Dimeric Form of Diphtheria Toxin: Purification and Characterization†

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Received August 14, 1985

ABSTRACT: Many preparations of diphtheria toxin were found to contain dimeric and multimeric toxin forms. The monomeric and dimeric forms were fractionated to greater than 98% purity, and their properties were compared. Dimeric toxin slowly dissociated to native monomers in solution at neutral pH and could be rapidly dissociated with dimethyl sulfoxide. In cell culture assays and rabbit skin tests, the dimer exhibited no significant toxic activity, except for that attributable to trace contamination by monomer, or partial dissociation to monomer during the incubation period. In guinea pig lethality tests, however, toxic activity varied depending upon the dose. At least 7-fold greater amounts of dimer than monomer (161 ng vs. 22 ng, respectively) were required to cause death at 18 h, whereas similar weights of the two toxin forms (22 ng) caused death at 120 h. This variability probably reflected slow dissociation of dimer to monomer in the animal. The dimer was unable to bind toxin receptors on the surface of susceptible cells, whereas it retained full activity in the ADP-ribosyltransferase, NAD-glycohydrolase, or ligand-binding assays. Thus, the lack of toxicity of the dimeric toxin may have resulted from distortion or occlusion of the receptor binding site on the B moiety. We propose that the dimer contains two monomeric units bound by hydrophobic interactions and that the points of contact involve regions of the B moieties that are normally buried in the native monomer.

To date we know of three primary modes in which the structure of whole diphtheria toxin (DT)[1] varies: (i) The intact, 535-residue toxin is readily cleaved by trypsin-like enzymes at an arginine-rich region within the first disulfide loop (Drazin et al., 1971; Gill & Pappenheimer, 1971). The "nicked" toxin, containing fragments A and B linked by a disulfide bridge, is found in variable amounts in purified toxin preparations, and there is evidence to suggest that the intact toxin must be nicked before it can inhibit protein synthesis in whole cells (Sandvig & Olsnes, 1980; Draper & Simon, 1980). (ii) DT, whether nicked or intact, often contains tightly bound endogenous dinucleotides, primarily adenylyl-(3′,5′)-uridine 3′-monophosphate (ApUp) (Barbieri et al., 1981). The source and functional significance of such dinucleotides are unknown. (iii) Finally, multimeric forms of DT have been detected. Goor (1968) reported a dimeric (6.8S) toxin form, which could be fractionated from the monomer by chromatography on Sephadex G-100. Dimeric and higher order multimeric forms were also reported by Relyveld (1970) and other investigators (Pappenheimer & Brown, 1968; Gill & Dinius, 1971; Pappenheimer et al., 1972; McKeever & Sarma, 1982), and we have found such forms in commercial and laboratory preparations. As an extension of our ongoing program to describe the structure and activity of DT fully, we have purified and characterized the dimeric form of the toxin, and we report the results here.

Experimental Procedures

Materials. The following isotopes were purchased from Amersham: [*adenylate*-^{32}P]NAD (1000 Ci/mmol), [*carbonyl*-^{14}C]NAD (53 mCi/mmol), [*adenine*-^{14}C]NAD (534 mCi/mmol), [^{35}S]methionine (1460 Ci/mmol), and iodine-125 (16.3 mCi/μg of I). Isotopes were quantified in a Beckman LS-100 liquid scintillation counter by using Cerenkov radiation for ^{32}P samples and ACS cocktail (Amersham) for the remaining isotopes. IHP and BSA were from Sigma. All other reagents and salts were of analytical grade.

High-Performance Size-Exclusion Chromatography. Samples were analyzed by HPSEC with a Waters system 272 HPLC apparatus equipped with a U6K injector and a Bio-Rad 1305 variable UV wavelength detector. A Bio-Rad TSK-250 column was used for all studies and was equilibrated in 20 mM NaH_2PO_4–100 mM Na_2SO_4, pH 6.8, at a flow rate of 1 mL/min.

Diphtheria Toxin and Antitoxin. DT (lot 442X) was obtained from Connaught Laboratories (Ontario, Canada) and stored at −78 °C. The monomeric and dimeric forms of toxin were purified by slight modification of existing procedures (Collier & Kandel, 1971). The commercial toxin (ca. 2 g) was first diluted with 5 volumes of loading buffer such that the final solution contained 50 mM Tris-HCl, pH 7.5, 1 μg/mL PMSF, 1 μg/mL DFP, and 0.02% NaN_3 and then applied by gravity feed to a 2.5 × 50 cm column of DEAE-Sephacel equilibrated in 25 mM Tris-HCl–1 mM EDTA, pH 7.5. The column was washed with equilibration buffer until the absorbance of the column effluent approached 0 at 280 nm, and the toxin was then eluted with a linear 0–0.25 M NaCl gradient (1.5 L) at a flow rate of 25 mL/h. Fractions of 7.5 mL were collected. Column fractions were analyzed by HPSEC, pooled into monomer-rich and dimer-rich frac-

† This work was supported by Grant AI 07877 from the National Institute of Allergy and Infectious Diseases, by Grant CA 30663 from the National Cancer Institute, and by The Shipley Institute of Medicine. J.T.B. was the recipient of Postdoctoral Fellowship AI-06607 from the National Institute of Allergy and Infectious Diseases.

