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EXISTENCE OF INTERMEDIATES IN THE REFOLDING OF T_4 LYSOZYME AT pH 7.4

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SUMMARY *GuHCl induced unfolding-refolding of T_4 lysozyme has been studied at equilibrium and by kinetic analysis using fluorescence emission and circular dichroïsm at 223 nm. Both approaches have indicated the occurrence of intermediates in the folding pathway. A plausible model has been proposed.*

INTRODUCTION

Domains in proteins have been described as folding units (1-6). We have tried to determine their role in the folding of T_4 lysozyme, a small protein (M.W. 18.500) built up of two structural domains joined by a helical segment, helix 60-79 (7). It has no disulfide bridges. The folding transition has been reported to be very cooperative and to follow a two-state behavior at lower pH (8, 9). We have reinvestigated the transition at equilibrium at pH 7.4, which is the optimum for enzymatic activity, and also performed kinetic analyses using fluorescence and circular dichroïsm signals to follow the transition.

MATERIAL AND METHODS

Phage T_4 lysozyme from strain B ac q e^+ (wild type), kindly provided by J. Owen and G. Streisinger (Eugene,Oregon) was prepared according to the procedure of Tsugita et al (10) slightly modified. The enzyme assay used was that by Elwell and Schellman (8).

Unfolding was induced by incubating T_4 lysozyme (final concentration 2 µM) in various concentrations of GuHCl at 23° in a 50 mM tris-HCl buffer, 1 mM $MgCl_2$, pH 7.4 containing either 1 mM β-mercaptoethanol or 100 µM DTT (for C.D. measurements). GuHCl concentrations were evaluated from refractive index using the tables of Nosaki (11). For equilibrium studies, the denaturing mixtures were incubated for 24 hours. For kinetic studies, zero time was determined by the introduction of the enzyme in the denaturation mixture.

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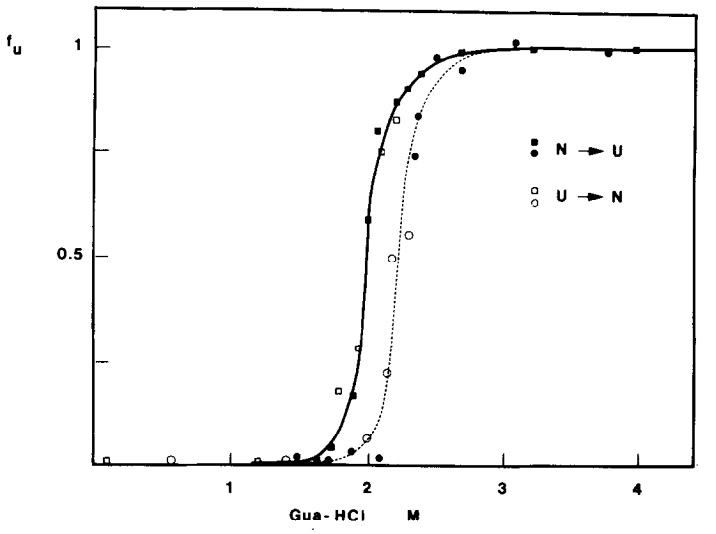


Figure 1 : Transition curves for the folding-unfolding of T_4-Lysozyme at pH 7.4, 23°, 2 µM final concentration.
——— : observed by variation of circular dichroïsm at 223 nm
---------- : observed by variation of wavelength of maximum fluorescence emission.

Refolding was monitored for solutions of T_4 lysozyme previously incubated in 6M GuHCl for 24 hours at 23° for complete unfolding and then diluted in the same buffer, with the GuHCl concentration varying from 1.5 M to 2.4 M. Incubation in regeneration mixture lasted 24 hours in equilibrium studies. For kinetic studies, zero time corresponded to the introduction of the denatured enzyme into the regeneration mixture.

Fluorescence was recorded with a Perkin-Elmer MPF 44B spectrofluorimeter. Circular dichroïsm was measured with a Jouan III super dichrograph. Temperature was kept constant at 23° ± 0.1° for all experiments.

