Observations on the Fluorescence and Toxicity of Botulinum Toxin*

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Many proteins exhibit a characteristic fluorescence which is believed to be due to the particular composition and structure of the protein. Boroff and Fitzgerald (1) and Boroff (2) have reported that when botulinum toxin is treated with alkali, certain iron salts, or with antiserum to the toxin, there is a decrease in fluorescence which correlates with the resulting decrease in toxicity. They suggested that the structure responsible for fluorescence and toxicity are the same or sufficiently related to react concurrently with a detoxifying agent. Any relationship between the biological activity of a protein and a physical property such as fluorescence would be exceedingly important and it was of interest therefore to determine if other agents which alter protein structure result in a similar correlation between biological activity and fluorescence.

EXPERIMENTAL PROCEDURE

Crystallized botulinum toxin (type A), crystalline bovine serum albumin (Armour and Company), and ovalbumin (Worthington Biochemical Corporation) were dissolved in 0.05 M acetate buffer at pH 3.8 and 0.05 M phosphate buffer at pH 6.8, at a concentration of 2 mg per ml. These pH values were chosen to determine the fluorescence on either side of the isoelectric points of the respective proteins. The experimental controls were made by diluting the protein solution with an equal volume of the appropriate buffer. The preparations treated with urea and guanidine were made up either by dissolving a calculated amount of the denaturant and then diluting to a fixed volume or by dilution with the appropriate volume of a concentrated solution. The preparations treated with sodium thioglycollate were prepared by mixing 1 ml of 0.2 M thioglycollate solution with 1 ml of protein solution containing 2 mg per ml. All studies were carried out at room temperature.

Fluorescence was measured at 330 mp with an activation wave length of 290 mp, with an Aminco-Bowman spectrophotofluorometer in conjunction with an IP 28 photomultiplier tube, mercury-xenon arc source, and the manufacturer's recommended slit combinations. The data are expressed as instrumental values and are uncorrected for the over-all spectral response of the instrument (see Duggan et al. (3)). Emission intensities were recorded from samples initially containing 1 mg of protein per ml, serially diluted down to 0.016 mg per ml with either the corresponding buffer or 6 M urea made up in the corresponding buffer.

The effect of urea treatment on the fluorescence of two other proteins has been studied in comparison with the toxin. When botulinum toxin was chromatographed on DEAE-cellulose, several fractions were obtained, although the starting material was homogeneous in electrophoresis and in the ultracentrifuge. Bioassay disclosed that some of the fractions were reduced in specific activity (LD50 per mg of N). Fluorescence

RESULTS

In the presence of 6 M urea, botulinum toxin preparations rapidly lost toxicity at both pH 3.8 and 6.8. Within 15 minutes, it was reduced to less than 5% of the original and after 1 hour to less than 1%. Some typical results are presented in Table I. It is seen that, despite the nearly complete loss of toxicity, the fluorescence did not change significantly and was substantially the same as a control without urea. After 1 hour, the urea was removed from the urea-treated toxin by dialysis; there was neither a restoration of toxicity nor a change in fluorescence. A plot of the fluorescence intensity as a function of protein concentration is shown in Fig. 1. Dissociation of fluorescence and toxicity was also observed when both were measured at varying concentrations of urea and guanidine (Fig. 2). Loss of toxicity began in solutions of urea between 3 and 4 M and was complete to the extent of 99% or more at 6 M and above. No appreciable loss of fluorescence was observed up to 10 M. However, some quenching appeared to take place in 10 M urea when the toxin concentration was 0.4 mg per ml or higher. Loss of toxicity in guanidine solutions began at about 1 M and was complete to the extent of 95% or more at 3 M or higher. Loss of fluorescence in guanidine solutions began at molarities of about 2 and was 90% complete at 7 M. These results parallel the denaturation and loss of biological activity observed with many proteins at similar urea concentrations (e.g. 7).

The effect of urea treatment on the fluorescence of two other proteins has been studied in comparison with the toxin. When exposed to 6 M urea for 1 hour, bovine serum albumin lost about 70% of its fluorescent intensity. Removal of the urea by dialysis resulted in the complete restoration of the fluorescence. Ovalbumin behaved similarly in the presence of urea, but its fluorescence was not restored completely upon dialysis. These results are shown graphically in Fig. 3.

