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Equilibrium and Kinetics of the Folding of Equine Lysozyme Studied by Circular Dichroism Spectroscopy

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The equilibrium unfolding and the kinetics of unfolding and refolding of equine lysozyme, a Ca^{2+}-binding protein, were studied by means of circular dichroism spectra in the far and near-ultraviolet regions. The transition curves of the guanidine hydrochloride-induced unfolding measured at 230 nm and 292.5 nm, and for the apo and holo forms of the protein have shown that the unfolding is well represented by a three-state mechanism in which the molten globule state is populated as a stable intermediate. The molten globule state of this protein is more stable and more native-like than that of α-lactalbumin, a homologous protein of equine lysozyme. The kinetic unfolding and refolding of the protein were induced by concentration jumps of the denaturant and measured by stopped-flow circular dichroism. The observed unfolding and refolding curves both agreed well with a single-exponential function. However, in the kinetic refolding reactions below 3 M guanidine hydrochloride, a burst-phase change in the circular dichroism was present, and the burst-phase intermediate in the kinetic refolding is shown to be identical with the molten globule state observed in the equilibrium unfolding. Under a strongly native condition, virtually all the molecules of equine lysozyme transform the structure from the unfolded state into the molten globule, and the subsequent refolding takes place from the molten globule state. The transition state of folding, which may exist between the molten globule and the native states, was characterized by investigating the guanidine hydrochloride concentration-dependence of the rate constants of refolding and unfolding. More than 80% of the hydrophobic surface of the protein is buried in the transition state, so that it is much closer to the native state than to the molten globule in which only 36% of the surface is buried in the interior of the molecule. It is concluded that all the present results are best explained by a sequential model of protein folding, in which the molten globule state is an obligatory folding intermediate on the pathway of folding.

Keywords: equine lysozyme; protein folding; molten globule; stopped-flow; folding intermediate

Introduction

Understanding how a protein folds from the unfolded state into its unique, functional structure under a native condition is a fundamental problem in molecular structural biology (Kim & Baldwin, 1990). Although this protein-folding problem has not yet been fully understood, it has been generally accepted that detection and characterization of intermediate conformational states between the native (N) and fully unfolded (U) states are useful for elucidating the mechanism of folding of globular proteins (Kuwajima, 1989; Kim & Baldwin, 1990; Schmid, 1992; Kiefhaber, 1995a).

The molten globule (MG) state has been known to be an intermediate between the N and U states for a number of globular proteins. This state has been observed in α-lactalbumin, cytochrome *c*, apo-

Abbreviations used: CD, circular dichroism; MG, molten globule; GdnHCl, guanidine hydrochloride; N, native; I, intermediate; U, unfolded.

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myoglobin, ribonuclease HI and several other proteins as an equilibrium unfolding intermediate under mildly denaturing conditions (Kuwajima *et al.*, 1976; Ohgushi & Wada, 1983; Griko *et al.*, 1988; Kuwajima, 1989, 1996a; Ptitsyn, 1995; Dabora *et al.*, 1996). The MG state is characterized by the presence of substantial secondary structure, a compact shape, formation of a hydrophobic core exposed to solvent, and the absence of rigid side-chain packing interactions (Kuwajima, 1989; Ptitsyn, 1995). Studies on the above proteins by a stopped-flow circular dichroism (CD) technique and by a pulsed hydrogen-exchange technique combined with 2D-NMR spectroscopy have shown that the MG state is identical with a transient kinetic intermediate formed at an early stage of the refolding from the U state (Kuwajima *et al.*, 1985, 1987; Ikeguchi *et al.*, 1986a; Roder *et al.*, 1988; Elöve *et al.*, 1992; Baldwin, 1993; Jennings & Wright, 1993; Yamasaki *et al.*, 1995; Arai & Kuwajima, 1996; Kuwajima, 1996a; Raschke & Marqusee, 1997). Therefore, it has been proposed that the protein folding is explained by a sequential model in which the MG state accumulates transiently as an obligatory on-pathway intermediate (Kuwajima, 1989; Ptitsyn, 1995).

However, in spite of the apparent existence of the MG-like kinetic folding intermediate in many globular proteins, whether or not the intermediate truly directs the folding reaction remains to be clarified. It has been suggested that the intermediate like the MG state is not obligatory in folding but may be a kinetically trapped or misfolded species (Dill *et al.*, 1995; Dill & Chan, 1997; Shakhnovich, 1997). The role of the MG state in protein folding is thus a topic that has become the subject of intense debate.

Equine lysozyme is a Ca^{2+}-binding lysozyme and is homologous to hen egg-white lysozyme and α-lactalbumin (Brew *et al.*, 1970; Qasba & Safaya, 1984; Nitta *et al.*, 1987; Nitta & Sugai, 1989). A crystallographic study has shown that the structure of equine lysozyme is very similar to those of non-Ca^{2+}-binding lysozyme (conventional lysozyme) and α-lactalbumin (Tsuge *et al.*, 1992). The structure of equine lysozyme thus consists of two domains, the α-domain and the β-domain, divided by a deep cleft. The α-domain consists mainly of four α-helices, while the β-domain is formed by two antiparallel β-sheets and a 3_{10}-helix (Tsuge *et al.*, 1992). In contrast to conventional lysozyme, however, equine lysozyme exhibits the MG state as a stable unfolding intermediate during the equilibrium unfolding and as a partially unfolded species at acidic pH (Nitta *et al.*, 1993; Van Dael *et al.*, 1993; Morozova *et al.*, 1995, 1997). Although the MG state of equine lysozyme is structurally similar to the MG state of α-lactalbumin, the equine lysozyme MG state is known to be much more native-like than the MG states of other proteins including α-lactalbumin, and stabilized by non-specific interactions and by specific side-chain packing interactions (Nitta *et al.*, 1993; Van Dael *et al.*, 1993; Morozova *et al.*, 1995, 1997). In understanding the role of the MG state in protein folding, it is thus intriguing to investigate whether or not the MG state of equine lysozyme is an intermediate of the kinetic refolding as observed in the other proteins and, if so, how fast the MG state is formed during the refolding.

In this study, we have investigated the equilibrium unfolding of equine lysozyme in GdnHCl by CD spectroscopy, and the kinetics of unfolding and refolding induced by concentration jumps of GdnHCl by stopped-flow CD measurements. It is shown that both the equilibrium and kinetics of folding and unfolding of this protein are well represented by a three-state mechanism that involves only the N, MG and U states, and that the MG state is identical with a transient folding intermediate formed within the dead-time of the stopped-flow measurement. These results are discussed in relation to possible models of protein folding, and it is concluded that the present results are best explained by the sequential model of folding.

