Twin-arginine-dependent translocases transport folded proteins across bacterial, archaeal, and chloroplast membranes. Upon substrate binding, they assemble from hexahelical TatC and single-spanning TatA and TatB membrane proteins. Although structural and functional details of individual Tat subunits have been reported previously, the sequence and dynamics of Tat translocase assembly remain to be determined. Employing the zero-space cross-linker N,N′-dicyclohexylcarbodiimide (DCCD) in combination with LC-MS/MS, we identified as yet unknown intra- and intermolecular contact sites of TatB and TatC. In addition to their established intramembrane binding sites, both proteins were thus found to contact each other through the soluble N terminus of TatB and the interhelical linker region around the conserved glutamyl residue Glu⁴⁹ of TatB from *Escherichia coli*. Functional analyses suggested that by interacting with the TatC N terminus, TatB improves the formation of a proficient substrate recognition site of TatC. The Glu⁴⁹ region of TatB was found also to contact distinct downstream sites of a neighboring TatB molecule and to thereby mediate oligomerization of TatB within the TatBC receptor complex. Finally, we show that global DCCD-mediated cross-linking of TatB and TatC in membrane vesicles or, alternatively, creating covalently linked TatC oligomers prevents TatA from occupying a position close to the TatBC-bound substrate. Collectively, our results are consistent with a circular arrangement of the TatB and TatC units within the TatBC receptor complex and with TatA entering the interior TatBC-binding cavity through lateral gates between TatBC protomers.

Twin-arginine-dependent translocation denotes the transport of folded proteins across the cytoplasmic membranes of bacteria and archaea and the thylakoidal membrane of plant chloroplasts. Substrate proteins of the twin-arginine translocation (Tat)³ pathway possess a conserved SRRXFLK sequence motif in their signal sequences. Although the name-giving RR motif is largely indispensable for Tat-specific recognition, other determinants of Tat substrates, such as the hydrophobicity of their signal peptides (1, 2) and sequence characteristics immediately downstream of the signal peptide (3), are required for a proficient interaction with the Tat translocase.

Tat translocases do not seem to preexist in the membrane but rather assemble upon contact with their substrates (4, 5). They assemble from two types of membrane proteins, TatC and one or several members of the TatA protein family. TatC consists of six helices that are tilted and mostly kinked, thus forming the concave structure of a cupped hand or baseball glove (6, 7). Two of the six transmembrane helices (TM)s are too short to span the membrane entirely, invoking local membrane perturbations. Similarly short single TM s are encountered at the N termini of all TatA-type proteins. According to NMR structures, the TM s are each followed by a short rigid hinge region and an amphipathic helix (APH) that is predicted to be 50% embedded in the bilayer (8–12). Many Tat translocases involve at least two isoforms of TatA, mostly termed TatA and TatB. TatA and TatB differ in the length of their C-terminal tails distal of the APH. Those tails are largely unstructured but, in the case of TatB, encompass two more helices (11) that are most likely involved in binding folded substrate proteins (3, 13). A broad range of bacterial Tat translocases involve another homolog of TatA, called TatE (14), that is shorter than TatA and TatB. Although TatE shares a high sequence identity with TatA, it represents an autonomous TatA-type subunit (14, 15). TatA-type proteins bind via overlapping binding sites to the hexahelical TatC (14, 16–20), leading to the formation of heterooligomeric Tat complexes. The TatB-binding sites of TatC known to date suggest that TatB intercalates between neighboring TatC monomers, thereby giving rise to circular TatBC complexes (17, 18) with the TM s of TatB associating...
with each other in the center of the complex (17). These TatBC arrays are reinforced by distinct TatC–TatC contacts (16, 17, 19). The TatBC oligomers provide a deep binding cavity for RR-containing signal sequences (17, 21–23) and thus allow their hairpin-like insertion into the plane of the membrane (3, 24, 25). Thus, TatB and TatC jointly function as the substrate receptor of Tat translocases.

TatC provides a specific recognition site for the RR-consensus motif that is found in the N-terminal part of Tat signal sequences, whereas all TatA-type proteins interact with downstream areas of Tat signal sequences (1, 2, 7, 15–17, 26–29). TatA, which seems to be expressed at superstoichiometric levels compared with the other Tat subunits (30–32), is unique in that its association with the TatBC complex and with an RR-signal sequence requires the proton-motive force (PMF) at the membrane (17, 33, 34). Circumstantial evidence suggested that the N terminus of TatA could destabilize the lipid bilayer (12, 35, 36), which was recently demonstrated to in fact occur in response to a bound substrate (37). This and a PMF-dependent homooligomerization of TatA (38–40) could be the triggers for the subsequent translocation of folded substrate proteins. Although Tat translocases can transport linear peptide sequences (41, 42), still unknown mechanisms link proficient transport to the folding degree of the substrate proteins (43–46). TatE shares functional properties with both TatA and TatB (5, 14, 15, 47, 48). Recent data suggest that it is part of the Tat substrate receptor complex and might play a role in the oligomerization of TatA (14).

