Cytolethal distending toxin (CDT) induces cell cycle arrest and apoptosis in eukaryotic cells, which are mediated by the DNA-damaging CdtB subunit. Here we report the first x-ray structure of an isolated CdtB subunit (Escherichia coli–II CdtB, EcCdtB). In conjunction with previous structural and biochemical observations, active site structural comparisons between free and holotoxin-assembled CdtBs suggested that CDT intoxication is contingent upon holotoxin disassembly. Solution NMR structural and 15N relaxation studies of free EcCdtB revealed disorder in the interface with the CdtA and CdtC subunits (residues Gly233–Asp242). Residues Leu186–Thr209 of EcCdtB, which encompasses tandem arginine residues essential for nuclear translocation and intoxication, were also disordered in solution. In stark contrast, nearly identical well defined α-helix and β-strand secondary structures were observed in this region of the free and holotoxin CdtB crystallographic models, suggesting that distinct changes in structural ordering characterize subunit disassembly and nuclear localization factor binding functions.

Cytolethal distending toxin (CDT) is a DNA-damaging bacterial toxin produced by a number of important bacterial pathogens (1, 2). The cytopathic effects associated with CDT intoxication are a direct result of chromosomal DNA damage inflicted by the CdtB subunit, a homolog of eukaryotic type I DNase (3, 4). CdtB-mediated chromosome strand breakage signals the induction of an ATM (ataxia telangiectasia-mutated)- or ATR (ataxia telangiectasia-mutated and Rad3-related)-dependent mitotic checkpoint resulting in the sequestration of Cdc25, a block in the cell cycle at the G2/M boundary, cellular distension, and ultimately cell death by endoreduplication or apoptosis (2, 5).

The CDT holotoxin is a heterotrimer composed of CdtA, CdtB, and CdtC subunits. Although the catalyst for CDT-mediated apoptosis is ultimately attributed to the DNase activity of CdtB, all three subunits are required for cellular entry of CDT (6, 7). The CdtA and CdtC subunits are lectin-like domains that bind carbohydrate residues on the target cell surface, whereby CDT is subsequently internalized by receptor-mediated endocytosis (8, 9). The Haemophilus ducreyi holotoxin structure (HdCDT) has demonstrated that CDT is an AB type toxin, with the catalytically active A subunit of the AB toxin corresponding to the CdtB subunit (10). Inspection of this structure and of the structurally similar CDT holotoxin from Actinobacillus actinomycetemcomitans (AaCDT) (11) revealed that one face of the CdtA-CdtC dimer binds the CdtB subunit. Mutagenesis studies of HdCDT suggest that another face of the dimer with a grooved ricin B-chain fold mediates cell surface binding (10).

Transporting of CDT to the nucleus is thought to involve retrograde transport through the Golgi and the ultimate translocation of CdtB from the endoplasmic reticulum to the nuclear envelope by an apparent ERAD (endoplasmic reticulum-associated degradation)-independent step (9). CdtB traverses the nuclear envelope by virtue of at least one nuclear localization sequence (NLS), which is recognized by unknown cognate nuclear localization factors (12). Although CdtB disassembly from the CDT holotoxin has not been established, direct deposition of the isolated EcCdtB subunit into the cytoplasm of HeLa cells by electroporation promotes cell cycle arrest at G2/M, demonstrating that free EcCdtB locates to the nucleus and retains toxicity (7). Significantly, toxicity determined by this avenue is dependent on the integrity of two EcCdtB arginine residues (Arg191–Arg192) within the characterized NLS (12).

Here, we report the first high-resolution structure of a free CdtB subunit. The nearly identical active site conformations in the free and holotoxin states of CdtB suggest that the much...
lower enzymatic activity of this subunit within assembled CDT is mediated by interactions with CdtC rather than intramolecular structural rearrangement within CdtB upon holotoxin disassembly (10). In addition, NMR 15N relaxation studies identify two regions of dynamic disorder within the ensemble of solution conformers of free EcCdtB, suggesting that distinct structural transitions involving ordered and disordered states underlie CdtB functional activation (holotoxin disassembly) and binding to cognate nuclear localization factors.

