Conformation of the Neurotoxin Crotoxin Complex and Its Subunits†

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ABSTRACT: Crotoxin, the neurotoxin from *Crotalus durissus terrificus* venom, and its two subunits, crotoxin A and crotoxin B, have been examined by fluorescence and circular dichroism techniques. Model spectra of crotoxin, generated from the expected contributions of the individual subunits, agreed with experimental results only at pH 2, where crotoxin was fully dissociated. From pH 4 to 10, crotoxin spectra could not be simulated by subunit contributions. Comparison of the results at pH values favoring either association or dissociation indicated that complex formation was accompanied by (1) a 70% decrease in fluorescent emission intensity and a 7-nm blue shift of the emission maximum, (2) an increase in the far-ultraviolet circular dichroism, and (3) a marked intensity enhancement of the near-ultraviolet circular dichroism spectrum. These observations were taken as evidence that tryptophan residues were masked by complex formation and that the crotoxin complex contained an increased proportion of ordered secondary structure, particularly β sheets. The results did not distinguish between conformational changes with subunit interaction or creation of ordered structure only at the subunit binding interface. Structural flexibility of the subunits was suggested from their ability to undergo reversible state changes by exposure to acid pH or guanidine hydrochloride. Examination of the concentration dependence of guanidine hydrochloride denaturation indicated that complex formation gave increased stability, although the subunits were individually quite resistant to guanidine hydrochloride denaturation. It is concluded that the ability of crotoxin and its subunits to exist in several reversible states is related to the control of the association–dissociation equilibrium of the complex and that conformational interconversion may be necessary for crotoxin to act on different target membranes.

Crotoxin, the major toxin in the venom of the neotropical rattlesnake *Crotalus durissus terrificus*, is a reversible complex of an acidic (crotoxin A) and a basic (crotoxin B) polypeptide[1] (Hendon & Fraenkel-Conrat, 1971). Purified crotoxin B exhibits weak lethality but becomes over 100 times more potent with the addition of the acidic component (Hendon & Fraenkel-Conrat, 1971). There is accordingly a toxic synergism of these two subunits in the crotoxin complex. Paradoxically, all of crotoxin's biological activities (phospholipase A_2 activity, hemolytic potency, and synaptic transmission blockage) reside on the B subunit (Hendon & Fraenkel-Conrat, 1971; Hawgood & Smith, 1977; Hanley, 1978). Thus, although required for full lethality, crotoxin A has no other identified function.

The nature of the subunit synergism has become more interesting with the recent recognition that crotoxin seems to belong to the category of presynaptic snake neurotoxins that share in vitro phospholipase A_2 (PhA)[2] enzymatic activity but, unlike other nonneurotoxic phospholipases, cause potent disruption of neuromuscular transmission (Chang & Lee, 1977; Chang et al., 1977). Two such neurotoxins, β-bungarotoxin and taipoxin, have structural similarities to crotoxin in that they consist of at least one polypeptide with sequence homology to phospholipase A enzymes (Eaker, 1978) and ancillary polypeptide chains which, in the case of taipoxin, also appear to augment lethal potency synergistically (Fohlman et al., 1976). However, these supernumerary polypeptide chains cannot be regarded as the key structural difference of neurotoxic vs. nonneurotoxic phospholipase A since a single-chain neurotoxic phospholipase A, notexin, has also been described (Halpert & Eaker, 1975). These comparative results do not clearly indicate how crotoxin A might act in the complex.

Recent binding experiments using radioactively labeled crotoxin have suggested that crotoxin A might function as a "chaperone", improving the targeting of the active B subunit by limiting nonspecific adsorption of the complex (Jeng et al., 1978; Bon et al., 1979). In binding to either erythrocytes or electroplaques, crotoxin A is released after the active B subunit has bound (Jeng et al., 1978; Bon et al., 1979). These data suggest that complex formation or dissociation may be important in understanding the mechanism of crotoxin action on target membranes. Thus, in order to identify any unique conformational aspects of the complex and to provide background data on the spectroscopic behavior of crotoxin, I have examined the fluorescent and circular dichroism spectra of crotoxin and its purified subunits.

Materials

Suppliers of reagents and materials were as follows: *Crotalus durissus terrificus* crude venom, Ross Allen Reptile Institute; guanidine hydrochloride (ultrapure), Heico; *N*-bromosuccinimide, 99% pure reagent grade, Aldrich; L-cystine and urea, Schwarz/Mann; *N*-acetyl-L-tryptophanamide, *N*-acetyl-L-tyrosinamide, and L-phenylalanine, Sigma; and *d*-10-camphorsulfonic acid, Eastman. *N*-Bromosuccinimide, L-cystine, urea, and *d*-10-camphorsulfonic acid (see Methods) were all freshly recrystallized before use. Crotoxin and its subunits were purified as described previously (Hendon & Fraenkel-Conrat, 1971, 1976).

Methods

UV absorption spectra were recorded on Cary 118C spectrophotometer with matched 1-cm quartz cuvettes. Corrected fluorescent spectra were taken on a Hitachi Perkin-Elmer MPF-3L recording fluorimeter calibrated against

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[1] Crotoxin A is also known as crotapotin and crotoxin B is also known as phospholipase A (Breithaupt et al., 1974).

[2] Abbreviations used: λ_{max}, wavelength of a spectroscopic extremum; CD, circular dichroism; Gdn·HCl, guanidine hydrochloride; MCD, magnetic circular dichroism; NBS, *N*-bromosuccinimide; PhA, phospholipase A_2 (EC 3.1.1.4); Trp, tryptophan; UV, ultraviolet.

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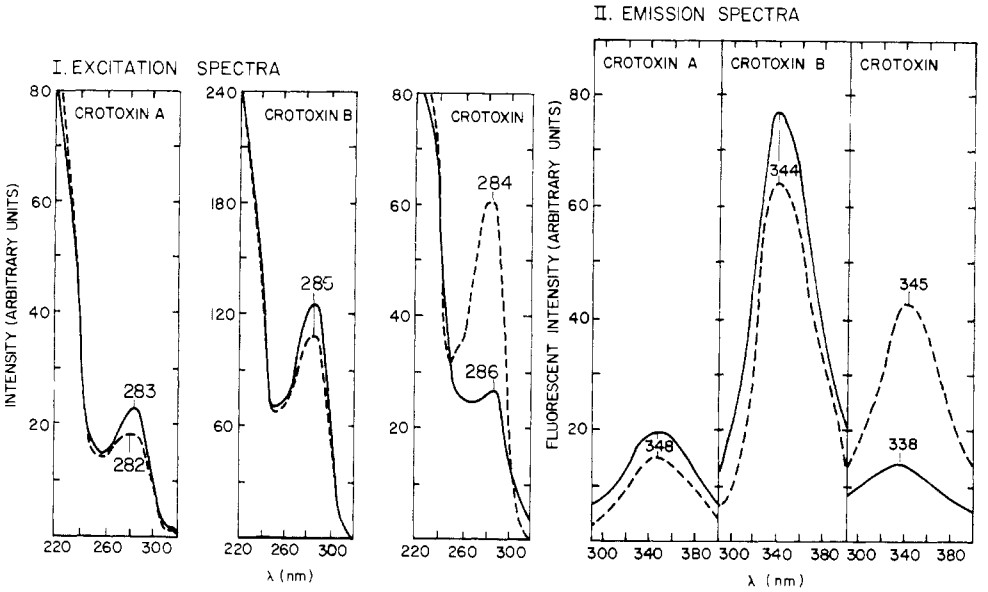

