YcdB from *Escherichia coli* Reveals a Novel Class of Tat-dependently Translocated Hemoproteins

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The Tat (twin-arginine translocation) system of *Escherichia coli* serves to translocate folded proteins across the cytoplasmic membrane. The reasons established so far for the Tat dependence are cytoplasmic cofactor assembly and/or heterodimerization of the respective proteins. We were interested in the reasons for the Tat dependence of novel Tat substrates and focused on two uncharacterized proteins, YcdO and YcdB. Both proteins contain predicted Tat signal sequences. However, we found that only YcdB was indeed Tat-dependently translocated, whereas YcdO was equally well translocated in a Tat-deficient strain. YcdB is a dimeric protein and contains a heme cofactor that was identified to be a high-spin Fe\(^{II}\)-protoporphyrin IX complex. In contrast to all other periplasmic hemoproteins analyzed so far, heme was assembled into YcdB in the cytoplasm, suggesting that heme assembly could take place prior to translocation. The function of YcdB in the periplasm may be related to a detoxification reaction under specific conditions because YcdB had peroxidase activity at acidic pH, which coincides well with the known acid-induced expression of the gene. The data demonstrate the existence of a class of heme-containing Tat substrates, the first member of which is YcdB.

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**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—*E. coli* MC4100 (8) or its tatAB-CDE-deficient derivative DADE (9) was selected for arabinose resistance (10) and used for physiological experiments. *E. coli* XL1-Blue MfR\(^\ddagger\) Kan (Stratagene) was used for cloning. Cells were grown aerobically on LB medium (1% Tryptone, 1% NaCl, and 0.5% yeast extract) in the presence of the appropriate antibiotics (100 \(\mu\)g/ml ampicillin and 12.5 \(\mu\)g/ml tetracycline).

**Plasmids and Genetic Methods**—For expression of ycdO and ycdB, the corresponding genes were amplified by PCR with genomic DNA as template using primer pairs YcdO-NcoI-F (5'-GTC TCG CCA TGG CCA TTA ACT TCC GCC GTA ACG CA-3') and YcdO-XhoI-R (5'-GCT TAC TCG CAT CCC AGC ACA CCG CGA AGT TG-3') and YcdB-NcoI-F (5'-TAC TCG AGT CCC AGC ACA CCG CGA AGT TG-3') and YcdB-XhoI-R (5'-CAT TAC TCG AGC CAT AAT AAC GCG CCT CCG AAA TAA TG-3'). The PCR products were gel-purified and then inserted into the corresponding sites of pBAD-His2- 

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**Biochemical Methods**—YcdO and YcdB were purified by affinity chromatography using Strep-Tactin Superflow\(^{TM}\) resin (IBA GmbH) following the manufacturer’s protocol, but EDTA was generally omitted from buffers. Protein concentrations were determined according to Lowry et al. (12), and SDS-PAGE analyses were carried out according to Laemmli (13). For native gels, SDS was omitted from all buffers, and samples were not reduced. The YcdO or YcdB constructs were

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**RESULTS**

YcdB is a Tat Substrate, whereas YcdO Is Not—YcdB from *E. coli* is distantly related to the dye-decolorizing peroxidase (DyP)\(^2\) identified in the fungus *G. candidum* (16, 17). There are bacterial DyP-like proteins in many bacterial species, but none of them has ever been biochemically analyzed. *E. coli* contains two of these DyP-like proteins, YfeX and YcdB, which share some sequence homology in their C-terminal halves. Of these, YcdB belongs to a subclass of DyP-like proteins with predicted Tat signals, one member of which has been shown to be a Tat substrate in the Gram-positive bacterium *Bacillus subtilis* (18). YcdB was a clear candidate for a novel class of Tat substrates. We thus clarified the Tat dependence of YcdB in *E. coli* and carried out the first purification and biochemical characterization of a bacterial YcdB homolog.

