from type II to type I (Mayne et al., 1975). In studies presented elsewhere (Pawlowski et al., 1981), the collagen mRNA population of BrdU-grown chondrocytes was examined by using a cell-free protein-synthesizing system. It was found that the BrdU-treated chondrocyte message would give an excess of α_1(I), indicating that in this system an altered subunit ratio is not due to translational effects. These seemingly contradicting findings may be the result of different types of controls being operable between cells starting to synthesize a new protein (BrdU system) and cells regulating the amounts of a protein they are already synthesizing. Direct examination of the mRNA levels is necessary, however, to precisely examine these findings.

The difference in the response to HIB among cells making different amounts of collagen presents several possibilities. Candidates for further study would include factors such as the presence of "cap" structures and translational control RNAs and the concentrations of protein initiation factors (Bergmann & Lodish, 1979; Heywood & Kennedy, 1976). Analysis, using cell-free protein-synthesizing systems, as well as direct examination of the mRNA concentration and structure should be able to distinguish among these possibilities.

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Identification and Characterization of the Direct Folding Process of Hen Egg-White Lysozyme†

Shingo Kato, Nobuo Shimamoto, and Hiroyasu Utiyama*

ABSTRACT: Refolding kinetics of hen egg-white lysozyme (HEWL) have been studied by means of the stopped-flow method with guanidinium chloride as the denaturant. We show here that the three-species model $U_1 \rightleftarrows U_2 \rightleftarrows N$ (U_1 and U_2 = unfolded; N = native) now established for pancreatic ribonuclease A is also valid for HEWL on the basis of the following lines of evidence: (1) refolding kinetics outside the transition region are biphasic; (2) dependence of the fractional amplitude for the fast phase on the ratio of the time constants of the two phases agrees with theory; (3) unfolding kinetics outside the transition region are of single phase; (4) direct evidence for the $U_2 \rightarrow U_1$ transformation is obtained by double-jump experiments; (5) the time constant of the binding reaction of a substrate analogue, 4-methylumbelliferyl *N*,*N'*-diacetyl-β-chitobioside, to HEWL molecules during refolding reaction agrees with the time constant of the direct refolding phase $U_2 \rightarrow N$. The characteristic properties of the nucleation-controlled reaction of refolding of small globular proteins are discussed in general. The results of the discussion are used to suggest that the direct folding process is nucleation controlled from the experimental results of the temperature dependence of the refolding rate.

The rate of folding reaction of small globular proteins usually ranges from 10 to 100 s^{-1}. Therefore, the folding mechanism is often discussed based on the kinetic results obtained by using the stopped-flow technique. The important character of the models put forward heretofore may be represented by reactions A and B.

$$U \underset{k_{21}}{\overset{\text{fast } k_{12}}{\rightleftharpoons}} I \underset{k_{32}}{\overset{\text{slow } k_{23}}{\rightleftharpoons}} N \qquad \text{(A)}$$

(U = unfolded; I = reaction intermediate; N = native.) Reaction A is biphasic: the faster reaction produces an in-

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termediate, I, on the direct folding pathway, which is then transformed at a slower rate into the native form, N.

$$U_1 \overset{\text{slow}}{\underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}}} U_2 \overset{\text{fast}}{\underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}}} N \qquad \text{(B)}$$

(U_1 and U_2 = unfolded; N = native.) Reaction B is also biphasic, but only the single, faster process is involved in the direct folding. Two unfolded forms are spectroscopically indistinguishable.

The model A was proposed by Ikai and Tanford (Ikai & Tanford, 1973; Ikai et al., 1973; Tanford et al., 1973) to interpret the experimental results obtained on horse heart ferricytochrome *c* and hen egg-white lysozyme (HEWL)[1] in a series of the first systematic study of protein folding from both theoretical and experimental viewpoints. Their work stimulated kinetic investigations on small globular proteins, because we can expect that the folding pathway may be elucidated by examining the properties of the intermediate and the two related processes; the equilibrium folding transition is known to be an essentially all-or-none process involving only N and U.

