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Role of Aromatic Residues in the Structure–Function Relationship of α-Bungarotoxin†

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ABSTRACT: The conformation of α-bungarotoxin and its cyanogen bromide cleaved and nitrated derivatives was studied by circular dichroism (CD). Native toxin contains no helices but some β forms and possibly β turns. Its ordered conformation is little affected when the peptide bond between Met-27 and Trp-28 is cleaved; however, the CD due to Trp-28 is abolished. The CNBr-cleaved derivative retains its immunoaffinity toward anti-toxin sera but loses its neurotoxicity toward the acetylcholine receptor. On the basis of both CD and fluorescence spectra, Trp-28 is probably stabilized by a short-range interaction with the carboxylate group of Asp-30. The ordered conformation of the toxin is also unaltered when one of the two tyrosine residues, identified as Tyr-54, is nitrated with tetranitromethane. This Tyr(NO_2)-54 derivative possesses both immunoaffinity and neurotoxicity. However, the toxin is denatured and loses its activities when the other tyrosine residue, Tyr-24, is also nitrated in 6 M guanidine hydrochloride, even after the denaturant is removed. Spectrophotometric titration of the toxin indicates that Tyr-54 has a normal apparent dissociation constant (pK_a = 9.7) and Tyr-24 ionizes at pH above 11.2. Both tyrosine residues are in a polar environment, but Tyr-24 is not readily accessible to reagents and is stabilized by long-range interactions, probably involving Glu-41.

Postsynaptic toxins (α-toxins) of snake venoms can be divided into two groups: short (type I) toxins contain 60–62 amino acid residues with four disulfide bonds and long (type II) ones 70–74 residues with five disulfide linkages (Lee, 1972; Tu, 1973; Yang, 1974; Karlsson, 1979). For instance, cobra neurotoxin of *Naja naja atra* (Taiwan), erabutoxin of *Laticauda semifasciata* (Okinawa), and toxin b of *Laticauda semifasciata* (Philippine) belong to type I, whereas α-cobratoxin of *Naja naja siamensis* and α-BuTX[1] of *Bungarus multicinctus* are type II toxins. The short toxin molecules consist of four cyclic polypeptides (loops) which show a high degree of sequence homology with the four major loops of the long toxin molecules, which have an extra minor loop. Because the five disulfide bonds are not fully established, α-BuTX can be tentatively assigned to have the following five loops (Figure 1): (1) residues 3–23 with a single charged group at Glu-20, (2) residues 16–44, which contain most of the invariant residues that recognize the acetylcholine receptor, (3) residues 48–59, (4) residues 60–65, and (5) residues 29–33, often termed as loop 2a which is inside loop 2. The role of this extra loop 2a in the long toxins has not been fully understood, al-

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[1] Abbreviations: α-BuTX, α-bungarotoxin; CB-I and CB-II, cyanogen bromide cleaved components I and II; NP-I and NP-II, mono- and dinitrotyrosyl derivatives; CD, circular dichroism; Tris, tris(hydroxymethyl)aminomethane.

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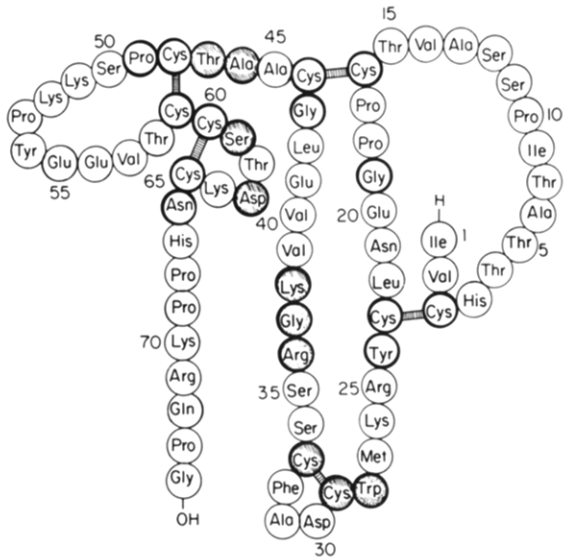

FIGURE 1: Primary structure of α-bungarotoxin from *Bungarus multicinctus*. Hatched bars represent possible disulfide linkages. According to Karlsson (1979), thick lines indicate invariant residues in short and long neurotoxins and cardiotoxins, hatched circles invariant residues only in long neurotoxins, and stippled circles invariant residues in long and short neurotoxins.

though recent X-ray diffraction studies of α-cobratoxin of *Naja naja siamensis* indicate that the protrusion of loop 2a at the end of the forked tongue configuraton of loop 2 makes this loop much broader in the long toxin than in the short toxin (Walkinshaw et al., 1980).

α-BuTX can tightly bind to the acetylcholine receptor and block the neuromuscular junction [see, for instance, Lee (1972)]. It has often been used to identify membrane preparations, which contain the receptor (Changeux et al., 1970; Miledi et al., 1971; Raftery et al., 1972; Brockes & Hall, 1975). Its structure–function relationship is therefore an important subject in molecular neurobiology. We report herein the conformation of α-BuTX and its cyanogen bromide cleaved and nitrated derivatives. The native toxin molecule contains a considerable amount of β form (and some β turns). An intact peptide bond between Met-27 and Trp-28 of the toxin is essential for its neurotoxicity, but CNBr-cleaved α-BuTX retains its immunoaffinity. Nitration of Tyr-54 does not affect the biological activities of α-BuTX, but the toxin is denatured and loses both its immunoaffinity and neurotoxicitiy when an NO_2 group is introduced to Tyr-24. Trp-28 is probably restricted by a short-range interaction with Asp-30, whereas Tyr-24 is an "internal" residue (i.e., exposed but not free to rotate), which is stabilized by long-term interactions, possibly involving Glu-41.

Experimental Procedures

Materials. Crude venom of *Bungarus multicinctus* was obtained from Cheng Hsin Tang Chemical Co., Taipei, Taiwan. CM-Sephadex was purchased from Pharmacia, Uppsala, Sweden, and CM-cellulose from Schleicher & Schuell, Dassel, West Germany. All other chemicals were of reagent grade. Water was double distilled.

Preparation of α-Bungarotoxin. Crude venom (300 mg) was dissolved in a minimum amount of 0.05 M ammonium acetate (pH 5.0) and applied to a CM-Sephadex C-25 column (1.2 × 25 cm). It was eluted with a nonlinear gradient; 1 M ammonium acetate (pH 6.8) was added to a mixing chamber containing 1 L of 0.05 M ammonium acetate (pH 5.0) to gradually increase the salt concentration. Four-milliliter fractions were collected and their absorbances at 280 nm recorded (Figure 2). Each fraction was assayed for its phospholipase A_2 activity (Strong et al., 1976) and tested for its inhibition of muscle contraction and blocking of the acetylcholine receptor activity (Ginsberg & Warriner, 1960). The purity of the fractions was tested by disc polyacrylamide gel electrophoresis in 0.35 M β-alanine at pH 4.5 (Reisfield et al., 1962).