In Fig. 1A, the signal sequence of YcdB from *E. coli* is compared with those of YcdB homologs from species of other phyla, including α-Proteobacteria, β-Proteobacteria, Bacilli, and Actinobacteria. As typically found for Tat substrates, YcdB homologs have rather long signal sequences with twin-arginine motifs at the end of a polar n-region of variable length, and the h-regions are rich in alamines and glycines (19). The conservation of these Tat signal sequence characteristics suggested to us that YcdB is indeed very likely to be translocated in a folded state across the cytoplasmic membrane by the Tat system.

When we analyzed the localization of recombinant YcdB in the cell, we detected mature YcdB in the periplasmic fraction (Fig. 1B). The co-detection of the biotin carrier protein in the cytoplasmic fraction, showed, as an internal control on the same blot, that no cytoplasm was leaking into the periplasmic fraction. Therefore, although the amount of periplasmic

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\(^2\)The abbreviation used is: DyP, dye-decolorizing peroxidase.
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cient derivative strain. Strikingly, there was not any transport of YcdB detectable without the Tat system (Fig. 1B). Thus, YcdB indeed requires the Tat system for translocation into the periplasm. Again, the precur-
sor accumulated in the membrane and cytoplasmic fractions. An inter-
esting aspect on which we did not focus in this study is that the detection of the precursor in the membrane fraction did not depend on the pres-
ence of the Tat system.

To complete the evidence for the Tat dependence, we tested the com-
plementation of YcdB translocation by tatABC, which was expressed in trans from its own promoter using the compatible low-copy vector pRK-
tatABC (15). We found that the translocation defect in the Tat-deficient strain was fully compensated by the tatABC genes in trans. The comple-
mentation system very much improved the translocation compared
with the wild-type strain (Fig. 1B). Such a positive effect of the increased
Tat system abundance on transport is typically observed with recombi-
nant Tat substrates (20). A significant portion of YcdB accumulated in
the cytoplasm, and a part of this was of mature size. Such a “mature”
species could point to a natural cytoplasmic subpopulation of YcdB, or
it simply could be the result of a translocon limitation: signal sequences
of accumulating Tat substrates are often found to be sensitive to pro-
teases, resulting in digestion of the signal sequence while the folded
mature part of the protein remains intact. We addressed this aspect by
lowering the ycdB induction (Fig. 1C). Strikingly, no precursor of YcdB
accumulated at lowered induction, and the cytoplasmic processing
decreased markedly relative to the amount of periplasmic YcdB. As
cytoplasmic maturation is enhanced by a limited translocation at higher
expression levels, it is unlikely to reflect some significant process under
natural conditions. Together, our results indicate that YcdB is a novel
Tat-dependently translocated periplasmic protein.

We also addressed the possible Tat dependence of the translocation
of YcdO. On the basis of signal sequence characteristics, YcdO has been
postulated to be a Tat substrate (3). However, the twin-arginine motif
in its signal sequence is not conserved in homologs from other phyla.
In addition, there is a deviation from the consensus Tat signal pat-
tern that is very unusual. The consensus twin-arginine pattern is
(S/T)RRFxFLK. In this pattern, the two arginines are almost invariable
with the exception of a rarely occurring exchange of the first Arg with
Lys (21). Hydrophobic residues are commonly not found at the S/T
position preceding the two arginines. YcdO has a hydrophobic Phe res-
idue at that position, which raises additional doubts regarding the pre-
diction that YcdO could be a Tat substrate.

When we examined the subcellular localization of YcdO, we found
that >50% of the detected recombinant YcdO was periplasmic. This
rather high translocation efficiency was not Tat-dependent, as YcdO
was equally well translocated in the absence of all Tat system compo-
nents (Fig. 2). In SDS-PAGE analyses, periplasmic YcdO migrated at the
same size as cytoplasmic YcdO. Again, as an internal marker on the
same blot, the detected biotin carrier protein of the cytoplasmic fraction
indicated that no cytoplasm had leaked into the periplasmic fraction.
We examined whether the detected periplasmic YcdO was correctly
processed. Recombinant YcdO was purified from the periplasmic frac-
tion by affinity chromatography using a Strep–Tactin matrix and was
subjected to electrospray mass spectrometry. We determined a molecu-
lar mass of 40,458 Da, which indicates a complete and homogeneous
cleavage of the signal sequence behind position 26 (supplemental Fig.
S1). The three residues preceding this cleavage site are ANA, which
constitute a typical LepB cleavage site. Therefore, all periplasmically
detected YcdO corresponded to translocated and correctly processed
protein. Analytical ultracentrifugation indicated that YcdO is mono-
meric in solution (data not shown). Together, the data show that YcdO is
a monomeric periplasmic protein that is not Tat-dependently translocated.
These data led us to focus on YcdB, as only YcdB was Tat-dependently
translocated.