Although the structures of individual Tat subunits have been solved (see above), current ideas about the possible conformation(s) of a substrate-bound TatABC translocase largely rest on data obtained by biochemical, genetic, and bioinformatics analyses. For a recent model of how the transmembrane parts of TatA, TatB, and TatC might assemble, see to Blümmel et al. (49). In that report, we introduced N,N′-dicyclohexylcarbodiimide (DCCD) as a new tool to identify contact sites of the Tat subunits. DCCD is a zero-space cross-linker forming isopeptide bonds between carboxyl and amino group-containing side chains of proteins (50). By means of a quantitative mass spectrometric analysis of DCCD-treated TatC, we identified distinct intramolecular contact sites of TatC, suggesting that the N-terminal and C-terminal tails of the molecule can tightly pack against the transmembrane core of TatC (49). Here, we have extended this approach onto the entire TatBC complex based on the premise that novel TatB–TatB and TatB–TatC contacts, if revealed by treatment with DCCD, might allow new insights into the composition and functioning of TatBC complexes.

Results

DCCD-dependent cross-linking reveals a novel TatBC contact site on the membrane surface

When Escherichia coli membrane vesicles were treated with DCCD and the membrane proteins were subsequently analyzed by SDS-PAGE and immunoblotting, anti-TatB and anti-TatC antibodies both recognized an about 50-kDa protein (Fig. 1A, black dot) in addition to the ~28-kDa TatB and the ~23-kDa TatC antigens. (Note that migration of the hydrophobic Tat proteins on SDS gels deviates from their actual molecular masses.) This 50-kDa TatBC-containing protein complex was strictly DCCD-dependent (compare lanes 1 and 2, and 3 and 4, respectively), suggesting that DCCD might be able to cross-link TatB and TatC to form a 1:1 complex. DCCD treatment of E. coli membrane vesicles yielded another prominent species of about 55 kDa (Fig. 1A, lane 2, green star), which, different from the 50-kDa product, was recognized only by anti-TatB antibodies. The 55-kDa product is therefore likely to represent a homodimer of TatB formed by DCCD.

Next, we purified the TatBC complex from membrane vesicles via a His tag on TatC, which allows the copurification of TatB when membrane vesicles are solubilized with N-lauroyl sarcosine (Fig. 1B, left lane). If the vesicles were treated with DCCD prior to solubilization, SDS-PAGE revealed three distinguishable 40–55-kDa species co-purifying with the TatB and TatC monomers (Fig. 1B, Dimers 1–3). By size and immunone-
Impact of a conserved glutamate of TatB

Figure 2. DCCD-sensitive carboxyl side chains of E. coli TatB. A, NMR structure of the first 101 residues of E. coli TatB (Protein Data Bank code 2MJO) (11). Highlighted are the TM and APH helices and helices 3 and 4 (x3 and α4) as well as residues addressed in this study. The membrane boundaries were sketched according to the NMR data (11). B, sequence coverage and sites of modification by DCCD of E. coli TatB determined by in-gel digestion of monomeric TatB using trypsin and chymotrypsin followed by LC-MS/MS analysis. For each amino acid of TatB from 14 to 171, MS intensities of peptides containing this residue were summed up and plotted against its sequence residue number. Relative quantification of peptides was performed based on detected ion intensities. Cumulative intensities of peptides identified with the modified sites (diamonds on vertical lines) according to the same logarithmic scale as the total sum of intensities including modified and unmodified peptides. Potential cleavage sites of trypsin and chymotrypsin (black) as well as actual cleavage sites observed in DCCD-treated (blue) and nontreated (green) TatB are indicated in the lower graph. C, vesicles were prepared from Δtat E. coli strains overexpressing TatABC, TatAB3A,4E9A, C, TatAB3A,4E9A, and TatAC. They were treated with either DCCD or DMSO before incubating with the fluorescent DCCD analog NCD-4. Proteins were separated by SDS-PAGE and analyzed under UV light. The gel was run without molecular mass standards because they could not be visualized using this technique of detection. The identity of the TatB band is indicated by its appearance being affected when using distinct TatB variants. Labeling of TatC by NCD-4 has been addressed previously (49). D, Western blot analysis using anti-TatB and anti-TatC antibodies to control for expression levels.

activity, these bands correspond to TatB dimers (J), 1:1 TatBC complexes (2), and TatC dimers (3). To determine the nature of these DCCD-dependent dimers, we subjected all bands marked by arrows in Fig. 1B to proteolytic digestion using trypsin alone or in combination with chymotrypsin and analyzed the resulting peptide mixtures by LC-tandem MS (LC-MS/MS).