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[1] Abbreviations: DT, diphtheria toxin; HPSEC, high-performance size-exclusion chromatography; PMSF, phenylmethanesulfonyl fluoride; DFP, diisopropyl fluorophosphate; IHP, inositol hexaphosphate; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Me_2SO, dimethyl sulfoxide; MLD, minimum lethal dose; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; NAD, nicotinamide adenine dinucleotide; IgG, immunoglobulin G; ApUp, adenylyl-(3′,5′)-uridine 3′-monophosphate.

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tions, and concentrated by ultrafiltration on Amicon YM-10 membranes. Each peak was subsequently chromatographed on a 5 × 80 cm column of Sephadex G-100 SF equilibrated in 50 mM Tris-HCl–1 mM EDTA, pH 7.5. The column was eluted at 80 mL/h, and 6-mL fractions were collected. Later experiments showed that Sephacryl S-200 SF provided greater separation and permitted higher flow rates to be used. Purified DT monomer and dimer so obtained were concentrated by ultrafiltration, dialyzed against 50 mM Tris-HCl, pH 7.5, and frozen in small aliquots at −78 °C. Prior to use in each assay, the purity of toxin preparations was analyzed by HPSEC. Antiserum against nicked, nucleotide-free DT was prepared in BALB/c mice by weekly intraperitoneal injection of toxin (3 μg) emulsified 1:1 with complete Freund's adjuvant.

Fractionation of Nucleotide-Free and Nucleotide-Bound DT. Monomeric or dimeric toxin (20 mg at 2 mg/mL) was applied by gravity feed to a 1.3 × 4.5 cm column of Matrex Gel Green A (Amicon) equilibrated in 50 mM Tris-HCl, pH 7.5, at 4 °C, and 1.0-mL fractions were collected. The nucleotide-bound form of DT passed through unretarded. After several column volumes of buffer were passed through the column, nucleotide-free toxin was eluted batchwise with 0.75 M NaCl in the same buffer. Each of the sample peaks was pooled, concentrated by ultrafiltration on an Amicon YM-10 membrane, dialyzed against 1000 volumes of 50 mM Tris-HCl, pH 7.5, for 16 h at 4 °C, and stored at −78 °C in small aliquots. The capacity of the column exceeded 10 mg of nucleotide-free toxin per milliliter of bed volume.

Assays. NAD-glycohydrolase, ADP-ribosyltransferase, cytotoxicity, and flow dialysis assays, as well as polyacrylamide gel electrophoresis, were performed as previously described (Kandel et al., 1974; Lory et al., 1980a). Dermonecrotic lesions were induced in 8–16-month-old New Zealand White rabbits by intradermal injection of DT in 50 μL of phosphate-buffered saline, pH 7.4, containing 0.2 mg/mL BSA (PBS–BSA). MLDs were determined following subcutaneous injection of DT (100 μL, diluted in PBS–BSA) into 275–350-g guinea pigs. The protocol for measuring binding of iodinated DT to Vero cells (see legend to Figure 7) will be described elsewhere (J. T. Barbieri and R. J. Collier, unpublished results).

Radioimmunoassay for DT. Fifty microliter portions of DT monomer (100 μg/mL) or dimer (60 μg/mL) in PBS were added to wells of flexible plastic 96-well microtiter plates (Dynatek), and the plates were incubated for 1 h at 25 °C and washed 3 times with 150 μL of RIA buffer (PBS containing 1% BSA and 0.02% NaN_3). Under these conditions equivalent weights of monomer or dimer remained associated with the plates following the washing procedure. Thirty microliters of competitor protein in PBS–BSA was added, followed by 30 μL of mouse anti-DT monomer antiserum diluted 1:5000 in PBS–BSA, and incubation was continued for 1 h. The wells were washed 3 times with RIA buffer, and 30 μL of rabbit IgG anti-mouse IgG (Miles), labeled with iodine-125 (Morrison, 1980) and diluted 1:100 in PBS–BSA, was added. After 1 h, the wells were again washed 3 times with RIA buffer, removed from the tray with scissors, and counted by liquid scintillation.

Results

Purification of Monomer and Dimer. When fractionated by HPSEC, commercial preparations of DT generally gave three peaks of 210-nm-absorbing material, with apparent M_r values of 48 000, 100 000, and >760 000 (Figure 1a). The relative sizes of the peaks varied from preparation to preparation. All three peaks had similar specific activities in the ADP-ribosyltransferase assay, and upon electrophoresis on SDS–polyacrylamide gels in the absence of reducing agents, all gave a single band corresponding in molecular weight to whole DT (see Figure 3). Freshly prepared toxin from either the PW-8 or the C7β strain of *Corynebacterium diphtheriae* gave only the M_r 48 000 peak on HPSEC. We inferred that the lowest molecular weight peak corresponded to monomeric toxin and the other two peaks to dimeric and multimeric forms similar to those reported previously (Goor, 1968; Relyveld, 1970). Since the true molecular weight of monomeric DT (58 342) is greater than the apparent molecular weight on HPSEC, the toxin may have been retarded by weak interactions with the column matrix. No retardation was observed with fragment A (M_r 21 164), which eluted with an apparent M_r of 22 000.

