The Isolated C-Terminal (F2) Fragment of the *Escherichia coli* Tryptophan Synthase β_2-Subunit Folds into a Stable, Organized Nonnative Conformation†

Alain Chaffotte,‡ Yvonne Guillou,‡ Muriel Delepierre,§ Hans-Jurgen Hinz,∥ and Michel E. Goldberg*,‡

Unité de Biochimie Cellulaire and Laboratoire de Résonance Magnétique Nucléaire, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France, and Institüt für Biophysik und Physikalische Biochemie, Universitätsstrasse 31, D-8400 Regensburg, Germany

Received March 6, 1991; Revised Manuscript Received May 22, 1991

ABSTRACT: Proteolysis of the β_2-subunit of *Escherichia coli* tryptophan synthase by the endoproteinase Glu C from *Staphylococcus aureus* V8 yields a peptide, F2, corresponding to the C-terminal 101 residues of the β-chain. The conformation and stability of isolated F2 in phosphate buffer at pH 7.8 (where native β_2 is stable) have been investigated. Circular dichroism spectra in the far-UV showed the presence of large amounts of secondary structure (19% α-helices, 34% extended β-structures). Circular dichroism spectra in the near-UV and sedimentation velocity studies indicated an open globular structure with the aromatic side chains in a symmetric (or disordered) environment. NMR spectra and rates of amide proton exchange showed that F2 fluctuates rapidly between several conformations. The thermal denaturation of F2 observed by the loss of far-UV circular dichroism with increasing temperature appeared noncooperative, and indicates a high thermal stability (T_m = 70 °C). Differential scanning microcalorimetry confirmed the absence of cooperativity and indicated a very low value for the calorimetric enthalpy of denaturation (ΔH = 17 kJ/mol). All these properties were compatible with a molten globule. However, the low sedimentation coefficient of F2 suggested a very hydrated and/or expanded structure, and the secondary structure content of isolated F2 (see above) differed widely from that reported in the literature for F2 within the context of native β_2 (49% α-helices and 13% extended β-structures). Thus, neither the secondary nor the tertiary structure of isolated F2 resembled those of native F2. In this respect, isolated F2 is not a "molten globule". It is proposed that the conformation of isolated F2 corresponds to a loosely packed, fluctuating, globule that may represent a precursor to the molten globule on the folding pathway of the C-terminal region of the β chains.

The folding of a polypeptide chain into its native conformation has long been considered as an "all or none" process, because of the high cooperativity of the denaturation–renaturation transition. Yet, in recent years, more and more evidence has been accumulated that suggests the existence of intermediates on the refolding pathway. One such intermediate, the "molten globule", is currently the subject of much attention. As early as 1973 (Ptitsyn, 1973), it was predicted that an intermediate should form rapidly in the folding of the polypeptide chain which should have the following properties: it would share with the native conformation a condensed globular structure with a hydrophobic core, a nearly native secondary structure, and an approximately correct (as compared to the native state) relative arrangement of the elements of secondary structure. It would, however, differ from the native state in that the hydrophobic inside of the molten globule would not be tightly packed. Equilibrium studies on the unfolding of several proteins at intermediate concentrations of denaturing agent have revealed the presence of states that are neither native nor fully unfolded and that exhibit many of the properties predicted for a molten globule [for a review, see Kuwajima (1989)]. For instance, their Stokes radii are only slightly larger than those of the native states, indicating globular structures; they have hydrophobic, yet not tightly packed, internal cores as shown by their ability to bind the bulky hydrophobic fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS);[1] their far-UV circular dichroism spectra are similar to those of the native proteins, indicating that the secondary structures are indeed nativelike; they exhibit no near-UV circular dichroism signal, and low tryptophan fluorescence polarization, indicating that the aromatic residues are highly mobile. Futhermore, transient intermediates with some of the properties reported above have also been observed during the folding of several proteins [reviewed by Kuwajima (1989)]. These observations lead to the conclusion that a molten globule state may be a general intermediate in protein folding (Ptitsyn et al., 1990). However, because of the cooperativity and rapidity of the folding process, studying the structural and thermodynamic properties of folding intermediates is a difficult task. This has up to now precluded a clear-cut identification of the molten globule [as originally defined by Ptitsyn (1973)] as an intermediate under conditions where the protein folds rapidly and is normally stable.

One approach has turned out to be quite fruitful for studying folding intermediates and might be used for experimental investigations of the properties of the molten globule. It consists of isolating protein fragments that would be able to undergo spontaneous folding independently of the rest of the polypeptide chain, thus providing insight into local events that

† This research was supported by funds from the Institut Pasteur, the Centre National de la Recherche Scientifique (URA D1129), the Université Paris 7, the Ministère de la Recherche et de la Technologie (Aide 87 C 0396), and the Fondation pour la Recherche Médicale. M.E.G. benefited from a von Humboldt award which permitted this collaboration.

* To whom correspondence should be addressed.
‡ Unité de Biochimie Cellulaire, Institut Pasteur.
§ Laboratoire de Résonance Magnétique Nucléaire, Institut Pasteur.
∥ Institüt für Biophysik und Physikalische Biochemie.

[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; NMR, nuclear magnetic resonance; pyridoxal-5′-P, pyridoxal 5′-phosphate; SDS, sodium dodecyl sulfate; UV CD, ultraviolet circular dichroism.

0006-2960/91/0430-8067$02.50/0

may be important for starting the folding of the complete polypeptide chain. Examples where this approach provided particularly original and valuable information are the early immunochemical studies of Anfinsen and co-workers on staphylococcal nuclease fragments (Sachs et al., 1972; Furie et al., 1975), the extensive search for, and attempts to characterize, "self-folding" fragments of thermolysin by Fontana and co-workers [reviewed by Fontana (1990)], and the elegant and detailed studies by Baldwin and co-workers on the conformation and stability of the C-peptide of bovine ribonuclease A (Bierzynski et al., 1982; Brems & Baldwin, 1984; Shoemaker et al., 1987; Osterhout et al., 1989). These and other studies suggested or confirmed that some isolated peptides are able to spontaneously adopt the secondary structure which they exhibit in the native protein.

