The YoeB Toxin Is a Folded Protein That Forms a Physical Complex with the Unfolded YefM Antitoxin

IMPLICATIONS FOR A STRUCTURAL-BASED DIFFERENTIAL STABILITY OF TOXIN-ANTITOXIN SYSTEMS*

The chromosomal YoeB-YefM toxin-antitoxin module common to numerous strains of bacteria is presumed to have a significant role in survival under stringent conditions. Recently we showed that the purified YefM antitoxin is a natively unfolded protein, as we previously reported for the Phd antitoxin in the Pl phage Doc-Phd toxin-antitoxin system. Here we report the purification and structural properties of the YoeB toxin and present physical evidence for the existence of a tight YoeB-YefM polypeptide complex in solution. YoeB and YefM proteins co-eluted as single peaks in sequential Ni-affinity FPLC and Q-Sepharose ion-exchange chromatography implying the formation of a YoeB-YefM complex. The unstable antitoxin was removed from the mixture by natural proteolysis, and the residual YoeB protein was purified using ion exchange chromatography. Fluorescence anisotropy studies of the purified YoeB and YefM proteins showed a 2:1 stoichiometry of the complex, providing direct evidence for a physical complex between the proteins. Near- and far-UV circular dichroism spectroscopy of the purified toxin revealed that, similar to the Doc toxin, YoeB is a well-folded protein. Thermal denaturation experiments confirmed the conformational stability of the YoeB toxin, which underwent reversible thermal unfolding at temperatures up to 56 °C. The thermodynamic features of the toxin-antitoxin complex were similar. Taken together, our results support the notion of a correlation between differential physiological and structural stability in toxin-antitoxin modules.

Several families of toxin-antitoxin (TA) modules have been identified on chromosomes of Archaea and bacteria, as well as on extrachromosomal DNA elements (plasmids), but not in eukaryotes (1–3). The toxin, typically a protein that can inhibit important cellular functions like translation or replication, is inactive when combined with the antitoxin.

When carried on plasmids, TA systems constitute a maintenance mechanism or “plasmid addiction” system ensuring a remarkably stable vertical plasmid inheritance to bacterial daughter cells upon division. Such an efficient inheritance pattern is achieved through toxin-mediated post-segregation killing (PSK) of bacteria that accidentally lose the plasmid (“cured” cells) after the segregation event (1). The PSK mechanism is based on the differential physiological stability of the protein components; namely, a stable toxin with a long half-life and an unstable antitoxin with a short half-life. Consequently, as neither de novo toxin or antitoxin expression occurs in cured cells, rapid proteolytic degradation of the antitoxin enables the retained stable toxin to execute its lethal function. The cellular targets of most plasmid-borne toxins are unknown. Cellular targets have been described in the TA systems of the ccd plasmid F, parDE of RK2 plasmid and his-kid/peml-pemK of plasmid R1/R100 (4–7). CcdB and ParE bind and neutralize DNA gyrase, whereas Kid (PemK) inhibits ColE1 replication by acting at the initiation of DNA synthesis and by acting as a sequence-specific endoribonuclease. The Escherichia coli chromosome contains at least six proteinaceous TA loci, including relBE (8, 9), mazEF (chpA) (10, 11), chpB (11), dinD-yafQ (9), yefM-yoeB (12–14), and ecnAB (15). The ecnAB TA system is exceptional because EcnB toxin activity does not depend on its release from the labile antitoxin (through the proteolysis of Ecna) but rather appears following synthesis (15). Recently, the yeeU-yeeV gene pair was suggested as a putative novel family of TA systems in E. coli (16).

The role of chromosomal TA systems in normal bacterial physiology is less understood. The most accepted hypothesis suggests that chromosomal TA systems fill a regulatory role under various stress conditions (1, 2, 17–20). In the reported relBE, mazEF, and chpB TA modules of E. coli, regulatory activity is displayed in the form of toxin-mediated synthesis inhibition, leading to growth arrest. Upon entering a stress condition like nutritional starvation, cellular proteases (Lon or Clp) activate RelE and MazF toxins by actively degrading their antitoxin partners (17, 19, 11). MazF and RelE act as endoribonucleases that cleave mRNA transcripts at the ribosomal A-site in specific codons, which causes the ribosomes to halt on a non-stop codon, thereby preventing them from terminating transcription and disassembling (19, 21–24). Stalled ribosomes are released by the activity of tmRNA molecules (25, 19) that specifically target-truncated mRNA/ribosome complexes (26) and facilitate their disassembly, hence allowing the initiation of a new cycle (27). Recently, the YoeB toxin was shown to trigger cell growth arrest by cleaving translated mRNAs in a similar manner (28).

