A Kinetic Model of Intermediate Formation during Assembly of Cholera Toxin B-subunit Pentamers*

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Cholera toxin is the most important virulence factor produced by Vibrio cholerae. The pentameric B-subunit of the toxin can bind to GM1-ganglioside receptors, leading to toxin entry into mammalian cells. Here, the in vitro disassembly and reassembly of CtxB₅ (the B subunit pentamer of cholera toxin) is investigated. When CtxB₅ was acidified at pH 1.0 and then neutralized, the B-subunits disassembled and could no longer migrate as SDS-stable pentamers on polyacrylamide gels or be captured by GM1. However, continued incubation at neutral pH resulted in the B-subunits regaining the capacity to be detected by GM1 enzyme-linked immunosorbent assay (tₕ/₂ ~ 8 min) and to migrate as SDS-stable pentamers (tₚ/₂ ~ 15 min). Time-dependent changes in Trp fluorescence intensity during B-subunit reassembly occurred with a half-time of ~8 min, similar to that detected by GM1 enzyme-linked immunosorbent assay, suggesting that both methods monitor earlier events than B-pentamer formation alone. Based on the Trp fluorescence intensity measurements, a kinetic model of the pathway of CtxB₅ reassembly was generated that depended on trans to cis isomerization of Pro-93 to give an interface capable of subunit-subunit interaction. The model suggests formation of intermediates in the reaction, and these were successfully detected by glutaraldehyde cross-linking.

Cholera toxin (Ctx)¹ and heat-labile enterotoxin (Etx) are the primary virulence factors produced by Vibrio cholerae and certain toxigenic strains of Escherichia coli, respectively (1–3). Both toxins are heterooligomeric proteins comprising an A-subunit that exhibits ADP-ribosyltransferase activity and five B-subunits that bind with high affinity to the glycolipid receptor, monosialoganglioside GM1, found in the plasma membranes of mammalian cells (4–6). The B pentamer components of both cholera toxin (CtxB₅) and E. coli heat-labile enterotoxin (EtxB₅) are widely thought of as carrier molecules principally involved in delivering the toxin A-subunit into cells (3). However, more recent studies have revealed that these receptor binding moieties possess striking immunomodulatory properties that can down-regulate inflammatory immune reactions (7–9). Such findings have prompted renewed interest in the B-subunit pentamers and led to their testing as a potential therapeutic agent for the treatment of inflammatory allergic and autoimmune disorders (10–13).

Assembly of Ctx and Etx into AB₅ complexes occurs in the periplasmic compartment of the bacterial cell envelope (14–16). Expression of either CtxB or EtxB in the absence of their corresponding A-subunits results in the formation of highly stable B-subunit pentamers that are devoid of enterotoxic activity. The in vivo pathway of B-subunit pentamerization is poorly understood, chiefly because of the difficulty of investigating such processes in the complex environment of the periplasmic space (17, 18). The use of in vitro conditions to study the disassembly and reassembly of the toxins was first reported by Finkelstein et al. (19) who showed that purified cholera toxin could be denatured in acid urea and subsequently reassembled into active toxin when neutralized. Similarly, when purified CtxB₅ or EtxB₅ were denatured in acid and subsequently neutralized, the B-subunits were shown to be able to reassemble into stable pentameric complexes (18, 20–22). The intrinsic stability of CtxB₅ or EtxB₅ in the presence of SDS has meant that pentamer formation can be investigated by use of SDS-PAGE, and this approach has been used to monitor the kinetics of EtxB pentamerization. However, nothing is known of the pathway of assembly intermediates that are formed during the assembly process. Many other bacterial pathogens also produce toxins with complex oligomeric structures (23, 24). Although proper acquisition of their quaternary structure is also known to be essential for the mode of action of these toxins, no stoichiometric intermediates have yet been isolated.

Here, we address the question of intermediate formation by studying the reassembly of CtxB pentamers after acid denaturation and subsequent neutralization at pH 7. The assembly of CtxB was followed using different signals, GM1 ELISA, capturing GM1-bound species, SDS-PAGE, measuring pentamer formation, and tryptophan fluorescence spectroscopy, which was found to monitor a structural transition, consistent with oligomer formation. The latter was subjected to a rigorous analysis by computational modeling.