Component III was further chromatographed on a CM-cellulose column (1.2 × 25 cm) under a linear 1-L gradient from 0.05 (pH 5.0) to 0.5 M (pH 6.8) ammonium acetate. The purified α-BuTX was free of phospholipase A_2 activity.

Toxicity. Eight albino mice each weighing 15–20 g were injected intraperitoneally with α-BuTX. The 50% lethal dosage, LD_{50}, was determined (Litchfield & Wilcoxon, 1949).

Immunization. An emulsion of one part of α-BuTX and four parts of complete Freund's adjuvant was weekly injected into the popliteal lymph node in the rear legs of rabbits for 6 weeks. The first dosage was one-third of LD_{50}; subsequent dosages were gradually increased but to no more than one-half of LD_{50}. Antisera were collected from the immunized animal 1 week after the last injection. Double immunodiffusion (Ouchterlony, 1958) and a quantitative precipitin test (Roitt et al., 1958) followed.

Reaction with Cyanogen Bromide. α-BuTX and its derivatives can be cleaved between Met-27 and Trp-28 by a modified method of Gross & Witkop (1962). To an ampule containing 20 mg of the toxin in 5 mL of 70% formic acid was added 100 M excess of CNBr with respect to methionine. The ampule was sealed and stirred for 24 h at room temperature. The reaction was stopped by adding 10 volumes of water. The solution was lyophilized. A control in which α-BuTX was incubated with formic acid but without CNBr was done by the same procedure.

The CNBr-cleaved toxin solutions were chromatographed on a CM-cellulose column (1.5 × 10 cm) and eluted with a 1-L linear gradient: 500 mL of 0.05 M ammonium acetate (pH 5.0) against 500 mL of 0.5 M ammonium acetate (pH 6.8). Four-milliliter fractions were collected and their absorbances at 280 nm recorded (Figure 3). The two nearly equal components CB-I and CB-II were homogeneous by disc polyacrylamide gel electrophoresis.

Nitration of α-BuTX. The tyrosine residues of α-BuTX were converted into 3-nitrotyrosines by a modified procedure of Sokolovsky et al. (1966). To 20 mg of α-BuTX in 10 mL of 0.05 M Tris-HCl (pH 8.0) was added 10-fold molar excess of tetranitromethane (in 95% ethanol) dropwise with vigorous stirring (experiment A). To 15 mg of α-BuTX in 7.5 mL of 0.05 M Tris-HCl and 6 M guanidine hydrochloride was added 150-fold molar excess of the reagent (experiment B). In both cases the formation of nitrotyrosine was followed by an increase in absorbance at 428 nm. The reaction was completed within 1 h. The mixture was immediately fractionated on a Sephadex G-50 column preequilibrated with 0.05 M ammonium acetate (pH 6.8): a major component NP-I or NP-II and two minor components NP-A and NP-B or NP-A′ and NP-B′ (Figure 4). Both components NP-I and NP-II were further purified on a CM-cellulose column, and each gave a single band by disc polyacrylamide gel electrophoresis.

Reduction of Disulfide Bonds and S-Carboxymethylation. The toxins were reduced by 40-fold excess of dithioerythritol in water at room temperature for 24 h. NaOH (0.1 N) was added to keep a constant pH of 8.0. The reduced product was carboxymethylated by adding 5-fold excess of iodoacetate at the same pH. The S-carboxymethylated product was desalted on a Sephadex G-50 column and lyophilized to dryness.

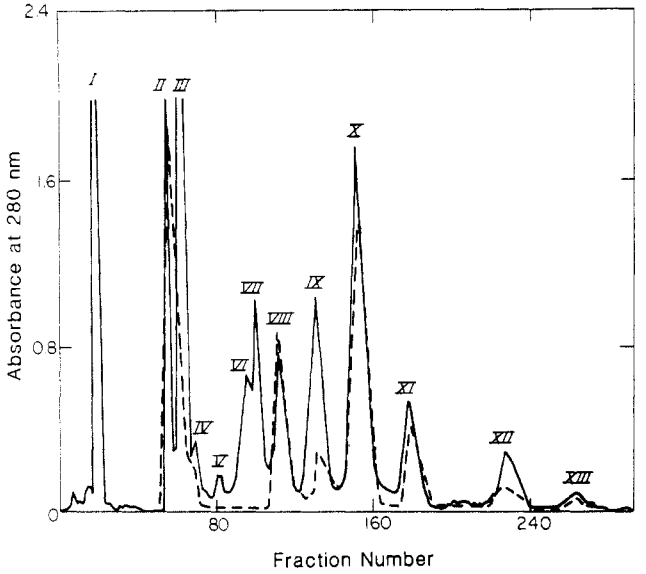

FIGURE 2: Purification of α-bungarotoxin (300 mg of crude venom on a CM-Sephadex C-25 column). Dashed line represents the phospholipase A_2 assay.

Amino Acid Analysis. Amino acid composition was determined by the method of Spackman et al. (1958) on a Yanagimoto LC-5A amino acid analyzer. Tryptophan residues were determined by ultraviolet absorption method (Goodwin & Morton, 1946).

The phenylthiohydantoin derivative of the NH_2-terminal amino acid (PTH amino acid) of CNBr-cleaved toxin was prepared by the Edman method (1970) and determined by gas–liquid chromatography on a Beckman Model GC-65 with a glass column (0.4 × 120 cm) packed with 10% SP-400 on Chromosorb.

Spectrophotometry. The concentration of α-BuTX was determined on a Cary 14 spectrophotometer. An absorption coefficient, $A^{1\%}_{1cm}$, of 1.20 at 280 nm was based on the dry weight of the protein (over P_2O_5 in a desiccator).

For spectrophotometric titration the pH of the α-BuTX solution was adjusted by NaOH solutions (0.1 N between pH 7.0 and 10.5, 1.0 N between pH 10.5 and 11.5, and 6.0 N for pH above 11.5); the pH increment was no more than 0.4 unit for each addition of NaOH. The differential molar absorbance at 295 nm, $\Delta\epsilon_{295}$, was calculated against a reference solution at pH 7.1.

