Characterization of High-Order Diphtheria Toxin Oligomers[†]

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ABSTRACT: In 3D domain swapping, a domain of a protein breaks its noncovalent bonds with the protein core and its place is taken by the identical domain of another molecule, creating a strongly bound dimer or higher order oligomer. For some proteins, including diphtheria toxin, 3D domain swapping may affect protein function. To explore the molecular basis of 3D domain swapping in a well-characterized protein system, domain-swapped oligomers of diphtheria toxin were produced by freezing and thawing under a variety conditions, including in various salts and buffers, and at various temperatures. Reaction yields were followed by high-performance size-exclusion chromatography. The traditional low pH pulse produced by freeze−thawing in mixed sodium phosphate buffer induces the oligomerization of DT, but the addition of alkali chloride salts was found to increase the yield in the order of $Li^+ > Na^+ > K^+$. Unexpectedly, oligomers also formed when DT was frozen and thawed in the presence of 1 M LiCl alone. Slower freezing and thawing of the mixture led to the production of more and larger oligomers. DT oligomers were also produced by exposure to acidic buffers, and were found by electron microscopy to adopt both linear and cyclized forms in a wide distribution of sizes. Upon the basis of these results, the model for the production of DT oligomers by freezing and thawing was expanded to include a salt-mediated pathway. We present a mechanism for the formation of high-order DT oligomers by acidification that takes into account domain swapping and hydrophobic interactions.

Diphtheria toxin (DT)[1] is a 535-residue protein that is the cytotoxic agent in the disease diphtheria. It is secreted by pathogenic strains of *Corynebacterium diphtheriae* as a monomer and contains three domains, each of which performs a distinct function in cellular intoxication. The receptor-binding domain (R, residues 389−535) attaches to a receptor on the target cell (*1*). After endocytosis and exposure to the acidic environment of the endosome, the translocation domain (T, residues 200−379) facilitates the passage of the catalytic domain (C, residues 1−188) into the cytosol. After translocation, the C domain inactivates an essential component of the cell's protein translation system, resulting in cell death (*2−4*). Residues 379−388 constitute the hinge loop, the extended chain that changes conformation when the R-domain swaps into the R-domain site of a second molecule in the process known as 3D domain-swapping.

DT monomers aggregate under a variety of conditions to form oligomers. The dimeric form was identified by Goor (*5*) after samples of monomer were frozen and thawed in the presence of phosphate buffer. Relyveld (*6*) reported the formation of DT dimers as well as higher-order oligomers after vigorously agitating a solution of monomer while it was being precipitated with ammonium sulfate. Carroll et al. (*7*) observed the dimer in commercial preparations of

diphtheria toxin that had been frozen and thawed in mixed phosphate buffer, and found the dimer to be metastable, requiring denaturation for quick separation of the chains.

A growing collection of evidence suggests that the active complex of the toxin that is capable of translocating the C domain across the cell membrane is an oligomer. Bell et al. showed that fragments of DT including the C and T domains form discreet oligomers of 20−24 subunits at the physiological pH of 5.0 (*8*). Silverman et al. demonstrated that a construct combining the C domain with helices 8 and 9 of the T domain was sufficient to form ion channels that are associated with translocation. The formation of an oligomer would be required to produce a transmembrane channel from two helices (*9*). In earlier studies, transmembrane channel activity was found to have a nonlinear dependence on toxin concentration, leading to the suggestion that the channel is formed by a DT oligomer (*10, 11*). The hydrophobic stretches of the transmembrane helices in DT are shorter than those of typical membrane proteins, resulting in the proposal that DT may oligomerize in order to form a large enough hydrophobic surface area to interact with a lipid bilayer (*12*). Finally, other toxins such as anthrax toxin (*13*) and α-hemolysin (*14*) have been shown to convert from a water-soluble monomer to a membrane-soluble oligomer.

The atomic basis of the adhesion of DT monomers in the DT dimer was revealed by comparison of the X-ray crystal structures of the DT monomer (*15*) and the DT dimer (*16*): the dimer is comprised of two monomers which swap their entire R domains, by a conformational change of the hinge loop. This type of oligomerization which conserves the "closed" interface between the R-domain and the rest of the

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[1] Abbreviations: DT, diphtheria toxin; EM, electron microscopy; HPSEC, high-performance size-exclusion chromatography; ApUp, adenylyl(3′→5′)uridine-3′-monophosphate; HB-EGF, heparin-binding epidermal growth factor.

DT molecule was termed "3D domain-swapping" and was later identified as a mechanism for the assembly of a variety of oligomeric proteins (*17*, *18*), including perhaps amyloid aggregates (*19*). DT was also found to be the dimeric, domain-swapped form in crystals of DT bound to a fragment of its receptor (*20*).

The formation of domain-swapped DT dimers requires initial disruption of a network of salt bridges and hydrogen bonds that form the "closed" interface between the C and the R domains of a DT monomer (*16*). The R domain then breaks its noncovalent bonds with the C and the T domains while remaining covalently tethered to them via the hinge-loop (residues 379−388) to form the "open monomer". Finally, the R domains of two open monomers bind with each other's C domains to reestablish the closed interfaces, forming a dimer.

The eutectic freezing and thawing process of an aqueous solution provides a means to transiently disrupt the closed interface and promote the formation of DT oligomers. As a solution decreases in temperature, an equilibrium between the solid and liquid phases of salt and water is maintained. Therefore, at any given temperature, the ratio of solutes in the liquid phase of this solution depends on their relative solubilities and is called the "eutectic composition" (*21*). In the case of freezing DT in the presence of mixed phosphate buffer, water freezes as the temperature is lowered, producing a concentrated, salty DT solution. Then, as the temperature is further lowered, the poorly soluble dibasic phosphate component precipitates, leading to a drop in pH from neutral to 3.6 by the time that the solution reaches its eutectic freezing point (*22*). This acidification leads to the protonation of the carboxylate residues in the salt bridges of the closed interface of DT and thus its disruption (*15*). Upon warming, these steps are reversed, first neutralizing the concentrated DT solution, which stabilizes the closed interfaces in newly formed dimers. Then the ice melts, leaving a neutral solution rich in dimers.