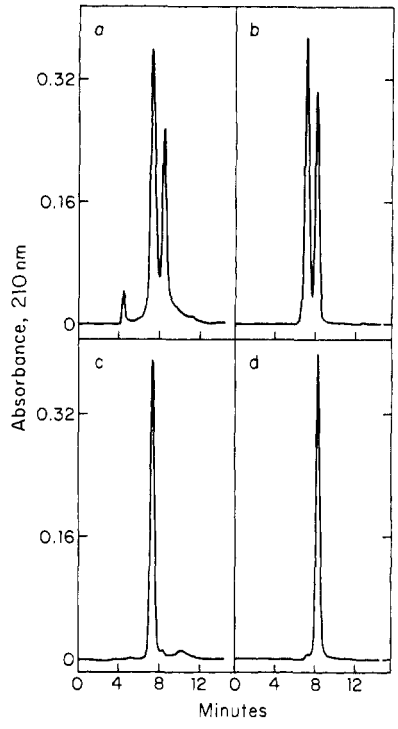

FIGURE 1: HPSEC elution profiles of DT samples taken at various stages of purification. At $t = 0$, aliquots were injected onto a Bio-Rad TSK-250 column equilibrated in 20 mM NaH_2PO_4–100 mM Na_2SO_4, pH 6.8, at 1.0 mL/min, and protein was monitored at 210 nm. (a) Commercial preparation of DT; (b) pooled DT obtained by chromatography on DEAE-Sephacel; (c) purified DT dimer obtained by Sephadex G-100 SF chromatography of the pooled dimer peak from DEAE-Sephacel (see Figure 2a); (d) purified DT monomer obtained as in panel c. Percentages of the various forms were determined from the relative peak heights.

Chromatography of commercial toxin on DEAE-Sephacel yielded two overlapping peaks (Figure 2a), the first corresponding to monomeric and the second to dimeric DT. Higher molecular weight forms were in trailing fractions of the second peak. Virtually homogeneous monomer (99% pure) and dimer (98% pure) were obtained by chromatography of individually pooled peaks from DEAE-Sephacel on Sephadex G-100 (Figures 2b, 1c,d) or Sephacryl S-200.

When either monomeric or dimeric DT was electrophoresed on SDS–polyacrylamide gels in the presence of reducing agents, the nicked molecules dissociated into fragments A and B (Figure 3). The multimeric forms of DT from any given preparation contained about the same relative percentages of nicked and intact molecules as the monomer from that preparation. Conversion of either monomer or dimer to the fully nicked state by treatment with trypsin did not alter the chromatographic behavior of either form on HPSEC.

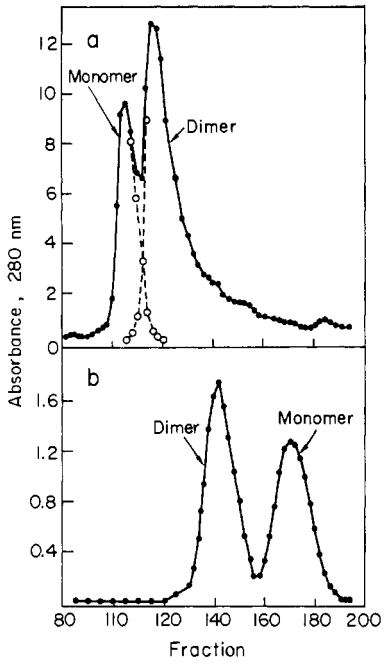

FIGURE 2: Purification of DT monomer and dimer. (a) A commercial DT preparation (2 g) was applied to a column of DEAE-Sephacel equilibrated in 25 mM Tris-HCl and 1 mM EDTA, pH 7.5, and eluted with a linear 0–0.25 M NaCl gradient. The effluent was monitored at 280 nm, and 7.5-mL fractions were collected. The relative percentages of monomer and dimer in column fractions were determined by HPSEC as described in Figure 1. (b) A sample (120 mg) of pooled material containing both monomer and dimer as described in panel a was concentrated by ultrafiltration and applied to a column of Sephadex G-100 SF equilibrated in 50 mM Tris-HCl–1 mM EDTA, pH 7.5. Fractions of 6 mL were collected.

Monomeric toxin (1–30 mg/mL) was unaffected by storage at 0–4 °C for several months or incubation at 37 °C for several days. Multimeric forms were less stable. Dimer showed slight conversion to monomer (5–10%) over a period of weeks at 4 °C, and higher order multimers dissociated to complex mixtures of oligomers within several days. At 37 °C dimer dissociated to monomer at about 5% per day. Addition of BSA (0.2 mg/mL) did not alter the stability of either form. When quick-frozen and stored at −78 °C in 50 mM Tris-HCl, pH 7.2, both monomeric and dimeric forms were stable for at least several months.

