Electron Transfer and Binding of the c-Type Cytochrome TorC to the Trimethylamine N-Oxide Reductase in Escherichia coli*

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Stéphanie Gon‡, Marie-Thérèse Giudici-Orticoni§, Vincent Méjean‡, and Chantal Iobbi-Nivol‡†

From the §Laboratoire de Chimie Bactérienne and ‡Laboratoire de Bioénergétique et Ingénierie des Protéines, Institut de Biologie Structurale et Microbiologie, Centre National de la Recherche Scientifique, 31 chemin Joseph Aiguier, BP 71, 13402 Marseille Cedex 20, France

Reduction of trimethylamine N-oxide ($E'_o$\textsubscript{TMAO/TMAO}) = +130 mV) in Escherichia coli is carried out by the Tor system, an electron transfer chain encoded by the torCAD operon and made up of the periplasmic terminal reductase TorA and the membrane-anchored penta-hem c-type cytochrome TorC. Although the role of TorA in the reduction of trimethylamine N-oxide (TMAO) has been clearly established, no direct evidence for TorC involvement has been presented. TorC belongs to the NirT/NapC c-type cytochrome family based on homologies of its N-terminal tetrahemic domain (TorC\textsubscript{N}) to the cytochromes of this family, but TorC contains a C-terminal extension (TorC\textsubscript{C}) with an additional heme-binding site. In this study, we show that both domains are required for the anaerobic bacterial growth with TMAO. The intact TorC protein and its two domains, TorC\textsubscript{N} and TorC\textsubscript{C}, were produced independently and purified for a biochemical characterization. The reduced form of TorC exhibited visible absorption maxima at 552, 523, and 417 nm. Mediated redox potentiometry of the heme centers of the purified components identified two negative midpoint potentials (−177 and −98 mV) localized in the tetrahemic TorC\textsubscript{N} and one positive midpoint potential (+120 mV) in the monohemic TorC\textsubscript{C}. In agreement with these values, the in vitro reconstitution of electron transfer between TorC, TorC\textsubscript{N}, or TorC\textsubscript{C} and TorA showed that only TorC and TorC\textsubscript{C} were capable of electron transfer to TorA. Surprisingly, interaction studies revealed that only TorC and TorC\textsubscript{N} strongly bind TorA. Therefore, TorC\textsubscript{C} directly transfers electrons to TorA, whereas TorC\textsubscript{N}, which probably receives electrons from the menaquinone pool, is involved in both the electron transfer to TorC\textsubscript{C} and the binding to TorA.

Bacterial respiration allows cells to grow in a changing environment (1). Under anaerobic conditions, several bacteria can use diverse electron acceptors such as nitrate, Me\textsubscript{2}SO or trimethylamine N-oxide (TMAO)\textsuperscript{1} (2, 3) for the oxidation of organic substrates. To reduce TMAO, Escherichia coli synthesizes two homologous systems, but one, the TorCAD system, is strongly induced by TMAO (4, 5), whereas the other, TorYZ, is expressed at a low level (6). Each of these systems comprises a periplasmic terminal reductase and a membrane-anchored c-type cytochrome. The membrane-bound Me\textsubscript{2}SO reductase, which is constitutively expressed in anaerobiosis, is also capable of TMAO reduction (7).

The TorCAD system is encoded by the torCAD operon, and its expression is under the control of the TorS/TorR two-component regulatory system, which mediates the response to the presence of TMAO in the medium (8). The torA gene encodes the periplasmic molybdenoreductase TorA (5). According to sequence homologies, TorA belongs to the Me\textsubscript{2}SO reductase family, a group of periplasmic enzymes that contain a molybdenum cofactor as a single prosthetic group and are capable of utilizing TMAO and/or Me\textsubscript{2}SO as electron acceptors (9). In the past few years, structures have been published for several members of this family (10–13). They are organized in four domains surrounding a bismolybdopterin guanine dinucleotide cofactor that is consequently buried in a deep depression of the protein surface. The insertion of the molybdenum cofactor into the TorA apoprotein is a cytoplasmic event (14) that probably involves the TorD cytoplasmic chaperone (15).