EXPERIMENTAL PROCEDURES

Crystallization and X-ray Data Collection—The cdtB gene used for these studies was derived from Escherichia coli strain 9142–88 (O128H−) (13) and cloned as described previously (14). Three distinct CDT operons have been characterized from various E. coli strains and are designated CDT-I, CDT-II, and CDT-III (2). E. coli 9142-88 produces CDT-II, and thus the CdtB used in this work is designated EcCdtB-II, referred to herein as EcCdtB. Recombinant EcCdtB protein corresponding to the mature subunit (residues 1–251) plus an 11-residue N-terminal linker including six histidine residues was crystallized by microbatch techniques as described elsewhere (15). The EcCdtB toxin preparation possessed identical toxicity and enzymatic activity as reported previously (7). X-ray data collected at Advanced Photon Source 22BM on a Mar300 charge-coupled device detector to 1.73 Å were processed by HKL2000 (16), and phases were determined by molecular replacement as implemented in AMoRe (17) using the H. ducreyi CdtB subunit from the holotoxin structure (hHdCdtB) as the search model (Protein Data Bank code 1SR4) (10). The hHdCdtB phasing model included residues 28–81, 93–134, 144–163, 194–211, 225–233, 237–241, and 267–281. The remaining residues were added by iterative cycles of manual model building in O (18) and model refinement by simulated annealing and conjugate gradient minimization as implemented in CNS (Crystallography and NMR System) (19). Solvent molecules were added using a combination of default parameters in CNS and ARP/wARP (20). Final refinement was performed using maximum likelihood with restrained refinement by Refmac5 (21) in the CCP4 suite (22). Secondary structure assignments were determined by the DSSP program (23). Root mean square deviations (r.m.s.d.) between CdtB structures were calculated using Swiss-PdbViewer, version 3.7 (24), and using Iterative Magic Fit to align the structures. After alignment, regions were manually defined for the r.m.s.d. calculation.

NMR Spectroscopy—All NMR data were recorded on a 14.1 T Varian Inova spectrometer (599.7 MHz for 1H). The recombinant EcCdtB used for all NMR studies was identical to that for crystallographic studies. The details of EcCdtB NMR sample preparation, resonance assignment determination, extent of assignments (25), and random coil chemical shifts (26, 27) have been reported. The general approach used for 15N relaxation data collection is that of Potter et al. (28). EcCdtB steady-state heteronuclear NOEs (15N-1H)NOE), 15N longitudinal (R1) and transverse (R2) relaxation time constants were measured by the steady-state, inversion-recovery, and Carr-Purcell-Meiboom-Gill methods, respectively (29–31) using standard water flipback methods (32). R1 and R2 spectra relaxation delays were as follows: 0.03, 0.05, 0.07, 0.09, 0.12, 0.15, 0.20, 0.35, 0.50, 0.65, 0.70, 0.90, 1.20, 1.60, 2.00, 2.35, 2.75 and 3.50 s for R1; 0.01, 0.03, 0.05, 0.07, 0.09, 0.11, 0.13, 0.15, 0.17, 0.21 and 0.25 s for R2.

| Table 1 |
| --- |
| Data collection and refinement statistics for E. coli cdtB |
| **Data collection** |
| Space group | P212121 |
| Unit cell (Å) | a = 39.88, b = 47.54, c = 114.89 |
| Molecules per asymmetric unit | 1 |
| Estimated solvent content (%) | 34.1 |
| Total reflections collected | 102,947 |
| Unique reflections | 23,348 |
| Completeness (%) | 99.1 (98.8) |
| Average redundancy | 16.8 (4.7) |
| Mosaicity (°) | 4.5 (3.5) |
| Rsym (%) | 0.42 |
| **Refinement statistics** |
| Resolution (Å) | 57.7-1.73 (1.78-1.73) |
| Rfactor, (%) | 18.4 (22.7) |
| Rfree (%) | 21.7 (29.1) |
| No. of protein atoms | 2098 |
| No. of water molecules | 160 |
| r.m.s.d. from ideal geometry | 0.02 |
| Bond lengths (Å) | 1.75 |
| Average B-factor (Å2) | 20.2 |
| Main chain B-factor | 18.4 |
| Side chain B-factor | 22.3 |
| Water molecules B-factor | 29.4 |