Earlier we showed that DT could be fractionated on ATP–agarose into two populations differing in the presence or absence of tightly bound endogenous dinucleotides (Lory & Collier, 1980), principally ApUp (Barbieri et al., 1981). Recently we found that Matrex Gel Green A, an agarose gel containing a modified triazine dye, can substitute for ATP–agarose for this fractionation. The Matrex Gel is superior to ATP–agarose in that it is more stable and has greater adsorptive capacity. We found that similar proportions of the monomeric and dimeric forms of DT from any given preparation bound to Matrex Gel Green A. Unless indicated otherwise, the studies described below were performed with the nicked, dinucleotide-free forms of toxin.

Solvent-Induced Dissociation of Dimer to Monomer. Urea at concentrations up to 2 M caused little dissociation of dimer, whereas higher concentrations converted the toxin to forms that ran as denatured high molecular weight structures on HPSEC. Similar results were obtained when dimer was treated with up to 6 M guanidine hydrochloride or 50% ethylene glycol. NaCl or NaSCN (up to 2 M) was also ineffective at dissociating the dimeric toxin into monomers.

The possibility that hydrophobic forces were involved in the association of DT multimers led us to test dimethyl sulfoxide (Me_2SO), a polar organic solvent with high dispersive capacity. Dimeric toxin was quantitatively converted to monomer by incubation in 40% (v/v) Me_2SO at 37 °C for 10 min (data not shown). Lower concentrations of Me_2SO were also effective, as was formamide, but longer incubations were necessary for complete dissociation. Monomeric toxin obtained after removal of Me_2SO by dialysis or gel permeation chromatography was indistinguishable from native monomer, as judged by cytotoxic or enzymic activities, or by reactivity with antitoxin in a radioimmunoassay. Endogenous dinucleotides remained associated with the toxin following such treatment, as evidenced by 280 nm/260 nm absorbance ratios or affinity chromatography on Matrex Gel Green A.

We confirmed the report of Goor (1968) that overnight incubation of dimeric DT with high concentrations of DTT (0.33 M) caused conversion to a form chromatographically indistinguishable from monomer. Monomeric toxin recovered after such treatments often aggregated upon dialysis or storage, however, and this reagent was judged less useful for conversion of dimer to native monomer.

Toxicity Studies. Initial measurements showed that the cytotoxic activity of dimeric DT against toxin-sensitive cell lines was at least 10-fold lower than that of monomer (Table I). Moreover, a correlation between toxicity and the content of residual monomer was apparent (Table II). Multiplying the ID_{50} toxin concentrations by the percentage of monomer yielded similar values (Table II, third column), indicating that (i) the toxicity of these preparations may result from con-

Table I: Inhibition of Protein Synthesis in Various Cell Lines by DT Monomer and Dimer[a]

cell line	ID_{50}[b] (mol/L) monomer	dimer
Vero	3.6×10^{-14}	9.1×10^{-13}
BSC-1	3.2×10^{-14}	4.4×10^{-13}
CV-1	3.8×10^{-14}	6.3×10^{-13}
WM-9	9.3×10^{-13}	2.9×10^{-11}
SW-620	1.9×10^{-12}	1.7×10^{-11}
HeLa	3.3×10^{-12}	8.1×10^{-11}
CHO	1.8×10^{-11}	6.3×10^{-10}

[a] Cells, plated at 5×10^4/vial and grown for 24 h, were incubated with increasing concentrations of purified monomer or dimer (diluted in PBS–BSA) for 23 h at 37 °C. The medium was then replaced with low-methionine medium containing 2 μCi/mL [^{35}S]methionine and incubated for an additional hour. The cells were washed 3 times with 3.0 mL of 10% TCA containing 1 mg/mL methionine and dried and the levels of protein synthesis assessed by liquid scintillation. The dimer preparations used in these studies contained 1.3–1.7% monomer. [b] Concentration of toxin that inhibited protein synthesis by 50%, relative to untreated controls. Molarities are expressed in terms of monomer equivalents per liter.

Table II: Correlation between Concentration of Monomer in Various Preparations and Ability To Inhibit Protein Synthesis[a]

% monomer[b]	ID_{50} (mol/L)[c]	concn of monomer at ID_{50}[d]
99.5[e]	3.5×10^{-14}	3.5×10^{-14}
22.4	1.2×10^{-13}	2.7×10^{-14}
5.6	3.0×10^{-13}	1.7×10^{-14}
3.3	8.1×10^{-13}	2.7×10^{-14}
1.2	1.1×10^{-12}	1.3×10^{-14}

[a] Vero cells were prepared as described in Table I and incubated at 37 °C with preparations of dimer containing various percentages of residual monomer. The inhibition of protein synthesis was assayed 23 h later. [b] Determined by HPSEC of each sample immediately prior to assay. [c] Molarities are expressed in terms of monomer equivalents per liter. [d] Determined by multiplying the ID_{50} of each preparation by the percentage of monomer present. [e] The values for a sample of purified monomer are given for reference.