Bennett et al. (*17*, *18*) suggested that domain-swapping can lead to the formation of linear or cyclized high-order DT oligomers, some of which may grow large enough to fall out of solution. In this work, we further characterize the conditions under which higher-order DT oligomers are formed and confirm the existence of both linear and cyclized oligomers. To describe this, we expand the model mechanism for the formation of DT dimers to include all oligomers and to take into account both an acid-mediated and our newly discovered salt-mediated reaction pathway.

EXPERIMENTAL PROCEDURES

Purification of DT. Unnicked DT was purchased from Connaught Laboratories and received in a frozen state. After slow thawing at 25 °C, 20% of the toxin was found to be dimeric and a trace amount of the toxin was trimeric. A 2-fold molar excess of ApUp (Sigma, no. A-4298) was added to the protein, which was then purified by the method of Carroll et al. (*23*).

Production of DT Oligomers by Freezing and Thawing. Samples of monomeric DT of 10 μL were prepared in 1.5 mL centrifuge tubes by diluting the protein stock solution with various concentrations of sodium phosphate, pH 7.0, lithium chloride, and/or sodium chloride.
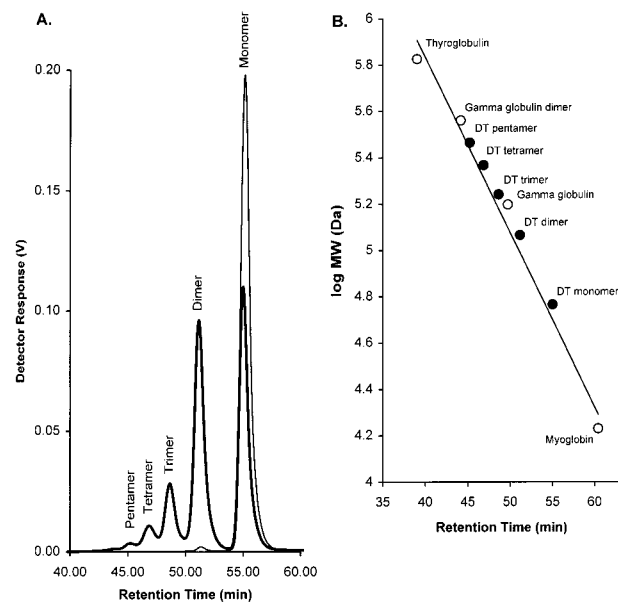

FIGURE 1: (A) Detection and molecular mass measurements of DT oligomers. The dark line is a HPSEC chromatogram of DT oligomers produced as a result of freezing and thawing a 10 μL mixture of 6 mg/mL monomer, 25 mM sodium phosphate, pH 7.5, and 100 mM LiCl. The light line is a chromatogram of the same mixture prior to freezing and thawing. Notice the conversion of monomeric DT to oligomers by the process of freezing and thawing in the selected buffer. The analysis was performed using two TSK3000SWxl silica columns (TosoHaas) in series with a running buffer comprised of 25 mM sodium phosphate and 100 mM sodium sulfate, pH 6.9, flowing at 0.4 mL/min. Injection volume was 3 μL, and absorbance was detected at 214 nm. Chromatogram voltages are normalized for baseline. (B) The molecular weight calibration of DT oligomers. Standard curve comparing peaks in panel A to the retention times of proteins from a set of HPSEC molecular weight standards (Bio-Rad), which were run under the same conditions. The dark line is a linear regression of the retention times versus the log molecular weights of the standards. The molecular weights of the standards are thyroglobulin, 670 kDa; γ globulin dimer, 316 kDa; γ globulin, 158 kDa; and myoglobin, 17 kDa. The molecular masses of the DT molecules are pentamer, 293 kDa; tetramer, 234 kDa; trimer, 175 kDa; dimer, 117 kDa; and monomer, 58 kDa.

The samples were frozen by one of two methods: slow or rapid. In the slow method, the samples were placed in fiberboard storage boxes (Revco, no. 5954), and then the boxes were placed into freezers at a variety of temperatures (−20, −40, and −80 °C). In the rapid method, samples were frozen by immersion into a bath of dry ice and 2-propanol, and then stored in boxes at −80 °C.

After 24 h, the samples were removed from the freezer and thawed by one of two methods: slow or rapid. In the slow method, the unopened boxes of samples were placed into either 25 or 4 °C air. In the rapid method, the samples were removed from the box and quickly placed into either a 25 or a 4 °C water bath.

Production of DT Oligomers in Acidic Buffer. Sample A: Following the hanging drop vapor diffusion technique used to grow protein crystals, droplets consisting of 2 μL of 0.04 mg/mL DT monomer in 10 mM Tris-HCl, pH 7.5, 2 μL of water, and 4 μL of 50 mM sodium citrate, pH 5.2, were suspended over 1 mL reservoirs of 50 mM sodium citrate, pH 5.2. Small quantities of precipitate were observed in the droplets after 12 h, and the samples were analyzed by negative stain electron microscopy after 4 days.