* Values indicated in parentheses are for the highest resolution bin.

** Crystal Structure of Free EcCdtB—** The structure of EcCdtB was determined to a resolution of 1.73 Å using x-ray diffraction analysis. Phasing information was obtained by molecular replacement using core regions of the CdtB subunit from the H. ducreyi holotoxin (10). A summary of x-ray data and refinement statistics for the EcCdtB structure is presented in Table 1. The final EcCdtB model consists of three CdtB structural subunits, residues 1–251 plus two residues of the 11-residue His tag linker, and 160 water molecules. A sample electron density map is shown in Fig. 1. Coordinates and structure factors have been deposited in the Protein Data Bank with accession code 2F1N.

The EcCdtB structure belongs to the α + β structural class and consists of a β-sandwich with α-helices on the exterior (Fig. 2A). Although EcCdtB has low sequence identity with the DNase-I family of enzymes including bovine DNase I (19.4% identity), human HapI DNA repair endonuclease (17.3% identity), and E. coli EXO III (18.6% identity), the protein adopts a very similar fold. Differences between the DNase I and CdtB...
family folds include the number of strands in the \( \beta \)-sheets and the number of connecting helices. As shown in Fig. 2B, the CdtB fold is composed of 8- and 6-stranded \( \beta \)-sheets (10, 11), whereas the canonical DNase I fold has two 6-stranded sheets (37). In the CdtB fold, two additional antiparallel \( \beta \)-strands (3 and 4) occur at the C-terminal end of \( \beta \)-sheet 1. The \( \alpha \)-helix connecting \( \beta \)-strands 2 and 3 in the DNase I family fold is missing in the corresponding region connecting \( \beta \)-strands 2 and 3 in the CdtB family fold.

**Active Site of EcCdtB**—The active site of EcCdtB contains two histidine residues (His\(^{136}\) and His\(^{243}\)) that are essential for catalysis of DNA phosphodiester cleavage (Fig. 3) (3, 4). Moreover, based on structural similarities to DNase I, catalytic enhancement attributed to a raising of the His\(^{243}\) \( pK_a \) in EcCdtB is likely to be contributed by Asp\(^{211}\) through side chain carboxylate-imidazole hydrogen bonding (37–39). The solvent structure surrounding these three conserved amino acids in EcCdtB and hHdCdtB shows striking structural conservation. The lower resolution of the *A. actinomycetemcomitans* CdtB subunit from the holotoxin structure (hAaCdtB) makes the comparison of solvent structure more difficult and is not included in Fig. 3. Three additional residues that make direct DNA substrate contacts in DNase I are located in structurally conserved positions in EcCdtB (Arg\(^{95}\), Arg\(^{120}\), and Asn\(^{176}\)). All of these important catalytic and substrate binding residues, which are absolutely conserved in all known CDTs, have remarkably similar side chain positions in the assembled holotoxin (10, 11) and free EcCdtB structures (Fig. 3).