**EXPERIMENTAL PROCEDURES**

Purification of CtxB—CtxB was purified from Vibrio sp. 60 (pTRH64) as described previously (9, 25) and stored at ~8 °C in phosphate-buffered saline, pH 7.2 (150 mM NaCl, 10 mM sodium phosphate, pH 7.2 (PBS)) at a concentration of 0.34–0.39 mM. The toxin concentration is calculated as the monomeric concentration.

SDS-PAGE—SDS-polyacrylamide (13.5%) gel electrophoresis was performed with a Bio-Rad Protean II system using the Laemmli

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method, as recommended by the manufacturer (Bio-Rad). Either 5 or 2 μg of protein were loaded into each well, and the gels were stained with Coomassie Blue or silver stain, respectively.

**Buffers and Solutions**—The buffers used were McIlvaine buffer (0.2 M disodium hydrogen phosphate, 0.1 M citric acid, pH 6–9), PBS, or KCl/HCl, pH 1. All buffers were filtered through sterile 0.22-μm filter before use.

**Disassembly and Reassembly of CtxB**—The conditions used for disassembly and reassembly of CtxB were adapted from those previously employed for studying the reassembly of *E. coli* heat-labile enterotoxin B-subunit (CtxB) (22). Briefly, the concentration of purified CtxB 5 was characteristic of the B-subunit pentamer. The percentage of reassembled CtxB 5 in SDS-polyacrylamide gels with an electrophoretic mobility specified times at 23°C. McIlvaine buffer, pH 7.0, and then incubated for a further 60 min at 23°C. Both immediately after neutralization and after incubation for specified times at 23°C samples were removed and diluted 100-fold in PBS to prevent further assembly, followed by analysis using a GM1 ELISA (see below, under “Experimental Procedures”). In addition, samples were also removed from the reaction mixture at specified time points and mixed at a ratio of 4:1 with 5× SDS-PAGE sample buffer. These were kept on ice for up to 1 h before applying them to SDS-polyacrylamide gels without prior heating of the samples. This later procedure permits identification of reassembled CtxB, since at ambient temperatures CtxB is stable in SDS-containing buffers and migrates in SDS-polyacrylamide gels with an electrophoretic mobility characteristic of the B-subunit pentamer. The percentage of reassembled CtxB 5 was determined by quantification of the amount of pentamer at each time point relative to the equivalent amount of native CtxB 5 as applied on the gel using the densitometry software (TL TotalLab V1.11) from Phoretix.

**Trp Fluorescence during CtxB Reassembly**—Reassembly of CtxB was monitored by measurement of Trp fluorescence in a PerkinElmer LS-50B spectrophotometer. Base-line data collection was initiated in a cuvette containing neutralizing McIlvaine buffer alone into which was added CtxB that had been subjected to a 10-min denaturation in SDS-PAGE sample buffer. This later procedure permits identification of reassembled CtxB, since at ambient temperatures CtxB is stable in SDS-containing buffers and migrates in SDS-polyacrylamide gels with an electrophoretic mobility characteristic of the B-subunit pentamer. The percentage of reassembled CtxB 5 was determined by quantification of the amount of pentamer at each time point relative to the equivalent amount of native CtxB 5 as applied on the gel using the densitometry software (TL TotalLab V1.11) from Phoretix.

**GM1 ELISA**—The amount of B-subunit in a reassembly mixture that had acquired the ability to bind to GM1 receptors was determined using a GM1 ELISA (21, 22, 26). Samples of the reassembly mixture were taken at specified time points, diluted 100-fold to a toxin concentration of 86 nM and then added to ELISA plates that had previously been coated with 200 ng of GM1 and subsequently blocked with 1% Marvel in PBS. Samples were serially diluted 2-fold in PBS, and bound B-subunits were detected using a polyclonal mouse anti-CtxB, antiserum (α12) used at a 1/10000 dilution. All other steps of the GM1 ELISA were as reported previously (26). For quantification of the amount of CtxB bound to the ELISA plates for each test sample, optical density readings corresponding to dilutions located on the linear part of the curve were compared with the dilution of a CtxB standard (1 μg/ml, diluted 2-fold), giving the same optical density reading.

