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Comparison of the Transient Folding Intermediates in Lysozyme and α-Lactalbumin†

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ABSTRACT: Refolding kinetics of two homologous proteins, lysozyme and α-lactalbumin, were studied by following the time-dependent changes in the circular dichroism spectra in the aromatic and the peptide regions. The refolding was initiated by 20-fold dilution of the protein solutions originally unfolded at 6 M guanidine hydrochloride, at pH 1.5 for lysozyme and pH 7.0 for α-lactalbumin at 4.5 °C. In the aromatic region, almost full changes in ellipticity that were expected from the equilibrium differences in the spectra between the native and unfolded proteins were observed kinetically. The major fast phase of lysozyme folding has a decay time of 15 s. The decay time of α-lactalbumin depends on the presence or absence of bound Ca^{2+}: 10 s for the holoprotein and 100 s for the apoprotein. In the peptide region, however, most of the ellipticity changes of the two proteins occur within the dead time (<3 s) of the present measurements. This demonstrates existence of an early folding intermediate which is still unfolded when measured by the aromatic bands but has folded secondary structure as measured by the peptide bands. Extrapolation of the ellipticity changes to zero time at various wavelengths gives a spectrum of the folding intermediate. Curve fitting of the peptide spectra to estimate the secondary structure fractions has shown that the two proteins assume a similar structure at an early stage of folding and that the intermediate has a structure similar to that of partially unfolded species produced by heat and, for α-lactalbumin, also by acid and a moderate concentration of guanidine hydrochloride. Thus, in spite of their known difference in equilibrium unfolding behavior and the difference of their biological functions, folding of the two proteins occurs via a similar structural intermediate. The thermal transition of the secondary structure in the equilibrium intermediate of α-lactalbumin was also investigated. The relevance of the present results to the equilibrium unfolding behavior of the proteins is described.

Lysozyme and α-lactalbumin are homologous proteins which have similar amino acid sequences (Brew et al., 1970; Imoto et al., 1972; Shewale et al., 1984; Qasba & Safaya, 1984), but their apparent physicochemical properties are markedly different. The unfolding equilibrium of lysozyme in the transition region when treated with guanidine hydrochloride (Gdn·HCl)[1] is expressed as a highly cooperative two-state reaction (Tanford et al., 1966; Aune & Tanford, 1969; Tanford, 1968, 1970). The unfolding of α-lactalbumin is less coopertive and shows a stable intermediate state at a moderate concentration of Gdn·HCl, and a similar conformational state exists also at acid pH (Kuwajima et al., 1976, 1981; Nozaka et al., 1978); in the following, each of these states is termed the A state. There are many comparative studies of the two proteins which have suggested similarity in their tertiary structures (Browne et al., 1969; Warme et al., 1974; Takase et al., 1978; Hill & Brew, 1975, and other references cited therein), but some authors have also reported nontrivial differences especially in their dynamic properties and have suggested a more flexible structure of α-lactalbumin than of lysozyme (Lin, 1970; Barman & Bagshaw, 1972; Iyer & Klee, 1973; Takesada et al., 1973; Pfeil, 1981a). The recent discovery that α-lactalbumin is a Ca^{2+}-binding protein and that it requires Ca^{2+} for structural stabilization in spite of low affinity of lysozyme to Ca^{2+} gave a new light for understanding the differences between the two proteins (Hiraoka et al., 1980; Permyakov et al., 1981; Murakami et al., 1982; Segawa & Sugai, 1983; Kronman & Bratcher, 1983) [see also Imoto et al. (1981) for lysozyme's affinity to Ca^{2+}].

Nevertheless, it has been postulated that not only the native structure but also the folding pathway of a protein are determined by its unique amino acid sequence (Anfinsen & Scheraga, 1975; Kim & Baldwin, 1982; Ghélis & Yon, 1982). It is also suggested that the pathways of folding of homologous proteins have been conserved during evolutional divergence (Ptitsyn & Finkelstein, 1980; Rossmann & Argos, 1981; Hollecker & Creighton, 1983; Krebs et al., 1983). Otherwise, a very large number of different mechanisms of folding must

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[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; CD, circular dichroism; UV, ultraviolet; RMS, root mean square.

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be imagined. Here, we ask a question of whether the differences in properties between lysozyme and α-lactalbumin are in conflict with the above proposal of the conserved folding pathway.

The A state of α-lactalbumin has a nativelike secondary structure of the peptide backbone but has lost the specific tertiary structure of the aromatic side chains (Kuwajima et al., 1975, 1976, 1981). The A state has a compact structure with a gyration radius as small as in the native state (~16 Å) (Dolgikh et al., 1981; Izumi et al., 1983). Equilibrium and kinetics of the unfolding and refolding of the protein have suggested that a state similar to the A state is also a kinetic intermediate formed at an early stage of folding. This was based on the following observations (Kita et al., 1976; Kuwajima, 1977; Nozaka et al., 1978): (i) the stability of the A state is higher than that of the completely unfolded (D) state under the native condition and (ii) the D to A transition that occurs within the dead time of stopped-flow measurement (~1 ms) is much faster than the transition from the A to the native (N) state, and hence, the folding proceeds in the order of D → A → N.

In order to investigate whether a similar structural intermediate accumulates during folding for lysozyme and α-lactalbumin, kinetics of refolding from the completely unfolded state are followed by measurement of time-dependent changes in CD spectra in the peptide and aromatic regions. Noncoincidence of refolding curves measured at different wavelengths demonstrates the existence of a transient structural intermediate formed at an early stage of refolding for both the proteins.