Results

CD spectra

Figure 1 shows CD spectra of the apo and holo equine lysozyme at pH 7.0 and 25°C. The holo protein with a bound Ca^{2+} is in the N state in the presence of GdnHCl up to 1.7 M, while only about 70% of the molecules are in the N state in the apo form even in the absence of GdnHCl (see below). Although equine lysozyme is destabilized in the

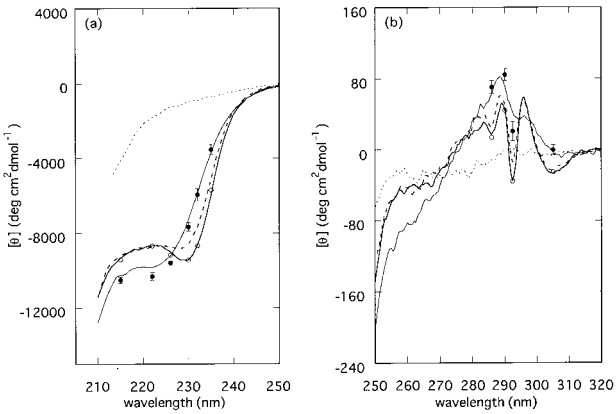

Figure 1. CD spectra of equine lysozyme in (a) the far and (b) the near-UV region (pH 7.0 and 25°C). The spectra of the holo protein in the N (0.5 M GdnHCl; thick continuous line) and the U states (6.0 M GdnHCl; dotted line), and those of the apo protein in the N (0 M GdnHCl; broken line) and the I states (1.0 M GdnHCl; thin continuous line) are shown. Filled circles show the ellipticity values at zero time and open circles show those at 300 seconds of the refolding of the holo equine lysozyme at 0.5 M GdnHCl.

apo form by removal of the bound Ca^{2+}, the addition of excess sodium ion (Na^{+}) stabilizes the protein to form the N state, and in this study, the protein solutions contained 0.1 M Na^{+}. The near-UV CD spectrum of the native protein shows characteristic positive Cotton effects at 283 nm, 289 nm and 296 nm, and troughs at 286 nm, 292 nm and 304 nm, indicating the presence of specific rigid packing interactions of aromatic (tryptophanyl) side-chains. Equine lysozyme has five tryptophan residues, at positions 28, 63, 64, 108 and 111. The far-UV CD spectrum shows negative ellipticity with a peak at 230 nm, which may arise from a Cotton effect of the aromatic side-chains in the far-UV region. These characteristics of the native CD spectra are completely lost in concentrated GdnHCl (6.0 M), where the protein is in the fully unfolded state. The addition of an intermediate concentration of GdnHCl to the protein solution, however, leads to a third conformational state (I), namely, the MG state, of lysozyme. The near-UV CD spectrum of the apo protein at 1 M GdnHCl shows enhancement of the positive Cotton effect at 289 nm and disappearance of the troughs observed in the native protein, indicating a conformational rearrangement around the aromatic residues in the I state. The far-UV CD spectrum shows enhancement of the negative ellipticity below 226 nm and appearance of a negative shoulder at 223 nm, indicating the presence of significant secondary structure in the I state (Figure 1). These characteristics of the CD spectra are in good agreement with those reported previously (Nitta *et al*., 1993; Van Dael *et al*., 1993).

Equilibrium unfolding

Figure 2 shows the GdnHCl-induced equilibrium unfolding transition curves of the apo and holo equine lysozyme measured by CD ellipticity at 230 nm and at 292.5 nm. The transition curves of the apo protein indicate the presence of two transitions, one occurring below 1 M GdnHCl and the other between 2.5 and 5 M GdnHCl. Although the transition curve of the holo protein measured at 230 nm apparently shows a single step transition, the curve measured at 292.5 nm clearly indicates the presence of two transitions, the first accompanied by an increase and the second accompanied by a decrease in ellipticity, giving rise to a maximum ellipticity at 3.4 M GdnHCl. For both the apo and holo lysozyme, the unfolding transition is not represented by a simple two-state mechanism, and at least three states, the N, I and U states, are required for interpreting the transition curves of Figure 2. Thus, the unfolding of equine lysozyme is assumed to be a three-state transition as:

$$\mathrm{N} \underset{}{\overset{K_{\mathrm{NI}}}{\rightleftharpoons}} \mathrm{I} \overset{K_{\mathrm{IU}}}{\rightleftharpoons} \mathrm{U}$$

Scheme 1.

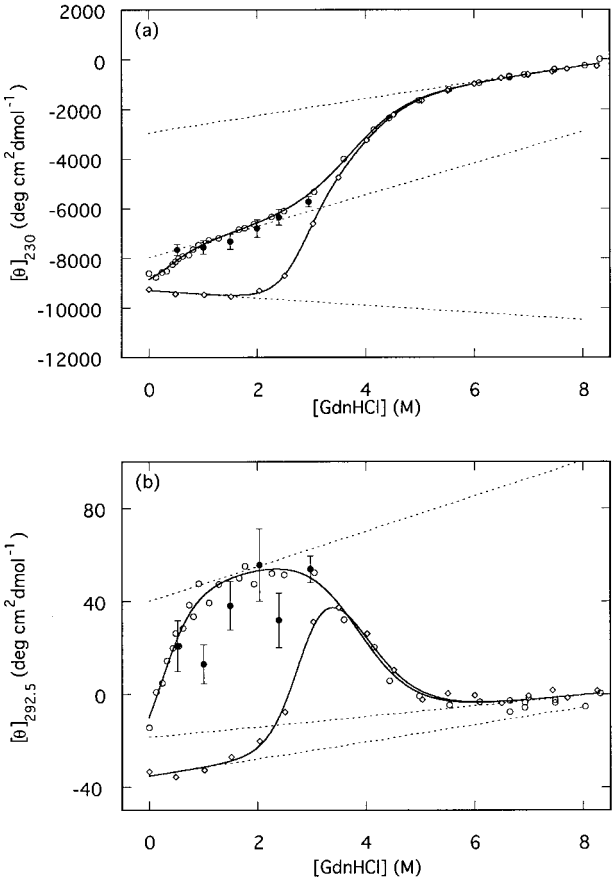

Figure 2. GdnHCl-induced equilibrium unfolding transition curves of the apo (open circles) and the holo (open diamonds) equine lysozyme measured by CD at (a) 230 nm and (b) 292.5 nm (pH 7.0 and 25°C). The ellipticity values of the burst-phase intermediate (filled circles) are shown. Continuous lines represent the theoretical curves based on equation (4) with the parameter values given in Table 1. Dotted lines indicate the ellipticity values of the pure N, I and U states.