FIGURE 1: Corrected excitation and emission fluorescence spectra of crotoxin and its subunits. Solid lines were spectra taken at pH 7.0 (0.1 M ammonium acetate), and broken lines were spectra taken at pH 2.0 (0.1 M ammonium acetate or 0.01 N HCl). Concentrations were 5.0 μM for crotoxin and crotoxin A and 4.5 μM for crotoxin B. Excitation and emission slit widths were 6 nm in all cases. Excitation and emission spectra were recorded by using the indicated λ_{max} determined experimentally for emission and excitation, respectively. Note the scale change for the crotoxin B excitation spectrum. The reported spectra were replicated by triplicate preparations from two sets of independently purified proteins.

a standard solution of anthracene in ethanol according to manufacturer recommendations. Fluorescent emission measurements, uncorrected for sensitivity vs. wavelength, were performed on a Hitachi Perkin-Elmer MPF-2A recording fluorimeter by using a blue-sensitive Hamamatsu R106 photomultiplier. Excitation and emission slit widths were 6 nm in all cases.

CD and MCD measurements were made on an instrument designed in this laboratory (Sutherland et al., 1974). Spectra were recorded from 190 to 250 nm (far-UV) and from 250 to 350 nm (near-UV) at 0.1-nm intervals by using a scan speed of 0.25 nm/s and an instrumental time constant of 0.3 s. For the far-UV, samples were approximately 0.01% protein (w/v) in 0.1-cm path-length cells with an instrumental slit width of 1.0 nm, and, for the near-UV, samples were 0.1% protein (w/v) in 1.0-cm path-length cells with an instrumental slit width of 0.5 mm. For MCD spectra, samples were prepared as suggested by Barth et al. (1972) and were 0.1% protein (w/v) in 1.0-cm path-length cells with a 2.0-mm instrumental slit width. For CD and MCD of both samples and the appropriate references, 10 passes through the experimental wavelength region were signal-averaged by an on-line computer and stored as sample-reference computer library files for subsequent manipulation and plotting. The CD intensity was calibrated with a 1 mg/mL *d*-10-camphorsulfonic acid standard (prepared gravimetrically from material twice recrystallized from benzene and stored over P_2O_5) and is expressed as $\Delta\epsilon$ $(M\ cm)^{-1} = \epsilon_L - \epsilon_R$ $(M\ cm)^{-1}$. The MCD intensity was expressed as $\Delta\epsilon/H$ $(M\ cm\ T)^{-1}$ where T = telsa = 10^4 G, and the magnetic field strength of the electromagnet (1.4 T) was calibrated with a potassium ferricyanide standard (Sutherland et al., 1974). CD and MCD results can be converted to mean residue ellipticity (Strickland, 1974) by $[\theta]_{MRW}$ $(deg\ cm^2\ dmol^{-1}) = 3305\Delta\epsilon n^{-1}$ where n = number of amino acid residues in the protein.

Protein concentrations were determined by using absorption at 280 nm and previously reported extinction coefficients for crotoxin, crotoxin A, and crotoxin B (Hendon & Fraenkel-Conrat, 1976) or, in the case of modified proteins, by weighing the lyophilized protein stored over P_2O_5 in vacuo. The spectra have no contribution from differential light scattering since the signals were independent of photomultiplier position (Philipson & Sauer, 1973). Molecular weights were assumed to be 8000 for crotoxin A, 15 000 for crotoxin B, and 23 000 for the crotoxin complex. Because these values are slightly different from those reported elsewhere (Horst et al., 1972; Breithaupt et al., 1974), it should be noted that appropriate corrections can be made when exact molecular weights from sequencing are available.

Results

Fluorescence Spectra. The UV absorption spectra for crotoxin and its subunits were typical protein spectra and resembled earlier published results (Breithaupt et al., 1974). In Figure 1, the corrected fluorescent excitation and emission spectra of crotoxin and its subunits indicate the presence of tryptophan residues which dominate the intrinsic fluorescence properties. Comparison of excitation and emission spectra at both acid and neutral pH for crotoxin A and crotoxin B shows little change other than a slight quenching at acid pH, unlike crotoxin spectra, which are accompanied by shifts in the positions of the excitation maximum (from 286 to 284 nm) and emission maximum (from 338 to 345 nm). Since acidic pH is known to reversibly dissociate the crotoxin complex (Hendon & Fraenkel-Conrat, 1971), it seemed likely that the increase in intrinsic fluorescence at acid pH was related to this dissociation. Accordingly, the pH dependence of emission intensity of crotoxin and its subunits was investigated in more detail (Figure 2). As the pH was lowered from 10, there was little change in the intensity until pH 4.5. Below pH 4.5, crotoxin underwent a progressive increase in emission intensity, with a parallel shift in the position of the emission maximum toward longer wavelengths (Figure 2a). At pH 2.5 and below, there were no further increases. The absolute intensity of crotoxin samples at the experimental pH was independent of the pH of the stock crotoxin preparation, and the identical pH profile of fluorescence could be generated by pH 2.0 crotoxin stock (dissociated) or pH 7.0 crotoxin stock solutions (as-

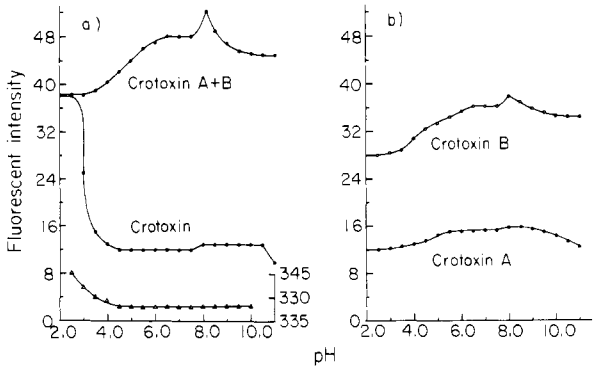

FIGURE 2: Dependence of uncorrected intrinsic fluorescence of crotoxin and its subunits on pH. (a) Crotoxin (4.4 μM) emission intensity at λ_{max} (●), crotoxin emission maximum (λ_{max}) (Δ), and summation of expected contributions of noninteracting subunits [data from (b)] to emission intensity (○). (b) Crotoxin A (5.0 μM) emission intensity at λ_{max} (●), and crotoxin B (4.3 μM) emission intensity at λ_{max} (○). Stock solutions were prepared in either pH 2.0 or 7.0 ammonium acetate buffer (0.1 M) titrated to the indicated pH. Other buffers tested did not alter the results. Excitation was at 286 nm in all cases by using 6 nm slit widths. Data are the average of six separate preparations of the proteins.