Circular Dichroism. CD spectra were measured on a Jasco J-20 spectropolarimeter under constant nitrogen flush. Fused-silica cylindrical cells of various light paths were used to keep the absorbance of the solution below 2. The temperature was controlled by connecting the water jacket of the cell to a Hotpack constant-temperature regulator. The solutions were clarified through a Gelman filter (5-μm pore size). Data were expressed as mean residue ellipticity, $[\theta]$, with a mean residue weight of 108.

Fluorescence. All spectra were measured on a Hitachi MPF-4 fluorescence spectrophotometer. A rectangular 1-cm cell was inserted in a thermostated cell holder.

Results

Preparation of α-BuTX. Crude snake venom can be fractionated into 13 well-defined peaks (Figure 2, solid line), although there are variations among the reported chromatograms (Lee et al., 1972; Clark et al., 1972; Eterovic et al., 1975; Abe et al., 1977). The distribution of the components also varies with the batches of venom. Only component I has a higher absorbance at 260 nm than at 280 nm. Components II–XIII are proteins but none of them is homogeneous. Phospholipase A_2 activity spreads over components II and VIII–XIII (Figure 2, dotted line). All components except component I inhibit the contraction activity of chicken cervix muscle, whereas components III–VIII block the activity of the acetylcholine receptor.

Components III and X corresponds to α- and β-BuTX (Lee et al., 1972). Occasionally the variations among batches of venom were too great to clearly define the two toxins. However, they can be identified by immunodiffusion because anti-α-BuTX shows no immunoaffinity toward components IV–XIII and anti-β-BuTX gives little, if any, precipitin with components IX and XI–XIII. Table I shows the amino acid composition of purified α-BuTX free of phospholipase A_2 (see Experimental Procedures), which agrees with that reported by Mebs et al. (1971).

CNBr-Cleaved α-BuTX. The two components of CNBr-cleaved α-BuTX (Figure 3) have the same amino acid composition as that of the parent toxin, except that Met-27 of components CB-I and CB-II is converted to homoserine and homoserine lactone, respectively (Table I). Component CB-I contains homoserine because it is eluted at a lower salt concentration than component CB-II, which apparently contains homoserine lactone. Because acidic hydrolysis of proteins may

Table I: Amino Acid Composition of α-Bungarotoxin and Its Derivatives

	α-BuTX[a]	CB-I	CB-II	NP-I	NP-II
Asx	4	4.2	4.3	3.9	4.1
Thr	7	7.0	6.8	6.5	6.4
Ser	6	5.6	5.7	5.4	5.3
Glx	5	5.1	5.3	4.8	4.7
Pro	8	8.4	8.1	7.5	7.7
Gly	4	3.9	4.1	4.0	4.0
Ala	5	5.2	5.3	5.1	4.9
½-Cysscy	10	9.1	9.2	9.2	9.1
Val	5	4.5	4.7	4.7	5.0
Met	1	0	0	0.8	0.9
Ile	2	1.8	1.9	1.7	1.6
Leu	2	2.0	2.0	1.6	1.8
Tyr	2	1.7	1.8	0.9	0
Phe	1	0.8	1.2	0.8	0.7
Trp	1	1.2[b]	1.2[b]		
Lys	6	5.6	5.5	6.1	5.8
His	2	2.0	1.9	1.7	1.6
Arg	3	2.6	2.7	2.9	2.8
3-NO_2-Tyr	0			0.9	1.9
homoserine and homoserine lactone	0	0.7	0.6	0	0

[a] Taken from Mebs et al. (1971, 1972). [b] Estimated by the method of Goodwin & Morton (1946).

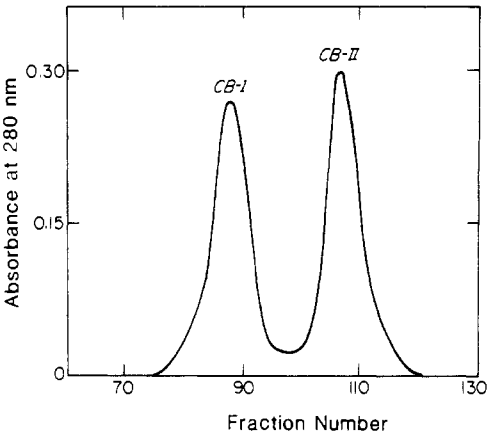

FIGURE 3: Separation of cyanogen bromide cleaved α-bungarotoxin on a CM-cellulose column. Starting material: 20 mg of toxin.

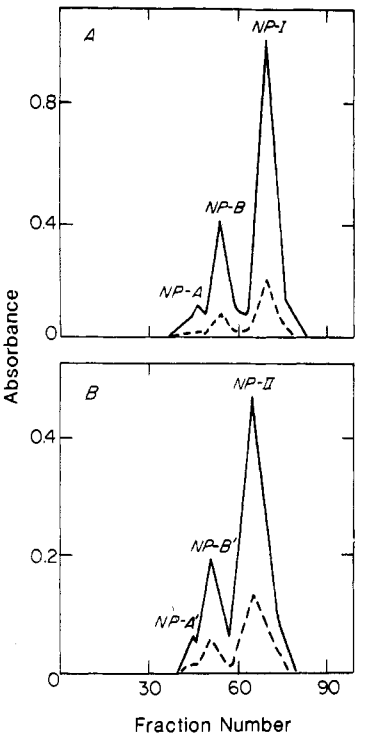

FIGURE 4: Elution of nitrated α-bungarotoxin on a Sephadex G-50 column preequilibrated with 0.05 M ammonium acetate (pH 6.8). Absorbances: (—) at 280 nm; (- - -) at 428 nm. Concentration of toxin used for nitration: 2 mg/mL in 0.05 M Tris-HCl (pH 8.0) with (B) and without (A) 6.0 M guanidine hydrochloride.

