TorT, a Member of a New Periplasmic Binding Protein Family, Triggers Induction of the Tor Respiratory System upon Trimethylamine N-Oxide Electron-acceptor Binding in Escherichia coli*§

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In anaerobiosis, Escherichia coli can use trimethylamine N-oxide (TMAO) as a terminal electron acceptor. Reduction of TMAO in trimethylamine (TMA) is mainly performed by the respiratory TMAO reductase. This system is encoded by the torCAD operon, which is induced in the presence of TMAO. This regulation involves a two-component system comprising TorS, an orthodox histidine kinase, and TorR, a response regulator. A third protein, TorT, sharing homologies with periplasmic binding proteins, plays a key role in this regulation because disruption of the torT gene abolishes tor expression. In this study we showed that TMAO protects TorT against degradation by the GluC endoprotease and modifies its temperature-induced CD spectrum. We also isolated a TorT negative mutant that is no longer protected by TMAO from degradation by GluC. Isothermal titration calorimetry confirmed that TorT binds TMAO with a binding constant of 150 μM. Therefore, we conclude that TorT binds TMAO and that this binding promotes a conformational change of TorT. We also showed that TorT interacts with the periplasmic domain of TorS in both the presence and absence of TMAO but the TorT-TMAO complex induces a higher GluC protection of TorS than TorT alone. These results support the idea that TMAO binding to TorT induces a cascade of conformational changes from TorT to TorS, leading to TorS activation. We identified several homologues of the TorT protein that define a new family of periplasmic binding proteins. We thus propose that the members of this family bind TMAO or related compounds and that they are involved in signal transduction or even substrate transport.

In anaerobiosis, Escherichia coli can use alternative terminal electron acceptors such as nitrate, nitrite, fumarate, dimethyl sulfoxide (Me2SO), or trimethylamine N-oxide (TMAO) for respiration (1). Reduction of TMAO in trimethylamine (TMA) mainly involves the TMAO reductase system. This respiratory system comprises three proteins: TorC, TorA, and TorD. The TorC protein is a pentahemicytochrome anchored to the membrane and oriented toward the periplasmic compartment. The TorA protein, located in the periplasm, is the terminal reductase and receives the electrons from TorC (2). TorD is a cytoplasmic protein that acts as both a specific chaperone and an escort protein for TorA, allowing TorA maturation and protection before its translocation into the periplasm by the Tat system (3–6).

The Tor respiratory system is encoded by the torCAD operon that is induced in the presence of TMAO (7). The TMAO control is strict because there is almost no expression of the tor operon in the absence of TMAO. The three genes torS, torT, and torR, located upstream of the tor operon, are involved in this regulation. Disruption of either one of these genes abolishes tor operon induction. The torS and torR genes encode a two-component regulatory system in which TorS is an orthodox sensor containing three phosphorylation sites and TorR a response regulator of the ompR family (8, 9). At its N-terminal extremity, TorS contains a large detector region of about 300 residues oriented toward the periplasm and flanked by two transmembrane segments. The cytoplasmic part of TorS comprises three domains, each of them containing a phosphorylation site: the N-terminal transmitter domain contains the primary site of phosphorylation (His443), the central receiver domain possesses a phosphorylatable aspartate (Asp723), and the C-terminal alternative transmitter domain bears the phosphodonor site for TorR (His850) (10). Upon detection of the signal, TorS autophosphorylates on His443. The phosphoryl group is then transferred to Asp723 and subsequently to His850. Thereafter, the phosphoryl group on His850 is transferred onto Asp53 of the N-terminal receiver domain of TorR (10). Once phosphorylated, TorR binds to decameric direct repeats, called tor boxes and located in the intergenic torC-torR region, to activate tor operon expression (11). TorS can also dephosphorylate

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§ The on-line version of this article (available at http://www.jbc.org) contains Table 3.

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3 The abbreviations used are: TMAO, trimethylamine N-oxide; PBP, periplasmic binding protein; ITC, isothermal titration calorimetry; DSG, disuccinimidyl glutarate.
phospho-TorR when TMAO is exhausted. Dephosphorylation is a rapid process occurring by reverse phosphorylation (12).

The torT gene is the third gene required for tor operon induction (13). It is located between the torS and torR genes and encodes a periplasmic protein of 36 kDa. Genetic evidence showed that TorT acts upstream of the TorS/TorR two-component system but its precise role is yet unknown. Interestingly, TorT shares weak homologies with periplasmic binding proteins (PBPs). These proteins are especially known to bind substrates and to allow their transport via ABC-type transporters (14, 15). Substrates include mono- and oligosaccharides, amino acids, oligopeptides, ions, metals, and vitamins (14). Many PBPs have been crystallized and their structures solved. These studies have revealed a common structure consisting of two globular lobes connected by a hinge region with the ligand-binding site located at the interface between the two domains (16–19). PBPs can be found in two conformations: a ligand-free open conformation and a ligand-bound closed conformation. There-fore PBPs undergo a conformational change upon binding of a substrate located at the interface between the two domains (16–19). PBPs can be found in two conformations: a ligand-free open conformation and a ligand-bound closed conformation. Therefore PBPs undergo a conformational change upon binding of the substrate. In addition to their role in transport, certain PBPs also proved to be involved in signal transduction by directly interacting with transmembrane sensors. The ChvE sugar-binding protein of Agrobacterium tumefaciens interacts with the VirA sensor to enhance induction of the vir genes in the presence of monosaccharides (20, 21). The PBPs were classified in several families according to the nature of the bound substrate (14). The PBPs of a given family share similar molecular masses and present sequence similarities located, in particular, in their N-terminal regions. TorT does not belong to any previously described family but shares weak homologies with the RbsB ribose-binding protein of E. coli and with uncharacterized PBPs (13).

