

Co-factor Insertion and Disulfide Bond Requirements for Twin-arginine Translocase-dependent Export of the *Bacillus subtilis* Rieske Protein QcrA*

Received for publication, October 23, 2013, and in revised form, March 5, 2014. Published, JBC Papers in Press, March 20, 2014, DOI 10.1074/jbc.M113.529677

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Background: The Rieske protein QcrA was recently shown to be exported by twin-arginine translocation (Tat) in *Bacillus subtilis*.

Results: QcrA has disulfide bond and co-factor requirements for effective Tat-dependent translocation.

Conclusion: A hierarchy exists between disulfide bonding and co-factor insertion in QcrA quality control and translocation.

Significance: First studies on Tat-dependent translocation where oxidative folding and co-factor attachment were addressed in a single native molecule.

The twin-arginine translocation (Tat) pathway can transport folded and co-factor-containing cargo proteins over bacterial cytoplasmic membranes. Functional Tat machinery components, a folded state of the cargo protein and correct co-factor insertion in the cargo protein are generally considered as prerequisites for successful translocation. The present studies were aimed at a dissection of these requirements with regard to the Rieske iron-sulfur protein QcrA of *Bacillus subtilis*. Notably, QcrA is a component of the cytochrome *bc*₁ complex, which is conserved from bacteria to man. Single amino acid substitutions were introduced into the Rieske domain of QcrA to prevent either co-factor binding or disulfide bond formation. Both types of mutations precluded QcrA translocation. Importantly, a proofreading hierarchy was uncovered, where a QcrA mutant defective in disulfide bonding was quickly degraded, whereas mutant QcrA proteins defective in co-factor binding accumulated in the cytoplasm and membrane. Altogether, these are the first studies on Tat-dependent protein translocation where both oxidative folding and co-factor attachment have been addressed in a single native molecule.

The Tat pathway is unique in that it is able to translocate correctly folded (1–3) and cofactor-attached cargo proteins across bacterial and chloroplast thylakoidal membranes (4–6). Apart from the globular nature of the Tat-dependent cargo, a second distinctive feature of the Tat system is the eponymous twin-arginine motif within its signal peptide (7–9). The current interpretation of the mechanism associated with Tat translocation is that the cargo proteins are first recognized by a docking complex composed of so-called TatC and TatA-like proteins (10–13). Importantly, it is thought that the cargo protein

undergoes proofreading and quality control at this point (1–3, 8, 14–17). After docking, the complex and cargo protein interacted, more TatA-like components are recruited such that multiple TatA-like components form a translocation pore (18). The proton-motive force then drives translocation (19, 20).

Bacillus subtilis is a non-pathogenic Gram-positive bacterium with a high capacity for protein secretion. Accordingly, it has become an important model organism for fundamental studies on protein export and secretion (7, 21–23). The Tat system in *B. subtilis* is composed of three TatA-like proteins (TatAd, TatAy, and TatAc) and two TatC proteins (TatCd and TatCy). TatAd-TatCd and TatAy-TatCy combine to form two independent parallel protein transport pathways, each with its own substrate specificities (24–26). The third TatA component, TatAc, has the intrinsic ability to form active Tat translocases both with TatCd and TatCy (27). The *tatAy-tatCy* operon is expressed under a broad range of conditions, which corresponds to the broad expression profile of the known substrates of the TatAy-TatCy translocase, namely the Dyp-type peroxidase EfeB (YwbN), the alkaline phosphatase YkuE, and the Rieske iron-sulfur protein QcrA (also known as PetC) (24, 28–31).

Rieske iron-sulfur proteins, in short Rieske proteins, form part of the cytochrome *bc*₁ complex in *B. subtilis*. Cytochrome *bc*₁ is a main component of the electron transport chain and, as a homolog of the complex III in mitochondria, it is structurally and functionally highly conserved in all three kingdoms of life (32, 33). In *B. subtilis*, the cytochrome *bc*₁ complex is a menaquinone:cytochrome *c* reductase composed of the Rieske protein (QcrA), cytochrome *b* (QcrB), and a larger than normal cytochrome *c*₁ (QcrC) (34). Notably, cytochrome *bc*₁ is a membrane protein complex that faces the extracellular side of the cytoplasmic membrane. The Sec pathway individually translocates the cytochrome *b* and cytochrome *c*₁ components across the membrane before they are processed, matured, and combined (32, 35–39). It is believed that cytochrome *b* and cytochrome *c*₁ form a primary protease-resistant complex, where the final step in complex maturation is the incorporation of the Rieske protein (32).