A. No salt added

[DT] (mg/ml)	[sodium phosphate] (mM)		
	25	100	400
6	M: 100% D: 0% H: 0%	M: 65% D: 31% H: 4%	M: 49% D: 47% H: 4%
25	M: 100% D: 0% H: 0%	M: 69% D: 21% H: 10%	M: 45% D: 44% H: 11%

B. 100 mM NaCl

[DT] (mg/ml)	[sodium phosphate] (mM)		
	100	200	400
25	M: 48% D: 35% H: 17%	M: 40% D: 37% H: 23%	M: 35% D: 42% H: 23%

C. 100 mM LiCl

[DT] (mg/ml)	[sodium phosphate] (mM)		
	12.5	50	200
11	M: 66% D: 25% H: 9%	M: 48% D: 41% H: 11%	M: 28% D: 53% H: 19%

D. 100 mM NaCl or LiCl

Salt (100 mM)	Sodium phosphate pH		
	7.0	7.5	8.0
LiCl	M: 35% D: 31% H: 34%	M: 39% D: 23% H: 38%	Precipitate
NaCl	M: 46% D: 28% H: 26%	M: 37% D: 34% H: 29%	Precipitate

E. 100 mM NaCl

[DT] (mg/ml)	[sodium phosphate] (mM)		
	25	50	100
6	M: 63% D: 35% H: 2%	M: 37% D: 59% H: 4%	M: 32% D: 63% H: 5%
12	N/A	M: 54% D: 42% H: 4%	M: 32% D: 61% H: 7%
25	N/A	N/A	M: 52% D: 42% H: 6%

F. 1M LiCl, no phosphate

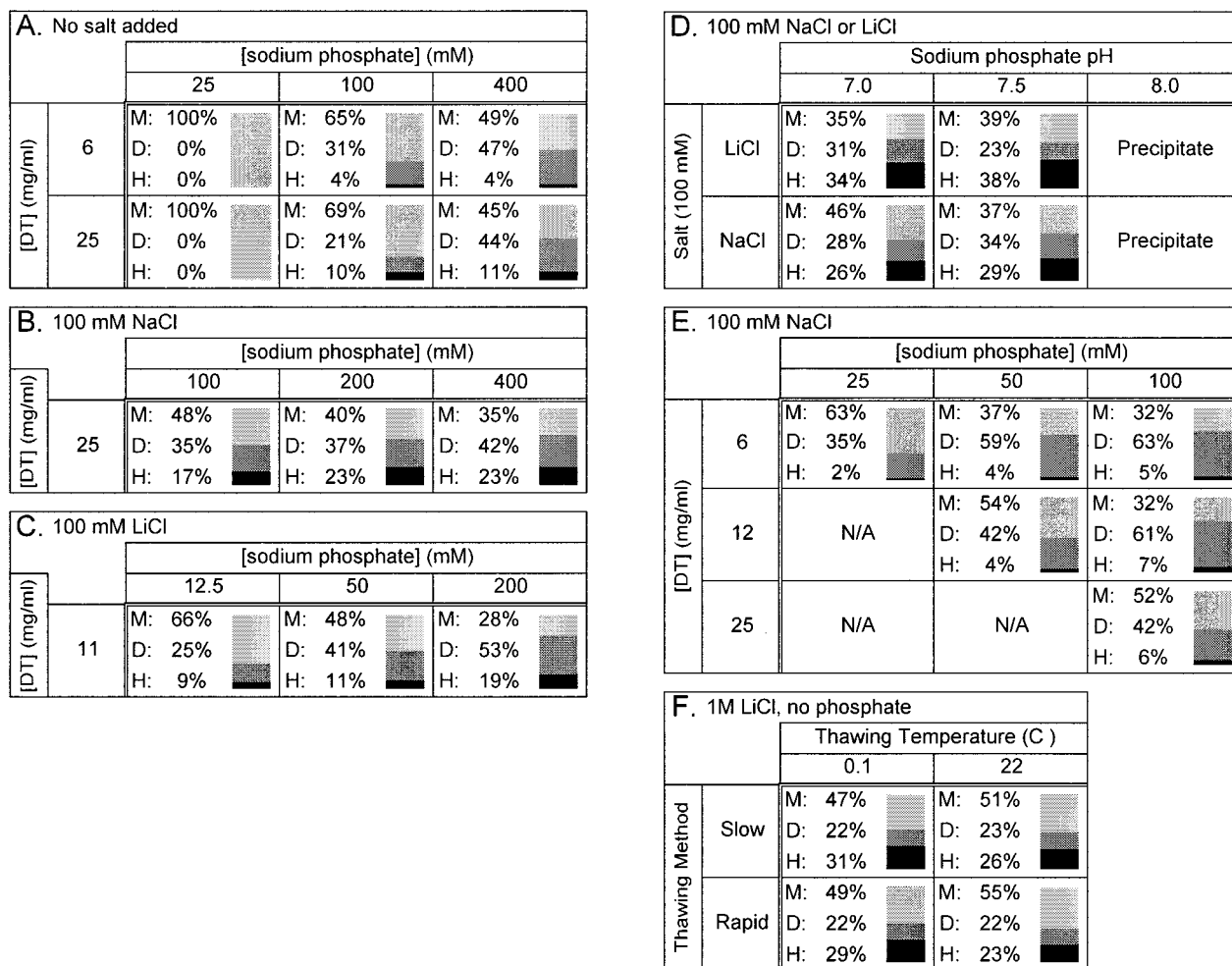| Thawing Method	Thawing Temperature (C)	
	0.1	22
Slow	M: 47% D: 22% H: 31%	M: 51% D: 23% H: 26%
Rapid	M: 49% D: 22% H: 29%	M: 55% D: 22% H: 23%

FIGURE 2: Relative distributions of DT monomer (M), dimer (D), and high-order oligomers of trimer size or larger (H) produced by freezing and thawing samples of monomer under different phosphate buffer, salt, and temperature conditions. Shaded bars show the proportion of monomer (light gray), dimer (medium gray), and heavier oligomers (black) in the sample. Unless noted otherwise, sample size was 10 μL, the pH was 7.5, freezing was at -20 to -30 °C using the "slow" method, and thawing was at 4 °C using the slow method. Slow and rapid methods are described in Experimental Procedures. Analysis performed by HPSEC. We estimate the error for these values to be $\pm 1.5\%$ based upon the reproducibility of similar experiments in our laboratory. (A) Varying sodium phosphate concentration vs DT monomer concentration. No salt added. Thawed at 22 °C. (B) Varying sodium phosphate concentration vs constant DT monomer concentration. 100 mM NaCl added. Thawed at 22 °C. (C) Varying sodium phosphate concentration vs constant DT monomer concentration. LiCl (100 mM) was added. (D) Varying sodium phosphate pH vs type of salt added. The respective salt (100 mM) was added to mixture. Sample volumes of 500 μL were frozen and thawed. (E) Varying sodium phosphate concentration vs DT monomer concentration. LiCl (100 mM) was added. Frozen at -80 °C by rapid method. Thawed at 22 °C. (F) Thawing temperature vs thawing method. DT monomer was 30 mg/mL and dialyzed into 1 M LiCl, pH 6.9. No sodium phosphate was present. Notice in particular the necessity of sodium phosphate buffer or salt in order for the oligomerization of DT to occur, and the increase in the yield of oligomers as the ionic strength is increased.