YcdB Contains FeIII-Protoporphyrin IX—Recombinant YcdB with a C-terminal Strep-tag II could be purified from the periplasmic fraction
and was analyzed by electrospray mass spectrometry (supplemental Fig.
S2). The mass was found to be 45,127 Da, which exactly matches the
mass of the mature protein when cleaved behind the typical LepB signal
cleavage site (AH3) at a distance of 35 residues from the N terminus.
A second mass of 43,814 Da corresponds to part of the purified mature
protein with its C-terminal Strep-tag II degraded. There was no precu-
sor detectable in the periplasmic fraction. When we analyzed the oligo-
meric state of YcdB by analytical ultracentrifugation, we determined an
apparent mass of 89.9 ± 0.8 kDa, which indicates that periplasmic YcdB
is a dimer (supplemental Fig. S3).

The periplasmic YcdB preparation was slightly reddish, pointing to
the presence of some chromogenic cofactor associated with YcdB. This
cofactor could not be covalently bound, as the mentioned mass analysis
did not show any alteration of the mass expected for the unmodified
mature protein. In addition to the protein absorption at 277 nm, we
identified a heme Soret band with a maximum at 406 nm and further
weak features between 485 and 660 nm in UV-visible absorption spectra
of periplasmic YcdB (Fig. 3A). The purified protein had an A406 nm/ A277 nm ratio near 1.5. The extinction coefficient of the 406 nm band was
calculated to be 55 M−1 cm−1. Upon reduction with sodium dithio-
nite, the 406 nm band slowly shifted within several minutes to 432 nm,
and the weak features at longer wavelengths somewhat condensed and
formed maxima at 558 and 622 nm. These data indicate that the heme in
YcdB is purified in an oxidized state. Furthermore, the slow reduction of
the heme iron by the strongly reducing dithionite suggests that transi-
tions other than the FeIII/FeII transition might be physiological.

Knowing that YcdB contains a noncovalent heme, we searched for
the heme signal by electrospray mass spectrometry. We detected a sig-
nal at 616 Da, which was likely to correspond to an iron-containing
protoporphyrin IX cofactor. Fragmentation analyses confirmed the
identity of the heme cofactor (Fig. 3B). The fragments deduced from
the 616 Da mass corresponded to two consecutive losses of 59 Da,
which are indicative of cleavages in the heme propionate side chains
(22). These data unequivocally demonstrate that YcdB contains an
FeIII-protoporphyrin IX complex.

The Heme Iron in YcdB Has a High-spin Electron Configuration—For
functional aspects, it is important to determine the spin state of the
heme iron in the hemoprotein. Cytochromes with electron transport

function are usually low-spin, and the heme iron has six ligands in oxidized and reduced states. High-spin hemes have either five or six ligands in their oxidized state and always only five ligands in their reduced state (23). Therefore, hemoproteins that transiently bind substrates as axial ligands of the heme iron usually have a high-spin electron conformation. Among these high-spin hemoproteins are enzymes such as oxygenases, catalases, peroxidases, and ligand carriers.