When botulinum toxin was chromatographed on DEAE-cellulose, several fractions were obtained, although the starting material was homogeneous in electrophoresis and in the ultracentrifuge. Bioassay disclosed that some of the fractions were reduced in specific activity (LD50 per mg of N). Fluorescence

* The terms "botulinus" and "botulinal," as well as "botulinum," have been used to describe the toxin from Clostridium botulinum. The term "botulinum toxin," shortened from Clostridium botulinum, has come into common use.
Fluorescence and Toxicity of Botulinum Toxin

TABLE I
Effect of treatment with urea on fluorescence and toxicity of botulinum toxin

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Relative toxicity*</th>
<th>Fluorescence†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Toxin in 0.05 M acetate at pH 3.8</td>
<td>100</td>
<td>$1.4 \times 10^8$</td>
</tr>
<tr>
<td>2. Toxin in 0.05 M phosphate at pH 6.8</td>
<td>100</td>
<td>$1.0 \times 10^8$</td>
</tr>
<tr>
<td>3. Solution 1 in 6 M urea for 1 hour</td>
<td>&lt;1.0</td>
<td>$1.2 \times 10^8$</td>
</tr>
<tr>
<td>4. Solution 2 in 6 M urea for 1 hour</td>
<td>&lt;1.0</td>
<td>$1.0 \times 10^8$</td>
</tr>
<tr>
<td>5. Solution 3 dialyzed free of urea</td>
<td>1.4 x 10³</td>
<td>$1.0 \times 10^8$</td>
</tr>
</tbody>
</table>

* The actual toxicity of the toxin solution which contained 2 mg per ml was $6 \times 10^7$ LD₅₀ per ml. The detoxification with urea was too rapid to permit a zero time sample to be obtained; therefore, it has been assumed that the assay obtained with the experimental controls ($3 \times 10^7$ LD₅₀ per ml) represented the 100% figure for the experiments.

† All samples were diluted to a range in which fluorescence was linear with respect to concentration. The values in the table were all made at 0.25 mg per ml.

measurements were also carried out, and the results of one experiment are summarized in Table II. It is apparent that the fraction eluted at a $\Gamma / 2$ of 0.32 had a significantly lowered toxicity and the fraction eluted at a $\Gamma / 2$ of 0.64 had lost 75% of its toxicity, yet both of these fractions had fluorescences even higher than the original material. Because it appeared likely that the loss of toxicity on the column was due to surface inactivation, an experiment was carried out in which a chromatographic separation was performed on toxin which was adsorbed on the column for 5 days. The elution pattern was changed markedly and most of the protein could not be recovered with the solvents used. The peak obtained at an ionic strength of 0.16 which had heretofore exhibited a very high specific toxicity was reduced more than 60% in toxicity with no loss in fluorescence.

It has also been found possible to reduce fluorescence without

FIG. 1. The effect of urea on the fluorescence intensity of type A botulinum toxin at different concentrations. △△, toxin in phosphate buffer, pH 6.8; △△--△△, toxin in phosphate buffer and 6 M urea; O--O, toxin in acetate buffer, pH 3.8; ●--●, toxin in acetate buffer and 6 M urea.

Fig. 2. Toxicity and fluorescence intensity of type A botulinum toxin in urea or guanidine. O--O, toxicity in urea; ●--●, fluorescence in urea; △△--△△, toxicity in guanidine; △△--△△, fluorescence in guanidine.

Fig. 3. The effect of urea on the fluorescence intensity of bovine plasma albumin and ovalbumin at different concentrations. △△--△△, bovine plasma albumin in phosphate buffer, pH 6.8; △△--△△, bovine plasma albumin after removal of urea by dialysis; O--O, ovalbumin in phosphate buffer, pH 6.8; O--O, ovalbumin in acetate buffer and 6 M urea; O--O, ovalbumin after removal of urea by dialysis.