At a higher level of structure, large protein fragments turned out to be of some use in showing that, as predicted by Wetlaufer (1973), distinct "domains" of a polypeptide chain can behave as independent folding units. Thus, as early as 1970, large N- and C-terminal fragments from *Escherichia coli* β-D-galactosidase were shown to behave as "self-folding" globular proteins that share specific antigenic sites with native galactosidase (Goldberg, 1969). A much more detailed study along the same line has been performed with two complementary fragments obtained by mild proteolysis of the *E. coli* tryptophan synthase β_2-subunit (Högberg-Raibaud & Goldberg, 1977a; Goldberg & Högberg-Raibaud, 1979; Zakin et al., 1980; Zetina & Goldberg, 1982).

The β_2-subunit is a dimer of identical polypeptide chains of molecular weight 44 000 each. It binds two molecules of pyridoxal-5′-P as a coenzyme. Mild proteolytic treatment results in "nicking" the polypeptide chain in a particularly protease-sensitive region between residues 270 and 296 (Högberg-Raibaud & Goldberg, 1977b; Crawford et al., 1978; Ahmed et al., 1986; Friguet et al., 1989). The nicked protein closely resembles native β_2 (Högberg-Raibaud & Goldberg, 1977b; Decastel & Goldberg, 1978); it is, however, a heterotetramer made of two F1 fragments (N-terminal, starting at residue 1) and two F2 fragments (ending at residue 397, the C-terminal residue of intact β-chains). The precise molecular weights of F1 and F2 depend on the enzyme used to generate the nick (Högberg-Raibaud & Goldberg, 1977b; Friguet et al., 1989), but do not differ much from 29–33K for F1 and 11–13K for F2. The F1 and F2 fragments can be isolated after various denaturing treatments (Högberg-Raibaud & Goldberg, 1977a) and were shown to refold independently of each other into globular structures sharing several features with their counterpart in the native protein (Högberg-Raibaud & Goldberg, 1977a; Goldberg & Högberg-Raibaud, 1979; Friguet et al., 1986). Furthermore, the refolded F1 and F2 fragments, when mixed in stoichiometric amounts, spontaneously reassemble into the heterotetrametric nicked β_2-protein (Högberg-Raibaud & Goldberg, 1977a). The conformation of isolated refolded F1 has been characterized quite extensively, including hydrodynamic properties, far-UV circular dichroism, fluorescence (Högberg-Raibaud & Goldberg, 1977a), reactivity of SH groups (Goldberg & Högberg-Raibaud, 1979), and immunoreactivity to conformation-specific monoclonal antibodies (Friguet et al., 1986). For lack of suitable probes, the conformation of isolated F2 has not been investigated in any detail. All that could be shown was that it is a fairly compact globular protein, that it exhibits a large far-UV CD signal (Högberg-Raibaud & Goldberg, 1977a), and that it reacts with one monoclonal antibody (mAb 93) that also recognizes native β_2 (Friguet et al., 1986). However, the extent to which this mAb is specific of the native conformation is not known.

In October 1988, while staying at the Institut Pasteur, Pr. Oleg Ptitsyn looked in detail into the 3D structure of tryptophan synthetase that had just been solved by Hyde et al. (1988). He noticed that in the region of the molecule corresponding to the F2 fragment, the side chains interact primarily with side chains of F1 and very little with other side chains of F2. He therefore predicted that isolated F2 should not be able to reach a tightly packed state and hence should behave, under "native" conditions (neutral pH, low temperature, no denaturing agent), as a molten globule. The study described here was aimed at testing this prediction.

The results we report show that the CD spectrum (in the far- and near-UV), the NMR spectrum, and the proton exchange properties of F2 (297–397) under native conditions indeed are characteristic of the molten globule. Futhermore, far-UV CD and microcalorimetric analyses of the reversible unfolding transition show that this fragment is fairly stable to thermal denaturation but that its unfolding transition is noncooperative. However, the amounts of α-helices and extended β-stretches in F2 differ considerably from those that exist in native F2 when associated to F1 within β_2. These properties are discussed in comparison with current views on the nature and role of the molten globule as a folding intermediate.

Materials and Methods

All the experiments employed 20 mM potassium phosphate/0.4 mM EDTA, pH 7.8, which will be designated "standard buffer".

Isolation of the F2 Fragment. The F2 fragment was obtained by proteolysis of native apo-β_2 using endoproteinase Glu C from *Staphylococcus aureus* V8 (Boehringer) following the general procedure of Friguet et al. (1989), with minor modifications including a scaling-up which allowed us to obtain larger amounts of the pure fragment. After proteolysis of 300 mg of apo-β_2 according to the conditions of Friguet et al. (1989), the protease was removed by FPLC (Pharmacia) chromatography with a preparative MonoQ column (1.6 × 10 cm). The cleaved protein was then heated at 80 °C for 6 min and the aggregated F1 removed by centrifugation (30 min at 12000*g*) after cooling. The free F2 remaining in the supernatant was finally precipitated with 2 M ammonium sulfate in 0.1 M potassium phosphate/2 mM EDTA, pH 7.8, and kept as a suspension (10–12 mg/mL) at 4 °C. No contaminants were detectable by SDS electrophoresis in a 20% homogeneous Phast gel (Pharmacia).

Before each experiment, F2 was collected from the ammonium sulfate suspension by centrifugation; the pellet was dissolved in the standard buffer and dialyzed extensively against the same buffer. Insoluble material was removed by centrifugation. The lack of aggregated material was confirmed by measuring the turbidity above 320 nm.

The concentration of F2 was calculated from the absorbance at 280 nm using 0.5 $mg^{-1} \cdot mL \cdot cm^{-1}$ as a specific extinction coefficient. This value was estimated based upon that previously published for the F2 obtained after proteolysis of holo-β_2 with trypsin (Högberg-Raibaud & Goldberg, 1977a) by subtracting the contribution of one phenylalanyl residue and one tyrosyl residue and correcting for the difference in molecular weight between the two fragments.

Analytical Centrifugation. Centrifugation was done in a Centriscan 75 (M.S.E. England) analytical ultracentrifuge, using the ultraviolet absorption scanning system at 280 nm. Single-sector cells with 20-mm optical path were used. The

centrifugation speed was 55 000 rpm, and the temperature was 20 °C. The time interval between successive scans was 30 min. The samples of F2 fragment were heated for 15 min at 80 °C and cooled just before the centrifugation. This heat step produces a preparation of F2 suitable for reversible denaturation–renaturation studies (see Results). Because the contribution of 20 mM potassium phosphate to the density and viscosity of the solvent is negligible, the standard ($s_{20,w}$) and the observed (s_{obs}) sedimentation coefficients were taken as equal.