A clear classification of the spin state of hemoproteins can be made with spectra of reduced hemes with bound carbon monoxide (CO). The strong ligand field with CO causes a shift in the reduced high-spin heme iron to the low-spin electron configuration upon CO binding. The dithionite-reduced YcdB precursor was incubated with CO, and CO binding resulted in a sharp Soret band at 419 nm and further alterations in the region between 530 and 600 nm (Fig. 4
A). The CO
red
minus-reduced difference spectrum clearly showed the CO
red
α-maximum at 572 nm, the β-maximum at ~536 nm, and the γ-maximum at 419 nm (Fig. 4B). The extinction difference between the peak at 419 nm and the trough at 437 nm was ~30-fold higher than the corresponding difference for the α-band, indicating that the heme of YcdB is high-spin in the reduced state. The Soret maximum of >418 nm in the CO-bound state is in agreement with the noncovalent binding mode of the heme cofactor (23). Together, the spectroscopic data confirm that YcdB contains a noncovalent high-spin heme. YcdB is therefore likely to function as an enzyme with oxygenase or peroxidase activity.

YcdB Has Peroxidase Activity at Low pH—The presence of a high-spin heme and the postulated relation to fungal DyP strengthened the hypothesis that YcdB might be a peroxidase. Peroxidases perform a Fe
III
/Fe
IV
transition during their catalytic cycle when they produce the compound I intermediate, in which oxygen forms a double bond with Fe
IV
(24). To examine the possible peroxidase activity of YcdB, we used the quantitative guaiacol assay. In this assay, the H
2
O
2
-dependent oxidation of the colorless guaiacol results in a guaiacol tetramer, which can be monitored at 470 nm (ε
260 nm = 26.6 mM
−1 cm
−1
). Because YcdB is induced under acidic conditions (7), we measured the activity at different pH values in the acidic to neutral region. We found that YcdB had significant guaiacol peroxidase activity at pH 4.0 (Fig. 5, A and B). The activity was somewhat lower but still significant at pH 3.0. Under less acidic conditions (at pH 5.0 or pH 6.0), the activity was markedly reduced. From the data, we calculated an activity of ~200 milliunits/mg purified YcdB with guaiacol as the H
2
O
2
-dependently oxidized substrate. As the natural substrate of YcdB is unknown, the specific peroxidase activity may be manifold higher in vivo. Having established the peroxidase activity of YcdB, we wondered what influence hydrogen peroxide might have on the spectral characteristics of YcdB. Interestingly, H
2
O
2
addition to oxidized YcdB caused major spectral alterations, indicating that H
2
O
2
could generate a ligand to the ferric iron. The Soret band shifted to 414 nm, and three signals appeared at longer wavelengths of 530, 555, and 603 nm (Fig. 5C). Such spectral changes might reflect the formation of an intermediate similar to compound I in the known peroxidase cycle. Some preparations of YcdB already partially showed the characteristics of this adduct (data not shown), implying that YcdB undergoes this type of reaction also in vivo. Together, the data suggest that YcdB may have a functional role as a periplasmic peroxidase at low pH.

YcdB Can Assemble Heme in the Cytoplasm—It was important to determine whether YcdB can assemble its cofactor in the cytoplasm prior to translocation. We therefore purified YcdB also from the translocation-deficient DADstrain and recorded the UV-visible spectra from these preparations. Any heme in YcdB from this strain would contain the noncovalent binding mode of the heme cofactor (23). Together, the spectroscopic data confirm that YcdB contains a noncovalent high-spin heme. YcdB is therefore likely to function as an enzyme with oxygenase or peroxidase activity.

FIGURE 3. YcdB is a hemoprotein. A, overlay of UV-visible spectra of oxidized YcdB-Strep purified by affinity chromatography (ox) and the same sample 2 min after reduction with sodium dithionite (red). Buffer-corrected spectra were recorded with protein in elution buffer (100 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 2.5 mM desthiobiotin). B, mass spectrometric fragmentation identification of protoporphyrin IX.
tion of MC4100 carrying pBAD-ycdB-Strep, and the heme content of the cytoplasmic YcdB species was assessed by native gel electrophoresis and subsequent activity or Coomassie Blue stains of the gel (Fig. 6B).

Three activity bands were separated. A single band corresponding to the mature protein was readily identified by comparison with mature periplasmic YcdB. The two other active bands could be assigned to the YcdB precursor and thus indicated the presence of the cofactor in the precursor. The two bands are likely to reflect two differently interacting populations. Interestingly, not all of the YcdB precursor was active: a significant population of the inactive precursor had not yet acquired the cofactor and migrated as a diffuse and inactive “smear” on native gels. The signal sequence was detected in this fraction by mass spectrometry (trypsin fragment K²DENGVNEPSR²).