* This work was supported in part by CEU projects PITN-GA-2008-215524 (TranSys), LSHG-CT-2006-037469 and -244093 (to V. J. G. and J. M. v. D.), and the transnational SysMO initiative through projects BACELL SysMO1 and -2 with funding from the Research Council for Earth and Life Sciences of the Netherlands Organization for Scientific Research (NWO-ALW).

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TABLE 1
Strains and plasmids used in this study

		Relevant properties	Ref.
Strains			
<i>B. subtilis</i> 168	<i>trpC2</i>		76
<i>B. subtilis</i> 168 <i>tatAdCd</i>	<i>trpC2, tatAd-tatCd::K_m; K_m^r</i>		55
<i>B. subtilis</i> 168 <i>tatAyCy</i>	<i>trpC2, tatAy-tatCy::Sp; Sp^r</i>		24
<i>B. subtilis</i> 168 <i>tatAc₁-tatAyCy</i>	<i>trpC2; tatAc::Em, tatAy-tatCy::Sp; Em^r; Sp^r</i>		55
<i>B. subtilis</i> 168 <i>tatCd-tatCy</i>	<i>trpC2; tatCd::K_m; tatCy::Sp; K_m^r; Sp^r</i>		25
<i>B. subtilis</i> 168 total- <i>tat₂</i>	<i>trpC2, tatAd-tatCd::K_m, tatAy-tatCy::Sp; tatAc::Em^r; Sp^r, K_m^r, Em^r</i>		24
<i>B. subtilis qcrA</i>	<i>trpC2, qcrA::pPP435</i>		28
<i>B. subtilis</i> ATCC6633	Subtilin producer		57
<i>L. lactis</i> MG1363	Plasmid-free derivative of NCDO 712		77
Plasmids			
pHB-201	<i>B. subtilis-E. coli</i> expression vector; ori-pBR322; ori-pTA1060; <i>cat86::lacZa</i> ; Em ^r ; Cm ^r		78
pHB-AyCy	pHB201 derivative carrying the <i>tatAy-tatCy</i> operon; Em ^r ; Cm ^r		79
pHB-TatC ^{aa}	pHB201 derivatives carrying <i>tatCy</i> genes that specify mutant TatCy proteins with specific single amino acid substitutions or C-terminal deletions; Em ^r ; Cm ^r		65
pHB-TatA ^{aa} TatCy ^{WT}	pHB201 derivatives carrying a wild-type copy of the <i>tatCy</i> gene plus <i>tatAy</i> genes that specify mutant TatAy proteins with specific single amino acid substitutions; Em ^r ; Cm ^r		53
pHB-QcrA	pHB201 derivative carrying wild-type <i>qcrA</i> ; Em ^r ; Cm ^r		28
pHB-QcrA ^{H102L}	pHB201 derivative carrying a mutant <i>qcrA</i> gene that specifies QcrA-H102L; Em ^r ; Cm ^r		This study
pHB-QcrA ^{H124L}	pHB201 derivative carrying a mutant <i>qcrA</i> gene that specifies QcrA-H124L; Em ^r ; Cm ^r		This study
pHB-QcrA ^{C123S}	pHB201 derivative carrying a mutant <i>qcrA</i> gene that specifies QcrA-C123L; Em ^r ; Cm ^r		This study
pNZ8910	SURE expression vector, P _{spas} ; Em ^r		57
pNZ-QcrA ^{H102L}	pNZ8910 derivative carrying a mutant <i>qcrA</i> gene that specifies QcrA-H102L; Em ^r		This study
pNZ-QcrA ^{C123S}	pNZ8910 derivative carrying a mutant <i>qcrA</i> gene that specifies QcrA-C123L; Em ^r		This study