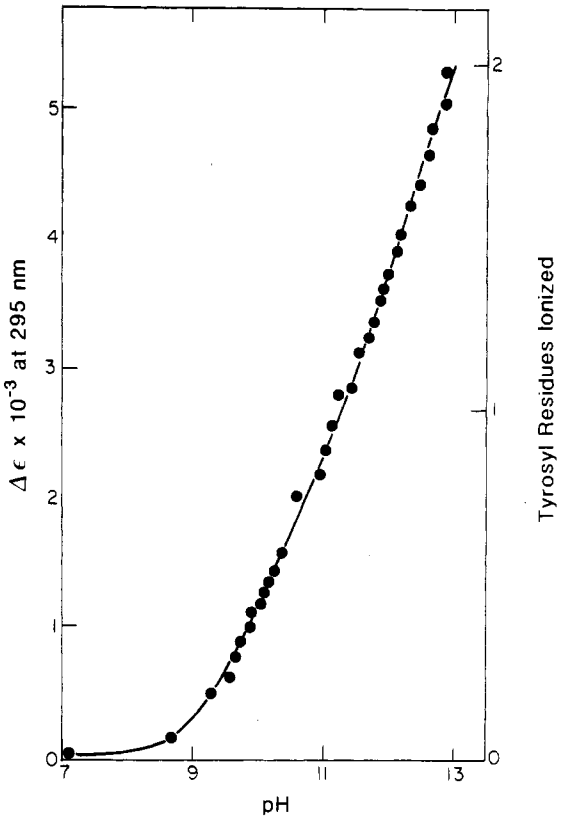

FIGURE 5: Spectrophotometric titration of α-bungarotoxin in 0.15 M KCl at 25 °C.

convert homoserine lactone into homoserine (Armstrong, 1949), components CB-I and CB-II cannot be quantitatively determined by conventional amino acid analysis. Suffice it to say, the methionine residues in both components are completely modified. (An anonymous reviewer points out that homoserine lactone should be readily obtainable, since it is resistant to carboxypeptidase A.)

Ile-1 and Trp-28 are located at the NH_2 termini of the two polypeptide chains of components CB-I and CB-II. However, Trp-28 does not react with phenyl isothiocyanate during the Edman degradation. If the five disulfide bonds of the toxin are first broken by reductive carboxymethylation, Trp-28 can be identified as the end group, and the two polypeptide chains of components CB-I and CB-II can be separated by chromatography (Sephadex G-50).

Nitrated α-BuTX. The nitrated components of α-BuTX (Figure 4) also have the same amino acid composition as that of native toxin (Table I). Component NP-I contains one nitrotyrosine and component NP-II two nitrotyrosines. Component NP-I shares the same elution volume as α-BuTX, and it is eluted slower than component NP-II. The minor components are probably polymerized substances through intermolecular cross-linking between tyrosine residues via phenoxide free radical intermediates (Boesel & Carpenter, 1970; Thomas et al., 1968).

Reactivity of Tyrosine Residues. The toxin has two tyrosine residues at positions 24 and 54, but only tyrosine can be titrated spectrophotometrically with a normal apparent pK_a of 9.7, and the other tyrosine begins to ionize at pH above 11.2 (Figure 5). The differential molar absorption coefficient at 295 nm for the ionized tyrosine is estimated to be 2800 M^{-1} cm^{-1}, which agrees with the literature value (Tanford et al., 1955). The titration profile seems to suggest that the "internal" tyrosine is in a polar environment and accessible to the solvent.

Assuming a molar absorption coefficient of 4100 M^{-1} cm^{-1} at 428 nm for 3-nitrotyrosine (Sokolovsky et al., 1966), component NP-I (Figure 4A) is estimated to contain one nitrotyrosine and component NP-II (Figure 4B) two nitrotyrosines, which agree with the amino acid analysis (Table I).

By CNBr cleaving of the nitrated component NP-I and then breaking of the disulfide bonds by reductive carboxymethylation, two polypeptides can be isolated: one polypeptide corresponding to residues 1–27 contains an intact tyrosine and the other corresponding to residues 28–74 has a nitrotyrosine. Thus, Tyr-54 is the normal one and Tyr-24 the internal one during spectrophotometric titration.

Biological Activities. Both components CB-I and CB-II are no longer lethal to mice which are injected intraperitoneally with as high as 5 μg of either component per g of body weight. But their immunoaffinity toward anti-α-BuTX remains strong; the maximum precipitin produced by the antisera and component CB-I or CB-II is about 70% of that by the antisera and α-BuTX. Apparently, the "native formats" of α-BuTX for the antibody binding are retained in both components. The sites for neurotoxicity and immunoaffinity may be located separately in the α-BuTX molecule.

Component NP-I is biologically active. However, its immunoaffinity toward anti-α-BuTX sera is less than that of α-BuTX (Figure 6). The equivalence points are about 2-fold apart, suggesting that some of the antigenic determinants have been lost or distorted upon nitration. The LD_{50} of component NP-I is 0.16 μg per g of body weight of mice as compared with 0.14 μg for α-BuTX. When 0.10 μg of α-BuTX or component NP-I was applied to a preparation of baby chicken biventer which was immersed in 20 mL of Krebs solution bubbled with a mixture of 95% O_2 and 5% CO_2, the muscle contraction was completely abolished by α-BuTX and component NP-I within 23.0 and 26.0 min, respectively. On the other hand, component NP-II is nontoxic even when the mice are injected with 2.2 μg of it per g of body weight. It does not block the activity of the acetylcholine receptor. Its immunoaffinity to anti-α-

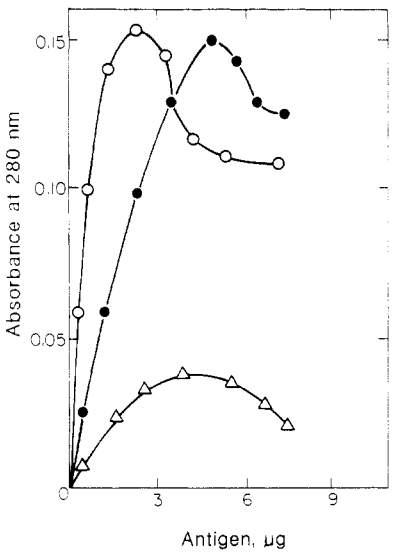

FIGURE 6: Precipitin assay of α-bungarotoxin and its nitrated derivatives. The precipitates were dissolved in 1 N NaOH. (○) α-BuTX; (●) component NP-I; (△) component NP-II.

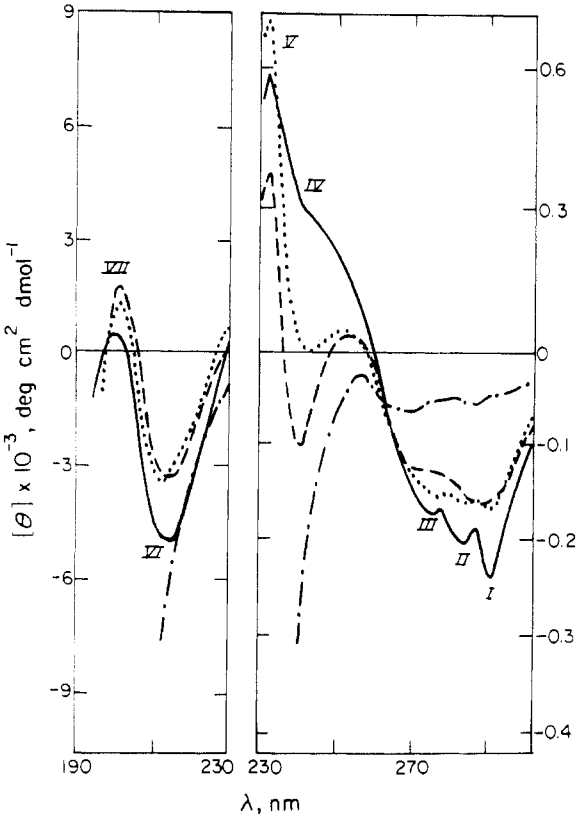

FIGURE 7: Circular dichroism of α-bungarotoxin. Solvents: (—) water; (···) 6 M guanidine hydrochloride (pH 7.0); (- - -) 1,2-ethanediol; (-·-) 1:1 (v/v) mixture of 1,2-ethanediol and 1-propanol.