The observed ellipticity of the protein ($A_{\mathrm{obs}}(c)$) at any concentration of the denaturant is given by the sum of the contributions from the three states as

$$A_{\mathrm{obs}}(c) = A_{\mathrm{N}} f_{\mathrm{N}}(c) + A_{\mathrm{I}} f_{\mathrm{I}}(c) + A_{\mathrm{U}} f_{\mathrm{U}}(c) \qquad (1)$$

where $f_{\mathrm{N}}(c)$, $f_{\mathrm{I}}(c)$ and $f_{\mathrm{U}}(c)$ are the fractions of the three states at a GdnHCl concentration of c ($f_{\mathrm{N}} + f_{\mathrm{I}} + f_{\mathrm{U}} = 1$), and A_{N}, A_{I} and A_{U} are the ellipticity values of the pure N, I and U states, respectively. The f_{N}, f_{I} and f_{U} terms are related to the equilibrium constants, K_{NI} and K_{NU}, of the unfolding transitions from N to I and from N to U, respectively, and hence are related to the corresponding free energy changes, ΔG_{NI}

and ΔG_{NU}, as:

$$\begin{aligned} f_N &= 1/(1 + K_{NI} + K_{NU}) \\ &= 1/[1 + \exp(-\Delta G_{NI}/RT) + \exp(-\Delta G_{NU}/RT)] \\ f_I &= K_{NI}/(1 + K_{NI} + K_{NU}) \\ &= \exp(-\Delta G_{NI}/RT)/ \\ &\quad [1 + \exp(-\Delta G_{NI}/RT) + \exp(-\Delta G_{NU}/RT)] \\ f_U &= K_{NU}/(1 + K_{NI} + K_{NU}) \\ &= \exp(-\Delta G_{NU}/RT)/ \\ &\quad [1 + \exp(-\Delta G_{NI}/RT) + \exp(-\Delta G_{NU}/RT)] \end{aligned} \quad (2)$$

where R and T are the gas constant and the absolute temperature, respectively. For many globular proteins, the free energy changes of unfolding are known to vary approximately linearly with c (Pace, 1986), so that:

$$\begin{aligned} \Delta G_{NI} &= \Delta G_{NI}^{H_2O} - m_{NI}c \\ \Delta G_{NU} &= \Delta G_{NU}^{H_2O} - m_{NU}c \end{aligned} \quad (3)$$

where $\Delta G_{NI}^{H_2O}$ and $\Delta G_{NU}^{H_2O}$ are the ΔG_{NI} and ΔG_{NU} at 0 M GdnHCl, respectively, and m_{NI} and m_{NU} represent the dependence of the respective free energy changes on c and thus the cooperativity indexes of the transitions. From equations (1) to (3), $A_{obs}(c)$ is given by:

$$A_{obs}(c) = \frac{A_N + A_I \exp[-(\Delta G_{NI}^{H_2O} - m_{NI}c)/RT] + A_U \exp[-(\Delta G_{NU}^{H_2O} - m_{NU}c)/RT]}{1 + \exp[-(\Delta G_{NI}^{H_2O} - m_{NI}c)/RT] + \exp[-(\Delta G_{NU}^{H_2O} - m_{NU}c)/RT]} \quad (4)$$

In general, A_N, A_I, and A_U are also dependent on c, and we assumed the linear dependence on c, as $A_N = a_1 + a_2c$, $A_I = a_3 + a_4c$, and $A_U = a_5 + a_6c$.

The data of Figure 2 were analyzed on the basis of equation (4) by the method of non-linear least-squares. In the analysis, we performed the global fitting, in which the transition curves at different wavelengths (230 nm and 292.5 nm) and for the apo and holo forms of the protein were fitted simultaneously, on the assumption that the A_N, A_I and A_U are common between the apo and holo forms. The fitting variables in the least-squares analysis were thus two sets of $\Delta G_{NI}^{H_2O}$, $\Delta G_{NU}^{H_2O}$, m_{NI} and m_{NU} for the holo and apo proteins, and two sets of a_1 to a_6 for the transition curves at the two different wavelengths (230 nm and 292.5 nm). The best-fit values of the equilibrium unfolding parameters thus obtained are given in Table 1.

The continuous lines in Figure 2 are theoretical curves drawn with the parameter values shown in Table 1. The theoretical curves show excellent agreement with the experimental data, indicating the validity of the three-state analysis. Therefore, only the three states, the N, I and U states, are sufficient and no other state is required for interpreting the unfolding transition of equine lysozyme. From the theoretical curves, apo equine lysozyme is partly unfolded and only about 70% of the molecules are in the N state even at 0 M GdnHCl (pH 7.0, 25°C), and more than 95% of the molecules are in the I state at 1.1 to 2.3 M GdnHCl. In the holo lysozyme, on the other hand, the N state persists up to 1.7 M GdnHCl, so that the accumulation of the I state is observed between 2.0 and 6.0 M of GdnHCl, and 64% of the molecules are in the I state at 3.3 M GdnHCl (Figure 2 and Table 1). From the equilibrium unfolding parameters in Table 1, $\Delta G_{NU}^{H_2O}$ for the holo protein in the presence of 10 mM $CaCl_2$ is larger than that for the apo protein by about 5 kcal/mol, indicating that the Ca^{2+}-binding stabilizes the N state. The stability of the I state, however, is not affected by the binding of Ca^{2+}, because the apo and holo equine lysozyme show identical $\Delta G_{IU}^{H_2O}$ values. These results demonstrate that the Ca^{2+}-binding site present in native equine lysozyme is not formed in the I state of the protein (Nitta *et al.*, 1993).

Kinetic folding and unfolding reactions

Stopped-flow CD experiments were carried out to investigate the kinetic folding and unfolding reactions of equine lysozyme. The refolding reactions of the holo protein were induced by concentration jumps of GdnHCl from 6.0 M to various concentrations between 0.5 and 3.0 M by the stopped-flow mixing technique, and the resultant kinetics were observed by rapid CD measurement at 230 nm and 292.5 nm. Typical refolding curves of the protein are shown in Figure 3. In both the refolding curves measured at the two wavelengths, the ellipticities of the refolded protein are identical with that in the N state, indicating the reversibility of the unfolding transition. Furthermore, both the refolding curves are well represented by a single exponential process. This behavior apparently suggests the absence of kinetic folding intermediates, and hence it is in contrast with the obser-

Table 1. Equilibrium unfolding parameters of equine lysozyme (pH 7.0 and 25°C)

	$\Delta G_{NI}^{H_2O}$ (kcal mol^{-1})	m_{NI} (kcal mol^{-1} M^{-1})	$\Delta G_{IU}^{H_2O}$ (kcal mol^{-1})	m_{IU} (kcal mol^{-1} M^{-1})	$\Delta G_{NU}^{H_2O}$ (kcal mol^{-1})	m_{NU} (kcal mol^{-1} M^{-1})
Holo protein	5.64 ± 0.32	2.00 ± 0.11	4.33 ± 0.59	1.13 ± 0.19	9.97 ± 0.49	3.13 ± 0.15
Apo protein	0.41 ± 0.07	1.97 ± 0.20	4.37 ± 0.33	1.15 ± 0.29	4.78 ± 0.33	3.12 ± 0.21