According to the NMR structure of E. coli TatB (Fig. 2A), its first 100 amino acids adopt a mostly α-helical conformation in membranes and membrane-mimetic environments, such that an APH is rigidly packed against the preceding short TM and is followed by the more flexibly arranged helices α3 and α4 (11). Downstream of this helical region of TatB, the remaining about 70 amino acids are predicted to be largely unstructured. As illustrated in Fig. 2B, the peptides that we identified by LC-MS/MS from the monomeric TatB band shown in Fig. 1B covered almost the entire amino acid sequence of TatB, except for the N-terminal 13 residues including the major part of the TM of TatB. Consistent with the helical part of TatB being exposed to a hydrophobic environment, the lipophilic compound DCCD modified predominantly carboxyl side chains located in that stretch of E. coli TatB. Thus, numerous peptides carrying the dicyclohexylurea (DCU) moiety at either an aspartyl or glutamyl side chain were obtained from DCCD-treated TatB, starting with Glu49 (Fig. 2B). These peptides are highlighted by the blue vertical bars at the positions of the DCU-modified residues, with the lengths of the bars reflecting the relative abundances of detection. TatB3A,4E9A is a conserved residue located in the linker region between APH and helix α3 (Fig. 2A). Its modification by DCCD occurred with one of the highest frequencies (Fig. 2B).

Residues Asp3 and Glu8 of E. coli TatB are the only negatively charged amino acids upstream of Glu49. Because residues 1–13 were not detected in our LC-MS/MS analyses, we addressed the susceptibility of TatB333 and TatBE8A to a modification by DCCD using labeling of membrane vesicles with the fluorescent analog of DCCD, N-cyclohexyl-N’-(4-dimethylamino-α-naphthyl)carbodiimide (NCD-4). As shown in Fig. 2C, NCD-4 stained WT TatB if not antagonized by a 10-fold molar excess of unlaabeled DCCD (lanes 1 and 2). Substitution of Glu99 by alanine (TatB3A,4E9A) still allowed labeling with NCD-4 (lane 3), which is consistent with the numerous DCCD target sites of TatB depicted in Fig. 2B. However, if Glu8 had also been exchanged against alanine, staining of this TatB3A,4E9A variant with NCD-4 in general was found to be decreased compared with WT TatB (lane 7). This was not due to a lack of expression of the tatBE8A,E9A double mutant (Fig. 2D, lane 4), although the double mutation led to an aberrant running behavior on SDS gels. These results might therefore hint toward the conserved Glu8 of TatB also being a target of DCCD, although we could not rigorously confirm this by other experimentation.

Next we searched for those DCCD-sensitive carboxyl side chains of TatB that DCCD would cross-link through amide bonds to free amino groups of nearby TatC residues, thereby generating the TatBC heterodimers shown in Fig. 1A. To this end, we screened the entire LC-MS/MS data sets derived from all bands highlighted in Fig. 1B (arrows) for spectra matching branched peptides composed of TatB and TatC peptide sequences. Because the transmembrane helix of TatB, which has been established as a hot spot for intramembrane contacts at the C-terminal domain of TatC (Fig. 3B), all lysyl residues of E. coli TatC high-
in an otherwise Cys-free TatABC background. When *E. coli* membrane vesicles carrying these variants of TatB and TatC were treated with either the sulfhydryl-specific cross-linker bismaleimidoethane (BMOE) or the disulfide-forming oxidant sodium tetrathionate (NaTT), TatBC heterodimers of ~50 kDa were in fact produced, as shown by immunoblotting using antibodies against TatB and TatC (Fig. 4A, black dot). The fact that, compared with BMOE (spacer arm length 8 Å), the zero-space disulfide cross-linker NaTT was not considerably less efficient in generating TatBC heterodimers (compare lanes 3 and 4) would be a further indication of the close molecular proximity of TatBE49 and TatCS2 in the TatBC complex. Fig. 4B (left) illustrates how this novel TatBC contact site would fit into a model of how TatB molecules intercalate between two neighboring TatC monomers as a basis for the generation of circular TatBC structures (17, 18).

**Multiple intra- and intermolecular TatB contacts detected by DCCD-dependent cross-linking**

Cysteine cross-linking of TatBE49C/TatCS2C membrane vesicles, in addition to yielding TatBC heterodimers, also resulted in the formation of TatB homodimers (Fig. 4A, green star). To pursue this further, we employed vesicles that contained the TatBE49C single mutation. The addition of BMOE to these vesicles caused the same TatB dimer formation as observed for TatBE49C/TatCS2C vesicles (Fig. 4C, left, lanes 4 and 6, green star), whereas TatBC heterodimers were not obtained from the TatBE49 vesicles due to the missing TatCS2C alteration (Fig. 4C, both panels, lanes 4 and 6, black dot). Homodimerization of TatB in the TatBE49C/TatCS2C and the TatBE49C vesicles therefore occurs through disulfide bonds between the Glu49 residues of two closely spaced TatB monomers, similar to what had previously been reported for numerous TatB residues upstream of Glu49 when replaced by cysteines (52).

Disulfide bonding between identical residues of two TatB monomers invokes their parallel, side-by-side orientation. We reasoned that if this indeed were the case, DCCD might cause the formation of isopeptide bonds between closely spaced carboxyl and amino groups of two neighboring TatB polypeptide chains. We therefore screened the LC-MS/MS-derived peptide sequences. As summarized in Fig. 5A, an unexpectedly large number of branched peptides was obtained after digesting the monomeric and dimeric TatB bands shown in Fig. 1B with trypsin (for detailed information, see Table S1, positions 1–402). However, a merely superficial glance at the residues of TatB that became cross-linked by DCCD reveals that except for the Glu49/Lys239 and Glu53/Lys103 pairs (Fig. 5B, red lines) all other DCCD-catalyzed isopeptide bonds had formed between residues that are too distant to explain them by a parallel alignment of two TatB monomers.