Circular Dichroism Measurements. The circular dichroism spectra were acquired with a Jobin-Yvon CD 6 dichrograph equipped with a thermostated cell holder. Each spectrum was the result of averaging at least three successive spectra.

When the temperature dependence of the dichroism was studied, the same protein sample in a hermetically sealed cell was equilibrated at the different temperatures for at least 45 min before the spectrum was acquired.

Deconvolution of far-UV CD spectra in terms of secondary structure was based on the method of Chang and colleagues (Chang et al., 1978; Yang et al., 1986).

NMR Experiments. NMR spectra were recorded and processed on a Bruker AC performance 300-MHz spectrometer equipped with an Aspect 3000 computer, a digital-phase shifter, and a QNP (^1H, ^{31}P, ^{15}N, and ^{13}C) probe. The temperature of the probe was regulated with a Brucker BVT 1000 temperature-control unit. The spectrum at 500 MHz was recorded at room temperature on a Varian Unity 500-MHz spectrometer.

The F2 fragment in ammonium sulfate suspension was dissolved and prepared as described above, but the standard buffer was either in H_2O or in ^2H_2O. Sample concentrations ranged from 1.2 to 0.012 mM, and the number of accumulated scans varied from 1000 to 22 000, respectively.

For the hydrogen exchange study, the protein was initially dissolved in the H_2O standard buffer at a concentration of 1.2 mM, and aliquots of this solution were diluted into the ^2H_2O standard buffer to concentrations of 0.12 and 0.012 mM.

The phase-sensitive two-dimensional correlated spectra (COSY) for the protein dissolved in the ^2H_2O buffer were recorded with presaturation of the solvent in order to suppress the water signal.

Microcalorimetry. Variation of the apparent specific heat capacity of the protein solutions with temperature was determined by using an adiabatic scanning microcalorimeter (DASM1). Due to the low magnitude of the transition enthalpies, protein concentrations ranging from 2 to 5 mg/mL were used. Heating rates of 1 and 2 K min^{-1} were employed to obtain maximal excess heat capacities. Neither the concentration nor the heating rate resulted in any significant change in the transition parameters. Data acquisition was computerized, and temperature and corresponding C_p values were measured every 0.1 °C. Each sample measurement was preceded by a base-line scan with buffer-filled cells. Electrical calibration was performed at 50 μW for 5 min. Integration of the transition peaks was done numerically. Molar thermodynamic quantities have been evaluated on the basis of M = 12 000 g/mL. van't Hoff enthalpies were determined from the calorimetric transition curves in the standard manner (Moses & Hinz, 1983).

Results

Hydrodynamic Properties of Isolated F2. In order to characterize its hydrodynamic volume, F2 was submitted to analytical ultracentrifugation, and its sedimentation coefficient was measured. The recording of the scans and the analysis for a typical run are shown in Figure 1. Gel filtration experiments (data not shown) had suggested that F2 undergoes an association–dissociation equilibrium. The sedimentation coefficient was therefore determined as a function of the protein concentration. Figure 2 shows that the apparent sedimentation coefficient increases with the F2 concentration. This confirms that F2 has a tendency to self-associate, presumably into dimers, in the concentration range investigated. Extrapolation of the apparent sedimentation coefficient to zero protein concentration yields a value of about 1.35 ± 0.1 S for $s_{20,w}$. This value is significantly smaller than that reported previously (1.8 S) for the F2 fragment obtained by proteolysis of β_2 with trypsin (Zetina & Goldberg, 1982), which is 21 residues longer than the F2 fragment studied here.

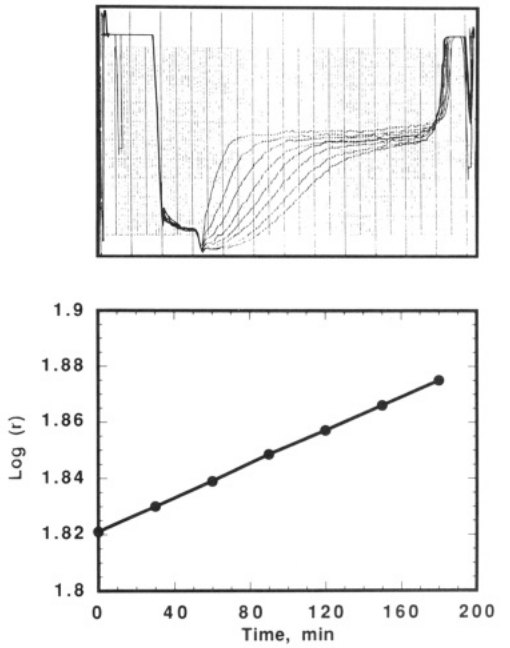

FIGURE 1: Determination of the sedimentation coefficient of F2. A sample of F2 (0.6 mg/mL in standard buffer) was centrifuged at 55 000 rpm and 20 °C in a 2-cm optical path single-sector cell. Scans of the absorbance at 280 nm were recorded at 30-min intervals. (Top) Direct recordings of absorbance vs distance to the axis of rotation. (Bottom) Natural logarithm of the distance from the axis of rotation to the midpoint of the boundary vs centrifugation time.

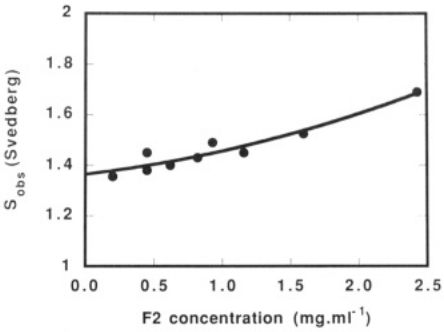

FIGURE 2: Concentration dependence of the sedimentation coefficient of F2. Samples of F2, at the concentrations indicated on the abscissa, were submitted to an analytical ultracentrifugation under the conditions described under Materials and Methods. For each sample, the sedimentation coefficient was determined and is plotted as a function of the F2 concentration.