TABLE 2
Primers used in this study

	Primer sequence	Underlined region
External primers		
QcrALnew	CGGAAGGTTACTAGTAGGGGTGACTTAGAGGGGG	SpeI
QcrARnew	CCG <u>GGATCC</u> GTGTAATATCAAGCCGCTCG	BamHI
Internal primers		
QcrAH102L-left	GTACACCCTAA <u>AG</u> CTTACAAATTG	CAT → CTT (His → Leu)
QcrAH102L-right	CAATTGTAA <u>GC</u> TTTtaggggtgTAC	CAT → CTT (His → Leu)
QcrAH124L-left	CCGTA <u>AG</u> GCATGGACAAAAGAATTTAT	CAT → CTT (His → Leu)
QcrAH124L-right	ATAAATTC <u>TTT</u> TGTCCATGCC <u>TT</u> TACGG	CAT → CTT (His → Leu)
QcrAC123S-left	CCGTAAT <u>GG</u> GATGGACAAAAGAATTTAT	GCC-CCC (Cys → Ser)
QcrAC123S-right	ATAAATTC <u>TTT</u> TGTCCAT <u>CC</u> ATTACGG	GCC-CCC (Cys → Ser)

All Rieseke proteins contain a well defined Rieseke domain. Within this domain there are specific residues involved in 2Fe-2S co-factor binding. In some Rieseke proteins, like QcrA of *B. subtilis*, the Rieseke domain contains Cys residues involved in disulfide bond formation (33, 40–42)(Fig. 1). Apart from QcrA of *B. subtilis*, other Rieseke proteins also share a strong association with the Tat pathway as was demonstrated in various bacteria and chloroplasts of green plants (43–47). Notably, the Gram-negative bacterium *Escherichia coli* does not contain a cytochrome *bc₁* complex, which precludes studies on Rieseke protein assembly in this important model organism for research on Tat-dependent protein translocation (32, 33). *B. subtilis* on the other hand is an attractive organism to explore the requirements and Tat dependence of Rieseke protein translocation. First, the electron transport chain in *B. subtilis* has two branches, creating a functional redundancy that allows for cytochrome *bc₁* mutation studies (32, 48, 49). Second, the *qcrABC* operon is highly expressed under a wide range of tested conditions and, consequently, it does not need to be artificially induced to investigate QcrA export in *tat* mutant strains (31, 50). The present studies were therefore aimed at analyzing the requirements for Tat-dependent export of QcrA in *B. subtilis*. For this purpose, QcrA was mutated to assess the roles of co-factor assembly and disulfide bonding in membrane translocation. Altogether, these studies reveal a hierarchy in the folding

events needed for productive QcrA translocation. Although folding and co-factor insertion of native Tat substrates have been investigated previously, this is the first example of an investigation into the importance of folding and co-factor assembly for Tat-dependent membrane translocation of a single native (non-engineered) cargo protein.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Growth Conditions—Bacterial strains and plasmids used in this study are listed in Table 1. Lysogeny Broth (LB) was composed of 1% tryptone, 0.5% yeast extract, and 1% NaCl. Bacterial cultures were grown in LB at 37 °C under vigorous shaking, or on LB agar plates incubated at 37 °C. *B. subtilis* cells were made competent in Paris medium (51). When appropriate, the cultures were supplemented with antibiotics: *Escherichia coli* cultures with 100 μg/ml of ampicillin (Ap) and *B. subtilis* cultures with 2 μg/ml of erythromycin (Em), 5 μg/ml of chloramphenicol (Cm), 10 μg/ml of tetracycline (Tc), 100 μg/ml of spectinomycin (Sp), or 20 μg/ml of kanamycin (Km).

Cloning and DNA Techniques—Cloning and ligation reactions were performed as described previously (52) using products from New England Biolabs. PCR were performed using the Phusion (New England Biolabs) or Pwo (Roche Applied Sciences) polymerases. Primers are listed in Table 2. The method-

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ology for site-directed mutagenesis has been described previously in detail (53). Briefly, although external primers were the same for constructing all *qcrA* mutants, the internal primers included nucleotide changes that translated into single amino acid mutations in the full QcrA sequence. External primers included sequences specifying restriction sites for BamHI and SpeI, which allowed for cloning into the *E. coli*-*B. subtilis* shuttle vector pHB-201. To overproduce mutant QcrA proteins, the respective mutated *qcrA* genes were PCR amplified from pHB-201-based plasmids, and cloned into plasmid pNZ8910 digested with NcoI and HindIII. Ligation mixtures were introduced into *Lactococcus lactis* by transformation, and pNZ8910-derivative plasmids thus obtained were then introduced into *B. subtilis* as previously described (29, 30).