In detail, over 80% of all DCCD-mediated TatB–TatB cross-links originated from the two glutamates Glu49 and Glu53 (Fig. 5A). In the vast majority of cases, these glutamates were found cross-linked to the two lysines Lys239 and Lys103 located at the distal end of helix α4 (Fig. 5B, thick blue lines). A smaller fraction of Glu49/Glu53-binding partners was represented by the more proximal lysines Lys55 and Lys93 within helix α3 of TatB.

### Figure 3. DCCD-mediated cross-links between TatB and TatC

**A**. The sites of *E. coli* TatB and TatC that were found cross-linked via DCCD. The data were extracted from Table S1. The terms “monomeric TatC” and “TatBC dimers” refer to Fig. 18. Frequency of identification refers to the number of cross-linked peptides identified by LC-MS/MS analyses (Table S1). 

**B**. Cross-linking of TatBE49C/TatCS2C membrane vesicles due to the missing TatCS2C alteration (Fig. 4C, both panels, lanes 4 and 6, black dot). Homodimerization of TatB in the TatBE49C/TatCS2C and the TatBE49C vesicles therefore occurs through disulfide bonds between the Glu49 residues of two closely spaced TatB monomers, similar to what had previously been reported for numerous TatB residues upstream of Glu49 when replaced by cysteines (52). Disulfide bonding between identical residues of two TatB monomers invokes their parallel, side-by-side orientation. We reasoned that if this indeed were the case, DCCD might cause the formation of isopeptide bonds between closely spaced carboxyl and amino groups of two neighboring TatB polypeptide chains. We therefore screened the LC-MS/MS-derived peptide sequences. As summarized in Fig. 5A, an unexpectedly large number of branched peptides was obtained after digesting the monomeric and dimeric TatB bands shown in Fig. 1B with trypsin (for detailed information, see Table S1, positions 1–402). However, a merely superficial glance at the residues of TatB that became cross-linked by DCCD reveals that except for the Glu49/Lys239 and Glu53/Lys103 pairs (Fig. 5B, red lines) all other DCCD-catalyzed isopeptide bonds had formed between residues that are too distant to explain them by a parallel alignment of two TatB monomers.

### Table 3. DCCD-mediated cross-links between TatB and TatC

| Positions cross-linked and frequency of identification from Table S1 | Recovered from monomeric TatC | Recovered from TatBC dimers |
|---|---|---|
| E49 | 12 | + |
| K239 | 3 | + |
| E53 | 9 | + |
| K239 | 3 | + |
| E56 | 6 | + |
| S2 | 4 | + |
| E77 | 4 | + |
| K239 | 4 | + |
| D100 | 5 | + |

**Figure 3.** DCCD-mediated cross-links between TatB and TatC. A, listed are the sites of *E. coli* TatB and TatC that were found cross-linked via DCCD. The data were extracted from Table S1. The terms “monomeric TatC” and “TatBC dimers” refer to Fig. 18. Frequency of identification reflects the number of cross-linked peptides identified by LC-MS/MS analyses (Table S1). B, illustration of the TatBC cross-links given in A. The four helices of TatB (TM, APH, α3, and α4) and the six TMs of TatC are indicated. Red dots, positions of lysyl residues of TatC.
Clearly, all of these contacts would require either a permanent or temporarily folded conformation of the TatB polypeptide chain or, alternatively, a crossover arrangement of two TatB monomers. For example, if the linker region connecting helices $H_4$ and $H_5$ of TatB allowed both helices to pack against each other, the residues Lys$_{93}$/Lys$_{103}$ would come into close proximity to the Glu$_{49}$/Glu$_{53}$ area of TatB (Fig. 5C). In fact, the data collection of Fig. 5A reveals that one-third of the branched peptides, in which Glu$_{49}$ was found cross-linked to Lys$_{93}$/Lys$_{103}$, were recovered from the monomeric TatB band of Fig. 1B. Even more frequently, the branched peptides originating from cross-links between Glu$_{53}$ and Lys$_{93}$/Lys$_{103}$ were recovered from the TatB monomer than from the dimer (Fig. 5A). These intramolecular contacts demonstrate that helix $H_4$ of TatB can form hairpin structures with preceding parts of the molecule, as depicted in Fig. 5C, which would be consistent with the high mobility of helix 4 observed in previous NMR studies (11).