Materials and Methods

Materials. Hen egg white lysozyme recrystallized 6 times was purchased from Seikagaku Kogyo Co., Ltd. Gdn·HCl was specially prepared reagent grade from Nakarai Chemicals Ltd. Androsterone and L-pantoyl lactone were obtained from Tokyo Chemical Industry Co., Ltd. and Wako Pure Chemical Industries, Ltd., respectively. Bovine α-lactalbumin was prepared from fresh milk by the method described previously (Kuwajima et al., 1976). This preparation (holo-α-lactalbumin) contains 1.1–1.2 ions of Ca^{2+} per molecule of the protein (Hiraoka et al., 1980; Segawa & Sugai, 1983). The Ca^{2+}-free apo-α-lactalbumin was obtained by the method of Hiraoka & Sugai (1984). Concentrations of lysozyme and α-lactalbumin were determined spectrophotometrically by using molar extinction coefficients at 280 nm of 37 600 (Imoto et al., 1972) and 28 500 M^{-1} cm^{-1} (Kronman & Andreotti, 1964), respectively. The concentration of Gdn·HCl was determined from the refractive index at 589 nm (Nozaki, 1972). For experiments of apo-α-lactalbumin, deionized pure water was used for preparation of buffer solutions, the buffers were further demetalized by a Chelex-100 column before use, and contamination of the solutions with Ca^{2+} was checked before and after each experiment by using a Hitachi 170-10 atomic absorption spectrometer. Since the concentrations of monovalent metal cations are also expected to affect the stability of apo-α-lactalbumin (Hiraoka & Sugai, 1984), the solutions for α-lactalbumin had a constant concentration of Na^+ (0.1 M) adjusted by NaCl and was buffered with 50 mM sodium cacodylate unless otherwise stated. For lysozyme, the solutions contained 0.07 M NaCl, and the pH was adjusted to 1.5 by a dilute HCl solution.

Methods. Equilibrium CD measurements were carried out in Jasco J-20 and J-500A recording spectropolarimeters. The spectropolarimeters were calibrated with androsterone that has a molar ellipticity at 304 nm of 11.2×10^3 deg·cm²·dmol⁻¹ in dioxane (Jasco's standard). This calibration gave CD values ~7% higher than the values previously reported for α-lactalbumin (Kuwajima et al., 1976). The path length of the optical cuvette was 10 mm in most cases, but a 1-mm path length was also used for measurement of the peptide spectra down to 200 nm. The temperature of solution was determined by dipping a thermistor probe in the cuvette with a Takara SPD 01-10A thermistor thermometer.

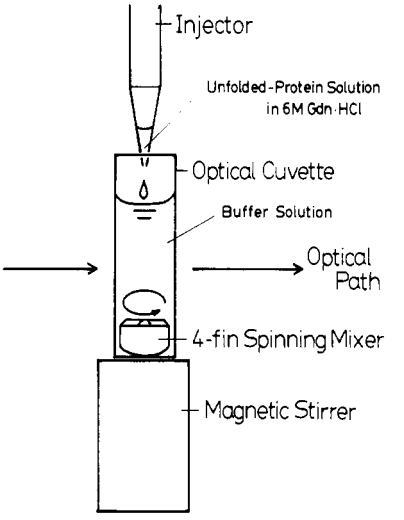

FIGURE 1: Illustration of the mixing device used in the present study. Spinning rate of the mixer was adjusted to give efficient mixing without disturbing the CD signal, and the mixing dead time was less than 3 s.

A mixing device (Figure 1) was attached to the CD apparatus or to a Union Giken SM-401 absorption spectrophotometer for the purpose of measuring the refolding kinetics of the proteins. Refolding was initiated by rapid mixing of the unfolded protein in 6 M Gdn·HCl with a buffer solution of 0 M Gdn·HCl, and the final concentration of Gdn·HCl was 0.3 M. The time constants of the CD apparatus and the absorption spectrophotometer were set at 0.5 or 1 s. Because of a low signal to noise ratio below 210 nm at this time constant, the kinetics were measured only at and above 210 nm. The dead time of the mixing device was estimated by measuring a change in a CD signal at 220 nm when 0.11% L-pantoyl lactone in 6 M Gdn·HCl was mixed with water in the same ratio of the Gdn·HCl solution to water as used for the refolding experiments [see Konno et al. (1975) for the CD spectrum of pantoyl lactone]. After density fluctuation had disappeared, the CD signal showed a constant value, and the dead time estimated was less than 3 s. All the kinetic experiments were done under the conditions where the rate of refolding is slow enough compared with the dead time and in addition where the refolding is essentially complete: at pH 1.5 for lysozyme and pH 7.0 for α-lactalbumin at 0.3 M Gdn·HCl and 4.5 °C. The pH values reported are pH meter readings at room temperature.

Curve fitting of the peptide CD data, to obtain estimates of structural fractions, was carried out for the equilibrium states and also for transient folding intermediates of the two proteins. The observed ellipticity, $[\theta]_{obsd}$, at any fixed wavelengths is expressed in terms of the reference values, $[\theta]_\alpha$, $[\theta]_\beta$, and $[\theta]_c$, for pure α-helix, β-form, and unordered form, respectively, as

$$[\theta]_{obsd} = f_\alpha[\theta]_\alpha + f_\beta[\theta]_\beta + f_c[\theta]_c \quad (1)$$

where f values are the fractions of the three forms in the protein molecule. A linear least-squares analysis was made

with a constraint, $f_\alpha + f_\beta + f_c = 1$. The reference curves used were the data of Greenfield & Fasman (1969), Saxena & Wetlaufer (1971), and Chen et al. (1974). The average helical length of 11 residues was used in the reference curve of Chen et al.

