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An early immunoreactive folding intermediate of the tryptophan synthase β_2 subunit is a 'molten globule'

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The refolding kinetics of the tryptophan synthase β_2 subunit have been investigated by circular dichroism (CD) and binding of a fluorescent hydrophobic probe (ANS), using the stopped-flow technique. The kinetics of regain of the native far UV CD signal show that, upon refolding of urea denatured β_2, more than half of the protein secondary structure is formed within the dead time of the CD stopped-flow apparatus (0.013 s). On the other hand, upon refolding of guanidine unfolded β_2, the fluorescence of ANS passes through a maximum after about 1 s and then 'slowly' decreases. These results show the accumulation, in the 1–10 s time range, of an early transient folding intermediate which has a pronounced secondary structure and a high affinity for ANS. In this time range, the near UV CD remains very low. This transient intermediate thus appears to have all the characteristics of the 'molten globule' state [(1987) FEBS Lett. 224, 9–13]. Moreover, by comparing the intrinsic time of the disappearance of this transient intermediate ($t_{1/2}$ 35 s) with the time of formation of the previously characterized [(1988) Biochemistry 27, 7633–7640] early imunoreactive intermediate recognized by a monoclonal antibody ($t_{1/2}$ 12 s), it is shown that this native-like epitope forms within the 'molten globule', before the tight packing of the protein side chains.

Tryptophan synthase β_2 subunit; Immunoreactive kinetic intermediate; Circular dichroism; Molten globule; Stopped-flow technique

1. INTRODUCTION

Refolding of the β_2 subunit of *E. coli* tryptophan synthase proceeds through a series of folding events, the nature and kinetics of which have been investigated extensively by different techniques [1–3], including the use of monoclonal antibodies. As a result, it was found [4] that one monoclonal antibody (IgG 19) recognizes specifically the refolding β chain even earlier than the first stage of native structure formation detected by the methods based on the fluorescence of the single tryptophanyl residue per β chain [2]. Yet, the appearance of the corresponding, native-like, antigenic site was shown to require a folding step, the rate constant of which ($k = 0.06\ s^{-1}$ at 12°C) could be determined during the refolding of β chains denatured by either acidic pH or GuHCl [5,6]. This pointed to the existence of a structural immunoreactive intermediate which is formed early during the folding process. However, the nature of this early immunoreactive intermediate state remained unclear.

Investigations on the refolding kinetics of some simpler, single-domain, monomeric proteins have shown the fast (within a subsecond time interval) formation of a transient intermediate state, already endowed with the majority of the secondary structure and the compactness of the native protein, but still missing the tight packing of the native tertiary structure [7–10]. These properties of such early kinetic intermediates are similar to those of the 'molten globule' intermediates which have been carefully studied at equilibrium during the last years [11–15]. The absence of tight packing in the 'molten globule' results in the high accessibility to the solvent of the non-polar groups within the hydrophobic core of the protein, and therefore leads to a strong binding of hydrophobic probes by the molten globule [8,16,17]. This renders quite convenient the use of a fluorescent hydrophobic probe, such as ANS, for monitoring the molten globule formation during protein folding [8,10,16]. Using this approach it has been shown that, in simple proteins, the molten globule intermediate appears in less than 1 s and disappears after several seconds or minutes, depending on the protein [8,10]. These time intervals are of the same order of magnitude as the time of formation of the early im-

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Abbreviations: β_2, the β_2 subunit of *Escherichia coli* tryptophan synthase; GuHCl, guanidine hydrochloride; CD, circular dichroism; ANS, anilino-8-naphthalene sulfonate; $t_{1/2}$, half-time of reaction; k, rate constant; mAb 19, a monoclonal antibody (IgG 19) specific for native β_2; EDTA, ethylene-diamine-tetraacetic acid

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munoreactive intermediate (see above) observed for β_2. We therefore tried to find out whether this immunoreactive intermediate appears together with the molten globule, or with the tight packing of the tertiary structure.

The present paper describes stopped-flow studies on the kinetics of ANS binding and of far and near ultraviolet CD changes during the refolding of β_2. The results obtained clearly indicate that the early immunoreactive intermediate recognized by IgG 19 appears within the molten globule, i.e. after the molten globule formation, but before the tight packing.

2. MATERIALS AND METHODS

The apo-β_2 protein was purified, crystallized and reactivated as described earlier [18], using as a starting material a culture of *E. coli* transformed with an expression plasmid directing the synthesis of tryptophan synthase (C.R. Zetina and A.F. Chaffotte, unpublished results). Protein concentrations in solution were measured spectrophotometrically, using an extinction coefficient at 280 nm of $A^{1\%} = 5.8$.