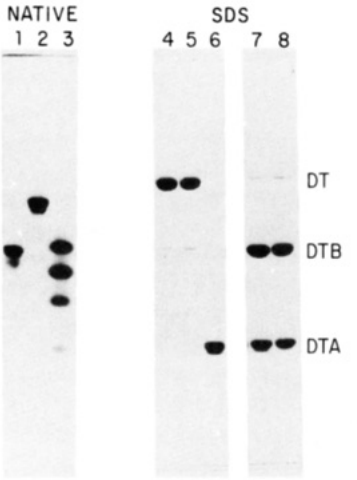

FIGURE 3: Polyacrylamide gel electrophoretic profiles of purified DT fractions. Samples (2–4 μg) were electrophoresed in 7.5% native gels (no detergent, lanes 1–3) or in 11.25% SDS gels in the absence (lanes 4–6) or presence (lanes 7 and 8) of 2-mercaptoethanol. Lanes 1, 4, and 7, purified DT monomer; lanes 2, 5, and 8, purified DT dimer; lanes 3 and 6, DT fragment A. The positions of intact DT and its constituent B (DTB) and A (DTA) fragments in SDS gels are shown along the right margin.

taminating monomer and (ii) the dimer does not inhibit the action of the monomer. These results raised the possibility that the dimer itself might be nontoxic.

To obtain a more accurate measure of the toxicity of the dimer, a procedure was devised to minimize conversion of dimer to monomer in the culture medium during incubation with cells. Samples of highly purified monomer or dimer were incubated with Vero cells for 2 h at 4 °C, which permitted toxin to bind under conditions where dissociation of dimer was negligible. The cells were washed to remove unbound toxin and then incubated in growth medium for 21 h at 37 °C prior to the assay of protein synthesis. The cytotoxic activity of dimer evaluated in this manner corresponded precisely with values predicted from the known amounts of monomer present (Figure 4). The purest preparation of dimer tested, with a monomer/dimer ratio of 1/245, had an LD_{50} 240-fold greater than that of monomer. We concluded that the dimeric form of DT had little, if any, toxic activity for cultured cells.

Results of rabbit skin tests were consistent with these findings. Intradermal injection of monomer or dimer produced dermonecrotic lesions with diameters linearly related to the logarithm of the quantity injected (Figure 5). The threshold amounts of toxin required to elicit a response were estimated by extrapolating to a lesion diameter of zero. The value obtained for monomer (56 fg) was 79-fold lower than that calculated for a sample of dimer (4.4 pg) containing 1.2% monomer.

DT monomer injected subcutaneously into guinea pigs consistently gave a minimum lethal dose (MLD) of 24 ng/250-g body weight. Higher doses yielded a relationship between dose administered and survival time similar to that described by Baseman et al. (1970). Replotting our data as log log MLD vs. survival time empirically gave a linear relationship (Figure 6a), which facilitated estimation of MLD from survival times between 13 and 120 h (Figure 6b).

Dimer gave different results than monomer in guinea pig lethality tests. Much larger amounts (ca. 8–10-fold) of dimer than of monomer were required to cause death within the first 1–2 days, but as the dose was decreased, and hence as survival time increased, the apparent specific toxicity of the dimer approached that of native monomer (22 ng). The basis of this

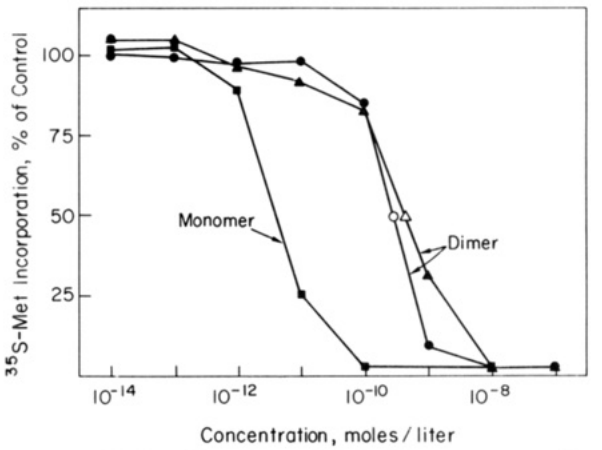

FIGURE 4: Inhibition of protein synthesis by DT monomer and dimer in cell culture. Vero cells, plated at 5×10^4/vial and grown for 24 h, were incubated with increasing concentrations of monomer (■) or dimer (●,▲) at 4 °C for 2 h to allow toxin binding and then washed twice with cold PBS. After incubation at 37 °C for 21 h in standard growth medium, the cells were pulse-labeled with low-methionine medium containing 2 μCi/mL [^{35}S]methionine for 1 h, and acid-precipitable radioactivity was determined. Values presented are relative to untreated controls. The open symbols represent LD_{50} values calculated for the two preparations of dimer and are based solely on their residual concentrations of contaminating monomer (1.2% and 0.9%, respectively). These calculations assume an LD_{50} for pure monomer of 3.9×10^{-12} mol/L.