In this study, we provide biochemical, biophysical, and genetic evidence that the TorT protein binds TMAO and interacts with the detector region of the TorS sensor. We also show that, upon binding of the TorT-TMAO complex, the TorS detector region undergoes a conformational change that probably activates it as a kinase. Finally we show that TorT belongs to a new family of PBPs.

### Experimental Procedures

**Strains, Plasmids, and Growth Conditions**—The strains and plasmids used in this work are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) medium at 37 °C. To maintain plasmid selection, ampicillin and kanamycin were added at 50 and 25 μg/ml, respectively. When necessary, isoprropyl β-D-thiogalactopyranoside was added at 1 mM to induce gene expression.

**β-Galactosidase Assays**—β-Galactosidase activities were measured on whole cells by the method of Miller (25). Strains were grown overnight anaerobically in LB at 37 °C. Values represent the average of three independent experiments with a variation of no more than 15% from the mean.

**Construction of the torT Deletion Mutant (LCB982)**—We used the method described by Datsenko and Wanner (26) to delete the chromosomal copy of torT gene of strain LCB620 (torA−·lacZ). This method consists of the replacement of the gene to be inactivated by a DNA fragment generated by PCR and allowing resistance to an antibiotic. The primers used for PCR were 60 nucleotides long and included 40-nucleotide homology extensions corresponding to the 5′ extremity or the 3′ region of the torT gene and 20-nucleotide priming sequences that hybridized to the resistance gene of plasmid pKD3 (CmR).

**Detailed information on the primer sequences are available on request.**

The PCR products were incubated with DpnI to eliminate remaining plasmid DNA and used to transform strain LCB620 harboring the λ Red helper plasmid pKD46, which permits recombination. The resulting strain was named LCB982 and has a deletion of the first 737 bases of the 1029 bases of the torT gene.

**Construction of Plasmids**—To construct plasmid pET-Tp, allowing overproduction of a C-terminal His6-tagged recombinant TorT protein, the torT coding sequence was amplified from MC4100 chromosomal DNA by PCR. We used a primer that contains a Smal site following by the 5′ coding sequence of torT and a primer that contains a Smal site followed by a sequence encoding a His6 tag and a sequence complementary of the 3′ end of torT. After enzymatic hydrolysis, the PCR products were cloned into the corresponding Smal site of vector pET21.

### Table 1

| Strain or plasmid | Genotype and/or characteristic | Ref. or source |
|-------------------|--------------------------------|---------------|
| Strain | | |
| MC4100 | araD139 Δ(lacPO72 yo-argF) U169 rpsL thi | Laboratory collection |
| LCB620 | MC4100 torA::MudII 1734 (torA−·lacZ) | 7 |
| LCB982 | ΔtorT derivative of LCB620 | This work |
| BL21(DE3) | F-ompT(hsdR mκ) gal dcm(DE3) | Novagen |
| Plasmid | | |
| pET21 | Vector containing the T7 promoter | Novagen |
| pET-Tp | torT cloned into pET21 (TorT protein tagged at the C terminus) | This work |
| pET-5p | Part of the torS gene cloned into pET21 (periplasmic TorS region tagged at the C terminus) | 28 |
| pET28 | Vector containing the T7 promoter | Novagen |
| pET-T_1Lg | torT_1Lg cloned into pET28 (TorT_1Lg protein tagged at the N terminus) | This work |
| pF119EH | Vector containing the pprom | 46 |
| pC6 | torT cloned into pF119EH (TorT protein) | This work |
| pE-Tp | torT cloned into pF119EH (TorT protein tagged at the C terminus) | This work |
| pE-T_1Lg | torT_1Lg cloned into pF119EH (TorT_1Lg protein tagged at the C terminus) | This work |

**ROLE OF TMAO-BINDING PROTEIN TOR T***

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To construct plasmid pET-T_{LSOP}, allowing overproduction of a N-terminal His_{6}-tagged recombinant TorT protein, the torT gene truncated of the sequence encoding the signal peptide was amplified from pJF-T_{LSOP} plasmidic DNA by PCR. We used a primer that contains an NcoI site followed by a sequence encoding a His_{6} tag and by the 5’ coding sequence of torT and a primer that contains a HindIII site followed by a sequence complementary of the 3’ end of torT. After enzymatic hydrolysis, the PCR products were cloned into the corresponding sites of vector pET28.

