Evidence for Identity between the Equilibrium Unfolding Intermediate and a Transient Folding Intermediate: A Comparative Study of the Folding Reactions of α-Lactalbumin and Lysozyme†

Masamichi Ikeguchi, Kunihiro Kuwajima, Masahiro Mitani, and Shintaro Sugai*

Department of Polymer Science, Faculty of Science, Hokkaido University, Kita-ku, Sapporo, Hokkaido 060, Japan

Received February 11, 1986; Revised Manuscript Received June 17, 1986

ABSTRACT: The refolding kinetics of α-lactalbumin at different concentrations of guanidine hydrochloride have been investigated by means of kinetic circular dichroism and stopped-flow absorption measurements. The refolding reaction consists of at least two stages, the instantaneous accumulation of the transient intermediate that has peptide secondary structure and the subsequent slow process associated with formation of tertiary structure. The transient intermediate is compared with the well-characterized equilibrium intermediate observed during the denaturant-induced unfolding. (i) Stabilities of the secondary structures against the denaturant, (ii) affinities for Ca^{2+}, and (iii) tryptophan absorption properties of the transient and equilibrium intermediates were investigated. In all of these respects, the transient intermediate is identical with the equilibrium one, demonstrating the validity of the use of the equilibrium intermediate as a model of the folding intermediate. Essentially the same transient intermediate was also detected in the folding of lysozyme, the protein known to be homologous to α-lactalbumin but whose equilibrium unfolding is represented as a two-state reaction. The stability and cooperativity of the secondary structure of the intermediate of lysozyme are compared with those of α-lactalbumin. The results show that the protein folding occurring via the intermediate is not limited to the proteins that show equilibrium intermediates. Although the unfolding equilibria of most proteins are well approximated as a two-state reaction, the two-state hypothesis may not be applicable to the folding reaction under the native condition. Two models of protein folding, *intermediate-controlled folding model* and *multiple-pathway folding model*, which are different in view of the role of the intermediate in determining the pathway of folding, are also discussed.

Elucidation of the mechanism by which proteins fold to their biologically active three-dimensional structures has been challenged by a number of investigators over the past two decades, and it has been suggested that protein folding is kinetically controlled with structural intermediates that occur on a limited number of the pathways uniquely determined by the primary sequence [reviewed in Kim and Baldwin (1982) and Ghélis and Yon (1982)]. Thus, major problems in protein folding are how to identify and how to characterize the intermediates populated kinetically but usually very unstable at equilibrium. Although chemical trapping is an effective approach to study the folding reactions accompanied by disulfide intermediates (Creighton, 1978; Creighton & Goldenberg, 1984), the effects of the chemical groups, introduced by the trapping reaction, on protein structure cannot be neglected (Goto & Hamaguchi, 1979; Chavez & Scheraga, 1980; White, 1982) [see also Kosen et al. (1983)] and very likely interfere with the structural characterization of the intermediates. One way out of this difficulty is to find out proteins that show stable structural intermediates during the equilibrium unfolding reactions so that the structures of the intermediates can be characterized at equilibrium (Wong & Tanford, 1973; Robson & Pain, 1976; Kuwajima, 1977; Adams et al., 1980; McCoy et al., 1980; Dolgikh et al., 1984; Mitchinson & Pain, 1985; Dalzoppo et al., 1985). However, general significance of such apparently exceptional cases has not yet been established, and a stringent test of identity of the equilibrium intermediate with the folding intermediate must be required (Creighton, 1979; Denton et al., 1982; Lynn et al., 1984).

Our previous studies have shown that there is a structural intermediate (A state) in the equilibrium unfolding of α-lactalbumin induced by treatment with Gdn·HCl[1] (Kuwajima et al., 1976, 1981; Kuwajima, 1977; Nozaka et al., 1978; Ikeguchi et al., 1986). Recently, in the kinetic CD study on refolding of α-lactalbumin, a transient folding intermediate was also detected and this intermediate exhibits CD spectra similar to those in the A state (Kuwajima et al., 1985).

In the present study, identity between the two intermediates, the A state and the transient intermediate of α-lactalbumin, is examined by investigating whether or not the secondary structure in the transient intermediate has the same stability and the same cooperativity of unfolding as does that in the A state. The experimental design to do this is as follows. Refolding from the unfolded (D) state is initiated by a concentration jump of Gdn·HCl, and the resultant kinetics are followed by CD spectra. According to the previous study (Kuwajima et al., 1985), the transient folding intermediate accumulates at a sufficiently low concentration of Gdn·HCl (0.3 M) and has an appreciable amount of the backbone secondary structure with the unfolded-like aromatic CD spectrum. This accumulation of the intermediate is succeeded by the tertiary structure formation as indicated by a time-

† This work was supported in part by Grants-in-Aid for Scientific Research [General Research (c), 58580200 and 60580217] from the Ministry of Education, Science and Culture of Japan.

[1] Abbreviations: CD, circular dichroism; Gdn·HCl, guanidine hydrochloride; $c_{Gdn\cdot HCl}$, concentration of guanidine hydrochloride; N and D states, native and fully unfolded states, respectively; A state, intermediate state of α-lactalbumin.

0006-2960/86/0425-6965$01.50/0

dependent change in the aromatic CD spectrum, and concomitant rearrangement of some secondary structure also occurs as indicated by a small change in the peptide CD spectrum. Because the formation of the transient intermediate is instantaneous in comparison with the dead time of the present CD measurement and also with the subsequent folding process, extrapolation of the time-dependent part of the CD change to zero time gives the ellipticity value of the folding intermediate. The refolding experiments are carried out at different values of final $c_{Gdn\cdot HCl}$ (Gdn·HCl concentration). Dependence of the ellipticity extrapolated to zero time on the final $c_{Gdn\cdot HCl}$ gives a Gdn·HCl-induced unfolding curve of the transient folding intermediate. This unfolding curve is compared with the transition curve between the A and D states, which has been obtained from equilibrium experiments.

In order to reinforce the identification of the A state with the folding intermediate, the refolding kinetics of α-lactalbumin are further investigated by the tryptophan absorption spectrum. In the previous studies, comparison of the Gdn·HCl-induced unfolding curve with the curve of transition between the native (N) and A states, both followed by the absorption at 293 nm, has shown that the difference in extinction coefficient between N and A is half that between N and D (Sugai et al., 1973; Kita et al., 1976; Kuwajima, 1977), and this may be useful for identification of the folding intermediate.