It was previously shown that the first gene of the tor operon, torC, encodes protein TorC, a 46-kDa pentahem c-type cytochrome (5, 16). TorC is anchored to the inner membrane by a sequence of about 20 hydrophobic residues, while a globular domain containing five hemes faces the periplasm. Heme binding to the five consensus CXCH motifs takes place in the periplasm by a mechanism involving the c-type cytochrome maturation machinery encoded by the ccm genes (17, 18). From sequence comparisons, TorC has been included in the NirT/NapC class of membrane-anchored multiheme c-type cytochromes. This family originally was composed of tetrahem c-type cytochromes of about 20 kDa that were involved in periplasmic nitrite and nitrate reduction (19). The N-terminal region of TorC, which is homologous to NirT and NapC, possesses the four heme binding sites, whereas the C-terminal part, which is present specifically in the Me\textsubscript{2}SO/TMAO respiratory systems, carries the fifth heme motif (5). Recently, two members of the NirT/NapC family, NapC and the TorC homologue DorC, have been characterized, and the redox potential values determined for the four hemes of NapC and the five hemes of DorC were all negative (20, 21).

In TMAO and Me\textsubscript{2}SO reduction systems, pentahem cytochromes proved to be essential for the electron transfer and thus appeared to be the intermediate components between the membrane quinone pool and the terminal reductase (6, 22). Since it has been shown in E. coli that TMAO reduction involves menaquinones (23), TorC should receive the electrons from the menaquinone pool ($E'_o$\textsubscript{quinoquinol/menaxdin}) = −74 mV) and transfer them to the active site of TorA, where TMAO reduction ($E'_o$\textsubscript{TMAO/TMAO}) = +130 mV) occurs.

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† To whom correspondence should be addressed. Tel.: 33-4-91-16-44-27; Fax: 33-4-91-71-89-14; E-mail: iobbi@ibsm.cnrs-mrs.fr.

¶ The abbreviations used are: TMAO, trimethylamine N-oxide; TMA, trimethylamine; PAGE, polyacrylamide gel electrophoresis.

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Interestingly, a recent genetic study showed that TorC in its unprocessed form is a negative regulator of the torCAD operon (18). Indeed, apo-TorC seems to inhibit the kinase activity of the TMAO sensor TorS by an unknown mechanism. This negative autoregulation probably means that the maturation of TorC is the limiting step for the Tor system biogenesis.

This paper provides in vivo and in vitro evidence that the mature form of TorC is directly involved in the electron transfer to the terminal reductase, TorA. We also show that TorC exhibits four negative redox potentials and a positive one corresponding to the fifth heme. The latter group is responsible for the terminal reductase, TorA. We also show that TorC binds TorA, and this interaction involves the C-terminal His6 tag and the complementary sequence of the 3’ coding region (Cct) that corresponds to a SmaI site followed by a His6 tag coding sequence inserted into pBAD24 (27).

**Plasmids**
- pJP119EH: Vector containing the P_tac promoter
- pBAD24: Vector containing the P_tac promoter
- pEC86: ccm ABCDEFGH inserted into pACYC184
- pTorAD: torAD coding sequence inserted into pJP119EH
- pTorADC: torC coding sequence inserted into pBAD24
- pTorADCN: torC coding sequence inserted into pTorAD
- pBC: torC coding sequence inserted into pBAD24
- pBCN: First half of torC coding sequence inserted into pBAD24
- pBCGC: Second half of torC coding sequence inserted into pBAD24

**Constructions Leading to TorC, TorCN, and TorCC Production**
To synthesize the periplasmic domain of TorC (TorCN), positions 1–194 relative to TorC) that includes the first four heme binding sites of TorC (Fig. 1B), the corresponding coding sequence was amplified by polymerase chain reaction using oligonucleotides C5Hc and a primer that corresponds to a SmaI site followed by a His6 tag coding sequence and the appropriate torC internal sequence.

