Bernheimer, A. W., and Schwartz, L. L. (1963), *J. Gen. Microbiol. 30*, 455.
Chesbro, W. R., Heydrik, F. P., Martineau, R., and Perkins, G. N. (1965), *J. Bacteriol. 89*, 378.
Chrambach, A., Reisfield, R. A., Wyckoff, M., and Zaccari, J. (1967), *Anal. Biochem. 20*, 150.
Cooper, L. Z., Madoff, M. A., and Weinstein, L. (1966), *J. Bacteriol. 91*, 1686.
Coulter, J. R. (1966), *J. Bacteriol. 92*, 1655.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.
Goshi, K., Cluff, L. E., and Norman, P. S. (1963), *Bull. Johns Hopkins Hosp. 112*, 15.
Harshman, S., Six, H. R., and Najjar, V. A. (1969), *Biochemistry 8*, 3417.
Inagami, T., and Murakami, K. (1972), *Anal. Biochem. 47*, 501.
Lehnhardt, W. F., and Winzler, R. J. (1968), *J. Chromatogr. 34*, 471.
Lominski, I., Arbuthnott, J. P., and Spence, J. B. (1963), *J. Pathol. Bacteriol. 86*, 258.
McNiven, A. C., Owen, P., and Arbuthnott, J. P. (1972), *J. Med. Microbiol. 5*, 113.
Mitchell, W. M. (1967), *Biochim. Biophys. Acta 147*, 171.
Moore, S. (1963), *J. Biol. Chem. 238*, 235.
Ouchterlony, O. (1949), *Acta Pathol. Microbiol. Scand. 28*, 507.
Rawitch, A. B., and Gleason, M. (1971), *Biochem. Biophys. Res. Commun. 45*, 590.
Reisfield, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature (London) 195*, 281.
Shapiro, A. L., Vinuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Six, H. R., and Harshman, S. (1973), *Biochemistry 12*, 2677.
The Beckman Protein Peptide Sequencer Instruction Manual (1969), Palo Alto, Calif., Beckman Instruments, Inc., Section 3.
Wiseman, G. M., and Caird, J. D. (1970), *Can. J. Microbiol. 16*, 47.
Yoshida, A. (1963), *Biochim. Biophys. Acta 71*, 544.
Zacharias, R. M., Zell, T. E., Morrison, J. H., and Woodlock, J. J. (1969), *Anal. Biochem. 30*, 148.

Physical and Chemical Studies on Staphylococcal α-Toxins A and B†

Howard R. Six‡ and Sidney Harshman*

ABSTRACT: Physical studies on purified A and B forms of staphylococcal α toxin show that the forms have similar minimal and functional molecular weights but differ from each other in charge. By isoelectric focusing, the A form is more acidic, pI 7.2, than the B form, pI 8.4. A comparison of mobilities of the two forms at pH 8.9 in analytical gel electrophoresis with varying gel concentrations gave parallel lines, indicating that the two forms are charge isomers, rather than size isomers. Both forms of the toxin gave identical mobilities in sodium dodecyl sulfate gel electrophoresis corresponding to a minimal mol wt of 27,500. Sedimentation equilibrium studies of the B form of α toxin in 8 M urea and in 6 M guanidine-HCl gave extrapolated values for the minimal mol wt of 28,700 and 31,000, respectively. Sedimentation velocity studies gave a value of 3.0 S from which a functional mol wt of 26,100 was calculated. Peptide mapping studies of the two forms gave 27 detectable peptides for the A form and 23 for the B form, 20 of which appear to be identical in both forms, indicating a large degree of homology in the amino acid sequences. The number of peptides obtained is in good agreement with a minimal mol wt of about 28,000. Automated N-terminal analysis of the A and B forms of staphylococcal α toxin permitted the unambiguous ordering of the first ten amino acids. Identical amino acid sequences were obtained for the two forms of toxin. Fractionation of the CNBr peptides from α-toxin A by Sephadex chromatography, following maleylation of the peptides, yielded seven peptides which eluted in positions identical with those obtained from the B form of toxin. Amino acid analysis showed each of the peptides to be very similar in composition to its B form counterpart. The absence of homoserine established peptide VII as the C terminus in both forms of the toxin.

Despite general agreement that staphylococcal α toxin in velocity centrifugation analysis has an $s_{20,w}$ of 2.8–3.0 S (Bernheimer and Schwartz, 1963; Lominski *et al.*, 1963; Coulter, 1966), other methods give divergent values for the molecular weight. Various laboratories using centrifugation and sodium dodecyl sulfate gel methods have reported values of 22,000 (Coulter, 1966), 36,000 (McNiven *et al.*, 1972), and 44,000 (Bernheimer and Schwartz, 1963) for purified α toxin. It has been suggested that variation in observed molecular weights is due to a mixture of molecules present in even highly purified preparations of α toxin (Coulter, 1966; Bernheimer, 1968). The mixture has been assumed to be caused by varying degrees of molecular association of α toxin (Arbuthnott, 1970). However, the possibility that high molecular weight aggregates of α toxin do not consist exclusively of α-toxin molecules cannot be excluded at present (Bernheimer, 1968).