**Kinetic Model of CtxB Reassembly**—All the fluorescence spectroscopic data were fitted using the following procedure (MicroMath Scientific Software) to perform a numerical simulation based on the kinetic pathway proposed in the model (Fig. 6A). The numerical simulation was performed using Euler integration, and least squares fitting was performed using the modified Powell method within the Scientist program (version 2.01, Micromath 1995). The dependent variables of the equations are M, D, T, TE, and F for the molar concentration of monomer, dimer, trimmer, tetramer, and pentamer with the proline in a cis conformation and U, M 2, M 3, M 4, M 5 for the molar concentration of monomer, dimer, trimmer, tetramer, and pentamer with the proline in a trans conformation. The kinetic equations are described as follows.

\[
U' = k_{\text{trans}} M + k_{\text{cis}} (M_2 + M_3 + M_4 + M_5) - U (k_{\text{cis}} + k_{\text{trans}} (M + D + T + TE)) \quad (\text{Eq. 1})
\]

\[
M' = k_{\text{cis}} U + k_{\text{trans}} (M_2 + M_3 + M_4 + M_5) + 2k_{\text{diss}} (D + T + TE) - M (k_{\text{trans}} + k_{\text{diss}} (U + M_2 + M_3 + M_4 + 2(M + D + T + TE)) \quad (\text{Eq. 2})
\]

\[
M_5' = k_{\text{ass}} (U + M + M_2 + M_3 + M_4 + M_5) - M_5 (k_{\text{diss}} (M + D + T) + k_{\text{ass}} (M + D + T + TE)) \quad (\text{Eq. 3})
\]

\[
D' = k_{\text{trans}} M_2 + k_{\text{cis}} (M_4 + M_5 + M_6 + M_7 + M_8 + 2(T + 2・\text{TE}) - D (k_{\text{trans}} + k_{\text{eff}} (U + M_2 + M_3 + 2(M + D + T) + D)) \quad (\text{Eq. 4})
\]

\[
M_7' = k_{\text{ass}} (U + D + M_3 + M_4 + M_5) - M_7 (k_{2 \text{trans}} (k_{\text{cis}} + h_{\text{ass}} (M + D + T + TE)) \quad (\text{Eq. 5})
\]

\[
T' = 2k_{\text{trans}} (M + D + k_{\text{cis}} (M_4 + M_5 + M_6 + M_7 + 2(T + 2・\text{TE}) - T (2k_{\text{trans}} + k_{\text{cis}}) \quad \text{and} \quad k_{\text{ass}} (U + M_2 + 2(M + D + T)) \quad (\text{Eq. 6})
\]

\[
M_8' = k_{\text{ass}} (U + D + M_3 + M_4 + M_5 + M_6 + M_7 + 2(M + D + T + TE) - M_8 (k_{\text{trans}} (k_{\text{cis}} + h_{\text{ass}} (M + D + T + TE)) \quad (\text{Eq. 7})
\]

\[
T' = 2k_{\text{trans}} (M + D + D + M_3 + M_4 + M_5 + M_6 + M_7 + 2(T + 2・\text{TE} + 5P) \quad (\text{Eq. 8})
\]

\[
P' = 2k_{\text{trans}} (M + D + T + D + D) \quad (\text{Eq. 9})
\]

\[
M_9' = k_{\text{ass}} (U + M + R(M_2 + 2M_3 + 3M_4 + 4M_5 + M_6 + M_7 + 2(T + 3・\text{TE} + 5P) \quad (\text{Eq. 10})
\]

where P is the fluorescence intensity at time 0, and R is the ratio of the fluorescence intensity of tryptophan at a properly formed interface by that at a free interface.

The fluorescence signal was carried by the pentamer as well as intermediates. The data for all protein concentrations were fitted globally. Differential equations were derived for each partial reaction and weighted statistically before being compiled into a Scientist equation file. All four rate constants, plus base line and the signal of fluorescence were allowed to vary in the fitting procedure.

**Chemical Cross-linking of CtxB Assembly Intermediates**—CtxB was acidified and neutralized at a final toxin concentration of 26 μM. The reassembly reaction was performed at 33°C, and aliquots were taken at discrete times (0, 2, 4, 10, and 30 min) after neutralization and mixed for 2 min with glutaraldehyde at a final concentration of 4% (v/v). 5× sample buffer was added at a ratio of 1:4 to quench the reaction, and the samples were analyzed by SDS-PAGE and silver-stained.

