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A Possible Initial Folding Intermediate: The C-Terminal Proteolytic Domain of Tryptophan Synthase β Chains Folds in Less Than 4 Milliseconds into a Condensed State with Non-native-like Secondary Structure†

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ABSTRACT: The isolated F2-V8 peptide corresponding to the 101 C-terminal residues of *Escherichia coli* tryptophan synthase β chains folds into a heat-stable, yet fluctuating, condensed state that contains a lot of secondary structure. However, this state has non-native-like secondary and supersecondary structures [Chaffotte, A., Guillou, Y., Delepierre, M., Hinz, H.-J., & Goldberg, M. E. (1991) *Biochemistry 30*, 8067–8074]. To characterize the rate of appearance of this state, stopped-flow studies on the far-ultraviolet circular dichroism (CD) and on the binding of 1-anilino-8-naphthalenesulfonate (ANS) have been conducted during the folding of guanidine-unfolded F2-V8. It was shown that both the CD signal at 222 nm and the ANS binding properties of folded isolated F2-V8 were regained, at 20 °C, within the dead time of the stopped-flow apparatus, which was 4 ms. At 12 °C, the binding of ANS was also completed within this dead time, but the ellipticity showed some minor later changes. After a rapid overshoot of the CD signal that occurred during the 4-ms dead time, a small readjustment of the ellipticity to the final value occurred more slowly and was completed after about 25 ms. Thus, even at 12 °C, the hydrophobic core and most of the secondary structure of folded F2-V8 were formed in less than 4 ms. These observations strongly suggest that the previously described condensed non-native-like state of F2-V8 results from a very rapid, nonspecific, hydrophobic collapse. It is proposed that such a state may be a general early intermediate in protein folding.

The mechanisms by which a polypeptide chain folds in vitro into its native conformation are currently the subject of active investigations. According to the general picture which has emerged in recent years (Kim & Baldwin, 1982, 1990; Jaenicke, 1991; Baldwin, 1991), the folding of a small monomeric protein made of a single domain would proceed as follows. First, the polypeptide chain would undergo local folding to give stretches of nativelike secondary structure (α helices or β strands) that build the "framework" (Kim & Baldwin, 1982) for forming the hydrophobic core of the native structure. Then, these elements of secondary structure would "adhere" (Kim

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& Baldwin, 1990) to one another to form an approximately native tertiary arrangement of the polypeptide chain. In an alternative model, the local nativelike secondary and tertiary structures of a "subdomain" (Oas & Kim, 1988) would be formed simultaneously, and different subdomains would then adhere to each other. The adhesion steps in both the framework and the subdomain models would lead to a condensed state, with secondary and supersecondary structures approximating those of the native state, but lacking the tight packing of atoms inside the hydrophobic core of the molecule. Such intermediates, loosely folded but already exhibiting several features of the native state, were originally predicted to exist on the basis of theoretical considerations (Ptitsyn, 1973) and were later named "molten globule" (Ohgushi & Wada, 1983). Equilibrium [see review by Kuwajima (1989)] as well as kinetic (Ptitsyn et al., 1990) studies on the renaturation of several proteins have indeed shown the existence of folding intermediates exhibiting some of the properties predicted for a molten globule. Such molten globules would then undergo local rearrangements leading to the tight packing of the atoms inside the protein core, and thus to the native state. At this point, it should be emphasized that in recent years some confusion has emerged in the literature concerning the exact nature of the molten globule. Indeed, the properties listed above as having been observed for both equilibrium and kinetic intermediates have been taken by some authors as defining the molten globule. Yet, they do not suffice to ascertain that the corresponding intermediates have, as originally proposed by Ptitsyn (1973), a conformation that already exhibits nativelike secondary and tertiary structure patterns. In the rest of this paper, and in line with the implicit conclusions of Ptitsyn (1987) and Ptitsyn et al. (1990), we shall keep the original concept of Ptitsyn which postulates that the experimentally observed molten globule should have nativelike secondary and supersecondary structures. To avoid any misunderstanding, we shall call this postulated intermediate the "specific" molten globule.