To synthesize the periplasmic C-terminal domain of TorC (TorCC, from position 198 to 390 that contains the fifth heme of TorC (Fig. 1C), the corresponding torC coding sequence was amplified by polymerase chain reaction using an oligonucleotide C5Hc and a primer that corresponds to a SmaI site followed by a His6 tag coding sequence and the appropriate torC internal sequence.

To synthesize the periplasmic C-terminal domain of TorC (TorCC, from position 198 to 390 that contains the fifth heme of TorC (Fig. 1C), the corresponding torC coding sequence was amplified by polymerase chain reaction using an oligonucleotide C5Hc and a primer that corresponds to a SmaI site followed by a His6 tag coding sequence and the appropriate torC internal sequence.

**Analytical Procedure**
Protein analysis was carried out using 12.5% or 10% SDS-PAGE. After electrophoresis, the presence of hemes within TorC and derivatives was revealed by staining the gel for peroxidase activity using 3,3’-diaminobenzidine as described by Thomas et al. (31); then Coomassie Blue staining of total proteins was performed (32). Protein concentrations were estimated using the technique of Lowry et al. (33).

**Spectroscopy and Mediated Redox Potentiometry**
Optical difference spectra and redox titration were performed on a Kontron Uvikon 932 UV-visible spectrophotometer. The UV-visible spectra of TorC, TorCN, and TorCC were carried out at 16 °C in 20 mM phosphate buffer (pH 7.4) with Triton X-100 0.05%. Optical redox titrations of TorC (5 μM) and TorCC (10 μM) were performed according to Dutton (34), and samples were kept at 20 °C under argon atmosphere. The following redox mediators were present at 5 μM: 1,4-benzo-

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**Table I**

| Strains or plasmids | Genotypes and/or characteristics | References or sources |
|---------------------|---------------------------------|-----------------------|
| E. coli MC4100 | araD139 ΔlacIPOZYA-argF1 U169 rpsL thi | M. J. Casadaban |
| DSS401 | MC4100 Δadsns Km’ | Ref. 25 |
| LC5B04 | MC4100 torC2 : φ1 Spc’ Δadsns Km’ | Ref. 6 |

Plasmids
- pJP119EH: Vector containing the P_tac promoter
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- pTorAD: torAD coding sequence inserted into pJP119EH
- pTorADC: torC coding sequence inserted into pBAD24
- pTorADCN: torC coding sequence inserted into pTorAD
- pBC: torC coding sequence inserted into pBAD24
- pBCN: First half of torC coding sequence inserted into pBAD24
- pBCGC: Second half of torC coding sequence inserted into pBAD24

**Plasmids pBC, pBCN, and pBCGC were digested with NheI and HindIII, and the resulting DNA fragments encoding the modified His tag TorC proteins were cloned just downstream of torD, into the compatible sites XbaI and HindIII of plasmid pTorAD (15), leading to plasmids pTorADC, pTorADCN, and pTorADC, respectively.**
quione \( (E^0 = +280 \text{ mV}) \), DCIP \( (E^0 = +217 \text{ mV}) \), 2,5-dimethoxybenzozquinone \( (E^0 = +180 \text{ mV}) \), 1,2-naphtoquinone \( (E^0 = +145 \text{ mV}) \), 1,4-naphtoquinone \( (E^0 = +60 \text{ mV}) \), duroquinone \( (E^0 = +5 \text{ mV}) \), 2-methyl, 1,4-naphtoquinone \( (E^0 = 0 \text{ mV}) \), pyocyanine \( (E^0 = -34 \text{ mV}) \), 2,5-dihydroxybenzoquinone \( (E^0 = -60 \text{ mV}) \), indigocarmine \( (E^0 = -125 \text{ mV}) \), 1,4-dihydroxynaphtoquinone \( (E^0 = -145 \text{ mV}) \), antraquinone 2-sulfonate \( (E^0 = -225 \text{ mV}) \), safranine \( (E^0 = -289 \text{ mV}) \), and neutral red \( (E^0 = -325 \text{ mV}) \). All potentials quoted are with respect to the normal hydrogen electrode. The absorbance change at 552 nm was plotted against redox potential, and theoretical Nernstian curves were fitted to the data using Sigma Plot.