**RESULTS**

**Disassembly and Reassembly of CtxB**—CtxB pentamers disassemble in acidic conditions, giving rise to monomeric B-subunits that can reassemble if placed in buffers neutral pH (18, 22). To investigate the reaction mechanism of reassembly, we analyzed the time course of CtxB oligomerization after acid denaturation and neutralization. First, we monitored the extent of disassembly and reassembly using a GM1 ELISA technique that captures assembled B-subunits on GM1-immobilized microtiter plates. CtxB was incubated for various times ranging from 0.3 to 60 min in HCl/KCl buffer, pH 1.0, then neutralized in McIlvaine buffer, pH 7.0, diluted and immediately tested in a GM1 ELISA. Short periods of acidification, e.g. 0.3 min was insufficient to achieve full disassembly of CtxB, since ~15% of the B-subunits were detected in the GM1 ELISA immediately after neutralization (Fig. 1A). However, it was found that acidification for a period of 10–60 min resulted in full disassembly of the B-subunits, since no protein could be detected in the GM1 ELISA (Fig. 1A; 0 min of neutralization).

If after neutralization, the samples were maintained at 23°C for 60 min before being diluted and then tested in the GM1 ELISA, it was evident that the B-subunits had reassembled and could now be detected by this technique (Fig. 1A). It was
noted that the extent of reassembly declined if the B-subunits were acidified for longer time periods. When CtxB5 was acidified for 10 min then neutralized and incubated for 60 min, ~75% of the B-subunits regained the ability to be detected in the GM1 ELISA. Because extended periods of acidification reduced the overall yield of assembled CtxB, all subsequent reassembly reactions reported were performed with a 10-min acidification step followed by neutralization.

To confirm that assembly of CtxB5 occurs during the 60-min incubation period, samples were taken both immediately and 60 min after neutralization and analyzed by SDS-PAGE without prior heating of the samples. As can be seen in Fig. 1B, after 60 min CtxB5 pentamers are clearly present, whereas immediately after neutralization they were absent (compare lanes 2 and 3). The electrophoretic mobility of reassembled CtxB5 was identical to that of native CtxB5 (lane 1).

Analysis of the kinetics of B-subunit reassembly was monitored by both GM1 ELISA and SDS-PAGE by sampling at various time points after neutralization (Fig. 2). In this experiment CtxB5 was acidified for 10 min and then neutralized to give a final concentration of 8.6 μM. By both techniques a time-dependent increase in formation of reassembled B-subunits was observed, but the half-times for reassembly were different, corresponding to ~8 or ~15 min by GM1 ELISA and SDS-PAGE, respectively. In addition the amount of assembled CtxB detectable by the two methods at each time point was also different. These discrepancies may be explained if the GM1 ELISA technique detects intermediate species in addition to CtxB5 pentamers, since SDS-PAGE monitors the amount of the pentamer alone. In this regard, the GM1 binding site is located at the interface between two subunits, and it is thus reasonable that a dimer, a trimer, or a tetramer as well as a pentamer may bind to GM1.

Use of Trp Fluorescence to Monitor CtxB Reassembly—To investigate the kinetics of the assembly process further, Trp fluorescence was employed as a continuous probe to yield data that could be used to model the reassembly reaction and, thus, explain the apparent discrepancy in the assembly kinetics observed by GM1 ELISA and SDS-PAGE. Each CtxB subunit contains a single Trp residue at position 88 that is located at the subunit interface in native CtxB5, and should therefore be a useful probe for studying CtxB assembly.