RESULTS

Equilibrium of Unfolding Induced by Gdn·HCl. Equilibrium CD spectra of native lysozyme and α-lactalbumin and their unfolded forms in 6 M Gdn·HCl are shown in Figure 4. The unfolding of lysozyme was followed by measuring the dependence of the ellipticity values at 287.5, 255, and 222 nm on the concentration of Gdn·HCl at the same pH and temperature as used for the kinetic experiment (pH 1.5, 4.5 °C). The transition curves measured at the three wavelengths coincide with each other, suggesting that the unfolding equilibrium of the protein under the present conditions occurs between two states in the transition region. The transition midpoint observed was at 2.3 M Gdn·HCl. Equilibrium unfolding of holo-α-lactalbumin by Gdn·HCl in the absence of excess Ca^{2+} was studied previously (Kuwajima et al., 1976). In contrast to the lysozyme unfolding, noncoincident transition curves measured at different wavelengths (270 and 222 nm) have demonstrated existence of a stable intermediate (A state). The temperature and pH dependence of the transition at each wavelength was also studied (Kuwajima et al., 1976, 1981; Kuwajima, 1977). At any temperature and any pH studied, the transition measured by the aromatic CD band (270 nm) occurs before the disruption of the backbone secondary structure measured by the far-UV CD band (222 nm). At neutral pH and 4.5 °C, the midpoint of the transition at 270 nm is at 2.0 M Gdn·HCl and that at 222 nm is higher by 0.2–0.3 M in the denaturant concentration. The previous study of the thermal unfolding of apo-α-lactalbumin has shown that at 0.1 M Na^+ the apoprotein is folded at 4.5 °C (Hiraoka & Sugai, 1984); its CD spectrum highly resembles that of the native holoprotein (Figure 4). The unfolding of apo-α-lactalbumin by Gdn·HCl at 0.1 M Na^+ and 4.5 °C was examined in this study. Also in this case, the transition is not a two-state reaction. Elimination of the bound Ca^{2+} decreases the transition midpoints at both wavelengths, but it affects more strongly the transition observed at 270 nm; the midpoint is at 1.5 M Gdn·HCl at 270 nm and at 1.9 M Gdn·HCl at 222 nm (pH 7.0 in 0.05 M NaCl–0.05 M sodium cacodylate, 4.5 °C).

Transient Structural Intermediate Observed in Refolding Kinetics. Figure 2 shows kinetic progress curves for lysozyme and for the two forms of α-lactalbumin at 4.5 °C measured by CD changes in the aromatic (250 and 287.5 nm for lysozyme and 270 nm for α-lactalbumin) and in the peptide (222 nm) regions. In spite of their apparent difference in equilibrium unfolding properties, the kinetics of folding of lysozyme and α-lactalbumin show a common feature. Although the changes in the aromatic CD occur in an observable time range, most of the change at 222 nm occurs within the dead time of the measurement. The noncoincidence of the kinetic curves measured at different wavelengths demonstrates existence of an early folding intermediate which is still unfolded when measured by the aromatic CD bands but has folded secondary structure as measured by the CD values at 222 nm. In most of the previous kinetic studies of lysozyme folding, the optical probe used was a change in absorption of tryptophan residues (Tanford, 1970; Tanford et al., 1973; Kato et al., 1981, 1982). Accordingly, time-dependent changes in absorption at 300 and 292 nm were also measured under the same condition as used for the CD measurements. The absorption decreased at 300 nm and increased at 292 nm during refolding, and their kinetics coincided with those observed by the ellipticity changes at 250 and 287.5 nm; however, the kinetic amplitude of the absorption change at 292 nm was smaller than the static absorption difference expected from the equilibrium unfolding curve (Kato et al., 1981). Thus, the changes in the aromatic