To construct plasmid pJF-Tp, the torT coding sequence was amplified from MC4100 chromosomal DNA by PCR. We used a primer that contains an EcoRI site followed by the 5’ coding sequence of torT and a primer that contains a SmaI site followed by a sequence encoding a His_{6} tag and a sequence complementary of the 3’ end of torT. After enzymatic hydrolysis, the PCR products were cloned into the corresponding sites of vector pJF119EH.

Detailed information on all primer sequences are available on request. All plasmid inserts were confirmed by DNA sequencing. Transformations were performed by the method of Chung and Miller (27).

Isolation of torT Negative Mutant—For random mutagenesis, the torT coding sequence was amplified from MC4100 chromosomal DNA by PCR using the same primers as for the construction of pJF-Tp. To allow the introduction of mutations in the torT sequence, three successive rounds of PCR amplification (30 cycles each) were performed. After enzymatic hydrolysis, the PCR products were cloned into the corresponding EcoRI and SmaI sites of vector pJF119EH. Ligation products were then transformed in strain LCB982 (torA^+/lacZ^+), whereas strains that produced TorT negative mutant proteins, that did not induce the torA^+/lacZ^+ fusion, yielded white or pale colonies (Lac-).

Purification of Recombinant Proteins—TorT was produced from strain BL21(DE3) harboring plasmid pET-Tp. The recombinant strain was grown aerobically until the optical density at 600 nm reached 0.8 unit. Overproduction of the protein was then induced with isopropyl β-D-thiogalactopyranoside (1 mM) for 4 h at 37 °C. The proteins were purified from the soluble fraction obtained after French press treatment of the cells in phosphate buffer (20 mM, pH 7.4) containing NaCl (500 mM) and imidazole (5 mM) and loaded onto a HiTrap^TM^-chelating Ni^{2+} column (Amersham Biosciences). The proteins were eluted with a step gradient of imidazole ranging from 20 to 500 mM. TorT was recovered in the 300 mM imidazole fraction. The proteins were then dialyzed against phosphate buffer (20 mM, pH 7.4) containing 10% glycerol.

TorT_{LSOP} was produced from strain BL21(DE3) harboring plasmid pET-T_{LSOP}. The proteins were purified with the same protocol used for TorT except that they were recovered from the pellet obtained after centrifugation (18,000 × g for 10 min) by using 8 M urea.

TorS_{N} was produced from strain BL21(DE3) harboring plasmid pET-Sp and purified as described by Gon et al. (28). TorS_{N} polypeptide corresponds to the His-tagged periplasmic N-terminal domain of TorS (position 17–319).

Circular Dichroism (CD) Spectra—CD measurements were performed with a Jasco J-810 instrument equipped with a Peltier-type temperature control system (model PTC-348WI) with a 0.2-mm path length. Far UV spectra of purified TorT protein (80 μg/ml in phosphate buffer, 20 mM, pH 7.4) were monitored at 25 °C from 195 to 260 nm at 0.2 nm/min and were averaged from three independent acquisitions. The thermal stability of TorT secondary structure (80 μg/ml) was monitored at 222 nm over the range 25–80 °C at 1 °C/min. The mean residue molar ellipticity at 222 nm was recorded at 0.2 °C intervals. The α-helical content was derived from the ellipticity at 222 nm as described in Morris et al. (29). The experimental data, i.e. plots of the observed ellipticity (θ) against temperature (T), were fitted with the data analysis program SigmaPlot to the equation reported by Tan et al. (30).

Limited Proteolytic Digestion—TorT (wild type or mutant) and TorS_{N}, together or separately, were subjected to proteolysis with endoprotease GluC (V8, type XVII-B; Sigma). Digestions were carried out with purified proteins in phosphate buffer (20 mM, pH 7.4) at 37 °C. TorT (7.4 μM) and/or TorS_{N} (7.8 μM) were incubated 10 min in the presence or absence of TMAO (10 mM) and endoprotease GluC was then added at a protein/enzyme mass ratio of 4/1 for TorT digestion, 20/1 for TorS_{N} digestion, and 4/1 for TorT/TorS_{N} co-digestion. The reactions were stopped after various incubation times (2, 10, 30, and 60 min) by adding SDS loading buffer (133 mM Tris-HCl, pH 8.8, 3.3 mM EDTA, 0.7 mM sucrose, 0.07% bromphenol blue, 6% SDS, 0.1 M dithiothreitol, and 1.7% β-mercaptoethanol) and by subsequently incubating the samples for 10 min at 95 °C. The samples were then analyzed on SDS-PAGE. For TorT digestion, the gel was revealed by Coomassie Blue staining, whereas for TorS_{N} digestion and TorT-TorS_{N} co-digestion, digestion products were first transferred to a Hybond^TM^-ECL^TM^- nitrocellulose membrane and detected with anti-TorS antibodies. Each experiment was repeated at least three times and gave the same profile of degradation.