BuTX is less than 20% of that formed by α-BuTX and its antisera (Figure 6). Thus, the nitration of Tyr-24 of α-BuTX destroys both its neurotoxicity and immunoaffinity.

Conformation of α-BuTX. α-BuTX is a small compact protein, which is sterically restricted by five –S–S– bonds and stabilized by side-chain interactions. Its CD spectra in water has at least seven CD bands: bands I–V in the near-UV region arise from nonpeptide chromophores (Figure 7, right side) and bands VI and VII are mainly due to peptide chromophores (Figure 7, left side). The lack of a double minimum at 222 and 208–210 nm that is characteristic of a helical conformation suggests that α-BuTX has no helices. Neither is α-BuTX

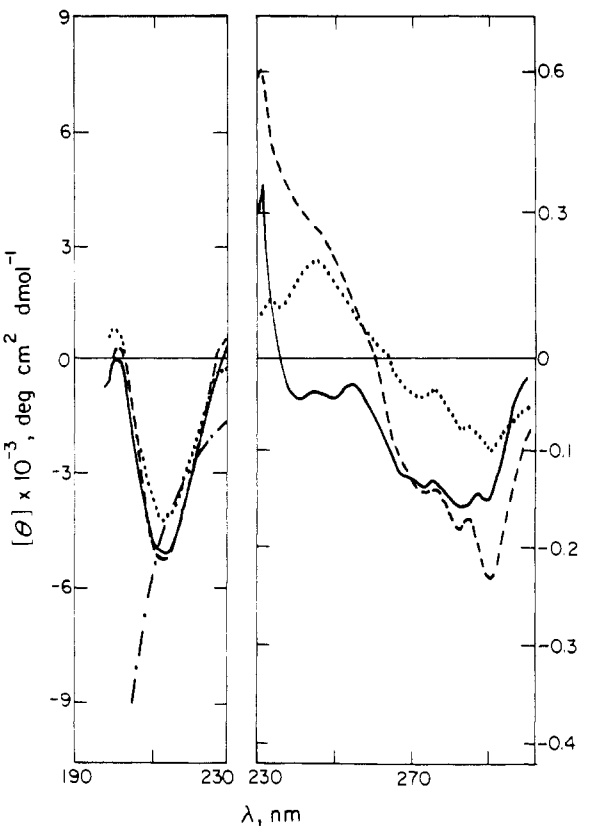

FIGURE 8: pH dependence of CD of α-bungarotoxin. Curves for pHs: (···) 2.7; (- - -) 7.0; (—) 10.8; (-·-) 12.4.

completely unordered, which would have shown a strong negative band near 197 nm. Band VI is a negative band at 214 nm, which bears some resemblance to that of a β form around 217 nm. Band VII is a weak positive one with a maximum at 200 nm, which is much smaller than that of the β form around 195 nm. Nevertheless, the α-BuTX molecule contains some ordered structure other than the helix, probably a mixture of β form, β turn, and unordered form (see Discussion). The CD of α-BuTX in 1 M KCl is the same as that in water (not shown).

The toxin is dentured in 6 M guanidine hydrochloride. All seven well-defined CD bands disappeared. Instead, the spectrum shows a strong negative band below 210 nm for an unordered form. In the near-UV region there remain some weak broad negative bands. Removal of the denaturant restores all the CD bands of the native toxin.

In 1,2-ethanediol and in a 1:1 (v/v) mixture of 1-propanol and 1,2-ethanediol the CD spectra of α-BuTX, except band IV, are nearly the same as the spectrum in aqueous solution, although the band positions slightly shift and the magnitudes of the extrema change to some extent. In the near-UV region the fine structures are blurred, whereas band IV between 235 and 250 nm greatly diminishes. Apparently, the secondary structure of α-BuTX in nonaqueous solution is virtually the same as that in aqueous solution, and the chromophore for band IV may be on the surface of the protein molecule and is therefore sensitive to the change in the solvent environment.

Assignment of Bands IV and V. The conformation of α-BuTX is stable over a wide range of pHs (Figure 8). Even at pH 2.7 or 10.8, the CD bands VI and VII of the peptide chromophores remain essentially the same as in neutral solution. In contrast, the CD due to nonpeptide chromophores is sensitive to pH. Band IV no longer exists when Tyr-54 is deprotonated at pH 10.8, but the profile of other bands still remains. Raising the pH to 12.4 denatures the protein, and

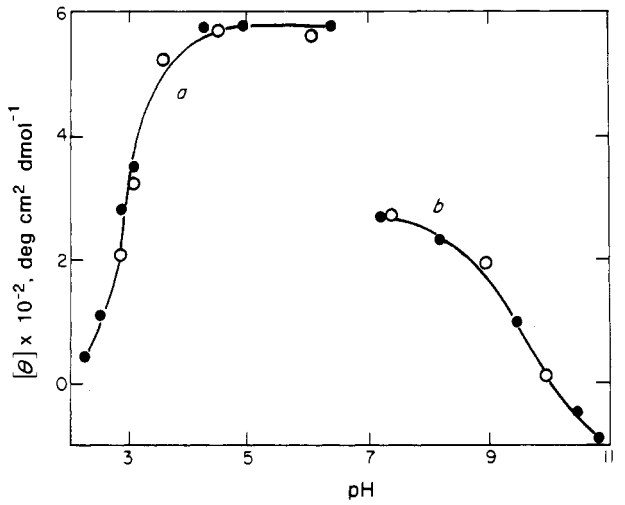

FIGURE 9: pH dependence of CD bands IV and V. Curve a for band V at 232 nm: (●) from pH 7.0 to 2.2; (○) back-titration to neutral pH. Curve b for band IV at 245 nm: (●) from pH 7.0 to 11.0; (○) back-titration to neutral pH.