The unfolding curve of the transient folding intermediate of lysozyme is also analyzed by the kinetic CD spectra. This protein is known to be homologous to α-lactalbumin (Shewale et al., 1984). Although the equilibrium unfolding of lysozyme in the transition zone is well represented as a two-state reaction without intermediates, the existence of a transient intermediate, similar to the intermediate of α-lactalbumin, in the kinetic folding process of lysozyme has been demonstrated (Kuwajima et al., 1985). Thus, the stability and cooperativity of unfolding of the transient folding intermediates are compared between the two proteins.

Materials and Methods

Materials. All the materials used were the same as reported in the previous paper (Kuwajima et al., 1985). Details of preparation of solutions and of determination of the concentrations of the proteins, Gdn·HCl, and Ca^{2+} have also been given previously (Kuwajima et al., 1985).

Methods. All measurements were made at 4.5 °C. The compartments that were directly in contact with the solutions for measurements, such as a mixing compartment, an observation cell, and reserviors of the apparatus used, were equipped with water jackets to control temperature by circulating water.

Equilibrium CD measurements were carried out as described previously (Ikeguchi et al., 1986). Difference absorption spectra were measured in a Hitachi Model 124 spectrophotometer with a pair of quartz cells of a 5-mm path length.

Kinetic CD measurements were carried out on a Jasco J-500A spectropolarimeter as described previously (Kuwajima et al., 1985). For the refolding experiments from the D state, the protein was originally unfolded in concentrated Gdn·HCl (>3.5 M), and for the unfolding experiments from the N state, the protein was originally dissolved in a buffer solution at 0 M Gdn·HCl. Concentration jump of Gdn·HCl was performed by rapid mixing of the protein solution that was injected from an injector with the diluent that had been prepared in a 10-mm path length optical cuvette set in the spectropolarimeter, using a mixing device given in the previous paper (Kuwajima et al., 1985), and the CD change associated with the refolding or the unfolding was measured. The dilution of the protein solution was kept 20- or 40-fold, and to adjust the denaturant concentration after the mixing to the preset value, an appropriate amount of the denaturant was added to the diluent; the high dilution ratio was found to be necessary for rapid, efficient mixing. The position of the nozzle of the injector was also important for efficiency of the mixing. For the refolding experiments from the D state, the injected solution had a higher density than the diluent, and it was injected downward from the nozzle placed just above the surface of the diluent solution. On the contrary, for the unfolding experiments or the refolding from the A state, in which the density of the diluent was higher, the nozzle was immersed in the diluent and placed close to the spinning mixer that sat on the bottom of the cuvette, and the nozzle was made sufficiently thin to prevent diffusion of the solution before injection. With these cautions, a dead time of mixing less than a few seconds was readily achieved.

The absorption kinetics at 292 nm associated with the refolding and the unfolding reactions of α-lactalbumin were measured on a Union Giken RA-1100 stopped-flow apparatus with a specific modification in its mixing compartment. The new mixing compartment, specially manufactured by Unisoku Co., Osaka, was designed to be efficient for mixing of the two solutions of very different densities (concentrated Gdn·HCl and water in this case) and also useful for a large concentration jump of the denaturant. The protein solution and the diluent were driven pneumatically, at about 5 kg/cm^2 pressure of nitrogen gas, through an eight-jet mixer. The dilution ratio of the protein solution and the diluent was made variable by means of a regulating needle valve that interposes resistance to the flow in the line from the reservoir of the protein solution to the mixer; 10-fold dilution of the protein solution was used in most cases. The protein solutions before mixing were the same as used in the kinetic CD measurements, except that a higher concentration of the protein was needed in the absorption measurements. The dead time of the stopped-flow measurement was determined by the method of Tonomura et al. (1978). The shortest dead time attained in the present experiments, at 5 kg/cm^2 driving pressure and with a 10-mm path length observation flow cell, was 6 ms. For the reactions having time constants longer than 20 s, the mixing device used in the kinetic CD measurements was also used, and the absorption change at 292 nm was monitored with a Union Giken SM-401 spectrophotometer.

In all the kinetic experiments, the data acquisition, storage, averaging, and analyses were made in a Sord M222 or a Sord M223-Mark-II microcomputer.

Results

Equilibrium Unfolding. Equilibrium unfolding reactions of apo-α-lactalbumin and lysozyme under the conditions used in the present study have been reported previously (Kuwajima et al., 1985). In the present study, the unfolding of α-lactalbumin induced by Gdn·HCl in the presence of excess Ca^{2+} (1 mM) at 4.5 °C was investigated by analysis of the ellipticity changes at 270 and 222 nm. In contrast to the three-state unfolding observed previously in apo-α-lactalbumin and holo-α-lactalbumin without excess Ca^{2+}, the unfolding transition curves measured at the different wavelengths were coincident with each other, suggesting that the unfolding reaction in the presence of 1 mM Ca^{2+} occurs between the two states. This result is essentially the same as that obtained previously by Ikeguchi et al. (1986) at 25 °C, and details of such an effect of Ca^{2+} on the equilibrium unfolding have been discussed therein.

Refolding Kinetics Followed by the Peptide CD Spectrum. Refolding kinetics from the D state were measured at different

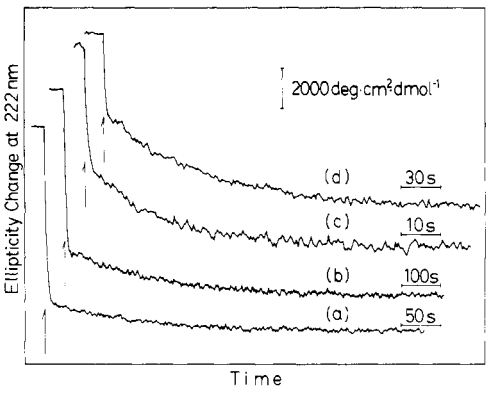


FIGURE 1: Kinetic progress curves of refolding of α-lactalbumin measured by the CD changes at 222 nm (4.5 °C). Vertical arrows indicate the zero times at which the refolding reactions were initiated by mixing the unfolded protein solutions in 6 M Gdn·HCl with the buffer solutions that contained appropriate amounts of Gdn·HCl to give the final folding conditions. The signal before the zero time shows the zero in ellipticity. Final conditions: (a) 0.30 M Gdn·HCl in the absence of Ca^{2+}; (b) 1.02 M Gdn·HCl in the absence of Ca^{2+}; (c) 1.77 M Gdn·HCl at 1 mM Ca^{2+}; (d) 2.52 M Gdn·HCl at 1 mM Ca^{2+}. The protein concentration after the mixing was 2.9–4.3 μM. All solutions contained 50 mM cacodylate–50 mM NaCl (pH 7.0, $[Na^+]$ = 0.1 M).