TABLE II
Toxicity and fluorescence of fractions of botulinum toxin eluted from DEAE-cellulose column

<table>
<thead>
<tr>
<th>Ionic strength of eluant</th>
<th>Protein recovered*</th>
<th>Specific toxicity†</th>
<th>Specific fluorescence†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>0.15†</td>
<td>$2.5 \times 10^7$</td>
<td>$1.1 \times 10^8$</td>
</tr>
<tr>
<td>0.16</td>
<td>0.21</td>
<td>$3.0 \times 10^7$</td>
<td>$6.6 \times 10^9$</td>
</tr>
<tr>
<td>0.32</td>
<td>0.53</td>
<td>$1.5 \times 10^7$</td>
<td>$6.1 \times 10^8$</td>
</tr>
<tr>
<td>0.64</td>
<td>0.28</td>
<td>$0.6 \times 10^7$</td>
<td>$6.8 \times 10^8$</td>
</tr>
<tr>
<td>Original</td>
<td>5.85</td>
<td>$2.3 \times 10^7$</td>
<td>$4.3 \times 10^8$</td>
</tr>
</tbody>
</table>

* By ultraviolet absorption at 277 and 290 μm.
† From peak tube.
‡ This value is of questionable accuracy because optical densities were not much greater than the blanks.
affecting toxicity. Botulinum toxin in 0.1 M thioglycollate solution showed very little fluorescence (<0.1% of original) without any notable loss in toxicity (85% of original). This thioglycollate-induced destruction of fluorescence was also observed with serum albumin and egg albumin, suggesting that the effect may be due to a nonspecific quenching rather than to any chemical change in the proteins. It is interesting to note that the fluorescence of tryptophan is not reduced to a great extent by the same concentration of thioglycollate.

**DISCUSSION**

These data indicate clearly that the molecular configuration of the botulinum toxin molecule necessary for toxicity can be altered to the point of almost complete loss of the toxicity without a change in the fluorescence. The hypothesis proposed by Boroff and Fitzgerald (1), that some specific region of the molecule responsible for toxicity is also responsible for fluorescence, must therefore be considered invalid. Their observations may be explained on the basis that the reagents caused a proportional destruction of two separate structures.

The statement by Boroff and Fitzgerald (1) that the fluorescence of botulinum toxin, serum albumin, and fibrinogen is much greater than can be accounted for by their tryptophan, tyrosine, and phenylalanine content is open to question. Weber (8) has measured the quantum yield of a series of proteins, including serum albumin, containing both tryptophan and tyrosine, and observed a maximal quantum yield of 0.3. In our hands also, serum albumin and botulinum toxin gave yields within the expected range.

The reduction of the fluorescence of serum albumin in the presence of urea and its complete restoration upon removal of the urea is directly comparable to changes in a series of properties measured by Neurath (9, 10) in studies on the reversible denaturation of this protein. Serum albumin recovered from urea treatment was shown to have approximately the same solubility, electrophoretic mobility, viscosity, anion-combining ability, and immunological specificity as the starting material. There is considerable evidence to demonstrate that the way urea denatures proteins is through the rupture of hydrogen bonds which are necessary for the maintenance of configurational stability. Upon removal of the urea, certain proteins, among them serum albumin, are able to return to their original configuration. The reduction in the fluorescence of serum albumin in the presence of urea is due, then, to a change in configuration. The fluorescence data on ovalbumin are also in accord with this interpretation. This protein is irreversibly denatured by urea. Its intrinsic viscosity, for example, is only partially restored by diluting out the urea (11). The fluorescence of ovalbumin followed a similar pattern, showing a great reduction in urea solution and a partial restoration upon removal of the urea. The reduction of fluorescence polarization in the presence of urea for many proteins containing tryptophan has been reported recently by Weber (8). He too has emphasized the importance of secondary structures in this phenomenon.

In the light of the interpretation that molecular configuration contributes so importantly to fluorescence, the retention of fluorescence by the toxin in the presence of 6 M urea is rather surprising, and even more so in view of indications (13-15) that even minor alterations in its hydrogen-bonded structure lead to a loss of toxicity, and for at least one of these changes (that due to alkali), to a loss of fluorescence. The structural configuration necessary for maximal fluorescence of the toxin must reside in a well protected portion of the secondary structure, whereas the structure responsible for toxicity is in a much more vulnerable position.

**SUMMARY**

The suggestion that the fluorescence and toxicity of botulinum toxin were due to a common structure within the molecule has been found to be invalid. It was found possible to inactivate the toxin by treatment with urea and by adsorption on a cellulose ion exchange resin without parallel reduction in fluorescence. The role of native configuration in protein fluorescence has been discussed.

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