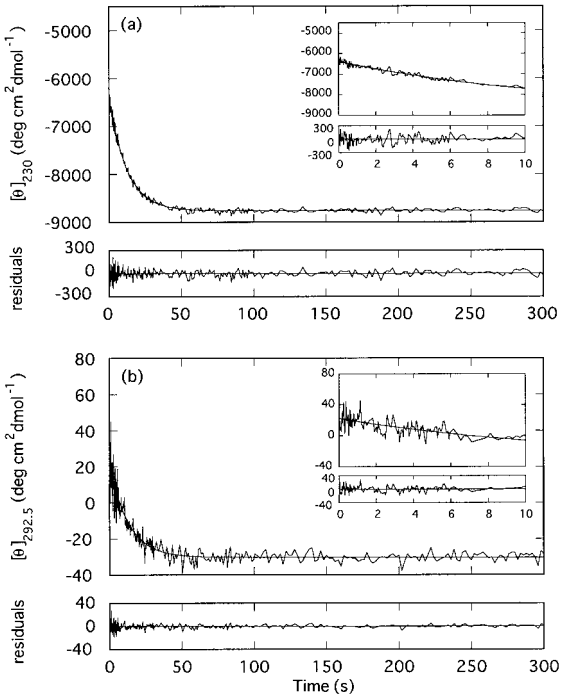

Figure 3. Representative kinetic refolding curves of the holo equine lysozyme measured by CD at (a) 230 nm and (b) 292.5 nm. The reaction was initiated by a GdnHCl concentration jump from 6.0 M to 2.5 M. The insets show the refolding curves within ten seconds. The curves were well fitted to a single exponential. The lower panels show the difference between the observed and fitted curves.

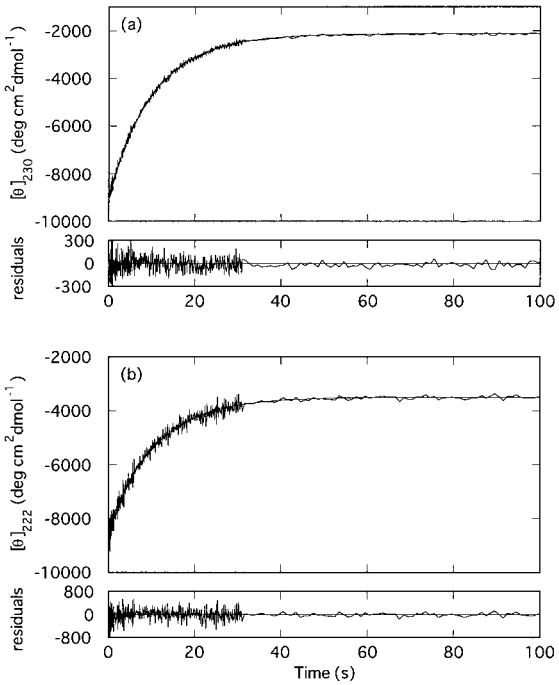

Figure 4. Kinetic unfolding curves of the holo equine lysozyme monitored by CD at (a) 230 nm and (b) 222 nm (pH 7.0 and 25°C). The reaction was initiated by a concentration jump of GdnHCl from 0 to 4.5 M. The curves were well fitted by a single exponential and the residual is shown in the lower panels.

vation of the I state in the equilibrium unfolding. However, more rigorous examination of the kinetic refolding curves has revealed the presence of a burst phase that occurs within the dead-time of the stopped-flow instrument. The extrapolation of the kinetic progress curve observed at 230 nm to zero time of the refolding yields an ellipticity value of -6350 deg cm^2 dmol^{-1}, and this value is significantly smaller than the baseline ellipticity for the U state, -2150 deg cm^2 dmol^{-1}, indicating the rapid ellipticity decrease (4200 deg cm^2 dmol^{-1}) attained in the burst phase. Similarly, the extrapolation of the kinetic progress curve at 292.5 nm to zero time leads to an ellipticity value of 32 deg cm^2 dmol^{-1}, which is much larger than the baseline value for the U state (-13 deg cm^2 dmol^{-1}). Both of these results thus demonstrate the presence of the burst-phase intermediate in the refolding of equine lysozyme.

The kinetic unfolding reactions of the holo equine lysozyme were induced by concentration jumps of GdnHCl from 0 M to various concentrations between 3.5 and 6.0 M. Typical unfolding curves measured by the far-UV CD at 230 nm and 222 nm are shown in Figure 4, and both the unfolding curves are well represented by a single exponential process.

The kinetic data were fitted by the non-linear least-squares method with the equation:

$$A(t) = A(\infty) + \Delta A \exp(-k_{app}t) \qquad (5)$$

where $A(t)$ and $A(\infty)$ are the CD ellipticity values at time t and the infinite time, respectively, and ΔA and k_{app} are the amplitude and the apparent rate constant of the observed phase, respectively. The apparent rate constants of refolding and unfolding reactions were obtained by fitting the observed reaction curves with equation (5), and they are shown as a function of c in Figure 5. The k_{app} for refolding decreases and that for unfolding increases with c, and the dependence of the logarithmic k_{app} on c is linear for both refolding and unfolding.

The burst-phase intermediate in the kinetic refolding

The above results demonstrate the presence of the burst-phase intermediate in the kinetic refolding of equine lysozyme. In order to characterize this intermediate in more detail, the kinetic refolding of the holo protein at 0.5 M GdnHCl was measured by stopped-flow CD at various wavelengths. Because the burst-phase process is kinetically separated from the subsequent folding events, the CD spectra of the burst-phase intermediate are given by the wavelength-dependence

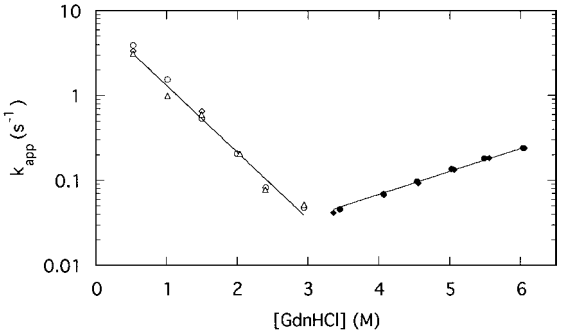

Figure 5. GdnHCl concentration-dependence of the apparent rate constants of the refolding (open symbols) and unfolding (filled symbols) reactions of the holo equine lysozyme measured by CD (pH 7.0 and 25°C) at 230 nm (circles), 222 nm (diamonds) and 292.5 nm (triangles).