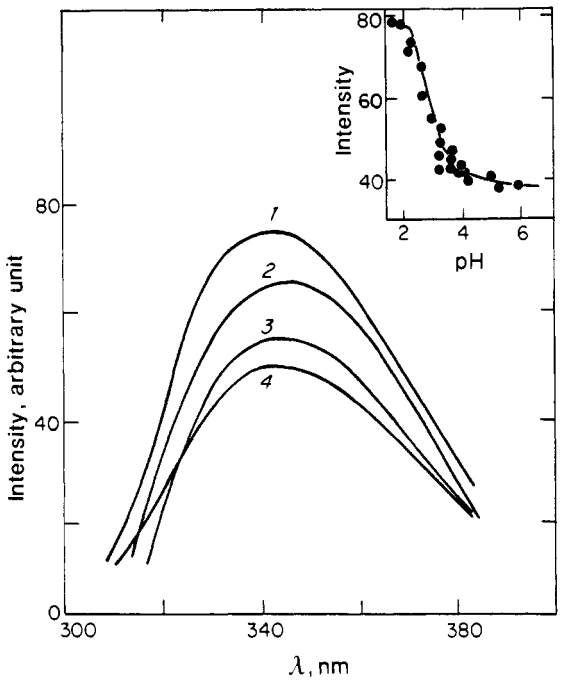

FIGURE 10: Fluorescence of α-bungarotoxin in aqueous solution at room temperature. Excitation wavelength: 295 nm. Curves for pHs: (1) 2.0; (2) 2.7; (3) 3.3; (4) 4.0. (Inset) Fluorescence at 345 nm vs. pH.

all bands disappear, except a new band below 200 nm due to an unordered form emerges. On the other hand, at pH 2.7 band V disappears and bands I, II, and III diminish, whereas band IV is unaltered.

Figure 9 shows the pH dependence of the CD band IV at 245 nm and band V at 232 nm. The magnitude of band IV drastically decreases above pH 9, and the sign changes from positive to negative around pH 10 with a middle point at pH 9.8 (curve b), which agrees with the pK_a of an exposed tyrosine residue of proteins. Accordingly, we assign band IV to Tyr-54.

The CD band V shows a sharp drop in magnitude with decreasing pH (Figure 9, curve a). Its midpoint at pH 3.2 coincides with the pK_a of an inaccessible acidic residue of proteins (Tanford, 1961). Very likely the asymmetric environment surrounding the band V chromophore is stabilized by a carboxylate ion of an acidic residue. This is supported by fluorescence studies of α-BuTX (Figure 10). When Trp-28

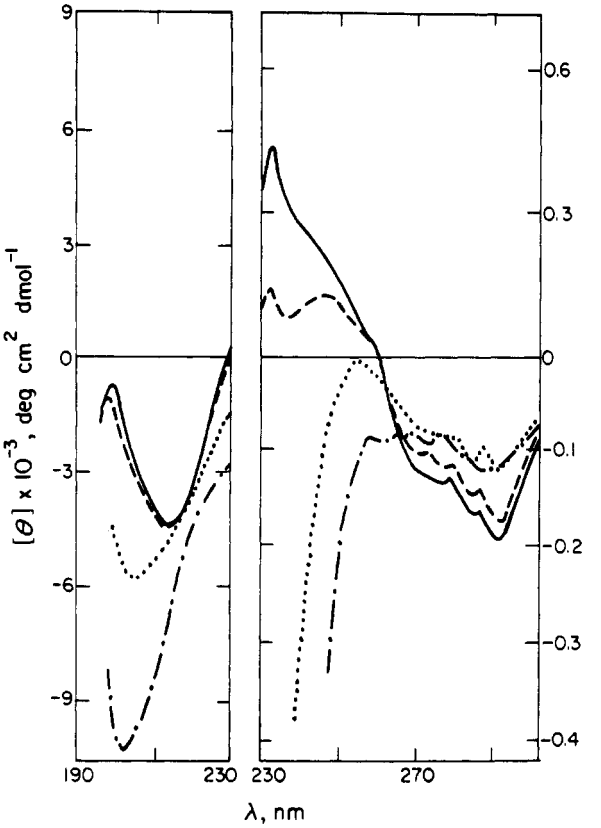

FIGURE 11: Temperature dependence of α-bungarotoxin in aqueous solution. Curves: (—) 40 °C; (- - -) 60 °C; (···) 80 °C; (-·-) 90 °C.

is excited at 295 nm, the emission spectrum has a maximum at 345 nm, and its intensity increases markedly with decreasing pH with a midpoint at pH 2.9 (Figure 10, inset), suggesting that the environment around Trp-28 is affected by the state of ionization of an acidic residue. Thus, we assign band V to Trp-28. According to the classification of tryptophan residues in proteins by fluorescence spectroscopy (Burstain et al., 1973), Trp-28 may be located on the surface of α-BuTX but have limited contact with the solvent.

Thermal Denaturation. The conformation of α-BuTX is temperature dependent (Figure 11) just like that of cobra neurotoxin (Chen et al., 1977) and cobra cardiotoxin (Hung & Chen, 1977). The CD bands VI and VII of the peptide chromophores and the bands in the near-UV region remain stable up to about 70 °C, except that band V diminishes above 60 °C. Drastic changes for all the bands occur between 80 and 90 °C. At 90 °C bands I, II, and III are blurred and bands IV and V disappear. A strong negative band at 202 nm indicates that the protein is denatured.

CD of Modified Toxins. Before α-BuTX was modified by CNBr, we did a control experiment by exposing the toxin to 70% formic acid in a sealed tube for 24 h, except no CNBr was added. This control α-BuTX in neutral solution had the same conformation and biological activity as the native toxin, suggesting that any possible denaturation by the formic acid treatment was reversed upon return to a normal aqueous environment. The CD spectrum of the CNBr-cleaved derivative of α-BuTX in neutral solution (Figure 12) indicates that both components CB-I and CB-II lose bands I–V in the near-UV region, but bands VI and VII due to peptide chromophores remain and the minimum at 214 nm is not shifted. Apparently, the scission of the peptide bond between Met-27 and Trp-28 in α-BuTX alters the local conformation around the aromatic residues but without completely unfolding the protein