Isothermal Titration Calorimetry—ITC experiments were performed at 25 °C using a MicroCal MCS instrument. After purification onto HiTrap^TM^-chelating Ni^{2+} column, the TorT and TorT_{LSOP} proteins were dialyzed against 50 mM Tris-HCl buffer (pH 7). The protein concentration was 25 μM in the calorimetric cell. Each experiment consisted of 10-μl injections of 10 mM TMAO or ribose prepared in the protein dialysis buffer. The heat of dilution was measured by injecting the ligand into the protein-free buffer solution or by additional injections of ligand after saturation; the value obtained was subtracted from the heat of reaction to obtain effective heat of binding. To obtain thermodynamic parameters, titration curves were fitted to a one-site model using MicroCal Origin software. Experiments were performed twice.

Native Gel Experiments—TorT (11.1 μM) and TorS_{N} (11.7 μM) were incubated together or separately in phosphate buffer...
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(20 mM, pH 7.4) in the presence or absence of 10 mM TMAO for 20 min at room temperature. Native sample buffer (200 mM Tris-HCl, pH 8.8, 5 mM EDTA, 1 mM sucrose, 0.1% bromphenol blue) was added to the samples before loading onto a 12% polyacrylamide gel. The migration pattern was revealed by Coomassie Blue staining.

Chemical Cross-link Studies—Disuccinimidyl glutarate (DSG, Pierce), which is a homobifunctional NHS-ester, was used as a cross-linker. TorT (8.3 μM) and TorSN (8.8 μM) were incubated for 30 min at room temperature in phosphate buffer with DSG (5 mM) in the presence or absence of 10 mM TMAO. The samples were analyzed by SDS-PAGE and revealed by Coomassie Blue staining.

Mass Spectrometry—Protein bands were excised from the polyacrylamide gel after electrophoresis. They were digested overnight by trypsin and subsequently analyzed by matrix-assisted laser desorption ionization time-of-flight (Voyager DE-RI). Proteins were then identified by comparison with the protein databases using the Mascot program.

Bioassay Experiments—The BLAcore apparatus (surface plason resonance) was used to analyze, in real time, the binding between TorSN and TorT. All experiments were carried out at 25°C. The TorSN polypeptide was dialyzed against 50 mM sodium formate buffer (pH 4.5) and subsequently immobilized on a sensor chip CM5 through amine coupling, using 20 mM phosphate (pH 7.4) as running buffer. For this purpose, the dextran matrix was first activated with 50 μl (5 μl/min) of a mixture of 200 mM 1-ethyl-3-(3-dimethylaminopropyl)carbo-diimide-hydrochloride and 50 mM N-hydroxysuccinimide. TorSN (4.7 μM) was injected during 6 min (5 μl/min) and the reaction was further stopped with 50 μl of 1 M ethanolamine hydrochloride, resulting in ∼4500 resonance units of immobilized protein. TorT at various concentrations, in 20 mM phosphate buffer (pH 7.4) containing 10% glycerol, was injected during 2.5 min (10 μl/min). The running buffer used was 20 mM phosphate buffer (pH 7.4) containing 100 mM NaCl. When indicated, TorT was first preincubated for 5 min with 0.1 or 1 mM TMAO prior to the injection. As a control, the same experiments were carried out without immobilizing protein on the sensor chip. Analysis of the sensogram, after subtraction of the control, was carried out with the evaluation software (BIAevaluaiton 2.1, BLAcore) to calculate the kinetic constants of the complex formation. The kinetic data were interpreted on the basis of the simple binding model A + B ⇌ AB (one analyte A reacts with a single site on the immobilized ligand B). The apparent equilibrium dissociation constant K_D was calculated from the ratio k_{off}/k_{on}.

Bioinformatical Procedures—Amino acid sequence of TorT from E. coli was used to perform a PSI-Blast on the non-redundant protein databases via the Infobiogen server. Multiple alignment was performed using ClustalW on the Infobiogen server.

RESULTS

Biochemical Evidence of the Formation of a TorT-TMAO Complex—TMAO is the inducer of the torCAD structural operon and this induction requires the TorS/TorR two-component system and the TorT periplasmic protein. We hypothesized that TMAO could be directly detected either by the TorS

sensor via its N-terminal region or by the periplasmic TorT protein. In the latter case, we assumed that the phosphorelay could then be activated by the interaction of the TorT-TMAO complex with the TorS sensor. To determine which of the TorT or TorS proteins binds TMAO, the TorT protein and the periplasmic domain of TorS (hereafter called TorS_p) were overproduced and purified. These two proteins were His-tagged at their C-terminal extremity to facilitate the purification procedure. The purified proteins were then subjected to limited proteolytic digestion by the endoprotease GluC in the presence or absence of TMAO. The samples were subsequently separated by SDS-PAGE.