The yefM-yoeB TA module was identified by the partial sequence similarity between the YefM gene (12–14), and the gene encoding the Phd antitoxin from the phd-doc TA plasmid ad-
The Phd-YefM protein family represents a case in which the natively unfolded state is physiologically relevant. The Phd antitoxin has a low intrinsic thermal stability \((T_m \approx 25 \, ^\circ C)\), expressed in the predominant random coil conformation found at physiological temperatures \(30\). As the Phd antitoxin is recognized and degraded by the ClpXP quality control machinery of the bacterial cell \(35\), the antitoxin is identified as a damaged protein. Accordingly, the unfolded state subjects the protein to constant degradation, thus ensuring the short half-life that is critical for physiological activity. The YefM protein exhibits even higher structural instability, with the random coil conformation predominating even at \(4 \, ^\circ C\) \(12\). Structural and thermodynamic studies have shown that other antitoxins are partially unstructured at physiologic temperatures as well. The CcdA antitoxin, for example, has a flexible and relatively unstructured region in its C-terminal portion \(36\). The investigators suggested that under physiological conditions, CcdA can adopt a partially unfolded conformation \(37\), the preferred substrate for the activity of the Lon protease, which is involved in the selective recognition and proteolytic removal of damaged proteins and protein turnover. The ParD antitoxin, encoded by the \(parDE\) operon of plasmid RK2, comprises a well structured N-terminal domain and a very flexible C-terminal domain \(38\). The crystal structures of the MazE-MazF TA complex \(39\) and the MazE antitoxin, stabilized with a specific camel antibody fragment \(40\), clearly showed the presence of a large unstructured C-terminal region in MazE. The absence of an ordered structure for the C-terminal region in the MazE can be explained by the crystal structure of the complex; the C-terminal domain of MazE achieves stability by wrapping itself around the MazF dimer, creating an extended polypeptide loop. Recently, the crystal structure of the archetypal TA RelB-RelE complex was determined \(41\). According to the results of that study, the aRelB antitoxin lacks a clear hydrophobic core and, similar to MazE, wraps around the aRelE toxin to acquire stability.

According to one hypothesis, the low physiological stability and low structural or thermodynamic stability of antitoxins are related because of the increased susceptibility of antitoxins to proteolysis \(42\). Despite the rapidly increasing number of identified TA systems and the accumulating number of solved crystal structures of TA systems \(39–41, 43–45\), comparative structural and thermodynamic data on their proteinaceous components are limited. Comparative studies in the \(phd-doc\) and \(ccd\) TA systems \(30, 42, 37\) showed that the stability of the toxin component is thermodynamically greater than that of its antitoxin partner and that the conformation of the toxin is more folded than the antitoxin at physiological temperature. Additional comparative data can be indirectly obtained by examining the solved crystal structures of TA complexes, albeit performed in non-physiological conditions. Both the MazE-MazF \(39\) and aRelB-aRelE \(41\) complexes display a well-ordered overall structure of the toxin in contrast to the significantly less ordered structure of the antitoxin, strongly suggesting that this structure occurs \textit{in vivo} as well.

An exceptional case is the \(\zeta\) TA system of plasmid pSM19035 \(45, 46\). Unlike other systems studied, the thermodynamic stability of the \(\zeta\) toxin is significantly lower than that of the \(\epsilon\) antitoxin. Yet, the observation that the \(\epsilon\) antitoxin maintains a short half-life compared with the toxin suggests that, at least in this system, factors other than a partially unfolded structure might be responsible \(46\). Nevertheless, the results of the above studies, together with the data accumulating from single protein experiments \(12, 36, 38, 44, 47, 48\), support the idea that across different TA systems the structural and thermodynamic instability of antitoxins compared with that of toxins is well preserved.