The fluorescence fast-kinetics studies were performed with the computer driven SFM-3 stopped-flow from Bio-Logic (Echirolles, France) equipped with a thermostat and connected to a PC/XT/AT Tandon microcomputer. The kinetics thus obtained for ANS binding were fitted to multiexponential functions with the Bio-Kine software supplied with the stopped-flow. Slow fluorescence kinetics were studied by manual mixing in the cuvette of a thermostated Perkin-Elmer LS-5B double monochromator spectrofluorimeter as described earlier [2]. The dead time of the stopped-flow was 0.005 s and that of the manual mixing was about 5 s. CD studies were performed with a stopped-flow attachment connected to a J-500A spectropolarimeter (Jasco, Japan) as described earlier [20]. The dead time of the stopped-flow was 0.013 s. Analysis of the kinetics of CD changes was done by the method of non-linear least-square fitting of the data with multiexponential functions in a NEC 9801 microcomputer [9].

For the fluorescence experiments, either 4 M GuHCl [20] or 5.5 M urea were used to obtain unfolded β_2. For the CD experiments, only 5.5 M urea was used, because sufficiently high dilutions of GuHCl

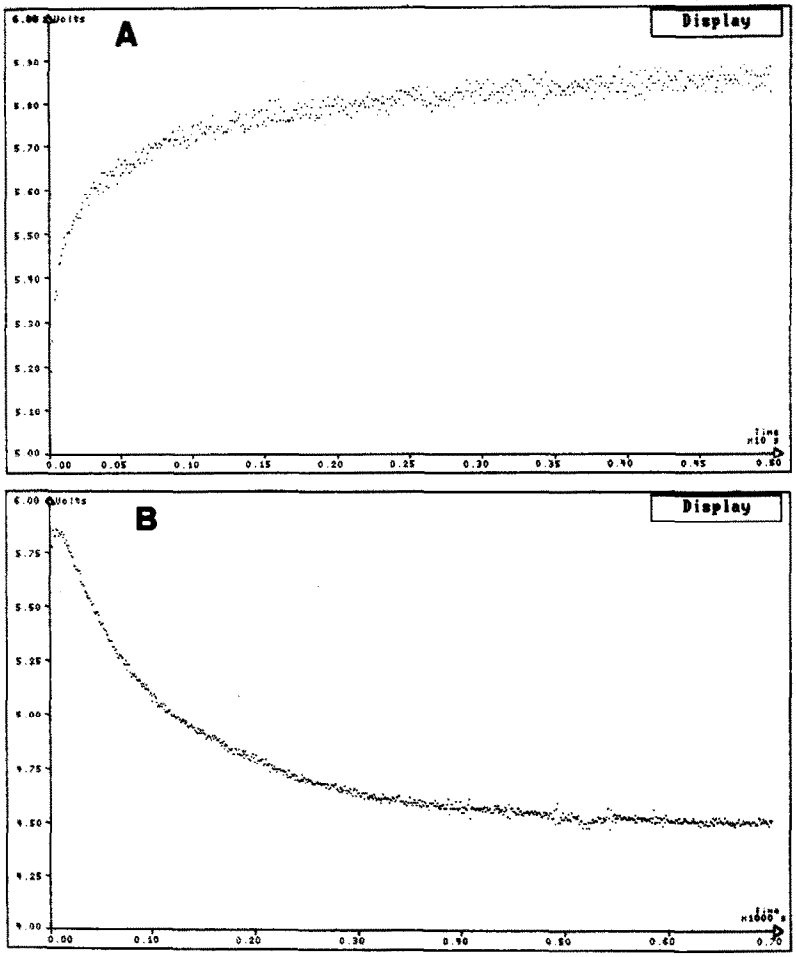

Fig. 1. Kinetics of refolding of GuHCl unfolded β_2 monitored by ANS fluorescence. β_2, unfolded in 6 M GuHCl, was first diluted to 4 M GuHCl and introduced into the small syringe (5 ml) of the stopped-flow equilibrated at 12°C. The second syringe (18 ml) was filled with buffer containing 30 μM ANS. At first injection of 6 μl of the unfolded protein and 244 μl of buffer was performed to rinse the chamber and speed up the syringes, and was immediately followed by a second, identical, injection. The actual concentrations after the last mixing were 0.04 mg/ml for the protein and 0.1 M for GuHCl. The fluorescence (excitation monochromator at 390 nm, emission above 460 nm through a high-pass filter) was recorded with different sampling times and the appropriate filtering constants. The results shown (in volts as a function of time) were obtained: (A) By averaging 16 identical experiments for the fast increase (full time scale shown: 5 s). (B) By averaging 21 identical experiments for the slower decrease (full time scale shown: 700 s).