Emission scans of native CtxB5 and of CtxB just after neutralization (referred to as CtxB (0 min)) are shown in Fig. 3A. The fluorescence intensity of CtxB5 immediately after neutralization was found to be 4-fold lower than that of native CtxB5 when measured at a concentration of 8.6 μM (Fig. 3A). This indicates that upon dissociation/unfolding and neutralization of CtxB, the environment around Trp-88 changes. Taking advantage of this, we investigated the time-dependent change in fluorescence intensity during a reassembly reaction (Fig. 3B). The experiment was initiated by recording the signal of McIlvaine pH 7 buffer alone, which was then followed by the addition of acidified toxin (arrow in Fig. 3B). The trace shows that there was an abrupt increase in the initial fluorescence intensity due to the addition of the toxin. Thereafter, a slower increase in fluorescence intensity was observed during the next 60 min. Such a slow increase in fluorescence is unlikely to be monitoring a conformational change associated with the early stages of folding (for review see Refs. 27–29).

To assess whether the slow increase in fluorescence intensity was attributable to an intrinsic slow folding process in the CtxB monomer or to the oligomerization event, the influence of CtxB concentration on the kinetics of fluorescence changes was investigated. CtxB5 was acidified at 86 μM for 10 min and diluted at concentrations ranging from 4.3 to 26 μM in buffer of appropriate pH values to give a final reaction of pH 7. As can be seen in Fig. 4 the rate of increase in fluorescence intensity increased with CtxB concentration. This demonstrates that the fluorescence measurements monitor a concentration-dependent process. A first-order reaction such as folding of the monomer or intramolecular rearrangement of the pentamer would be concentration-independent. We therefore conclude that the changes in Trp fluorescence monitor the progress of a multi-molecular reaction in which association events were rate-limiting.

Interestingly, the half-time of the oligomerization reaction obtained from fluorescence measurements at a CtxB concentration of 8.6 μM was 7.6 ± 0.5 min, which was virtually identical to the half-time of reassembly determined by GM1 ELISA (Fig. 2). This suggests that both techniques are reporting the same reaction. To test if this is the case, the half-time for reassembly at various CtxB concentrations was determined by GM1 ELISA and compared with the half-times obtained using Trp fluorescence (Table 1). A striking concurrence of half-times for assembly was obtained using the two techniques.
We therefore conclude that the GM1 ELISA and changes in Trp fluorescence measure the same reaction.

A Kinetic Model of CtxB Assembly—Because of the accuracy of the kinetic measurements of CtxB assembly obtained by Trp fluorescence, a kinetic model of the reaction mechanism was generated. Given that the changes in Trp fluorescence appear to be reporting the same events as those involved in B-subunit captured by GM1, we speculated that oligomer formation (i.e. dimer, trimer, tetramer, and pentamer) is the event being monitored. As can be seen on the x-ray crystallographic structure of CtxB₅ reported by Zhang et al. (30, 31), the subunit interface between adjacent monomers is formed primarily through hydrogen bond interactions between β-strand number 3 of one subunit (i.e. amino acids Phe-25 to Ala-32) and the terminal portion of β-strand number 6 of the adjacent subunit (i.e. amino acids Ala-97 to Asn-103) (30, 31). For these β-strands to align, proline at position 93 must be in a cis configuration, so that β-strand number 6 orients toward the adjacent subunit to form the inter-subunit interface (Fig. 5). In generating a kinetic model of CtxB assembly we have thus assumed that a cis proline conformation is required for formation of the inter-subunit interface. Therefore in this model (Fig. 6A), the monomer exists in two states, a trans configuration (filled circles) and a cis configuration (open circles), and the rate-limiting step in the oligomerization reaction is the rate of proline isomerization. These two states of the monomer have different physical properties with respect to their ability to interact with another monomer and to assemble into oligomeric complexes. Each monomer has two surfaces that are able to interact with adjacent monomers. Thus, if one considers mon-
omer $M$, a stable subunit interface can be formed through a hydrogen bond network between $\beta$-strand number 3 of $M$ and $\beta$-strand number 6 of subunit $M + 1$ or it can be formed between $\beta$-strands number 6 of $M$ and $\beta$-strand number 3 of subunit $M - 1$. However, if $M$ is in a trans configuration, only one of its surfaces can interact with another monomer since in this case $\beta$-strand number 6 is not in the proper orientation to interact with $\beta$-strand number 3 of $M - 1$. Thus, if $M$ is in a trans configuration it can only associate with $M + 1$, which must be in a cis configuration, whereas if $M$ is in a cis configuration, both of its surfaces are available for interaction with $M + 1$ (in a cis configuration) and $M - 1$ (in cis or trans states). Hence only one subunit in the trans configuration can be present in any oligomeric complex, with proline isomerization to a cis configuration required for further association of other monomers. The equilibrium between trans and cis states in any complex formed during the assembly reaction is represented by the vertical arrows in Fig. 6A. The horizontal and the diagonal arrows represent all possible association and dissociation reactions with monomer in a trans or cis configuration (upper pathway), whereas association and dissociation with monomer only in a cis configuration is represented by the horizontal arrows of the lower pathway.