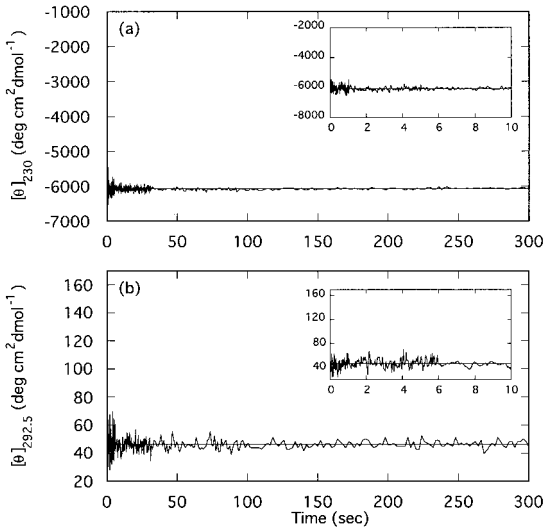

Figure 6. Kinetic refolding curves of the apo equine lysozyme monitored by CD at (a) 230 nm and (b) 292.5 nm. The reaction was initiated by a concentration jump of GdnHCl from 6.0 to 2.5 M. The insets show the curves within ten seconds.

of the ellipticity obtained by extrapolating the kinetic progress curves to zero time (Kuwajima *et al.*, 1985; Kuwajima, 1996b). The CD spectra of the burst-phase intermediate obtained are compared with the spectra in the N, I and U states in Figure 1. The spectra at 300 seconds after the initiation of refolding, where the protein is fully refolded, are shown for comparison. It can be seen that both the far and near-UV CD spectra of the burst-phase intermediate coincide well with those of the I state, which is almost fully populated at 1 M GdnHCl in the apo form at equilibrium. Particularly to be noted is the fact that the burst-phase intermediate or the I state of equine lysozyme has pronounced secondary structure as indicated by the far-UV CD spectrum, and significant tertiary structure as characterized by the near-UV CD spectrum (see Discussion). It has been found that kinetic refolding curves measured at various wavelengths are all well represented by a single exponential process and that the rate constant of refolding is independent of the wavelength used.

The dependence of the zero-time ellipticity, which has been obtained by extrapolating the kinetic refolding curve to zero time, on GdnHCl concentration gives information about the stability of the burst-phase intermediate (Ikeguchi *et al.*, 1986a; Arai & Kuwajima, 1996; Kuwajima, 1996b). Such dependence of the ellipticity of the burst-phase intermediate is shown in Figure 2. Although the experimental errors are large, especially for the data in the near-UV region, the ellipticity values of the burst-phase intermediate are reasonably coincident with those of the equilibrium I state. Therefore, the burst-phase intermediate in the kinetic refolding and the equilibrium unfolding intermediate state are identical with each other in the shapes of their CD spectra as shown above and in their stabilities. We thus conclude that the burst-phase intermediate is identical with the I state, and that almost all molecules convert into the I state without rapid formation of the N state in the burst phase.

In order to confirm the identity between the burst-phase intermediate and the I state, the refolding reaction from the U to the I state was investigated by the stopped-flow CD in apo equine lysozyme (Figure 6). The reaction was induced by a concentration jump of GdnHCl from 6.0 to 2.5 M. The apo equine lysozyme is in the I state at 2.5 M GdnHCl (Figure 2). The ellipticity changes at both 230 nm and 292.5 nm are complete within the dead-time of the stopped-flow instrument, and no change is observed in a time range up to 300 seconds after the instrumental dead-time. The result thus indicates that the I state is formed rapidly within the dead-time, supporting the identity between the I state and the burst-phase intermediate.

Discussion

For a number of globular proteins, including α-lactalbumin, cytochrome *c*, apomyoglobin and ribonuclease HI, a transient intermediate, having characteristics of the MG state, is known to accumulate at an early stage of kinetic refolding from the U state (Kuwajima *et al.*, 1985; Ikeguchi *et al.*, 1986a; Roder *et al.*, 1988; Elöve *et al.*, 1992; Jennings & Wright, 1993; Yamasaki *et al.*, 1995; Arai & Kuwajima, 1996; Raschke & Marqusee, 1997). For these proteins, an on-pathway sequential folding model (or intermediate-controlled folding model) in which the MG state is generated as an obligatory state on the pathway of folding has been postulated (Kuwajima, 1989; Ptitsyn, 1995). However, the sequential model is not the only

model that can explain the folding reactions of the proteins. In a multiple-pathway folding model, protein molecules fold through multiple different pathways, and these pathways may include a funnel-like throughway path on which the protein directly folds into the N state without accumulation of any intermediates (Dill *et al*., 1995; Dill & Chan, 1997; Shakhnovich, 1997). The transient folding intermediate observed experimentally may correspond to a kinetically trapped species at a local energy minimum in the conformational space of the unfolded state. In support of this model, the thermodynamic studies of the MG state of bovine α-lactalbumin (Yutani *et al*., 1992; Griko *et al*., 1994; Pfeil, 1998) and the kinetic refolding studies of the disulfide-intact and three-disulfide species of the protein (Ikeguchi *et al*., 1998) have recently suggested that the MG state of this protein is not stabilized by native-like cooperative interactions and not distinct thermodynamically from the fully unfolded U state. Therefore, there is still a question about the significance of the MG state as a state to direct the folding reaction.

The present results show that the equilibrium unfolding of equine lysozyme is well represented by the three-state mechanism involving the MG state as a stable intermediate (Figure 2 and Table 1) and that the burst-phase intermediate in the kinetic refolding of the protein is identical with the equilibrium MG state (Figures 1, 2 and 6). The previous studies by CD spectra and hydrogen-exchange reactions combined with NMR spectra have shown that the overall structure of the MG state of equine lysozyme is similar to that of α-lactalbumin (Nitta *et al*., 1993; Van Dael *et al*., 1993; Morozova *et al*., 1995, 1997). However, unlike α-lactalbumin, the MG state of equine lysozyme is stabilized by nonspecific interactions and by native-like specific side-chain interactions (Morozova *et al*., 1995, 1997). Apparently, the MG state of equine lysozyme is thermodynamically distinct from the U state and much more native-like than that of α-lactalbumin (see below). Therefore, for fully understanding the role of the MG state in protein folding, it is important to address the following questions: (1) is the kinetic folding of equine lysozyme represented by the sequential folding model with the MG state as a specific obligatory intermediate? (2) If so, what has brought about the apparent difference in the folding behavior between α-lactalbumin and equine lysozyme? In order to answer these questions, we first describe more details about similarities and differences of the MG state between α-lactalbumin and equine lysozyme, and then discuss the folding mechanism of equine lysozyme in comparison with those of α-lactalbumin and other globular proteins.

Similarities and differences of the MG state between α-lactalbumin and equine lysozyme

All the experimental data of the equilibrium and the kinetics of folding and unfolding of equine lysozyme in the present study are well represented by the three-state mechanism that involves only the N, MG and U states. The three-state mechanism is analogous to a three-state equilibrium unfolding of α-lactalbumin induced by GdnHCl but apparently different from the unfolding mechanism of conventional non-Ca^{2+}-binding lysozyme that is known to show a two-state unfolding without accumulation of the MG state at equilibrium (Ikeguchi *et al*., 1986b). Thus, in the unfolding behavior, equine lysozyme is more similar to α-lactalbumin than to conventional lysozyme. The structures of α-lactalbumin and equine lysozyme in the MG state are also similar to each other. In both these proteins, the MG state has ordered secondary structure in the α-domain with the β-domain being less organized, and α-helical segments including the A and B helices are packed together to form a hydrophobic cluster in the α-domain (Schulman *et al*, 1995, Morozova *et al*., 1995, 1997).