could not be achieved with the stopped-flow used. The residual concentrations of denaturing agent during refolding were 0.1 M for GuHCl, and 0.5 M for urea. The final protein concentration (after mixing) was 0.04 mg/ml for the fluorescence measurements and 0.4–0.6 mg/ml for the CD measurements. All experiments were performed at 12°C in 0.1 M potassium-phosphate buffer, pH 7.8, containing 0.002 M EDTA and 0.005 M 2-mercaptoethanol.

3. RESULTS

Fig.1 shows the change in binding of the hydrophobic fluorescent probe ANS to the protein during the refolding of GuHCl unfolded β_2. The first process, which can be observed only with the stopped-flow, is a rapid increase in ANS fluorescence, reflecting an increase in the protein chain affinity for the hydrophobic probe. This is followed by a slower decrease in the ANS fluorescence, i.e., a decrease in ANS binding to the protein chain, probably as a consequence of the side chain tight packing. Thus, the binding of ANS to the protein molecule passes through a maximum, some 3–5 s after the initiation of the folding process. Similar results have been obtained for urea unfolded β_2 (data not shown). This strongly suggested that a molten globular intermediate may exist during the refolding of unfolded β chains.

To ascertain that the intermediate with high ANS binding indeed corresponds to a molten globule, it appeared essential to verify that it has already acquired most of the secondary structure, but not yet the tight packing, of the native state. The only existing technique that could be used for that purpose was CD stopped-flow [9,15,19]. Unfortunately, it was not possible, with the CD stopped-flow apparatus we used, to achieve the 35–50-fold dilution necessary for studying the refolding of GuHCl unfolded β chains. Because the rates of formation and disappearance of ANS binding were found to be the same during the refolding of GuHCl and urea unfolded β chains (see above), and because for most of the elementary steps involved in the folding of β_2 that were previously observed by fluorescence techniques, the rate constants were found to be the same during refolding of the GuHCl unfolded and of the acid-denatured protein [3], we supposed that they would also be the same for urea unfolded β_2, and that the steps detected by CD changes would also have similar rate constants regardless of the denaturing agent used for unfolding β chains. We therefore studied, by the stopped-flow technique, the kinetics of regain of the near and far UV CD during the refolding of urea-unfolded β chains.

Fig.2A shows that, in 5.5 M urea, β chains exhibit a far UV CD spectrum characteristic of a random coil conformation, as opposed to native β_2 which contains a high amount of secondary structure. Fig.2B represents the kinetics of regain of the far UV CD signal during the folding process. It can be seen that more than half of the native protein ellipticity at 225 nm is restored during the dead time of the stopped-flow apparatus (0.013 s). The remaining ellipticity is regained much more slowly (several minutes). Similar kinetics were observed at 220 nm.

Fig.3 shows the regain of the near UV CD during the folding of urea denatured β_2. This signal reflects the restoration of the native-like tight packing of the aromatic side chains. The increase in near UV CD is clearly much slower than the *increase* in ANS binding