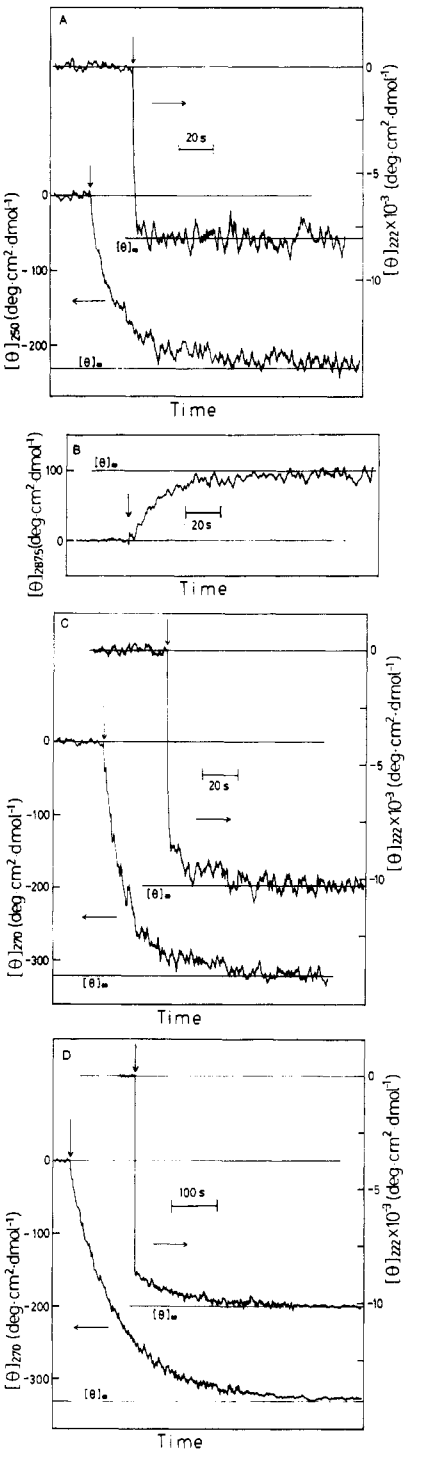

FIGURE 2: Kinetic progress curves of refolding measured at different wavelengths at 4.5 °C. The refolding was initiated by a concentration jump of Gdn·HCl from 6.0 to 0.3 M. (A) Lysozyme at 250 and 222 nm (pH 1.58); (B) lysozyme at 287.5 nm (pH 1.58); (C) holo-α-lactalbumin at 270 and 222 nm (pH 7.0); (D) apo-α-lactalbumin at 270 and 222 nm (pH 7.0). Vertical arrows indicate the zero time at which the mixing occurred. $[\theta]_\infty$ denotes the ellipticity at equilibrium. Protein concentrations are the following: (A) 4.43×10^{-5} M for 250 nm and 2.52×10^{-6} M for 222 nm; (B) 4.12×10^{-5} M; (C) 4.49×10^{-5} M for 270 nm and 2.83×10^{-6} M for 222 nm; (D) 5.19×10^{-5} M for 270 nm and 5.22×10^{-6} M for 222 nm. The time constant of the spectropolarimeter was changed from 1 to 5 s at 340–380 s after mixing for (D).

Table I: Curve Fitting of Peptide CD Spectra of Lysozyme and α-Lactalbumin with Various Reference Data

	Greenfield & Fasman (1969)				Saxena & Wetlaufer (1971)				Chen et al. (1974)			
	f_α (%)[b]	f_β (%)	f_c (%)	RMS[a]	f_α (%)	f_β (%)	f_c (%)	RMS	f_α (%)	f_β (%)	f_c (%)	RMS
Lysozyme												
equilibrium states												
native, pH 1.5, 4.5 °C	22.7	21.5	55.8	429	31.4	15.3	53.3	364	30.9	10.5	58.6	580
thermally unfolded, pH 1.5, 76.6 °C	7.7	31.2	61.1	237	19.6	21.6	58.8	444	17.5	13.0	69.5	452
transient intermediate, pH 1.5, 4.5 °C	15.2	28.9	55.9	379	22.9	24.6	52.5	262	24.9	16.0	59.1	504
α-Lactalbumin												
equilibrium states for the holoprotein												
native, pH 7, 4.5 °C	28.6	18.0	53.4	642	36.7	11.8	51.5	570	38.0	2.8	59.2	545
A state, pH 2.0, 4.5 °C	22.1	31.8	46.1	813	31.2	20.7	48.1	899	33.3	25.2	41.5	709
thermally unfolded, pH 7, 77.6 °C	15.8	27.4	56.8	583	24.8	20.4	54.8	472	24.3	18.3	57.4	499
equilibrium states for the apoprotein												
folded, pH 7, 4.5 °C	25.9	24.1	50.0	402	34.4	15.9	49.7	428	37.0	8.9	54.0	511
thermally unfolded, pH 8.0, 41.5 °C	20.8	27.0	52.2	652	28.6	20.2	51.2	592	30.0	20.1	49.9	500
transient intermediate, pH 7.0, 4.5 °C	20.0	29.3	50.7	867	28.8	20.9	50.2	848	30.0	22.7	47.4	974

[a] RMS error, expressed in deg·cm²·dmol⁻¹. CD data between 210 and 250 nm were used for calculation. [b] Structural fractions are expressed as percent of chain length.

Table II: Curve Fitting of Peptide CD Spectra Using the Data Including Those below 210 nm

	Greenfield & Fasman (1969)				Saxena & Wetlaufer (1971)				Chen et al. (1974)			
	f_α (%)	f_β (%)	f_c (%)	RMS	f_α (%)	f_β (%)	f_c (%)	RMS	f_α (%)	f_β (%)	f_c (%)	RMS
α-Lactalbumin												
native holoprotein,[a] pH 7, 4.5 °C	24.6	26.1	49.3	708	34.8	14.7	50.5	729	37.8	3.2	59.0	548
A state, pH 2.0,[b] 4.5 °C	26.8	24.3	48.9	1248	37.4	10.6	52.0	1189	35.6	9.4	54.9	1351

[a] Wavelength range used was from 202 to 245 nm. [b] Wavelength range used was from 200 to 245 nm.

CD bands represent the folding process of lysozyme studied so far by other researchers. The coincidence between the CD and absorption kinetics was also observed for α-lactalbumin as already discussed earlier (Nitta et al., 1977).

Figure 3 shows semilogarithmic plots for the kinetic data measured by the aromatic CD shown in Figure 2. For lysozyme and holo-α-lactalbumin, the kinetics are biphasic, while it is much closer to a single reaction for apo-α-lactalbumin; a least-squares analysis of the refolding curve of the apoprotein with a two-exponential decay function gives rate constants differing only by a factor of 2. The biphasic refolding kinetics of lysozyme agree with the results of Kato et al. (1982), in which it is suggested that the slow phase is caused by an interconversion between two unfolded species associated with proline isomerization [see Kim & Baldwin (1982)]. In the biphasic kinetics of lysozyme and holo-α-lactalbumin, the major folding occurs in the fast phase, and its decay time is 15 s for lysozyme (pH 1.5, 4.5 °C) and 10 s for holo-α-lactalbumin (pH 7.0, 4.5 °C, 4.5×10^{-5} M protein). The refolding rate of holo-α-lactalbumin is dependent on the protein concentration, and an ~1.5-fold decrease in the rate was observed when the concentration was 3×10^{-6} M (a decay time of ~15 s for the kinetics of the aromatic CD). This is not unexpected considering the effect of the bound Ca^{2+} on stabilization of native α-lactalbumin. In fact, our preliminary study shows that the refolding rate of the protein is accelerated by Ca^{2+} (unpublished data). For apo-α-lactalbumin, the refolding has a decay time of ~100 s.