In the present work, we investigated the secondary structure and thermodynamic stability of the purified YoeB toxin. We show that the purified toxin, unlike the natively unfolded YefM antitoxin, is a well folded protein at physiologic temperatures. We provide evidence that the YoeB toxin protein forms a tight complex with the YefM antitoxin and describe the thermodynamic stability of the complex. As in the Phd-Doc TA system, we found a marked difference in the thermodynamic stability of the YefM antitoxin and YoeB toxin proteins and discuss its relevance for the proper functioning of toxin-antitoxin systems.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of YefM/YoeB-His**—A DNA fragment containing the coding sequence of the \(yefM-yoeB\) genes was produced by PCR using the chromosomal DNA of \(E. coli\) K-12 MC1061 as template and the primers ATGYEFM \((5'-AGTAACTGTACAAAGGCCAGG-3')\) and YOE-B-NOSTOP \((5'-ATATGTAACGACGCTG-3')\). The PCR fragment was cloned into the ptRcHis2 vector \(49\), fused to a c-Myc epitope and His-tagged to generate a pTMB plasmid, which was then transformed into \(E. coli\) TOP10 strain bacteria \(49\). The transformed bacteria were grown in \(2YT\) broth at \(37 \, ^\circ C/200\) rpm to an \(A_{600}\) of \(\approx 0.4\). Protein expression was induced by the addition of \(2 \, \text{mM}\) isopropyl-\(\beta\)-\(D\)-thiogalactopyranoside and incubation for 1 h. The cells were harvested and resuspended in buffer \(A\) \((50 \, \text{mM}\ NaHPO}_4, \text{NaOH}, \text{pH} 8.0, 0.5 \, \text{mM}\) NaCl) and lysed by three passages through a French press cell \((1400\) psi).

The insoluble material was removed by centrifugation for 20 min at \(20,000 \times g\) and the supernatant applied onto an \(XK 16/20\) fast protein liquid chromatography column \(\text{Amersham Biosciences}\), packed with \(\text{Ni-CAM HC affinity resin (Sigma) and pre-equilibrated with buffer A. Following extensive washing, the bound proteins were eluted from the column using buffer A, containing } 25 \, \text{mM}\ \text{NiCl}_2\). Fractions containing the co-eluting YoeB-His and YefM proteins, identified by SDS-PAGE analyses, were combined and dialyzed against buffer \(B\) \((20 \, \text{mM} \text{Tris- HCl, pH} 8.0)\). The protein solution was applied onto a HiPrep 16/10 Q-Sepharose XL ion exchange column \(\text{Amersham Biosciences}\) pre-equilibrated with buffer \(B\). The fraction eluting from the column at \(635 \, \text{mM}\) NaCl, using a gradient of \(0.2 \, \text{to} 1 \, \mu\text{M}\) NaCl in buffer \(B\), contained both YoeB-His and YefM. Following dialysis against PBS \(10 \, \text{mM}\ \text{NaHPO}_4, 1.5 \, \text{mM}\ \text{KHPO}_4, 140 \, \text{mM}\ \text{NaCl, 2.7 mM KCl, pH} 7.3)\), the fraction was subjected to analytical reversed-phase high performance liquid chromatography \(\text{RP-HPLC (Vydac), using a gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid.}

**Purification of YoeB-His Protein**—Incubation of the Ni-affinity fraction, containing YefM-YoeB-His, for \(2–6\) weeks at \(4 \, ^\circ C\) or at room temperature removed the YefM portion. The incubation time varied from one production to another. The preparation was dialyzed against buffer \(B\) and loaded onto a HiTrap Q XL ion exchange column \(\text{Amersham Biosciences}\). The YoeB-His protein was eluted in a single peak at \(730 \, \text{mM}\) NaCl using a gradient of \(0.1–1 \, \mu\text{M}\) NaCl gradient in buffer \(B\). At this point, the YoeB-His protein was at least \(95\%\) pure, as estimated by Coomassie Blue staining of SDS-PAGE gels. YoeB-His was dialyzed against PBS, pH 7.3 before analysis.

**Purification of YefM**—The YefM antitoxin was expressed and partially purified as previously described \(12\) with a few changes. Briefly, a glutathione \textit{S}-transferase \((\text{GST})\)-\(YefM\) conjugate protein was overexpressed in \(E. coli\) BL21(DE3) \(p\text{LysS (Novagen). Following cell lysis, GST-}\(\text{(His)}_6\)-\(YefM\) was purified on a glutathione-Sepharose column \(\text{Amersham Biosciences}\). YeM proteins were separated from the GST-
His tags using 16 units of factor Xa protease (Novagen)/1 mg of YeFM fusion. To further purify YeFM, the GST-His and YeFM protein mixture was dialyzed against buffer B and applied onto a 1-ml HisTrap nickel affinity column (Amersham Biosciences). Pure YeFM proteins were washed out with buffer B, and 1-ml fractions were collected. YeFM was dialyzed against PBS, pH 7.3 for characterization analyses.