Despite this considerable number of intramolecular TatB cross-links obtained by treatment with DCCD, the majority of

Figure 4. TatB Glu$_{49}$ contacts both the N terminus of TatC and Lys$_{93}$ of a neighboring TatB molecule. A, membrane vesicles of E. coli containing TatA and the indicated cysteine variants of TatB and TatC in a Cys-less TatC background (TatABC ΔCys) were either mock-treated, alkylated using IAA, or incubated with the Cys cross-linkers BMOE and NaTT. Membrane proteins were resolved by SDS-PAGE and probed by immunoblotting using anti-TatB and anti-TatC antibodies. TatB-immunoreactive bands of the size of 3x TatB and TatB x TatC, which were obtained even in conditions that block the formation of disulfides (lane 1, DTT; lane 2, IAA), probably represent background material. The TatC-immunoreactive band labeled with a blue star corresponds in size to a dimer of TatC. Sizes of marker proteins (M) are given. B, models of how TatB intercalating between two TatC monomers promotes circular TatBC arrays. The suggested position of TatB is supported by the established contact between TatBE$_{49}$ and the N terminus of TatC (left; the RR-recognition site of TatC consisting of its N-terminal domain and TM 2/TM 3 loop lies behind the Glu$_{49}$-proximal end of the TatB APH). This position of TatB is also consistent with two neighboring TatB monomers interacting with each other via Glu$_{49}$ and Lys$_{93}$ (right). TatC is represented by its six numbered TMs, and TatB is represented only by its TM and APH (left) or by its four helices (right). C, as in A using vesicles with the indicated Cys variants of TatB and TatC. All lanes shown on either of the two blots are derived from a single SDS gel each. Two of the blotted lanes showing results of an additional mutant were excised between lanes 6 and 7. To visualize stained molecular mass standards, which had been run to the left of the lane labeled 1, the blot was first photographed before exposing it to the antibodies. This lane was aligned with the decorated blots.
the cross-links between Glu\textsuperscript{49} and Lys\textsuperscript{93}/Lys\textsuperscript{103} were, however, recovered from the dimer bands of Fig. 1B, as the data presented in Fig. 5A reveal. In other words, Glu\textsuperscript{49} predominantly formed intermolecular contacts with the Lys\textsuperscript{93} and Lys\textsuperscript{103} residues of a neighboring TatB monomer. These cross-links actually represent the largest fraction of all DCCD-mediated TatB–TatB contacts detected (Fig. 5A). They suggest that a considerable fraction of the TatB molecules copurifying with TatC adopt a nonparallel, branched orientation such that TatB monomers intersect at their Glu\textsuperscript{49} and Lys\textsuperscript{93}/Lys\textsuperscript{103} residues. If, for example, two TatB monomers became cross-linked via the side chains of Glu\textsuperscript{49} and Lys\textsuperscript{93}, both coupled monomers would still retain a free Lys\textsuperscript{93} and a free Glu\textsuperscript{49} residue, respectively, to form cross-links with two more adjacent TatB monomers. Continuation of this process would lead to TatB oligomers covalently linked through their Glu\textsuperscript{49}/Lys\textsuperscript{93} sites. To address such an oligomerization of TatB experimentally, we constructed a TatB variant carrying both the E49C and K93C alterations. If membrane vesicles obtained from this tatB double mutant were treated with the sulfhydryl-specific cross-linker BMOE, a considerable fraction of TatB-immunoreactive molecules displayed sizes much larger than that of a TatB dimer (Fig. 4C, left, lane 7). We therefore conclude that the major fraction of TatB molecules copurifying with TatC are arranged in an oligomeric manner such that their Glu\textsuperscript{49} residues are positioned in the immediate vicinity of the distal end of helix α4 of an adjacent TatB monomer (Fig. 4B, right). These novel intermolecular TatB contacts would thus support the idea of circular TatBC receptor structures, which has been put forward on the basis of distinct contact sites between TatB and TatC (17, 18), TatC and TatC (17), TatB and TatB (17), and TatB and substrate (13, 17).

**TatB improves the formation of a functional substrate-binding site on TatC**

The data presented thus far indicate that when TatB is bound to TatC, the Glu\textsuperscript{49}/Glu\textsuperscript{53} linker region of TatB is predominantly found in contact both with downstream regions of TatB as well as with the substrate-binding site of TatC. The latter is composed of the N-terminal domain and the juxtaposed TM 2/TM 3 loop of TatC (16, 27, 53). Therefore, the question arose of whether the negatively charged Glu\textsuperscript{49}/Glu\textsuperscript{53} linker region of
TatB was directly involved in substrate recognition and binding by the TatBC complex.

As demonstrated several times before (14, 17, 28), binding of a Tat substrate to the TatBC complex of membrane vesicles can be visualized by the use of Tat precursor proteins that carry the photoactivatable cross-linker \( p \)-benzoylphenylalanine (Bpa) in their signal sequences. Thus, Bpa engineered at position Phe14 in the RR-consensus motif of the model Tat precursor protein TorA-mCherry (TmC) cross-links to TatC of TatABC-containing vesicles if activated by UV light (Fig. 6A, lanes 1 and 2, blue star). In contrast, when Bpa is positioned at position Leu27 in the hydrophobic core of the signal sequence, it yields cross-links to TatB and TatA (lanes 7 and 8, green and pink stars). In vesicles that carried an alanine exchange of TatBE49, both Bpa-containing variants of TorA-mCherry showed an impaired association with TatC, TatB, and TatA (lanes 2, 4, 8, and 10; the differing sizes of the TatC-TmC adducts of lanes 2 and 4 are explained by a His tag on the TatC of the control vesicles). Only when Glu49 and Glu53 of TatB were simultaneously replaced by alanine did cross-linking to TatA, TatB, and TatC seem to be diminished (lanes 6 and 12). The failure to cross-link to TatB can be explained by the drastically reduced level of TatB that we repeatedly observed for vesicles carrying the TatBE49A/E53A variant (Fig. 6B, lane 3). Obviously, the simultaneous loss of both glutamyl residues Glu49 and Glu53 causes TatB to become unstable, most likely because of an impaired association with TatC and neighboring TatBs. In contrast to TatB, the level of TatC was not substantially altered in the TatBE49A/E53A vesicles (Fig. 6B, lane 3), and yet, precursor binding to TatC was strongly diminished (Fig. 6A, lanes 4 and 6). Whereas these cross-linking data do not support a direct involvement of the Glu49/Glu53 linker region of TatB in substrate binding, they nevertheless suggest that proficient precursor binding to TatC depends on the presence of TatB.