The kinetics of refolding were measured at various wavelengths. The refolding rate is independent of the wavelength for all the cases. The ellipticity values of the early folding intermediate were obtained by extrapolation of the ellipticity changes to zero time by using the semilogarithmic plots as shown in Figure 3. The wavelength dependence of the ellipticity value extrapolated, then, gives a CD spectrum of the folding intermediate. The results are shown in Figure 4. Also shown in the figure for comparison are the spectra of the native proteins, the unfolded proteins in 6 M Gdn·HCl, and the partially unfolded states of the proteins, namely, the thermally unfolded states of lysozyme and α-lactalbumin and the A state of α-lactalbumin.

Curve Fitting of the Peptide CD Spectra. The results of curve fitting of the peptide CD spectra are shown in Table I. The early intermediates of the two forms of α-lactalbumin give an essentially identical spectrum, showing that the structural fractions of the intermediate are not affected by Ca^{2+} under the present condition, although the folding rate and the stability of the native state are markedly enhanced by Ca^{2+}. The spectrum of the intermediate of lysozyme is similar to that of α-lactalbumin. They show a high content of β-structure, a low content of α-helix, and invariability of the unordered fraction, compared with the spectra of the native molecules (Table I); since the kinetic data below 210 nm were not measured, the comparison of the calculated structural fractions is made by using the CD data between 210 and 250 nm (see below for possible deviations of the calculated fractions that would appear if we could use the data below 210 nm). Thus, at the first stage of folding detectable in the present experiments, both lysozyme and α-lactalbumin assume a similar structural intermediate. The spectra of the intermediates of the two proteins also resemble the spectra of the partially unfolded states of the proteins. The native tertiary structure is almost completely disrupted in all these species as measured by the aromatic CD spectra, but there is a significant amount of the backbone secondary structure as measured by the far-UV CD spectra. The calculated fractions of the partially unfolded species are very similar to those of the folding intermediates, although the thermally unfolded proteins at high temperatures show a little less amounts of the ordered secondary structure (Table I).

In order to test the goodness of the fit of the observed CD curves to the calculated ones, the spectra of the native and the A states of α-lactalbumin were taken down to 202 and 200 nm, and the least-squares curve fitting was made by using the

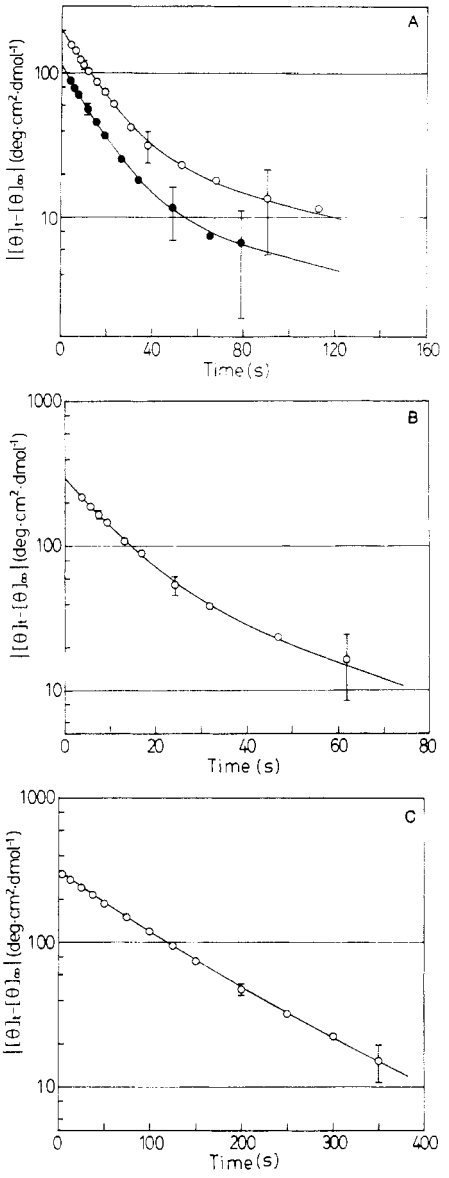

FIGURE 3: Semilogarithmic plots of the refolding curves measured by aromatic CD bands. (A) Lysozyme at 250 nm (○) and at 287.5 nm (●); considering the level of the experimental error, the semilogarithmic plots at these two wavelengths are coincident with each other. (B) Holo-α-lactalbumin at 270 nm. (C) Apo-α-lactalbumin at 270 nm. Solid curves are theoretically calculated to give the best fits to the observed data on the basis of the nonlinear least-squares analysis with the observational equation with two exponential terms as $[\theta]_t = [\theta]_\infty + \Delta[\theta]_1 e^{-k_1 t} + \Delta[\theta]_2 e^{-k_2 t}$, where $\Delta[\theta]_i$ and k_i are the amplitude and the rate constant of each term. The following are the values of these kinetic parameters: (A) $\Delta[\theta]_1 = 174.7$, $\Delta[\theta]_2 = 27.6$, $k_1 = 6.66 \times 10^{-2}$ s^{-1}, $k_2 = 8.44 \times 10^{-3}$ s^{-1} at 250 nm and $\Delta[\theta]_1 = -103.0$, $\Delta[\theta]_2 = -11.7$, $k_1 = 6.91 \times 10^{-2}$ s^{-1}, $k_2 = 8.25 \times 10^{-3}$ s^{-1} at 287.5 nm; (B) $\Delta[\theta]_1 = 235.8$, $\Delta[\theta]_2 = 60.2$, $k_1 = 9.70 \times 10^{-2}$ s^{-1}, $k_2 = 2.33 \times 10^{-2}$ s^{-1}; (C) $\Delta[\theta]_1 = 265.1$, $\Delta[\theta]_2 = 45.1$, $k_1 = 1.07 \times 10^{-2}$ s^{-1}, $k_2 = 4.79 \times 10^{-3}$ s^{-1}.