However, it is true that there are marked differences in the structure and the thermodynamic properties of the MG state between α-lactalbumin and equine lysozyme, and these differences may be important for understanding the folding reaction of equine lysozyme. The enhanced near-UV CD band around 289 nm demonstrates the presence of packing of aromatic side-chains in the MG state of equine lysozyme (Figure 1), while the absence of the near-UV CD bands indicates the absence of such tertiary packing interactions in the MG state of α-lactalbumin (Kuwajima *et al*., 1976). A hydrophobic cluster involving three tryptophanyl residues, Trp28, Trp108 and Trp111, seems to be more densely packed in the MG state in equine lysozyme than in α-lactalbumin, and this may be responsible for the enhanced near-UV CD band (Morozova *et al*., 1995, 1997). The thermodynamic analysis of the GdnHCl-induced unfolding of equine lysozyme gave a $\Delta G_{IU}^{H_2O}$ value of 4.33 $\sim$ 4.37 kcal mol^{-1} and an m_{IU} value of 1.13 $\sim$ 1.15 kcal mol^{-1} M^{-1} (Figure 2 and Table 1), and these values are significantly larger than the corresponding parameter values of bovine α-lactalbumin ($\Delta G_{IU}^{H_2O}$ 1.42 kcal mol^{-1}, m_{IU} 0.75 kcal mol^{-1} M^{-1}; Ikeguchi *et al*., 1986b), indicating that the interactions stabilizing the MG state are more extensive and cooperative in equine lysozyme.

Previous calorimetric studies have revealed the difference between α-lactalbumin and equine lysozyme (Van Dael *et al*., 1993; Griko *et al*., 1995). In α-lactalbumin, the MG state shows a diffuse thermal transition without any observable heat absorption peak in the scanning calorimetric measurement, and the thermal unfolding from the N state is well represented as a two-state reaction between N and the thermally unfolded state (Yutani *et al*., 1992; Griko *et al*., 1994; Pfeil, 1998). Apparently, the MG state and the U state belong in the same thermodynamic species. In a recent study by Ikeguchi *et al*. (1998), it was possible to describe the GdnHCl-induced unfolding transition of

bovine α-lactalbumin as a two-state transition by regarding the MG and U states as the same thermodynamic species. In equine lysozyme, however, the thermal unfolding of the MG state is accompanied by a large enthalpy increase, and the unfolding from the N state results in two partly overlapping heat absorption peaks, one produced by the transition from N to MG and the other by the transition from MG to the thermally unfolded state (Van Dael *et al.*, 1993; Griko *et al.*, 1995). Apparently, the MG state of equine lysozyme is a thermodynamic state that is distinct from both the N and U states. This property of the MG state of equine lysozyme is responsible for the three-state kinetic refolding of this protein, in which the MG state accumulates as a specific folding intermediate (see below).

Models for kinetic folding of equine lysozyme and characterization of the transition state

The MG state of equine lysozyme is a stable thermodynamic state that is formed much faster than the N state in the kinetic refolding from the U state (Figures 3 and 6; see above). Thus, a two-state folding model in which the MG and U states belong in the same thermodynamic state should be rejected for equine lysozyme, although such a model previously explained the kinetic refolding of bovine α-lactalbumin (Ikeguchi *et al.*, 1998). Further, almost all molecules are shown to convert into the MG state within the burst phase (see above), so that we did not perform the kinetic analysis based on a triangular folding model (Wildegger & Kiefhaber, 1997). Therefore, there are at least two possible alternative models to explain the refolding of equine lysozyme. One is an on-pathway model (sequential model):

$$\mathrm{U} \underset{k_{\mathrm{IU}}}{\overset{k_{\mathrm{UI}}}{\rightleftharpoons}} \mathrm{I} \underset{k_{\mathrm{NI}}}{\overset{k_{\mathrm{IN}}}{\rightleftharpoons}} \mathrm{N}$$

Scheme 2.

in which the MG state is an obligatory state to reach the N state. The other is an off-pathway model:

$$\mathrm{I} \underset{k_{\mathrm{UI}}}{\overset{k_{\mathrm{IU}}}{\rightleftharpoons}} \mathrm{U} \underset{k_{\mathrm{NU}}}{\overset{k_{\mathrm{UN}}}{\rightleftharpoons}} \mathrm{N}$$

Scheme 3.

in which the MG state has to be fully disrupted and returns to the U state before reaching the N state.

The derivative, m, of the natural logarithm of the rate constant with respect to the GdnHCl concentration reflects a difference in the surface exposed to solvent between the initial and the transition states of refolding or unfolding reaction, and can be compared with the equilibrium m values to determine a relative degree of structural organization in the transition state ($\alpha_{\ddagger}$). The GdnHCl concentration-dependence of the rate constants of folding and unfolding were thus investigated and used for assessment of the on-pathway or off-pathway model of folding. Since the formation of the burst-phase intermediate occurring within the dead-time of the measurements is kinetically uncoupled with the subsequent folding reaction, we assume a rapid pre-equilibrium between U and I (=MG). Thus, the apparent rate constant of the unfolding and refolding reactions is represented by the following equations:

$$k_{\mathrm{app}} = k_{\mathrm{NI}} + [f_{\mathrm{I}}/(f_{\mathrm{I}} + f_{\mathrm{U}})]k_{\mathrm{IN}} = k_{\mathrm{NI}} + [1/(1 + f_{\mathrm{U}}/f_{\mathrm{I}})]k_{\mathrm{IN}} \quad (6)$$

for the on-pathway model, and:

$$\begin{aligned} k_{\mathrm{app}} &= k_{\mathrm{NU}} + [f_{\mathrm{U}}/(f_{\mathrm{I}} + f_{\mathrm{U}})]k_{\mathrm{UN}} \\ &= k_{\mathrm{NU}} + [1/(1 + f_{\mathrm{I}}/f_{\mathrm{U}})]k_{\mathrm{UN}} \end{aligned} \quad (7)$$

for the off-pathway model.