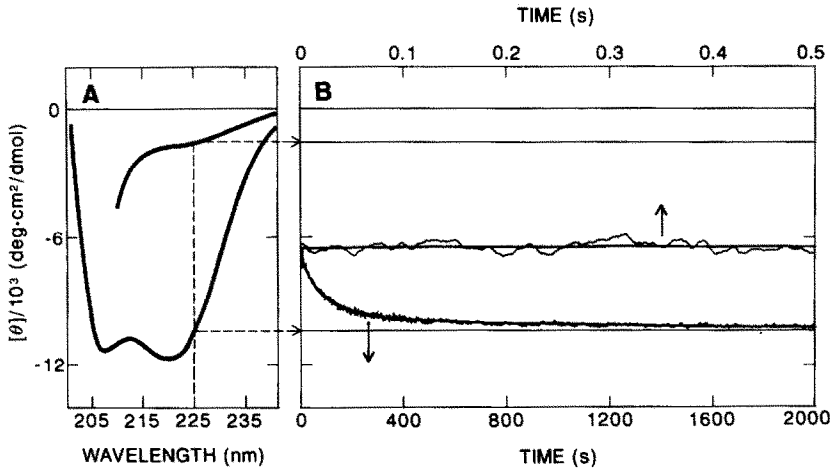

Fig. 2. Kinetics of refolding of urea unfolded β_2 monitored by far UV CD. β_2, either native or unfolded in 5.5 M urea, was introduced in one of the syringes of the stopped-flow equilibrated at 12°C, while the second syringe contained the buffer (with or without 5.5 M urea). After the mixing 75 μl of protein with 750 μl of buffer in a total time of 67 ms, the protein concentration in the observation chamber was 0.42 mg/ml. Data acquisition was triggered when the flow stopped. (A) Far UV CD spectra of the native and unfolded protein obtained by mixing native β_2 with urea free buffer, and urea unfolded β chains with buffer containing urea. (B) Kinetics of far UV CD change after mixing urea unfolded β chains with urea free buffer; the residual urea concentration was 0.5 M. The ellipticity at 225 nm was recorded as a function of time. The curves shown were obtained by averaging 15 experiments. Upper tracing: short sampling time (0.5 s full scale); lower tracing: long sampling time (2000 s full scale).

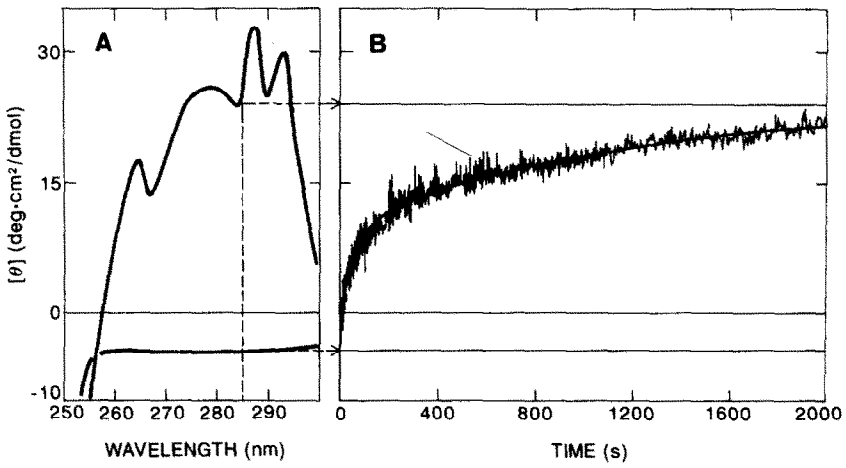

Fig. 3. Kinetics of refolding of urea unfolded β_2 monitored by near UV CD. Experiments similar to those described in Fig. 2 were conducted. The experimental conditions were the same, except for the final protein concentration in the observation chamber which was 0.57 mg/ml. (A) Near UV CD spectra of native and urea unfolded β_2. (B) Kinetics of near UV CD change upon refolding of unfolded β chains. The ellipticity at 285 nm was recorded as a function of time. Averaging over 10 experiments gave the curve shown.

(see Fig. 1). Moreover, the near UV CD changes at a rate comparable to that of the *decrease* in ANS binding (Fig. 1) and of the slow phase of restoration of the far UV ellipticity.

Taken together, the results above clearly show the transient accumulation of an early folding intermediate endowed with pronounced secondary structure, absence of tight packing of the aromatic side chains and high binding capacity for hydrophobic probes (here, ANS), i.e. all the characteristics of the 'molten globule'.

Table I represents the results of the best (within noise) approximations by multi-exponential functions of all the kinetics which were investigated in this work. By comparing the rate constants of these various processes with that of the appearance of the first antigenic intermediate recognized by mAb 19 [5,6], one can see that the antigenic determinant is formed well after the appearance of the molten globule, but well before the tight packing of the side chains and even before the complete native secondary structure is achieved. From this, it can be concluded that the antigenicity appears within the molten globular state.

4. DISCUSSION

The results reported above give new information on several aspects of the folding mechanism of the tryptophan synthase β_2 subunit. The first aspect is the demonstration that, like for the folding of the few other proteins thus far studied by CD stopped-flow, a large part of the far UV ellipticity (and hence of the secondary structure) of the native state is regained very rapidly, i.e. within the dead time of the stopped-flow device [9,15,19,21,22]. However, in this case (see also [21]), nearly one half of the native far UV CD signal appears much more slowly, together with later steps in the folding process. Thus, the slowest phase (7% of the total signal) of the far UV CD regain coincides with the slowest phase of near UV CD regain and of ANS desorption. We cannot exclude the possibility that this part of the 'slowly relaxing' far UV CD signal is related