CD values including those below 210 nm. The results are shown in Table II. For native holo-α-lactalbumin, the fractions are essentially the same as those obtained previously. However, the structural fractions in the A state deviates from the previous values with a significant increase in the RMS error. This indicates that there is some spectral contribution(s), which is not well fitted by the reference spectra used in the calculations, in the A state. Thus, it should be emphasized that, when we compare the calculated structural fractions, it is particularly important whether the same wavelength region is chosen in the calculations. Although this result rather suggests that the calculated fractions in the partially unfolded species shown in Table I are not very accurate, extent of the deviation of the structural fractions does not harm the conclusion that these species including the folding intermediates have an appreciable amount of the backbone secondary structure.

Refolding from the A State. In order to investigate whether or not the A state of α-lactalbumin is kinetically distinct from the folding intermediate, the refolding kinetics from the A state was also examined. The refolding was initiated by a pH jump from 2.2 to 7.0 in the presence of 0.3 M Gdn·HCl at 4.5 °C. The results have shown that the rate constants and the ellipticity values extrapolated to zero time excellently agree with those in refolding from the completely unfolded state (data not shown). This indicates (i) that the A state is not the folding intermediate but that the interconversion from the A state to the folding intermediate occurs within the dead time of the measurement or otherwise (ii) that the A state itself corresponds to the intermediate state attained at an early stage in refolding from the completely unfolded state.

Diffuse Thermal Transition of the Secondary Structure in the A State. From all of the results shown above and also from the results in the previous studies (Kuwajima, 1977; Nozaka et al., 1978), it is safe to conclude that the partially unfolded species belong to the same conformational species as observed at the first stage of folding. Since at acid pH, the A state of α-lactalbumin is a stable equilibrium state (Kuwajima et al., 1975, 1976), examination of the temperature dependence of the peptide CD spectrum in the A state may give some insight into mechanisms of stabilization of the secondary structure in the folding intermediate. Figure 5 shows the temperature dependence of the ellipticity at 222 nm for the A state (pH 1.2–2.5) and also for the native states of lysozyme and holo- and apo-α-lactalbumin (pH 1.5, 7.0, and 8.0, respectively). The native proteins show a cooperative thermal transition which is reflected in a sigmoidal shape of the temperature dependence. The midpoints of the sigmoidal parts, 47 °C for lysozyme, 28 °C for apo-α-lactalbumin, and 58 °C for holo-α-lactalbumin, coincide with the transition temperatures observed by the aromatic CD or by the tryptophan absorption spectra (Kuwajima & Sugai, 1978; Hiraoka & Sugai, 1984). On the contrary, the A state of the protein shows no cooperativity. The negative ellipticity at 222 nm decreases monotonically with increasing temperature. The spectrum at a high temperature in the A state coincides with the spectrum of the thermally unfolded protein at neutral pH (Figure 4). It is concluded that the diffuse thermal transition in the A state is not a definite conformational transition but rather corresponds to a variation in an ensemble of the conformational species that is partially folded but does not have cooperative forces to stabilize the native conformation. Such a variation in the partially folded species has been discussed in our previous studies (Nozaka et al., 1978; Kuwajima & Sugai, 1978).

The temperature dependence of the ellipticity at 222 nm at pH 2.5 in the presence of Gdn·HCl was also investigated for α-lactalbumin. The results are also shown in Figure 5. At this pH and at a moderate concentration of Gdn·HCl (~3 M), the conformational state of the protein is between the A and the completely unfolded state (Kuwajima et al., 1976). A striking feature is that the ellipticity decreases with decreasing temperature below 20–30 °C. The disruption of some ordered backbone structure is brought about by cooling under these conditions.

DISCUSSION

There has been a great deal of accumulation of the data on the reversible unfolding of globular proteins. The thermo-