values of $c_{Gdn\cdot HCl}$ by means of the CD changes at 222 nm. The results for α-lactalbumin are shown in Figure 1. Similar refolding curves were also observed in lysozyme, except for dependence of the refolding rate on Ca^{2+} concentration observed only in the α-lactalbumin folding (see below). The refolding reactions of the two proteins occur in the two stages, the instantaneous change in ellipticity occurring on initiation of the refolding (the first stage) and the subsequent slow process occurring in an observable time range (the second stage) (Figure 1). As shown in the previous study of the refolding reactions at 0.3 M Gdn·HCl (Kuwajima et al., 1985), the first stage corresponds to formation of the transient intermediate that has folded secondary structure but has unfolded tertiary structure. With increasing $c_{Gdn\cdot HCl}$, however, the magnitude of the change in the peptide ellipticity observed in the first stage decreases and concomitantly the proportion of the change observed in the second stage to the total difference in ellipticity between the D and N states increases (Figure 2). Thus, at a high $c_{Gdn\cdot HCl}$, the transient intermediate begins to unfold and the tertiary interaction becomes important for stabilization of the peptide secondary structure. On the other hand, when the unfolding reaction from the N state was monitored by the CD change at 222 nm, only a single reaction was observed for both the proteins, and the observed CD change in the kinetics was in fair agreement with the difference in ellipticity between the D and N states expected from the equilibrium unfolding curve, indicating that there is no detectable intermediate in the unfolding reaction (data not shown).

Extrapolation of the time-dependent part of the refolding curve to zero time has been made by the method shown in the previous study (Kuwajima et al., 1985). Dependence of the extrapolated value of ellipticity on the final $c_{Gdn\cdot HCl}$ gives an unfolding transition curve of the transient folding intermediate. Such transition curves for the two proteins are shown in Figure 2 and compared with the unfolding curves at the final equilibria. The transition of the intermediate is less cooperative than the equilibrium unfolding transitions. At 1.8 M Gdn·HCl for α-lactalbumin at 1 mM Ca^{2+}, and at 1.5 M Gdn·HCl for lysozyme, the ellipticity change in the first stage of refolding is only 30–40% of the difference in ellipticity between the D and N states although the refolding reactions of the two proteins under these conditions are essentially complete. It should also be noted that the transition curve of the transient intermediate of α-lactalbumin at 1 mM Ca^{2+} coincides well with that at 0 M Ca^{2+}, and this forms a contrast to remarkable dependence of the apparent equilibrium unfolding curve on Ca^{2+} concentration. This behavior of the intermediate is analogous to the independence of the A ⇌ D transition on Ca^{2+} concentration observed previously (Ikeguchi et al., 1986) and suggests that there is no detectable Ca^{2+} binding to the transient folding intermediate.

The rate constants of refolding in the second stage have also been calculated, and they are shown in Figure 3, together with some unfolding rate constants and, for α-lactalbumin, also with

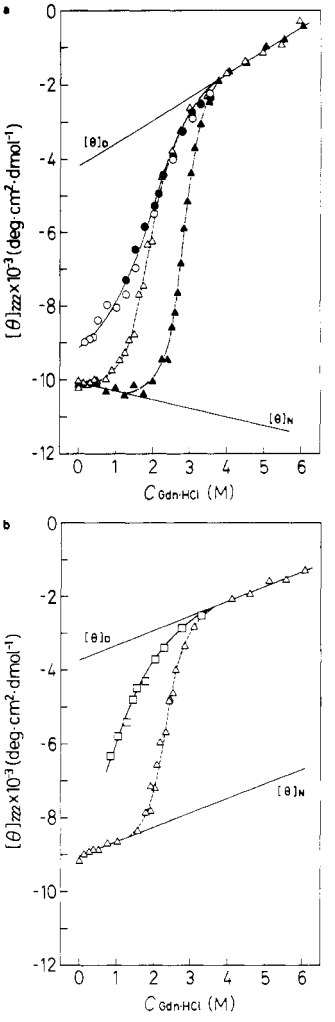


FIGURE 2: Unfolding transition curves of the transient folding intermediates (solid curves) of α-lactalbumin (a) and lysozyme (b), which were obtained from the dependence on $c_{Gdn\cdot HCl}$ of the ellipticity at 222 nm extrapolated to zero time of the refolding curve, shown in comparison with the equilibrium unfolding curves (broken curves and a dot–dash curve). (a) α-Lactalbumin in the absence of Ca^{2+} (open symbols) and in the presence of 1 mM Ca^{2+} (filled symbols). Ellipticity values: (○, ●) obtained by extrapolation of the refolding curves to zero time; (△, ▲) obtained by the equilibrium measurement. In the equilibrium measurements, protein concentration is 3.0–3.2 or 30–37 μM. Other experimental conditions are the same as in Figure 1. (b) Lysozyme at 4.5 °C and pH 1.5. Ellipticity values: (□) obtained by extrapolation of the refolding curves to zero time; (△) values from the equilibrium measurement. Protein concentration is 3.1–3.4 μM in the kinetic experiments and is 3.0 or 45 μM in the equilibrium experiments. All solutions contain 0.07 M NaCl, and the pH is adjusted by a dilute HCl solution. In (a) and (b), $[\theta]_N$ and $[\theta]_D$ represent the ellipticity values in the N and the D states, respectively, obtained by linear extrapolations of the dependence of the values on $c_{Gdn\cdot HCl}$ before and after the transition.