Sample B: Droplets consisting of 1.5 μL of 10 mg/mL DT monomer in 10 mM Tris-HCl, pH 7.5, and 1.5 μL of 50 mM sodium citrate, pH 5.2, were suspended over a 1 mL reservoir of 50 mM sodium citrate, pH 5.2. Small amounts of light brown precipitate were observed in the bottom of the drop after 3 days, but the protein in solution remained stable for several months at room temperature after that. Thirty days after preparation, the drop was diluted 300-fold in reservoir buffer and analyzed by negative stain electron microscopy.

Sample C: Droplets consisting of 2 μL of 4.0 mg/mL DT monomer in 10 mM Tris-HCl, pH 7.5, 2 μL of water, and 4 μL of 50 mM sodium acetate, pH 4.3 were suspended over a 1 mL reservoir of 50 mM sodium acetate, pH 4.3. No precipitate was observed in the drop, and the samples were analyzed by negative stain electron microscopy after 36 days.

Analysis of DT Oligomers. HPSEC was used to analyze DT oligomers after their production by freezing and thawing.

The oligomers and the size standards (BioRad #151$-$1901) against which they were calibrated were separated with two TSK-3000SWxl silica columns (Toso-Haas) in series using a Waters 510 pump and detected at an absorbance of 214 nm using a Waters 484 detector. A buffer of 25 mM sodium phosphate and 100 mM sodium sulfate, pH 6.9, was run at a flow rate of 0.4 mL/min. The relative distribution of the DT oligomers was determined by the integration of the absorbance peaks using the BASELINE 810 software package.

Electron microscopy was used to analyze DT oligomers after their production in acidic buffer. The sample in Figure 3A was negatively stained with 2% uranyl acetate, then imaged at 60000× magnification on a Philips CM120 electron microscope using an acceleration voltage of 120 kV and a slow-scan CCD camera. The samples in Figure 3, panels B and C, were negatively stained with 1% uranyl acetate, then imaged at 50000× magnification on a Hitachi
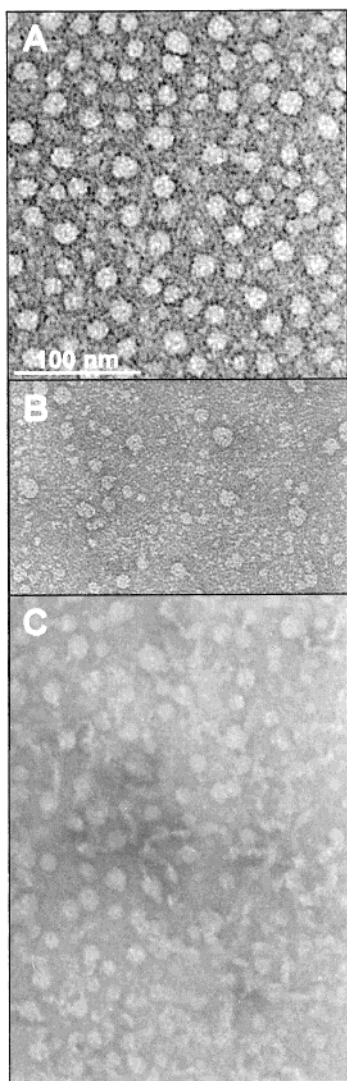
FIGURE 3: Negative stain electron micrographs of DT oligomers under acidic conditions. Oligomers were produced by diluting monomeric DT in 10 mM Tris-HCl, pH 7.5, with acidic buffer in a 1:1 ratio to form a hanging drop, which was suspended, over a 1 mL reservoir of the acidic buffer solution. All images are set to the same scale. (A) Cyclic oligomers were produced by the dilution of DT with 50 mM sodium citrate, pH 5.2. Sample was stained with 2% uranyl acetate and was imaged at 60000× magnification 1 day after preparation. These DT oligomers are completely cyclized and appear in a variety of diameters. (B) Highly heterogeneous distribution of cyclic oligomers was produced by the dilution of DT with 50 mM sodium citrate, pH 5.2, and 50 mM sodium phosphate, dibasic. Sample was stained with 1% uranyl acetate and was imaged at 50000× magnification 1 month after preparation. Some medium and large-sized oligomers exhibit concentrations of dark uranium stain near their centers. (C) These oligomers of DT were produced by the dilution of DT with 50 mM sodium acetate, pH 4.3. Sample was stained with 1% uranyl acetate and was imaged at 50000× magnification 1 day after preparation. Notice that these DT oligomers appear in both linear and cyclized forms.

H7000 electron microscope using an acceleration voltage of 75 kV and photographic film. It was essential for the binding of the protein that the graphite grids not be glow-discharged before the sample was applied to them.

RESULTS

Formation of Diphtheria Toxin Oligomers by Freezing and Thawing. Samples of diphtheria toxin monomer, which were frozen and thawed in the presence of salts and/or sodium phosphate buffer, yielded a variety of distributions of oligomers. These oligomers were separated at pH 6.9 by high-performance size-exclusion gel chromatography with a peak resolution fine enough to observe aggregates of up to heptamer size, quantify aggregates of up to pentamer size, and isolate aggregates of up to tetramer size. Unresolved aggregates larger than heptamer size comprised the void volume. The identity of each chromatogram peak in a distribution of oligomers was determined by comparing its retention time to a standard curve constructed from gel filtration size standards (Figure 1).