Far- and Near-UV Circular Dichroism of the Isolated F2 Fragment. In order to compare the secondary structure of the isolated F2 fragment to that of the corresponding region in the native β-chain obtained from the crystallographic data (Hyde et al., 1988), the far-UV circular dichroism spectrum of the isolated fragment was recorded and analyzed (Chang

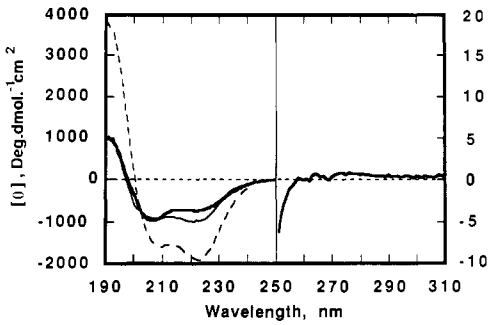

FIGURE 3: CD spectrum of the isolated F2. (Left) Far-UV region. The spectrum was recorded at 25 °C in a 0.5-mm path-length sample cell. The concentration of F2 was 0.44 mg/mL in standard buffer. The solvent contribution was measured with the buffer in the same conditions and subtracted from the sample spectrum. (Thick line) Recorded spectrum; (thin line) theoretical spectrum recomposed from the result of the quantitative analysis using the method of Chang (Chang et al., 1978; Yang et al., 1986); (dashed line) theoretical spectrum recomposed from the secondary structure content of the F2 region in native $\alpha_2\beta_2$ deduced from crystallographic data. (Right) Near-UV region. The spectrum was recorded at 25 °C in a 1-cm path-length sample cell. The concentration of F2 was 1.45 mg/mL in the standard buffer.

et al., 1978; Yang et al., 1986). Figure 3 shows the spectrum of F2 between 190 and 260 nm, after subtracting the contribution of the buffer. The general shape of this spectrum reveals a relatively high proportion of secondary structure. The shoulder at 195 nm and the ratio of the amplitudes at the two minima (207 and 222 nm) indicate a rather large proportion of extended β-structures. The quantitative decomposition shows that the isolated fragment has a secondary structure content (19% α-helix and 34% β-structure) which is quite different from that of the corresponding region in the total native protein (49% α-helix and 13% β-structure). These observations demonstrate that the average conformation of the isolated F2 domain is significantly different from the structure of F2 when it is complexed with F1.

The CD spectrum of F2 in the near-UV region is presented in Figure 3. This spectrum, which is quite flat between 250 and 300 nm, clearly indicates that the aromatic residues in this fragment (three phenylalanines and three tyrosines) do not exist in a well-defined structural state.

NMR Properties of Isolated F2. The F2 fragment contains five histidines, three phenylalanines, and three tyrosines. As shown in the ^{1}H NMR spectrum of F2 (Figure 4a), there is no significant dispersion in chemical shifts, indicating the absence of a tightly packed hydrophobic core and of strong side-chain interactions. Futhermore, the chemical shift values obtained from the COSY experiment (data not shown) for each residue type are very similar to the values predicted for a random-coil structure (Bundi & Wüthrich, 1979). However, a significant line broadening was observed, which was not expected for a protein of such a small size. In a protein spectrum, the broadening of resonances can result from at least three distinct contributions: molecular associations, restricted rotational motion, or chemical exchange phenomena. The line widths were found to be independent of concentration over the range 1.2–0.012 mM, ruling out molecular association. Comparative measurements of the overall tumbling correlation time for the isolated F2 fragment and for the native protein can give information on the contribution of restricted rotational motion to line broadening. Unfortunately, the large difference in the size of the F2 fragment as compared to β_2 renders this analysis useless. Finally, a broadening resulting from chemical exchange processes can be evidenced from line-width variation with temperature but also with the spectrometer frequency,

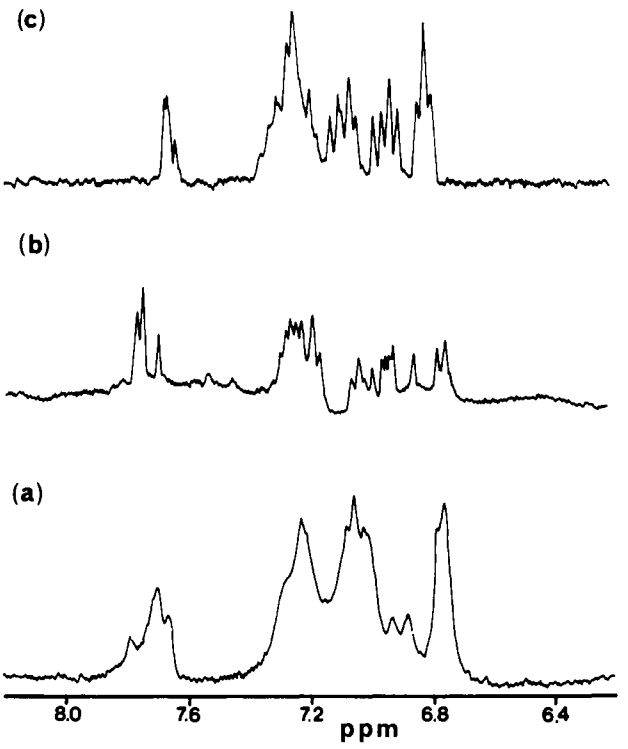

FIGURE 4: Aromatic region of the ^{1}H NMR spectra of 2.2 mg/mL F2 fragment in deuterated standard buffer, pH 7.8, at 300 MHz (a) at 17 °C (b) in the presence of 8 M urea at 17 °C (c) at 71 °C.

as a field-dependent broadening is typical of intermediate exchange processes. For isolated F2, increasing the temperature to 71 °C induced only a small change in chemical shifts (≈0.1 ppm), but all resonances became sharper (Figure 4c), indicating an increased mobility of the polypeptide chain. Reducing the temperature to 17 °C resulted in a spectrum similar to the spectrum obtained before heating (data not shown). Thus, the line-width effect was fully reversible. In addition, in the presence of 8 M urea at 17 °C, all resonances sharpened up to the same extent as at high temperature (Figure 4b). However, the proton spectrum in the presence of 8 M urea was significantly different, as far as chemical shift values are concerned, from the spectrum obtained at high temperature. Two reasons can account for these differences. First, small chemical shift changes can be due to local perturbations resulting from specific binding of urea to the protein. Second, the extended unfolding induced by urea can lead to a different state than that induced by the high temperature, particularly since the temperature, 71 °C, is not high enough to completely unfold the protein (see below). A spectrum obtained at room temperature at 500 MHz indicated, for most signals, a substantial (about 2-fold) line broadening (Figure 5). Due to the overlap of most signals, it was not possible to measure the line widths of individual peaks except for the histidine C_2 peaks. The field-dependent broadening results from exchange processes occurring at an intermediate rate on the NMR time scale. From these results, it was possible to give an upper limit of 150 s^{-1} for the exchange rate of the histidine C_2 protons.