The model B was proposed by Baldwin and co-workers based on later but more extensive investigations on bovine pancreatic ribonuclease A (RNase A)[1] (Garel & Baldwin, 1973, 1975a,b; Hagerman & Baldwin, 1976; Garel et al., 1976; Nall et al., 1978). According to the proposal by Brandts et al. (1975), the two unfolded forms, U_1 and U_2, are considered to differ in the cis and trans configuration of proline residues in a molecule. The model B, which is fully established now, indicates that the kinetic folding transition is also a highly cooperative phenomenon in contrast to the model A. Therefore, it is important to reinvestigate if the model A is really valid for HEWL and ferricytochrome *c*. In the present paper we present lines of clear evidence on HEWL, which support the model B. The similar conclusion was recently reported on ferricytochrome *c* by Ridge et al. (1981).

Now that the model B is expected to be applicable to small globular proteins in general, we focus attention on the mechanism of the direct folding reaction, $U_2 \leftrightarrow N$. The involvement of a nucleation process in the rate-limiting step of the direct folding reaction has been discussed [for example, Wetlaufer (1973)], but clear experimental evidence has not been obtained yet. In the second half of this paper we discuss the characteristics of the nucleus in the protein folding. By *nucleus* we mean to express a conformation comprising a small domain of characteristic structure and size. The rate-limiting step of protein folding is assumed to be the formation of a nucleus. Here we propose, by a reasoning analogous to the nucleation reaction in condensed systems (Turnbull & Fisher, 1949), that the rate of nucleation-controlled refolding reaction may exhibit a maximum at a temperature. We also present experimental evidence on HEWL that the temperature dependence of the direct folding rate in fact shows a maximum but that of the slow isomerization reaction does not.

Experimental Procedures

Materials. Hen egg-white lysozyme, grade I, was obtained from Sigma Chemical Co. The further purification and characterization of the sample used in the present study were described previously (Kato et al., 1981). Protein concentration was determined spectrometrically on the basis of $A_{280}^{1\%} = 26.3$ in 0.1 M acetic acid (Saxena & Wetlaufer, 1970). Special-grade GdmCl for biochemical use was purchased from Wako Chemical Industries, and its concentration was determined by the refractive index measurement (Nozaki, 1972). A substrate analogue for HEWL, 4-methylumbelliferyl *N,N'*-diacetyl-β-chitobioside [$(GlcNAc)_2$-MeU][1] was a gift from Dr. K. Hamaguchi and Dr. Y. Yang of Osaka University. This sample was used after purification by gel filtration with Sephadex G-15 to remove traces of contaminated 4-methylumbelliferone. Concentration of $(GlcNAc)_2$-MeU was determined by using a molar absorbance of 1.24×10^4 M^{-1} cm^{-1} at 316 nm (Van Landshoot et al., 1977). Other reagents were guaranteed reagent grade from Nakarai Chemicals, Kyoto.

Stopped-Flow Measurements. The folding kinetics of HEWL were measured with a stopped-flow UV spectrophotometer, Model RA-401 (Union Giken Co., Osaka). The absorbance due to tryptophan residues (301 nm) was used as the probe for folding reactions. The details of the apparatus, its use, and the analysis of the data were described previously (Kato et al., 1981). The standard initial and final refolding conditions were 4 and 0.5 M GdmCl, respectively, in 50 mM glycine–HCl buffer, pH 2.6. The protein concentration (final) was 28 μM.

Binding of $(GlcNAc)_2$-MeU to HEWL. Both equilibrium and kinetic measurements were made fluorometrically in a Hitachi fluorescence spectrophotometer, Model MPF-4. In equilibrium measurements, a standard quartz cell (10 × 10) was used, whose temperature was kept constant at 25.0 ± 0.1 °C by circulating thermostated water in a jacketed cell holder. Fluorescence emission spectra were measured with an excitation light of wavelength 330 nm. This light does not excite aromatic residues in protein molecules. The monochromator bandwidths for excitation and emission were 4 and 6 nm, respectively. The spectra were measured on a solution containing 3.35 μM $(GlcNAc)_2$-MeU, 0.5 M GdmCl, and 50 mM glycine–HCl buffer, pH 2.6, in the presence and absence of 69 μM HEWL. Kinetic measurements were made by using a stopped-flow attachment, together with a Union Giken 77 microcomputer. A quartz cell used in the attachment was 2 × 2 mm and thermostated at 25.0 ± 0.1 °C. Fluorescence intensity at 372 nm (peak of the emission spectrum) was measured with excitation at 330 nm. The monochromator bandwidths for excitation and emission were 15 and 20 nm, respectively. The emission fluorescence below 350 nm was cut off with an appropriate filter to avoid contamination with stray excitation light. In the measurements of the binding rate of $(GlcNAc)_2$-MeU to HEWL molecules in the native form, the final condition was similar as in equilibrium measurements. Kinetic measurements of the binding during HEWL refolding were also made in similar final conditions except HEWL molecules were initially unfolded in 4 M GdmCl.