sociated). Thus, the pH-induced changes were completely reversible and exhibited no "hysteresis" from the previous pH state of the toxin. In Figure 2b, the pH profiles of the isolated A and B subunits' fluorescence show no such increases at acid pH. The fact that the increase occurs only with the complex is emphasized by a computed pH profile of emission intensity for the sum of the expected contributions of the isolated subunits (crotoxin A + B), which is compared to the experimental crotoxin profile in Figure 2a. The computed intensity agrees with the experimental only at pH 2.5 and below, where the crotoxin complex is fully dissociated. In both crotoxin and crotoxin B, there is a small but reproducible decrease in intensity from pH 8.0 to 7.5. Exposure of any of the proteins to a pH above 10 results in irreversible changes in fluorescent properties and increases in light scattering, consistent with denaturation.

If the fluorescent quenching can be represented by the equilibrium $X + nH^+ \rightarrow Y$, where X = crotoxin complex (represented by fluorescence intensity above pH 4.5) and Y = dissociated crotoxin (represented by fluorescence intensity measured at pH 2.0), then, if A = the proportion of complexed crotoxin and we assume that the quenching is a measure of the amount of complex formation (Wells, 1971), the plot of log $[A/(1-A)]$ against pH should yield a straight line where the slope gives n, the number of protons released with association. Analysis of the data in Figure 2 gives a value of three protons by this method. This is quite interesting, because the number of fixed charges on other toxic PhA homologues, expected to strongly resemble crotoxin B, is 3 (notexin) or 4 (*Notechis* II-5) at neutral pH (Eaker, 1978). The neutralization of fixed positive charges on crotoxin B with complex formation produces the acidic charge character and low isoelectric point of crotoxin (Horst et al., 1972).

The origin of the pH-dependent fluorescent changes in crotoxin was pursued by selective chemical modification of the tryptophan residues, the dominant fluorophors. Several procedures have shown that there are three Trp residues in crotoxin B and one in crotoxin A (Hendon & Fraenkel-Conrat, 1971; Breithaupt et al., 1974; see later MCD results). The pH-induced quenching and shift in λ_{max} in crotoxin were consistent with the removal of Trp residues from a hydrophilic to a hydrophobic environment with formation of the crotoxin complex at permissible pH. To test this hypothesis, accessible

Table I: *N*-Bromosuccinimide Oxidation of Tryptophans in Crotoxin, Crotoxin A, and Crotoxin B

reactant	conditions[a]	no. of Trp modified[b]	NBS consumed[c] (mol)
crotoxin, associated	pH 6.0	1.2	6
crotoxin, dissociated	pH 3.0	3.2	15
crotoxin, dissociated and denatured	pH 6.0 + 8 M urea	4.0	15
crotoxin B	pH 6.0	2.3	10
crotoxin B	pH 6.0 + 8 M urea	3.0	9
crotoxin A	pH 6.0	1.0	3

[a] Reaction followed the protocol of Spande & Witkop (1967). Stepwise additions of NBS were monitored by changes in absorbance in a Cary 118. Buffers were as follows: pH 3.0, 0.1 M sodium acetate, and pH 6.0, 0.1 M ammonium acetate. Crotoxin samples precipitated at pH 6.0 unless additions were made slowly at 5-min intervals with 10-fold more diluted NBS stock. [b] Quantitated spectrophotometrically as recommended by Spande & Witkop (1967). An independent quantitation and a check on the specificity of the reaction was made by hydrolyzing 0.5 mg of desalted and lyophilized NBS-reacted crotoxin in 4 N methanesulfonic acid. Tryptophan values of crotoxin samples oxidized at pH 3.0 and 6.0 agreed within 5% of the spectrophotometric values, and there was no apparent degradation of other amino acids. [c] Total amount of NBS added when the change of absorbance with incremental addition reached a plateau. No attempt was made to extend the additions of NBS beyond the plateau.

tryptophans were oxidized by *N*-bromosuccinimide (NBS) at pH 3.0 (complex dissociated) and pH 6.0 (complex formed). From Table I, it is evident that dissociation of the crotoxin complex by either low pH or the addition of urea exposes at least two Trp that are not accessible in the native complex. Analysis of NBS-oxidized crotoxin (pH 6.0, associated) showed that the Trp on crotoxin A and one Trp on crotoxin B was modified. Therefore, two inaccessible Trp residues in the complex must be on crotoxin B. After preparation of NBS-crotoxin modified at pH 6.0, the pH sensitivity of the residual fluorescence was investigated. Above pH 4.5, there was virtually no fluorescence, indicating that the modified Trp residues must, in the native complex, be the source of the pH-insensitive emission (Figure 3). Reconstitution of the crotoxin complex from NBS-modified crotoxin A gave very similar results (Figure 3), suggesting the crotoxin A Trp alone was largely responsible for the fluorescence observed above pH 4.5 from the native complex. Modification of either the single Trp on crotoxin A or both the crotoxin A Trp and the pH 6 accessible Trp on crotoxin B did not affect the stepwise increases in fluorescent intensity with incremental decreases of the sample pH below 4.5.

Far-UV CD Spectrum. Study of the far-UV CD spectrum reveals information on the secondary structure of a given protein (Greenfield & Fasman, 1969; Chen et al., 1974). Examination of the far-UV CD of crotoxin samples at pH 2–4 showed an increase in negative ellipticity as the pH was raised (Figure 4). As with the fluorescence effects, these changes were independent of the starting pH of the sample and were completely reversible. Since these results pointed to changes in the proportion of ordered secondary structure with complex formation, the far-UV CD of the isolated subunits was examined (Figure 5) and gave no evidence of a change of similar magnitude, albeit purified crotoxin A exhibited a small reversible CD diminution at low pH. Summation of the individual subunits' CD spectra at pH 2.0 and 4.0 supported the fluorescence results in that the computer-generated spectrum agreed closely with the experimental only at pH 2.0 (Figure

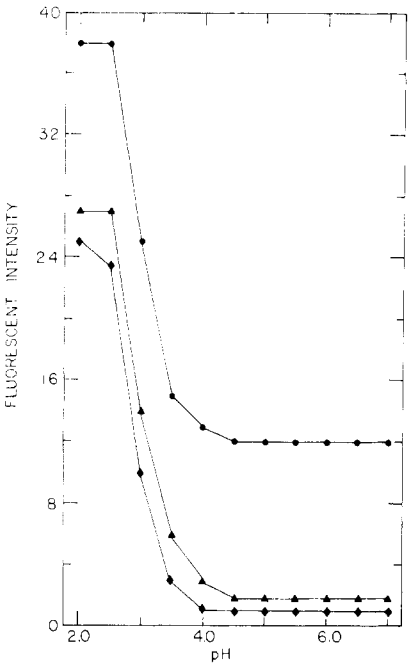

FIGURE 3: Dependence of uncorrected intrinsic fluorescence of *N*-bromosuccinimide-modified crotoxin on pH. Native crotoxin (4.5 μM) emission intensity at λ_{max} (●), crotoxin (5.1 μM) modified by NBS at pH 6.0 (♦), and crotoxin (5.0 μM) reconstituted from NBS-oxidized crotoxin A (▲).