**Comparison of Free EcCdtB with CdtB in Holotoxins**—The 1.02-Å r.m.s.d. measured for 216 C\(^{\alpha}\) atoms from aligned, non-gapped residues of free EcCdtB and the homologous hHdCdtB subunit (48% identity) crystal structures demonstrates that conformational rearrangements accompanying holotoxin assembly are not global (Fig. 4). If only residues within regular secondary structure elements are considered (111 residues), the r.m.s.d. between C\(^{\alpha}\) atoms from the two CdtB proteins drops to 0.70 Å. Given the 97% CdtB sequence identity between *H. ducreyi* and *A. actinomycetemcomitans* CdtB subunits, it is not surprising that over the entire structure, the C\(^{\alpha}\) r.m.s.d. from these holotoxin-associated proteins is somewhat lower (0.43 Å, see Fig. 4). Indeed, the backbone topology of all three subunits from these two CDT holotoxins is nearly identical (10, 11).

Although the structures of CdtB in the free and assembled forms are very similar, the CdtB crystal structures show significant protein backbone variation between free (EcCdtB) and holotoxin (hHdCdtB and hAaCdtB) subunits in the loop region joining the two C-terminal \( \beta \)-sheet 2 strands \( \beta 13 \) and \( \beta 14 \) (residues Ile\(^{230}\)–Pro\(^{245}\) of EcCdtB, Fig. 4). Close inspection of the free EcCdtB structural alignments with hHdCdtB and hAaCdtB revealed that most of this region of CdtB is in the subunit inter-
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face with CdtA and CdtC (supplemental Fig. 1). This interface is occupied largely by residues Leu<sup>263</sup>–His<sup>274</sup> in hHdCdtB and hAaCdtB (corresponding to EcCdtB residues Tyr<sup>232</sup>–His<sup>243</sup>) and contains a three-residue α-helix (residues Leu<sup>263</sup>–Gln<sup>265</sup>) in both proteins that is not formed by the corresponding residues of the free EcCdtB subunit (Tyr<sup>232</sup>–Ala<sup>234</sup>). Although the r.m.s.d. between Ca atoms of hHdCdtB and hAaCdtB in this region are only 0.30 Å, the corresponding Cα r.m.s.d. between EcCdtB and hHdCdtB is 5.64 Å in the same region.

**NLS Regions**—A tandem arginine sequence located in helix E of EcCdtB (Arg<sup>191</sup>–Arg<sup>192</sup>, Fig. 5), which is part of a larger monopartite or bipartite NLS (40), is essential for nuclear translocation and CDT-mediated intoxication (12). Although a corresponding tandem arginine sequence within an NLS for HdCdtB and AaCdtB has not been characterized experimentally, the expanded structural alignment in the vicinity of the EcCdtB NLS region with the holotoxin subunits shown in Fig. 5 reveals that the hHdCdtB and hAaCdtB Arg<sup>249</sup>–Arg<sup>250</sup> side chain positions face out into solution in close proximity to the EcCdtB Arg<sup>191</sup>–Arg<sup>192</sup> side chains, even though the locations of these arginine pairs in the backbone topology of the respective CdtB orthologs are quite different.

**Solution NMR Structural and 15N Relaxation Studies of Free EcCdtB**—To identify potential ensemble variations in structure and backbone motions that could be related to CdtB-dependent CDT biological function, NMR solution studies of EcCdtB were carried out with an identical EcCdtB protein to that used for crystallographic studies (15, 25). Although the quality of the NMR spectra were generally good for the uniformly <sup>2</sup>H<sup>-</sup>, <sup>15</sup>N-, and <sup>13</sup>C-labeled EcCdtB protein, 29 residues concentrated primarily within the first 100 N-terminal residues of free EcCdtB were characterized by μs-ms time scale exchange broadening to the extent that the corresponding <sup>1</sup>H–<sup>15</sup>N-HSQC resonances were unobservable (25).