Purified α toxin has also been shown to be heterogeneous in

† From the Department of Microbiology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232. *Received December 15, 1972.*

‡ Present address: Department of Pharmacology, Washington University School of Medicine, St. Louis, Mo. 63110.

electrophoretic analysis (Bernheimer and Schwartz, 1963; Wadstrom, 1968; McNiven *et al.*, 1972). Purified α toxin was resolved by electrofocusing into three peaks with isoelectric points of 8.0–8.7, 6.5–7.5, 4.5–5.5 (Wadstrom, 1968). The observation that focusing of each peak yielded the original pattern led Wadstrom to suggest that the multiple forms represented an associating–dissociating system. In contrast, Arbuthnott (1970) reported that similar α-toxin forms, also obtained by electrofocusing, were stable upon refocusing and did not generate the original pattern.

Since a procedure for isolating two pure forms of α toxin has been developed (Six and Harshman, 1973), it was of obvious interest to investigate their molecular and electrophoretic properties.

Materials and Methods

Reagents. Urea, sucrose, and guanidine hydrochloride were obtained from Schwarz/Mann as the Ultra Pure grade. Sodium dodecyl sulfate was supplied by Sigma Chemical Co. L-(1-Tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin was purchased from Worthington Biological Corp. and used without purification. The A and B forms of α toxin were prepared as described previously (Six and Harshman, 1973). Fc-43 (perfluorotributylamine) was supplied by Minnesota Mining Corp. Cyanogen bromide was supplied by Eastman Chemical Co. and was stored in the dark at 4°. Maleic anhydride was obtained from Mallinckrodt Chemical Co. and used without further purification.

Electrofocusing. The electrofocusing column used has a volume capacity of 440 ml and was equipped with double-cooling jackets (LKB-Produktor). The pH 3–10 gradient was achieved using ampholyte LKB 8141. The procedure used followed that of Vesterberg *et al.* (1967), except that the initial sucrose gradients were established by a continuous pumping technique (Ayad *et al.*, 1968). The linear nature of the gradient was confirmed by refractive index analysis using the refractomotor Model Abbe-3L (Bausch & Lomb Optical). The "dense" solution contained 200 ml of sucrose, 7.5 ml of ampholyte (40% w/v), and 7.5 ml of water, while the "light" solution contained 212.5 ml of water, 2.5 ml of ampholyte (40% w/v), and the sample. The run was started at constant milliamperage (5.0 mA) which required approximately 300 V. After 14 hr the current was adjusted to a constant voltage of 900 V and the milliamperage fell eventually to 2.0. Total running time was 60 hr (Harshman and Six, 1969).

Relative Mobility. Samples of the toxins were electrophoresed at gel concentrations of 3–8%, using the procedure described by Hendrick and Smith (1968). The ratio of *N,N'*-methylenebis(acrylamide) to acrylamide was 1/38 in all cases. Electrophoresis was carried out at pH 8.9 and the mobility is relative to the tracking dye Bromophenol Blue.

Sodium Dodecyl Sulfate Electrophoresis. Chain weight estimations were carried out using essentially the method of Shapiro *et al.* (1967). The samples were lyophilized and dissolved in 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol and incubated at 37° for 3 hr and then dialyzed against the appropriate electrophoresis buffer containing sodium dodecyl sulfate. Bromophenol Blue and sucrose were added to yield a final concentration of 0.003% and 0.2 M, respectively.

The samples were then layered under the buffer, on the top of the gel. To compensate for any swelling during staining, the gels were cut at the tracking dye and stained according to the method described by Weber and Osborn (1969). Thus, the relative mobility becomes the distance migrated divided by the length of the gel. In some cases the proteins used as standards were first reduced and carboxymethylated (Crestfield *et al.*, 1963). For comparative purposes some preparations of the α-toxins A and B were submitted to this procedure even though amino acid analysis showed them to be devoid of cysteine residues (Six and Harshman, 1973).

Peptide Mapping. The toxins were denatured by dialysis against water followed by lyophilization. The protein was then suspended in 0.01 M phosphate, pH 7.5. Trypsin was added in three aliquots each at 1% of the weight of the toxin. The aliquots were added at 0, 2, and 6 hr. The incubation was carried out at room temperature for 18 hr. Electrophoresis was done at 40 V/cm for 2 hr at pH 3.5 (Ryle *et al.*, 1955). The papers were dried and chromatographed in the second dimension in butanol–acetic acid–water (4:1:5) for 20 hr. Peptide maps were performed on 46 × 57 sheets of Whatman 3 chromatography paper. The peptides were detected with Ninspray (Nutritional Biochemical Co.).

Sedimentation Velocity. Sedimentation velocity studies were performed on a Spinco Model E ultracentrifuge. In most cases, Kel-F double sector synthetic boundary cells and schleiren optics were used. Studies were done at a rotor speed of 56,000 rpm and at 20°. In some cases, a 2.5° angle cell was placed in the counterbalance hole so that two samples could be run simultaneously. Protein concentrations ranged from 2 to 6 mg/ml. The photographic plates were analyzed with the aid of a Nikon Model 6C microcomparator.