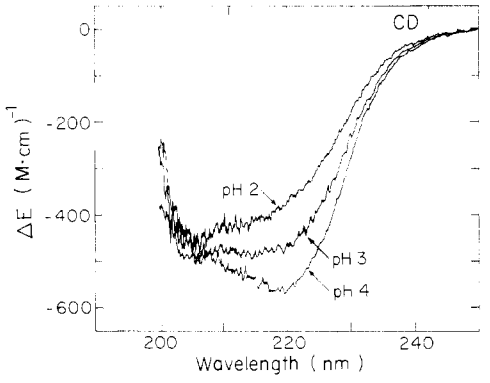

FIGURE 4: Far-ultraviolet circular dichroism spectra of crotoxin as a function of pH. Spectra were recorded as described under Methods in 0.01 M ammonium acetate titrated to the indicated pH. Identical spectra were obtained regardless of whether the samples were dissolved as a lyophilized powder directly in the final buffer or were prepared from pH 2.0 or 7.0 concentrated stock solutions diluted into the final buffer. Noise levels from the average of 10 wavelength passes are indicated.

6b). At pH 4.0, the computer sum of the crotoxin A and crotoxin B contribution to the far-UV CD gave a substantial underestimate of the true crotoxin CD (Figure 6a). To specify the nature of this disagreement, the difference spectrum was computed (Figure 6a). It resembled the model spectrum for β structure (Greenfield & Fasman, 1969; Saxena & Wetlaufer, 1971; Chen et al., 1972), but the red shift of the negative extremum toward 220 nm, the displacement of the crossover point to below 200 nm, and the pronounced shoulder at 208 nm are all unusual features. There may be several complicated or superimposed elements contributing to these distortions, including a negative disulfide band below 210 nm (Takagi & Ito, 1972), a hydrophobic perturbation of the β-structure spectrum (Raghavendra & Ananthanarayanan, 1976), or a contribution from β bends (Brahms et al., 1977). By use of two procedures for estimating the proportions of secondary structures, an increase in the content of ordered

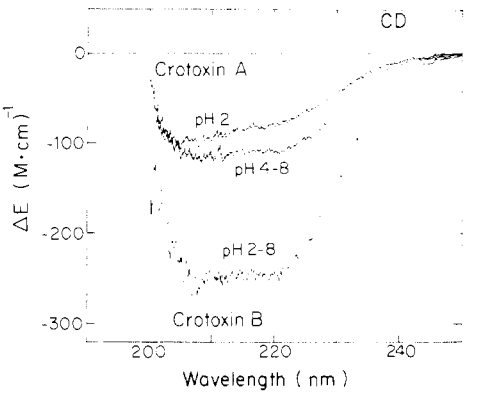

FIGURE 5: Far-ultraviolet circular dichroism spectra of crotoxin A and crotoxin B as a function of pH. Details as in the legend to Figure 4, with the addition that pH 6.0 and above samples were prepared in 0.05 M sodium phosphate buffers; all others were prepared in 0.01 M ammonium acetate buffer titrated to the appropriate final pH.

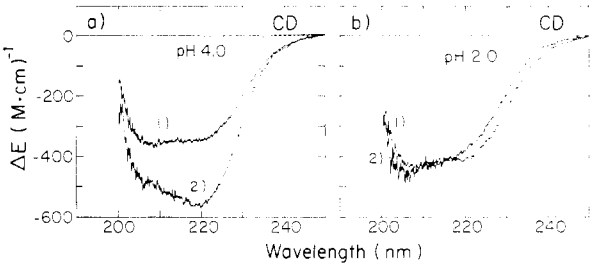

FIGURE 6: Comparison of crotoxin far-ultraviolet circular dichroism spectra with the contributions from its subunits. Spectrum 1 is the computer sum of individual spectra for the isolated subunits (see Figure 5). Spectrum 2 is the experimental result for crotoxin (Figure 4). Results are reported at pH 2.0 (b) and 4.0 (a). Sample pH was maintained by 0.1 M ammonium acetate titrated to the final pH. At pH 4.0 the dashed lines are the computer-generated difference spectrum of 2 minus 1.

Table II: Secondary Structure Estimations of Crotoxin and Its Subunits from Far-Ultraviolet CD Spectra

		method of analysis					
		Greenfield & Fasman (1969)			Chen et al. (1974)		
toxin	pH	α helix (%)	β structure (%)	random (%)	α helix (%)	β structure (%)	random (%)
crotoxin	2.0	30	15–20	50–55	36	31	33
crotoxin	4.0–9.0	35	40–50	15–25	40	45	15
crotoxin A	2.0	none	50	50	6[b]	41.5	47.5[a] (5% β bend)
crotoxin A	4.0–8.0	5[b]	45	50	7.5[b]	37.5	50[a] (5% β bend)
crotoxin B	2.0–8.0	12	40	48	18	37	45

[a] Best fit was obtained by using a four-component summation of α helix, β structure, and residual structure ("random") as in Chen et al. (1974) but including a β-bend term with standard values from Brahms et al. (1977). [b] The proportion is too small to realistically generate α-helical structure. Previously uncharacterized forms of ordering or far-UV transitions of aromatic or disulfide chromophores may be responsible for this contribution.

forms with formation of the crotoxin complex was clearly shown (Table II). The increase in ordering was largely attributable to a β or β-like structure, in agreement with the difference spectrum (Figure 6a). The proportions of ordered secondary structures in Table II should only be considered a conceptual guide, and not a rigorous prediction, because such determinations have a very large uncertainty (Barela & Darnall, 1974).