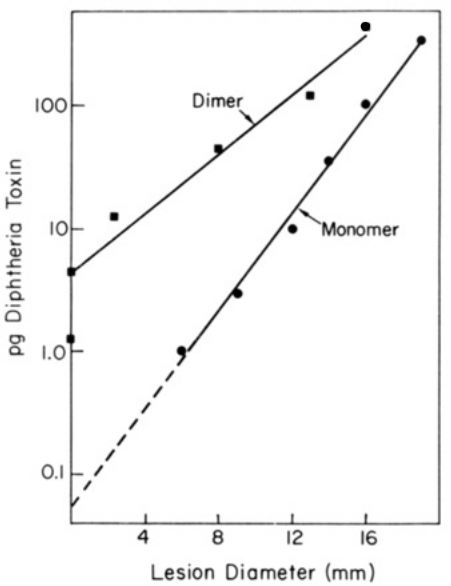

FIGURE 5: Induction of dermonecrotic skin lesions by DT monomer and dimer. The shaved backs of New Zealand White rabbits were injected intradermally with increasing amounts of monomer (●) or dimer (■) dissolved in 50 μL of PBS–BSA. Forty-eight hours later, lesion diameters were determined and plotted as a function of the amount of toxin injected. Each point represents the average of at least two animals and injections.

paradox may be that the dimer per se is nontoxic, but it dissociates to toxic monomers in the animal over a period of hours to days. This may explain the report of Goor (1968) that the 6.8S form of DT had approximately the same lethal activity for guinea pigs as the monomer.

Enzymic and Ligand-Binding Properties of Monomer and Dimer. On a weight basis both monomer and dimer showed identical specific activities in the NAD-glycohydrolase and ADP-ribosyltransferase reactions (data not shown). Using flow dialysis, we found that monomer and dimer had similar affinities for NAD (K_d = 10 and 16 μM, respectively) and that a molecule of dimeric toxin contained about twice the number

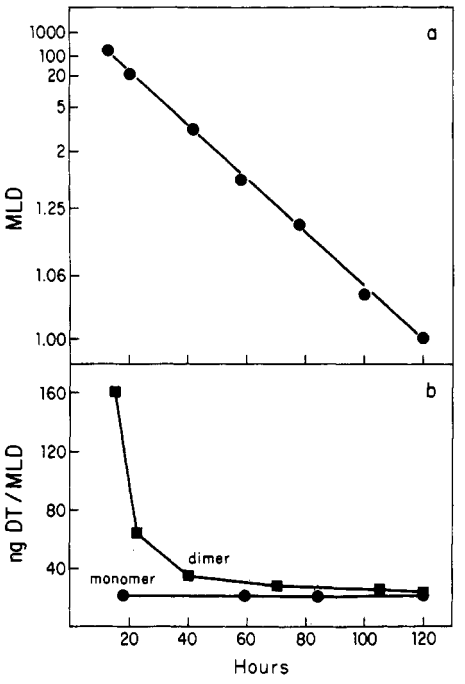

FIGURE 6: Lethality of DT monomer and dimer in guinea pigs. Animals were injected subcutaneously with increasing amounts of monomer or dimer in PBS–BSA, and survival times were recorded. Values have been normalized to those for 300-g guinea pigs. An MLD is defined as the amount of toxin required to kill a 300-g guinea pig at 120 h. (a) Standard curve for the lethality of monomer, showing the dependence of survival time on MLD injected (plotted in terms of log log MLD); (b) values for the amount of toxin (in nanograms) per MLD as a function of survival time, as calculated from the standard curve in panel a and the amounts of toxin injected.

of binding sites (2.1 mol^{-1}) as monomeric toxin (1.1 mol^{-1}). Also, the affinity of ApUp for dimer at 25 °C (310 pM) and the number of binding sites (2.2 mol^{-1}) correlated with values found for the monomer (300 pM, 0.9 mol^{-1}) (Collins et al., 1984).

Binding of Monomer and Dimer to Vero Cells. We measured the abilities of monomeric and dimeric DT to compete with radiolabeled monomer for receptors on Vero cells (Figure 7). Binding of ^{125}I-DT monomer was inhibited by 50% in the presence of an equal concentration (by weight) of unlabeled monomer, but an 18-fold higher concentration of dimer was required for the same level of inhibition. One-seventeenth of this dimer preparation (5.6%) was monomer.

The binding of ^{125}I-DT dimer to Vero cells was also measured directly and found to be at least 20-fold lower than that of monomer (data not shown). The low level of binding observed was nonspecific, since inclusion of a 100-fold excess of unlabeled dimer or monomer did not reduce the amount of label that became associated with cells.

Immunoreactivity of DT Monomer and Dimer. In a solid-phase radioimmunoassay (data not shown) monomer was effective at inhibiting the binding of polyclonal antimonomer antibody to immobilized monomer or dimer. Free dimer readily blocked binding with immobilized dimer but was much less effective against immobilized monomer. We estimated that about 30% of the antigenic determinants on monomer are inaccessible in the dimeric form. Variations in the immunoreactivity of monomeric and dimeric DT were also detected by Pappenheimer et al. (1972).