Sedimentation Equilibrium. Low-speed sedimentation equilibrium studies were done using a Spinco Model E ultracentrifuge equipped with a photoelectric scanner and multiplex accessory. An AN-G rotor accommodated five different protein concentrations; ordinarily these concentrations ranged from 0.1 to 0.5 mg/ml. The protein solutions were equilibrated with the appropriate solvent by dialysis and then diluted to their final concentration using the dialysate as the diluent. The samples were loaded in 12-mm double sector cells (sapphire windows) on top of small amounts of FC-43 to form columns of about 4 mm in height. Studies were done at a rotor speed of 16,000 rpm. Scans were made at 280 nm after 16 and 24 hr to ensure that equilibrium had been reached. Small corrections were made (when necessary) for base-line absorption by accelerating the rotor to 40,000 rpm, thereby depleting the meniscus of protein. A built-in calibration mode and a reference cell allowed direct calculation of ln c and r^2 from the chart paper. The method is described in more detail by Chevenka (1969). The density of each solvent was determined with the aid of 50-ml Leach pycnometers. A partial specific volume of 0.72 ml/g was computed for both forms of the toxin from their amino acid compositions (Cohn and Edsall, 1943); this value was used in all molecular weight calculations.

Amino-Terminal Analysis. AUTOMATED EDMAN DEGRADATION. Each toxin (7 ml) was subjected to Edman degradation in the Beckman automatic sequencer. Quadrol buffer and a double cleavage cycle were used. The products were investigated by gas–liquid chromatography on a Beckman GC-45 instrument (The Beckman Protein Peptide Sequencer Instruction Manual, 1969). The phenylthiohydantoin derivative indicated by the gas–liquid chromatography determinations was confirmed by thin layer chromatography. Three different solvent systems were used (Inagami and Murakami, 1972). In most cases, the phenylthiohydantoin derivative was chromatographed in only one of these solvents, the one best suited to resolve the phenylthiohydantoin amino acid suspected from gas–liquid chromatography. The phenylthiohydantoin amino acid was located by ultraviolet absorption and by ninhydrin.

In both cases, an estimate of the released phenylthiohydantoin alanine in the first cycle was 140 nmol which was made by a comparison of the gas–liquid chromatography peak to the peak of a known amount of authentic phenylthiohydantoin alanine.

Cyanogen Bromide Cleavage. Cyanogen bromide cleavage at methionine residues was performed as described by Gross and Witkop (1962). Samples of 3–15 mg were dissolved in 1–3 ml of 70% formic acid. Cyanogen bromide was added and the mixture was allowed to stand at room temperature for 16–20 hr in the dark. The reaction was terminated by diluting the mixture tenfold with water and aspirating for 10 min followed by lyophilization. The conversion of methionine to homoserine was confirmed by amino acid analysis (*i.e.*, the absence of methionine and the presence of an equivalent amount of homoserine). Such a 40:60 ratio of homoserine to homoserine lactone is found after acid hydrolysis (Armstrong, 1949), the homoserine value was corrected by this factor. In all the preparations checked the conversion was greater than 95%.

Maleylation of Peptides. In some cases, the peptides were solubilized by maleylation. The procedure was essentially as described by Butler *et al.* (1969). The peptides were suspended in 2–5 ml of 0.1 M borate buffer, pH 8.3. Maleic anhydride (equal in weight to the peptides or protein) was added in three aliquots and the pH was maintained between 8.5 and 9.5 by the addition of 0.5 M NaOH. The reaction was complete in 10–15 min. To obtain a quantitative estimate of the reaction, lyophilized toxin was subjected to maleylation followed by reaction with fluorodinitrobenzene. Samples were taken for amino acid analysis before and after this treatment; the recovery of lysine was 100%, indicating that all the ε-amino groups of lysine had been covered and were unavailable during the fluorodinitrobenzene reaction. It was therefore assumed that the maleylation of the peptide was quantitative.

Results

Electrofocusing. Isoelectric focusing analysis, as expected, showed that α-toxin A was more acidic than α-toxin B. Each toxin gave a single peak of hemolytic activity with the A form focusing at pH 7.2 and the B form focusing at pH 8.4 (Table I). No evidence for interconversion of the two forms was obtained. The purified toxins were stable during electrofocusing and greater than 85% of the hemolytic activity could be recovered. In contrast, electrofocusing of material chromatographed on Sephadex G-100-SF (Six and Harshman, 1973), which is now known to be contaminated with a protease, gave recoveries of only about 20%. Moreover, in addition to the pH 8.4 and 7.2 hemolytic peaks, this material contains a third peak of activity (1–2% of the total) which is detected at pH 5.1. The pH 5.1 material was not found in either the α-toxin A or B preparations and was not investigated further.

Relative Migration in Gel Electrophoresis. In order to establish if the observed difference in mobility between α-toxin A and α-toxin B was due to an intrinsic difference in charge or to differences in molecular size, relative mobilities in gels of differing acrylamide concentration were investigated (Hendrick and Smith, 1968). In this procedure, it is expected that molecules of similar size but different charge will give parallel lines when mobility is plotted against per cent concentration of the gel, whereas molecules of different size and charge will give divergent lines. The data show that at pH 8.9 α-toxins A and B both have linear relative rates of mobility at gel concentrations from 3–8% (Figure 1). The fact that the lines for α-toxins A and B are parallel indicates that they are of similar molecular size and thus neither represents a polymeric form of the other.

TABLE I: Isoelectric Focusing of the α Toxins.

