ISOLATION OF TOXIC SUBUNITS FROM TWO MURINE-TOXIC PROTEINS FROM PASTEURELLA PESTIS

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Received September 24, 1968

Two mouse-toxic proteins, Toxin A (240,000 molecular weight) and Toxin B (120,000 molecular weight), were dissociated in sodium dodecyl sulfate to small molecular weight subunits. Subunit size from 10,000 to 12,000 molecular weight is proposed based on gel electrophoresis, ultracentrifugation and dialysis experiments. The subunits retained approximately 60% of the toxic activity of the large molecular weight toxins.

Two mouse-toxic proteins have been isolated from lysates of Pasteurella pestis, strain Tjiwidej (1). Employing Sephadex G-200 gel filtration after the method of Andrews (2), the molecular weight of Toxin A has been estimated to be 240,000 and Toxin B 120,000 (3). Because of their large molecular weights, the very similar amino acid composition (3) and the fact that Toxin B is approximately half the molecular weight of Toxin A, we suspected that these proteins were composed of similar subunits.

Treating either toxin with citric acid (pH 2.5) resulted in the formation of small molecular weight inactive and somewhat unstable subunits of very similar size (4). As an alternative approach to disaggregate the toxic protein and to obtain stable subunits, toxin was treated with sodium dodecyl sulfate (SDS) after the method of Shapiro, Vineula and Maizel (5). This method has been used to determine approximate subunit molecular weight by disruption of hydrogen and hydrophobic bonds and at the same time saturating proteins with SDS so that separation by gel electrophoresis is based on size rather than

charge (5). Toxic protein was treated with 1% SDS solution at 37°C for 3 hours followed by dialysis for 16 hours against 0.1% SDS in 0.01 M sodium phosphate buffer (pH 7.1). Ninety percent of the protein remained in the dialysis bag. Protein remaining in the dialysis bag was assayed by SDS acrylamide gel electrophoresis using bovine albumin monomer and dimer (Pentex, crystallized) and ribonuclease (2X crystallized, Worthington) as standards. Toxin A or B gave a single major band of the same electrophoretic mobility as ribonuclease (Fig. 1). In some experiments mercaptoethanol was added to the SDS solution, but no change in electrophoretic pattern was observed.

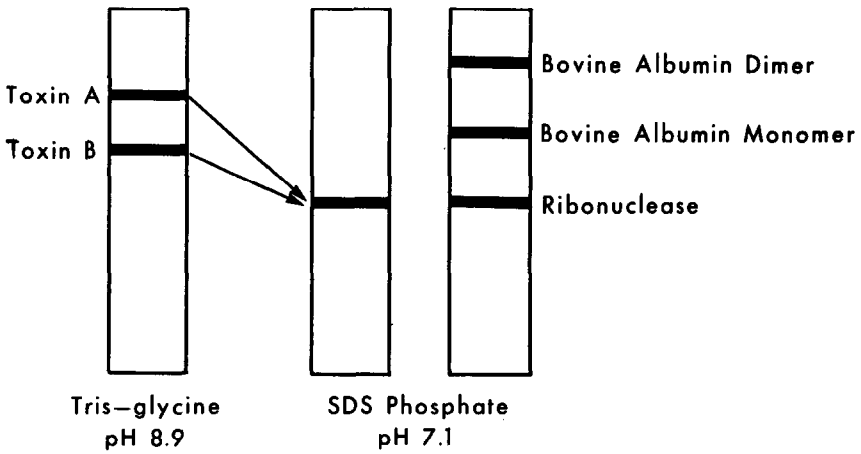

Figure 1. Acrylamide gel electrophoresis of Toxins A and B and toxin subunits. The patterns of separation of control Toxins A and B are diagramed as indicated on the gel on the left. The toxins routinely are separated by acrylamide gel electrophoresis in tris glycine buffer (pH 8.9) using a technique modified from Ornstein and Davis (3). Arrows indicate the toxin subunit position after SDS treatment in the center gel (see text). Protein (150-200 µg) was applied to the gel. SDS treated protein was subjected to electrophoresis for 2 hours at 10 ma/gel. Gels and buffer contained 0.1% SDS and sodium phosphate (0.1 M; pH 7.1) after the method of Maizel (5). Standard proteins are shown in the right hand gel and were treated in same manner as the toxins. Protein bands were identified with amido black stain. The origin and the positive pole are at the top; the negative pole at the bottom of the gels.