**Protein Identification and Characterization**—The identity of YeFM and YoeB-His was verified by the mass spectrometry analysis of protein spots isolated from Coomassie Blue-stained gels, according to established protocols (49). Briefly, protein spots were excised from stained gels and treated with trypsin solution (Promega) for 16 h at 37 °C. Peptides were extracted from the gel onto a sample plate for MALDI-MS. Peptide mass was determined in the positive ion reflector mode in a Voyager-DE STR mass spectrometer (Applied Biosystems). Peptide mass fingerprints were compared with databases using the MS-Fit program (prospector.ucsf.edu). To determine YeFM and YoeB-His concentrations, we measured tyrosine and tryptophan absorbance in alkaline (0.1 M KOH) conditions. The YeFM concentration was calculated using an extinction coefficient of 2381 m−1 cm−1 at 293.2 nm for single tyrosine (4 Tyr). For YoeB-His, we used an extinction coefficient of 1507 m−1 cm−1 for single tyrosine (6 Tyr) and 5377 m−1 cm−1 for single tryptophan (4 Trp) at 280 nm. As the exact proteinaceous content of the complex was unknown, we estimated the protein concentration using the Coomassie Plus protein assay reagent (Pierce) according to the manufacturer’s instructions.

Circular dichroism (CD) spectra were obtained using an AVIV 202 spectropolarimeter equipped with a temperature-controlled sample holder. Delta epsilon (Δε) was calculated as in Equation 1,

$$\Delta\varepsilon = \varepsilon (c \times L \times 3298)$$

(Eq. 1)

where \(\varepsilon\) is the observed ellipticity, \(c\) is the concentration in mol/liter, and \(L\) is the path length in cm. For thermal denaturation experiments, the sample temperature was equilibrated for 30 s at each temperature interval, and the ellipticity at each wavelength was averaged for at least 1 min. Following the thermal denaturation cycle, samples were examined for turbidity (protein aggregation) by centrifuging for 10 min at 10,000 × g. If turbidity was found, then fresh protein samples were used to avoid faulty experiments and misrepresentation of results.

To examine the induction of conformational transitions upon YeFM and YoeB-His interactions, we analyzed their far-UV CD spectra using a tandem CD cuvette (Hellma). YeFM and YoeB-His were added to separated tandem cells and the sum of ellipticity measured. Subsequently, the individual samples were mixed, and ellipticity was measured again.

**Fluorescence Labeling and Spectroscopy**—Purified YeFM or YoeB-His proteins were reacted for 2 h with 10 equivalents of N-hydroxysuccinimide (NHS) fluorescein (Pierce) on ice in the dark. Unreacted dyes were separated from labeled protein solutions by overnight dialysis in the dark against PBS, pH 7.3, with four buffer exchanges. Fluorescein anisotropy measurements were made using a PerkinElmer LS-50B luminescence spectrometer. Anisotropy of the labeled solution of YeFM or YoeB-His proteins (0.5 μM) was excited at 490 nm and monitored at 520 nm. Tryptophan anisotropy measurements were carried out using an ISS K2 fluorometer. YeFM-His protein (0.35 μM) was excited at 280 nm, and emission was monitored at 360 nm. For each single point, at least five measurements were collected, and their average values were used for calculation. All experiments were performed in PBS, pH 7.2.

**YeFM Stability Assay**—Overnight cultures of E. coli strain MC4100 carrying the pBAD-yeFM or pBAD-yeFMycB plasmid (12) were grown at 37 °C/200 rpm in LB broth to mid-log phase (A900 = 0.4). YeFM expression was then induced for 10 min with 0.02% arabinose and subsequently treated with 0.2% glucose to repress expression from the pBAD promoter and 1 mg/ml serine hydroxamate (SHX) to inhibit translation and induce amino acids starvation condition. At 20-min intervals after repression, aliquots (2 μl) were removed and analyzed by Western blot analysis (see below) to assess the quantity of YeFM. Densitometer assessment of YeFM was achieved using an Image Scanner (Amersham Biosciences) and the Image-Master one-dimensional prime program (Amersham Biosciences). Exponential trendline function was fitted to the first six time points (0–100 min) to estimate the YeFM half-life, using the Microsoft Excel program.

**Western Blot Analysis**—To detect YeFM, aliquots of E. coli strain MC4100 (2 ml) were centrifuged for 2 min at 20,000 × g at 4 °C and resuspended in 80 μl of double-distilled water. Samples of 60 μl were added to 20 μl of 4× sample buffer, and the remaining 20 μl were used to quantify the total protein using the Coomassie Blue Plus protein assay reagent (Pierce). Aliquots containing equal total protein amounts were loaded on a Tris-Tricine, SDS 16% polyacrylamide slab gel. After electrophoresis, the proteins were electroblotted to polyvinylidene difluoride membrane filters (Bio-Rad). The detection of YeFM was performed using anti-YeFM serum raised in rabbit. The membrane was then incubated with peroxidase-conjugated anti-rabbit antibodies, and YeFM proteins were detected through the enhanced chemiluminescence reaction after exposure to a sensitive film.