Peaks of Hemolytic Act.	Toxins from Sephadex G-100: Isoelectric pH	Distribution of Act. (%)	Isoelectric pH: A	Isoelectric pH: B
1	5.1	1		
2	7.25	15	7.20	
3	8.40	84		8.45

Sodium Dodecyl Sulfate Gel Electrophoresis. A plot of relative mobility *vs.* log molecular weight for a set of standard proteins and for α-toxins A and B is shown in Figure 2. The two forms do not separate under these conditions and an apparent mol wt of 27,500 is obtained for both α-toxins A and B. Additional experiments were done in 0.01 M phosphate, pH 7.1, containing either 0.1 or 1.0% sodium dodecyl sulfate. In such determinations, the range of values of the mol wt observed was 26,000–29,000 with an average of 27,500. It is of interest that upon extended exposure to sodium dodecyl sulfate, 48 hr at 4°, both α-toxins A and B tend to aggregate as evidenced by their failure to penetrate the gels. We have tentatively interpreted this finding as indicating that lipid binding by α toxin facilitates aggregation of the protein. Hexamer forms of α toxin have been observed after interaction with red cell ghosts and polar lipid containing artificial liposomes (Freer *et al.*, 1968).

Sedimentation Velocity Studies. To determine the $s_{20,w}$ values for the purified toxins, samples of the toxins were dialyzed against 0.10 M phosphate buffer, pH 7.2, and subjected to ultracentrifugation analysis. All values were corrected to standard conditions of $s_{20,w}$. A sedimentation coefficient of 3.0 S was obtained for both A and B. The peaks were symmetrical indicating homogeneity. Further, when samples containing equal parts of A and B were run, no distortion of the peak was observed. This result is consistent with the observation that both α-toxins A and B are eluted at the same position from G-100 Sephadex columns. Using a partial specific volume of 0.72 ml/g and a diffusion constant of 1×10^{-6} cm²/sec (Forlani *et al.*, 1971), a mol wt of 26,100 was computed for both forms of α toxin.

Sedimentation Equilibrium Studies. In view of the relative instability of the toxins at low salt concentrations, the prolonged

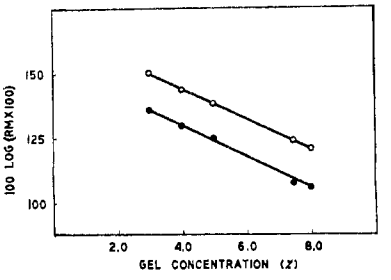

FIGURE 1: Relative mobility *vs.* gel concentration. Electrophoretically purified α-toxins A and B were used. The buffer was Tris-Gly, pH 8.9; the ratio of bis:monomer was 1:38; RM = relative mobility; (○) α-toxin A; (●) α-toxin B. For further details, see Materials and Methods.

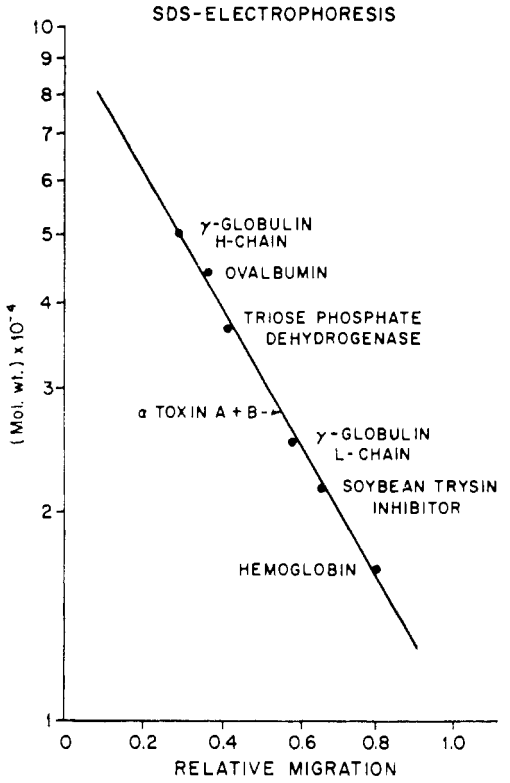

FIGURE 2: Sodium dodecyl sulfate electrophoresis. The standards were carboxymethylated. The gels were 10% acrylamide. The buffer was 0.04 M Tris-HCl, pH 8.1, containing 0.1% sodium dodecyl sulfate. For further details, see Materials and Methods.

period of time required for equilibrium sedimentation analysis was deemed unsuitable for determining the molecular weight. However, the method is suitable for determining the minimum molecular weight since dissociating solvents are employed in such as analysis and insoluble forms of the toxin do not accumulate during the run. Accordingly, samples of α-toxin B were run in both 8 M urea and 6 M guanidine hydrochloride. Plots of ln c *vs.* r^2 were linear in all cases indicating that the solutions were homogeneous with respect to molecular size over the entire solution column. Least-squares analysis gave correlative coefficients greater than 0.999 except in the 0.1-mg/ml samples. Since in both the urea and the guanidine solvents the 0.1-mg/ml samples were least reliable, they were not

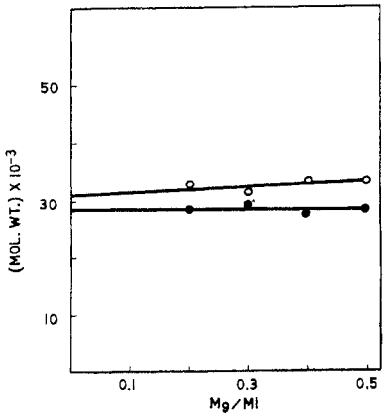

FIGURE 3: Molecular weight *vs.* protein concentration: (○) α-toxin B in 6 M guanidine-HCl–0.02 M phosphate, pH 7.2, (●) α-toxin B in 8 M urea–0.02 M phosphate, pH 7.2.