Hence, all the intermediates thus far postulated (framework, subdomain, specific molten globule) should exhibit secondary and supersecondary patterns present in the native state. In a recent report, we suggested that an intermediate with nonnative secondary and supersecondary structures might also exist. Indeed, we showed that a 101-residue polypeptide fragment (F2-V8)[1] corresponding to the C-terminal extremity of the β chain of *Escherichia coli* tryptophan synthase (Friguet et al., 1989; Kaufmann et al., 1991) folds into a stable globular condensed state which, however, contains secondary and supersecondary structures that differ considerably from those of the F2-V8 domain in the native protein (Chaffotte et al., 1991). This clearly ruled out that isolated F2-V8 might share major nativelike structural features with the native state. Yet, the large far-UV CD signal, the absence of a near-UV CD signal, the lack of effective protection of amide protons against exchange with deuterium, the absence of an induced chemical shift in the NMR spectrum, and the very low enthalpy change and lack of cooperativity of the unfolding transition exhibited by isolated F2-V8 are features commonly observed for molten globules (Kuwajima, 1989). This led us to propose that the conformation of isolated F2-V8 might result from a hydrophobic collapse of the polypeptide chain into a loosely packed, fluctuating, globule that might be a precursor of the specific molten globule on the folding pathway (Chaffotte et al., 1991). To further test this model, it seemed important to study the kinetics of folding of isolated F2-V8, and to compare them with the kinetics of some of the steps already identified in protein folding. Here, we report stopped-flow kinetic studies on the appearance of the far-UV CD signal and on the binding of ANS during the refolding of guanidine-unfolded F2-V8. The results obtained, which are compatible with the model proposed earlier (Chaffotte et al., 1991), will also be shown to offer a simple plausible explanation for the complex kinetics often observed for the transient binding of ANS during protein folding.

Materials and Methods

Chemicals. Guanidine hydrochloride, ultrapure grade, was purchased from Schwarz/Mann Biotech, and 1-anilino-8-naphthalenesulfonic acid was from Sigma. The *Staphylococcus aureus* endoproteinase Glu C (V8) was from Boehringer. All other chemicals were reagent grade.

Buffer. Unless otherwise stated, the buffer used (buffer A) was 20 mM potassium phosphate/0.4 mM EDTA (sodium salt), pH 7.8. When needed, buffer A was supplemented with 2-mercaptoethanol as indicated.

Preparation of the F2-V8 Fragment. Isolation of F2-V8 was achieved by limited proteolysis of the β_2 subunit of tryptophan synthase using 0.1% (w/w) V8 protease according to the procedure previously described (Friguet et al., 1989). The isolated F2-V8 fragment was stored at 4 °C as a suspension in 2 M ammonium sulfate.

The denaturation of F2-V8 was performed as follows: the desired volume of F2-V8/ammonium sulfate suspension was centrifuged, and the pellet was dissolved in 100 mM potassium phosphate, 2 mM EDTA, and 5 mM 2-mercaptoethanol, pH 7.8, and then dialyzed overnight at 4 °C against the same buffer. It was then dialyzed against 6 M GuHCl in buffer A supplemented with 100 mM 2-mercaptoethanol (final pH 7.8) during at least 24 h at 4 °C. The protein concentration after the dialysis was determined using the assay of Bradford (1976).

Fluorescence Spectra. The fluorescence emission spectra of free and bound ANS were recorded in an LS5B (Perkin Elmer) spectrofluorometer. The temperature of the samples was 20 °C.

Stopped-Flow Measurements. All the kinetic experiments were carried out with an SFM3 mixing device from Bio-Logic (Pont de Claix, France) equipped with two large (18 mL) syringes injecting into the first mixer and a small syringe (5 mL) injecting into the second mixer. For fluorescence measurements, the mixing device, equipped with an F15 (1.5 mm × 1.5 mm cross section) fluorescence cell, was combined with the optical bench and detection module of Bio-Logic. Two photomultipliers were used, one for the detection of the emitted light through an Oriel 450-nm long-pass filter (LG-450-F) and the second for the detection of the excitation light. The ratio of the two signals was electronically determined in the Bio-Logic dual amplifier, and recorded and analyzed by means of the Bio-Kine software package of Bio-Logic. For circular dichroism measurements, the Bio-Logic mixing device, equipped with a T50/15 (5-mm optical path) transmission cell, was coupled to the CD6 spectrodichrograph from Jobin-Yvon (Longjumeau, France), using the mechanical, optical, electronic, and software accessory kit supplied by Jobin-Yvon.