Table I

Kinetic parameters of β_2 folding steps

Stages of protein structure formation	Technique	Time interval									
		<0.013 s		0–5 s		0–50 s		0–200 s		0–3000 s	
		k (s^{-1})	A (%)	k (s^{-1})	A (%)	k (s^{-1})	A (%)	k (s^{-1})	A (%)	k (s^{-1})	A (%)
Secondary structure	far UV CD	>100	57	–	–	0.13	7	0.01	29	0.001	7
Molten globule	ANS binding	–	–	22	40	–	–	–	–	–	–
		–	–	4	28	–	–	–	–	–	–
		–	–	0.7	32	–	–	–	–	–	–
Immunoreactivity	mAb 19 binding	–	–	–	–	0.06	100	–	–	–	–
Tight packing of	ANS desorption	–	–	–	–	–	–	0.02	30	0.001	70
side chains	near UV CD	–	–	–	–	–	–	0.02	43	0.001	57

to the contribution of aromatic residues to the CD spectrum in the far UV region [13,23–25]. However, it also can reflect the real process of formation of some secondary structure elements in the β chain during its refolding. Moreover, secondary structure formation is very probably responsible for the major part of the 'slowly relaxing' far UV CD signal (phase with k = 0.01 s^{-1} corresponding to 29% of the total signal), since aromatic residues are highly unlikely to contribute to such an important extent to the far UV CD signal. It would be of interest to know whether these slow changes in secondary structure are associated with interdomain or intersubunit interactions in this complex, multi-domain, oligomeric protein. To answer this question, the rate constants of the different phases of secondary structure regain should be carefully compared to the rate constants previously determined for subunit or domain assembly during the renaturation of the GuHCl unfolded protein. To this effect, CD stopped-flow studies on the refolding of GuHCl unfolded β chains have just been undertaken.

The second important observation is that the early immunoreactive intermediate which carries the native-like epitope recognized by mAb 19 appears within the molten globular state (see above), and has its overall properties: large amount of secondary structure and presence of a hydrophobic core, but no tight packing of the side chains. In other words, this means that the rate limiting step in the formation of the mAb 19 specific, native-like, epitope is a rearrangement of the polypeptide chain that occurs within the molten globular state. Moreover, as seen from the data presented in Table I, some changes in the secondary structure also may occur within the molten globular state. This suggests that the 'molten globule' conformation may be more heterogeneous than initially expected, in that secondary and tertiary structure rearrangements can take place within the molten globular state. Alternatively, because β is a large multidomain polypeptide chain, one may propose that only one of its domains rapidly forms a homogeneous molten globule, and that the later appearance of the antigenicity as well as the far UV CD signal increase reflect different steps in the slower folding of the other domain. Though the latter hypothesis seems unlikely in view of preliminary unpublished results on the kinetics of ANS and mAb 19 binding during the folding of the isolated N-terminal (F_1) and C-terminal (F_2) domains of the β chain, it cannot be entirely ruled out without further experiments.

The last aspect of the β_2 folding process on which the present experiments bring important new information deals with the mere significance of our previous studies on β_2 folding. Indeed, all the folding steps previously investigated occurred with $t_{1/2}$ larger than 20 s. This had cast some doubts on their being essential steps in the folding process, and they could be considered as slow, minor rearrangements occurring when the protein is already almost native. These restrictions no longer hold since very fundamental steps such as the tight packing of side chains (near UV CD and ANS desorption) and perhaps even the completion of a significant part of the native secondary structure (far UV CD) also occur slowly, in time ranges comparable to several of the various signals previously used for monitoring the folding process.

Finally we wish to point out that, though the CD studies reported here were done with urea unfolded β chains while all previous observations were made with the GuHCl unfolded protein, the main conclusions just discussed remain valid. Indeed, very similar results were obtained for the binding of ANS during the refolding of these two denatured forms of β_2. Moreover, there is very little doubt that the large changes observed for ANS binding during the folding process reflect the formation and disappearance of the molten globular state. Comparing the kinetics of formation of the mAb 19 specific epitope with the kinetics of ANS binding and release therefore suffices to support our conclusions. Furthermore, the assumption that most of the elementary steps involved in the refolding of β_2 do not depend on the denaturing agent used, is supported by studies comparing the renaturation of GuHCl unfolded and acid-denatured β chains [3]. However, because of the uncertainties of the effects of the urea treatment on the rates of some CD phases, attempts to correlate the various phases in the complex kinetics of ANS desorption, near UV CD increase, or slow secondary structure (far UV CD) increase, with the kinetics previously observed with other signals have not been made. A more precise picture of the folding process will hopefully emerge, when CD stopped-flow experiments on the refolding of GuHCl β_2 will be achieved.

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