TABLE II: Amino-Terminal Sequence of α-Toxins A and B.

Residue No.	α-Toxin A	α-Toxin B
1	Ala	Ala
2	Asp	Asp
3	Ser	Ser
4	Asp	Asp
5	Ile	Ile
6	Asn[a]	Asn[a]
7	Ile	Ile
8	Lys	Lys
9	Pro	Pro
10	Gly	Gly

[a] Under the conditions used in this study, phenylthiohydantoin asparagine is relatively unstable and partially converts to phenylthiohydantoin aspartic acid. Therefore, when both phenylthiohydantoin asparagine and phenylthiohydantoin aspartic acid were detected the residue was assigned to be asparagine. For further details, see Materials and Methods. Average repetitive recovery based on Ile at positions 5, 7, and 14 (data not shown) is 93%; based on Ile positions 5 and 7 only, it is 95%.

used in calculating the molecular weight. A plot of the molecular weight *vs.* protein concentration showed little concentration dependence in 8 M urea and gave 28,7000 for the mol wt (Figure 3). The determinations made in 6 M guanidine hydrochloride did show a slight concentration dependence and an extrapolated value of 31,000 was obtained for the mol wt (Figure 3). These values for the minimal molecular weight of α-toxin B are in good agreement with each other and also with the value of 27,500 for both α-toxins A and B obtained above by sodium dodecyl sulfate gel analysis.

Peptide Mapping. To obtain information as to the subunit molecular weight and the extent of the homology of the electrophoretic forms of α toxin, the technique of peptide mapping was applied. Typical patterns are shown in Figures 4 and 5.

The pattern is quite similar for both forms, indicating that at least 20 of the tryptic peptides occur in both forms. However, despite the apparent larger number of amino acid residues in the B form (Six and Harshman, 1973), the A form has consistently yielded more peptides, 23–27, than the B form, 21–24. There are a number of explanations for this result. The most obvious is that a small portion of the amino acid sequence in the two forms is different, permitting a separation of some peptides from the A form that remain unresolved in the maps of the B form.

From the amino acid composition (Six and Harshman, 1973), based on a mol wt of about 28,000, the number of trypsin-sensitive bonds predicted is about 30 in both forms. Since the number of peptides detected with each form ap-

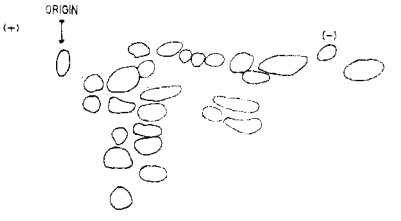

FIGURE 4: Peptide map of α-toxin A.

TABLE III: Comparison of the CNBr Peptides of α-Toxins A and B.[a]

	I		II		III		IV		V		VI		VII	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Aspartic acid	8.6	8.6	7.8	7.7	9.2	9.4	4.2	5.0	3.6	4.0	4.7	5.0	2.4	1.7
Threonine	4.5	4.5	3.6	2.5	5.7	6.2	2.9	3.7	1.9	2.0	1.7	1.1	1.3	1.3
Serine	4.1	3.8	3.7	3.4	3.6	3.4	1.7	1.7	1.9	2.8	1.9	1.8	2.7	2.0
Glutamic acid	6.1	5.2	5.6	5.4	6.2	5.9	2.0	1.0	1.0	0.7	2.1	2.1	2.7	3.0
Proline	2.2	2.0	2.2	2.1	1.3	0.9	0.7	0.5	1.0	1.1	1.0	1.0	1.3	0.9
Glycine	6.4	5.4	4.4	3.6	4.6	4.5	2.5	2.7	2.6	1.4	4.2	3.0	3.0	2.9
Alanine	3.4	2.8	3.0	3.0	2.2	1.5	1.2	1.1	2.0	2.2	1.0	1.0	2.0	2.1
Valine	3.8	3.4	3.0	3.2	2.3	2.6	1.2	1.1	1.2	0.9	1.4	1.1	1.2	1.1
Isoleucine	3.3	3.2	2.7	3.2	2.7	3.0	1.5	1.7	1.2	1.0	0.8	0.8	1.0	0.8
Leucine	3.6	3.3	2.9	3.2	2.0	2.2	1.1	1.0	1.6	1.9	1.0	1.1	1.8	1.4
Tyrosine	2.6	2.8	2.4	1.7	2.3	2.5	0.6	0.6	0.7		0.7	0.7		
Phenylalanine	2.2	2.1	1.7	1.9	0.9	1.1	0.7	0.6	1.1	1.7	0.9	0.7	0.6	0.6
Lysine	5.3	5.0	5.4	6.0	6.2	6.2	*2.0*	*2.0*	*2.0*	*2.0*	1.7	1.8	1.5	1.6
Histidine	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	0.3						0.7	0.6
Arginine	1.2	1.2	2.0	2.1	3.1	2.8	0.3	0.2			*1.0*	*1.0*	*1.0*	*1.0*
Homoserine	0.8	1.0	1.0	0.7	0.9	1.1	0.7	1.0	0.7	1.0	1.0	1.0		

[a] Values were obtained from samples hydrolyzed for 20 hr. Amino acids of less than one-tenth residue were omitted. The italicized values are assigned as integers.

proaches that number, it can be concluded that both exist as single polypeptide chains or, if smaller subunits exist, they cannot be identical.