In all the experiments, refolding was initiated by a concentration jump from 6 to 0.075 M GuHCl, mixing 10 μL (from the small syringe) of denatured protein (in 6 M GuHCl)

[1] Abbreviations: ANS, 1-anilino-8-naphthalenesulfonic acid; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; F2-V8, C-terminal 101-residue fragment obtained by proteolysis of *E. coli* tryptophan synthase β_2 subunits with the V8 endoproteinase from *S. aureus*; GuHCl, guanidine hydrochloride.

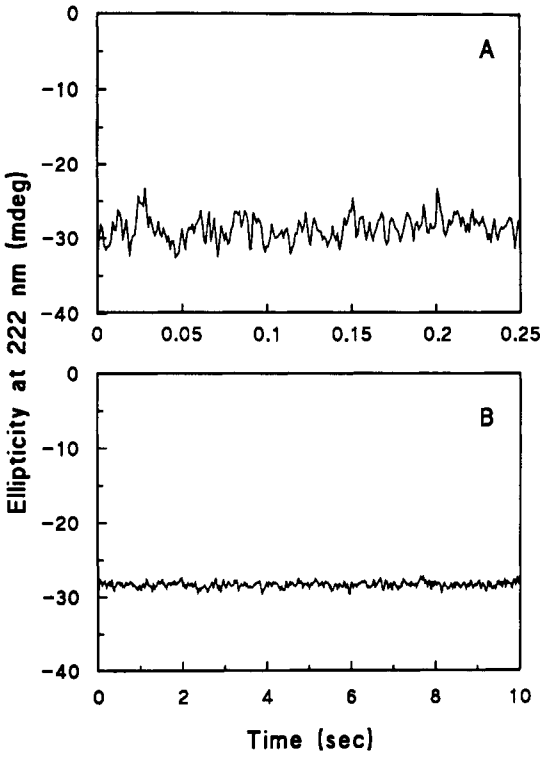

FIGURE 1: Kinetics of folding of F2-V8 at 20 °C monitored by far-UV circular dichroism. Unfolded F2-V8 (8 mg/mL in buffer A containing 6 M GuHCl and 100 mM 2-mercaptoethanol) was diluted 80-fold with buffer A supplemented with 4 mM 2-mercaptoethanol. The temperature was adjusted to 20 °C. The CD signal at 222 nm was monitored from the moment the syringes were stopped. The dead time was 4 ms. (A) Average of 40 successive kinetics, each acquired with a sampling interval of 1 ms and a filtering time constant of 1 ms. (B) Average of 40 successive kinetics, each acquired with a sampling period of 20 ms and a filtering time constant of 10 ms.

with 790 μL (395 μL from each large syringe) of buffer A, in 100 and 140 ms for CD and fluorescence measurements, respectively. The resulting flow rate (8 and 5.7 mL/s) corresponds, with both types of observation cells, to a dead time of 4 ms.

The stopped-flow temperature was controlled by means of an external thermostated water bath and a high flux pump to circulate the water between the bath and the stopped-flow apparatus. The temperature of the thermostat was adjusted so that the reservoirs and observation cell were, within ±0.1 °C, at the desired temperature.

Results

The circular dichroism spectrum of isolated F2-V8 shows no signal in the near-UV region (Chaffotte et al., 1991). This indicates that the aromatic residues of isolated F2-V8 are in a symmetrical environment and hence do not contribute to the far-UV CD spectrum of the isolated fragment. Thus, this spectrum depends exclusively on the secondary structure elements present in isolated F2-V8. The CD signal at 222 nm was therefore a reliable probe for monitoring the kinetics of appearance of the secondary structure during the folding of isolated F2-V8. Figure 1 shows the variation of the ellipticity at 222 nm as a function of time in a stopped-flow experiment in which F2-V8, unfolded in 6 M GuHCl, was diluted 80-fold in buffer A at 20 °C. Even at the shortest sampling interval used (1 ms per data point in Figure 1A), no variation of the ellipticity could be detected during the first second, and the CD signal remained constant thereafter (Figure 1B). Moreover, the value of the ellipticity obtained immediately after the dead time of the apparatus, i.e., 4 ms under the experimental conditions used, coincided with that expected from the CD spectrum of isolated F2-V8. Thus, all the secondary structure of isolated F2-V8 was formed in less than 4 ms when the folding took place at 20 °C.