Equilibrium CD Measurements. CD measurements in the ultraviolet region were made with a Jasco spectropolarimeter, Model J-40, equipped with a data processor, Model J-DPZ. A jacketed quartz cell of a 0.1-mm path length was used. The solution temperature was kept constant to ±0.1 °C by circulating thermostated water in the jacket. The spectral bandwidth of the monochromator was set at 1 nm. The apparatus was calibrated with a dioxane solution of epiandrosterone on the basis of $[\Theta] = 1.12 \times 10^4$ at 364 nm at 25 °C (a datum from Jasco).

Results and Discussion

Refolding Kinetics in Various Refolding Conditions. In a previous report (Kato et al., 1981) we showed that the re-

[1] Abbreviations: HEWL, hen egg-white lysozyme with the intact disulfide bonds; RNase A, bovine pancreatic ribonuclease A with disulfide bonds intact; GdmCl, guanidinium chloride; $(GlcNAc)_2$-MeU, 4-methylumbelliferyl *N,N'*-diacetyl-β-chitobioside; τ, time constant of a recation; *A*, amplitude of a reaction; CD, circular dichroism.

Table I: Kinetic Parameters for the Refolding of HEWL at 25 °C Measured at 301 nm[a]

final GdmCl concn (M)	τ_1 (s)	τ_2 (s)	A_1 (M^{-1} cm^{-1})	A_2 (M^{-1} cm^{-1})	$A_2/(A_1 + A_2)$
0.5	16	1.3	+160	+1350	0.89
1.0	25	4.3	+240	+1210	0.83
1.5	39	13.9	+340	+910	0.73
2.0	68		+1100		≃0

[a] The initial GdmCl concentration was 4.0 M. All solutions contained 50 mM glycine–HCl buffer, pH 2.6. The positive and negative signs of amplitude indicate that the absorbance was decreasing and increasing, respectively, during refolding.

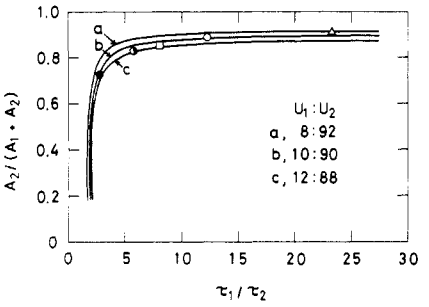

FIGURE 1: Theoretical curves for the relation between the fractional amplitude of the fast refolding process, $A_2/(A_1 + A_2)$, and the ratio of the time constants, τ_1/τ_2, predicted by the model $U_1 \leftrightarrow U_2 \leftrightarrow N$. The ratio of $[U_1]$ and $[U_2]$ used for the calculation is indicated in the figure for each curve. The experimental data are plotted as follows: open circle, 0.5 M GdmCl; half-filled circle, 1.0 M GdmCl; filled circle, 1.5 M GdmCl; the triangle, 5% HOAc; open square, 0.5 M GdmCl and 5% HOAc. The last two data were taken from Kato et al. (1981).

folding kinetics of lysozyme were biphasic for the three refolding conditions which contained 0.5 M GdmCl or 5% acetic acid or both and that the kinetic parameters obtained (τ's and the fractional amplitudes) were independent of wavelength of measurement. The latter result resolved the difficulty contained in the results of Tanford et al. (1973) that the refolding kinetic result at 292 nm did not display any biphasic character in contrast to the data obtained at 301 nm. The fractions of the amplitude in the fast phase, $A_2/(A_1 + A_2)$, for the above three refolding conditions roughly agree with each other, but a slight dependence on the ratio τ_1/τ_2 was noticed on a closer inspection. In fact, this dependence can provide a useful criterion to judge which of the above two models, A or B, is valid (Hagerman & Baldwin, 1976; Hagerman, 1977). In this work we examined the dependence more expicitly by using refolding conditions containing various concentrations of the denaturant.