Amino-Terminal Sequence of the Toxins. Samples of α-toxin A and α-toxin B were subjected to sequential Edman degradation as described under Materials and Methods. In both experiments, the degradation was carried through 23 cycles. The results of the first ten cycles are summarized in Table II. Unequivocal interpretations of the amino acid residues detected in cycles 11–23 are hampered by background noise and are at present tentative; however, no differences in the sequence of the A and B forms were detected. The results show that the composition and sequence of the amino-terminal ends of both α-toxins A and B are identical and thus indicate that no protease modifications has occurred at the amino terminal of either form.

Comparison of the CNBr Peptides Derived from α-Toxins A and B. To obtain a detailed comparison of the amino acid sequence of the two forms of α toxin, the cyanogen bromide peptides of the A and B forms were maleylated and chromatographed on Sephadex G-50-F. Both forms give similar elution patterns. That obtained with the B forms of toxin is shown in Figure 6. The peaks obtained were pooled, lyophilized, and rechromatographed. Peptides I–V were rechromatographed on Sephadex G-50-F. Peptides VI and VII were rechromatographed on Sephadex G-25-SF and G-10, respectively, to remove the contaminating maleic acid.

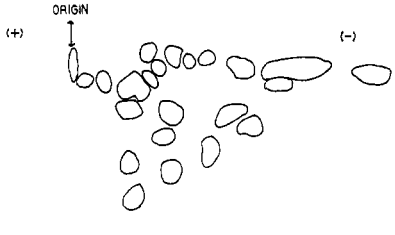

FIGURE 5: Peptide map of α-toxin B.

The amino acid composition of each of the purified peptides is given in Table III. Recoveries for each peptide ranged from 56 to 83%. However, this represents an underestimate since in most cases only the peak region of the peptide was pooled. An overall survey of the data clearly indicates that the amino acid compositions of the cyanogen bromide derived peptides from both forms of toxins are very similar. In both forms of α toxin, peptides I, II, and III, which collectively represent about 60% of the total molecule, are distinguished by their ratios of histidine, arginine, and homoserine which are 1:1:1, 1:2:1, and 1:3:1, respectively. Similarly, peptides A-IV and B-IV can be distinguished from peptides A-V and B-V by their distinctive ratios of alanine, isoleucine, and leucine being 1:2:1 in the former case and 2:1:2 in the latter. Peptide VII from both the A and B forms of α toxin is devoid of homoserine and is thus presumed to be the C-terminus peptide in

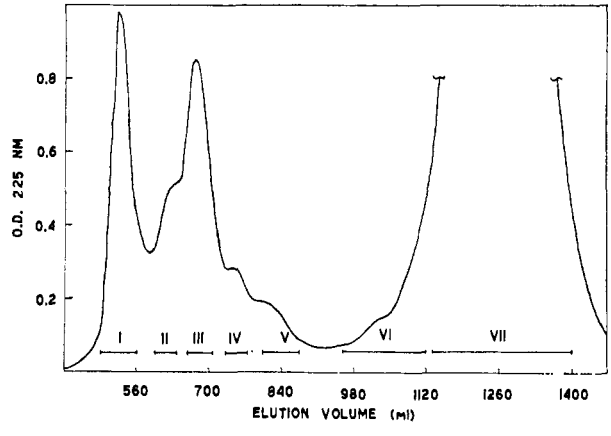

FIGURE 6: Sephadex chromatography of the maleylated cyanogen bromide peptides. Twelve milligrams of CNBr-cleaved α-toxin B was maleylated and chromatographed on Sephadex G-50-F. The column was 5 × 83 cm and the elution buffer was 0.1 M ammonium bicarbonate. The flow rate was maintained at a constant 42 ml/hr and fractions were collected at 10-min intervals. The fractions were monitored at 225 nm on a Zeiss PM Q II spectrophotometer.

each molecule. Despite these broad similarities a detailed examination of the compositions of the peptides from the two forms of α toxin reveals selective differences in composition, particularly with respect to the amino acids Asp, Glu, Thr, Gly, and Tyr. The significance of these differences must await more detailed chemical studies.

Discussion

Data obtained by electrofocusing show that α-toxin A corresponds to the pH 7.2 form of α toxin reported by Wadstrom (1968) and Arbuthnott (1970) and the α-toxin B to the pH 8.4 form. In contrast to partially purified preparations of α toxin, pure A and B forms are stable to refocusing, permitting recoveries of greater than 85%. Moreover, contrary to Wadstrom's findings (1968), no evidence of interconversion of the A and B forms was observed. These results are consistent with the observed relative mobilities of α-toxins A and B in gel electrophoresis at pH 8.9 and 4.5 and their elution patterns in preparative gel electrophoresis (Six and Harshman, 1973), suggesting that the mobility differences observed in electrofocusing are not due to differences in ampholyte binding.