Sodium phosphate buffer at pH 7.5 is sufficient to induce oligomerization of DT by freeze−thawing, so long as the buffer is more concentrated than 50−100 mM (Figure 2A). The yield of all oligomers increased by 60% with the addition of 100 mM NaCl to 100 mM phosphate buffer, and the yield of trimers and higher order structures doubled with the addition of the same amount of salt to 400 mM phosphate buffer (Figure 2B).

The cation present during the freezing and thawing of DT has a significant effect on the degree of oligomerization of the sample. LiCl was found to be a more potent enhancer of oligomerization than was NaCl (Figure 2C). KCl, on the other hand, was found to have little effect as an additive (M. Bennett, unpublished data). Further, for fixed concentrations of protein and sodium phosphate, the addition of LiCl led to the production of more high-order aggregates than did NaCl (Figure 2D). This relationship in the contribution to oligomerization of $Li^+ > Na^+ > K^+$ suggests a mechanistic role for the cation related to size or solubility. The addition of divalent cations at concentrations similar to those used for the monovalent salts leads to the immediate precipitation of the protein; an effect that had been observed previously (*24*).

The initial pH of the phosphate buffer influences the degree of oligomerization as well, contributing to the production of more oligomers as the buffer is made more basic. In the extreme case at pH 8.0, aggregates of such large sizes were produced that a precipitate appeared in the thawed solution and remained there for several days until breaking down into smaller oligomers and redissolving, as predicted by Bennett et al. (*18*). The greater degree of oligomerization induced by a higher initial pH might seem surprising given the acid-catalyzed model for the oligomerization of DT, but it should be noted that the initial pH of a phosphate buffer solution has no effect on the pH of the solution at the eutectic freezing point, which is always 3.6 (*22*).

When the availability of salt and phosphate ion is limited, the degree of oligomerization is reduced as the concentration of diphtheria toxin is increased (Figure 2E). However, in the presence of a sufficiently high ratio of salt and phosphate ion to DT (∼ 0.010 mol/g protein), increasing the concentration of toxin leads to the production of more oligomers (Figure 2, panels A and E). These data underscore the role of salt and phosphate ion as limiting reagents in oligomerization.

The speed of freezing and thawing affects the amount of oligomerization as well as the distribution of the sizes of its products. A sample that was slowly frozen in a freezer yielded a greater proportion of high-order oligomers than did a sample that was rapidly frozen by immersion into a

bath of liquid 2-propanol and dry ice (Figure 2, panel B, left, vs panel E, lower right). Slow thawing led to slightly greater degrees of oligomerization than did rapid thawing (Figure 2F), and all of that increase was expressed in the formation of high-order oligomers. Diphtheria toxin, which was supercooled to −20 °C and incubated for 24 h, exhibited no oligomerization, indicating that the formation of crystalline ice (and presumably the resulting high concentration of DT) is a requirement for the reaction.

Finally, oligomers were produced by freezing and thawing DT in solutions of 1 M LiCl (Figure 2F). No mixed phosphate buffer was required to facilitate the reaction, and thus no pH change. This result, in combination with the oligomerization of DT by precipitation in ammonium sulfate by Relyveld (*6*), suggests that a salt-mediated reaction pathway exists in addition to the acid-mediated reaction pathway described by Carroll et al. (*7*) and Bennett et al. (*17*).

Formation of Diphtheria Toxin Oligomers by Acidification. Diphtheria toxin exposes hydrophobic surfaces under acidic conditions and often forms precipitates, which remain insoluble even upon return to neutral pH (*25, 26*). These characteristics make DT difficult to study under most low-pH conditions. However, partially soluble aggregates of the DT fragment CRM-45 (the C and T domains) were characterized by Bell et al. (*8*), who also determined that the whole toxin and this fragment shared the same solubility response to pH. Using buffer conditions similar to those employed by Bell et al., and in the absence of detergent, we produced partially soluble aggregates of whole DT and then examined them by negative-staining electron microscopy.

Diphtheria toxin almost exclusively forms cyclized oligomers near its narrow, salt-dependent pH transition point at pH 5.2 (Figure 3A). These oligomers are highly hydrophobic and bind to the carbon-coated electron microscopy grids only if not polarized by glow discharge. This preference suggests that the oligomers bind to the grid via patches of hydrophobic residues exposed by the pH-induced conformational change, possibly orienting them with respect to the film surface in the same direction that one would expect them to relate to a hydrophobic bilayer. As with DT aggregates produced by freeze−thaw methods, the oligomers occur in a wide distribution of sizes, and as such are not suited for analysis by image reconstruction. Near the transition pH, these aggregates gradually increase in size until they precipitate from solution (data not shown).

Under conditions of low ionic-strength just above its transition pH, DT slowly forms a very fine precipitate, particles of which were observed to be in near-constant motion likely induced by thermal drafts in the solution. An electron micrograph of the solution taken after negative staining with 1% uranyl acetate shows a highly heterogeneous mixture of cyclized DT oligomers (Figure 3B).

Below pH 5.2, a transition point where the DT becomes markedly insoluble, 1 mg/mL of diphtheria toxin in 25 mM sodium acetate at pH 4.3 formed no precipitate even in the absence of detergent. A negative stain electron micrograph of the sample indicates that the toxin aggregated into both linear and cyclized oligomers of varying orders (Figure 3C). This coexistence of the two forms and the high stability of the solution imply an equilibrium relationship among the linear and cyclized conformations of the DT oligomer.

DISCUSSION

Formation of DT Oligomers by Freezing and Thawing in Mixed Phosphate Buffer. At concentrations above ∼50mM, sodium phosphate buffer alone allows DT to oligomerize during freezing and thawing. Because eutectic formation concentrates the DT and reduces the pH to 3.6 (*23, 24*), the aggregation of DT is initiated by the protonation of its carboxylic and histidyl groups and the subsequent disruption of the salt bridges holding together the closed interface (*16*). However, because sodium phosphate buffer at pH 7.5 is about 85 mol % dibasic, most of the dibasic phosphate in that solution will have precipitated before the solution cools enough to drop below the transitional pH. Given a 25 mg/mL sample of DT, it would require a minimum of 200 mM sodium phosphate, pH 7.5, to provide an acidic proton to the 131 carboxylates and 32 histidyls on each DT molecule after the dibasic component of the phosphate buffer has precipitated. Because DT was oligomerized using half that amount of sodium phosphate, there must be a characteristic of phosphate buffer other than cooling-induced acidity that also facilitates the formation of the open monomer. We propose that that characteristic is direct ionic interaction with the protein through charge shielding.