**Kinetics of Electron Transfer**

The kinetic assays were achieved using anaerobic cuvettes, typically filled with phosphate buffer \( (20 \text{ mM}, \text{pH } 7.4) \), TMAO \( (50 \text{ mM}) \), and either TorC \( (10 \mu \text{M}) \), TorC \( (5 \mu \text{M}) \), or TorC \( (5 \mu \text{M}) \) previously reduced by dithionite. Before adding TMAO reductase \( (350 \text{ units} \cdot \text{ml}^{-1}) \), the cuvette mixture was flushed with argon \( (10 \text{ min}) \). The oxidation rate of the cytochrome was determined from the absorption band at 552 nm.

**Interaction Experiments**

**Interaction Studies on PAGE under Native Conditions**—TorA \( (5 \mu \text{M}) \) and either TorC \( (5 \mu \text{M}) \), TorC \( (5 \mu \text{M}) \), or TorC \( (10 \mu \text{M}) \) were incubated for 30 min at room temperature in 20 mM phosphate buffer \( (\text{pH } 7.4) \), Triton X-100 \( 0.05\% \). The interactions were analyzed by 10 or 12.5% PAGE for 30 min at room temperature in 20 mM phosphate buffer \( (\text{pH } 7.4) \), Triton X-100 \( 0.05\% \). For identification, the proteins were stained for heme with 3,3'-Triton X-100 0.05%. The interactions were analyzed by 10 or 12.5% PAGE for 30 min at room temperature in 20 mM phosphate buffer \( (\text{pH } 7.4) \), Triton X-100 0.05%. The interactions were analyzed by 10 or 12.5% PAGE for 30 min at room temperature in 20 mM phosphate buffer \( (\text{pH } 7.4) \), Triton X-100 0.05%.

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**RESULTS**

**The Intact TorC Protein Is Required for TMAO Respiration**—To establish that the c-type cytochrome TorC is required for electron transfer to the terminal enzyme TorA, we cloned the torAD genes alone \( (15) \) or together with torC under the control of the \( F_{\text{PAC}} \) promoter of the plasmid pJF119. The resulting pTorAD and pTorADC plasmids were introduced into strain LCB504, and the growth rate was monitored. The recombinant strains containing pTorAD did not grow significantly in the presence of TMAO \( (\text{Fig. } 2) \), and the cells containing pTorADC grew very slowly only after 36 h of incubation. These results strongly suggest that both domains of the TorC protein are required for the TMAO respiratory process.

**Overproduction and Purification of TorC and of Its N- and C-Terminal Domains**—To study the biochemical characteristics of the TorC protein, we constructed recombinant plasmids that encode either TorC; TorC \( (\text{Fig. } 1) \), which corresponds to the N-terminal domain of TorC; or TorCN, which corresponds to the second half of the TorC protein \( (\text{Fig. } 1) \). DNA fragments encoding TorC, TorCN, and TorCC were cloned into the pBAD24 vector, giving rise to plasmids pBC, pBCCN, and pBCC with His tags added at the C-terminal extremity of each protein. Moreover, since the heme incorporation occurs in the periplasm, we introduced the TorC signal peptide in front of TorCC to assure its periplasmic localization.

**Overproduction of mature c-type cytochrome, and especially multiheme c-type cytochromes, is difficult in \( E. coli \), due to the limited capacity for synthesis of the c-type cytochrome of this organism. To overcome this problem, we used a strain containing the plasmid pEC86 that carries the c-type cytochrome maturation \( ccm \) genes and thus improves production of c-type cytochromes \( (17) \). Nevertheless, the amount of mature cytochrome produced is rather low even in the presence of this plasmid, and the expression of cytochrome-encoding genes has to be controlled to prevent saturation of the maturation machinery. Accordingly, the TorC genes were cloned into the pBAD24 vector under the control of the \( F_{\text{PBD}} \) promoter for controlled expression of TorC proteins. Plasmids pBC, pBCCN, and pBCC were then introduced into strain MC4100 carrying the compatible plasmid pEC86. TorC and TorCC expression was induced with 0.0005% arabinose because concentrations higher than 0.001% apparently overloaded the heme delivery machinery and led to degraded proteins. Higher levels of the mature TorCC protein were obtained by using 0.1% arabinose, probably because TorCC is a monoheme protein. Heme-staining proteins of 47 and 24 kDa were present in membrane extracts of the strains containing pBC and pBCC, respectively, indicating that TorC and TorCN were matured (data not shown). Since these proteins were exclusively located in the membrane fraction (data not shown), detergent Triton X-100 was used to