Table I summarizes the numerical data obtained. As the GdmCl concentration in the final refolding condition was increased, the time constants for both the fast and the slow phases increased; the increment was greater for the fast phase. The relative amplitude for the fast phase decreased gradually with an increase in GdmCl concentration, and it suddenly vanished at 2.0 M. These results can be explained quantitatively by the model B as compared in Figure 1. The theoretical curves were calculated on the assumptions that the U_1 and U_2 species have the same extinction coefficient, $k_{32} = 0$, and the equilibrium constant for $U_1 \rightleftharpoons U_2$ transformation, that is, the ratio k_{12}/k_{21}, is maintained constant during refolding. The comparison shows that the data points fit very well with a theoretical relation with $[U_2]:[U_1] = 90:10$. The theory also predicts that the fractional amplitude for the fast phase decreases sharply as the ratio τ_1/τ_2 approaches unity. This

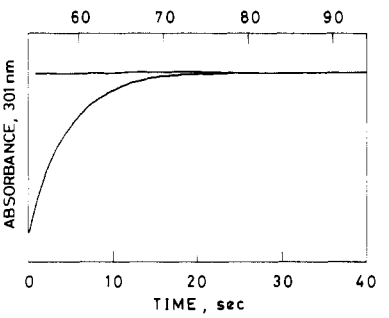

FIGURE 2: Kinetic progress curves for HEWL unfolding at 25 °C, as measured by absorbance at 301 nm after a stopped-flow concentration jump from 1.5 to 6.0 M GdmCl in 50 mM glycine–HCl buffer, pH 2.6. The lower and upper traces show the changes over the time ranges 0–40 s (lower abscissa scale) and 50–100 s (upper abscissa scale), respectively. The final protein concentration was 28 μM.

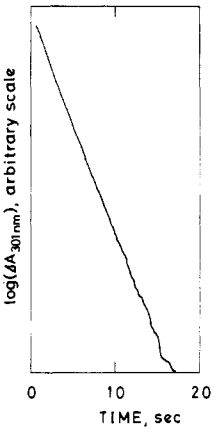

FIGURE 3: Semilogarithmic plot of the net absorbance change evaluated from the results as shown in Figure 2.

explains why the amplitude for the fast phase suddenly vanished at 2.0 M GdmCl and a pseudo-single-phase unfolding reaction resulted. The theoretical relation for the model A, on the contrary, predicts that the fractional amplitude for the fast phase increases to unity when $\tau_1/\tau_2 = 1$ (Hagerman & Baldwin, 1976; Hagerman, 1977). Thus, we can conclude that the refolding kinetics of HEWL obey the model B.

A value of 0.9 for the ratio $[U_2]/([U_1] + [U_2])$ may be compared with a theoretical value of 0.64 for proteins in unfolded conformation at equilibrium with two proline residues in the trans configuration. This result may indicate that one of the two residues could be permissive [see a recent discussion by Stellwagen (1979) and Levitt (1981)]. Furthermore, the equilibrium constant for the cis–trans isomerization may deviate from the value of 0.8 for the unfolding condition, because unfolded molecules in the initial unfolding condition are quickly transformed into a transient unfolded conformation in a refolding condition.

Single-Phase Unfolding Kinetics. The experimental results of Tanford et al. (1973) show that the unfolding kinetics outside the transition zone are biphasic and the amplitude of the fast phase increases with GdmCl concentration. Since these results are not consistent with the model B, we reinvestigated the unfolding reaction. Figure 2 shows the kinetic progress curves for HEWL unfolding in 6.8 M GdmCl and 50 mM glycine–HCl buffer, pH 2.6, at 25 °C. No absorbance change was observed at times longer than 30 s. The semilogarithmic plot of the net absorbance change (Figure 3) gives a straight line, indicating that the unfolding reaction is of single phase. This behavior is consistent with the model B, where U_1 and U_2 species are spectrometrically indistinguishable and the rate constant k_{23} is negligible.