When the TorT protein was incubated with GluC in the absence of TMAO, it was progressively degraded (Fig. 1A). The amount of the entire protein diminished and several proteolytic products appeared. After 1 h of incubation, the band corresponding to intact TorT had almost disappeared. In contrast, when the TorT protein was incubated with 10 mM TMAO prior to the addition of GluC, it was subjected to less degradation as after 1 h of incubation a large amount of the entire protein was still present (Fig. 1A). Moreover, in the presence of TMAO, the digestion pattern of TorT remained unchanged from 2 to 30 min of incubation with GluC. This result shows that TorT

![Endoproteinase GluC digestion of TorT (A) and TorS_p (B) in the presence or absence of TMAO. Purified TorT (7.4 μM) or TorS_p (7.8 μM) were subjected to proteolysis with endoproteinase GluC at a protein/enzyme mass ratio of 4/1 for TorT or 20/1 for TorS_p in the presence or absence of 10 mM TMAO. Times of incubation are indicated above the gel. A, digestion products were separated by SDS-PAGE and stained with Coomassie Blue. Intact TorT was also run as a control (left lane). The position of TorT protein is indicated by an arrow. B, digestion products were separated by SDS-PAGE, transferred to nitrocellulose, and visualized using antibodies raised against TorS. Intact TorS_p was also run as a control (left lane). The position of the TorS_p polypeptide is indicated by an arrow.](image-url)
becomes resistant to endoproteinase GluC digestion in the presence of TMAO. This strongly suggests that TorT binds TMAO and that this binding modifies the conformation of TorT, allowing TorT to resist proteolysis.

In contrast, TorS
N exhibits the same sensitivity to GluC with or without TMAO. Indeed, the TorS
N polypeptide was already cleaved into several products and the full-length polypeptide was almost undetectable after 10 min of incubation with GluC, with or without TMAO (Fig. 1B). When TorS
N was incubated for 1 h with GluC, it was entirely degraded whether TMAO was present or not prior to the addition of GluC. The fact that TMAO did not modify the patterns of TorS
N proteolysis suggests that TorS does not detect TMAO directly (Fig. 1B).

**Genetic Evidence That TorT Is a TMAO-binding Protein**—If TorT is a TMAO-binding protein, it should be possible to isolate TorT mutant proteins unable to bind TMAO and therefore to activate the tor operon expression. We performed random mutagenesis of the torT gene by successive rounds of PCR amplification. The mutated PCR products were cloned under the control of the ptac promoter and the resulting plasmids were introduced into strain LC8982, which is deleted of the torT gene and contains a torA′−lacZ fusion. Transformants were screened on MacConkey/lactose medium containing TMAO (see “Experimental Procedures”). Several negative mutants were isolated. One of these mutants was further characterized. Sequencing indicated that a T was substituted for a C in position 80 of the protein (hereafter called TorTL80P). To confirm that the TorTL80P mutant of a leucine by a proline in position 80 of the protein in position 239 in the torT operon expression, we measured the β-galactosidase activities of strain LC8982 containing the pJF-TL80P plasmid in anaerobiosis in the presence or absence of TMAO. As shown in Fig. 2A, no significant β-galactosidase activity could be detected in strain LC8982 containing either the pJF119EH vector or the pJF-TL80P plasmid whether TMAO was present or not. In contrast, the lacZ fusion of strain LC8982 was normally induced in the presence of TMAO when a wild type copy of torT was provided in trans whether the encoded TorT protein contained a C-terminal His
6 tag (pJF-Tp) or not (pCJ6) (Fig. 2A). This result confirms that TorTL80P is unable to induce tor operon expression in the presence of TMAO.

To test the ability of TorTL80P to bind TMAO, the matured protein was overproduced and subsequently purified. The purified TorTL80P protein was then subjected to limited proteolytic digestion by the endoproteinase GluC. The sample was separated by SDS-PAGE and stained with Coomassie Blue. Intact TorT protein contained a C-terminal His
6 tag (pJF-Tp) or not (pCJ6) (Fig. 2B). This indicates that TMAO at 10 mM has no effect on the overall secondary structure of the protein. An estimate of 35% α-helical content in TorT was calculated from these spectra, in agreement with the 37% of α-helix predicted from the primary sequence of TorT.

The CD spectra of the TorT protein at 25 °C in the presence or absence of 10 mM TMAO were essentially superimposable (Fig. 3A). This indicates that TMAO at 10 mM has no effect on the overall secondary structure of the protein. An estimate of 35% α-helical content in TorT was calculated from these spectra, in agreement with the 37% of α-helix predicted from the primary sequence of TorT.

Temperature-induced CD changes of TorT were then monitored at 222 nm and a modification of the CD spectrum of TorT was observed when 10 mM TMAO was added. Indeed, the protein was more stable in the presence of TMAO as indicated by the increase of T
m (Fig. 3B). Values of T
m were extracted from the melting curves by fitting the data to the equation reported by Tan et al. (30). T
m values are 57 ± 1.02 °C and 62 ± 1.5 °C in the presence and absence of 10 mM TMAO, respectively.