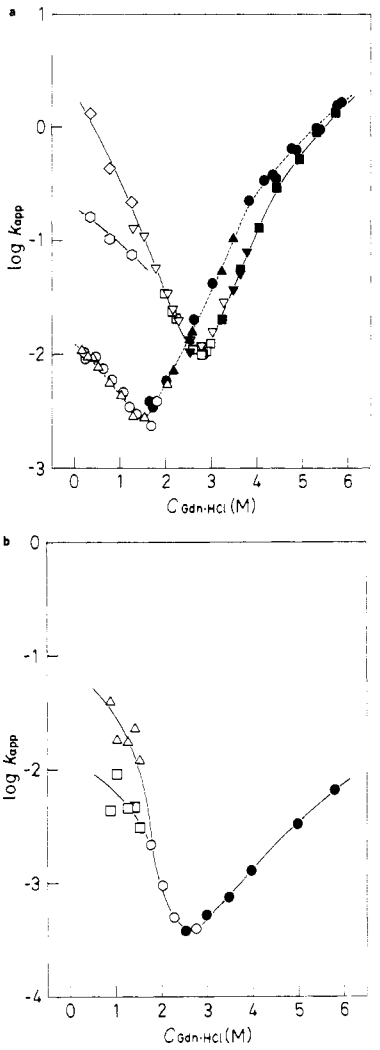


FIGURE 3: Dependence of the apparent rate constants (in s^{-1}) of refolding and of unfolding on $c_{Gdn\cdot HCl}$, in α-lactalbumin (a) and lysozyme (b). Open symbols refer to the refolding reactions, and filled symbols, to the unfolding reactions. (a) Apparent rate constants: of the apo protein measured by the ellipticity changes at 222 nm (△, ▲) and by the absorption changes at 292 nm (○, ●); of the holo protein at 1 mM Ca^{2+} measured by the ellipticity changes at 222 nm (▽, ▼) and by the absorption changes at 292 nm (◇, ○, □, ■). Diamonds and hexagons indicate the rate constants of the fast- and the slow-refolding phases, respectively, observed at low values of $c_{Gdn\cdot HCl}$. In the measurements of the absorption kinetics, the protein concentration after the mixing is 20–35 μM. Other experimental conditions are the same as in Figure 1. (b) Apparent rate constants: of the fast- (△) and the slow-refolding (□) phases at low values of $c_{Gdn\cdot HCl}$; of the single-phase refolding at $c_{Gdn\cdot HCl}$ values >1.6 M (○) and of the unfolding (●). The data for lysozyme are from the kinetic CD measurements at 222 nm. The conditions are the same as in Figure 2b.

the rate constants measured by changes in the tryptophan absorption spectrum (see below), as a function of $c_{Gdn\cdot HCl}$. For α-lactalbumin at 1 mM Ca^{2+} and for lysozyme, the second stage of refolding is further composed of two first-order phases at a low $c_{Gdn\cdot HCl}$ (<1.5 M) and the faster phase of the two phases has a larger kinetic amplitude. This behavior was discussed in our previous study (Kuwajima et al., 1985) and also in the studies by Kato et al. (1982). With increasing $c_{Gdn\cdot HCl}$, for all the cases, the apparent refolding rate decreases up to the value at the midpoint of the corresponding equilibrium transition curve, and the apparent unfolding rate increases beyond the midpoint. The rate constant profile for lysozyme is essentially the same as that reported in earlier published work, although the data in the earlier work have been based

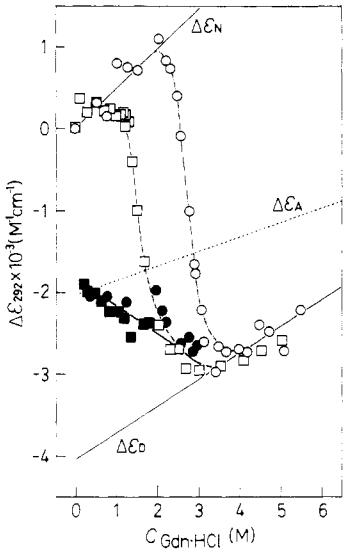


FIGURE 4: Equilibrium unfolding transition curves of α-lactalbumin, at 0 M (broken curve) and 1 mM Ca^{2+} (dot–dash curve), and the unfolding transition curve of the transient folding intermediate (solid curve), as measured by the tryptophan absorption at 292 nm, at pH 7.0 and 4.5 °C. Equilibrium data (open symbols) were obtained from the difference absorption measurements, in which protein concentration was 65–80 μM. The data are represented as the difference molar extinction coefficients, taking the N state in the absence of Gdn·HCl as the reference state. Filled symbols indicate the difference molar extinction coefficients in the transient folding intermediate, which were estimated by subtracting the kinetic absorption change, in the refolding, from the corresponding equilibrium value of the difference absorption on the transition curve. Squares and circles represent the data for the apo protein in the absence of Ca^{2+} and for the holo protein in the presence of 1 mM Ca^{2+}, respectively. A dotted line shows the difference molar extinction coefficient expected for the pure A state, $\Delta\epsilon_A$, which was obtained by linear extrapolation of the dependence of the difference absorption of the A state on $c_{Gdn\cdot HCl}$ between 0 and 0.5 M at acid pH (pH 2.0). $\Delta\epsilon_N$ and $\Delta\epsilon_D$ denote the difference molar extinction coefficients of the N and the D states, respectively, obtained by linear extrapolations from the regions before and after the transition.

on changes in tryptophan absorption (Tanford et al., 1973; Kato et al., 1982). Comparison of the results in Figure 3 with those in Figure 2 shows some correlation between the decrease in the apparent folding rate and the decrease in population of the transient intermediate both brought about by an increase in $c_{Gdn\cdot HCl}$, while such dependence of the folding rate on $c_{Gdn\cdot HCl}$ can be interpreted also in terms of a change in stability of the activated state lying along the pathway of the second stage of refolding (see Discussion). The other remarkable result in Figure 3 is that the refolding rate of α-lactalbumin is strongly dependent on Ca^{2+} concentration. About a 100-fold increase in the rate is observed at 1 mM Ca^{2+}, although the unfolding rate does not show such dependence. More detailed descriptions of the Ca^{2+}-dependent folding of the protein will be presented elsewhere, however.

Unfolding Equilibrium and Refolding Kinetics of α-Lactalbumin Followed by the Tryptophan Absorption Spectrum. Equilibrium unfolding curves of the holo form at 1 mM Ca^{2+} and the apo form of α-lactalbumin measured by the tryptophan absorption at 292 nm are presented in Figure 4. By assuming linear dependence of the difference molar extinction coefficient in the D state, $\Delta\epsilon_D$, on $c_{Gdn\cdot HCl}$, the $\Delta\epsilon_D$ at 0 M Gdn·HCl is estimated to be -4.0×10^3 M^{-1} cm^{-1}. This value agrees well with the value obtained in the previous study (Sugai et al., 1973). On the other hand, $\Delta\epsilon_A$ in the A state is known to be -1.9×10^3 to -2.1×10^3 M^{-1} cm^{-1}, evaluated from the acid transition curve (Kronman et al., 1972; Kita et al., 1976; Kuwajima, 1977); the acid state has been shown to be identical with the A state (Kuwajima et al., 1976, 1981). The broken