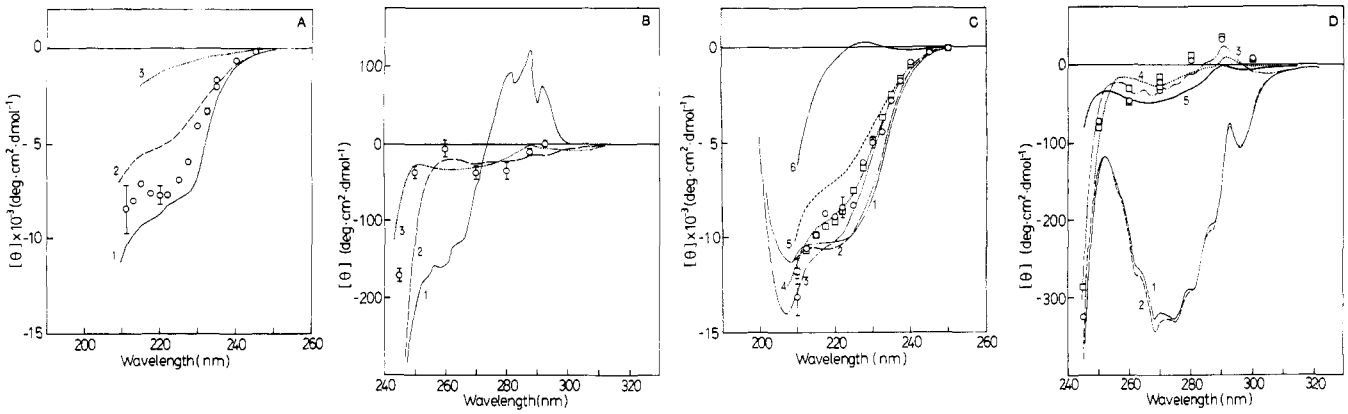

FIGURE 4: CD spectra of lysozyme (A, B) and α-lactalbumin (C, D) in various conformational states. (A, B) Lysozyme at pH 1.5 throughout; curve 1, the native state at 4.5 °C; curve 2, the thermally unfolded state at 69 °C; curve 3, the unfolded state in 6 M Gdn·HCl at 4.5 °C; (○) the transient folding intermediate at 4.5 °C. (C) Curve 1, native holo-α-lactalbumin at pH 7.0 and 4.5 °C; curve 2, folded apo-α-lactalbumin at pH 7.0 and 4.5 °C; curve 3, the A state at pH 2.0, 0.1 M NaCl, and 4.5 °C; curve 4, the thermally unfolded state of the apoprotein at pH 8.0, 10 mM sodium borate ([Na^+] = 10 mM) at 41 °C; curve 5, the thermally unfolded state of the holoprotein at pH 7.0 and 78 °C; curve 6, the unfolded state in 6 M Gdn·HCl at pH 7.0 and 4.5 °C; the transient folding intermediate for the holoprotein (○) and apoprotein (□) at pH 7.0 and 4.5 °C. (D) The conformational states and solvent conditions for the curves from 1 to 3 and for the two symbols (○, □) are the same as those for the corresponding ones in (C); curve 4, the thermally unfolded states of the apoprotein at pH 7.0 and 62.5 °C; curve 5, the unfolded state in 6 M Gdn·HCl at pH 7.0 and 4.5 °C; the spectrum of the thermally unfolded holoprotein, which is not shown because of complication of the figure, is essentially the same as curve 4.

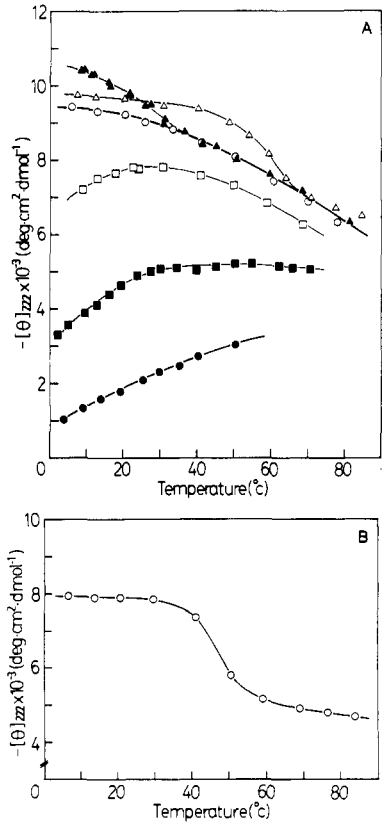

FIGURE 5: Thermal transition curves of lysozyme (B) and α-lactalbumin (A) measured by the ellipticity changes at 222 nm. (A) the A state of α-lactalbumin at pH 2.0 and 0.1 M NaCl (○); the holoprotein at pH 7.0 (△); the apoprotein at pH 8.0 and 10 mM sodium borate ([Na^+] = 10 mM) (▲); the unfolded states at pH 2.5 in the presence of Gdn·HCl, at 2.9 M Gdn·HCl and 0.1 M NaCl (□), at 4.0 M Gdn·HCl and 0.1 M KCl (■), and at 6.7 M Gdn·HCl and 0.1 M KCl (●). (B) Lysozyme at pH 1.5 and 0.07 M NaCl.

dynamic analysis of unfolding can now provide good criteria for the stability of the native conformations of proteins (Tanford, 1968, 1970; Privalov, 1979; Pfeil, 1981b). However, it is also true that there is no simple correlation between the Gibbs energy changes in unfolding and the primary sequences of proteins. The native structure of a protein is only marginally stable as determined by a delicate balance between stabilizing and destabilizing contributions. In this sense, lysozyme and α-lactalbumin are typical proteins, which have similar primary sequences but have different unfolding behavior and different stabilities of the native conformations. On the other hand, increasing accumulation of the crystallographic data of proteins has made it possible to analyze the structural and functional similarities of proteins in terms of taxonomical or evolutional aspects (Ptitsyn & Finkelstein, 1980; Rossmann & Argos, 1981; Richardson, 1981). From these analyses, we can expect with little doubt that homology in amino acid sequence leads to analogy in three-dimensional structure. In fact, although the X-ray structure of α-lactalbumin is not yet available, it is reasonably possible to fold the α-lactalbumin backbone based on the known structure of lysozyme (Browne et al., 1969; Warme et al., 1974). Therefore, it is important to ask the question of what is a key factor to fold these homologous proteins if simple energetics deduced from the unfolding studies cannot explain the anticipated structural similarities.