New Salt-Mediated Oligomerization Pathway. Three main observations regarding salts can be drawn from the results of this study: (1) The addition of monovalent salts to solutions of DT and phosphate buffer substantially increased the yield of oligomers. (2) The identity of the cation in the added salt affected how much the yield was increased, in the order of $Li^+ > Na^+ > K^+$. (3) The presence of 1 M LiCl alone was sufficient to induce DT to oligomerize when frozen and thawed.

These three observations, along with the inability to account for the oligomerization of DT in low concentrations of sodium phosphate using the acid-mediated reaction model alone, suggest that a salt-mediated reaction pathway also exists (Figure 4). We propose that the salt-mediated pathway differs from the acid-mediated pathway in the means by which the salt bridges connecting the C and the R domains are broken: the ionic interactions are disrupted by the shielding effects of the salt instead of by the neutralization of their carboxylate components by protonation. This interpretation of the role of salt in weakening ionic forces in the closed monomer is supported by the fact that salt raises the transition pH of diphtheria toxin.

The rate of freezing and thawing affects both the production of DT oligomers and the distribution of their sizes. In general, the slower these processes are, the greater the degree of oligomerization and the size of the products. Solutions of monomer in phosphate buffer or salt require the formation of crystalline ice in order to oligomerize. Bennett et al. (*18*) describe the role of freezing in the reaction as creating high concentrations of toxin in the spaces between regions of growing ice crystals, where the acidic conditions induced by cooling phosphate buffer lead to the formation of open monomers, which then oligomerize as the solution thaws. The high concentration of toxin in these temporary spaces promotes oligomerization by lowering the entropic barrier to aggregation.