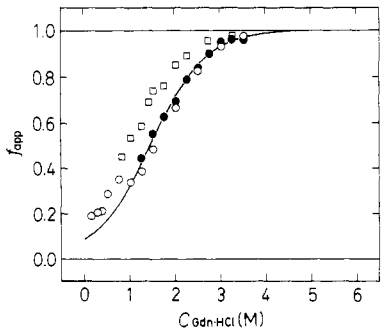


FIGURE 5: Normalized unfolding curves of the transient folding intermediates of α-lactalbumin (○, ●) and lysozyme (□), as compared with the equilibrium A ⇌ D transition (solid curve) for α-lactalbumin. Symbols are the same as in Figure 2. The apparent fractional extent of unfolding, f_{app}, for the transient folding intermediate of each protein was calculated from the data in Figure 2, on the assumption that the ellipticity in the intermediate is the same as that in the N state. The solid line is a theoretical curve of the A ⇌ D transition, obtained on the basis of the three-state analysis of the unfolding equilibria of α-lactalbumin.

line in Figure 4 shows dependence of $\Delta\epsilon_A$ on $c_{Gdn\cdot HCl}$, which has been obtained by linear extrapolation of the dependence of the $\Delta\epsilon_A$ in the acid state observed at low $c_{Gdn\cdot HCl}$ values.

The refolding and unfolding kinetics of the protein were followed by absorption measurements at 292 nm. The rate constants of the reactions are shown in Figure 3a, and they show excellent agreement with the rate constants obtained by the CD measurements. Also in accord with the results of the CD kinetics, the refolding from the D state has been found to be composed of at least two stages: namely, the instantaneous absorption change occurring within the dead time and the subsequent time-dependent absorption change. Thus, the transient intermediate has an intermediate absorption between those of the N and D states. On the other hand, the unfolding from the N state has been found to be a single reaction that has a kinetic absorption change comparable to the absorption difference expected from the equilibrium unfolding curve. The unfolding transition curve of the transient folding intermediate, represented by dependence of the $\Delta\epsilon$ extrapolated to zero time on $c_{Gdn\cdot HCl}$, has been obtained in the same manner as described above, and it is also shown in Figure 4. The transition curve is roughly coincident with the curve measured by the ellipticity at 222 nm (Figure 2a), although scattering of the data points from the absorption measurement does not allow us to make a more quantitative comparison. At a low $c_{Gdn\cdot HCl}$, the $\Delta\epsilon$ of the transient intermediate has a value of -2.0×10^3 M^{-1} cm^{-1}, which is in good agreement with $\Delta\epsilon_A$. The result supports the proposal that the transient folding intermediate is identical with the A state. Since the present stopped-flow measurement had a dead time of several milliseconds, it is also concluded that the formation of the transient intermediate is complete within the millisecond time range.

Comparison of the Unfolding Transition of the Transient Folding Intermediate with the A ⇌ D Transition. Normalized transition curves, given by the apparent fractional extents of unfolding, f_{app}, as a function of $c_{Gdn\cdot HCl}$, for the transient folding intermediates of the two proteins are shown in Figure 5, and they have been calculated from the results of the ellipticity at 222 nm (Figure 2). A prerequisite for making such a transition curve is knowledge of the ellipticity in each pure state (the intermediate or the D state) in the transition region as a function of $c_{Gdn\cdot HCl}$. For the D state, this is explicit as shown in Figure 2. For the transient intermediate, we have assumed that the ellipticity value is the same as that in the N state. If the intermediate is identical with the A state, this assumption is valid because the A state is known to have the ellipticity approximately the same as that in the N state at 222 nm (Kuwajima et al., 1976, 1981).

The solid line in Figure 5 shows a theoretical curve of the A ⇌ D transition, which is derived from the equilibrium unfolding data of apo-α-lactalbumin at 4.5 °C using a three-state analysis [see Ikeguchi et al. (1986) for details of the analysis]. Excellent coincidence between the theoretical curve for the A ⇌ D transition and the experimental points for the transition of the transient intermediate is found. This is strong evidence for identity between the transient intermediate and the A state of α-lactalbumin.

From Figure 5, the Gibbs energy change at 0 M Gdn·HCl, ΔG^{H_2O}, and the cooperativity index, m, for the transient intermediate of lysozyme are estimated by assuming the two-state transition of the intermediate to the D state and the linear dependence of the Gibbs energy change on $c_{Gdn\cdot HCl}$ (Ikeguchi et al., 1986). The ΔG^{H_2O} value (0.86 kcal/mol) is a little smaller than the corresponding value for α-lactalbumin (1.33 kcal/mol). Since the A state of α-lactalbumin has been known to be slightly more stable at acid pH than at neutral pH (Kuwajima, 1977), the stability of the intermediate in α-lactalbumin is higher than that in lysozyme. On the other hand, the m values show an agreement between α-lactalbumin and lysozyme (0.91 and 0.89 kcal/mol per M, respectively), indicating that the transient folding intermediates of the two proteins are similar to each other.

Discussion

The aim of the present study is to compare the equilibrium unfolding intermediate (the A state) and the transient folding intermediate of α-lactalbumin to establish the identity between them. The results obtained are summarized as follows: (i) Both intermediates show the same stability and the same cooperativity in the Gdn·HCl-induced unfolding (Figure 5). (ii) The transient folding intermediate has no binding capacity for Ca^{2+}, and this is consistent with the lack of the binding capacity in the A state (Ikeguchi et al., 1986). (iii) The tryptophan absorptions at 292 nm are identical for both the states. (iv) In accord with rapidity of the A ⇌ D transition (Kuwajima, 1977), the transient folding intermediate is formed rapidly within the stopped-flow dead time. Similarity of the two intermediates with regard to their CD spectral features has also been shown previously (Kuwajima et al., 1985). These results may be sufficient to make the identification of the A state with the folding intermediate convincing, and the validity of the use of the equilibrium unfolding intermediate as a model of folding intermediate is demonstrated. α-Lactalbumin is known to be fully in the A state at acid pH or by removal of the bound Ca^{2+} in the absence of other metal ions at neutral pH (Kuwajima et al., 1976; Mulqueen & Kronman, 1982; Segawa & Sugai, 1983). Thus, it is now experimentally feasible to isolate the folding intermediate at equilibrium and to characterize its structure. Some of the uniqueness of the structure in the A state was discussed in the previous paper (Ikeguchi et al., 1986).