RESULTS

1 - Study of the unfolding-folding equilibrium

Figure 1 illustrates the transition curves obtained by measuring the shift of the fluorescence maximum and the change in the circular dichroïsm at 223 nm in going from the folded to unfolded state. For both variables, complete reversibility of the transition was observed. Obviously the transition curves observed by fluorescence and by C.D. do not coïncide. This indicates that intermediate species are present in the unfolding-folding process, in spite of the strong cooperativity of the transition curves. Furthermore, the transition followed by C.D. is asymmetrical. A very abrupt change in ellipticity corresponding to 60% of the total amplitude change is observed

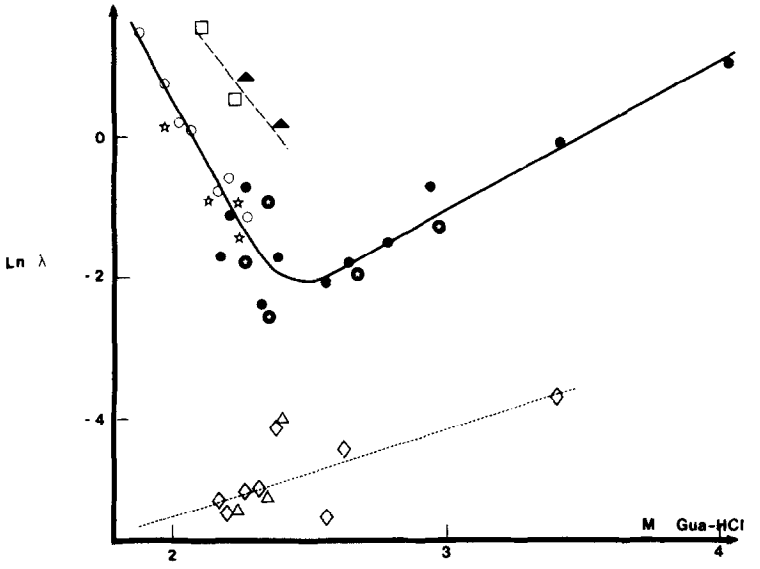


Figure 2 : Variation of macroscopic rate constants λ versus Gu-HCl concentrations for the three phases observed in the kinetics : slow phase of denaturation observed by variation of fluorescence intensity (◇) and variation of circular dichroïsm at 223 (△) ; rapid phase of denaturation observed by variation of fluorescence intensity (●) and variation of ellipticity at 223 nm (⭘); very rapid phase of denaturation observed by CD at 223 nm (▲);renaturation phase observed by fluorescence intensity (○) and CD at 223 nm (☆) ; very rapid phase observed by fluorescence intensity (□).

between 1.8 and 2 M GuHCl, where no significant change in fluorescence has occurred. Above 2M GuHCl, the last 40% of the total ellipticity change take place in the region where the totality of fluorescence change occurs.

2 - Kinetic study of the GuHCl induced unfolding and refolding of T_4 lysozyme

Kinetics were determined by recording the variation of fluorescence intensity at 326 nm, and the changes of C.D. at 223 nm. The results are shown in figure 2.

To account for the data obtained by C.D. and fluorescence, the observed kinetics must be described by at least three exponential terms :

$$f = P_1 e^{-\lambda_1 t} + P_2 e^{-\lambda_2 t} + P_3 e^{-\lambda_3 t} \qquad (1)$$

The amplitudes of the fastest and the slowest process are very small for refolding , which is rapid and can be reasonably described by a single exponential term. As clearly indicated in figure 2 , the

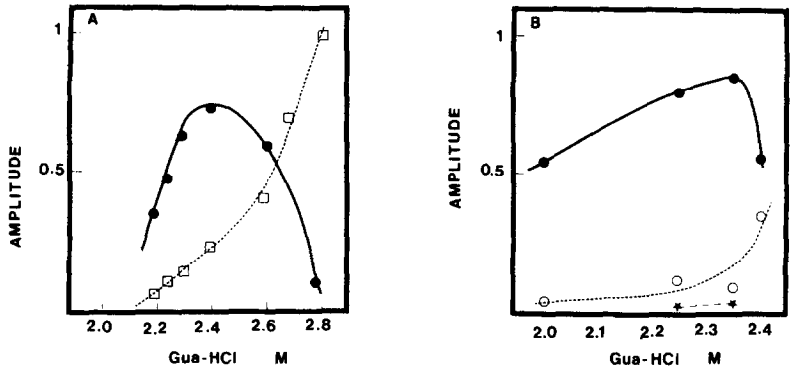


Figure 3 : Variation of amplitudes of denaturation kinetic phases versus Gu-HCl concentration :
A : from fluorescence data : rapid phase (□) and slow phase (●).
B : from CD measurement : very rapid phase (✱), rapid phase (○) and slow phase (●).

macroscopic rate constants for the three phases, including the slowest , vary with GuHCl concentrations. Figure 3 gives the amplitudes of the unfolding process. It was checked that the slowest phase is not due to an artefact, such as aggregation (it does not depend on lysozyme concentration) or adsorption on glass (the same data were obtained in experiments in plastic tubes where aliquot have been taken out at various time and fluorescence measured). It was also checked that the faster phase was not due to an artefact of mixing : the same experiment was done with a change from 6M to 4M GuHCl (where the protein is completely denatured) and no signal was observed.