Aliquots containing the YeFM-YoeB-His complex were resolved using a Tris-Tricine SDS 16% polyacrylamide gel electrophoresis, and proteins were electroblotted to polyvinylidene difluoride membrane filters. The detection of YoeB-His protein was performed using mouse anti-c-Myc (Sigma) and peroxidase-conjugated anti-mouse antibodies through the enhanced chemiluminescence reaction after exposure to a sensitive film.**

**RESULTS**

**YefM and YoeB Form a Complex in Solution**—For studying the potential interaction between the proteins, the YefM anti-toxin and YoeB toxin were expressed in a single operon in E. coli strain TOP10 under the regulation of a Ptrc promoter. For detection and purification, YoeB was expressed in fusion to the c-Myc epitope and His tag. SDS-PAGE analysis revealed the presence of both expressed proteins, YoeB-His (≈14 kDa)
and YefM (~10 kDa), in a single peak eluting from a nickel affinity column (Fig. 1A). We verified the identity of each protein using Western blot analysis and mass spectrometry of protein spots excised from the gel (data not shown). The YefM-YoeB interaction appeared to be stable because the Ni-(His)6-YoeB/YefM proteins could not be separated by an extensive 24-h wash of the nickel affinity column or by Q-Sepharose ion-exchange chromatography (Fig. 1B). The analytical RP-HPLC elution profile of the Q-Sepharose fraction containing both proteins showed a single sharp peak at ~50% acetonitrile (Fig. 1C). However, further chromatography of the purified proteins revealed that YefM and YoeB-His have proximate hydrophobicity levels (data not shown). Therefore, their co-elution in RP-HPLC analysis does not necessarily imply that the complex is preserved under denaturing conditions.

YoeB Toxin Purification—Because previous attempts to express the YoeB toxin in fusion to GST (12) resulted in very low protein yield, here we isolated YoeB-His from the complex by taking advantage of the expected proteolytic instability of YefM. Following long term incubation of the YoeB-His/YefM fraction eluting from the Ni-affinity column, SDS-PAGE analysis (Fig. 2A) revealed a selective degradation of the antitoxin component, probably because of the presence of minute amounts of specific proteases in the solution. Q-Sepharose ion-exchange chromatography yielded a highly purified YoeB-His protein fraction, displaying a single band with an estimated molecular mass of ~15 kDa upon SDS-PAGE under reduced denaturing conditions (Fig. 2B).

Binding Stoichiometry—To determine the stoichiometry of the YefM-YoeB interaction, we monitored changes in the anisotropy signal obtained from the fluorescein-labeled antitoxin upon binding to different molar ratios of the toxin (Fig. 3A) and from the reciprocal anisotropy assay using fluorescein-labeled toxin and unlabeled antitoxin (Fig. 3B). The anisotropy assay was clearly consistent with a stoichiometry of YefM:YoeB2.

Because only YoeB-His contains tryptophan residues, we monitored tryptophan anisotropy during complex formation to substantiate the stoichiometry analysis (Fig. 3C). The stoichiometry of YefM:YoeB2 was supported by the densitometric assessment of the YefM and YoeB-His bands as they appeared in SDS-PAGE analyses (Fig. 2A), which yielded a YoeB-His:YefM ratio of 1.92 ± 0.31.

Structural Characterization of YoeB—The structure of the purified YoeB-His protein obtained from far-UV circular dichroism (CD) spectroscopy is consistent with a well folded protein containing at least 50% α-helical secondary structures (Fig. 4A), according to the K2d program for prediction of protein secondary structure from CD spectra (50). The secondary structure content of the toxin remained almost unchanged between 4 °C and physiological temperatures. To study thermodynamic stability of the toxin, we performed a thermal denaturation experiment, monitoring the ellipticity at 222 nm between 2 and 80 °C. As shown in Fig. 4B, a slight increase in ellipticity between 2 and 60 °C indicated a moderate decrease in the
helical content of YoeB. At 60 °C, a further increase in the slope of ellipticity indicated a melting temperature of 65 °C. Cooling from 80 °C back to 2 °C did not result in refolding (data not shown). Moreover, samples extracted from the cuvette after the experiment were always turbid.

To determine the exact melting point at which the toxin loses its conformational reversibility, YoeB-His was melted using a series of temperature ranges and then cooled back repeatedly, raising the target temperature by no more than 5 °C in each experiment. Conformational changes were monitored by measuring CD ellipticity at 222 nm. According to this analysis, YoeB-His demonstrated complete structural reversibility in all melting-cooling cycles between 4 and 56 °C (Fig. 4C and D). YoeB-His thermal melt (filled symbols) and reverse (empty symbols) experiments from 4 to 56 °C (C) and 5 to 60 °C (D), monitored by CD ellipticity at 222 nm. Temperature step was 2 or 2.5 °C, respectively, averaged for 1 min. The results suggest that YoeB folding is irreversible at temperatures above 56 °C.