**Figure 6. Requirement of TatB for substrate recognition by TatC.** A, autoradiography. Two variants of the Tat precursor (\( p \)) protein TmC with Bpa incorporated at positions Phe14 and Leu27 were synthesized and radioactively labeled *in vitro*. Control membrane vesicles (TatABC ΔCys) and vesicles harboring the indicated alanine exchanges in TatB were added. Cross-linking was initiated by irradiation with UV light. Marked are adducts of \( p \)TorA-mCherry to TatA, TatB, and TatC. B, contents of TatB and TatC of the vesicles as determined by immunoblotting with anti-TatB and anti-TatC antibodies. C, as in A, comparing cross-linking of \( p \)TorA-mCherry to TatC, TatB, and TatA in the presence (TatABC) and absence (TatAC) of TatB. Control samples with no vesicles added are shown in lanes 1, 2, 7, and 8. D, TatC content of the vesicles used in C as determined by immunoblotting against TatC.
To directly demonstrate this, we compared binding of TorA-mCherry to membrane vesicles in the presence and absence of TatB (Fig. 6C). As expected, in vesicles harboring only TatA and TatC, cross-linking between the L27Bpa variant of TorAmCherry and TatB was missing (compare lanes 10 and 12). As reported previously (17), in the absence of TatB, Leu27 of TmC was found in proximity to TatC (lane 12). TatAC vesicles, however, showed an impaired cross-linking also of the F14Bpa variant of TmC to TatC (compare lanes 4 and 6), although their TatC content was even higher than that of TatABC vesicles (Fig. 5D). A possible explanation for this finding is that an efficient formation of the substrate recognition site from the two known epitopes of TatC, its N-terminal domain and the TM 2/3 loop, depends on the presence of TatB. Through juxtaposition to the Glu99/53 linker region of TatB, the per se flexible N-terminal domain of TatC (6, 7) might become more stably positioned next to the TM 2/3 loop.

**Lateral access of TatA to TatBC-bound substrate**

It is experimentally well-established (7, 17, 18, 20, 51) that TatB and TatC also contact each other within the lipid bilayer through the TM of TatB and the TM 5 of TatC. The latter is part of the substrate-binding cavity in the interior of the TatBC complex (17). Recent data suggest that upon substrate binding, the TM of TatB shifts its position from TM 5 of TatC to TM 6 and that this shift goes along with the reciprocal recruitment of TatA from the outside position on TM 6 to TM 5 of TatC (20). This position switching between TatB and TatA would require a movement of the TatA and TatB TMs in between two neighboring TatC monomers. We tried to address this experimentally by cross-linking the two residues, Phe69 and Ala133, of TatC that are predicted to flank the path of exchange of the TatA and TatB TMs (Fig. 7A). When these residues were each replaced by cysteines, the addition of the cross-linker BMOE caused a considerable formation of dimers, tetramers, and hexamers of TatC (Fig. 7B), providing evidence for the molecular proximity of Phe69 and Ala133 in the oligomeric TatBC complex. The simultaneous F69C and A133C substitutions of TatC allowed for a reduced, yet still substantial, translocation activity into membrane vesicles, as assayed by the amounts of proteinase K–resistant precursor (p) and mature form (m) of TatA-mCherry (lane 2) were drastically reduced when the ATP pool of the in vitro reaction was depleted by the addition of hexokinase in combination with glucose (lane 4) but could largely be restored by the addition of NADH (lane 6). In analogy, the PMF-sensitive contact between TatA and the signal peptide of TorA-mCherry L27-Bpa largely disappeared after ATP deple- tion (Fig. 7F, lanes 2 and 3, pink star) and could be re-installed by NADH (lane 4). If, however, under these PMF-generating conditions, DCCD was added, the precursor–TatA contact was not established (lane 5). Fig. S1 verifies the NADH-dependent generation of an H+-gradient in membrane vesicles treated with DCCD. Thus, DCCD prevents TatA from contacting the Tat substrate much like disulfide bridges between neighboring TatC molecules do (Fig. 7D). Collectively, these results strongly suggest that to contact the Tat substrate, TatA enters the TatBC-binding cavity through lateral gates between TatBC protomers.