As described above, the far-UV CD spectrum of F2 demonstrates the presence of a large amount of secondary structure. This seemed to be in contradiction with the lack of definite conformation as seen by NMR. Thus, the stability of the secondary structures was tested via hydrogen exchange studies. In the first series of experiments, an aliquot of the protein solution (1.2 mM) in standard buffer was diluted 10- or 100-fold in a deuterated standard buffer, and the spectrum

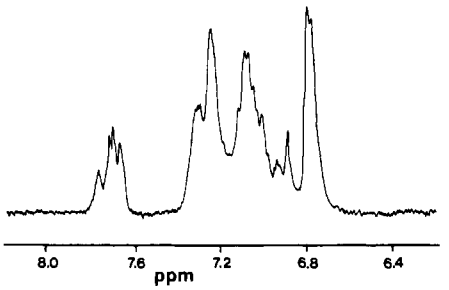

FIGURE 5: Aromatic region of the ^1H NMR spectrum of the F2 fragment (0.2 mM) in deuterated standard buffer, pH 7.8, at 500 MHz and room temperature.

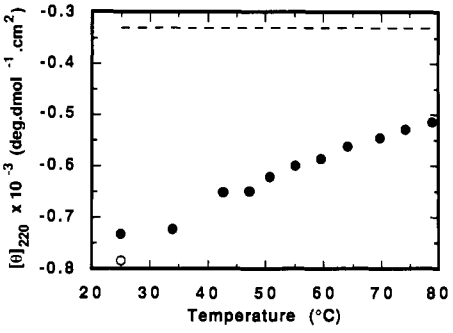

FIGURE 6: Thermal CD transition of F2. The amplitude at 220 nm was taken from the CD spectrum recorded at the corresponding temperature; the conditions of acquisition of the spectra were the same as in Figure 2. The open circle corresponds to the amplitude measured at 25 °C after 80 °C. The dashed line corresponds to the dichroism amplitude at 220 nm of F2 at the same concentration, in 4 M guanidinium chloride in the same buffer, at 25 °C.

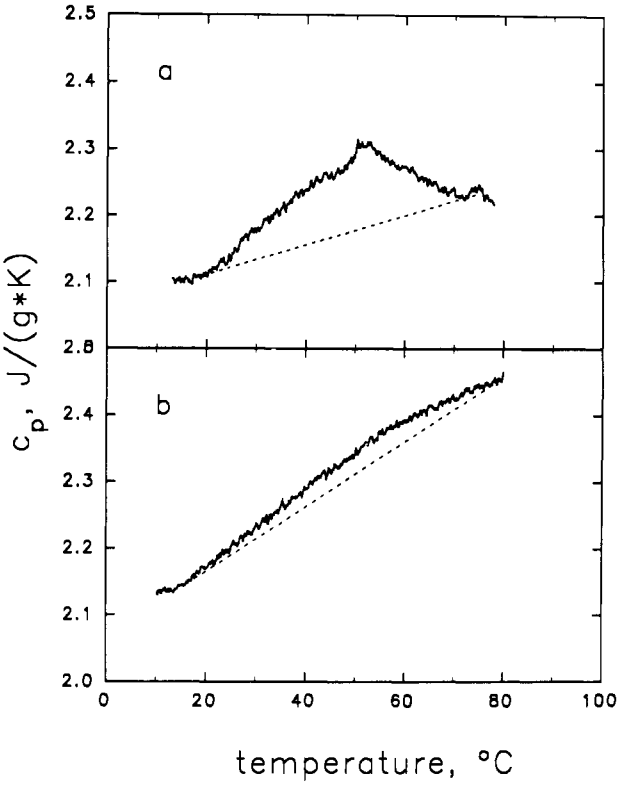

FIGURE 7: Microcalorimetric scans of F2. The heating rate was 1 K/min; the F2 concentration was 5.3 mg/mL; the buffer was 20 mM potassium phosphate, pH 7.8. (a) First scan; (b) second scan. Changes in heating rate (2 K/min) and concentration (2.8 mg/mL) did not affect the results.

was recorded after a 30-min incubation at room temperature. No amide protons could be detected, thus showing a complete exchange of all these protons for deuterium in 30 min. To improve the time resolution of the exchange experiments, a sample of F2, extensively dialyzed against the standard buffer, was diluted 3-fold with the deuterated buffer. NMR spectra were then recorded after various times of incubation at 9 °C. In the first spectrum, recorded after only 2 min at 9 °C, the amide NH to ND ratio was that expected from the composition of the buffer (i.e., 1:2), suggesting that the exchange was already completed. That the NMR spectrum did not change during further incubation (up to 2 h) indeed confirmed this conclusion. Thus, the exchange observed for the F2 amide protons was very much faster than the exchange of the lysozyme amide protons, a protein of similar size, at the same pH (Delepierre, 1983).

Thermal CD Transition of F2. The thermal stability of the isolated fragment was examined at the secondary structure level by following the evolution of the far-UV CD spectrum as a function of temperature. Between 25 and 80 °C, the general shape of the spectrum was maintained, but a slight decrease of the absolute amplitude at all wavelengths was observed (not shown), indicating a small temperature-induced loss in secondary structure. The spectrum recorded after reaching 80 °C and returning at 25 °C was superimposable with the original spectrum recorded at 25 °C, and indicated that the phenomenon is totally reversible.

As shown in Figure 6, the thermal transition observed at 220 nm is not cooperative. Similarly, no cooperativity was detectable at 192 or 207 nm.

Thermal Differential Scanning Calorimetry Transition of F2. Determination of the excess heat capacity as a function of temperature is an excellent probe of the presence of intra- and intermolecular interactions in proteins. Figure 7 reports the first and second scans of a solution of F2 in standard buffer containing 5.3 mg/mL protein. The transition curves are characterized by extreme broadness which is indicative of a structural change involving low cooperativity. The calorimetrically determined enthalpy values are 38 ± 5 and 17 ± 5 kJ/mol for the first and second scan, respectively. Subsequent measurements with the same sample reproduce the second scan. The corresponding van't Hoff enthalpies are 104 ± 10 and 84 ± 10 kJ/mol. The ratio of $\Delta H_{uH}/\Delta H_{cal} > 0$ implies the occurrence of intermacromolecular interactions. This result is in accordance with the increase of the sedimentation constant with protein concentration. Rescanning the sample after heating results in a smaller transition enthalpy than in the first scan and renders the transition even less cooperative. The width and the low cooperativity of the calorimetric transition curves correspond to similar observations with CD. The extremely low transition enthalpies compared to ΔH values for native small globular proteins (Pfeil, 1986) are a clear indication of the presence of only weak secondary structural interactions.