**Fig. 1. Schematic diagram of proteins TorC, TorCN, and TorCC.** Representation of the membrane-anchored TorC (A), its N-terminal domain, TorCN (B), and the soluble form of its C-terminal domain, TorCC (C). The signal peptide is removed in the mature protein. Black circles and gray squares correspond to the position of the heme binding sites and of the His tag, respectively.
solvabilize them. A 23-kDa heme-staining protein was detected in the periplasmic extract of the strain containing pBC C (data not shown). The detergent-solubilized fractions containing TorC or TorC N or the periplasmic fraction containing TorC C were loaded onto Ni$^{2+}$-columns, and the His-tagged proteins were eluted from the column at about 150 mM imidazole. Analysis by SDS-PAGE revealed the presence of TorC, TorC N, and TorC C as a single major band on gels stained for heme-dependent peroxidase activity (Fig. 3A). No other c-type cytochrome copurified with the proteins and no apparent degradation occurred. The same gels stained with Coomassie Blue (Fig. 3B) revealed one major band; moreover, this band cross-reacted with anti-His antibodies in Western blots (data not shown), confirming that His-tagged derivatives of TorC had been purified.

**Characterization of TorC by Optical Spectroscopy Shows That TorC Exhibited Positive and Negative Heme Redox Potentials**—The UV-visible spectrum of purified TorC shows a Soret peak at 411 nm in the oxidized state and peaks at 417 (Soret), 411 (Soret), 404, and 385 nm (Q bands) in the reduced state (Fig. 4). These peaks are characteristic of c-type cytochromes and confirm that TorC is a c-type cytochrome as predicted by the presence of c-type heme binding sites (CXXCH) in the amino acid sequence. TorC was totally reduced by dithionite, but it was only partially reduced by ascorbate (Fig. 4A). These results indicate that at least one of the TorC hemes exhibits a positive redox potential, whereas the remaining hemes display more negative redox potentials. Similar UV-visible spectra were obtained with membrane extracts from strain DSS401 containing native TorC after reduction with ascorbate or dithionite (Fig. 4B), indicating that the purified His tag TorC cytochrome has spectral properties similar to those of the unmodified native TorC protein.

To determine which domain of TorC contains a positive redox potential center, we carried out UV-visible spectra with the purified TorC N and TorC C domains (Fig. 4, C and D). Fig. 4C shows that the hemes of TorC C are totally reduced by dithionite but not by ascorbate. In contrast, Fig. 4D shows that the unique heme of TorC C is totally reduced by both ascorbate and dithionite. These findings strongly suggest that the fifth heme of TorC has a positive potential, whereas the first four hemes exhibit negative potentials.

**Determination of the Redox Potentials of the TorC Protein**—To define more precisely the heme potentials, a mediated redox potentiometry was carried out on the purified TorC protein. This analysis revealed three distinct redox type centers, one at −177 mV (two hemes), one at −98 mV (two hemes), and one at +114 mV (one heme) (Table II). These potentials are in agreement with the previous spectral evidence for one positive heme potential in the TorC N domain and four negative heme potentials in the TorC C domain. Mediated redox potentiometry of TorC C (Table II) yielded a value of +120 mV, in complete agreement with the spectral evidence for a positive heme in the TorC C domain of TorC (Fig. 4D) and with the heme potential values obtained with the intact TorC protein.