We expand this model by proposing that salts in the sample are concentrated by the same crystallization mechanism,
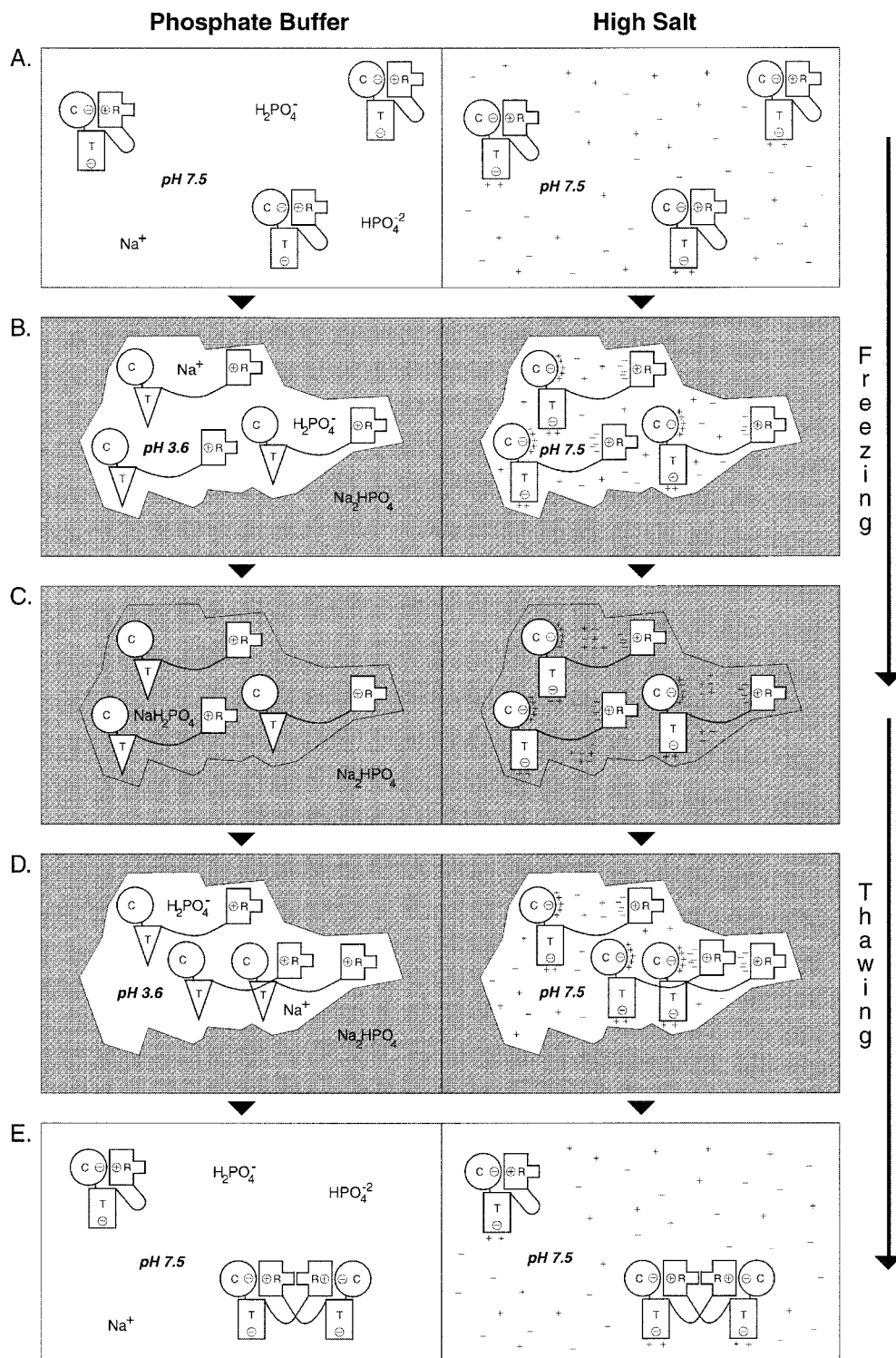
FIGURE 4: Two hypothetical pathways to the formation of domain swapped DT oligomers by freezing and thawing. (A) DT monomers aggregate into oligomers (E) when frozen and thawed in the presence of neutral sodium phosphate buffer (left), or a monovalent salt such as LiCl (right). (B) As the solution cools, ice crystals begin to form and the solutes become highly concentrated as the volume of liquid water available to them decreases. The dibasic component of sodium phosphate buffer solidifies, leaving the more soluble monobasic form in the liquid phase to acidify the solution (*22*) and neutralize the protein's carboxylates by protonation. In the salt-mediated pathway, all charged protein residues are shielded by the high concentrations of dissolved ions. As a result of the disruption of the ionic interactions between the C and the R domains in both pathways, the DT monomers adopt the more entropically favored open conformation. (C) The solution reaches the eutectic frozen state, where it can remain for long periods of time with no effect on the yield of the reaction. (D) When thawing begins, those regions containing high concentrations of solutes melt first; allowing oligomerization reactions to occur until thawing has proceeded to the point where the pH or salt conditions once again overwhelmingly disfavor the open form. The high concentrations of salt are likely to contribute to increased concentrations of dehydrated protein through "salting out", which has in itself been shown to oligomerize DT (*6*), possibly through the reduction of the entropic barrier to oligomerization (*17*, *18*). In both pathways, oligomers form as the ionic interactions between the C and R domains of the DT molecules are restored in a reversal of the events in panel B. (E) Upon the completion of thawing, the ionic interactions of the closed interfaces in both monomers and oligomers are returned to their full strength, stabilizing the closed forms of the molecules.
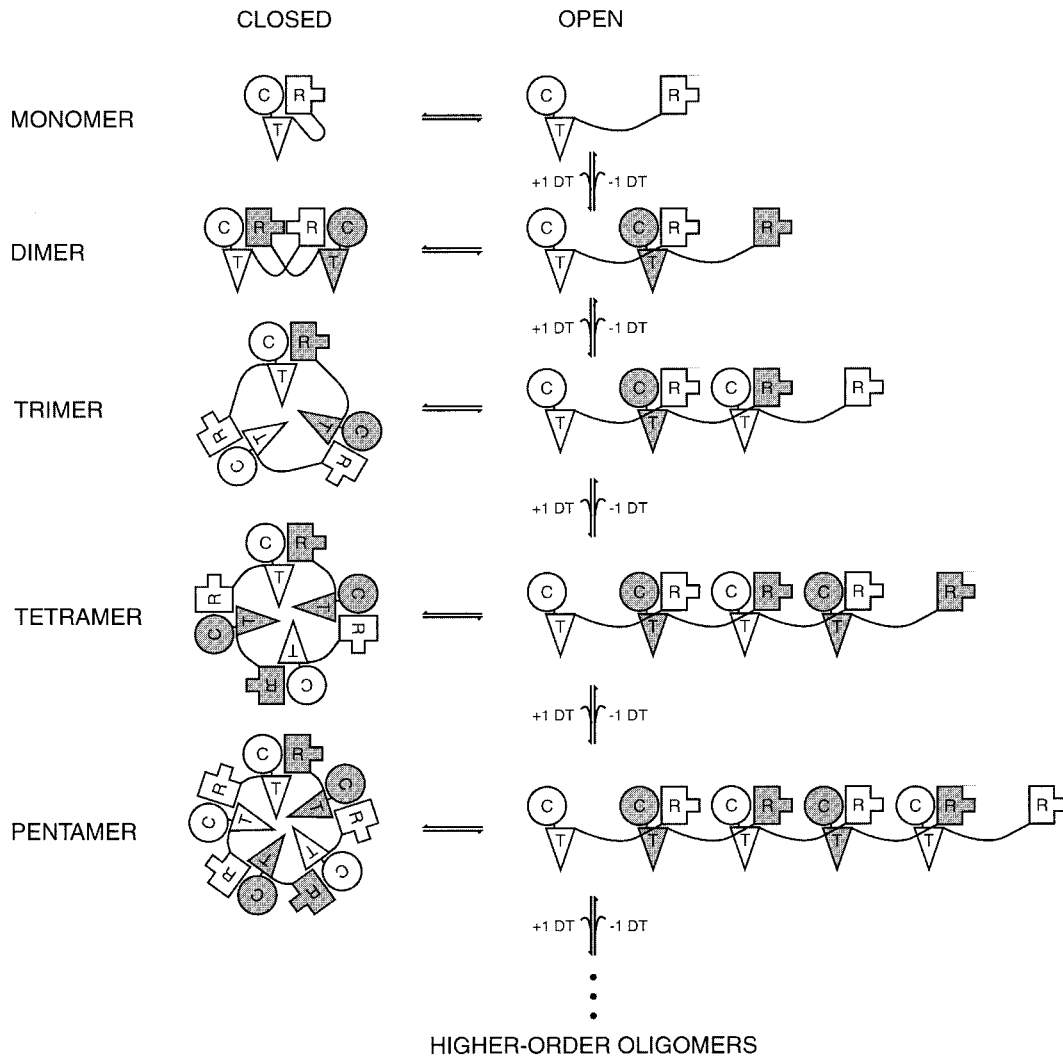
FIGURE 5: Molecular model for the formation of DT oligomers. The confirmation of the existence of both linear and cyclic DT aggregates at acidic pH leads to a model that expands upon the suggestion of Bennett et al. (*17*) that high-order DT oligomers may be comprised of chains of domain swapping monomers. Upon the disruption of the closed interface, molecules of DT form open monomers, which are stabilized by acidic or high-salt conditions. These open monomers combine by re-forming the closed interface with other open monomers, creating linear "open oligomers" which carry an exposed C domain half of the closed interface at one terminus and an exposed R domain half of the closed interface at the other. In turn, these open oligomers can extend by adding another open monomer to one end, break up into two smaller open oligomers by the disruption of an internal closed interface, or cyclize by the formation of a closed interface by the C and R domains at their termini. Conversely, cyclized oligomers can linearize by the disruption of any one of the closed interfaces that hold them together. As the domain swapping interactions take place on the C and the R domains, and the pH-dependent hydrophobic character of the molecule is on the T domain, this model does not preclude additional nonpolar interactions between adjacent monomers comprising an oligomer. Indeed, given the increase in the hydrophobicity of DT under acidic conditions, DT oligomers at or below the transition pH are likely to depend on hydrophobic interactions for their structure.