The comparison of backbone EcCdtB residue <sup>1</sup>H/<sup>15</sup>N, <sup>13</sup>Cα, and <sup>13</sup>CO NMR chemical shift differences with those of the corresponding residues in small “random coil” peptides shown in Fig. 6A is a good indicator of protein secondary structure (26, 27). For the majority of the EcCdtB protein, the secondary structure predictions match those observed in the crystallographic model of the protein (Fig. 2). However, the <sup>13</sup>Cα and <sup>13</sup>CO chemical shift differences from random coil in the regions of EcCdtB encompassing helix A (E20-I31), β-strand 2 (Ile<sup>39</sup>–Gln<sup>43</sup>), and β-strand 5 (Gln<sup>81</sup>–Ser<sup>87</sup>) are not completely consistent with the observed secondary structural elements in the crystal structure, although these regions have large gaps in chemical shift information because of conformational exchange (Fig. 6A) (25). More significantly, the region of EcCdtB encompassing residues Leu<sup>186</sup>–Thr<sup>209</sup> (Fig. 6A, blue bars) has backbone <sup>1</sup>H/<sup>15</sup>N, <sup>13</sup>Cα, and <sup>13</sup>CO NMR chemical shifts remarkably similar to those of random coil. This disorder (blue region in Fig. 6C) is somewhat unexpected given the elements of regular secondary structure (helix E and β-strand 11) observed in the same region of the EcCdtB crystal structure (Fig. 2A). Similar trends were observed for residues Glu<sup>213</sup>–Asp<sup>242</sup> of EcCdtB (red regions in Fig. 6, A and C), although, in general, the chemical shifts in solution are larger than those in the other disordered region (residues Leu<sup>186</sup>–Thr<sup>209</sup>).

Complementary <sup>15</sup>N relaxation NMR spectra were recorded and analyzed for the free EcCdtB protein in solution (Fig. 6B). The majority of EcCdtB residues are characterized by fast internal ns–ps (<sup>15</sup>N-{<sup>1</sup>H}NOE) and slower “tumbling” ns (<i>R</i><sub>t</sub>/<i>R</i><sub>p</sub>) solution dynamic properties consistent with a 29-kDa protein. This <sup>15</sup>N relaxation data fit well to an isotropic hydrodynamic model for EcCdtB, which produced a rotational correlation time of 13.6 ns.
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In the final model, 110 (43%) make crystal contacts. Regions showing symmetry contacts are spread throughout the protein (Fig. 6D). The disordered regions at Ser74–Gln80 and Gly233–Asp242 in the solution state of the protein contain significant crystal contacts. In the Gly233–Asp242 region, all residues make crystal contacts with symmetry related molecules, with five of the 10 residues forming hydrogen bonds with symmetry-related molecules. In contrast, in the longest disordered region (Leu186–Thr209, blue in Fig. 6, A–C), crystal contacts are localized around the five-residue helix E.

DISCUSSION

Although it has been established that CdtB is the catalytically active subunit of the CDT toxin and that CdtA and CdtC are necessary for cell surface binding and cellular entry (1, 2), the molecular basis and temporal ordering of putative holotoxin disassembly and delivery of CdtB to the nucleus where intoxication occurs is not well understood. In conjunction with previous in vivo and biochemical assays of CdtB in free and holotoxin forms, comparisons presented here between two orthologous CDT holotoxin subunits (hHdCdtB and hAaCdtB) and our structural and dynamic studies of the free EcCdtB subunit provide a more detailed basis from which answers to these questions of CDT function can begin to be synthesized.

Implications of Similar Free and Holotoxin CdtB Active Sites for Cellular Intoxication—In the HdCDT and AaCDT holotoxin structures, the N-terminal “tail” of CdtC encompassing residues 21–35 of the subunit extends out from a globular β-trefoil motif in an apparent occlusion of the CdtB active site (10, 11). A possible “autoinhibitory function” for this extended CdtC N-terminal region has been suggested based on the observation that the DNase activity of hHdCdtB within the assembled holotoxin was nearly absent in an in vitro assay, in contrast to the much greater activity of the isolated HdCdtB subunit and a mutant form of the holotoxin in which the N-terminal tail of CdtC was truncated (CdtCΔ21–35) (10). By contrast, in vivo studies of wild type and CdtCΔ21–35 CDTs revealed similar cellular intoxicating effects. Considering the nearly identical active sites and global folded structures of free and holotoxin CdtB, it seems unlikely that intramolecular conformational rearrangements account for the discrepancies between the in vitro enzymatic activities of free and holotoxin-associated CdtB (10). The observations discussed above suggest that autoinhibition of CDT intoxication by CdtC is disabled by holotoxin disassembly, which, in turn, produces a catalytically active free CdtB subunit. Although an alternative autoregulatory mechanism involving displacement of the N-terminal CdtC polypeptide tail to activate CdtB in the context of an intact holotoxin cannot be ruled out from existing data, the cellular intoxicating effects of isolated EcCdtB described previously underscores the plausibility of the subunit disassembly model (7, 12).