Moreover the binding of TMAO to TorT was measured by ITC (Fig. 4). This binding was slightly exothermic and the K
D found to be 150 ± 10 μM. The same experiment was performed with ribose as a control. Ribose showed no detectable binding (data not shown). Using the same approach, we confirmed that the TorTL80P mutant was unable to bind TMAO (data not shown). Altogether, the significant modification of
the temperature-induced CD spectrum of TorT by TMAO and ITC results confirm that TorT is a TMAO-binding protein.

**TorT and TorSN Form a Complex and Addition of TMAO Leads to a TorSN Conformational Change**—Our results show that TMAO signal transduction starts by the binding of TMAO to TorT and we propose that the TorT-TMAO complex then activates the TorS sensor by interacting with its periplasmic detector region. The interaction between TorSN and TorT was first tested by cross-linking experiments. The TorSN (34 kDa) and TorT (36 kDa) proteins were incubated with the DSG cross-linker. The samples were then separated by SDS-PAGE and the proteins were revealed by Coomassie Blue staining. As shown in Fig. 5A, when TorSN was incubated in the presence of DSG, a band appeared. The estimated molecular mass of this band is in agreement with the formation of a dimer of TorSN. When TorSN and TorT were incubated together in the presence of DSG, a band was observed in addition to the band corresponding to the TorSN dimer. The estimated molecular mass fits well with the formation of a TorSN-TorT complex. In fact, the presence of TorSN and TorT in this band was confirmed by mass spectrometry. The TorSN and TorT proteins were then incubated together with the DSG cross-linker in the presence of TMAO. Surprisingly, the addition of TMAO led to the same pattern, meaning that TMAO did not affect the formation of the TorSN-TorT complex in vitro (Fig. 5B). These results indicate that TorSN can interact with TorT alone or in complex with TMAO.

The interaction between TorSN and TorT was then confirmed by native gel electrophoresis. The TorSN and TorT proteins were incubated together or alone in the presence or absence of TMAO. The samples were then analyzed by native-PAGE and the proteins were revealed by Coomassie Blue staining. As expected, the TorSN polypeptide seemed to be unaffected by the presence of TMAO as the migration pattern was the same in the presence or absence of TMAO (Fig. 6). Unfortunately, the TorT protein could not be detected in these conditions, because of its high pl (>9), it remained in the well. Nevertheless, when the TorSN and TorT proteins were incubated together prior to electrophoresis, the band corresponding to the TorSN polypeptide faded and a new band appeared that may correspond to a complex between TorSN and TorT (Fig. 6). The presence of TorSN and TorT in this band was confirmed by mass spectrometry. This complex was observed whether the proteins were incubated in the presence of TMAO or not. These results not only confirm the interaction between TorSN and TorT, but also indicate that these proteins interact with each other in the absence as well as in the presence of TMAO. Similar experiments were performed with various protein concentrations. The formation of the complex was again observed irrespective of the presence of TMAO (data not shown). This result indicates that the affinity of TorT for TorS is not affected by the presence of TMAO.

To determine the kinetic constants of the TorSN-TorT complex formation, we performed biosensor experiments (surface plasmon resonance). For this purpose, purified TorSN polypeptide was coupled to the dextran matrix of a sensor chip, and TorT was injected into the TorSN-containing sensor chip. The sensogram reflected the association and dissociation of the
two proteins, confirming that TorT interacts with TorSN (data not shown). The association \(k_{\text{on}}\) and dissociation \(k_{\text{off}}\) rate constants of TorT to TorSN were determined at three different concentrations of TorT (Table 2). This allowed calculation of an average value for the apparent equilibrium dissociation constant \(K_D\) of 8.2 nM (Table 2). Moreover, in our conditions, preincubation of TorT with TMAO prior to the injection did not significantly change the kinetic constants (Table 2). These results clearly demonstrate that TorSN interacts strongly with TorT alone or in complex with TMAO.

To determine whether TMAO induces a cascade of conformational changes from TorT to TorSN when it binds to TorT, we carried out limited proteolytic digestions with GluC of the TorSN polypeptide incubated with TorT in the presence or absence of TMAO. The samples were then separated by SDS-PAGE and the TorSN digestion products were revealed by anti-TorS antibodies after membrane blotting. Comparison of the proteolytic products of TorSN incubated with TorT in the presence or absence of TMAO shows that after 2 min of digestion, the patterns were similar in the absence and presence of TMAO but that after 10–60 min of digestion, the patterns were clearly different (Fig. 7). The major difference was the persistence of uncleaved TorSN in the experiment performed in the presence of TMAO, whereas the TorSN amount rapidly decreased in the experiment performed in the absence of TMAO. These results show that binding of the TorT-TMAO complex to TorSN increases its resistance to GluC digestion and we therefore propose that TMAO indirectly induces a conformational change of TorS when it binds to TorT.