Table II: Kinetic Parameters for the Unfolding of HEWL at 25 °C Measured at 301 nm[a]

final GdmCl concn (M)	τ (s)	A (M^{-1} cm^{-1})
6.0	9.7	−1330
6.3	6.8	−1250
6.8	4.2	−1420
7.3	2.8	−1450
7.8	1.3	−1380

[a] The initial GdmCl concentration was 1.5 M. The initial and final solutions contained 50 mM glycine–HCl buffer, pH 2.6. The negative sign of the amplitude indicates that the absorbance was increasing during unfolding.

Table II summarizes the numerical results of the unfolding kinetics together with the data obtained in other unfolding conditions. At all the GdmCl concentrations investigated, the unfolding reactions were strictly of single phase. The time constant decreased with GdmCl concentration. The amplitudes as expressed by the molar absorbance change were almost constant and agreed with the static absorbance changes.

Interconversion Reaction between the Two Unfolded Forms in an Unfolding Condition. The process by which the slow refolding form U_1 is produced from the optically identical, fast refolding form U_2 can be measured by double-jump experiments (Brandts et al., 1975). In this method, the fast unfolding of N is monitored spectroscopically in the first unfolding jump and the slow formation of U_1 is monitored by the amplitude of the slow refolding reaction in the second refolding jump. This assay procedure was extremely useful for RNase A, because the fractional amplitude for the slow phase amounted to as large as 80% for the unfolded solution at equilibrium. Moreover, the rate of interconversion $U_2 \rightarrow U_1$ can be made negligibly small compared with that of $N \rightarrow U_2$ by carrying out the first unfolding reaction at 0 °C (Cook et al., 1979). This is due to the fact that the activation energy for the isomerization reaction in unfolding conditions is larger than that for the unfolding reaction. Therefore, the species present in the system is virtually U_2 only in the beginning of the isomerization reaction at 0 °C, which makes the data analysis very simple.

For the HEWL unfolding reaction at 0 °C in 6.8 M GdmCl, the time constant was measured as 250 s. A comparison of this result with that at 25 °C (4.2 s from Table II) gives a rough estimate of the activation energy as 26 kcal/mol. Since the activation energy for the isomerization reaction in unfolding conditions is about 20 kcal/mol, the two rates come closer as the temperature is decreased. What makes double-jump experiments more difficult for HEWL is the small amplitude (10%) of the slow refolding reaction. Therefore, it is unavoidable that the results of such experiments are only semiquantitative, but yet they are important for evaluation of the models.

Double-jump experiments were carried out as follows. The first unfolding jump was made from 1.5 to 6.9 M GdmCl, pH 2.6, at 25 °C. The unfolding reaction was completed within 15 s. After a certain duration, the second jump was made to 0.7 M GdmCl, pH 2.6, at 0 °C, and the refolding process was measured in a Hitachi spectrophotometer, Model 320. The refolding reaction was carried out at 0 °C to make the refolding rate slow enough so that mixings could be carried out manually. The fractional amplitude for the slow phase in refolding after 15 s of unfolding was roughly 5%. It increased to 10% after a sufficiently long time, 45 min (data not shown). These results yielded a time constant of about 20 s for $U_2 \rightarrow U_1$ interconversion, which is comparable to the value of 40 s for RNase A (Cook et al., 1979). Thus, the model B is also favored by the double-jump experiment.

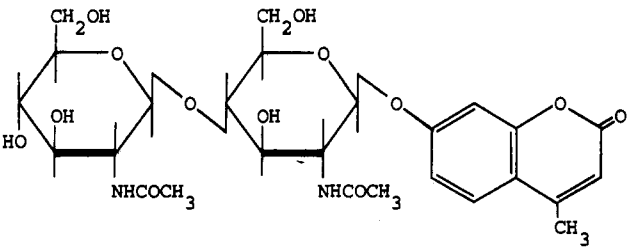

FIGURE 4: Structural formula of 4-methylumbelliferyl *N,N'*-diacetyl-β-chitobioside [$(GlcNAc)_2$-MeU].