leading to both an increase in the disruption of the protein's ionic interactions and a depression of the eutectic freezing point of the reaction mixture. This depression allows more time between the concentration of the solutes and their freezing for open monomers to form. Similarly, the presence of additional salt allows these frozen regions containing open monomers to liquefy sooner during thawing, which provides more time for oligomers to form before the entire sample melts and the regions of concentrated reactants disperse. This model for the role of ice formation in the duration of the reaction is supported by the increase in oligomerization that results from a slowing in freezing and thawing speeds.

    The formation of DT oligomers by 3D domain swapping is related to the intoxication pathway of DT because both processes depend on the conformational change of the

molecule from its closed form to its open form. The separation of the C domain from the R domain is necessary during intoxication to free the C domain for translocation across the cell membrane and is necessary during oligomerization to expose the closed interface so that domain swapping can take place. In both processes, the interactions that bind the C domain to the R domain must be broken. Our observation that domain swapping, and therefore the formation of the open monomer, can be induced at high concentrations of salt and protein in the absence of acidifying buffer provides experimental evidence that the interactions holding the C–R interface together are ionic in nature and do not necessarily require acidic pH to break. This conclusion is supported by the observation by Louie et al. that the presence of a fragment of the cell-surface target of diphtheria

toxin, heparin-binding epidermal growth factor (HB-EGF), is sufficient to convert DT monomers into domain-swapped dimers at neutral pH (*20*).

Formation of DT Oligomers by Acidification. Diphtheria toxin forms heterogeneous mixtures of linear and cyclized oligomers under acidic conditions. Near the transition pH of the toxin, the reaction is shifted heavily in favor of producing the cyclized forms, but under conditions such as 25−50 mM sodium acetate, pH 4.3, the linear and cyclized oligomers are produced in more equal proportions. Bennett et al. (*17*) suggested that higher-order oligomers "are formed by domain swapping, making linear or cyclized aggregates in which each molecule interacts with two neighboring molecules by providing an R domain to one and accepting an R domain from the other." We find both linear and cyclized DT oligomers, confirming Bennett's model and expanding it to propose an equilibrium relationship between the two forms (Figure 5). Computer models show that the hinge loop that links the T to the R domain is long enough to accommodate the formation of both linear and cyclized oligomers of DT.

However, the work of Bell et al. (*8*) shows that there must be interactions other than domain swapping which hold oligomers together at low pH. CRM-45, a fragment of DT lacking the R domain but having the same hydrophobic response to pH as the whole toxin, was found to oligomerize at pH 5.0. Because the R domain is required for domain swapping, and as CRM-45 oligomers almost entirely reversed back to monomers upon return to neutral pH, it is likely that exposure of hydrophobic surfaces on the T domain stabilizes DT oligomers at or below the transition pH. Given that domain swapping in DT oligomers is well established (*16*), and considering the much higher stability of whole DT oligomers at neutral pH as compared to CRM-45 oligomers, we propose that both domain swapping between C and R domains and hydrophobic interactions between T domains contribute to the stability of whole toxin oligomers. The relative contribution of each type of interaction is dependent on the pH, with the hydrophobic interactions dominant below the transition point, domain swapping dominant above it, and both interactions significant near it.

This study adds to the evidence that the biologically active DT complex that translocates the C domain across the cell membrane is an oligomer by showing that the whole protein assembles into objects of discreet size (100 ± 25 nm diameter) and circular shape at the physiologically active pH of 5.2 (Figure 3, panels A and B), These aggregates have not been imaged before due to the difficulty of keeping DT in solution under acidic conditions. Our use of low concentrations of DT (0.5−5 mg/mL) under low ionic strength conditions (5−30 mM salt, including buffer) enhanced the stability of the protein at low pH long enough for electron microscopy studies to be performed. We observed that decreasing the protein concentration and ionic strength of DT solutions slowed but ultimately did not prevent the eventual precipitation of the toxin at low pH. The most dilute combinations of protein and ionic strength led to samples of DT at the endosomal pH of 5.2−5.4 that were partially soluble at room temperature for months, particularly when acetate buffer was used instead of citrate buffer.

While Louie et al. note the conversion of DT monomer into domain-swapped dimer by a fragment of the HB-EGF receptor (*20*), Carroll et al. observe that the domain-swapped DT dimer does not bind to the HB-EGF receptor, rendering it nontoxic (*7*). These two results can be integrated with the findings of this work by proposing that the binding region (residues 106−147) of the HB-EGF receptor disrupts the ionic interactions that hold the C and the R domains together upon the binding of the receptor to monomeric DT, causing the formation of the open monomer. We propose that this complex then binds with other open monomers to form an oligomeric translocation assembly. Much of the receptor-binding surface of the DT R-domain is sterically blocked in the crystal structure of the domain-swapped dimer (*16*), so HB-EGF cannot dock and disrupt the dimer's C−R interface. Because we propose that higher-order oligomers are formed by the same domain-swapping process that DT dimers are and would therefore have similarly blocked R domains, we predict that they too will be found to be nontoxic.

Some cyclic oligomers near the transition pH exhibit single, dark spots of accumulated uranyl stain near their centers (Figure 3B). These spots suggest dense regions of negative charge to which uranyl cation complexes, which are of the general formula $UO_2(OUO_2)_{n+1}^{2+}$, preferentially bind (*27, 28*). Cation channels also tend to have centers of dense, negative charge, but the resolution of the electron micrographs and the heterogeneity of the samples allow only speculation that the dark spots could conceivably be related to the cation channels formed by DT oligomers in membranes at acidic pH.

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