The structural difference between free and holotoxin CdtB proteins in the region encompassing residues Tyr232–His243 of EcCdtB (Fig. 4) and the solution backbone disorder in the same loop region (Gly233–Asp242, red regions of Fig. 6, A–C) suggest a link to subunit disassembly. This region forms the most significant interactions between subunit B and both of the other subunits, A and C. The hHdCdtB and hAaCdtB residue side chain using those regions of defined solution secondary structure predicted from the NMR chemical shift data shown in Fig. 6A. However, the disordered regions of EcCdtB evident from chemical shift comparisons (Leu186–Thr209 and Gly233–Asp242) were likewise characterized by fast internal and tumbling dynamic properties characteristic of disordered polypeptides (41). Residues Ser74–Gln80 of EcCdtB, which connect β-strands 3 and 4 (green regions in Fig. 6, A–C) also had 15N relaxation properties consistent with a flexible loop. Model-free analysis (34, 35) of fast internal motions within well structured regions of CdtB reveal consistent small amplitude (mostly <100 ps) motions, in contrast to larger amplitude motions in the three regions described above characterized by higher ns-ps mobility (data not shown). We note that sufficient electron density for model determination was evident in all three regions with solution disorder (Fig. 1 and data not shown). Backbone φ and ψ dihedral angles from the solution structure were calculated for those residues of EcCdtB in ordered regions for which a reliable angle could be obtained using the TALOS method (42). A comparison of 100 such TALOS-derived φ and ψ dihedral angles with those extracted from the crystal structure shown in supplemental Fig. 2 suggests an EcCdtB ensemble average solution conformation very similar to that in the crystal state for the ordered regions of the protein.

Analysis of Crystal Contacts and Correlation to Regions of Solution Disorder—Given the NMR results indicating solution disorder in the three regions of EcCdtB, crystal contacts were analyzed as a possible explanation for the structural order in the same regions of the corresponding crystallographic model. The EcCdtB crystals used for x-ray diffraction analysis have a relatively low solvent content of 34% (Table 1). Of the 253 residues
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FIGURE 6. Solution structure and dynamics of EcCdtB. A, bar plot of backbone 1H, 15N, and 13C EcCdtB chemical shift differences from random coil values (26, 27) for each residue. Labeled secondary structure schematic drawings are indicated above the plot. B, bar plot of steady-state heteronuclear NOE [15N-{1H}NOE] and R2/R1 time constant ratios as a function of residue number. Error bars for 15N-{1H}NOE measurements correspond to the standard error from five experimental data sets, and error bars for R2/R1 ratios are calculated uncertainties to the exponential fit. C, schematic representation of EcCdtB with regions of structural and/or dynamic disorder highlighted in green (Ser74–Gln60), blue (Leu186–Thr209), or red (Gly233–Asp242). Similar coloring of corresponding residues is shown in A and B. Characterized (Arg191–Arg192 and Arg235–Arg236) and putative (Arg208) arginine residues that mediate cellular trafficking are annotated in A and C.D, overlay of regions of solution disorder and crystal symmetry contacts. Regions of disorder identified by dynamics solution studies are shown in cyan, and residues with atoms less than 5 Å of symmetry-related molecules in the crystal structure are in orange. Regions that overlap both dynamic disorder and crystal contacts are in yellow.