The present study also shows that essentially the same transient intermediate accumulates in lysozyme folding. In accord with the homology of the two proteins, the cooperativity index in the Gdn·HCl-induced unfolding of the intermediate of lysozyme is essentially the same as that of α-lactalbumin. The apparent difference in their equilibrium unfolding has been shown to be ascribable to a difference in the intrinsic stability of the N state between the proteins (Ikeguchi et al., 1986). Therefore, folding processes of these two homologous proteins may be similar to each other. The protein folding occurring

via the transient intermediate is not limited to the proteins that show equilibrium intermediates, and the two-state hypothesis is not applicable to the kinetic folding under the native conditions.

A number of models of protein folding have been presented either theoretically or experimentally, and in general it has been postulated that structural segments of the native or other ordered structures are present before the tertiary structure assembles (Anfinsen & Scheraga, 1975; Tanaka & Scheraga, 1977; Kuwajima, 1977; Crippen, 1978; Lim, 1978; Karplus & Weaver, 1976, 1979; Ptitsyn & Finkelstein, 1980; Lesk & Rose, 1981; Kim & Baldwin, 1982; Ghélis & Yon, 1982; Go, 1983, 1984). On the one hand, it is also assumed that the incipient structures of folding must be very unstable, and the folding intermediates have been considered to be not readily detected even kinetically (Anfinsen & Scheraga, 1975; Karplus & Weaver, 1976). This assumption of instability of folding intermediates primarily results from a logical solution of the requisite of the two-state hypothesis of the folding transition [reviewed by Go (1983)]. On the other hand, in an alternative model, the folding occurs in a unique and definite sequence of steps under suitable folding conditions, and the presence of well-populated structural intermediates has been demonstrated (Kim & Baldwin, 1982; Ghélis & Yon, 1982; Brems & Baldwin, 1985; Kim, 1985). The results in the present study support the latter model and show that the intermediate is stable enough to detect kinetically or even at equilibrium in some cases.

Denton et al. (1982) and Lynn et al. (1984) have shown that ribonuclease A in concentrated $LiClO_4$ has a structure similar to that in the A state of α-lactalbumin and have suggested that this partially ordered state is not the intermediate that contributes to the pathway of folding, on the basis of the fact that some of the ordered structure in $LiClO_4$-denatured ribonuclease A becomes disordered instantaneously on initiation of the refolding. This behavior is apparently in contrast with the refolding behavior of α-lactalbumin under a strongly native condition at 0.3 M Gdn·HCl (4.5 °C). Under such a condition, the state identical with the A state is accumulated instantaneously, whether the refolding has been started from the D state or from the A state [see Kuwajima et al. (1985)]. In this context, however, it is worthy to be considered what is expected in the refolding under a marginally native condition where the folding intermediate is relatively unstable; such cases are found in the refolding of α-lactalbumin at 1.8 M Gdn·HCl in the presence of 1 mM Ca^{2+} and in the refolding of lysozyme at 1.5 M Gdn·HCl. Under these conditions, the ellipticity change at 222 nm occurring in the first stage of the refolding from the D state is only 30–40% of the total difference between the D and N states (Figure 2). Therefore, if the refolding begins with the pure A state that has the same ellipticity as in the N state at 222 nm (this is attainable in α-lactalbumin), the CD intensity is expected to be first decreased instantaneously to the value attained in the first stage of the refolding from the D state and then to be increased to the initial value during the second stage of the folding; the refolding curve of α-lactalbumin from the A state is shown in Figure 6, and the result accords with this expectation. This behavior is the same as what has been observed by Denton et al. (1982) in their ribonuclease folding studies. Obviously, there are two explanations for the above phenomenon, and distinction between them is important in assessing significance of the A state as a folding intermediate. In the following, the two models that are distinctive in this respect are discussed.

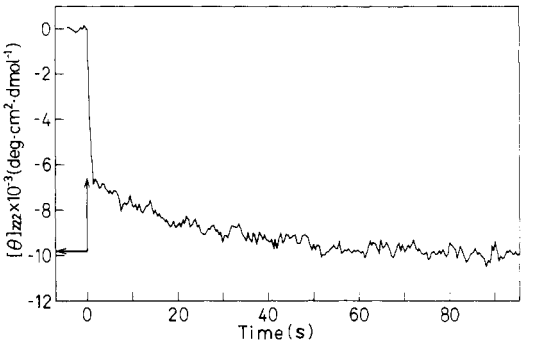


FIGURE 6: Kinetic progress curve of the refolding of α-lactalbumin from the A state, under a marginally native condition at pH 7.0 in the presence of 1.8 M Gdn·HCl and 1 mM Ca^{2+}, at 4.5 °C, measured by the CD change at 222 nm. The protein solution in the pure A state at pH 1.7 in the absence of Gdn·HCl was mixed with the diluent that contained appropriate amounts of alkali and Gdn·HCl. The ventrical arrow indicates the zero time at which the refolding was initiated, and the horizontal arrow indicates the ellipticity in the A state. The signal before the zero time shows the zero in ellipticity. The protein concentration after the mixing is 3.1 μM.

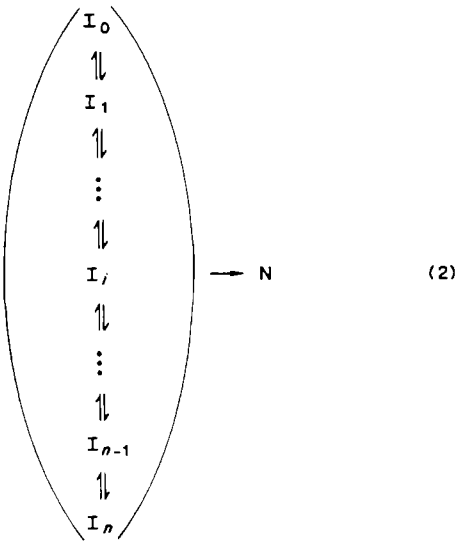
(*i*) *Intermediate-Controlled Folding Model.* In this model, we assume that the A state is an obligatory intermediate existing on the reaction pathway at an early stage of folding. Interconversion between the A and D states is much faster than the subsequent folding in the second stage, and hence rapid preequilibrium between A and D is attained before the second stage. However, there must be some activation barrier between A and D because the two different states are macroscopically distinguishable. The total folding reaction is written by

$$D \overset{\text{rapid}}{\rightleftharpoons} A \xrightarrow{k_f} N \qquad (1)$$

The decrease in the CD intensity at the first stage in the refolding from the A state, under a marginally native condition observed above, is due to a shift of the equilibrium between A and D under such a folding condition but not due to partial disruption of a localized region of the secondary structure in the molecule. Thus, the observation by Denton et al. (1982) can also be interpreted simply in terms of the A-like state of ribonuclease A being less stable than the A state of α-lactalbumin under the folding condition. This model predicts that the apparent folding rate in the second stage, k_{app}, is decreased with decreasing the stability of the A state and represented as $k_{app} = [K_{DA}/(1 + K_{DA})]k_f$, where K_{DA} is the equilibrium constant of the D $\rightleftharpoons$ A reaction and k_f is the intrinsic folding rate of the second stage. Therefore, according to this model, the decrease in k_{app} with increasing $c_{Gdn\cdot HCl}$ shown in Figure 3 must, at least in part, result from destabilization of the A state caused by an increase in $c_{Gdn\cdot HCl}$. The present results show that there is some cooperativity in the transition between the D state and the transient intermediate as evidenced by sigmoidal transition curves in Figure 5 and also show that the transition of the intermediate measured by the tryptophan absorption is approximately coincident with that measured by the CD at 222 nm. Apparently, these are consistent with the intermediate-controlled folding model, but not sufficient to justify its validity.