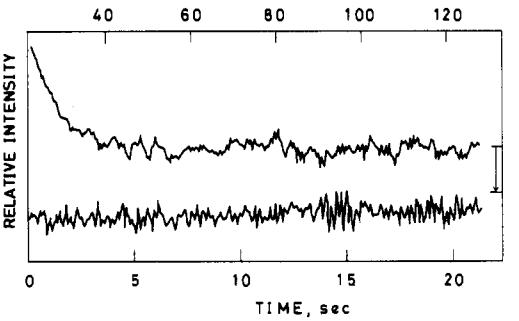

FIGURE 5: Changes in the fluorescence intensity with time during refolding of HEWL in the presence of $(GlcNAc)_2$-MeU at 25 °C. The final solution contained 3.35 μM $(GlcNAc)_2$-MeU, 0.5 M GdmCl, 50 mM glycine–HCl buffer, pH 2.6, and 69 μM HEWL. HEWL molecules were first unfolded in 4 M GdmCl. An effect of photochemical reaction was corrected for (see the text). The upper and lower traces show the changes over the time ranges 0–20 s (lower abscissa scale) and 30–130 s (upper abscissa scale), respectively. The lower trace was shifted downward as indicated by the arrow.

Binding of $(GlcNAc)_2$-MeU to HEWL. As in the case of RNase A (Nall & Baldwin, 1977), binding of a substrate analogue during refolding is one of the most critical experiments to test which of the two models, A or B, applies to the refolding reaction. In the model A the binding will take place in the slow phase but in the model B in both the fast and slow phases. This type of experiment suits HEWL, because the amplitude for the fast, refolding reaction is very large (90%). Figure 4 shows the chemical structure of a substrate analogue, 4-methylumbelliferyl *N,N'*-diacetyl-β-chitobioside, in which a fluorescent probe is covalently bonded to a chitobioside. This compound was shown to bind to the B, C, and D positions in the active site of HEWL (Yang & Hamaguchi, 1980). A single molecule of $(GlcNAc)_2$-MeU binds to a molecule of HEWL in the native form.

Fluorescence emission spectrum on $(GlcNAc)_2$-MeU in 0.5 M GdmCl and 50 mM glycine–HCl buffer, pH 2.6, gave a peak at 372 nm with an excitation wavelength of 330 nm. For the $(GlcNAc)_2$-MeU·HEWL complex, the peak wavelength remained the same but the intensity was diminished by the binding. The equilibrium constant for the binding in the present condition was measured as 3.0×10^3 M^{-1}. This result may be compared with a value of 1.5×10^4 M^{-1} obtained in a dilute buffer solution at pH 5.2 (Yang & Hamaguchi, 1980). The binding reaction of $(GlcNAc)_2$-MeU to HEWL in the native form was shown to be completed within the mixing time (15 ms). This result indicates that the rate constant of the binding is larger than 1×10^6 M^{-1} s^{-1}. In the binding experiment during refolding, HEWL molecules were initially unfolded in 4 M GdmCl and then brought to 0.5 M GdmCl by rapid mixing with a buffer containing $(GlcNAc)_2$-MeU. Of the refolded, native form of HEWL, the proportion of molecules bound with the analogue was checked to be constant during the refolding reaction.

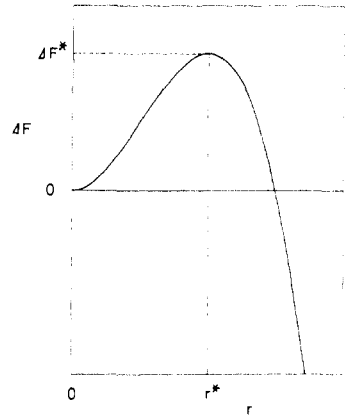

FIGURE 6: Schematic representation of the dependence of the free energy of formation of a nucleus, ΔF, on radius r for spherically symmetric nuclei (see eq 2).