Discussion

Converging evidence from several laboratories, studying a variety of proteins through different experimental approaches, supports the view that the folding of a polypeptide chain into its native structure proceeds along a sequence of states that obeys the following general pattern:

(1) Stretches of native secondary structure (α-helices, β-strands, perhaps β-turns) are first formed within milliseconds (Udgaoncar & Baldwin, 1988; Röder et al., 1988) and constitute the framework of the native conformation.

(2) The polypeptide chain then condenses into a compact structure, the "molten globule". As indicated in the introduction, the molten globule was originally predicted on the-

oretical grounds (Ptitsyn, 1973), and then experimentally observed at equilibrium or as a transient folding intermediate for quite a few proteins (Ptitsyn et al., 1990). The general properties and significance of the molten globule have been reviewed recently (Kuwajima, 1989). Briefly, the molten globule has the following characteristics: it is nearly as compact as the native state, has a secondary structure content similar to that of the native state (as judged from the UV CD or infrared spectroscopy), and has a hydrophobic core (as judged from the emission spectrum of aromatic residues). However, unlike the native state, the molten globule is not tightly packed: large fluorescent probes like ANS have access to its hydrophobic core; the aromatic side chains are mobile (as judged from their low fluorescence polarization) and in a symmetrical environment (they exhibit no near-UV CD). Furthermore, it is considerably more flexible than the native state: most exchangeable protons indeed are readily exchangeable, and the NMR spectrum exhibits only few small induced chemical shifts as compared to the native state.

(3) The side chains then pack tightly, thus stabilizing the native tertiary conformation into their nativelike fold.

(4) Folded domains and subunits finally assemble to generate the native state. In several instances, the assembly has been shown to produce a "conformational feedback" (Goldberg, 1990) that modulates the conformation of the polypeptide chain within the domains or subunits.

In this sequence of steps, the molten globule has been thought to play a key role in that it was believed to be the first stable condensed globular state of the polypeptide chain. In the work reported here, we come to the conclusion that stable condensed globular intermediates may precede the molten globule on the folding pathway.

Indeed, some but not all of the properties observed for isolated F2 fit those originally predicted for a molten globule. The structural features that fit a molten globule are the following:

(1) Its sedimentation coefficient ($s_{20,w}$ = 1.35 S) suggests that it is not a compact globular protein. Indeed, let us assume that F2 is a tightly packed, nonhydrated molecule with a partial specific volume equal to 0.73 mL/g. Then one can calculate its frictional coefficient, $f = 3.8 \times 10^{-8}$ g/s, from the values of the molecular weight and of the sedimentation coefficient. One can also calculate the frictional coefficient, f_0, of a spherical particle with the same molecular weight and partial specific volume as F2; one obtains 2.7×10^{-8} g/s. The ratio f/f_0 would then be equal to 1.4. Such a high value might suggest that the isolated F2 fragment adopts an extremely extended conformation with an axial ratio of 8, a value by far higher than that one can estimate from the X-ray structure of tryptophan synthase (Hyde et al., 1988) for the F2 domain in the context of the native protein. Alternatively, the unexpectedly high value of f/f_0 might suggest that isolated F2 is significantly hydrated. The latter hypothesis seems by far more likely, and is compatible with the "wet" molten globule model.

The value of the sedimentation coefficient of the monomeric F2 fragment, obtained by proteolysis of β_2 with the V8 endoproteinase, is significantly smaller than that previously reported (1.8 S) for the monomeric F2 fragment obtained by trypsin treatment (Zetina & Goldberg, 1982). This could be explained by two factors. One is that the extrapolation of the observed sedimentation coefficient to zero protein concentration is much more reliable in the present study, because measurements were made in a lower protein concentration range. The second, more likely, explanation is that the structures of the two fragments might be different, because the trypsin–F2 fragment carries a 21-residue N-terminal extension (as compared to V8–F2) that brings about a significant increase of the molecular weight and might play an important role in the conformation of the F2 domain.

(2) A large circular dichroism signal is present in the far-UV. This indicates that the isolated F2 fragment is folded into a conformation that contains an important amount of secondary structure.

(3) No circular dichroism signal can be detected in the near-UV region of the spectrum, indicating that the side chains of the aromatic residues (here, tyrosines and phenylalanines since F2 contains no tryptophan) are not tightly packed in an asymmetric environment.

(4) The NMR spectrum at 300 MHz shows no significant deviation in chemical shift for the cyclic amino acid side chains (histidines and aromatic residues) as compared to a random coil. A similar observation was made by Dolgikh et al. (1985) on the A-state of α-lactalbumin, the typical molten globule.

(5) The amide protons are not, or only poorly, protected against exchange with the solvent, again as observed for the molten globule state of α-lactalbumin (Dolgikh et al., 1985). This points to a rather large conformational mobility of isolated F2. The increase in line width with increasing frequency in the NMR spectrum also suggests that F2 undergoes rapid fluctuations between different conformations.

The thermodynamic properties of isolated F2 also seem to fit the usual behavior of a molten globule. Indeed, though the secondary structure of the isolated F2 fragment appears to be heat-resistant up to temperatures that are unusually high for a small protein with no disulfide bridges (the T_m, defined as the temperature at which 50% of the native far-UV CD signal has disappeared, is about 70 °C), the circular dichroism transition curve, as well as the microcalorimetric analysis of the heat of unfolding of F2, clearly indicates that the denaturation–renaturation transition of the isolated fragment is a noncooperative process. Futhermore, the enthalpy of denaturation of isolated F2, over the temperature range investigated, is about 20–30 kJ/mol, a value smaller by a factor about 10 than that expected for a native, compact globular protein of similar size. These properties are compatible with the conclusion that isolated F2 behaves as a molten globule. Thus, as pointed out by Kuwajima (1989), the temperature dependence of the molecular properties of the A-state of α-lactalbumin (the prototype of a molten globule) shows diffuse thermal unfolding without significant cooperativity. Also, several studies (Dolgikh et al., 1985; Pfeil & Sadowski, 1985; Pfeil et al., 1986) indicate that the heat absorption during the thermal transition of the A-state of α-lactalbumin is very small. This can be understood by the absence, in the molten globule, of the cooperative network of specific, long-range interactions that stabilize the native state. It also correlates with the observation that in the equilibrium transition between the molten globule and the native state, the changes in both enthalpy and heat capacity are important (Kuwajima, 1977; Damaschun et al., 1986; Pfeil et al., 1986).