In addition, when we compared the patterns of TorSN proteolysis after GluC digestion, we observed that the TorSN polypeptide was totally degraded within 1 h in the absence of TorT (Fig. 1B), whereas in the presence of TorT significant
Role of TMAO-binding Protein TorT

![Graph: GluC-digestion time (min) vs. TorS\(_N\) for TMAO and + TMAO](image)

amounts of proteolytic products of the TorS\(_N\) polypeptide were still detected after 1 h of digestion by GluC (Fig. 7). Therefore the addition of the TorT protein even in the absence of TMAO leads to a slower degradation of the TorS\(_N\) polypeptide by GluC. This partial protection of TorS\(_N\) by TorT confirms that TorT interacts with TorS even in the absence of TMAO. In conclusion, we propose that TMAO binding to TorT induces a cascade of conformational changes from TorT to TorS.

TorT Belongs to a New Family of PBPs—We have previously observed that the TorT protein shares weak homologies with the RbsB ribose-binding protein within restricted regions (13). Because TorT is clearly not a sugar-binding protein, we wondered if TorT could belong to a new family of PBPs. A search to identify homologues of the \(E.\ coli\) K12 TorT protein (TorT\(_{E.\ coli}\)) revealed 44 proteins of unknown function in addition to TorT\(_{E.\ coli}\) (see supplemental materials Table 3) and RbsB was not found among these proteins. This result indicates that TorT belongs to a new family different from that of the sugar-binding proteins. All the proteins identified are found in proteobacteria. The sizes of those proteins are similar and range from 329 to 365 amino acids, except for three of them. The TorT proteins from \(E.\ coli\), \(Shigella\), and \(Salmonella\) strains show between 66 and 99% identity with TorT\(_{E.\ coli}\); whereas the remaining 30 proteins share between 29 and 41% identity with TorT\(_{E.\ coli}\). As shown in Fig. 8, conserved amino acids are found throughout the entire protein and a conserved motif is present in the N-terminal region. Interestingly, the residue that is mutated in TorT\(_{E.\ coli}\) is located in this region. This result strongly suggests that TorT belongs to a new family of PBP and that the proteins of this family bind TMAO or related compounds.

DISCUSSION

Although the periplasmic TorT protein was shown to be strictly required for tor operon induction, its precise role has not been deciphered so far. Due to sequence homologies, TorT was thought to be a periplasmic binding protein. In this study we identified TMAO as a ligand of the TorT protein and clearly established the role of this novel PBP in signal transduction. We showed in particular that TMAO modifies the temperature-induced CD spectrum of the TorT protein (Fig. 3). In the presence of TMAO, the \(T_m\) is increased, a phenomenon that was also observed in the case of the \(\alpha\)-glucose/\(\alpha\)-galactose-binding protein of \(E.\ coli\) when the protein was incubated with 10 mM glucose (31). TMAO binding therefore leads to a tertiary structural modification of TorT. Moreover we showed that TMAO protects TorT from digestion by the GluC endoproteinase (Fig. 1A). This effect is highly specific because TMAO protects neither TorS\(_N\) (Fig. 1B) nor bovine serum albumin used as a control (data not shown). Moreover a mutated TorT protein is no longer protected from GluC digestion in the presence of TMAO (Fig. 2B). These results confirm not only that TMAO binds to TorT but also that TMAO binding to TorT induces a conformational modification of TorT thereby increasing its resistance to proteolysis \textit{in vitro}. Therefore, as already observed for several PBPs, TorT undergoes a conformational change upon binding to its dedicated substrate. Despite their rather diverse sequences, the PBPs seem to share a conserved common structure with two globular domains linked by an hinge region (16–19). An hypothesis would be that TorT has a similar fold and that TMAO binding to TorT occurs in the cleft between those two domains promoting their closing, as observed for other PBPs.

The ITC experiments not only confirmed that TorT binds TMAO, but also revealed a \(K_D\) of \(150 \pm 10\ \mu M\) for TMAO binding (Fig. 4). It is interesting to note that this binding constant, and the apparent Michaelis constant of TMAO for TorA, the TMAO reductase terminal enzyme (\(K_m = 70\ \mu M\)), are in the same range (32). Indeed it makes sense that expression of the Tor respiratory system occurs only when enough TMAO is present in the medium.