To determine whether a molten globule state forms during thermal melting, we used near-UV CD to investigate the tertiary structure of YoeB-His. The graph in Fig. 5A shows that the CD spectrum of YoeB-His is consistent with an ordered tertiary structure. The spectra remained nearly unchanged during a temperature shift from 4 and 60 °C. At 60 °C and above, however, toxin aggregation occurred. The results were supported by a CD thermal denaturation experiment from 4 to 80 °C (Fig. 5B). The changes in tertiary structure were monitored at 274 nm (representing near-UV CD spectra extreme points), respectively corresponding to tyrosine and phenylalanine absorbance. As shown in Fig. 5B, no significant change occurred until 60 °C, when ellipticity sharply decreased. The tertiary structure of YoeB-His was fully reversible (and practically unchanged) after heating to 37 °C (Fig. 6A) and to 56 °C (Fig. 6B). This finding was supported by similar near-UV CD spectra at 4 °C before and after a heating-cooling cycle to 56 °C (Fig. 6C). The results of these experiments indicate that YoeB-His retains both its secondary and tertiary conformations during temperature increases until it begins to
aggregate. We could not determine the existence of a molten globule stage during the melting-cooling cycles.

Structural Characterization of the YefM\textsubscript{YoeB-His} Complex—Far-UV CD analysis of the purified YefM\textsubscript{YoeB-His} proteins at 4 °C (Fig. 7\textsubscript{A}) displayed two minima in the vicinity of 220 and 208 nm, indicating the considerable presence of \(-\)helices, whereas the 208-nm minimum point was lower, suggesting the presence of unstructured regions as well. Although this structure was retained at physiological temperature (Fig. 7\textsubscript{A}), at temperatures above 60 °C, sediments resulting from protein aggregation formed. This finding was supported by a CD thermal denaturation assay monitored between 4 and 80 °C at 222 nm (Fig. 7\textsubscript{B}). At 62 °C, a switch of the slope of ellipticity from a moderate to a sharp increase indicated that the complex underwent denaturation, with a melting point of \(70 \degree C\). After the complex was exposed to 70 °C, SDS-PAGE analysis of the supernatant fraction revealed that only the YefM antitoxin portion remained soluble (Fig. 7\textsubscript{A}, inset), whereas the pellet contained YoeB-His (data not shown). Far-UV CD spectroscopy of the soluble fraction (at 25 °C) demonstrated that the conformation of YefM was predominantly unstructured (Fig. 7\textsubscript{A}), as expected in an uncomplexed state (12). Therefore, we inferred from this finding that only YoeB-His had undergone aggregation following thermal melting. The lack of aggregation by its antitoxin partner reflects the remarkable solubility of YefM.

We studied the structural reversibility of the complex by monitoring the CD ellipticity signal of YefM\textsubscript{YoeB-His} along different melting-cooling cycles. Between 4 and 37 °C, YefM\textsubscript{YoeB-His} demonstrated complete structural reversibility (Fig. 8\textsubscript{A}). Nevertheless, only partial refolding of the complex (Fig. 8\textsubscript{B}) occurred after a melting-cooling cycle between 4 and 60 °C, accompanied by a slight formation of protein sediments. When the target temperature was elevated to 65 °C, however, the partial refolding was lost as well (data not shown). Far-UV CD spectra at 4 °C, 55 °C and again at 4 °C (Fig. 8\textsubscript{C}) demonstrated that similar to YoeB-His, the YefM\textsubscript{YoeB-His} complex also displays complete secondary structure reversibility below 60 °C. This reversibility was also valid for the tertiary structure of the complex. As shown in Fig. 9, a near-UV CD temperature melt to 56 °C and wavelength scan analyses clearly demonstrated that the tertiary structure of YefM\textsubscript{YoeB-His} is stable during such a melt. The results of these experiments suggest that the thermodynamic stability of the complex is largely determined by the stability of the toxin component.

Structural Analysis of the YefM\textsubscript{YoeB-His} Interaction upon Binding—We examined whether the YefM and YoeB-His interaction induces folding of the natively unfolded YefM protein portion of YefM\textsubscript{YoeB-His} by determining the sum of their far-UV CD spectra before and after interaction (Fig. 10), using...
a toxin:antitoxin ratio of 2:1 (Fig. 10A). Interestingly, mixing the YefM and YoeB-His proteins did not significantly change the CD spectra (Fig. 10B). Additionally, SDS-PAGE analysis of a YefM-YoeB-His sample analyzed in CD and subjected to Ni-affinity chromatography confirmed that mixing the YefM and YoeB-His proteins results in complex formation (Fig. 10C).