The present results clearly show that both lysozyme and α-lactalbumin assume a similar transient intermediate at an early stage of folding. The intermediate is stable enough to detect kinetically and has an appreciable amount of backbone secondary structure that is comparable to that found in the native state. Similarity of the apparent strucutral fractions of the intermediates of the two proteins may result from the homology of the primary sequences of the proteins. The conformational spaces available for the two proteins are already restricted in a similar way at an early stage of folding. The results are consistent with the proposal that the protein folding is kinetically controlled with a transient structural intermediate and that the kinetic pathway of folding is similar for the homologous proteins (Kim & Baldwin, 1982; Hollecker & Creighton, 1983). At this time, however, this concept is not necessarily in conflict with the thermodynamic determination of protein structure (Anfinsen & Scheraga, 1975). It might be a plausible assumption that the native conformation is the one of lowest Gibbs energy but is reached through kinetically controlled intermediates (Ghélis & Yon, 1982).

Relation of the Present Results to Equilibrium Unfolding of the Proteins. It has been well documented that the equilibrium unfolding transitions of most small globular proteins

are well represented, to first approximation, as a cooperative two-state reaction without any stable intermediates (Privalov, 1979; Pfeil, 1981b) [see also Saito & Wada (1983) for illustrations and exceptions to this rule]. Lysozyme is a typical protein which shows a two-state unfolding (Tanford et al., 1966; Aune & Tanford, 1969; Tanford, 1968, 1970). This is not in conflict with the present results of kinetic accumulation of the folding intermediate. Equilibrium methods can only detect the species that are stably populated in the transition region. If a protein possesses a higher stabilizing energy in the native state, intermediate species detected kinetically are practically nondetectable by equilibrium methods. Similarly, apparent discrepancy in equilibrium unfolding behavior between lysozyme and α-lactalbumin can be accounted for in terms of a difference in stability of the native state between the proteins. As discussed by Tanford (1970) and by Robson & Pain (1976), whether or not the equilibrium intermediate occurs during the transition is highly sensitive to the stabilities of the intermediate against the native and completely unfolded states. Thus, if the native state of lysozyme is more stable than that of α-lactalbumin, the intermediate detected for α-lactalbumin is practically nondetectable in the unfolding of lysozyme. In fact, stabilization Gibbs energy of the native state is 3–5 kcal/mol higher for lysozyme than for α-lactalbumin at neutral pH and 25 °C (Kuwajima et al., 1976), and native lysozyme at pH 1.5 and 4.5 °C is still more stable than α-lactalbumin at pH 7.0 and 4.5 °C as shown by the present results. The difference in equilibrium unfolding behavior is not against the conserved nature of the folding mechanism but should rather reside in higher stability of native lysozyme after folding.

Identity of ΔH_{cal} determined from calorimetric measurements and ΔH_{vH} from van't Hoff plots obtained by equilibrium studies gives a criterion of the two-state behavior (Privalov & Khechinashvili, 1974; Privalov, 1979). Recently, Pfeil (1981a) reported that the thermal unfolding of α-lactalbumin satisfies this criterion, even in the presence of Gdn·HCl, in spite of the known spectral evidence for the presence of the stable intermediate (A state) in the unfolding by Gdn·HCl (Kuwajima et al., 1976). A similar puzzle is also found for lysozyme unfolding. Calorimetric data of lysozyme unfolding have demonstrated that the thermodynamic states of both heat- and Gdn·HCl-unfolded lysozyme are indistinguishable (Pfeil & Privalov, 1976) although various spectroscopic studies have shown that the thermal unfolding of lysozyme leads to a partially unfolded species (Sophianopoulos & Weiss, 1964; Hamaguchi & Sakai, 1965; Aune et al., 1967; Chistyakova et al., 1976; Kugimiya & Bigelow, 1973); e.g., at 80 °C about half of the helical structure of lysozyme is still preserved. In this context, it is noteworthy that the A state of α-lactalbumin shows a diffuse thermal unfolding (Figure 5). Such a noncooperative gradual change is presumably not reflected explicitly in calorimetric recording. The value of the ratio $\Delta H_{cal}/\Delta H_{vH}$ found for several proteins is known to be about 1.05, and the difference from 1.00 may be experimentally significant (Privalov & Khechinashvili, 1974; Freire & Biltonen, 1978; Privalov, 1979).

Comparison with Other Work. Kato et al. (1981) have studied the refolding kinetics of lysozyme by following aromatic absorption at 301, 292, and 250 nm by means of the stopped-flow method. They found that the total kinetic amplitude does not coincide with the static absorption difference between the initial unfolded and the final native states and have concluded that the unfolded species assumes a new transient conformation within the mixing dead time. Our results are consistent with their finding and also have revealed that this transient intermediate has an appreciable amount of the backbone secondary structure.

Proteins that are known to exhibit a similar transient folding intermediate as detected by CD spectra include bovine carbonic anhydrase B (McCoy et al., 1980), swine pepsinogen (McPhie, 1982), the C_L fragment of human immunoglobulin light chain (Goto & Hamaguchi, 1982), and bovine ribonuclease A (Schmid, 1984). Application of the peptide amide proton exchange to detect and characterize a folding intermediate of ribonuclease A was studied by Schmid & Baldwin (1979) and by Kim & Baldwin (1980). They have shown that an early hydrogen-bonded intermediate is populated during folding under strongly native conditions. It is still open, however, whether or not the existence of the early transient intermediate that has backbone secondary structure is a general phenomenon in protein folding.

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Registry No. Ca, 7440-70-2; lysozyme, 9001-63-2.

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