Properties of Multimeric Forms Other Than Dimer. Several preparations of DT composed primarily of tetramer or hexamer were also examined (data not shown). Each was significantly less toxic in cell culture than purified monomer,

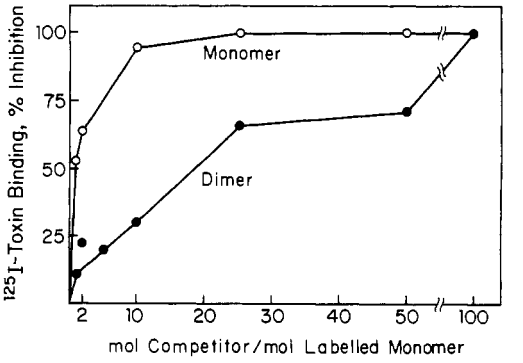

FIGURE 7: Inhibition of ^{125}I-DT monomer binding to cells in culture. Vero cells (5×10^4/well) were treated at 4 °C with 0.5 mL of binding buffer containing ^{125}I monomer (0.12 μg/mL; 4.8×10^7 cpm/μg) and increasing concentrations of unlabeled monomer (○) or dimer (●). After 7 h, the cell lawns were washed 4 times with cold PBS and dissolved in 0.4 mL of 0.1 N NaOH, and the cell-associated toxin was measured by liquid scintillation.

and each could be converted to the fully toxic monomer by treatment with Me_2SO. Due to their instability, these forms were not characterized in detail.

Discussion

The dimeric form of DT characterized here behaves as a distinct molecular species and is sufficiently stable to withstand standard analytical and chromatographic procedures. Furthermore, we have found that highly ordered crystals, originally believed to be of monomeric DT (Collier et al., 1982), are actually of the dimeric form (unpublished data). That dimer forms such highly ordered lattices represents the best evidence that it is in fact a distinct molecular species and not a collection of similar, but not identical, molecular aggregates. Crystals formed from purified dimer are currently being analyzed by X-ray crystallography.

Purified dimeric DT was essentially nontoxic but could be readily dissociated into the fully toxic, monomeric form. The dimer must therefore represent a noncovalent association of two potentially toxic monomers. What forces and which regions of the toxin molecule are involved in the association? A definitive answer awaits solution of the three-dimensional structure, but we believe that dimers (and probably higher order aggregates as well) form when hydrophobic regions of the B moiety, normally buried in the monomeric toxin, come into contact. Evidence in support of this conclusion is as follows:

(1) Dimethyl sulfoxide or formamide quantitatively dissociated the dimeric toxin into monomers, whereas NaCl, NaSCN, urea, or Gdn-HCl did not. This suggests that the interactions between monomeric units are primarily hydrophobic, and not ionic. Hydrophobic interactions have also been implicated in other multimeric systems, such as the dimerization and multimerization of insulin (Pocker & Biswas, 1981), in the H2A–H2B dimer and H3–H4 tetramer of histone core complexes (Eickbush & Moudrianakis, 1978), and in certain antibody–antigen interactions (van Oss et al., 1979).

(2) The B fragment is known to contain long stretches of hydrophobic sequences believed to function in the insertion of DT into lipid bilayers and possibly in transmembrane transport of the A fragment into the cytosol (Lambotte et al., 1980). Although normally buried in the native molecule, at least some of these sequences become exposed when DT is incubated at pHs below 5 (Blewitt et al., 1984; Collins & Collier, unpublished data). These are conditions under which toxin insertion into membranes is maximal (Donovan et al., 1981; Kagan et al., 1981).

(3) A noncovalent dimer of a hydrophobic cyanogen bromide fragment from the B moiety (CNBr-1) has been reported (Falmagne et al., 1978), whereas no noncovalent dimers of fragment A have been found. Dimers of intact fragment B are also frequently observed during purification of fragment A, even in the presence of reducing agents and 4–6 M urea (unpublished data).

(4) The inability of dimeric DT to inhibit protein synthesis in susceptible cells appears to be due to the loss of receptor recognition, a function generally attributed to the B moiety (Eidels et al., 1983). Labeled dimer showed no specific binding to intact cells, and unlabeled dimer was unable to compete with labeled monomer for receptor binding. The slight competition observed with dimer preparations was attributable to traces of contaminating monomer.

(5) All functions related to the A fragment were unaltered in the dimeric toxin. The NAD site on each monomeric toxin within the dimer was found to bind and hydrolyze NAD, and treatment with thiols liberated a fully functional A fragment capable of ADP-ribosylating EF-2 in vitro. Dimer also bound ApUp with the expected stoichiometry (2 mol/mol) and the same affinity as native monomer. The latter implies that the composite nucleotide binding site, formed by the NAD site on fragment A and the P site on fragment B (Lory et al., 1980b; Proia et al., 1980), is unaffected in the dimer. Nucleotide-free dimer was also found to bind Matrex Gel Green A, an immobilized triazine dye believed to interact with the composite NAD site/P site of whole toxin.

The structure of the monomeric components of the dimeric toxin molecule thus appears to be locally altered within hydrophobic and receptor-binding regions of the B domain, while large portions of the molecule are apparently unperturbed. Consistent with this view, the far-ultraviolet circular dichroism spectra of the monomer and dimer are identical, but perturbations of aromatic residues within the dimeric structure are indicated (Collins & Collier, 1984) from near-ultraviolet spectra. Studies are currently in progress to determine the membrane insertion activity of the dimeric form.