All of the data obtained indicate that both α-toxins A and B are composed of single polypeptide chains with a minimal and functional mol wt of about 28,000. The separation of 24 peptides following tryptic digestion and peptide mapping shows that the molecule cannot consist of identical subunits. Moreover, the earlier demonstration that each form contains a single N-terminal alanine and a single C-terminal lysine tends to exclude the possibility that each form consists of multiple nonidentical peptides.

The fact that both α-toxins A and B emerge from Sephadex G-100 columns at the same position led us to assume that their respective molecular weights would be the same. This was confirmed in sodium dodecyl sulfate gel electrophoresis where both forms of the toxin give single bands corresponding in migration to a mol wt of 27,500. Further confirmation of this value was obtained with the B form of α toxin by sedimentation equilibrium in urea and guanidine hydrochloride where values of 28,700 and 31,000, respectively, were obtained. Since Tris, which is known to be bound to both forms of the toxin (Six and Harshman, 1973), is released upon exposure to urea or guanidine, the close agreement of the molecular weight estimates by these three methods with that obtained using nondenaturing conditions indicates that bound Tris does not significantly influence the molecular weight of the toxin.

Velocity centrifugation under nondenaturing conditions gives an $s_{20,w}$ value of 3.0 S for both α-toxins A and B. This value is in good agreement with that obtained by others (Bernheimer and Schwartz, 1963; Coulter, 1966; Lominski *et al.*, 1963). Using values of 0.72 for the partial specific volume (see Materials and Methods) and 1×10^{-6} for the diffusion constant (Forlani *et al.*, 1971), a mol wt of 26,100 is obtained from the Svedberg equation (Svedberg and Pederson, 1940). Since this value agrees closely with the minimal molecular weight obtained under denaturing conditions, it is concluded that the functional molecular weight and the minimal molecular weights are the same. It is of interest that upon standing a small amount (8%) of material with an $s_{20,w}$ value of 10.9 S was observed in the preparations of α-toxins A and B. It is assumed that this material corresponds to the 12S inactive hexamer form reported previously (Bernheimer and Schwartz, 1963; Lominski *et al.*, 1963). The formation of the 12S form remains poorly understood since no evidence of intermediate sized complexes is observed in velocity sedimentation or in analytical disc gel electrophoresis.

Since most laboratories have obtained an $s_{20,w}$ value of 3.0 S for the functional form of α toxin, the basis for the wide range of molecular weights reported is not clear. It is reasonable that the overestimate of 44,000 obtained by the pseudo-equilibrium method (Bernheimer and Schwartz, 1963) was due to the presence of high molecular weight contaminants in the original preparations. Since a lower value, 32,000, has recently been reported after additional purification of the preparation (Forlani *et al.*, 1971), this interpretation appears justified. The discrepancy between 36,000 (McNiven *et al.*, 1972) and 27,500 obtained here is less well understood since both forms were derived from sodium dodecyl sulfate gel analysis. The value of 22,000 obtained by equilibrium centrifugation by Coulter (1966) is perplexing since this is lower than that observed here even in the presence of urea and guanidine. It may be that the protease has partially degraded the preparation.

The data obtained from the sequential degradation of the two toxins show that amino-terminal sequences are identical, at least for ten residues. This is taken as strong evidence that no protease degradation has taken place at the amino terminal of either form. Moreover, CNBr peptides obtained from α-toxin A are in close agreement with those obtained from the B form. Sephadex chromatography after maleylation shows that all seven peptides elute in positions identical with their B counterparts. The amino acid composition of each of these peptides is in good agreement with that obtained from the B form. No homoserine is found in peptides A-VII and B-VII indicating that peptide VII represents the C terminus of both forms of the toxin. Despite these broad agreements, it is not possible from the data at hand to assign either differences in amide content or amino acid composition as the basis for the observed difference in charge of the A and B forms of α toxin. Such an assignment must await more detailed amino acid sequence data.

Acknowledgment

The authors acknowledge with pleasure the assistance of Dr. Tadashi Inagami in performing the amino-terminal analysis during the automated Edman degradation procedures.

References

Arbuthnott, J. P. (1970), *in* Microbial Toxins, Vol. III, Montie, T. C., Kadis, S., and Ajl, S. J., Ed., New York, N. Y., Academic Press, p 189.

Armstrong, M. D. (1949), *J. Amer. Chem. Soc. 71*, 3399.

Ayad, S. R., Bonsall, R. W., and Hunt, S. (1968), *Anal. Biochem. 22*, 533.

Bernheimer, A. W. (1968), *Science 159*, 847.

Bernheimer, A. W., and Schwartz, L. L. (1963), *J. Gen. Microbiol. 30*, 455.

Butler, P. J. G., Harris, J. I., Hartley, B. S., and Leberman, R. (1969), *Biochem. J. 112*, 679.

Chevenka, C. H. (1969), A Manual of Methods for the Ultracentrifuge, Palo Alto, Calif., Beckman Instruments, Inc.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N. Y., Reinhold, p 370.

Coulter, J. R. (1966), *J. Bacteriol. 92*, 1655.

Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.
Forlani, L., Bernheimer, A. W., and Chiancone, E. (1971), *J. Bacteriol. 106*, 138.
Freer, J. H Arbuthnott, J. P., and Bernheimer, A. W. (1968), *J. Bacteriol. 95*, 1153.
Gross, E., and Witkop, B. J. (1962), *J. Biol. Chem. 237*, 1856.
Harshman, S., and Six, H. R. (1969), *Biochemistry 8*, 3423.
Hendrick, J. L., and Smith, A. J. (1968), *Arch. Biochem. Biophys. 126*, 155.
Inagami, T., and Murakami, K. (1972), *Anal. Biochem. 47*, 501.
Lominski, I., Arbuthnott, J. P., and Spence, J. B. (1963), *J. Pathol. Bacteriol. 86*, 258.
McNiven, A. C., Owen, P., and Arbuthnott, J. P. (1972), *J. Med. Microbiol. 5*, 113.
Ryle, A. P., Sanger, F., Smith, L. F., and Kitai, R. (1955), *Biochem. J. 60*, 541.
Shapiro, A. L., Vinuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Six, H. R., and Harshman, S. (1973), *Biochemistry 12*, 2672.
Svedberg, T., and Pederson, K. O. (1940), The Ultracentrifuge, Oxford, Clarendon Press.
The Beckman Protein Peptide Sequencer Instruction M anual (1969), Section 3, Beckman Instruments, Inc., Palo Alto, Calif.
Vesterberg, O., Wadstrom, T., Vesterberg, K., Svensson, H., and Malmgren, B. (1967), *Biochim. Biophys. Acta 135*, 435.
Wadstrom, T. (1968), *Biochim. Biophys. Acta 168*, 228.
Weber, K., and Osborn, M. J. (1969), *J. Biol. Chem. 244*, 4406.

Spectral Properties of Human Lysozyme and Its Inhibitor Complexes. Fluorescence and Difference Spectra†

Roderick S. Mulvey, Richard J. Gaultieri, and Sherman Beychok*

ABSTRACT: The fluorescence spectra and fluorimetric and spectrophotometric titrations of human lysozyme and its complexes with oligomers of *N*-acetyl-D-glucosamine (AcGlcN) have been investigated. The results are compared with a number of parallel studies on hen egg-white lysozyme. The spectrophotometric titration and alkaline difference spectra of the human lysozyme $(AcGlcN)_4$ complex show that the pK_{int} of Tyr-63 (-62) is lowered from approximately 10.5 to 10.0 when the inhibitor is bound. The fluorescence of human lysozyme has a lower quantum efficiency (0.04) than that of hen egg-white lysozyme and its emission maximum is at a shorter wavelength (330 nm). The spectrofluorimetric titrations of human and hen egg-white lysozymes are very similar. In contrast their complexes do not exhibit the same dependence of fluorescence on pH in the acid region. Below pH 5.5 the fluorescence of the hen egg-white lysozyme–$(AcGlcN)_3$ complex is strongly quenched whereas the fluorescence of the human lysozyme complex is as intense as the fluorescence of the free enzyme. The marked enhancement of enzyme fluorescence at pH 7.5 by inhibitor binding was used to determine the association constant of a series of saccharides from di- to penta-*N*-acetyl-D-glucosamine. At room temperature the K_a of $(AcGlcN)_3$ with human lysozyme is 1.9×10^4. Compared to hen egg-white lysozyme, this represents a difference of about 800 cal/mol in the free energy of binding. These different properties of the two lysozymes may largely be related to the substitution of Trp-62 of hen egg-white lysozyme by Tyr-63 (-62) in human lysozyme.

Human and hen egg-white lysozyme possess a high degree of homology, 78 positions out of a total of 130 having identical residues (Canfield, *et al.*, 1971; Jolles and Jolles, 1972). Alignment of the two sequences to maximize homology requires only a single deletion in the hen egg-white lysozyme between residues 47 and 48. X-Ray crystallographic studies of hen egg-white lysozyme at 2-Å resolution (Blake *et al.*, 1965) and of human lysozyme at 6-Å resolution (Blake and Swan, 1971) suggest that the two enzymes have very similar secondary structures, as do studies of the far-ultraviolet circular dichroism (Halper *et al.*, 1971; Ikeda *et al.*, 1972). Other investigations (Osserman and Lawlor, 1966; Jolles *et al.*, 1968; Cohen, 1969) point to similarities in the structure and function of the active sites of these enzymes.

Despite the overall similarity of these two lysozymes, a number of differences in enzymatic and physical properties have been noted. Human lysozyme is more active than hen egg white (Osserman Lawlor, 1966; Mouton and Jolles, 1969; Isaka *et al.*, 1971), the measured difference in activity depending on the type of assay employed. Using a turbidimetric assay with *Micrococcus lysodeikticus* (Gorin *et al.*, 1971), we found the human enzyme to be four to five times more active than hen egg-white lysozyme. The near-ultraviolet circular dichroic spectrum of human lysozyme (Halper *et al.*, 1971; Ikeda *et al.*, 1972) is quite distinct from that of hen egg-white lysozyme reflecting the altered arrangements of aromatic residues in the two enzymes.

Of the 51 different residues in hen egg-white and human lysozymes 27 occur at external[1] positions. Most of the remaining 24 changes, at surface and internal positions, are

† From the Departments of Biological Sciences and Chemistry, Columbia University, New York, New York 10027. *Received January 15, 1973.* This work was supported in part by Grant GB 29481x1 from the National Science Foundation and Grant 5 R01 CA 13014 from the National Institutes of Health.

[1] The terms "internal," "surface," and "external" are used as defined by Browne *et al.* (1969).