The results of further CD experiments using antitoxin:toxin molar ratios of 1:1 and 2:1 were similar (data not shown).

**In Vivo Proteolytic Stabilization of YefM—Coupled binding and folding of unstructured proteins was suggested to be the manner by which unstructured proteins gain proteolytic stability in cell (33).** The YefM protein was previously reported to have a short physiological half-life when expressed alone (12). As we did not observe a significant conformational change following complex formation, we examined whether YefM antitoxin is metabolically stabilized when expressed together with its toxin partner, YoeB, under translation-inhibition conditions. Such conditions were suggested to free YoeB to execute its detrimental activity (28). We used SHT to induce amino acid starvation, which simulates the inhibition of translation. Western blot analysis showed that when expressed together with YoeB, the half-life of YefM increases from about 50 to ~70 min (Fig. 11). Thus, under the conditions imposed in this experiment, YefM displayed a 50% increment in proteolytic stability when complexed with YoeB. In both cases, the amount of YefM decay was consistent for at least 100 min, whereas at 120 min the amount of antitoxin increased by ~30%, presumably caused by a renewal of chromosomal yefM-yoeB promoter activity. A delayed increase in TA promoter activity during starvation was reported for the relBE system (17).

**DISCUSSION**

Toxin-antitoxin modules are widespread throughout the entire prokaryotic world (1–3, 17, 18). Such systems appear to play a central role in the response of bacteria and Archaea to environmental stress and their ability to survive harsh conditions. Studying the mechanisms of TA module activity is important from a physiological point of view because of their abundance. The ubiquity of TA systems in prokaryotic cells and their absence from eukaryotic organisms make them very attractive for developing novel types of antibacterial agents.

The YefM-YoeB *E. coli* system has close homologues in several major pathogens including *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Mycobacterium tuberculosis* (12). We previously demonstrated that the YefM antitoxin is a natively unfolded protein (12), like its distinct homologue Phd antitoxin (30). We suggested that the natively unfolded state of YefM implies that the YoeB toxin recognizes a linear rather than a conformational determinant within the YefM antitoxin (12). This observation could be useful for targeting the YefM-YoeB system for antibacterial therapy.

The results of the current study provided strong evidence for a stable physical interaction between the YoeB and YefM toxin-antitoxin proteins and determined the stoichiometry of the complex. The co-expression of histidine-tagged YoeB together with YefM implied that YefM and YoeB combine to form a tight complex in solution. Furthermore, despite the relative acidic and basic amino acid composition of YefM (theoretical pI, 4.88) and YoeB-His (theoretical pI, 6.31), respectively, the YefM-YoeB-His complex remained stable during ion exchange chromatography (Fig. 1B). Complex formation was further supported by fluorescence anisotropy studies (Fig. 3). Character-
YoeB Stability and Molecular Interactions

Although lacking any sequence homology, a comparison between the YoeB and Doc proteins is inevitable in light of our results. Similar to YoeB, the Doc toxin is a well ordered protein at physiological temperatures, with an α-helical content of ∼50% and a $T_m$ of 60 °C (42). Comparable biophysical characteristics fit with the observation that Doc and YoeB fill analog roles.

The structural stability of YoeB-His was retained at the level of tertiary structure as well. The finding in near-UV CD analyses that only marginal changes occur upon temperature increase until the point at which YoeB-His aggregates indicates that the YoeB toxin did not switch into a clear molten globule stage. Hence, the conformational changes in secondary structure (Fig. 4) did not affect the overall three-dimensional structure of the toxin (Figs. 5 and 6). At 60 °C and above, YoeB-His lost the capacity for reversibility, and the rapid aggregation of the toxin from this temperature onward resulted in a strong irreversibility.