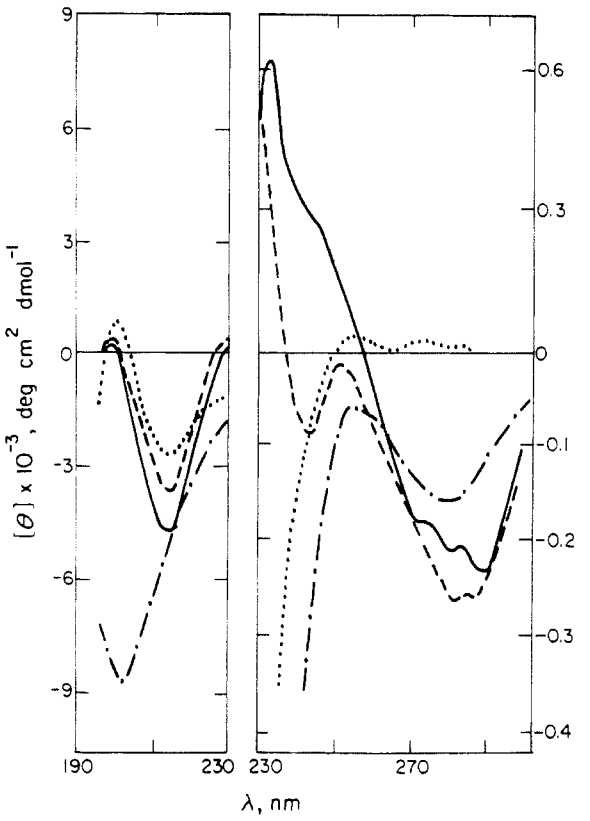

FIGURE 12: Circular dichroism of modified α-bungarotoxins. Curves: (—) α-BuTX in water (control); (---) (···) nitrated components NP-I and NP-II in 0.05 M Tris-HCl (pH 8.0); (···) CNBr-cleaved component CB-I or CB-II in 0.05 M phosphate buffer (pH 7.0).

molecule. This may account for the retention of immunoaffinity of components CB-I and CB-II and the loss of their blocking activity to the acetylcholine receptor.

The CD spectra of nitrated derivatives of α-BuTX (Figure 12) indicate that component NP-I does not have band IV just as α-BuTX in alkaline solution (pH 10.5) or in nonaqueous solution (cf. Figures 7 and 8), but all other bands are almost the same as those of native neutral solution (cf. Figure 7). (The pK_a of 3-nitrotyrosine is about 6.8; thus, the nitrated tyrosine residue in α-BuTX is largely ionized at pH 8.0). This supports our assignment of band IV to Tyr-54, the nitration of which does not destroy the ordered conformation of the toxin or the local conformation surrounding Trp-28. In contrast, component NP-II loses both bands VI and VII and resembles α-BuTX in strongly alkaline solution (pH 12.8; cf. Figure 8). In the near-UV region bands I–V are replaced by a weak broad band with a minimum near 280 nm. Thus, the nitration of the internal Tyr-24 not only alters the local conformation surrounding Tyr-24 and Trp-28 but also prevents the toxin from refolding into its native conformation even after the denaturant (6 M guanidine hydrochloride) is removed.

Discussion

This work mainly concerns the role of Tyr-24, Trp-28, and Tyr-54 in preserving the structural integrity of α-BuTX or, more properly, the role of segments of the polypeptide chain that include aromatic residues, the conformation of which can be monitored by physicochemical methods. The sequence homologies indicate that several residues including Tyr-24, Trp-28, and Arg-36 are invariant amino acid residues and Asp-30 is a highly conserved residue [see, for instance, Karlsson (1979)]. In the absence of X-ray diffraction studies of α-BuTX, we can superimpose the primary structure of the long α-BuTX onto the three-dimensional structure of the long α-cobratoxin from *Naja Naja siamensis* venom (Walkinshaw et al., 1980) and make some educated guesses. If both toxins have similar structures, Glu-41, Glu-55, and Glu-56 are expected to be close to Tyr-24 and Trp-28, and Asp-30 is also near Trp-28 (cf. Figure 1). Both Tyr-24 and Trp-28 in loop 2 may be located in a triple-stranded β-pleated sheet consisting of portions of loops 1, 2, and 3. By structural analogy to α-cobratoxin, the –NH– and –CO– groups of Cys-33 may be hydrogen bonded to the –CO– of Asp-30 and =NH of the guanidino group of Arg-36, respectively. In contrast, the short toxins, Philippine sea snake toxin b (Tsernoglou & Petsko, 1977) and erabutoxin b (Low, 1979), may have a hydrogen bond between the side chains of Arg-37 and Asp-31 according to Tsernoglou et al. (1978).

The structure–function relationship of many α-toxins has been deduced from chemical modification studies of invariant residues [for two recent reviews, see Karlsson (1979) and Low (1979)]. The X-ray diffraction studies of several α-toxins further indicate the importance of the triple-stranded β-pleated sheet, which maintains the shape of the toxin molecules and preserves the protruding region of loop 2 that most probably binds to the acetylcholine receptor. Thus, both Tyr-24 and Trp-28 of α-BuTX are important in maintaining its structural integrity and neurotoxicity, although no generalizations about the reactivity of these aromatic residues in toxins can be made at present. We have postulated that Trp-28 may be stabilized by short-range interactions with an acidic residue; Asp-30 appears to be a probable candidate, and its carboxylate group can interact with the indole group of the tryptophan residue. This is in accord with the quenching of fluorescence due to Trp-28 by a neighboring protonated acidic group (Figure 10). In contrast, the conformation of Trp-29 in cobra neurotoxin from *Naja naja atra* venom, a short toxin, is not affected by the protonation of acidic residues according to our CD (Chen et al., 1977) and fluorescence (Y.-H. Chen, unpublished data) studies. It seems that the local conformation of the invariant tryptophan residue in a long toxin may differ from that in a short toxin. On the other hand, the microenvironment of Trp-29 in erabutoxin, another short toxin, appears to be in a rigid conformation on the basis of NMR studies (Inagaki et al., 1980).

We have also postulated that Tyr-24 is an internal residue in a strongly polar rather than nonpolar environment (see Figure 5). It is stabilized by long-range interactions with other residues and probably surrounded by carboxylate groups. Because the NMR studies of several short toxins indicate that the invariant Tyr residue is close to the carboxylate group of an acidic residue (Fung et al., 1979; Inagaki et al., 1980), the sequence homologies between the long and short toxins may point to a similar proximity between Glu-41 and Tyr-24 in α-BuTX. This tyrosine residue can only be nitrated in the presence of a denaturant. Tyr(NO_2)-24 is mostly deprotonated at pH 8.0 and cannot be hydrogen bonded to a carboxylate group. The electrostatic repulsion between ionized Tyr-24 and Glu-41 or the steric hindrance due to the introduction of nitro groups or both may weaken the long-range interactions. Accordingly, component NP-II of α-BuTX is unfolded (see Figure 7), even after the removal of 6 M guanidine hydrochloride.