While it is doubtful that the soluble dimeric form, as such, plays any direct role in the action of DT, detailed structural analysis of the dimer may yield important information about the process by which the toxin enters susceptible cells. In studies to be presented elsewhere, we have found that dimerization and multimerization can occur when DT is exposed to low pH. Low pH is known to have physiologically important effects on DT; namely, it induces (i) exposure of hydrophobic surfaces of the B moiety (Blewitt et al., 1984; Collins and Collier, unpublished data), (ii) insertion of the toxin into artificial lipid bilayers and formation of ion-conductive channels (Donovan et al., 1981; Kagan et al., 1981), and (iii) transfer of the A moiety across plasma membranes (Sandvig & Olsnes, 1980; Draper & Simon, 1980) and, presumably, across endosomal membranes as well. In the normal entry process DT experiences low pH conditions only once it has bound to its receptor, and in this state, exposure of hydrophobic surfaces apparently leads to membrane insertion. Exposure of such surfaces while the toxin is free in solution leads instead to formation of dimers and multimers. It may be, therefore, that the structure of the partially unfolded DT monomer within the dimer resembles a potential intermediate in the process of membrane insertion.

ACKNOWLEDGMENTS

We thank Patricia Bernard and Denise Owens for excellent technical assistance.

REFERENCES

Barbieri, J. T., Carroll, S. F., Collier, R. J., & McCloskey, J. A. (1981) *J. Biol. Chem. 256*, 12247–12251.

Baseman, J. B., Pappenheimer, A. M., Jr., Gill, D. M., & Harper, A. A. (1970) *J. Exp. Med. 132*, 1138–1152.

Blewitt, M. G., Zhao, J.-M., McKeever, B., Sarma, R., & London, E. (1984) *Biochem. Biophys. Res. Commun. 120*, 286–290.

Collier, R. J., & Kandel, J. (1971) *J. Biol. Chem. 246*, 1496–1503.

Collier, R. J., Westbrook, E. M., McKay, D. B., & Eisenberg, D. (1982) *J. Biol. Chem. 257*, 5283–5285.

Collins, C. M., & Collier, R. J. (1984) *Biochim. Biophys. Acta 828*, 138–143.

Collins, C. M., Barbieri, J. T., & Collier, R. J. (1984) *J. Biol. Chem. 259*, 15154–15158.

Donovan, J. T., Simon, M. I., Draper, R. K., & Montal, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 172–176.

Draper, R. K., & Simon, M. I. (1980) *J. Cell Biol. 87*, 849–854.

Drazin, R., Kandel, J., & Collier, R. J. (1971) *J. Biol. Chem. 246*, 1504–1510.

Eickbush, T. H., & Moudrianakis, E. N. (1978) *Biochemistry 17*, 4955–4964.

Eidels, L., Proia, R. L., & Hart, D. A. (1983) *Microbiol. Rev. 47*, 596–620.

Falmagne, P., Lambotte, P., & Dirkx, J. (1978) *Biochim. Biophys. Acta 535*, 54–56.

Gill, D. M., & Dinius, L. L. (1971) *J. Biol. Chem. 246*, 1485–1491.

Gill, D. M., & Pappenheimer, A. M., Jr. (1971) *J. Biol. Chem. 246*, 1492–1495.

Goor, R. S. (1968) *Nature (London) 217*, 1051–1053.

Kagan, B. L., Finkelstein, A., & Colombini, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4950–4954.

Kandel, J., Collier, R. J., & Chung, D. W. (1974) *J. Biol. Chem. 249*, 2088–2097.

Lambotte, P., Falmagne, P., Capiau, C., Zanen, J., Ruysschaert, J.-M., & Dirkx, J. (1980) *J. Cell Biol. 87*, 837–840.

Lory, S., & Collier, R. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 267–271.

Lory, S., Carroll, S. F., & Collier, R. J. (1980a) *J. Biol. Chem. 255*, 12016–12019.

Lory, S., Carroll, S. F., Bernard, P. D., & Collier, R. J. (1980b) *J. Biol. Chem. 255*, 12011–12015.

McKeever, B., & Sarma, R. (1982) *J. Biol. Chem. 257*, 6923–6925.

Morrison, M. (1980) *Methods Enzymol. 70*, 214–220.

Pappenheimer, A. M., Jr., & Brown, R. (1968) *J. Exp. Med. 127*, 1073–1086.

Pappenheimer, A. M., Jr., Uchida, T., & Harper, A. A. (1972) *Immunochemistry 9*, 891–906.

Pocker, Y., & Biswas, S. B. (1981) *Biochemistry 20*, 4354–4361.

Proia, R. L., Wray, S. K., Hart, D. A., & Eidels, L. (1980) *J. Biol. Chem. 255*, 12025–12033.

Relyveld, E.-H. (1970) *C. R. Seances Acad. Sci., Ser. 3 270*, 410–413.

Sandvig, K., & Olsnes, S. (1980) *J. Cell Biol. 87*, 828–832.

van Oss, C. J., Absolom, D. R., Grossberg, A. L., & Neumann, A. W. (1979) *Immunol. Commun. 8*, 11–29.