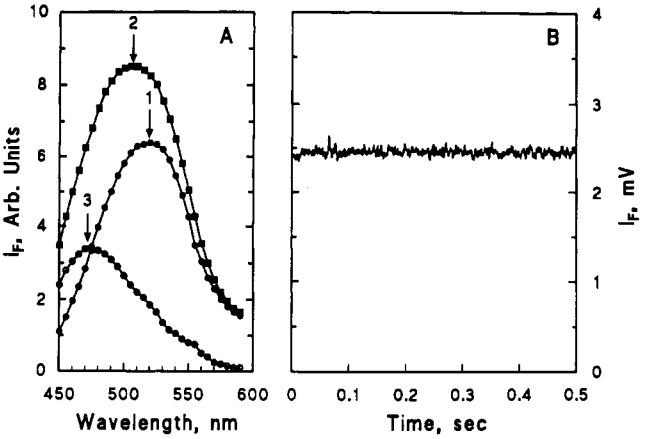

FIGURE 2: Binding of ANS to F2-V8. (A) Fluorescence emission spectrum of 0.1 mM ANS with and without F2-V8: The spectra of 0.1 mM ANS in buffer A supplemented with 75 mM GuHCl and 4 mM 2-mercaptoethanol were recorded at 20 °C, using a 2.5-nm bandwidth slit for both the excitation and the emission monochromators. The excitation wavelength was 370 nm. (1) Spectrum of ANS alone; (2) spectrum of ANS in the presence of 40 μg/mL F2-V8; (3) calculated difference spectrum between spectra 2 and 1, representing the transfer of bound ANS from buffer to its environment in F2-V8. The vertical arrows indicate the maximum of each spectrum. (B) Kinetics of ANS binding during the folding of F2-V8 at 12 °C: F2-V8 (3.2 mg/mL in buffer A containing 6 M GuHCl and 100 mM 2-mercaptoethanol) was diluted 80-fold with buffer A supplemented with 4 mM 2-mercaptoethanol and 0.1 mM ANS. The fluorescence signal was monitored, through a high-pass filter (see Materials and Methods), and recorded from the time the syringes came to a stop. The dead time was 4 ms. The sampling interval and the filtering time constant were 1 ms. The trace shown corresponds to the average of 95 successive experiments.

We then tried to determine whether this rapid formation of the secondary structure is, as observed for most proteins, followed by a slower ($t_{1/2}$ over 50 ms) step leading to a state that binds ANS maximally. From the fluorescence spectrum of isolated F2-V8 in the presence of ANS (Figure 2A), it was verified that ANS does bind to isolated F2-V8. Following the experimental approach described by Stryer (1965), we "calibrated" the wavelength of the emission maximum and the quantum yield of free ANS as a function of the composition of different ethanol/water mixtures. Results essentially identical to those reported by Stryer were obtained (data not shown). From the calibration of the maximum emission wavelength as a function of the solvent composition, and from the position of the emission maximum of ANS bound to F2-V8 (474 nm, see Figure 2A), we could estimate that the average polarity of the hydrophobic core to which ANS binds is equivalent to that of 92% ethanol in water. By comparing the intensity of the fluorescence emitted at 474 nm by the ANS bound to F2-V8 to that of a standard solution of ANS in 92% alcohol, we could estimate that about 0.02 mol of ANS was bound per mole of F2-V8 fragment.

A fluorescence stopped-flow experiment was then performed to analyze the rate of formation of the hydrophobic core during the folding at 20 °C of isolated F2-V8. The polypeptide, unfolded in 6 M GuHCl, was diluted 80-fold in buffer A containing 10^{-4} M ANS, and the fluorescence (λ_{exc} = 370 nm; λ_{em} above 450 nm) was recorded as a function of time. The maximal ANS fluorescence was obtained within the dead time of the machine, and no evolution of the fluorescence (i.e., ANS binding) could be detected at later times (data not shown).