Thus, the microscopic rate constants of the refolding from I to N, k_{IN}, and of the unfolding from N to I, k_{NI}, for the on-pathway model are given by:

$$k_{\mathrm{IN}} = k_{\mathrm{app}}/[f_{\mathrm{I}}/f_{\mathrm{N}} + f_{\mathrm{I}}/(f_{\mathrm{I}} + f_{\mathrm{U}})] \quad (8)$$

$$k_{\mathrm{NI}} = (f_{\mathrm{I}}/f_{\mathrm{N}})k_{\mathrm{IN}} \quad (9)$$

(Kuwajima *et al.*, 1989; Ikeguchi *et al.*, 1998), and, similarly, those for the off-pathway model are given by:

$$k_{\mathrm{UN}} = k_{\mathrm{app}}/[f_{\mathrm{U}}/f_{\mathrm{N}} + f_{\mathrm{U}}/(f_{\mathrm{I}} + f_{\mathrm{U}})] \quad (10)$$

$$k_{\mathrm{NU}} = (f_{\mathrm{U}}/f_{\mathrm{N}})k_{\mathrm{UN}} \quad (11)$$

Figure 7(a) shows the microscopic rate constants, k_{IN} and k_{NI}, of the holo equine lysozyme calculated from equations (8) and (9), assuming the on-pathway model. In the calculation, the f_{N}, f_{I}, and f_{U} terms were obtained from the equilibrium unfolding parameters given in Table 1. The microscopic rate constant k_{UN} based on the off-pathway model was calculated, and it is shown by a dotted line for comparison in Figure 7(a). As there is no reason to expect linear dependence of ln k_{ij}, where i and j stand for N, I or U ($i \neq j$), on GdnHCl concentration, small deviations from linearity at low or high concentration of GdnHCl may not be exceptional (Kuwajima *et al.*, 1989; Matouschek *et al.*, 1994) and are observed in the ΔG of equilibrium unfolding transition (Schellman, 1987; Johnson & Fersht, 1995). To simplify the following analysis, however, we assumed the linear dependence of ln k_{ij} on GdnHCl concentration, c (Tanford, 1970), as:

$$\ln\ k_{ij} = \ln\ k_{ij}^{\mathrm{H_2O}} + (m_{i\ddagger}/RT)c \quad (12)$$

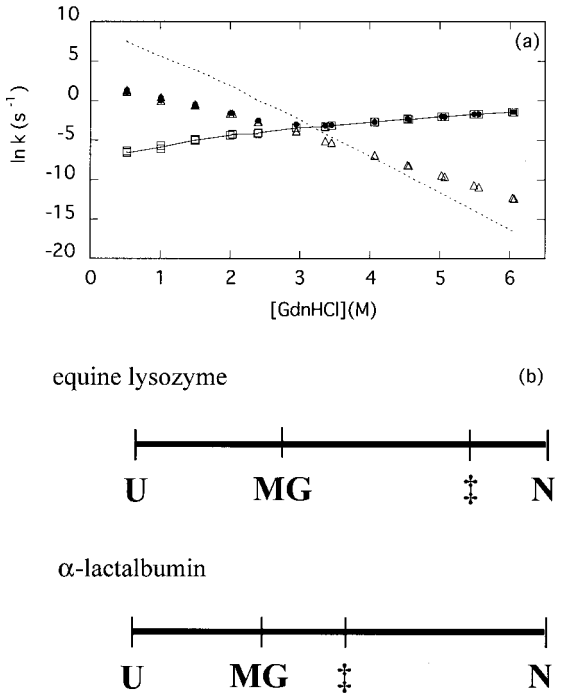

Figure 7. (a) GdnHCl concentration-dependence of the microscopic rate constants of the holo equine lysozyme assuming the on-pathway and the off-pathway models. Open triangles, open squares and filled circles denote k_{IN}, k_{NI} and k_{app}, respectively (on-pathway model), and continuous and dotted lines denote k_{UN} and k_{NU}, respectively (off-pathway model). (b) Positions of the MG and the transition states of equine lysozyme and bovine α-lactalbumin in the reaction coordinate.

where $k_{ij}^{H_2O}$ is the k_{ij} in water, and $m_{i\ddagger}$ is the m value for the activation process from i to the transition state. The m values for the individual activation processes thus obtained are summarized in Table 2.

From Figure 7(a) and Table 2, the rate constant (k_{UN}) for direct folding from U to N based on the off-pathway model shows much steeper dependence on c than k_{IN} for the folding from I to N based on the on-pathway model, and the k_{UN} is as large as 2×10^4 s^{-1} in the absence of the denaturant. Thus, if we take the off-pathway model to explain the folding of equine lysozyme, there must be a very fast track of folding on which the protein folds in a submillisecond range. Because experimentally it has been observed that the U to I (=MG) step occurs much faster than the subsequent folding to the N state, the formation of the MG state must be much faster than the fast-track folding in the off-pathway model. Although this possibility may not be excluded, it seems more likely that the folding of equine lysozyme follows the on-pathway model. The native-like structure formed in the MG state is maintained during the activation process of folding, and the MG state is an obligatory folding intermediate in this model.

The relative degree of structural organization in the transition state of folding ($\alpha_\ddagger$) was evaluated from the m values in Tables 1 and 2, and it is given by $\alpha_\ddagger = 1 - (m_{N\ddagger}/m_{NU})$. This value is 83% for the holo equine lysozyme (Table 2). On the other hand, the relative degree of the structural organization in the MG state (α_I) is estimated at 36% from m_{IU}/m_{NU} (Table 2), which is coincident with the value of the heat capacity increment for the thermal unfolding of the MG state, which amounts to about 35% of the total heat increment (Griko *et al.*, 1995). The results thus indicate that the transition state of folding is more organized than the MG state, being consistent with the on-pathway model in which the structure organized in the MG state is retained during the subsequent process to the N state.

Comparison of equine lysozyme folding with those of α-lactalbumin and other globular proteins

Comparison of the relative degrees of structural organization (α_I and $\alpha_\ddagger$) between equine lysozyme and bovine α-lactalbumin is particularly useful for understanding the folding mechanism of equine lysozyme. Figure 7(b) shows the positions of the

Table 2. Kinetic and equilibrium m values and the relative degree of structural organization in the I and the transition states (pH 7.0 and 25°C)

	$-m_{I\ddagger}$ (A) or $-m_{U\ddagger}$ (B) (kcal mol^{-1} M^{-1})	$m_{N\ddagger}$ (kcal mol^{-1} M^{-1})	m_{IU} (kcal mol^{-1} M^{-1})	m_{NU} (kcal mol^{-1} M^{-1})	m_{IU}/m_{NU} (%)	$1 - m_{N\ddagger}/m_{NU}$ (%)
A. *On-pathway model*						
Equine lysozyme[a]	1.47 ± 0.03[b]	0.53 ± 0.03	1.13 ± 0.19[c]	3.13 ± 0.15[c]	36 ± 6	83 ± 1
Bovine α-lactalbumin[d]	0.65 ± 0.02[e]	1.14 ± 0.04[e]	0.75[f]	2.39[f]	31	52
B. *Off-pathway model*						
Equine lysozyme[a]	2.61 ± 0.03	0.52 ± 0.03	1.13 ± 0.19[c]	3.13 ± 0.15[c]	36 ± 6	83 ± 1

[a] Holo protein at 10 mM Ca^{2+}.
[b] Standard deviations.
[c] From Table 1.
[d] Holo protein at 1 mM Ca^{2+}.
[e] Kuwajima *et al.* (1989).
[f] Ikeguchi *et al.* (1986b).