**DISCUSSION**

**YcdB Is the First Member of a New Class of Tat Substrates**—This study was initiated to reveal the translocation mode and structural characteristics of two postulated protein substrates of the Tat system, YcdB and YcdO. We have demonstrated that, of these two proteins, only YcdB is a Tat substrate in *E. coli*, and this protein turned out to be the first example of a Tat-dependently translocated hemoprotein.

In *E. coli*, all other periplasmic hemoproteins analyzed so far are Sec substrates, which fold and assemble their cofactors after translocation.
The biogenesis of periplasmic c-type cytochromes, which are proteins with two cysteine-derived thioether links to the vinyl groups of protoporphyrin IX, has been well studied (25). All c-type cytochromes contain a heme attachment pattern (CXXCH) that forms a disulfide in the oxidative periplasmic environment and therefore has to be reduced by specific reductases to allow heme ligation to the protein. The histidine in this pattern serves as an axial ligand for the heme iron. The ccmAB-CDEFGH genes are responsible for heme delivery and attachment to c-type cytochromes in the periplasm of E. coli. They are also involved in the reduction of the target CXXCH motif prior to heme ligation. However, none of these genes could be demonstrated to carry out heme transport across the membrane (25). The same heme that is used for c-type cytochromes is also used for b-type cytochromes, which do not attach their heme covalently. As b-type cytochromes can assemble their heme in the periplasm without involvement of the ccm genes, heme transport is an unanswered question (26).

With YcdB, the first protoporphyrin IX-containing protein transported by the Tat system has been identified. Tat substrates such as iron-sulfur proteins, molybdoproteins, flavoproteins, and nickel proteins assemble their cofactors in the cytoplasm (19). As heme biogenesis takes place in the cytoplasm and as no periplasmic machinery is known to be required for noncovalent heme assembly, there is no argument against heme cotransport with Tat substrates. YcdB is exceptional among the heme-containing proteins in its requirement for cytoplasmic heme transport. Heme is available on either side of the cytoplasmic membrane; and therefore, the cofactor insertion may not be the reason for the Tat-dependent translocation of YcdB. We clearly detected heme in the cytoplasmic YcdB precursor, suggesting that the holoprotein can be translocated. We also detected an apoprecursor, corresponding to the fraction that had not yet assembled its cofactor. If the apoprecursor can fold without the heme, it is possible that it assembles the heme after translocation. As heme insertion can also occur in the periplasm, other folding requirements that make the Tat system necessary have to be considered. YcdB does contain four cysteines. One of these is positioned in the hydrophobic region of the signal sequence, and the others are separate cysteines in the mature domain. Folding in the periplasm could result in undesired disulfide formation, making cytoplasmic folding necessary. Another explanation may be that YcdB has to be folded outside even under conditions of acid stress, as the protein is induced under acid conditions and may thus function in response to acid stress (7). Folding in the periplasm could be less effective under such conditions, making translocation as a folded protein necessary. These explanations show that many factors have to be considered as causative for the Tat dependence of YcdB, and further yet unknown circumstances may turn out to be important.

Why is YcdB not translocated by the Sec system? The Tat dependence indicates a requirement for cytoplasmic folding, but not necessarily for cytoplasmic heme insertion. YcdB is exceptional among the heme-containing proteins in its requirement for cytoplasmic folding. Heme is available on either side of the cytoplasmic membrane; and therefore, the cofactor insertion may not be the reason for the Tat-dependent translocation of YcdB. We clearly detected heme in the cytoplasmic YcdB precursor, suggesting that the holoprotein can be translocated. We also detected an apoprecursor, corresponding to the fraction that had not yet assembled its cofactor. If the apoprecursor can fold without the heme, it is possible that it assembles the heme after translocation. As heme insertion can also occur in the periplasm, other folding requirements that make the Tat system necessary have to be considered. YcdB does contain four cysteines. One of these is positioned in the hydrophobic region of the signal sequence, and the others are separate cysteines in the mature domain. Folding in the periplasm could result in undesired disulfide formation, making cytoplasmic folding necessary. Another explanation may be that YcdB has to be folded outside even under conditions of acid stress, as the protein is induced under acid conditions and may thus function in response to acid stress (7). Folding in the periplasm could be less effective under such conditions, making translocation as a folded protein necessary. These explanations show that many factors have to be considered as causative for the Tat dependence of YcdB, and further yet unknown circumstances may turn out to be important.