To further confirm the presence of small subunits after SDS treatment, Toxin A or B was treated with SDS as described for electrophoresis (37°C for 3 hrs followed by 16 hrs dialysis at room temperature) and subjected to ultracentrifugation. One per cent toxin solutions were used (Fig. 2). Toxin A or B in SDS showed single identical peaks with sedimentation coefficients of 1.4 S. Toxin A in buffer sedimented at 10.8 S and Toxin B in buffer sedimented at 7.8 S. Ribonuclease in SDS gave a

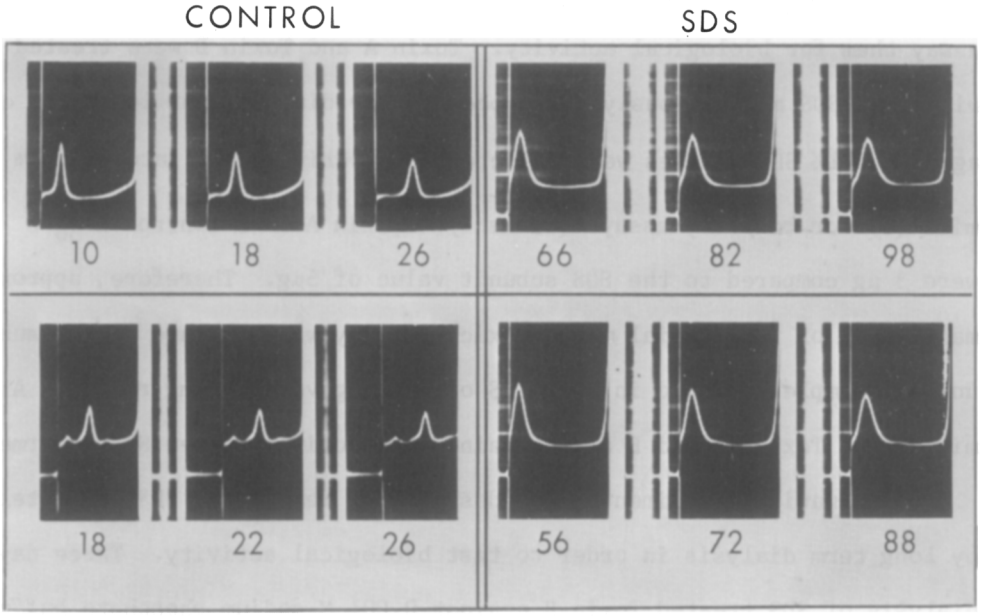

Figure 2. Ultracentrifugation of native toxins and toxins in SDS. Sedimentation velocity experiments were performed at 20°C in a Spinco Model E Ultracentrifuge equipped with a schlieren optical system at 59,780 rpm. Toxin A or B was pretreated with 1.0% SDS for 3 hrs at 37°C followed by dialysis for 16 hrs against 0.1% SDS, sodium phosphate buffer (0.01 M, pH 7.1). Patterns for Toxin B in 0.002M phosphate buffer pH 7.6 are shown in the upper left and Toxin B in SDS, 0.01M phosphate buffer pH 7.1 upper right. Toxin A in 0.002M phosphate buffer pH 7.6 is shown in the lower left and Toxin A in SDS, 0.01M phosphate buffer pH 7.1 lower right. Toxin concentrations were one per cent. Time in minutes is indicated at the bottom of each photograph.

value of 1.6 S. The $S_{20,w}$ values obtained were in agreement with electrophoresis experiments indicating a subunit molecular weight of approximately 10,000 to 12,000 from both Toxins A and B.

Treatment of Toxin B with 0.1% SDS for 30 minutes at 27°C followed by ultracentrifugation resulted in the appearance of two distinct peaks. Protein was distributed approximately equally between an intermediate size subunit (2.5 S) and a peak sedimenting at 7.6 S. This indicated that very brief treatment with low concentrations of SDS was sufficient to induce partial dissociation of Toxin B to probably a dimer or tetramer form.