Transmembrane sensors often utilize an extracytoplasmic domain to sense external stimuli either directly or indirectly. The periplasmic detector region of the sensors PhoQ of \(Salmonella\) \textit{typhimurium}, CiaT of \(Klebsiella\) \textit{pneumoniae}, and DcuS of \(E.\ coli\) directly binds \(Mg^{2+}\), citrate, and fumarate, respectively (33–35). Here we showed that the periplasmic domain of the TorS sensor detects indirectly the presence of TMAO by binding the TorT-TMAO complex. Interaction of the TorS detector region (TorS\(_N\)) with the TorT protein was established by different biochemical experiments: (i) TorS\(_N\) became partially protected from digestion by GluC in the presence of TorT indicating that TorS\(_N\) interacts with TorT (Fig. 7); (ii) when TorS\(_N\) and TorT were incubated in the presence of a chemical cross-linker, a complex of the two proteins was observed (Fig. 5); (iii) the TorS\(_N\)-TorT complex was also visualized after migration of the mixed proteins onto native PAGE (Fig. 6); (iv) a strong interaction between TorS\(_N\) and TorT was detected by biosensor experiments (Table 2). In the last three experiments, the TorS\(_N\)-TorT complex was detected whether TMAO was present or not. Therefore TMAO does not seem to promote the formation of the TorS\(_N\)-TorT complex but rather induces a conformational change of TorT that is subsequently transduced to TorS\(_N\). Indeed, when TorS\(_N\) was subjected to digestion by GluC in the presence of TorT, addition of TMAO clearly improved the resistance of TorS\(_N\) to proteolysis. An
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Other PBPs proved to be involved in signal transduction via two-component regulatory systems by directly contacting their sensor partners. Two different mechanisms were first described: in the first case, the ligand-loaded PBP interacts with the sensor and activates it; whereas, in the second, the unliganded PBP controls and inhibits the sensor. In *A. tumefaciens*, the VirA/VirG two-component system induces expression of the *vir* genes in the presence of certain plant phenols and sugars. The sugars act via a periplasmic protein called ChvE. Genetic evidence suggests that the ChvE-sugar complex binds to the VirA sensor, which transmits the signal to the VirG response regulator (21). In *B. pertussis*, the periplasmic BctC protein binds and interacts with the BctE sensor (23). Upon binding of the BctC-citrate complex, BctE autophosphorylates and then transphosphorylates its partner BctD, leading to induction of the *bct* operon. In contrast to TorT, unliganded BctC protein does not interact with its sensor partner BctE and citrate loading is necessary for BctC to interact with BctE. In *V. furnissii*, regulation of the chitinolytic genes involves the periplasmic CBP protein and the ChiS sensor. It has been proposed that, in the absence of the sugar ligand, CBP interacts with the ChiS sensor, locking it into an inactive conformation (22). Genetic evidence indicates that chitin disaccharide or oligosaccharide dissociates the complex by binding to CBP, releasing ChiS and leading to the expression of chitinolytic genes. In this case, binding of the unliganded PBP to its sensor partner inhibits kinase activity, whereas the ligand-loaded PBP no longer interacts with the sensor, a situation again different from that of the TorT/TorS system.

In *V. harveyi*, the periplasmic LuxP protein is involved together with the LuxQ/LuxU/LuxO phosphorelay system in the regulation of quorum-sensing communication. It was recently shown that LuxP associates with the LuxQ hybrid sensor in both the presence or absence of its ligand, autoinducer-2. In the absence of autoinducer-2, LuxQ is active as a kinase, whereas in its presence, LuxQ acts as a phosphatase (24). Interestingly, the Tor system exemplifies another regulatory mecha-
anism. Indeed, although both TorT and LuxP interact with their sensor partner whether their ligand is present or not, binding of the ligand to the PBP has an opposite effect on the sensor: binding of TMAO to TorT activates TorS as a kinase, whereas binding of autoinducer-2 to LuxP renders LuxQ active as a phosphatase. Therefore our study reveals a new regulatory mechanism and illustrates the diversity of signal detection by two-component systems. In fact, signal detection by the Tor regulatory system is much more complex than previously thought, not only because TMAO detection is indirect, but also because immature cytochrome TorC inhibits the kinase activity of TorS by binding to TorSN (28, 36). TorSN can thus interact with both TorT and apoTorC. The effect of apoTorC binding on TorSN-TorC complex is under investigation.

In this study, we identified several homologues of the TorT protein in different proteobacteria. Finding TorT homologues in E. coli, Shigella flexneri, S. typhimurium, Vibrio cholerae, and Shewanella oneidensis was not surprising because these bacteria possess a functional Tor system (37–42). However, depending on the organism, the genetic organization of the tor locus is different. In E. coli, Shigella, Salmonella, and Shewanella, all tor genes are clustered, whereas in Vibrio, the tor genes are scattered onto the chromosome by pairs, namely torT/torS, torD/torR, and torC/torA. Interestingly, the localization of the genes encoding the four TorT homologues found, respectively, in Bradyrhizobium, Marinomonas, Rhodoferax ferriferducens, and Silicibacter is completely different. In these four bacteria, the torT-like gene is located immediately upstream of two genes encoding an ATP-binding protein and a membrane-bound protein of a putative ABC-type transporter. We therefore hypothesize that these four TorT-like proteins are the third component of an ABC-type transporter and that the transported substrate is TMAO or a related compound. A TMAO-specific transporter was proposed to exist in the bacterium Aminobacter aminovorans but the components of this transporter were not identified (43). In E. coli, TMAO transport has not yet been investigated because the TMAO reductase (TorA) is located in the periplasm. However, this question deserves to be addressed because TMAO is not solely an electron acceptor but also a powerful chemical chaperone stabilizing proteins in stressful conditions (44, 45).

In conclusion, we propose that TorT belongs to a new family of periplasmic binding proteins. We hypothesize that the proteins of this family can bind TMAO or related compounds and can be involved in signal transduction and perhaps transport.

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