**Discussion**

One major secret yet to be disclosed about the functioning of Tat translocases is the interplay between the apparently disparate TatC- and TatA-type proteins. Knowledge of their molecular interactions might be key to understanding how they cooperate in allowing folded proteins to cross cellular membranes. Although individual structures of TatC and TatA/TatB have been published, structural information on complexes formed from the Tat subunits is missing. This is particularly true for the association of TatB with TatC, both of which have long been known to concertedly form a substrate receptor complex.
Because TatA, which is required for the actual translocation of the substrate protein, is a structural homolog of TatB, it will bind to TatC in a similar way as TatB, although the consequence of this interaction would be translocation rather than substrate recognition and binding.

One approach of identifying contact sites between two proteins is chemical cross-linking. Here we have explored the potential of DCCD to form isopeptide bonds between adjacent proteins. In this way, we discovered novel contact sites between TatB and TatC that do not involve the transmembrane parts of both proteins but rather domains assumed to be attached to the membrane surface. The area around the conserved glutamyl residue Glu⁴⁹ of E. coli TatB was thus found in immediate proximity to the N- and C-terminal ends of TatC. TatB⁴⁹ being...
located in the linker region between the APH and helix 3 is likely to be positioned on the surface of the lipid bilayer. Its vicinity to either end of TatC is consistent with the previously described molecular proximity of the N-terminal and C-terminal domains of TatC to each other and to the TM 2/TM 3 loop on the cis-surface of the membrane (49).

Such a conformation of both terminal domains of TatC excludes the possibility that they could function as permanent connecting points between neighboring TatC monomers in the oligomeric TatBC complexes. Rather, these and previous studies revealed the trans-sided TM 2 residues Asp93 and Phe96 of E. coli TatC associating with the trans-sided TM 4 residue Ala123. These intermolecular TatC contacts are likely to reflect a side-by-side (i.e. head-to-tail) assembly of adjacent TatC monomers as the basis for circular TatC scaffolds of the TatBC receptor complex. Other sites in TatC were found to cause a face-to-face dimerization of TatC (17, 27, 51, 55), but it is not clear to what extent these dimers reflect functional units.

Besides the newly discovered contacts between TatB and TatC on the membrane surface, site-specific cross-linking and sequence co-evolution analysis had shown that both proteins associate with each other within the lipid bilayer through the TM of TatB and residues clustering on TM 5 and 6 and the intervening loop of TatC. Those studies also revealed contacts between TatB and TM 1 and 2 of TatC, which constitute the other flank of TatC opposite to TM 5/6. Thus, the TM of TatB is obviously intercalated between two adjacent TatC monomers, and the continuous packing of one TatB between two TatC monomers would favor the formation of circular TatBC complexes. The concept of circular TatBC complexes again is strongly supported by our novel finding of intermolecular TatB contacts being a prerequisite for the N-proximal end of a neighboring TatB monomer. In principle, an intermolecular contact might then still be possible between Glu49 of one TatB and Lys93 located at the N-proximal end of a neighboring TatB APH (cf. Fig. 4B). In fact, DCCD-caused Glu49–Lys93 isopeptide bonding was obtained (Fig. 5A), yet with dramatically lower frequency than that between Glu49 and Lys93. An alternative explanation for the DCCD-dependent intramolecular TatB cross-links therefore would be that they reflect the high flexibility of the C terminus of TatB revealed by NMR analysis. In theory, this flexibility could be linked to conformational changes of TatB during substrate binding.

Disulfide cross-linking of two TatB monomers as a consequence of replacing Glu49 by cysteines (this study) as well as multiple residues within the TM and APH of TatB (52) involves the existence of TatB subpopulations with an at least partially linear, registered alignment of two or even more TatB molecules. The DCCD-mediated cross-linking of two TatBs through Glu49/Lys55 and Glu90/Lys93 (cf. Fig. 5) suggests that a nearly parallel oligomerization of TatB seems to occur at some frequency even for TatC-associated TatB molecules. At this point, it is not clear whether such oligomers would populate the inside or the periphery of a circular TatB structure.

Early Bpa-dependent cross-linking studies had revealed the proximity between TatB and the N-terminal domain of TatC (16), which is part of the RR-signal peptide recognition site of TatC (27). We have now identified the Glu49/Thr53 linker region of TatB as a contact site for the N terminus of TatC, which in turn comes close to the TM 2/TM 3 loop of TatC (49) to constitute the RR-recognition site. Despite this molecular vicinity, we were not able to demonstrate a direct involvement of the Glu49/Thr53 linker region of TatB in the substrate recognition process. We found, however, that the interaction of the RR-signal peptide with its recognition site on TatC was disturbed in the absence of TatB. This could mean that the Glu49/Thr53 linker region of TatB functions as a clamp for the N terminus of TatC to secure its juxtaposition to the other RR-interacting epitope of TatC (i.e. the TM 2/TM 3 loop).