The observation that SDS induced stable subunits enabled us to assay them for biological activity. Toxin A and Toxin B were treated with 1.0% SDS as previously described. After dialysis for 16 hours against 0.1% SDS samples were injected intraperitoneally into 20 gram mice (6) for toxicity assays (Table I). Toxin A or B control LD_{50}'s were 3 μg compared to the SDS subunit value of 5μg. Therefore, approximately 60% of the initial mouse toxic activity was retained in SDS subunits. Samples diluted in 0.1% SDS or water gave the same result. A mixture of Toxins A and B also retained 60% activity after SDS treatment.

Alternative experiments were designed to remove the SDS completely by long term dialysis in order to test biological activity. Three day dialysis of SDS treated Toxin B against 0.001 M sodium phosphate buffer with five changes of dialysate resulted in a 90% reduction in the protein concentration inside the viscose tubing dialysis bag. This is in contrast to 16 hour dialysis where a 10 percent reduction was noted. The dialysis results reinforced the conclusion that the subunits were of small molecular weight and did not readily reaggregate upon dilution of SDS solutions.

Estimates of the size of the basic structural subunits of murine toxin range from 10,000 to 12,000. This figure is derived from data

TABLE 1. Mouse-toxic activity of SDS subunits. Toxins (2 to 3 mg) were dissolved directly in 1% SDS (pH 7.1) and agitated at 37°C for 3 hours followed by dialysis against SDS (0.1%) in 0.1 M sodium phosphate buffer pH 7.1 for 16 hours at room temperature. Samples were diluted and injected intraperitoneally into 18-20 gm female Swiss albino mice. Deaths were recorded after 24 hours. D=deaths, S=survivors of 6 mice injected per dilution. SDS injected up to 1% gave no killing.

Sample	Micro-grams protein injected[a]				
	40	20	10	5	2
	D-S	D-S	D-S	D-S	D-S
Control					
Toxin A	6-0	6-0	6-0	6-0	2-4
Toxin B	6-0	6-0	5-1	4-2	-
SDS subunits					
Toxin A	6-0	6-0	5-1	3-3	-
Toxin B	6-0	6-0	6-0	2-4	-

[a]based on a modified Lowry protein technique (7).

including sedimentation velocity experiments, SDS gel electrophoresis, and total amino acid minimal molecular weight (3) based on one cysteine residue per subunit. Data from gel filtration and vacuum dialysis measurements of subunits formed in acid indicate the molecular weight is close to 10,000 (4). This would support the concept of ten or twelve subunits per 120,000 molecular weight Toxin B. Toxin A appears to contain the same size subunits.

The data showing retention of biological activity in these small subunits after SDS treatment of both Toxin A or B seems quite remarkable. The possibility exists that the subunits reassociate to an active aggregate in the mouse upon dilution of SDS. However, this appears unlikely since dilution of SDS subunits with water or various concentrations of SDS in which subunits remain completely dissociated did not cause any

variation in specific toxic activity. The small amount of protein actually injected would also tend to shift the equilibrium toward dissociation. Attempts are being made to determine the specific conditions required for reassociation. Since SDS binds tightly to proteins, complete removal of SDS may be required to initiate reassociation.

Isolation of biologically active subunits also indicates that quarternary and possibly tertiary structure is not required for toxicity. In this connection, investigations with thyroglobulin by Steiner and Edelhoch (8) showed that unlike guanidine HCl or urea, SDS was the only dissociating agent which did not disturb the α helix of the resulting subunits. Klee (9) found that dissociation of thetin homocysteine methylpherase to form 50,000 molecular weight subunits resulted in active subunits if excess detergent was not present. Large structural proteins appear to be particularly susceptible to SDS. Cytochrome B (10) and mitochondrial structural protein (11) have been dissociated with SDS and appear to retain much of their "native" character. Large virus polymers (12), erythrocytes (13) and bacterial membranes (14) are reduced to subunits by very strong binding of SDS to hydrophobic regions in some cases created by removal of lipid from these sites (13). Susceptibility of the large toxin proteins to detergent supports earlier findings indicating toxin is part of the P. pestis membrane structure (15).

Acknowledgment. This research was supported by Grant 5 ROl AI-03866-08 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service; the National Science Foundation Grant GB-6162; and by United States Air Force Grant AF-AFOSR-68-1343.

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