hydrogen bonds shown in supplemental Fig. 1 that appear to stabilize interactions with CdtA and CdtC involve only Arg208 (hydrogen bonds to residue CdtA-Phe77 and CdtA-Ser75) and Gln236 (hydrogen bonds to CdtC-Arg39 and CdtA-Ser160) from CdtB (corresponding to EcCdtB residues Arg235 and Gln238, respectively), which are both completely conserved residues in the Cdt family. In addition, residues Leu266–Ile270 of hHdCdtB and hAaCdtB (corresponding to EcCdtB residues Arg235–Ile239) is the only region to make extensive van der Waals contacts with both the CdtA and CdtC in the tripartite interface (10, 11). There is a three-residue 310 helix found only in hHdCdtB and hAaCdtB in this region (residues Leu263–Gln265), which may also be important for subunit disassembly, because additional van der Waals contacts to CdtA involve all three helical residues (10, 11). Extensive crystal contacts with symmetry-related molecules in the subunit interface region of the free EcCdtB crystal structure (Gly233–Asp242, Fig. 6D) strongly suggest that different protein-protein interactions are stabilizing this region of the free and holotoxin CdtB proteins (10, 11). When considered together, the x-ray and NMR studies strongly suggest that the CdtB conformational flexibility in solution at the subunit interface is stabilized through an order-to-disorder conversion mediated by protein-protein contacts associated with putative holotoxin disassembly and concomitant CdtB catalytic activation.

Distinct EcCdtB Solution and Crystal States Provide Insights into Nuclear Trafficking—Unexpectedly, a 24-residue region of EcCdtB (residues Leu186–Thr209) had profoundly different solution and crystal characteristics. The Ca atoms of residues within this region of the free EcCdtB crystal structure superimpose very well with the corresponding Ca atoms of the hHdCdtB (0.62 Å) and hAaCdtB (0.60 Å) holotoxin structures. This similarity is in stark contrast to the random coil structural and dynamic properties of this region of EcCdtB in solution (Fig. 6, A and B), which encompasses two arginine residues (Arg191–Arg192) essential for CdtB catalytic activation. Such contacts could stabilize the helix, and possibly stabilize the entire Leu186–Thr209 region. Moreover, given the strong tendency among different protein-protein interactions, outlined below, suggest that the extended conformation of this surface located NLS region in the crystal states of the free and holotoxin CdtBs may resemble the conformation adopted in complex with a nuclear localization factor such as importin-α (also known as karyo-
Flexible and contiguous NLS regions within target proteins ranging in length from 5 to 25 amino acids (40) are typical. Crystal structures of NLS peptides bound to importin-α reveal extended backbone conformations (43–46), similar to the putative NLS region of the EcCdtB crystal structure. In the EcCdtB crystal structure, the formation of helix E results in the NLS Arg191-Arg192 side chains being adjacent tandem arginine side chains (Arg249-Arg250) in the EcCdtB crystal (40), similar to the putative NLS region of the EcCdtB crystal structure. The overlap of the Arg191-Arg192 residues involved in nuclear transport with the N-terminal end of the Leu186–Asn48 sequence (40) as the site for an NLS, although no candidate Arg/Lys residues were found in this region (47). A second tandem arginine sequence (Arg191-Arg192) in EcCdtB, which is required for cellular entry or cytoplasmic trafficking but is not absolutely required for nuclear localization, is located in the putative EcCdtB-EccdtC interface (12). Therefore, another scenario for a bipartite NLS in EcCdtB, is one in which Arg191-Arg192 and Arg235-Arg236 comprise a single bipartite sequence in EcCdtB, which is consistent with the flexibility observed in the vicinity of both of these arginine pairs (Fig. 6, A–C, blue and red regions). Although both tandem arginine regions are on the same surface of EcCdtB, such a bipartite arrangement would be unusual, because these two tandem arginine sequences are separated by more than the 20-residue limit of a consensus importin-α bipartite sequence (40).

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