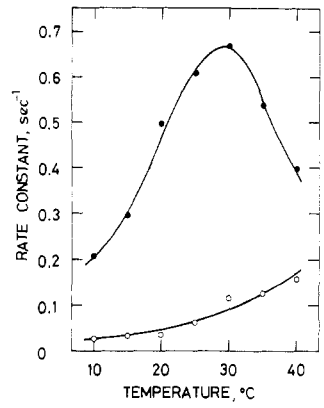

FIGURE 7: Dependences of rate constants (reciprocal of the time constant, τ^{-1}) of HEWL refolding on temperature for the fast phase (closed circles) and the slow phase (open circles). The final condition was 0.5 M GdmCl and 50 mM glycine–HCl buffer, pH 2.6.

Kinetic progress curves of the binding (Figure 5) show clearly a major phase of binding with a time constant of 1.2 s. This value is in good agreement with the time constant of the fast, refolding reaction (1.3 s) in the same refolding condition. The binding process corresponding to the slow, refolding reaction is not detected because of the large noise. Thus, the results of the binding reaction of $(GlcNAc)_2$-MeU to HEWL during refolding are consistent with the model B.

Consideration on the Characteristic Properties of Nucleation-Controlled Reaction. It is clear now that the model B applies to refolding kinetics of small globular proteins such as RNase A, ferricytochrome *c*, and hen egg-white lysozyme. It should be emphasized, therefore, that the folding of small globular proteins is highly cooperative in kinetic as well as in equilibrium measurements. Under the circumstances we have to invent a new experimental approach to elucidate the properties of the direct folding process, or we need to employ different experimental techniques to further resolve the elementary steps of folding.

Here we consider what evidence we should present to critically show that the direct folding process is nucleation controlled. It is proven useful to examine the classical theory on the rate of nucleation in condensed systems, i.e., liquid–solid or solid–solid transformations. The nucleation rate is given by (Turnbull & Fisher, 1949)

$$v = J \exp[-(\Delta f^* + \Delta F^*)/(kT)] \qquad (1)$$

where ΔF^* denotes the free energy for the formation of the critical size nucleus, Δf^* the activation energy for diffusion of molecules across the liquid–solid interface, k the Boltzmann constant, T the absolute temperature, and J a constant. For spherically symmetric nuclei, the free energy of formation of a nucleus with radius r is given by

$$\Delta F = -(4/3)\pi r^3(\Delta f_v) + 4\pi r^2\sigma \qquad (2)$$

where Δf_v denotes the bulk free energy change per unit volume and σ the surface free energy per unit area. The dependence of ΔF on r is depicted in Figure 6. Then ΔF^* and r^* for the critical size nuclei are given by

$$r^* = 2\sigma/\Delta f_v \qquad \Delta F^* = (16\pi\sigma^3/3)/(\Delta f_v)^2 \qquad (3)$$

Since Δf_v is related to the melting temperature T_m for the solid phase by $\Delta f_v = (\Delta H_v/T_m)(\Delta T)$ (ΔH_v is the enthalpy change for the liquid–solid transformation, and $\Delta T = T_m - T$), the magnitude of ΔF^* becomes increasingly large as the temperature approaches T_m. Therefore, the nucleation rate as given by eq 1 tends, after passing through a maximum, to decrease with temperature near T_m. This type of behavior has been observed not only on systems of small molecules but also on homogeneous nucleation processes of synthetic polymers such as polyethylene (Mandelkern, 1964).

Next we consider the case of protein folding. We assume that the process is nucleation controlled and that the rate is given by a similar expression as eq 1 but with different meanings. A nucleus in protein folding represents a conformation comprising a domain of characteristic structure and size, and ΔF^* in eq 1 denotes the free energy of formation of such nuclei from unfolded proteins. As a possible configuration of the domain we suggest a hydrogen-bonded chain elements with a hydrophobic surface or surfaces. This configuration may ensure a further rapid folding up of the rest of the molecule. In addition, since a large, positive heat capacity change (Tanford, 1973) and a negative entropy change are expected for the nuclei formation stated above, ΔF^* may increase with temperature and become exceedingly large near T_m. On the other hand, as pointed out previously (Kato et al., 1981), the unfolded protein in refolding conditions differs in its conformation from those in unfolding conditions at equilibrium and represents a kinetic intermediate with a free energy of local minimum. Some hydrophobic residues, if not all, make hydrophobic contacts in the intermediate. Therefore, so that a domain can be made, the hydrophobic contacts are first broken before a hydrogen-bonded structure is formed and hydrophobic residues are made to contact directly with water.