DISCUSSION

Kinetic data as well as equilibrium results clearly indicate the occurrence of intermediates in the folding pathway of T_4 lysozyme. In fact, transition obtained by ellipticity at 223 nm is asymmetric and can be decomposed in two transitions, the first one (c_m = 1.95 M) is very cooperative (n = 26) and corresponds to a variation of 60% of the signal. The second one with C_m = 2.25 M, identical to C_m obtained from fluorescence measurements, may be correlated to events occurring in the tryptophan region. Equilibrium data strongly suggest that C-terminal domain must refold first. This domain contains all

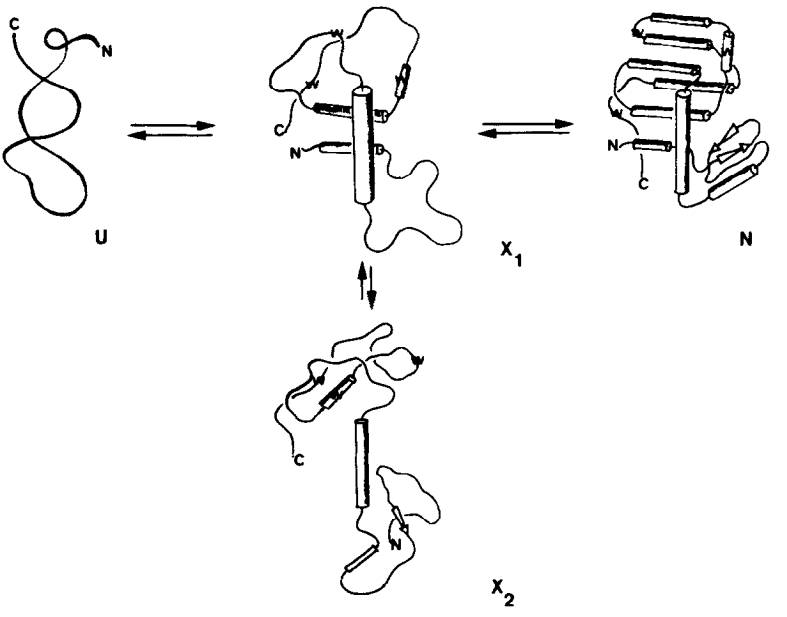
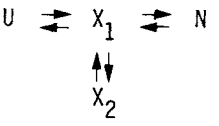


Figure 4 : A possible pathway of folding for T_4-Lysozyme.

three tryptophan residues and variation in tryptophan fluorescence begins at higher GuHCl concentrations than are required for loss of a great part of the secondary structure. The structured region which unfolds with C_m = 2.25 M contains at least one, and probably all three tryptophans and represents 20% of the whole helix content of the protein. These results have to be compared with those obtained by Elwell and Schellman with mutant enzymes ; in the enzyme from strain e R_1 1-75 (Trp 138 → Tyr 138), a single mutation leads to a change in ΔH corresponding to 20% of the entire ΔH of unfolding. This tryptophan is entirely buried in the native wild type enzyme, and strongly interacts with Met 102 and Met 106, perhaps forming a nucleation center (12), the Met pertaining to helix 95-106.

Additional information is provided by kinetic data which allow us to distinguish three phases, which implies that there are at least two intermediates. To determine the best model for the kinetic pathway that accounts for the results, various models have been analyzed by a simulation procedure using the method of Kübicek and Visnack (13). The most

plausible model involves an intermediate X_1 in the folding pathway and a side-reaction generating dead-end species X_2, as follows :



This scheme is not compatible with an independent refolding of each of the domains, but may be consistent with a sequential refolding. The C-terminal domain may refold independently, but the other domain needs its presence for folding and stabilization.

Stabilization of the two domains seems to be due mainly to the formation of a hydrophobic pocket produced by the interaction of three helical segments, helix 3-11, helix 60-79 and helix 95-106 (12). Helix 3-11 strongly interacts with the C-terminal domain. Therefore, in this protein, the two domains depend on their mutual interaction for their stabilization.

We suggest that X_1 is an intermediate in which the C-terminal domain is partially (or totally) folded. On X_2 , we have little information, except that it is not completely unfolded and that it has to revert to X_1 in order to achieve the native structure. Its structure might be held fixed by the interaction of helix 3-11 with other parts of the molecule so that the correct interactions cannot take place. Figure 4 illustrates schematically a plausible pathway which takes into account equilibrium and kinetic data and also an analysis of interactions in the native structure (12).

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