Habersetzer et al. (20) recently found that overproduction of substrate in whole E. coli cells caused the exchange of TatA for TatB at TM 5 of TatC and a corresponding positional switch of both proteins at TM 6 of TatC. This implies that TatA moves from a more peripheral position around the TM 6 toward the concave face of TatC, which was actually experimentally verified for the chloroplast Tat translocase (19). Our data lend further support to such a functional movement of TatA to the interior of circularly arranged TatC protomers. Thus, we found that contacts between TatA and substrate being a prerequisite for a TatA-mediated translocation event are impaired by locking the lateral entrance of TatA between neighboring TatC protomers. The same effect was achieved by DCCD, which we found to form numerous cross-links between TatB/TatC and TatB/TatB and thereby most likely prevents TatB from vacating the TatA-binding sites on TatC. At this point, however, we cannot exclude the possibility that DCCD, besides cross-linking TatB and TatC to each other, might also influence the con-
Impact of a conserved glutamate of TatB

tact between substrate and TatA that was reported previously (56).

Subsequent events following the association of TatA with substrate are still hypothetical. Further experimentation will have to address whether the circular arrangement of TatC persists and TatA oligomers form a translocation platform within an expanding TatC ring or whether TatC monomers undergo a substantial reorientation (20, 55) or whether a TatA pore laterally buds off from a circular TatB receptor (18).

Experimental procedures

Plasmids

Plasmids used in this study are listed in Table S2. Plasmid p8737 was used to introduce the Ala codon GCG into the tatB gene and plasmid pUNITATCC4 (52) to introduce the Cys codon TGT into the tatB and tatC genes according to the QuikChange site-directed mutagenesis kit protocol (Stratagene). All primers used in this study are listed in Table S3. Amber stop codon mutations in the gene encoding TorA-mCherry of plasmid pPJ3 have been described (17).

Membrane vesicles

Inside-out inner membrane vesicles (INV) were prepared as described (57) from E. coli strains BL21(DE3)* (Novagen) or BL21(DE3)ΔTat (kindly provided by B. Ize and T. Palmer) transformed with plasmid p8737 or pUNITATCC4 and derivatives thereof.

In vitro reactions

The RR-precursor proteins TorA-mCherry and pSufI were synthesized in vitro transcription/translation using plasmids pPJ3 and pEJ. Cell extracts used for the in vitro reactions were prepared by incubating the Ala codon GCG into the tatB gene and plasmid pUNITATCC4 (52) to introduce the Cys codon TGT into the tatB and tatC genes according to the QuikChange site-directed mutagenesis kit protocol (Stratagene). All primers used in this study are listed in Table S3. Amber stop codon mutations in the gene encoding TorA-mCherry of plasmid pPJ3 have been described (17).

Cross-linking using DCCD

To detect contacts between the Tat subunits using DCCD, 2 µl of each INV preparation (~100 A280 units/ml) were diluted with 47.5 µl of INV buffer (57). After adding either 0.5 mM DCCD or 0.5 µl of DMSO, INV were incubated for 10 min at 37 °C and 300 rpm. Proteins were precipitated with 5% TCA and resuspended in 40 µl of SDS-loading buffer. Aliquots of 20 µl were each analyzed by SDS-PAGE (10% gels) and Western blotting using either anti-TatB or anti-TatC antibodies.

Disulfide cross-linking

Two µl of each INV preparation (~100 A280 units/ml) were diluted with 98 µl of DTT-free INV buffer. Disulfide formation was initiated through the addition of either 0.1 mM BMOE or 1 mM NaTT. Control samples were mock-treated with either 20 mM DTT or 10 mM iodacetamide (IAA). Samples were incubated for 30 min at 25 °C. The BMOE reaction was stopped with 20 mM DTT for 5 min at 37 °C. For detecting contacts between the Tat subunits, the treated INV were subsequently precipitated with 5% TCA and resuspended in 42 µl of SDS-loading buffer in the case of NaTT without DTT. Aliquots of 20 µl were each analyzed by SDS-PAGE (10% gels) and Western blotting using either anti-TatB or anti-TatC antibodies. For use in in vitro assays, the BMOE-treated INV were centrifuged at 90 000 × g for 45 min, and the pellet was resuspended in 5 µl of INV buffer containing 20 mM DTT (14,000 rpm, 30 min).

Purification of DCCD-modified TatB and TatC

Purification of TatC and of TatC-associated TatB was performed as described (49).

LC-MS/MS

In-gel digestion of proteins was performed essentially as described (49). In brief, proteins in bands excised from SDS-polyacrylamide gels were destained and subjected to reduction and alkylation of cysteine residues followed by digestion overnight with either trypsin as the sole protease or in combination with chymotrypsin. Peptide mixtures of three biological replicates were analyzed by LC-MS/MS using an UltiMate 3000 RSLC nano coupled to a Q Exactive Plus (Thermo Fisher Scientific) mass spectrometer followed by peptide identification and quantification using the program MaxQuant and identification of cross-linked peptides using the program Link as described (49). DCU-modified peptides were identified as variable modification of aspartate or glutamate (+206.17830 Da) and a modification-specific neutral loss of −125.08406 Da. Cross-linked sites were visualized using the program xiNET (version 2.0) (59).

Identification of DCCD-binding sites

The binding of DCCD was detected directly by MS analysis (see above) or indirectly using the fluorescent DCCD analogue NCD-4 (Synchem) as described (49).

Acknowledgment—We thank Bettina Knapp for excellent technical assistance.

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