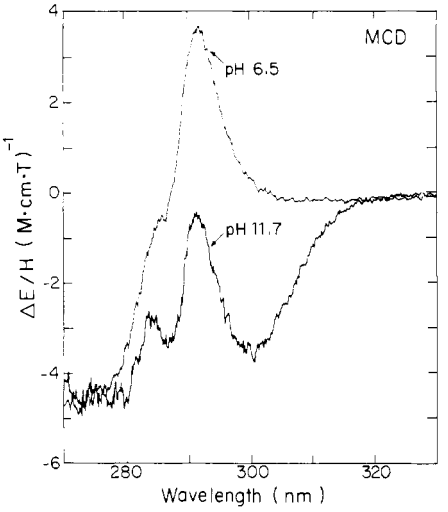

FIGURE 7: Magnetic circular dichroism spectra of crotoxin at pH 6.5 and 11.7. Spectra were recorded as described under Methods.

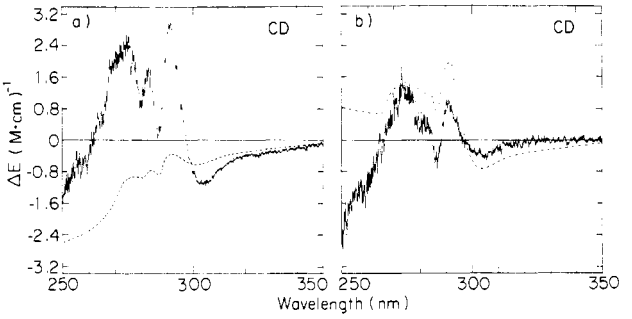

FIGURE 8: Near-ultraviolet circular dichroism spectra of crotoxin and its comparison to the contributions from its subunits. (a) Experimental results for crotoxin at pH 2.0 (---) and pH 4.0–8.0 (—). The computer summation of crotoxin A + crotoxin B spectra at pH 2.0 (not shown) is identical with the experimental crotoxin result at the same pH. (b) Computer summation of crotoxin A + crotoxin B (see Figures 9 and 10) spectra at pH 4.0–8.0 (—) and the difference spectrum of crotoxin minus (crotoxin A + crotoxin B) over the same pH range (–·–·). Spectra were recorded as described under Methods, and the noise levels are shown in the solid lines.

MCD Spectrum. Although it gives no conformational information, MCD is a useful approach for obtaining an independent estimate of the tryptophan content of proteins (Barth et al., 1972; Holmquist & Vallee, 1973). In agreement with previous results from other techniques (Hendon & Fraenkel-Conrat, 1971; Breithaupt et al., 1974), four Trp residues are present in the complex (Figure 7). MCD spectra (not shown) of the subunits gave a single Trp in crotoxin A and three Trp in crotoxin B.

Near-UV CD Spectra. Since the fluorescence data implicated aromatic residues as reporters on the formation of the complex, the near-UV CD spectra were expected to indicate changes in local structure around dichroic chromophores. In Figure 8, the near-UV CD spectra for crotoxin are shown at pH 2.0 and 4–8. Significant changes in intensity and positions of the maxima and minima were seen over the transitional pH range 2–4 but not above, in agreement with other techniques. To establish that these changes could not be accounted for by the behavior of the isolated subunits, their spectra were summed at the reference pH values (Figure 8b) and compared to the crotoxin results (Figure 8a). As had been seen before, the summed and experimental spectra agreed only at pH values where the complex was dissociated. The difference spectrum (Figure 8b) of crotoxins minus (crotoxin A + crotoxin B) emphasized that the bands were increased in intensity, but not

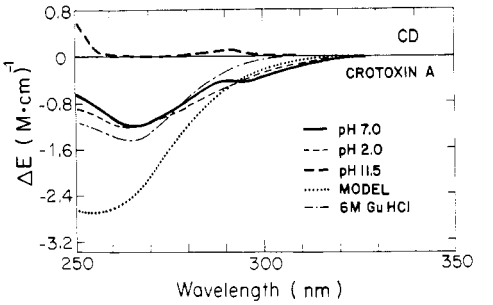

FIGURE 9: Near-ultraviolet circular dichroism spectra of crotoxin A as a function of pH and the effect of 6 M guanidine hydrochloride. The model spectrum was that of a mixture of *N*-acetyl-L-tryptophanamide, *N*-acetyl-L-tyrosinamide, L-phenylalanine, and L-cystine equimolar to their expected concentrations in crotoxin A samples. L-Cystine alone was responsible for the observed spectrum.

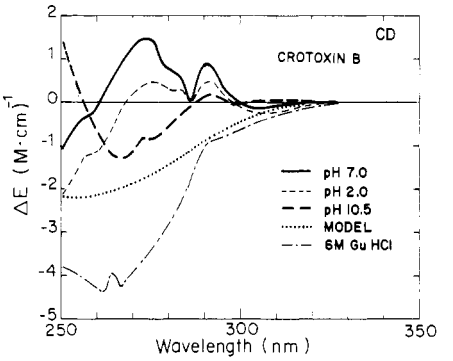

FIGURE 10: Near-ultraviolet circular dichroism spectra of crotoxin B as a function of pH and the effect of guanidine hydrochloride. Model spectrum was produced as described in the legend for Figure 9.

qualitatively altered, by association of the subunits.

The examination of the individual near-UV CD of crotoxin A and crotoxin B indicated the origin of components of the crotoxin CD. Crotoxin A (Figure 9) has a single broad band with λ_{max} at approximately 266 nm and a weak shoulder at 288 nm. The spectrum is relatively unchanged by acidic pH or exposure to 6 M Gdn·HCl. Since 6 M Gdn·HCl, a denaturant abolishing the far-UV ordered-structure contributions in crotoxin A, was ineffective, it seemed likely that the near-UV CD arose largely, if not exclusively, from the intrinsic chirality of the disulfide chromophores (Beychok, 1966). In keeping with this, exposure of crotoxin A to pH 11.5 gave rise to an irreversible loss of the major signal and its replacement by weaker positive signals at 250 and 291 nm (Figure 9). A simple model mixture of CD-active amino acids contributing to the near-UV region (tryptophan, tyrosine, phenylalanine, and cystine), at the same concentration as in crotoxin A, was compared to the experimental crotoxin A data. The mixture gave a single broad band with λ_{max} at 255 nm which was exclusively attributable to L-cystine (Takagi & Ito, 1972). The differences in intensity and λ_{max} in crotoxin A vs. the model compound L-cystine are very similar to those observed with bulky substitutions (mimicking a polypeptide environment) in chemical derivatives of L-cystine (Casey & Martin, 1972) or to those in denatured, disulfide-rich proteins (Takagi & Izutsu, 1974). Although the far-UV CD indicated pH-dependent changes in structure, the disulfide chiroptical properties are apparently insensitive to these changes.