(*ii*) *Multiple-Pathway Folding Model.* In this model, we assume that the structure of the transient folding intermediate is highly sensitive to the final folding condition, and hence there are multiple pathways of folding depending on the intermediate structure formed at the first stage. The transition between the A and D states is not a cooperative two-state reaction, but in its extreme case, it is represented as a one-dimensional transition, being analogous to helix-coil transitions of homo-

polypeptides (Brandts, 1969; Poland & Scheraga, 1970), and the macroscopic state of the transient intermediate is an average of various microscopic structural species. The folding reaction in this model is expressed by



(2)

The averaged conformation of the microscopic species enclosed by parentheses above has spectral features of the A state at 0 M Gdn·HCl andd has those of the D state in concentrated Gdn·HCl, and hence there is essentially no distinct energy barrier between the two subsets, each of which is averaged over a number of the microscopic species that belong to the same conformational ensemble. Therefore, the decrease in the CD intensity at the first stage in the refolding from the A state is due to a shift in the average of the species usually accompanied by disruption of some localized part(s) of the secondary structure in a particular molecule. The k_{app} in this model cannot be expressed explicitly using the stability of the intermediate structural state but may rather be better represented by the stability of the activated state lying on the pathway that depends on the final folding condition. As evident from the assumption, the model also predicts extreme rapidity of the first stage of refolding, being in accord with the rapidity of the A $\rightleftharpoons$ D transition observed experimentally. This model has been discussed in our previous studies as an explanation for the presence of multiple unfolded states in human α-lactalbumin and also for diffuse thermal transition of the A state (Nozaka et al., 1978; Kuwajima & Sugai, 1978; Kuwajima et al., 1985). However, the compact globularity of the molecule in the A state requires a deviation from the one dimensionality of the A $\rightleftharpoons$ D transition (Kuwajima et al., 1975; Dolgikh et al., 1981; Izumi et al., 1983), because the interactions other than the short-range interactions must be taken into account in interpreting the stabilization of the compact structure in the A state.

At present, which of the mechanisms represented by the above two models is predominant remains to be clarified, and it might also be plausible that both of them contribute to the real folding reaction. Since we can investigate the D $\rightleftharpoons$ A transition, equivalent to the first stage of folding, by various structural probes at equilibrium, the time has been set to settle this problem.

ACKNOWLEDGMENTS

We are grateful to Dr. K. Nitta, Dr. Y. Hiraoka, and Y. Harushima for their helpful discussions. K.K. also thanks Professor R. L. Baldwin and Dr. P. S. Kim for sending copies of their papers before publication.

Registry No. Lysozyme, 9001-63-2.

REFERENCES

Adams, B., Burgess, R. J., Carrey, E. A., Mackintosh, I. R., Mitchinson, C., Thomas, R. M., & Pain, R. H. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 447–467, Elsevier/North-Holland Biomedical, Amsterdam and New York.

Anfinsen, C. B., & Scheraga, H. A. (1975) *Adv. Protein Chem. 29*, 205–300.

Brandts, J. F. (1969) in *Structure and Stability of Biological Macromolecules* (Timasheff, S. N., & Fasman, G. D., Eds.) pp 213–290, Marcel Dekker, New York.

Brems, D. N., & Baldwin, R. L. (1985) *Biochemistry 24*, 1689–1693.

Chavez, L. G., Jr., & Scheraga, H. A. (1980) *Biochemistry 19*, 1005–1012.

Creighton, T. E. (1978) *Prog. Biophys. Mol. Biol. 33*, 231–297.

Creighton, T. E. (1979) *J. Mol. Biol. 129*, 235–264.

Creighton, T. E., & Goldenberg, D. P. (1984) *J. Mol. Biol. 179*, 497–526.

Crippen, G. M. (1978) *J. Mol. Biol. 126*, 315–332.

Dalzoppo, D., Vita, C., & Fontana, A. (1985) *Biopolymers 24*, 767–782.

Denton, J. B., Konishi, Y., & Scheraga, H. A. (1982) *Biochemistry 21*, 5155–5163.

Dolgikh, D. A., Gilmanshin, R. I., Brazhnikov, E. V., Bychkova, V. E., Semisotnov, G. V., Venyaminov, S. Y., & Ptitsyn, O. B. (1981) *FEBS Lett. 136*, 311–315.

Dolgikh, D. A., Kolomiets, A. P., Bolotina, I. A., & Ptitsyn, O. B. (1984) *FEBS Lett. 165*, 88–92.

Ghélis, C., & Yon, J. (1982) *Protein Folding*, Academic, New York and London.

Go, N. (1983) *Annu. Rev. Biophys. Bioeng. 12*, 183–210.

Go, N. (1984) *Adv. Biophys. 18*, 149–164.

Goto, Y., & Hamaguchi, K. (1979) *J. Biochem. (Tokyo) 86*, 1433–1441.

Ikeguchi, M., Kuwajima, K., & Sugai, S. (1986) *J. Biochem. (Tokyo) 99*, 1191–1201.

Izumi, Y., Miyake, Y., Kuwajima, K., Sugai, S., Inoue, K., Iizumi, M., & Katano, S. (1983) *Physica B+C (Amsterdam) 120B*, 444–448.

Karplus, M., & Weaver, D. L. (1976) *Nature (London) 260*, 404–406.

Karplus, M., & Weaver, D. L. (1979) *Biopolymers 18*, 1421–1437.

Kato, S., Shimamoto, N., & Utiyama, H. (1982) *Biochemistry 21*, 38–43.

Kim, P. S. (1986) *Methods Enzymol.* (in press).

Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–489.