Apart from the monomer in a trans state that cannot associate on one side before proline isomerization, all pathways to the pentamer are allowed, and the rate constants for cis-trans isomerization ($k_{\text{cis-trans}}$ for cis-to-trans and $k_{\text{trans-cis}}$ for trans-to-cis), association ($k_{\text{ass}}$), and dissociation ($k_{\text{diss}}$) are the same at any stage in the pathway. It is assumed that the dissociation of pentamers is infinitely slow. The fluorescence data were fitted based on the model shown in Fig. 6A. The equations describing how each species appears and disappears in time are given under “Experimental Procedures.”

Based on those equations, a numerical simulation of all the fluorescence data was undertaken, as described under “Experimental Procedures,” and a fit between experimental and modeled data is indicated in Fig. 6B. The fit is optimal when $k_{\text{trans}}$ and $k_{\text{cis}}$, the rates of isomerization of the prolines, which determine whether subunits can interact, are $0.1 \text{ s}^{-1}$ and $0.02 \text{ s}^{-1}$, respectively. The association rate is slow for a purely diffusion limited process, suggesting the proportion of productive collisions is low. However, the precise conformational requirements for the productive collision of two subunits interfaces may be sufficiently rigorous that such events are relatively infrequent.

When the model was used to determine the kinetics of intermediate formation in a reassembly reaction at a toxin concentration of 26 $\mu\text{M}$, intermediates were predicted to accumulate within the first 5 min after neutralization (Fig. 6C). In this respect, dimers accumulated the most rapidly (~2 min), whereas trimers and tetraders formed within 4 min after neutralization. The half-times of the decay of the dimer, trimer, and tetramer were $t_{1/2} = 5, 13, 11 \text{ min}$, respectively, indicating that the dimer disappeared faster than trimer and tetramer, which decreased in approximately similar time. In addition, it seems that the dimer has two rates of decay, one fast at the beginning of the reaction and one slower 5–10 min after neutralization, the latter very similar to the decay observed for the trimer and tetramer. All the species remained present even 30 min after neutralization, although at any time point there is always more dimer present during the reassembly reaction than trimer and tetramer.

To assess the robustness of the model, the kinetics of total oligomer formation (i.e. the sum of pentamers and all intermediates, including dimers, trimers, and tetraders) was compared with the kinetics of oligomer formation as determined by GM1 ELISA (Fig. 6D; compare open circles and filled circles for model and GM1 ELISA data, respectively). A reassembly reaction at a final toxin concentration of 26 $\mu\text{M}$ is shown as an example. A good fit was obtained between the calculated and the experimental data, supporting the view the GM1 ELISA technique traps all intermediates as well as pentamers. No better fit was obtained when other combinations of oligomers were computed from the model. To further validate the model, the kinetics of formation of pentamer alone was calculated and compared with that determined by SDS-PAGE when reassembly was carried out at a final toxin concentration of 26 $\mu\text{M}$ (Fig. 6D; compare open circles and filled diamonds for the model and SDS-PAGE data, respectively). Again there was good agreement between the predicted and experimentally derived data, lending further support to the proposed reaction scheme.