**Transfer of Electrons from TorC to the TMAO Reductase TorA**—In the model of the Tor electron transfer pathway, TorC transfers electrons from the menaquinones, which are embedded in the cytoplasmic membrane, to the periplasmic TMAO reductase TorA. To demonstrate directly that TorC can act as an electron shuttle from the inner membrane to TorA, we studied electron transfer between purified TorC and TorA. TorC was previously reduced by dithionite under anaerobic conditions (Fig. 4A). Oxidation of TorC, followed at 552 nm (Fig. 5) was specifically dependent upon TMAO reductase, since no oxidation occurred before the addition of TorA (data not shown).

Based on the redox potentials of TorC C (−177 and −98 mV) and TorC C (+120 mV) and on $E'_{\text{S/O^2MADO/TMAO}}$ (+130 mV), we hypothesized that electrons are transferred from TorC C to TorC C and from TorC C to TorA. In this model, TorC C is the direct electron donor to TorA. Purified TorC N and TorC C were tested as electron donors for TMAO reductase. The monoheme TorC C domain was oxidized by the addition of TorA, whereas the tetraheme TorC N domain was not (Fig. 5). Although oxidation of TorC N was slower than that of TorC, these results indicate that the electron donor for TorA is most probably located in the C-terminal part of TorC. When a mixture of purified TorC N and TorC C was tested, the electron transfer was slightly higher than that of TorC C alone but slower than that of TorC. These results indicate that TorC N also plays a role in the electron transfer pathway.

**Binding of TorC to TorA Mainly Involves the TorC N-terminal Domain**—The above results with purified components imply a direct interaction between these proteins. To characterize the binding between TorC and TorA, the formation of a TorC–TorA complex was studied under nondenaturing conditions by native PAGE and by BLAcore experiments. TorA and TorC were mixed together and loaded in the same conditions onto two polyacrylamide gels. After electrophoresis under native conditions, one gel was stained for heme (Fig. 6A, lanes 1 and 2), and a Western blot with anti-His antibodies was performed.
with the second gel (Fig. 6A, lanes 3 and 4). Significantly, the migration of TorC was retarded in the presence of TorA (Fig. 6A, lanes 1 and 3 with lanes 2 and 4). This result suggested that TorC can bind to TorA. To confirm this result, we studied the interaction between TorA and TorC by using the BIAcore technique (surface plasmon resonance). For this purpose, purified TorA protein was coupled to the dextran matrix of a sensor chip, and TorC was injected into the TorA-containing sensor chip. The sensorgram reflected the association and dissociation of two proteins, indicating that TorC directly interacts with TorA (Fig. 7A) in agreement with the above result.

The interaction of the individual domains with TorA was also studied by the same two approaches. Surprisingly, no binding between TorCN and TorA was detected on a native gel stained for heme or by Western blot with anti-His antibodies. Indeed, whatever the conditions used, TorC migrated at exactly the same position in the presence and in the absence of TorA (Fig. 6B, compare lanes 1 and 3 with lanes 2 and 4). Similarly, no binding between TorCN and TorA was detected by the BIAcore procedure (Fig. 7B). As a control, we coupled TorCN to the dextran matrix and injected TorA, and again, no formation of a complex corresponding to the association of TorCN and TorA was detected (data not shown). When TorCN and TorA were mixed and loaded onto a polyacrylamide gel under non-denaturing conditions, TorCN migration was retarded by the presence of TorA (Fig. 6C), suggesting that the N-terminal domain of TorC is sufficient for TorA binding. The fact that TorCN can bind to TorA was confirmed by using the BIAcore technique. Indeed, an increase in the amount of recovered resonance units was observed when the TorCN protein was

Table II

| Midpoint potentials of the | Contribution | Midpoint potential of the |
|---------------------------|--------------|---------------------------|
| pentahemic TorC protein   | %            | monohemic TorCN protein   |
| mM                        |              | mM                        |
| +114 ± 10                 | 10           | +120 ± 20                 |
| -98 ± 8                   | 40           |                           |
| -177 ± 9                  | 50           |                           |

Fig. 4. UV-visible spectra of purified TorC (A) and membranous extracts containing TorC (B), purified TorCN (C), and TorCC (D). Visible spectrum of the different cytochromes are shown, in the oxidized state (solid lines) and reduced by ascorbate (dashed lines) or dithionite (dotted lines).