YcdB Is a Structural Homolog of Fungal DyP—Bacterial proteins that share some sequence homology with fungal DyP-type peroxidases have been identified in the past. This resulted in the creation of the term “DyP-type peroxidase family,” which has been used in many genome annotations. A Hidden Markow Model-based algorithm was created in 2002 at The Institute of Genomic Research, which recognizes...
most of these proteins (accession number TIGR01413, available at www.tigr.org). The Institute of Genomic Research already noted in the description of this postulated family that "a distinct, uncharacterized branch (TIGR01412) of this superfamily has a typical twin-arginine dependent signal sequence characteristic of exported proteins with bound redox cofactors." The assignment of YcdB as a DyP-like protein was indirect, and no bacterial member of this family has ever been characterized. The only characterized peroxidase of the DyP family, an enzyme from the fungus *G. candidum*, has only 19% sequence identity to YcdB (Fig. 7), which is slightly above the identities of ~13–14% often found between structurally unrelated proteins of similar size (e.g. TatC and TatD).

The sequence alignment of YcdB and DyP shows that the postulated ligand of the heme of *G. candidum* DyP (28) is not at a similar position in YcdB (Fig. 7). His164 of mature DyP has been tentatively assigned as the axial ligand histidine (29), and an H164A exchange of DyP results in a significant decrease in activity, attributed to loss of the heme (28). If His164 is indeed the axial ligand in DyP, then there are major differences in ligandation of the hemes in DyP and bacterial DyP-family proteins. This is surprising, as our data strongly suggest that heme binding is similar in DyP and YcdB. The oxidized heme in fungal DyP has a Soret maximum at 406 nm (16), as we found for YcdB (Fig. 3). Another similarity is that YcdB has peroxidase activity at acidic pH with guaiacol as substrate (Fig. 5), which has also been found for DyP (16). It will thus be a very interesting future task to compare the structures of DyP and YcdB. The similarities between DyP and YcdB reside mainly in the C-terminal half of these proteins, and some conserved patterns can be found here, suggesting that this domain comprises the conserved parts of the heme-binding pocket (Fig. 7).

**YcdB May Function as a Peroxidase under Acid Stress Conditions**—YcdB has been shown to have its heme iron in a high-spin configuration (Fig. 4). This is functionally important, as it implies a five-ligand coordination in the reduced state, whereas the oxidized heme iron may bind either five or six ligands. The high-spin configuration suggests that a substrate ligand can be bound directly to the heme, such as in oxygenases, peroxidases, and ligand-transporting hemoproteins. It was therefore an important observation that YcdB has peroxidase activity, as does fungal DyP (Fig. 5). The natural substrates are not known for DyP or YcdB. However, it is intriguing that peroxidase activity is significantly enhanced at acidic pH, and acidic conditions have been shown previously to induce transcription of the ycdNOB operon, suggesting that YcdB may fulfill some function as a peroxidase under acidic stress conditions (7). We also consider it possible that YcdB could be capable of catalyzing other redox reactions with small ligands under acidic stress conditions. However, our results suggest that such redox reactions are unlikely to involve FeIII/FeII transitions at the heme center. Furthermore, we observed adduct formation with H2O2, which may well reflect the typical compound I stage of the peroxidase cycle (Fig. 5C). From the physiological point of view, the characterization of YcdB as a peroxidase sheds light on the conserved ycdNOB locus. Although the exact functions of the other encoded components remain to be revealed, the present results on YcdB are surely the first step toward the understanding of this locus on a biochemical level.

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