Crotoxin B has a more complicated near-UV CD spectrum that is similar to that of the crotoxin complex (Figure 10). Exposure to acid pH produced a reversible change in the magnitude of the contributing signals. As with crotoxin A, base denaturation produced an irreversible abolition of the

Table III: Comparisons of Secondary Structures of Presynaptic Snake Neurotoxins and Snake Phospholipase A_2 Isoenzymes by Both Experimental (CD) and Predictive[e] Techniques

toxin	family	measured (%)			predicted (%)		
		α helix	β structure	ref	α helix	β structure	ref
crotoxin	Crotalidae	37.5[a]	45[a]	this work			
crotoxin A		6[b]	41[b]	this work			
crotoxin B		15[a]	38.5[a]	this work			
β-bungarotoxin	Elapidae	34, 40[c]	18[c]	Hamaguchi et al. (1968)	7[d]	36[d]	Kondo et al. (1978b)
heavy subunit					10	33[d]	Kondo et al. (1978b)
light subunit					none	40	Kondo et al. (1978b)
notexin	Elapidae				8[c]	25[c]	
phospholipase A_2							
Naja melanoleuca	Elapidae	16, 17, 20	40[d]	Joubert & van der Walt (1975)			
Bitis gabonica	Viperidae	22	45	Viljoen et al. (1976)	20	36	Viljoen et al. (1976)
Vipera ammodytes	Viperidae						
toxic isoenzyme		21	32[d]	Gubenšek & Lapanje (1974)			
nontoxic isoenzyme		28	33[d]				

[a] Average from values in Table II. [b] Average weighted to lower values by rounding 7.5 to 7.0 and 37.5 to 37. [c] Hanley, unpublished experiments. [d] Recalculated from literature. [e] Chou & Fasman, 1974.

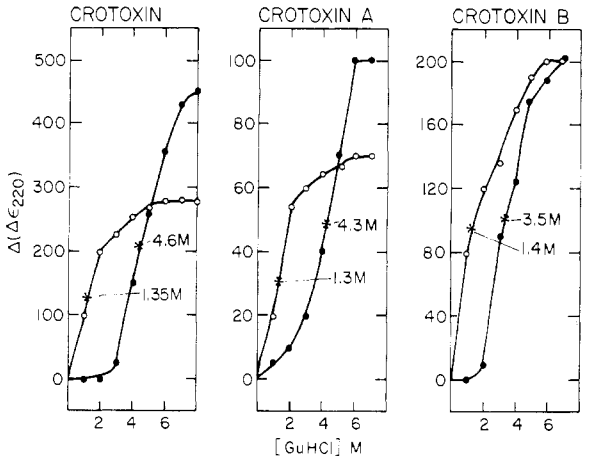

FIGURE 11: Dependence of $\Delta\epsilon_{220}$ on concentration of guanidine hydrochloride at 25 °C for crotoxin and its subunits. Filled circles give the results at pH 7.4 (0.1 M sodium phosphate buffer) and open circles give the results at pH 2.0 (0.1 N HCl). Stocks were prepared in either pH 2.0 or 7.4 buffers and diluted into the appropriate guanidine hydrochloride solution or in 8 M guanidine hydrochloride at either pH 2.0 or 7.4 and diluted with buffer alone to the final guanidine hydrochloride concentration. Indicated data points were independent of the method used and are the average of two determinations by each procedure. Spectra were recorded between 210 and 240 nm in a 0.5-cm path-length cell. Asterisks (*) mark the points of half-maximal denaturation determined by a log-probit graphical transform of the indicated data. All spectra were recorded immediately after dissolution of samples and time-dependent changes are not reported.

characteristic spectrum and its replacement by weaker bands centered at 250, 267, 277, and 292.5 nm. Unlike crotoxin A, however, crotoxin B was sensitive to spectral perturbation by 6 M Gdn·HCl in the near-UV region. Exposure to the denaturant led to a reversible increase in the negative ellipticity, accompanied by the loss and addition of several fine structural features of the spectrum. These changes are difficult to interpret since they could arise either by the removal of stronger masking signals by denaturation, suggesting that the residual spectrum arises from a Gdn·HCl resistant structure, or by induction of a new chromophore environment in Gdn·HCl, suggesting that the spectrum arises from a Gdn·HCl-dependent stabilization. Comparison of the Gdn·HCl spectrum with that of the model amino acid mixture suggests a large contribution from cystine residues. These results are consistent with the general conclusion that disulfide-rich proteins can exhibit novel or persistent structures under denaturing conditions (Cortijo et al., 1973) even though secondary structure is eliminated.

Guanidine Hydrochloride Denaturation. Quantitative aspects of the potency of Gdn·HCl as a denaturant report on the stability of proteins (Bull & Breese, 1975), and, by using far-UV CD as the means of detecting denaturation, the loss of global structure can be followed (Cortijo et al., 1973). Accordingly, crotoxin and its subunits were exposed to varying concentrations of Gdn·HCl, up to 8 M, in both acid and neutral pH media. The intensity of the ellipticity at 220 nm was used to monitor disruption because both α and β structures contribute to the signal at this wavelength. At acid pH, crotoxin, crotoxin A, and crotoxin B were all readily, but reversibly, denatured (Figure 11). The denaturation vs. [Gdn·HCl] profile of crotoxin agreed closely with an average profile generated from the measurements of the separate subunits. At neutral pH, however, crotoxin is markedly more stable than either of its subunits, as judged from its half-maximal denaturation value (4.6 M for crotoxin vs. 4.3 M for crotoxin A and 3.5 M for crotoxin B) or by its denaturation threshold (3 M for crotoxin vs. <1.0 M for crotoxin A and 2 M for crotoxin B). By extrapolation procedures discussed elsewhere (Pace, 1975), it is possible to determine the apparent free energy of unfolding in the absence of denaturant, $\Delta G°$. By use of a linear extrapolation based upon $\Delta G_{app} = \Delta G° + m\cdot[\text{Gdn·HCl}]$, at pH 7.4, $\Delta G° = 4.50$ kcal/mol [slope = −0.86 kcal/(mol M)] for crotoxin, 2.02 kcal/mol [slope = −0.62 kcal/(mol M)] for crotoxin B, and 2.20 kcal/mol [slope = −0.44 kcal/(mol M)] for crotoxin A. Apparently, stability is conferred on the subunits with association into the complex. At pH 2, all three polypeptides are more readily denatured, as indicated by their threshold and half-maximal values.

Discussion

Crotoxin has been shown to block neuromuscular transmission by prejunctional interference with neurotransmitter release (Vital Brazil et al., 1973; Chang & Lee, 1977; Chang et al., 1977; Hawgood & Smith, 1977) and, in common with other snake toxins of this type, has a phospholipase A_2 en-

zymatic activity (Hendon & Fraenkel-Conrat, 1971; Breithaupt, 1976a). Crotoxin also exhibits indirect hemolytic (Hendon & Fraenkel-Conrat, 1971; Jeng et al., 1978), myotoxic (Breithaupt, 1976b), and postsynaptic (Hanley, 1978; Bon et al., 1979) activities. Thus the toxin can exert a disruptive influence on several membranes. In the red blood cell and electroplaque membranes, the interaction of crotoxin with the surface leads to its dissociation, releasing the A subunit into the medium (Jeng et al., 1978; Bon et al., 1979). However, the full expression of crotoxin's lethal action requires the participation of both the acidic and basic subunits (Hendon & Fraenkel-Conrat, 1971, 1976). The relationship between crotoxin's activities and the precise role of the complex in each remain mysterious. One consideration is whether any of these activities are qualitatively different in the complex, as opposed to the B subunit (which can exhibit all of the crotoxin activities). Under certain in vitro conditions, the A subunit appeared to inhibit the expression of the phospholipase activity (Breithaupt, 1976a), but it is unlikely that this occurs under physiological conditions (Jeng et al., 1978). To date, no other qualitative *functional* change upon association of crotoxin A and B into the complex has been described.