CD spectroscopy is one of the most sensitive physical methods for studying protein conformation in a solution. The α helix, β form, and unordered form all display their characteristic CD bands in the UV region (Greenfield & Fasman, 1969; Chen et al., 1974). More recently, the CD of the β turn has been theoretically calculated by Woody (1974). Quan-

titative analyses of CD spectra of proteins usually give a good to excellent estimate of the helicity (Chen et al., 1974). But the estimates of the β form remain to be equivocal. The CD of the β turn bears some resemblance to that of the β form, and the computed β_t values seem to be not significantly related with the X-ray results of proteins of known three-dimensional structure (Chang et al., 1978). Our CD results of α-BuTX, which qualitatively are similar to those reported by Hamaguchi et al. (1968), indicate the absence of the helical conformation, unless the optical activity due to nonpeptide chromophores is so large as to obscure that of peptide chromophores, a situation that is highly unlikely and lacks any experimental evidence. The position and sign of band VI of α-BuTX suggest the presence of a considerable amount of the β form, although the presence of β turns cannot be ruled out. This agrees with the predicted secondary structure based on amino acid sequence (Chou & Fasman, 1974a,b, 1978), by which α-BuTX contains about 30% β form and 40% β turn (Chen et al., 1975; Hseu et al., 1977).

The CD of the β form usually shows a negative band near 217 nm and a positive one around 195 nm; the ratio of the magnitude of the positive to negative band is about 2. This is not the case for the CD of α-BuTX (Figure 7), which shows a weak positive band. One possibility is that a combination of β form and unordered form could result in a weak positive or negative band around 200 nm. Furthermore, we still do not know much about the CD of nonpeptide chromophores in the far-UV region, which might cancel the positive CD of the β form in α-BuTX. The status of CD of the β turns is even more uncertain at present. Therefore, we do not attempt to make any quantitative analysis of the CD spectra of α-BuTX.

In the near-UV region the fine CD structures are attributed to the aromatic groups of a protein: the wavelength ranges for tryptophan, tyrosine, and phenylalanine are usually located between 280 and 295, 265 and 290, and 250 and 270 nm, respectively (Strickland, 1974). For instance, the tyrosine residues of ribonuclease A show three bands at 276, 283, and 288.5 nm (Horwitz et al., 1970), and the tryptophan residues of carboxypeptidase have bands at 286 and 293.5 nm (Fretto & Strictland, 1971). The CD spectrum of α-BuTX (Figure 7) suggests that band I at 290 nm may be assigned to Trp-28, as has been band V, and band III near 275 nm arises from Tyr-24, as does band IV (the contribution of Tyr-54 might be ruled out because band III is retained when this tyrosine residue is nitrated in component NP-I). The assignment of band II is equivocal because either Tyr-24 or Trp-28 or both could have been responsible for this band. The CD of phenylalanine residues in a protein is usually weak; thus, Phe-32 of α-BuTX may not contribute to bands I, II, and III. The contributions of the five cystine residues are probably minor because all the CD bands of native α-BuTX disappear when the toxin is denatured. Suffice it to say, the folding of the polypeptide chain is necessary to maintain the asymmetrical environment of the aromatic residues, which gives rise to bands I–V in the near-UV region.

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Quantitation of Aromatic Residues in Proteins: Model Compounds for Second-Derivative Spectroscopy†

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ABSTRACT: The ultraviolet absorption spectrum of proteins in 6 M guanidine is approximately that of the sum of the spectra of the constituent aromatic amino acids, phenylalanine, tyrosine, and tryptophan, plus contributions from light scattering and disulfides. A multicomponent analysis of the spectrum would theoretically permit simultaneous quantitation of each aromatic amino acid in the protein. In practice, this has not been possible, because of the similarities of the spectra of the amino acids, large differences in molar absorptivity, variable absorption by the disulfides, light scattering, and wavelength shifts which occur when the amino acids are incorporated into proteins. We describe a method for the simultaneous quantitation of the aromatic amino acids in purified proteins. We used second-derivative ultraviolet spectroscopy coupled with a statistically weighted multicomponent analysis. Use of the second derivative virtually eliminated interference from light scattering and from cystine. Empirical selection of model compounds obviated the problem of wavelength shifts. The models are *N*-acetylphenylalanine ethyl ester in 6 M guanidine for phenylalanine, *N*-acetyltyrosine ethyl ester in 55% methanol for tyrosine, and mellitin in 6 M guanidine for tryptophan. This method permits accurate, rapid quantitation of phenylalanine, tyrosine, and tryptophan in intact, denatured proteins.

The ultraviolet spectrum of proteins approximates that of its constituent aromatic amino acids (Wetlaufer, 1962). The near-ultraviolet spectrum of a denatured protein can be closely, but not exactly, mimicked by an equimolar mixture of its constituent aromatic amino acids plus cystine (Edelhoch, 1967). The differences between the observed and "synthesized" spectra remain of interest. Exact reconstruction of protein spectra from simpler models is of particular interest because one might then derive rapid spectroscopic methods for quantitation of the aromatic residues within proteins. Edelhoch (1967) introduced a simple method for determination of the tryptophan content of proteins from their absorbance spectra, although the analysis gives erroneous values for some proteins (Hugli & Moore, 1972; Levine, 1982). Tyrosine values are less reliable, and phenylalanine cannot be estimated.

Derivative spectroscopy might provide a more powerful technique for quantitative analysis of amino acid residues in proteins (Balestrieri et al., 1978, 1980; Federici & Levine, 1980). The potential of increased resolution and precision was pointed out by Giese & French (1955). Others subsequently provided theoretical and experimental contributions to derivative spectroscopy (Olson & Alway, 1960; Butler & Hopkins, 1970; Grum et al., 1972; Brandts & Kaplan, 1973; Shibata, 1976; O'Haver & Green, 1976; Hawthorne & Thorngate, 1978). With the addition of microprocessors or computers to commercial spectrophotometers, the calculation of derivative spectra became a simple task. The first derivative ($dA/d\lambda$) is the rate of change of absorbancy with wavelength; the second derivative ($dA^2/d\lambda^2$) is the velocity of that change. A derivative spectrum thus enhances small dips, peaks, and shoulders compared to the direct spectrum.

Balestrieri et al. (1978) took advantage of this enhancement to develop a technique for quantitating phenylalanine with proteins. They added known amounts of *N*-acetylphenylalanine ethyl ester to the protein solution and were able to determine the content of phenylalanine in the protein by regression analysis. The second derivative obeys Beer's law. If the second derivative spectrum of a protein could be dissected to isolate the contributions of the three aromatic amino acids, then it would be possible to quantitate all three amino acids in that protein. We used simple compounds to provide models whose spectra match those of the aromatic residues. Using second-derivative spectra, we were able to accurately quantitate

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