Kita, N., Kuwajima, K., Nitta, K., & Sugai, S. (1976) *Biochim. Biophys. Acta 427*, 350–358.

Kosen, P. A., Creighton, T. E., & Blout, E. R. (1983) *Biochemistry 22*, 2433–2440.

Kronman, M. J., Jeroszko, J., & Sage, G. W. (1972) *Biochim. Biophys. Acta 285*, 145–166.

Kuwajima, K. (1977) *J. Mol. Biol. 114*, 241–258.

Kuwajima, K., & Sugai, S. (1978) *Biophys. Chem. 8*, 247–254.

Kuwajima, K., Nita, K., & Sugai, S. (1975) *J. Biochem. (Tokyo) 78*, 205–211.

Kuwajima, K., Nitta, K., Yoneyama, M., & Sugai, S. (1976) *J. Mol. Biol. 106*, 359–373.

Kuwajima, K., Ogawa, Y., & Sugai, S. (1981) *J. Biochem. (Tokyo) 89*, 759–770.

Kuwajima, K., Hiraoka, Y., Ikeguchi, M., & Sugai, S. (1985) *Biochemistry 24*, 874-881.
Lesk, A. M., & Rose, G. D. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4304-4308.
Lim, V. I. (1978) *FEBS Lett. 89*, 10-14.
Lynn, R. M., Konishi, Y., & Scheraga, H. A. (1984) *Biochemistry 23*, 2470-2477.
McCoy, L. F., Jr., Rowe, E. S., & Wong, K. P. (1980) *Biochemistry 19*, 4738-4743.
Mitchinson, C., & Pain, R. H. (1985) *J. Mol. Biol. 184*, 331-342.
Mulqueen, P. M., & Kronman, M. J. (1982) *Arch. Biochem. Biophys. 215*, 28-39.
Nozaka, M., Kuwajima, K., Nitta, K., & Sugai, S. (1978) *Biochemistry 17*, 3753-3758.
Poland, D., & Scheraga, H. A. (1970) *Theory of Helix-Coil Transitions in Biopolymers*, Academic, New York and London.
Ptitsyn, O. B., & Finkelstein, A. V. (1980) *Q. Rev. Biophys. 13*, 339-386.
Robson, B., & Pain, R. H. (1976) *Biochem. J. 155*, 331-344.
Segawa, T., & Sugai, S. (1983) *J. Biochem. (Tokyo) 93*, 1321-1328.
Shewale, J. G., Sinha, S. K., & Brew, K. (1984) *J. Biol. Chem. 259*, 4947-4956.
Sugai, S., Yashiro, H., & Nitta, K. (1973) *Biochim. Biophys. Acta 328*, 35-41.
Tanaka, S., & Scheraga, H. A. (1977) *Macromolecules 10*, 291-304.
Tanford, C., Aune, K. C., & Ikai, A. (1973) *J. Mol. Biol. 73*, 185-197.
Tonomura, B., Nakatani, H., Ohnishi, M., Yamaguchi-Ito, J., & Hiromi, K. (1978) *Anal. Biochem. 84*, 370-383.
White, F. H., Jr. (1982) *Biochemistry 21*, 967-977.
Wong, K. P., & Tanford, C. (1973) *J. Biol. Chem. 248*, 8518-8523.

Molecular Order, Dynamics, and Ionization State of Phosphatidylethanolamine Bilayers As Studied by ^{15}N NMR†

Serge Akoka, Charles Tellier,* and Serge Poignant
Laboratoire de RMN et Réactivité Chimique, UA CNRS No. 472, Centre de Recherche en Biologie et Physico-Chimie Cellulaire, 44072 Nantes, France

Received March 21, 1986; Revised Manuscript Received July 24, 1986

ABSTRACT: Dipalmitoylphosphatidylethanolamine (DPPE) and dipalmitoylphosphatidylcholine (DPPC), ^{15}N-labeled in the polar head group, were synthesized. The proton-decoupled ^{15}N spectra of DPPC and DPPE in aqueous dispersion have exactly the form anticipated for powder line shapes governed by an axially symmetric shielding tensor. The chemical shift anisotropy ($\Delta\sigma$) of DPPC is lower than 0.4 ppm at 30 °C and vanished when the temperature or the half-height line width is increased; DPPE always exhibits an asymmetric line shape, and ^{15}N NMR spectra of DPPE are obtained at various temperatures and simulated to measure exactly the chemical shift anisotropy. At each temperature, the order parameter of the C-N bond segment is derivated from $\Delta\sigma$ and reveals that the average orientation of the C-N bond around the axis of rotation is near the "magic angle" (54.7°). Isotropic correlation times are derived from T_1, which are higher than values obtained for phosphatidylcholine by other nuclei. Arrhenius plots of T_1 and T_2 allowed us to calculate the activation energy for the motion of the DPPE and the DPPC C-N bond. The value of this activation energy for the DPPE (53 kJ/mol) is higher than the one found for the DPPC C-N bond (32 kJ/mol). These differences agree with the capacity of the ethanolamine head groups to bind noncovalently to their neighbors in the plane of the membrane surface. A direct titration curve of the amino group is achieved by the variation of the chemical shift with the bulk pH, and the interfacial pK_a is calculated to be 11.1. At pH 11, two distinct protonation states of DPPE are observed, which are in slow exchange compared to the NMR time scale. The present results clearly show the great discriminating power of ^{15}N spectroscopy in terms of environmental changes around the nitrogen atom at the interfacial region of membranes.

Phospholipid bilayers are an integral part of biological membranes. Knowledge of the molecular structure and dynamics of phospholipids is therefore essential for an understanding of their functional role in biological membranes. Of the many techniques being used to probe the molecular environment within membranes, nuclear magnetic resonance (NMR)[1] has proved to be one of most useful methods for investigating the dynamical state of the membrane [for reviews, see Seelig and Seelig (1980) and Jacobs and Oldfield (1981)].

At the hydrophilic part of the membrane, polar head groups play an important role in ions, drug binding, and ionization properties of lipid bilayers (Siminovitch et al., 1984). It is also known that modification of the polar head group cause changes in the cooperative properties of membranes. For example, the gel to liquid-crystal phase transition occurs at a significantly higher temperature for DPPE than for DPPC (Ladbrooke & Chapman, 1969). In order to understand the influence the

† This work was supported by a research fellowship from Ministère de la Recherche et de la Technologie (to S.A.).
* Author to whom correspondence should be addressed.

[1] Abbreviations: NMR, nuclear magnetic resonance; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; NOE, nuclear Overhauser effect.

0006-2960/86/0425-6972$01.50/0