This indicated that the hydrophobic core of isolated F2-V8 was completed in less than 4 ms and that after the formation of this core the polypeptide chain did not undergo any further rearrangement of the structure that would give rise to an increase in the fluorescence of bound ANS.

In an attempt to slow down the appearance of the hydrophobic core and of the secondary structure of isolated F2-V8 beyond the dead time of the stopped-flow, folding experiments similar to those described above were repeated at a lower temperature (12 °C). Figure 2B shows that all the binding of ANS again occurred during the 4-ms dead time. However, some changes in the far-UV CD signal could indeed be observed after the mixing. Figure 3A,B shows that the ellipticity first decreases (the ellipticity is negative; thus its amplitude increases), during the dead time, to a value below the final signal but then increases to the equlibrium value following kinetics that could be fitted to a single-exponential process with a half reaction time of about 4–6 ms.

In order to determine the amplitude of the change occurring during the dead time of the stopped flow, and to verify that no phase was missed either before or after the observation period, the following control experiment was performed: renaturation buffer, 6 M GuHCl, F2-V8 in 6 M GuHCl diluted 80-fold with 6 M GuHCl, and finally F2-V8 in 6 M GuHCl diluted 80-fold with renaturation buffer were successively injected in the stoppped flow. After each injection, the CD at 222 nm was recorded for at least 1 s (Figure 3C). The difference between the ellipticity measured for regions 3 (F2-V8 in GuHCl) and 2 (GuHCl) represents the intrinsic ellipticity of unfolded F2-V8. When this (negative) value was added to the CD signal of the buffer (region 1), one obtained the ellipticity of unfolded F2-V8 in the renaturation buffer, i.e., the initial ellipticity at the very beginning of the folding process. This value is marked by the horizontal arrow in Figure 3, at the time when the folding was initiated after the last injection. The difference between this initial value and the plateau observed after about 25 ms in Figure 3A,B coincides with the difference between the ellipticities of unfolded and folded F2-V8 observed at equilibrium. This ascertains that all the CD change was indeed observed during the kinetic experiments reported above and that no slow folding step was overlooked. Thus, it can be concluded that even at 12 °C the hydrophobic core and a major part of the secondary structure of isolated F2-V8 were formed in less than 4 ms, with only small further rearrangements of this initial secondary structure occurring with a half reaction time of about 5 ms.

Discussion

The main finding reported in this paper is that the condensed, globular, stable state with nonnative secondary and supersecondary structures previously described for isolated F2-V8 (Chaffotte et al., 1991) is reached at 20 °C in less than 4 ms after initiation of folding. This conclusion is based on the observation of two signals, the far-UV CD and the binding of the hydrophobic fluorescent probe ANS. Both criteria deserve some comments.

There is clear-cut evidence that the asymmetrical environment of aromatic side chains and of cysteine residues may influence the far-UV CD signal of proteins (Sears & Beychok, 1979; Manning & Woody, 1989). Isolated F2-V8 exhibits no circular dichroism in the near-UV, indicating that its side chains are in a symmetrical environment and hence cannot contribute to the far-UV CD signal. Therefore, the far-UV CD spectrum of F2-V8 reflects essentially the secondary structure of the polypeptide chain. The CD signal at a single wavelength surely does not suffice to characterize in detail the