Identification of CtxB Assembly Intermediates—Although the model indicated that intermediates will accumulate in a time period sufficient for them to be studied, no intermediates...
have previously been experimentally identified. The dissociation rate shows that oligomeric intermediates ought to be stable once formed ($K_D = 540$ nM), and therefore, it should be possible to isolate them. In an attempt to trap such intermediates, reassembling B-subunits were exposed to the covalent cross-linking agent, glutaraldehyde, and subsequently subjected to analysis by SDS-PAGE. Ordinarily, SDS-PAGE analysis of B-subunits that are undergoing reassembly detects only monomeric and pentameric species (Fig. 1B). However, it is likely that only the B-subunit pentamer can withstand the denaturing activity of SDS, and thus, any oligomeric intermediates would collapse to the monomeric state. Before seeking to trap intermediates with glutaraldehyde, we investigated the effect of glutaraldehyde treatment on native CtxB$_5$. Incubation of 26 $\mu$M CtxB$_5$ for 2 min at 23 °C with 4% (v/v) glutaraldehyde followed by the addition of SDS-PAGE sample buffer resulted in the B-subunits migrating on SDS-PAGE as cross-linked tetramer (TE), cross-linked pentamers (CLP), and higher molecular weight cross-linked aggregates (A) (Fig. 7, lane 3). Importantly, no molecular weight species corresponding to dimers or trimers were present. However, when CtxB$_5$ was acidified for 10 min, neutralized at final protein concentration of 26 $\mu$M, and then, after various time periods, incubated with 4% (v/v) glutaraldehyde, it is evident that the reassembling B-subunits contain dimeric, trimeric, and tetrameric species in addition to cross-linked pentamers and aggregates (Fig. 7, lanes 4–8). The sample taken at the earliest time point after neutralization and cross-linked with glutaraldehyde contained more of the di-
The Trp fluorescence measurements were used to build a kinetic model of the reassembly process in which we assumed that all intermediates species could exist, but that formation of a subunit interface necessitates Pro-93 to be in a cis-configuration. Given that proline can undergo isomerization between \textit{trans} and \textit{cis} states, the model assumes that B-subunits in which Pro-93 is in the \textit{trans}-configuration would not be competent to form a subunit interface until isomerization to a \textit{cis} configuration had occurred. Based on these assumptions a model was generated that closely fit the experimental data and which allowed us to determine the kinetics of formation of oligomeric intermediates. The isomerization rate constants ($k_{\text{cis}}$ and $k_{\text{trans}}$) as calculated from the fit were in good agreement with previously described values, thus supporting the validity of the model (32). This revealed that oligomeric intermediates maximally accumulate within the first 5 min of the reassembly reaction and then decline as stable pentamer formation occurs. In support of this, cross-linking studies showed that soon after neutralization dimers, trimers and tetramers could be detected in a reassembly mixture and that these declined with time, consistent with their assembly into pentamers. By comparing the kinetics of oligomer formation, as determined by GM1 ELISA, to that calculated from the model, the best fit was obtained when pentamer as well as all intermediates were taken into account in the calculation, thus supporting the hypothesis that the GM1 ELISA and the Trp fluorescence data report oligomer formation and not pentamer formation alone. Further support for the model was indicated by the close fit obtained between the kinetics of pentamer formation, as determined by SDS-PAGE analysis and as calculated from the model.

Previous studies on the reassembly of the closely related B-subunit of \textit{E. coli} heat-labile enterotoxin revealed that acidification for periods of greater than 1 min resulted in a dramatic loss of competence to reassemble (22). This was attributed to \textit{cis} to \textit{trans} prolyl isomerization occurring during the acidification step that could not be isomerized to an assembly-competent \textit{cis}-configuration upon return to neutral pH. By contrast, our results show that CtxB can be acidified for up to 1 h with only a 1.5-fold loss in the amount of B-subunits subsequently able to bind to GM1. This difference between the two toxins might be due to a one-residue difference at position 94, which is a histidine in CtxB but an asparagine in EtxB (22, 30, 31, 33, 34). The histidine may have an effect on the environment of Pro-93 and the \textit{cis}-\textit{trans} isomerization reaction (35).

Our results show for the first time that assembly of CtxB$_5$ can occur via ordered formation of oligomeric species and that they can equally go through two alternative pathways (i.e. addition of dimer and trimer to make pentamer or addition of tetramer and monomer to make pentamer). Studies on the \textit{in vitro} assembly of the AB$_5$ holotoxin reveal that the A subunit cannot directly assemble with native CtxB$_5$ rather, only with B-subunits that are in the process of reassembling (18). This led to the suggestion that the A-subunit normally interacts with a B-subunit assembly intermediate. Moreover, Hardy et al. (18) show that the half-time of EtxB pentamer formation \textit{in vitro} was shorter in cells that also expressed the A-subunit, a finding that lent further support to the idea that the A-subunit interacts with and stabilizes a B-subunit assembly intermediate (18). Interestingly, when a mixture of reassembling CtxB subunits was added to GM1-coated microtiter plates, and then an excess of CtxA was added, a very high level of CtxA bound to the plates. This contrasted with the negligible amount of CtxA that was detected if added to GM1 plates coated with