From the above discussion we may suggest that the refolding rate of small globular proteins exhibits a maximum at a temperature below T_m. A decrease in the direct folding rate with temperature has been reported for RNase A (Hagerman & Baldwin, 1976).

Temperature Dependence of the Direct Refolding Rate of HEWL. Figure 7 shows the dependence on temperature of the rate constants (reciprocal of time constant, τ^{-1}) for the fast and slow refolding reactions in 0.5 M GdmCl and 50 mM glycine–HCl buffer, pH 2.6. In this refolding condition the temperature of half-transition was determined as 51 °C from static CD measurements at 222 nm (Figure 8). It is clearly seen that the direct refolding rate exhibited a maximum at about 29 °C. Thus, from the reasoning stated under Consideration on the Characteristic Properties of Nucleation-Controlled Reaction, we may conclude that the direct folding reaction is nucleation controlled. In sharp contrast, the slow refolding rate increased monotonously and an Arrhenius plot of the results yielded a straight line, consistent with the postulate that the process is related to the cis–trans isomerization of proline residues. An activation energy of 11 kcal/mol was estimated from the slope. This value may be compared with

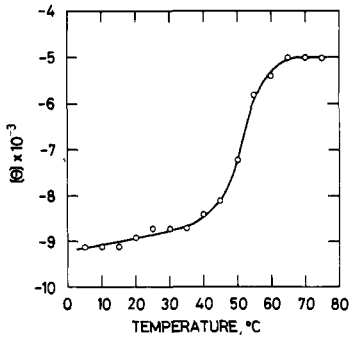

FIGURE 8: Thermal unfolding transition of HEWL in 0.5 M GdmCl and 50 mM glycine–HCl buffer, pH 2.6, as measured by the static CD measurements.

2.3 and 17.6 kcal/mol for RNase A at 0.1 M and 2.0 M GdmCl, respectively, at pH 6 (Nall et al., 1978).

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Enniatin Synthetase, a Novel Type of Multifunctional Enzyme Catalyzing Depsipeptide Synthesis in *Fusarium oxysporum*†

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ABSTRACT: Enniatin synthetase, a multifunctional enzyme catalyzing depsipeptide formation in *Fusarium oxysporum* was purified to 98% homogeneity as judged by analytical disc gel electrophoresis. The enzyme consists of a single polypeptide chain of a molecular weight of about 250 000. Similar to a number of peptide synthetases and to fatty acid synthetase the enzyme contains 4′-phosphopantetheine as a prosthetic group. Studies on substrate specificity revealed that the enzyme is capable of synthesizing enniatins A–C and also mixed-type enniatins containing more than one species of amino acid. A linear dependence of rate of enniatin synthesis on enzyme concentration was observed, indicating that depsipeptide formation is an intramolecular process. Omission of the methyl donor *S*-adenosyl-L-methionine resulted in the formation of unmethylated enniatins with a reaction rate of about 10% of that observed in the case of enniatins. Sulfhydryl-directed reagents generally had an inhibitory influence on enniatin synthesis. However, inhibition studies with iodoacetamide revealed that it behaves differently toward the hydroxy acid site(s) and amino acid site(s). This indicates the presence of chemically distinct thiol groups in the active sites for the two substrates.

Enniatins constitute a class of depsipeptides produced by several strains of *Fusarium* (Plattner et al., 1948). They consist of three residues of one of the branched-chain amino acids L-valine, L-leucine, or L-isoleucine and three residues of D-2-hydroxyisovaleric acid. (D-Hyiv).[1] The constituents of the depsipeptides are linked together by amide and ester bonds in an alternating fashion. In addition, amide bonds are

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Hyiv, hydroxyisovaleric acid; SAM, *S*-adenosyl-L-methionine; PEI, poly(ethylenimine).

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