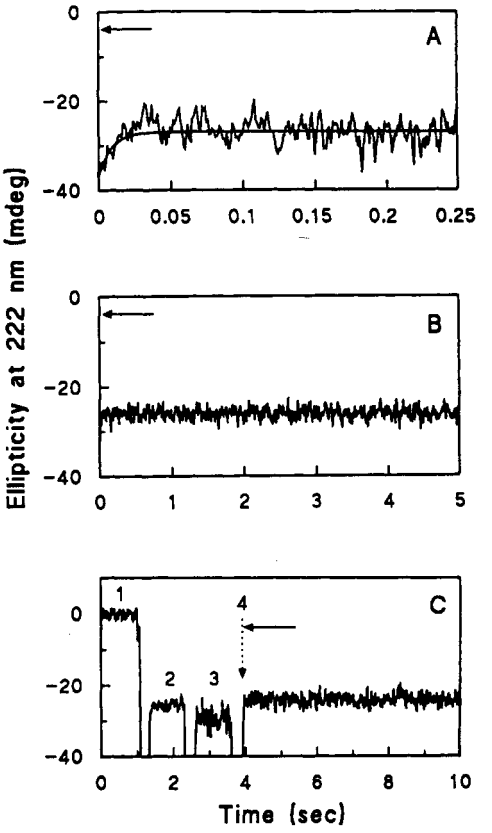

FIGURE 3: Kinetics of folding of F2-V8 at 12 °C monitored by far-UV circular dichroism. Except for the temperature, which was 12 °C, these experiments were performed as described in the legend to Figure 1. (A) Averaged tracing of 40 succesive kinetics acquired with a sampling interval and a filtering constant of 1 ms. (B) Averaged tracing of 40 successive kinetics acquired with a sampling interval and a filtering constant of 5 ms. (C) Determination of the CD signal of unfolded F2-V8 in renaturation buffer: Syringe 1 contained buffer A supplemented with 4 mM 2-mercaptoethanol. Syringe 2 contained 6 M GuHCl and 4 mM 2-mercaptoethanol in buffer A. Syringe 3 contained F2-V8 (8 mg/mL) in buffer A supplemented with 6 M GuHCl and 100 mM 2-mercaptoethanol. The following injection sequence was used: 800 μL from syringe 1 was first injected in 300 ms, and the recording was triggered. After 1 s, during which the CD of buffer A was recorded (region 1), 800 μL from syringe 2 was injected over 300 ms, and the CD of 6 M GuHCl was recorded during 1 s (region 2). Next, 790 μL from syringe 2 and 10 μL from syringe 3 were injected over 300 ms, and the CD of unfolded F2-V8 in 6 M GuHCl was recorded for 1 s (region 3). Finally, 790 μL from syringe 1 and 10 μL from syringe 3 were injected over 300 ms, and the CD signal of refolding F2-V8 (region 4) was observed. The figure shows the average of 10 successive kinetics, each acquired with a sampling interval and a filtering constant of 10 ms. The large difference between the signals observed for 6 M GuHCl (region 2) and for buffer A (region 1) originates from the fact that the light beam across the observation cell is not parellel. Therefore, the illuminated portion of the surface of the photomultiplier depends on the refractive index of the solution in the cell, and because of the inhomogeneity of the photomultiplier surface, it is not possible to simultaneously adjust the CD base line at zero for different refractive indexes. This results in the observed shift in the base line when the refractive index of the solvent is changed. This shift, however, does not affect later quantitative measurements of CD changes as long as the solvent is not changed again. The tracings in regions 2 and 3 were fitted to horizontal straight lines; the difference between the values obtained for these two regions corresponds to the CD of unfolded F2-V8. When one subtracts this difference from the CD of buffer A, one obtains an estimate of the CD signal of unfolded F2-V8 in buffer A, i.e., the starting value of the folding kinetics. This value is indicated by the horizontal arrows in panels A–C. The vertical arrow in panel C corresponds to the initiation of the folding process. The negative "spikes" during the injection phases correspond to optical artifacts presumably due to flow birefringence when 6 M GuHCl solutions, which have a high refractive index and viscosity, flow through the observation cell.

secondary structure. Yet, the ellipticity at 222 nm has often been used as a unique criterion for monitoring the kinetics of changes in secondary structure [see the many references cited in Kuwajima (1989)]. Indeed, because of the sensitivity of the signal at 222 nm to the contents in β structures and, mainly, in α helices, even relatively small changes in secondary structure can be detected. Thus, the lack of detectable CD change after the dead time in the stopped-flow experiments reported above appears as strong evidence that the secondary structure of isolated F2-V8 does not undergo significant changes at later stages, and hence is indeed already formed within the 4-ms dead time of the stopped-flow machine. That this secondary structure does not coincide with the one existing in the F2 region of native tryptophan synthase has been established previously (Chaffotte et al., 1991).