All the properties reported above fit the initial version of the molten globule model. Some other properties, however, do not fit this model. First, as discussed above, the very high value of f/f_0 points either to a nonglobular, extended conformation or to an elevated degree of hydration. Neither is consistent with the molten globule model. Second, while the molten globule model explicitly states that the nature and extent of secondary structure should be about the same in the molten globule as in the native state, this seems not to be the case for F2. Indeed, because the far-UV circular dichroism

spectrum has been recorded down to 190 nm (see Figure 2), a reliable estimate of the F2 secondary structure content could be made. The calculated secondary structure (19% of α-helix and 34% of extended β-structure) is quite different from that observed for F2 within native tryptophan synthase [49% α-helix and 13% extended β-structure, as seen from the X-ray structure solved by Hyde et al. (1988)]. In a previous report, we had concluded that the isolated F2 fragment obtained by proteolysis with trypsin contains about 15% α-helix and 15–25% β-structure (Högberg-Raibaud & Goldberg, 1977a). Since at that time the three-dimensional structure of tryptophan synthase was not known, and because no change in the total circular dichroism was detected upon association of the complementary F1 and F2 fragments when they generate the "native" reconstituted protein, we had assumed that the isolated fragments already had built up their nativelike secondary structures. This is clearly not the case, in view of the large discrepancy between the amounts of secondary structure estimated for the circular dichroism spectra and from the X-ray studies. Thus, while containing a large amount of stable secondary structure, both the isolated V8–F2 and trypsin–F2 fragments have a framework of secondary structure which differs from that which they have within native β_2.

In summary, it can be concluded that the isolated F2 fragment adopts a conformation (or, more correctly, a set of conformations) which shares many structural and thermodynamic features with the molten globule but lacks one that is essential to the molten globule model: the presence of nativelike secondary and supersecondary structures. Compared to other cases where similar studies were conducted [see the review by Kuwajima (1989)], the main difference is the unusually high hydrodynamic volume of isolated F2, and a far-UV circular dichroism spectrum for isolated F2 that differs drastically from that predicted from the structure of the F2 region of native β_2. How can one explain these differences between the properties of F2 and those of a molten globule? In isolated F2, the nativelike pattern of supersecondary structure (Richardson, 1981) cannot be completed because it requires interaction with stretches of secondary structure carried by the complementary F1 region (see the introduction). In the molten globule model, however, the formation of the nativelike supersecondary structure is essential, since it is the loose, hydrophobic packing which is the driving force that brings about the stabilization of the secondary structure. Therefore, this model predicts that the rates of formation of the secondary structure and of the molten globule during protein folding should be essentially the same. Yet, the experimental results on a variety of proteins indicate that a large fraction of the secondary structure appears in less than 10–15 ms while the molten globule appears with a half-reaction time on the order of 50 ms (Ptitsyn et al., 1990). This apparent contradiction has already been pointed out by Baldwin (1990). One is therefore led to the conclusion that the secondary structure that forms very early in protein folding exists within an intermediate structure, or group of structures, which precede the molten globule, and in which potential secondary structures are stabilized, presumably by creating a more hydrophobic environment around the polypeptide chain. Such an intermediate, resulting from a nonspecific hydrophobic collapse, was postulated by Levitt and Warshel (1975) to play a major role in the early steps of protein folding. Isolated F2 might well represent an intermediate, formed from such a nonspecific collapse, which fails to proceed further on the folding pathway because it lacks the side chains (of F1) required for creating the specific contacts that lead to the nativelike supersecondary structure of the molten globule and, later, to the native state. High-resolution circular dichroism stopped-flow studies on the kinetics of formation of secondary structures during F2 folding are underway in our laboratory to test this hypothesis.

References

Ahmed, S. A., Fairwell, T., Dunn, S., Kirschner, K., & Miles, E. W. (1986) *Biochemistry 25*, 3118–3124.

Baldwin, R. L. (1990) *Nature 346*, 409–410.

Bierzynski, A., Kim, P. S., & Baldwin, R. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2470–2474.

Brems, D. N., & Baldwin, R. L. (1984) *J. Mol. Biol. 180*, 1141–1156.

Bundi, A., & Wüthrich, K. (1979) *Biopolymers 18*, 285–298.

Chang, C. T., Wu, C.-S. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 12–31.

Crawford, I. P., Decastel, M., & Goldberg, M. E. (1978) *Biochem. Biophys. Res. Commun. 86*, 309–316.

Damaschun, G., Gernat, Ch., Damaschun, H., Bychkova, V. E., & Ptitsyn, O. B. (1986) *Int. J. Biol. Macromol. 8*, 226–230.

Decastel, M., & Goldberg, M. E. (1978) *FEBS Lett. 89*, 64–68.

Delepierre, M. (1983) Thèse d'Etat, Université d'Orsay.

Dolgikh, D. A., Abaturov, L. V., Bolotina, I. A., Brazhnikov, E. V., Bychkova, V. E., Bushuev, V. N., Gilmanskin, R. I., Levedev, Y. O., Semisotnov, G. V., Tiktopulo, E. I., & Ptitsyn, O. B. (1985) *Eur. J. Biophys. 13*, 109–121.

Fontana, A. (1990) in *Peptides. Chemistry, Structure and Biology* (Rivier, J. E., Marshall, G. R., Eds.) ESCOM-Leiden.

Friguet, B., Djavadi-Ohaniance, L., & Goldberg, M. E. (1986) *Eur. J. Biochem. 160*, 593–597.

Friguet, B., Djavadi-Ohaniance, L., & Goldberg, M. E. (1989) *Res. Immunol. 140*, 355–376.

Furie, B., Schechter, A. N., Sachs, D. H., & Anfinsen, C. B. (1975) *J. Mol. Biol. 92*, 497–506.

Goldberg, M. E. (1969) *J. Mol. Biol. 46*, 441–446.

Goldberg, M. E. (1990) in *Protein Folding: Deciphering the Second Half of the Genetic Code* (Gierasch, L., & King, J., Eds.) pp 141–154, AAAS, Washington, D.C.

Goldberg, M. E., & Högberg-Raibaud, A. (1979) *J. Biol. Chem. 255*, 7752–7757.

Högberg-Raibaud, A., & Goldberg, M. E. (1977a) *Biochemistry 16*, 4014–4020.

Högberg-Raibaud, A., & Goldberg, M. E. (1977b) *Proc. Natl. Acad. Sci. U.S.A. 74*, 442–446.

Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W., & Davies, D. R. (1988) *J. Biol. Chem. 263*, 17857–17871.

Kuwajima, K. (1977) *J. Mol. Biol. 114*, 241–258.

Kuwajima, K. (1989) *Proteins: Struct., Funct., Genet. 6*, 87–103.

Levitt, M., & Warshel, A. (1975) *Nature 253*, 694–698.

Moses, E., & Hinz, H.-J. (1983) *J. Mol. Biol. 170*, 285–317.

Osterhout, J. J., Baldwin, R. L., York, E. J., Stewart, J. M., Dyson, H. J., & Wright, P. E. (1989) *Biochemistry 28*, 7059–7064.

Pfeil, W. (1986) in *Thermodynamic Data for Biochemistry and Biotechnology* (Hinz, H.-J., Ed.) pp 349–399, Springer Verlag, Berlin.

Pfeil, W., & Sadowski, M. L. (1985) *Stud. Biophys. 109*, 163–170.

Pfeil, W., Bychkova, V. E., & Ptitsyn, O. B. (1986) *FEBS Lett. 198*, 287–291.

Ptitsyn, O. B. (1973) *Vestn. Akad. Nauk SSSR 5*, 57–68.

Ptitsyn, O. B., Pain, R. H., Semisotnov, G. V., Zerovnik, E., & Razgulyaev, O. I. (1990) *FEBS Lett. 262*, 20-24.
Richardson, J. (1981) *Adv. Protein Chem. 34*, 167-339.
Roder, H., Elöve, G. A., & Englander, S. W. (1988) *Nature 335*, 700-704.
Sachs, D. H., Schechter, A. N., Eastlake, A., & Anfinsen, C. B. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3790-3794.
Shoemaker, K. R., Fairman, R., Kim, P. S., York, E. J., Stewart, J. M., & Baldwin, R. L. (1987) *Cold Spring Harbor Symp. Quant. Biol. 52*, 391-398.
Udgaoncar, J. B., & Baldwin, R. L. (1988) *Nature 335*, 694-699.
Wetlaufer, D. B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 697-701.
Yang, J. T., Wu, C.-S. C., & Martinez, H. M. (1986) *Methods Enzymol. 130*, 208-269.
Zakin, M., Boulot, G., & Goldberg, M. E. (1980) *Eur. J. Biochem. 10*, 16-21.
Zetina, C. R., & Goldberg, M. E. (1982) *J. Mol. Biol. 157*, 133-148.

Multiplicity of Acidic Subunit Isoforms of Crotoxin, the Phospholipase A_2 Neurotoxin from *Crotalus durissus terrificus* Venom, Results from Posttranslational Modifications†

Grazyna Faure,‡ Jean-Luc Guillaume,§ Luc Camoin,§ Bernard Saliou,‡ and Cassian Bon*,‡

Unité des Venins, Unité Associée Institut Pasteur/INSERM 285, and Laboratoire de Microséquence, Unité de Biologie Moléculaire des Récepteurs URA 1336, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France

Received September 18, 1990; Revised Manuscript Received May 28, 1991

ABSTRACT: Crotoxin, the major toxin of the venom of the South American rattlesnake, *Crotalus durissus terrificus*, is made of two subunits: component B, a basic and weakly toxic phospholipase A_2, and component A, an acidic and nontoxic protein that enhances the lethal potency of component B. Crotoxin is a mixture of isoforms that results from the association of several isoforms of its two subunits. In the present investigation, we have purified four component A isoforms that, when associated with the same purified component B isoform, produced different crotoxin isoforms, all having the same specific enzymatic activity and the same lethal potency. We further determined by Edman degradation the polypeptide sequences of these four component A isoforms. They are made of three disulfide-linked polypeptide chains (α, β, and γ) that correspond to three different regions of a phospholipase A_2 precursor. We observed that the polypeptide sequences of the various component A isoforms all agree with the sequence of an unique precursor. The differences between the isoforms result first by differences in the length of the various chains α and β, indicating that component A isoforms are generated from the proteolytic cleavage of the component A precursor at very close sites, possibly by the combined actions of endopeptidases and exopeptidases, and second by the possible cyclization of the α-NH_2 of the N-terminal glutamine residue of chains β and γ. These observations indicate that the component A isoforms are the consequence of different posttranslational events occurring on an unique precursor, rather than the expression of different genes.

Crotoxin, the major toxic component from the venom of the South American rattlesnake, *Crotalus durissus terrificus*, is a potent neurotoxin that possesses a phospholipase A_2 activity (Slotta & Fraenkel-Conrat, 1938) and exerts its lethal action by blocking neuromusclar transmission (Vital-Brazil & Excell, 1971). At this level, crotoxin acts on nerve terminals by altering the quantal release of acetylcholine (Hawgood & Smith, 1977; Chang & Lee, 1977; Hawgood & Santana de Sa, 1979). These effects are similar to those observed with other snake venom phospholipase A_2 neurotoxins such as β-bungarotoxin, taipoxin, notexin, ammodytoxin A, agkistrodotoxin, caudoxin, etc., which belong to the class of β-neurotoxins [for a review, see Hawgood & Bon (1991)]. Crotoxin has also been reported to act postsynaptically (Vital-Brazil, 1966; Bon et al., 1979; Lee & Ho, 1980), causing the pharmacological desensitization of the acetylcholine receptor (Bon et al., 1979) and possesses myotoxic properties when injected intramuscularly (Gopalakrishnakone et al., 1984; Kouyoumdjian et al., 1986).

Crotoxin is made up of two nonidentical subunits: a basic and weakly toxic phospholipase A_2 subunit, component B, and an acidic, nontoxic and nonenzymatic subunit, component A (Rübsamen et al., 1971; Hendon & Fraenkel-Conrat, 1971). When mixed together, components A and B spontaneously associate into a one-to-one complex that possesses all the properties of native crotoxin, in particular its high lethal potency [Horst et al., 1972; Breithaupt et al., 1975; for a review, see Breithaupt (1976)].

Binding experiments indicated that crotoxin dissociates when it interacts with biological membranes: component B binds

† This research was supported in part by grants from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, and the Direction des Recherches Etudes et Techniques.
* Address correspondence to this author.
‡ Unité des Venins, Unité Associée Institut Pasteur/INSERM 285.
§ Laboratoire de Microséquence, Unité de Biologie Moléculaire des Récepteurs URA 1336.

0006-2960/91/0430-8074$02.50/0