Since acidic pH is known to reversibly dissociate the complex (Hendon & Fraenkel-Conrat, 1971, 1976), the fluorescent, near-UV CD, and far-UV CD spectra were monitored as a function of pH. In all cases, pronounced changes were seen in the pH range from 2 to 4 that could, with restoration of the starting conditions, be completely reversed and were, moreover, independent of the starting pH of the crotoxin stock. The inability of the independent spectra of the isolated subunits to mimic crotoxin's behavior indicated that these changes were unequivocally correlated with the association state of the complex. With association of subunits A and B, the following spectral changes were noted: a quenching and shift of the emission maximum of the Trp fluorescence and a strong enhancement of both the far- and near-UV CD band intensities. There are two possible origins for the effects noted. One is that these spectral changes reflect merely the passive association of the two subunits. The second is that the association either induces or is induced by conformational changes in one or both subunits. For example, the far-UV changes in negative ellipticity with complex formation could be accounted for by either stabilization of the A–B interaction by an intersubunit ordered domain or conformational changes. On the other hand, the near-UV CD and fluorescence changes, and the accessibility to NBS oxidation of Trp residues, support the immobilization of aromatic residues (Trp residues in particular) in a hydrophobic domain created by the binding of the two subunits to form the complex and do not suggest conformational changes.

Remarkably similar fluorescence data were reported for the neurotoxic viper complex from *Vipera ammodytes*, which is considered analogous to crotoxin in that it also consists of an acidic subunit and a basic PhA homologue (Tchorbanov et al., 1977). Tchorbanov et al. (1977) concluded, in agreement with the results here, that Trp residues were located in the subunit interaction zone and that fluorescence changes reflected the pH modulation of complex stability.

The literature offers two perspectives on the presence or absence of conformational changes on complex formation. The first, based on observations of the interactions of subunits in oligomeric enzymes (Perham, 1975), suggests small but critical structural shifts result in functional differences. However, as a membrane-targeted "synergistic" system, crotoxin might be more properly compared to interacting polypeptides that work at or bind to membrane surfaces. CD changes observed with subunit association of luteinizing hormone (Bewley et al., 1972), with neurophysin–peptide interaction (Breslow, 1970), or with shifts between membrane-bound and self-assembled states of fd virus coat protein (Nozaki et al., 1978) establish an ample precedent for conformational changes between association–dissociation states of oligomeric membrane-active proteins. In crotoxin, acid pH dissociation may mimic a natural membrane-mediated effect whose consequence would be the release of the A subunit from the surface-bound B subunit.

The Gdn·HCl denaturation curves for crotoxin and its subunits indicated a relatively high resistance to the action of this perturbant (Bull & Breese, 1975), attributable to the high content of disulfides (Bull & Breese, 1975; Pace, 1975). Formation of crotoxin from its subunits apparently confers some stabilization, since at neutral pH the midpoint of the denaturation profile is shifted to a higher Gdn·HCl concentration and very little disruption occurs up to 3 M; for comparison, both isolated subunits are significantly affected by 3 M Gdn·HCl. Calculation of the apparent free energies of denaturation indicated that the purified subunits were less stable than their Gdn·HCl denaturation midpoints implied. At neutral pH, both crotoxin A and crotoxin B gave approximately 2 kcal/mol by an extrapolation procedure (Pace, 1975), although this value should be considered a minimum estimate (Pace, 1975). Surprisingly, the free energy of unfolding of crotoxin (4.5 kcal/mol) was apparently a simple sum of the subunit contributions and did not reveal any quantitative information on the free energy of dissociation of the complex. From a preliminary estimate of the apparent K_d of crotoxin dissociation ($K_d = 1.3 \times 10^{-10}$; Paradies & Breithaupt, 1975), a $\Delta G_{app} = 12.2$ kcal/mol is calculated, which would make the stability of the complex comparable to that of the dimer of hemoglobin (Chothia & Janin, 1975). Current work is aimed at elucidating quantitative aspects of the crotoxin A to crotoxin B interaction and regulating crotoxin complex stability by physiological effectors. It is obvious that a substantial perturbation must take place with crotoxin binding to erythrocyte or electroplaque membranes (Jeng et al., 1978; Bon et al., 1979) in order for the stable complex to dissociate.

The spectral data gives insight into the structures of the individual subunits. Crotoxin A consists of three polypeptide chains cross-linked by disulfides (Horst et al., 1972; Breithaupt et al., 1974). The near-UV CD is almost exclusively attributable to the disulfide content (Takagi & Ito, 1972; Takagi & Izutzu, 1974) since it is stable to acid and Gdn·HCl, sensitive to alkali (pH 11.5), and has the characteristic broad negative extremum with $\lambda_{max} = 255$ nm. Because the far-UV CD is sensitive to both acid and Gdn·HCl, the near-UV disulfide signal must be conformationally insensitive. The secondary structure of crotoxin A is best approximated by a summation of roughly equal "random-coil" and β-structure contributions, at both acid and neutral pH, with a small additional component of α helix at neutral pH. Of the currently described presynaptic snake toxins (Eaker, 1978), only β-bungarotoxin has a two-subunit structure comparable to that of crotoxin (Kondo et al., 1978a). The recently completed amino acid sequence of β-bungarotoxin revealed that the light subunit is, however, unlike crotoxin A as it is a highly basic single polypeptide chain and cross-linked to the heavy subunit by a single disulfide. Intriguingly, application of the secondary structure prediction rules of Chou & Fasman (1974) to the light subunit's sequence gives roughly 40% β sheets (Kondo et al., 1978b), very close to the 50% value for

crotoxin A reported here from CD measurement. Despite obvious differences, crotoxin A and the light chain of β-bungarotoxin may have similar solution structures and similar interaction domains with the larger, PhA-homologous subunits. Otherwise, β-bungarotoxin and crotoxin are not alike in either their near- or far-UV CD spectra (Hamaguchi et al., 1968).