Fig. 5. Oxidation kinetics of purified TorC, TorCN, and TorCC by TorA. Decrease in the normalized absorbance of the $\alpha$-band (552 nm) of TorC (5 $\mu$m), TorCN (5 $\mu$m), or TorCC (10 $\mu$m) in 20 mM phosphate buffer (pH 7.4), 0.05% Triton X-100, 50 mM TMAO, and 45 units of TorA.

Fig. 6. Analysis of interactions between TorA and TorC (A), TorCC (B), or TorCN (C) by PAGE under native conditions. Cytochrome $c$ alone (lanes 1 and 3) or mixed with TorA (lanes 2 and 4) was loaded onto polyacrylamide gels. After the electrophoresis, the interactions between proteins were checked by staining the gels for heme (lanes 1 and 2) or by a Western blot with anti-His antibodies (lanes 3 and 4). The arrows indicate retarded migration of TorC or TorCN in the presence of TorA. A, 5 $\mu$m TorC alone (lanes 1 and 3) or mixed with 5 $\mu$m TorA (lanes 2 and 4); B, 10 $\mu$m TorCC alone (lanes 1 and 3) or mixed with 5 $\mu$m TorA (lanes 2 and 4); C, 5 $\mu$m TorCN alone (lanes 1 and 3) or mixed with 5 $\mu$m TorA (lanes 2 and 4).
FIG. 7. Sensorgrams of interactions between immobilized TorA and TorC (A), TorCN (B), or TorCC, (C) proteins. TorC (5 μM), TorCN (5 μM), and TorCC (5 μM) were injected (50 μl) into a sensor chip with dextran matrix coupled either to TorA (a) or to no protein as a control (b).

The results clearly show that the TorCN domain interacts with TorA. The association rate constants ($k_{on}$) and the dissociation rate constant ($k_{off}$) of TorC and TorCN to TorA were determined at four different concentrations between 2.5 and 10 μM (data not shown). TorCN exhibited a $K_d$ value of 4.5·$10^{-8}$ M (Table III). Significantly, the sensorgram of intact TorC binding to TorA exhibits at least two steps leading to the determination of two constants, $K_{d1} = 1.7·10^{-8}$ M and $K_{d2} = 3.0·10^{-8}$ M (Table III). These two steps could correspond to the fixation of TorC N and TorC C, but TorCN is mainly responsible for the binding of intact TorC to TorA because TorC C alone does not bind TorA. The low affinity between TorC N and TorA could also explain the poor catalytic efficiency in electron transfer to TorA exhibited by TorCN compared with that of the intact TorC protein (Fig. 5).

DISCUSSION

This study has revealed that TorC is made up of two domains of similar size, corresponding to its N- and C-terminal half (TorC N and TorC C, respectively) (Figs. 1 and 3). As the other members of the NirT/NapC family with which it shares sequence homologies, TorC N contains a membrane anchor segment followed by four heme-binding sites (5, 20). Production of the isolated TorC N domain resulted in a stable mature protein. Unfortunately, and in contrast to what was described previously for NapC (20), the removal of the membrane anchor of TorC and TorCN led to an unstable protein (data not shown). The second domain, TorC C, contains the fifth heme-binding motif of TorC (Fig. 1C) and presents sequence homologies with the corresponding region of pentahemic cytochromes specifically involved in TMAO/Me2SO respiration of various bacteria (6, 35). Although the fifth heme-binding site is located at the C-terminal extremity (positions 329–333 relative to TorC), we were unable to produce a stable protein starting from position 285 of TorC, i.e., just upstream from the fifth heme-binding site (data not shown). The region located between the fourth and the fifth heme-binding sites probably plays an essential role in the stability and/or the folding of the TorC C domain.

