between guinea pig and bovine α-lactalbumin in their native state may be due to the difference in the stability of the intermediate conformational state between the two proteins. The higher stability of residual structure in the thermally unfolded state would make the smaller enthalpy change of the thermal unfolding of guinea pig α-lactalbumin than those of the other α-lactalbumins (Figure 1). It is important for the exact understanding of the dynamic nature of a protein to characterize the transiently unfolded state; therefore, further investigation is needed in the future.

Hydrogen Exchange of Trp60 in Human α-Lactalbumin. In human α-lactalbumin, the deviation of the observed exchange rate of Trp60 from the calculated one becomes larger at lower temperature (Figure 3d). The activation energy of the exchange reaction is only 14 kcal/mol higher than that of Ac-Trp-OEt (Table II). This value is small when the value of ΔH_u (about 45 kcal/mol; see Figure 1) is compared. Therefore, Trp60 in human α-lactalbumin exchanges through the low activation energy process at a low temperature in contrast with the high activation energy process observed for the same tryptophan residue in bovine α-lactalbumin.

It is often observed that the protons in the highly protected stable region of a protein exchange by the low activation energy process at a low temperature. In lysozyme, the imino protons exchange through the fully unfolded state at a high temperature, and the low activation energy mechanism is dominant for all of the imino protons below 55 °C (Wedin et al., 1982). The change in the exchange mechanism can be explained as follows. There are various dynamic processes in a protein, and a labile proton exchanges through the most rapid pathway. Whether a selected pathway involves the global unfolding of the protein or not depends on temperature, because the global unfolding equilibrium is temperature sensitive while the local fluctuation is temperature insensitive (Englander & Kallenbach, 1984).

Although we did not observed the low activation energy process of Trp60 and Trp26 in the other species, it would appear at a lower temperature range than that studied here. The difference in the exchange behavior of Trp60 between human and bovine (goat) α-lactalbumin would be caused by the difference in the exchange rates of the low activation energy process. The exchange rate of the low activation energy process would be very sensitive only to the local environment around the observed proton even in the highly protected region. There might be no relation between the exchange rate of the low activation energy process and the global thermodynamic properties. Both human and bovine α-lactalbumins are the same in the global thermodynamic properties: the enthalpy change of the thermal unfolding and the metal binding ability. Therefore, the difference in the exchange behavior of the imino protons between bovine α-lactalbumin and lysozyme at a low temperature should be due to the difference in the local environment of the individual imino protons between the two proteins.

Hydrogen Exchange of Trp104 and Trp118. Since Trp104 and Trp118 are conserved in all α-lactalbumins which have been fully sequence-analyzed, we cannot distinguish the two residues by the method described here. Both Trp104 and Trp118 exchange through the low activation energy process in all α-lactalbumins, because the difference in the apparent activation energy between these tryptophans residues and free residues is small (see Table II). Trp104 and Trp118 in both bovine and goat α-lactalbumin exchange relatively fast with the same rate constants. Both tryptophan residues may locate near the surface of the protein. In guinea pig and human α-lactalbumin, one of these residues exchanges at the same rate as in bovine and goat α-lactalbumin. This reflects the high homologies among the proteins. The exchange rates of Trp104 and Trp118 of the bovine protein are independent of the protein stability (Harushima et al., 1988). However, the differences in the local environment in α-lactalbumins may affect their exchange rates.

In human α-lactalbumin, Trp104 (or Trp118) exchanges with comparable rates to the freely solvent-accessible tryptophan as shown in Figure 3d. The activation energy of the exchange reaction is significantly smaller than that of Ac-Trp-OEt (Table II). This suggests that the tryptophan residue is not simply more exposed to solvent than that of bovine (and goat) α-lactalbumin but is involved in a structure which accelerates the catalytic reaction of OH^-, e.g., strong H bonding to a solvent water molecule. On the other hand, one of the tryptophan residues in guinea pig α-lactalbumin exchanged 30 times slower than in bovine (and goat) α-lactalbumin. Although the exchange is slow, the activation energy is only 7.4 kcal/mol higher than that of Ac-Trp-OEt (see Table II). The exchange of Trp118 (or Trp104) in guinea pig α-lactalbumin is still mediated through the low activation energy process.

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