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{assembly_of_cholera_toxin_pentamers_in_vitro}
\caption{Identification of assembly intermediates using glutaraldehyde cross-linking. CtxB$_5$ was acidified and neutralized at a final toxin concentration of 26 $\mu$g/mL. At 0, 2, 4, 10, and 30 min after neutralization, samples were mixed with glutaraldehyde at a final concentration of 4\% (v/v) and incubated for 2 min before quenching the reaction by addition of SDS sample buffer. Each of the samples, 0 min (lane 4), 2 min (lane 5), 4 min (lane 6), 10 min (lane 7), and 30 min (lane 8) was applied to an SDS-polyacrylamide gel without boiling. As a control native CtxB$_5$ (lane 3) was cross-linked under the same conditions to establish that dimer or trimer are not present when such a sample is analyzed without boiling. Upon boiling of cross-linked CtxB$_5$ (lane 2), dissociation occurs, releasing the migration positions of monomer (CLM), dimer (D), trimer (T), tetramer (TE), cross-linked pentamer (CLP) as well as cross-linked aggregates (A). native, non-cross-linked CtxB$_5$ (lane 1).}
\end{figure}

\section*{DISCUSSION}

Here, we have investigated the pathway of CtxB reassembly \textit{in vitro}. It was evident by both GM1 ELISA and SDS-PAGE analysis that, immediately after assembly was initiated, the B-subunits were in a dissociated state with no pentamers present. Continued incubation resulted in the formation of SDS-stable B-subunit pentamers that had an indistinguishable electrophoretic mobility from native CtxB$_5$. Moreover, during the incubation at pH 7.0, B-subunits re-acquired the ability to be captured by GM1 and to be detected in a GM1-based ELISA. Previous studies suggest that these two techniques are useful for monitoring formation of B-pentamers (18). However, when the kinetics of CtxB assembly were experimentally determined using SDS-PAGE, it was found that half-time for B-pentamer formation was considerably slower than the half-time for acquisition of the capacity to bind to GM1. Given that the GM1 receptor binding pocket, as seen in the crystal structure, is formed by two adjacent monomers, it is conceivable that assembled dimers and other higher oligomeric species, in addition to CtxB$_5$, would bind to GM1.

When Trp fluorescence spectroscopy was used to investigate the reassembly process, it was found that a time-dependent change in fluorescence intensity occurred. Interestingly, there was a striking concurrence in the half-times of the change in fluorescence intensity with the half-times for the acquisition of the ability of the B-subunits to bind to GM1. To test whether the two methods reported similar events, the reassembly process was monitored at different B-subunit concentrations. We found that the two signals followed the same kinetic pattern, exhibiting an increased rate of assembly with increased protein concentrations. We therefore conclude that the Trp fluorescence and the ability to bind to GM1 ganglioside report the formation of the same species. Furthermore, our observations that the half-time of the reassembly reactions decreased with increasing protein concentration implies that a multi-molecular event is being monitored rather than intramolecular refolding.
native CtxB5. Such an observation suggests that GM1 recognizes CtxB assembly intermediates in addition to B-subunit pentamers and that the intermediates retain the capacity to bind CtxA. This observation supports the findings reported here, namely that the GM1 ELISA technique can be used to monitor the half-time of formation of assembly intermediates.

Altogether the results validated a model in which very few mechanistic constraints, namely prolyl isomerization and association/dissociation processes had been imposed, showing that a numerical model based on simple physical constraints accurately describes these complex biological reactions. Such an approach may be helpful in deciphering other assembly mechanisms. In many respects the events associated with the oligomerization of cholera toxin B-subunit pentamer are similar to other assembly processes in which molecules go through unfolding and folding steps during which hydrophobic surfaces are formed as the driving force for oligomerization or aggregation. Such kinetic modeling could therefore be used to study a wider variety of assembly reactions.

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