The comparison between the midpoint redox potential values obtained for the soluble form of the Paracoccus denitrificans NapC (n = 1, –235, –207, –181, and –56 mV) (20) and TorC C, the N-terminal domain of TorC (n = 2, –177 and –98 mV) (Table II), highlights that they are all negative and in the same range. This result confirms that NapC and TorC C are highly related. It is also striking that, for TorCN, each of the midpoint redox potentials corresponds to two heme centers. This feature could be representative of a symmetry in the N-terminal domain of TorC. This hypothesis agrees with the previous proposal of Roldan et al. (20), that the tetrahemic domain of NirT/NapC c-type cytochromes comes from a gene duplication leading to two related diheme subdomains. If true, then the redox potential of the first and third hemes of TorC or that of the second and the fourth ones should be very similar.

The midpoint redox potential described by Shaw et al. (21) for purified DorC, a TorC pentahemic homologue, is in the same range of values (–276, –185, –184, –128 mV, and –34 mV) as those detailed above for NapC. Surprisingly, all the potentials exhibited by DorC are negative, while a positive one has been detected for TorC. Indeed, we have clearly shown that the redox potential of the fifth heme is about +120 mV, and the presence of a positive heme center has been observed in both the isolated TorC C domain and the intact TorC protein (Table II). Moreover, this result has been confirmed by a study performed directly on native TorC using membrane extracts (Fig. 4B). These results are also in agreement with the fact that ascorbate reduced partially TorC and TorC C completely (Fig. 4). The midpoint redox potentials are often associated to His-Met ligated hemes (36). The comparison of the sequences surrounding the fifth heme-binding site of the pentahemicytochrome family reveals the presence of one highly conserved residue of methionine (position 353 in the TorC amino acid sequence) (35). This residue is a good candidate for the axial ligand, although a conserved histidine residue (position 340) might also play such a role.

As shown by the in vitro electron transfer experiments, the
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TorC\(_C\) domain donates the electrons directly to TorA (Fig. 5). This finding fits well with the positive midpoint redox potential exhibited by the TorC\(_C\) heme (\(E^\circ_{0} = +120 \text{ mV}\)) and that of the reduction reaction of TMAO (\(E^\circ_{0,TMAO/TMA} = +130 \text{ mV}\)). The rate of electron transfer is slightly but significantly enhanced by the presence of the tetrahemic domain TorC\(_N\) in the reconstitution system. The enhanced rate in the presence of TorC\(_N\) probably means that TorC\(_N\) transfers the electrons to TorC\(_C\), since TorC\(_N\) alone cannot directly feed TorA with electrons (Fig. 5). This result, together with the fact that the presence of both TorC\(_N\) and TorC\(_C\) domains are required for bacterial growth with TMAO as a sole exogenous electron acceptor (Fig. 2), support a model in which TorC\(_N\) receives the electrons from the menaquinone pool and then transfers them to TorC\(_C\), which gives them to TorA. In the Nir and Nap respiratory systems, the TorC\(_N\) homologues (NirT and NapC) and their associated diheme cytochromes (NirB and NapB, respectively) constitute distinct proteins (19, 37). In the case of TorC, the TorC\(_N\) tetrahemic domain is fused to the TorC\(_C\) monohemic domain, and it is clear from our experiments that the intact TorC protein is more efficient than a mixture of the two isolated domains.

Although the simplest model of interaction between TorC and TorA would have been that TorC\(_C\) binds TorA, since it transfers the electrons to TorA, we have shown by two different approaches that TorC\(_C\) does not significantly bind TorA, whereas TorC\(_N\) binds TorA efficiently (Figs. 6 and 7). The apparent equilibrium dissociation constant obtained by the analysis of the BIACore data is similar for TorC\(_N\) and TorC\(_C\), although a second \(K_d\) with a higher value can also be calculated for TorC (Table III). This latter might correspond to a weak interaction between TorC\(_N\) and TorA that takes place after the TorC\(_N\)-TorA binding, allowing the electron transfer. Based on these findings, we proposed a model in which the tetrahemic domain of TorC transfers the electrons to TorC\(_N\) and binds TorA in such a way that TorC\(_N\) is correctly positioned to transfer them to TorA (Fig. 8). This may also explain why TorC\(_N\) and TorC\(_C\) are fused. Finally, the binding of the TorC\(_N\) domain to TorA raises the question of a possible binding of TorC\(_N\) and also of the tetrahemoproteins of TorC to their associated terminal reductase.

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