MG and the transition states of the two proteins along the reaction coordinate in terms of the relative degree of structural organization. The results show that the α_I of equine lysozyme is larger than that of α-lactalbumin, consistent with the fact that the MG state of equine lysozyme is more organized than that of α-lactalbumin (Morozova *et al*., 1995, 1997). Moreover, a more significant difference is observed in $\alpha_\ddagger$ between the two proteins. Although about 50% of the hydrophobic surface exposed in the U state is buried in the transition state of α-lactalbumin, more than 80% of the surface is buried in the transition state of equine lysozyme (Table 2 and Figure 7(b)). This suggests that the structure of the transition state of equine lysozyme is much more ordered than that of α-lactalbumin. Because the native-like packing of side-chains is already present in the MG state of equine lysozyme, it is likely that such specific structure formed in the kinetic MG state is retained in the transition state of folding. The specific hydrophobic cluster in the MG state may play a crucial role in stabilizing the α-domain and guiding the subsequent structural formation in equine lysozyme.

The presence of specific native-like side-chain packing in the MG state has been suggested for apomyoglobin (Lin *et al*., 1994; Kay & Baldwin, 1996) and for cytochrome *c* (Marmorino & Pielak, 1995; Colón & Roder, 1996; Colón *et al*., 1996; Marmorino *et al*., 1998). Colón *et al*. (1996) have studied the role of packing interactions in cytochrome *c* folding, and found that the non-specific hydrophobic interactions are sufficient for stabilizing the early folding intermediate, while the specific side-chain packing interactions are required for stabilizing the subsequent partially folded intermediate at intermediate stages of folding. Recent studies of the MG state of α-lactalbumin have suggested that native-like side-chain packing interactions can also stabilize the MG state of this protein (Wu & Kim, 1997, 1998; Song *et al*., 1998). Thus, the difference in the folding mechanism between α-lactalbumin and equine lysozyme may be rather superficial, and may depend on how much the MG state is stabilized by the native tertiary interactions.

The present results, together with the known results for α-lactalbumin, thus suggest that the difference in the stability of the MG state is important in determining the apparent folding behavior of a globular protein. If the MG state is stable enough and sufficiently accumulates early in the refolding, almost all molecules are converted into the MG state in the burst phase, and the folding reaction is represented by the on-pathway sequential model. However, if the MG state is not stable enough, it accumulates only partially even under native conditions, and either the three-state or two-state model can explain the folding reaction. In such a case, a triangle folding mechanism can explain the folding reaction (Kiefhaber, 1995b; Wildegger & Kiefhaber, 1997). If the MG state is unstable and does not accumulate in the refolding process, then the folding becomes a two-state reaction (Khorasanizadeh *et al*., 1996; Baldwin, 1996), as observed in very small globular proteins such as chymotrypsin inhibitor 2 (Jackson & Fersht, 1991) and cold shock protein B (Schindler *et al*., 1995).

Chevron plot

A plot of the dependence of $\ln k_{app}$ on c is called a chevron plot (Figures 5 and 7). For many globular proteins, a bent in the chevron plot at low c values is observed and referred to as a ''rollover'' (Baldwin, 1996). The rollover can be caused by transient accumulation of a folding intermediate, and the absence of the rollover has been interpreted as an indication of a two-state folding reaction (Jackson & Fersht, 1991). According to this criterion, the apparent linear chevron plot of the equine lysozyme refolding may be interpreted in terms of a two-state-like folding reaction (Figure 5). However, the stopped-flow CD results clearly demonstrate the accumulation of the MG intermediate with sufficient structure and stability (Figures 1 and 2). Such apparent contradiction is due to the high stability of the MG state of this protein. Because the population of the MG state is much higher than that of the U state at $0 \sim 3$ M GdnHCl, i.e. f_U/f_I is almost zero, the apparent rate constant, k_{app}, is nearly equal to k_{IN} below 3 M GdnHCl, and thus the rollover is not observed (Figure 7(a), and see equation (6)). Thus, the absence of the rollover in the chevron plot may not be a safe criterion in determining the folding mechanism, and hence must be used carefully.

Conclusions

We have shown that the unfolding equilibrium of equine lysozyme is well represented by the three-state mechanism in which only the N, MG and U states are populated, and the MG state is identical with the burst-phase intermediate in the kinetic refolding of the protein. The MG state of equine lysozyme is more stable than that of α-lactalbumin, and stabilized by the non-specific hydrophobic interactions and by the native-like side-chain packing interactions (Morozova *et al*., 1995, 1997). The higher stability of the MG state of equine lysozyme brings this protein to obey the sequential folding model in which the MG state is formed as a specific folding intermediate before formation of the N state. The relative degree of structural organization in the transition state is much higher than that of the MG state in equine lysozyme, so that the structure organized in the MG state may be retained in the transition state.

Materials and Methods

Materials

Equine lysozyme was purified as described (Bell *et al.*, 1981). The apo equine lysozyme was obtained by chromatography on a Bio-Gel P-4 column equilibrated with 0.01 M HCl, followed by lyophilization. The contamination of the solutions for the apo protein with Ca^{2+} was checked by using Quin-2 from Wako Pure Chemical Industries, Ltd. (Osaka, Japan: see Nitta & Watanabe, 1991). Protein concentration was determined by measuring the absorbance at 280 nm using an extinction coefficient of $E_{1\%} = 23.5$. Buffer solutions for the holo protein contained 50 mM sodium cacodylate (pH 7.0), 50 mM NaCl and 10 mM $CaCl_2$, while those for the apo protein contained 50 mM sodium cacodylate (pH 7.0), 50 mM NaCl and 1 mM EGTA and were demetalized by a Chelex-100 column, which has a strong attraction for divalent metals (BioRad, CA). All solutions were filtered through membrane filters (pore size 0.45 μm) before measurements.

CD measurements

Equilibrium and kinetic CD measurements were carried out in a Jasco J-720 spectropolarimeter. The temperature of the cuvette was maintained at 25°C by circulating water. The mean residue ellipticity, [θ] (deg cm^2 $dmol^{-1}$), was obtained using a mean residue weight of 112.7 Da for equine lysozyme. The path-length of the optical cuvettes for equilibrium measurements were 1 and 10 mm for far and near-UV regions, respectively. Kinetic CD measurements were carried out using a stopped-flow apparatus (specially constructed by Unisoku, Inc., Osaka, Japan; volume ratio 1:10.5) attached to the spectropolarimeter as described (Kuwajima, 1996b). The path-lengths of 1.0 and 3.8 mm with a dead-time of 20 and 25 ms were used for far and near-UV CD measurements, respectively. The protein concentrations for the equilibrium and kinetic measurements were 21 ~ 31 μM and 6.3 ~ 57.0 μM.

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