The fluorescence spectrum of isolated F2-V8 in the presence of ANS shows that this fragment can bind this hydrophobic probe. It allows us to characterize the average polarity around bound ANS as equivalent to that of a 92% solution of ethanol in water. One could propose that ANS binding takes place directly onto hydrophobic patches formed by superficial β structures of F2-V8, as reported by Semisotnov et al. (1991) for polylysine. However, the wavelength of the fluorescence emission maximum of ANS bound to F2-V8 was 474 nm, a value very close to that (about 470 nm) reported for ANS bound to the equilibrium molten-globule forms of carbonic anhydrase, α-lactalbumin and β-lactamase, and quite distinct from that (about 500 nm) of ANS bound to polylysine (Semisotnov et al., 1991). This strongly suggests that isolated F2-V8 contains a loosely packed hydrophobic core to which ANS can bind. However, the fluorescence intensity of bound ANS corresponds to an extremely small average number of ANS molecules bound per F2-V8 fragment (only 2%). This value is much smaller than that observed (under similar conditions, i.e., 10^{-4} M ANS) for the molten globules of bovine carbonic anhydrase (about 1 bound ANS per protein) and of *S. aureus* β-lactamase (about 0.5 bound ANS per protein molecule in addition to the 1 ANS already bound to the native enzyme). To account for this small value, one could assume that the binding constant of isolated F2-V8 for ANS is significantly smaller than that of about 10^4 M^{-1} reported for other molten globules (Semisotnov et al., 1991). This does not appear very likely in view of the relatively low polarity of the "binding site" for ANS, as revealed from the fluorescence emission spectrum of bound ANS (see above). This low polarity should result in a partition of ANS between water and the protein comparable to that reported for molten globules (Semisotnov et al., 1991). Alternatively, one could assume that only a small fraction of the F2-V8 molecules is, at a given time, in a conformation that can bind the hydrophobic probe efficiently. The latter hypothesis is compatible with our previous report (Chaffotte et al., 1991) indicating that F2-V8 rapidly oscillates between several conformers.

The formation both of the loose hydrophobic core (detected by the binding of ANS) and of the secondary structure (detected by the CD at 222 nm) of isolated F2-V8 is completed in less than 4 ms at 20 °C, and nearly completed in the same time even at 12 °C. The high rate of appearance of these structural features is compatible with our previous proposal that the loosely packed, non-native-like, globular state of isolated F2-V8 may represent an early folding intermediate that would precede the specific molten globule (Chaffotte et al., 1991). This conclusion is in line with Dill's model according to which the first stage of protein folding would be a hydrophobic effect-driven collapse of the polypeptide chain resulting in the nonspecific formation of local secondary structure elements (Dill, 1985). Indeed, the isolated F2-V8 fragment turns out to be an appropriate model system for testing Dill's proposal because it cannot form the native long-range interactions responsible for the stabilization of the native supersecondary and tertiary structures and hence fails to reach the specific molten globular state (Chaffotte et al., 1991). This renders it possible to observe the nonspecific collapse in the absence of specific long-range interactions. That the collapsed, loosely packed globular conformation of isolated F2-V8 appears much more rapidly than the specific molten globules (Ptitsyn et al., 1990) or intermediates with nativelike secondary structure elements (Udgaoncar & Baldwin, 1988; Roder et al., 1988; Bycroft et al., 1990) thus far characterized lends solid experimental support to Dill's model. We emphasize that this conclusion could be reached because of the improved time resolution of the CD stopped flow used in this study. Indeed, the upper limit for the time of formation of the initial burst of observable secondary structure elements has been reduced from 18 ms (Kuwajima et al., 1991) to 4 ms. This permitted us to clearly resolve the nonspecific collapse studied here ($t_{1/2}$ below 4 ms) from the formation of the first observable intermediates with stable nativelike secondary structure elements, which have been shown for several proteins to appear with half reaction times of the order of 20 ms (Roder et al., 1988; Udgaoncar & Baldwin, 1988; Bycroft et al., 1990).