Crotoxin B can be classed both structurally and functionally with the class of PhA isoenzymes (Eaker, 1978). It can undergo a reversible transition to another structural form at acid pH, detectable only by changes in the near-UV CD, that is similar to changes in the CD and fluorescence spectra of *Crotalus adamanteus* PhA (Wells, 1971) correlated with monomer–dimer interconversion. Consistent with this, pH- and concentration-dependent equilibria between monomers, dimers, and tetramers of crotoxin B have been described (Paradies & Breithaupt, 1975). Assignment of the near-UV CD signals must be deferred until the Gaussian components have been resolved. However, that the unequivocal assignment of the signals may be complex is indicated by the uninterpretable near-UV CD changes in *Bitis gabonica* PhA with selective chemical modification of important amino acid residues (Viljoen et al., 1976, 1977).

The recently published X-ray diffraction structure of bovine pancreatic PhA (Dijkstra et al., 1978) revealed that 50% of the structure was α helix and 10% was β structure. If the secondary sturcture estimates for crotoxin and its B subunit prove to be correct, this would indicate different folding patterns for crotoxin B and pancreatic PhA, two quite similar enzymes. This will be a key point for furture examination inasmuch as it suggests a major conformational difference between toxic and nontoxic forms of PhA. Further, the several interconverting states of crotoxin must be closely examined for their relevance to actions on different target membranes.

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References

Barela, T. D., & Darnall, D. W. (1974) *Biochemistry 13*, 1694.

Barth, G., Bunnenberg, E., & Djerassi, C. (1972) *Anal. Biochem. 48*, 471.

Bewley, T. A., Sairam, M. R., & Li, C. H. (1972) *Biochemistry 11*, 932.

Beychok, S. (1966) *Science 154*, 1288.

Bon, C., Changeux, J.-P., Jeng, T.-W., & Fraenkel-Conrat, H. (1979) *Eur. J. Biochem.* (in press).

Brahms, S., Brahms, J., Spach, G., & Brack, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3208.

Breithaupt, H. (1976a) *Toxicon 14*, 221.

Breithaupt, H. (1976b) *Naunyn-Schmiedeberg's Arch. Pharmacol. 292*, 271.

Breithaupt, H., Rübsamen, K., & Habermann, E. (1974) *Eur. J. Biochem. 49*, 333.

Breslow, E. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 493.

Bull, H. B., & Breese, K. (1975) *Biopolymers 14*, 2197.

Casey, J. P., & Martin, R. B. (1972) *J. Am. Chem. Soc. 94*, 6141.

Chang, C. C., & Lee, J. D. (1977) *Naunyn-Schmiedeberg's Arch. Pharmacol. 296*, 159.

Chang, C. C., Su, M. J., Lee, J. D., & Eaker, D. (1977) *Naunyn-Schmiedeberg's Arch. Pharmacol. 299*, 155.

Chen, Y., Yang, J. T., & Martinez, H. M. (1972) *Biochemistry 11*, 4120.

Chen, Y., Yang, J. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350.

Chothia, C., & Janin, J. (1975) *Nature (London) 256*, 705.

Chou, P. Y., & Fasman, G. D. (1974) *Biochemistry 13*, 222.

Cortijo, M., Panijpan, B., & Gratzer, W. B. (1973) *Int. J. Pept. Protein Res. 5*, 179.

Dijkstra, B. W., Drenth, J., Kalk, K. H., & Vandermaelen, P. J. (1978) *J. Mol. Biol. 124*, 53.

Eaker, D. (1978) *International Symposium on Proteins*, Taipei, Taiwan.

Fohlman, J., Eaker, D., Karlsson, E., & Thesleff, S. (1976) *Eur. J. Biochem. 68*, 457.

Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108.

Gubenšek, F., & Lapanje, S. (1974) *FEBS Lett. 44*, 182.

Halpert, J., & Eaker, D. (1975) *J. Biol. Chem. 250*, 6990.

Hamaguchi, K., Ikeda, K., & Lee, C.-Y. (1968) *J. Biochem. (Tokyo) 64*, 503.

Hanley, M. R. (1978) *Biochem. Biophys. Res. Commun. 82*, 392.

Hawgood, B. J., & Smith, J. W. (1977) *Br. J. Pharmacol. 61*, 597.

Hendon, R. A., & Fraenkel-Conrat, H. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1560.

Hendon, R. A., & Fraenkel-Conrat, H. (1976) *Toxicon 14*, 283.

Holmquist, B., & Vallee, B. L. (1973) *Biochemistry 12*, 4409.

Horst, J., Hendon, R. A., & Fraenkel-Conrat, H. (1972) *Biochem. Biophys. Res. Commun. 46*, 1042.

Jeng, T.-W., Hendon, R. A., & Fraenkel-Conrat, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 600.

Joubert, F., and van der Walt, S. J. (1975) *Biochim. Biophys. Acta 379*, 317.

Kondo, K., Narita, K., & Lee, C.-Y. (1978a) *J. Biochem. (Tokyo) 83*, 91.

Kondo, K., Narita, K., & Lee, C.-Y. (1978b) *J. Biochem. (Tokyo) 83*, 101.

Nozaki, Y., Reynolds, J. A., & Tanford, C. (1978) *Biochemistry 17*, 1239.

Pace, N. (1975) *CRC Crit. Rev. Biochem. 3*, 1.

Paradies, H. H., & Breithaupt, H. (1975) *Biochem. Biophys. Res. Commun. 66*, 496.

Perham, R. N. (1975) *Philos. Trans. R. Soc. London, Ser. B 272*, 123.

Philipson, K. D., & Sauer, K. S. (1973) *Biochemistry 12*, 3454.

Raghavendra, K., & Ananthanarayanan, V. S. (1976) *Biochem. Biophys. Res. Commun. 70*, 1042.

Saxena, V. P., & Wetlaufer, D. B. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 969.

Spande, T., & Witkop, B. (1967) *Methods Enzymol. 11*, 498.

Strickland, E. H. (1974) *CRC Crit. Rev. Biochem. 2*, 113.

Sutherland, J. C., Vickery, L. E., & Klein, M. P. (1974) *Rev. Sci. Instrum. 45*, 1089.

Takagi, T., & Ito, N. (1972) *Biochim. Biophys. Acta 257*, 1.

Takagi, T., & Izutsu, T. (1974) *J. Biochem. (Tokyo) 75*, 441.

Tchorbanov, B., Aleksiev, B., Bukolova-Orlova, T., Burstein, E., and Atanasov, B. (1977) *FEBS Lett. 76*, 266.

Viljoen, C. C., Visser, L., & Botes, D. P. (1976) *Biochim. Biophys. Acta 438*, 424.

Viljoen, C. C., Visser, L., & Botes, D. P. (1977) *Biochim. Biophys. Acta 483*, 107.

Vital Brazil, O., Excell, B. J., & Santana de Sa, S. (1973) *J. Physiol. (London) 234*, 63P.

Wells, M. A. (1971) *Biochemistry 10*, 4078.