The YefM-YoeB-His complex did not appear to show further thermodynamic stabilization. Both far- and near-UV CD analyses, with an approximate $T_m$ of 70 °C and partial refolding after heating to 60 °C (Figs. 7–9), indicated only slightly elevated thermal stability of the complex. The selective aggregation of the toxin at 60 °C suggests that partial refolding actually represents full reversibility of the soluble fraction of the complex, albeit abrogated by the rapid aggregation of the toxin at 65 °C. Consistent with the thermal denaturation characteristics of YoeB-His alone, the YefM-YoeB-His complex also displayed full structural reversibility below 60 °C. From the results of these experiments, we deduce that the thermal stability of the YefM-YoeB-His complex is dependent upon the
YoeB Stability and Molecular Interactions

stability of the toxin. Therefore, as the interaction of YefM antitoxin with YoeB toxin does not appear to involve significant structural changes, we can assume that such interaction cannot increase the stabilization of the toxin, either alone or when complexed with YefM. We further postulate that at high temperatures, an increase in the dissociation rate between YefM and YoeB leads to a situation in which the toxin spends more time in solution as the uncomplexed state, eventually resulting in self aggregation. Such weak interactions at elevated temperatures might be ascribed to a state in which the unfolded YefM antitoxin has little effect on YoeB-His structural packing but rather wraps itself around the toxin, as described for the aRelB and MazE antitoxins (39, 41). Supporting this hypothesis is the lack of a significant conformational change in secondary structure upon the YefM-YoeB-His interaction (Fig. 10). We were surprised at this finding, however, because of the predominantly unfolded nature of YefM (12). Many intrinsically disordered proteins have been reported to undergo disorder-to-order structural transition upon binding to their biological target ligands (31, 33, 52). Prior structural studies of TA interactions demonstrated an induction of the structure of unfolded (or partially unfolded) antitoxins upon binding to the toxin partner, represented by the YefM distant homologue Phd (42) and the MazE antitoxins (39, 40). The latter system offers a detailed description of MazE conformational changes at the amino acid level, made possible by the availability of the three-dimensional structures of both the MazE antitoxin (40) and the MazE-MazF complex (39). According to these structures, MazE binds to MazF via its C-terminal domain, which is unstructured in the uncomplexed state but becomes stabilized after wrapping around the MazF homodimer. Notably, although gaining order, the MazE C-terminal domain does not acquire a considerable amount of secondary structure but rather adopts an extended polypeptide conformation embedded with only a very short α-helix (5 residues long) (39, 40). Therefore, we can postulate that the YefM flexible regions become similarly stabilized by YoeB merely by being embraced by the toxin and without adopting additional secondary structure. In light of their recent discovery that MazF functions as an endoribonuclease, Zhang et al. (23) elegantly speculated that the MazE unstructured region can actually mimic the RNA strand and hence block the MazF RNA binding site. As YoeB is suggested to act as an endoribonuclease as well (28), we can speculate that YefM could be bound in a similar manner. Although hypothetical, such a simple binding seems to be enough to increase the half-life of YefM in vivo (Fig. 11). We suggest that by constraining its exposed unfolded regions, the YefM antitoxin can avoid recognition and consequent degradation by cellular quality control proteases.

The findings that both Phd-Doc (14, 29) and YefM-YoeB (12) are functional TA systems suggests that the YefM and Phd antitoxins evolved from a common ancestor system that branched out to establish new TA systems having different toxins (12, 14). Unlike their antitoxin partners, the Doc and YoeB toxins do not share sequence homology (12). Despite the divergence of these systems, the conservation of their common structural and thermodynamic features is rather intriguing. Such a phenomenon was described for the parD (kis, kid) TA system of plasmid R1 (53) and the ccd (ccdB, ccdB) TA system of plasmid F (54). The parD and ccd systems have been proposed to derive from a common ancestor on the basis of low sequence homology between the antitoxins (55). Yet, a clear sequence alignment between the toxins was not found. Nevertheless, the structure of the Kid toxin resembles that of CcdB (44), despite having different toxic activity (43, 6). A significantly lower thermodynamic stability of the antitoxin compared with the toxin was reported for the ccd system (37). Based on their structural resemblance, the Kis and Kid proteins should be likely to determine similar behavior as well, although such a comparative study has not yet been carried out. We suggest that the structural data presented here for the YoeB-YefM module correlate well with the functional demands of a TA system. Whereas the key structural requirements of the YefM/Phd antitoxins are proteolytic susceptibility and a toxin binding determinant, the YoeB/Doc toxins require a much more complex conformation for executing their cytotoxic activity.

In conclusion, we propose that the structural instability of the YefM antitoxin combined with the stable, well-ordered structure of the YoeB toxin is prerequisite for the proper functioning of this system. The thermodynamic stability of the YefM-YoeB complex mirrors the stability of the toxin. We speculate that upon complex formation, parts of the flexible un-folded regions of YefM are confined to the surface of YoeB, adopting an extended coil conformation at the most. Such differential structural features are consistent with differential physiological stability. The folded and evolutionarily conserved profile of the YoeB toxin is consistent with the structural complexity that should be required for such an elaborate toxic activity as RNA cleavage.

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