The last point we wish to discuss deals with the usual interpretation of the kinetics of ANS binding during protein folding. Maximal binding of ANS to F2-V8 is achieved within less than 4 ms after initiation of the folding process. Previous stopped-flow studies on ANS binding during the folding of a variety of proteins had also shown a rapid phase that occurred during the dead time (Ptitsyn et al., 1990), but its importance was rarely discussed. Rather, the emphasis was put on slower phases of ANS binding, which were taken as corresponding to the formation of the molten globular state (Ptitsyn et al., 1990). By analogy with what we observed for F2-V8, we propose that the initial rapid phase of ANS binding corresponds to a nonspecific hydrophobic collapse of the polypeptide chain into a condensed, loosely packed, non-native-like globular state, with overall features similar to those of isolated F2-V8. From this state, the folding would then proceed toward the specific molten globule, along with an increased amount of bound ANS. Thus, the slower phases leading to maximal ANS binding ($t_{1/2}$ about 50–200 ms) would indeed correspond, as commonly accepted, to the formation of the specific molten globule.

Registry No. Tryptophan synthase, 9014-52-2.

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Antimicrobial Peptides from *Amaranthus caudatus* Seeds with Sequence Homology to the Cysteine/Glycine-Rich Domain of Chitin-Binding Proteins†

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ABSTRACT: Two antimicrobial peptides (*Ac*-AMP1 and *Ac*-AMP2) were isolated from seeds of amaranth (*Amaranthus caudatus*), and their physicochemical and biological properties were characterized. On the basis of fast atom bombardment mass spectroscopy, *Ac*-AMP1 and *Ac*-AMP2 have monoisotopic molecular masses of 3025 and 3181, respectively. Both proteins have p*I* values above 10. The amino acid sequence of *Ac*-AMP1 (29 residues) is identical to that of *Ac*-AMP2 (30 residues), except that the latter has 1 additional residue at the carboxyl terminus. The sequences are highly homologous to the cysteine/glycine-rich domain occurring in many chitin-binding proteins. Both *Ac*-AMP1 and *Ac*-AMP2 bind to chitin in a reversible way. *Ac*-AMP1 and *Ac*-AMP2 inhibit the growth of different plant pathogenic fungi at much lower doses than other known antifungal chitin-binding proteins. In addition, they show some activity on Gram-positive bacteria. The antimicrobial effect of *Ac*-AMP1 and *Ac*-AMP2 is strongly antagonized by cations.

Chitin [poly(β-1,4-*N*-acetyl-D-glucosamine)] is a polysaccharide occurring in the cell wall of fungi and in the exoskeleton of invertebrates. Although plants have not been reported to contain chitin or chitin-like structures, proteins exhibiting strong affinity to this polysaccharide have been isolated from different plant sources [for a review, see Raikhel and Broekaert (1992)]. Examples of such chitin-binding proteins are basic chitinases from bean (Boller et al., 1983), wheat (Molano et al., 1979), and tobacco (Shinshi et al., 1987), chitin-binding lectins from wheat (Rice & Etzler, 1974), barley (Peumans et al., 1982), rice (Tsuda, 1979), and stinging nettle (Peumans et al., 1983), and a small protein from rubber tree latex, called hevein (Van Parijs et al., 1991). All these chitin-binding proteins share a homologous cysteine/glycine-rich domain of about 40-43 amino acids, which is repeated either 2-fold (in the nettle lectin) or 4-fold (in wheat, barley, and rice lectins) or fused to an unrelated domain (in basic chitinases).

Although the exact physiological role of these proteins remains uncertain, they all have been shown in in vitro experiments to exert antibiotic activities, suggesting a defense-related function. Indeed, antifungal properties have been ascribed to chitinases (Schlumbaum et al., 1986; Broekaert et al., 1988), nettle lectin (Broekaert et al., 1989), and hevein (Van Parijs et al., 1991). On the other hand, the wheat lectin causes deleterious effects on the development of insect larvae (Murdock et al., 1990; Czapla & Lang, 1990).

In the present paper, we describe the isolation and characterization of two antimicrobial peptides from the seeds of amaranth (*Amaranthus caudatus*) that are homologous to the cysteine/glycine-rich domain of chitin-binding proteins. The amaranth peptides inhibit the growth of fungi to a much higher

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