Crude Cell Fractionations—Crude cell protein extractions were performed after cultures were grown to early stationary phase. Culture aliquots (2 ml) were treated with Complete protease inhibitor (Roche Applied Science) and pelleted. The extracellular fraction (1.5 ml) was removed, and proteins in this fraction were precipitated overnight with trichloroacetic acid (TCA; final concentration 10%). The cell pellet was resuspended in 100 μ l of lithium dodecyl sulfate gel loading buffer and reducing agent (NuPAGE, Invitrogen) before disruption by bead-beating three times with glass beads at 6500 rpm for 3 s with 30-s intervals (Precellys 24 lysis and homogenization, Bertin Technologies). The TCA-precipitated extracellular fraction was acetone washed and resuspended in 50 μ l of lithium dodecyl sulfate gel loading buffer and reducing agent. Samples were heated at 95 $^{\circ}$ C and, if necessary, stored at -20° C. Crude cell extract aliquots of 10 μ l and growth medium aliquots of 20 μ l corresponding to 2 A_{600} units were used for NuPAGE and Western blotting.

Subcellular Fractionations—Subcellular fractionation studies were performed as described previously (28). However, for the present experiments cultures were grown to early stationary phase. The protein concentration of samples was estimated using the DCTM Bio-Rad Assay (Bio-Rad) and samples were stored at -20° C until further use. Aliquots of 10 μ g of protein were used for sample analysis by NuPAGE and Western blotting.

Pulse-Chase Labeling Experiments—Pulse-chase labeling experiments were performed as described previously with minor adaptations (54, 55). Cells were grown overnight in an adapted MBD medium (56) in which the Soytone was replaced with 3.5% maltodextrin and the glucose concentration was reduced to 2.1%. Overnight 2-ml cultures with an optical density at 600 nm (A_{600}) of ~ 1 were labeled with 40 μ Ci of [³⁵S]methionine/cysteine (PerkinElmer Life Sciences). After 2 min labeling of proteins (*i.e.* the pulse), any further incorporation of [³⁵S]methionine/cysteine was stopped by addition of an excess non-radioactive methionine/cysteine mixture (*i.e.* the chase; 2.5 mg/ml final concentration). Next, samples were withdrawn and immediately mixed with ice-cold TCA (10% final concentration). Upon 30 min incubation on ice, the precipitated proteins were collected by centrifugation and QcrA was immunoprecipitated with specific antibodies and protein A-Sepharose beads as previously described (54). The immunoprecipitated QcrA was analyzed by NuPAGE and subsequent autoradiogra-

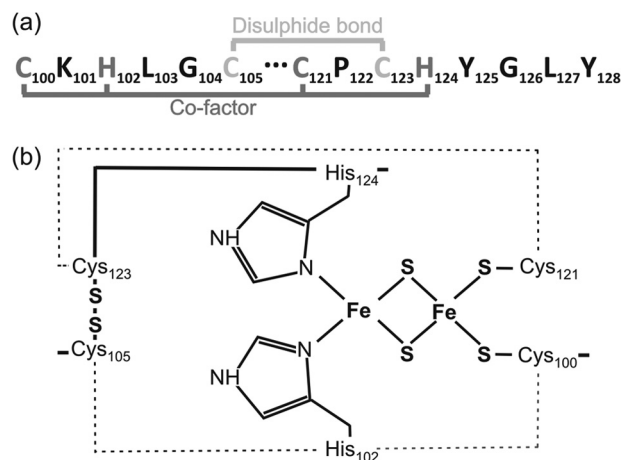


FIGURE 1. The Rieske domain of *B. subtilis* QcrA. *a*, annotated amino acid residues involved in co-factor attachment and disulfide bond formation. *b*, schematic representation of 2Fe-2S co-factor-binding by Cys-100, His-102, Cys-123, and His-124.

phy on a PhosphorImager (Cyclone[®] Plus storage phosphor system, PerkinElmer Life Sciences).

Overproduction of QcrA Mutant Proteins—*B. subtilis* cells containing pNZ-derivative plasmids with a subtilin-inducible mutated *qcrA* gene were grown in LB medium at 37 $^{\circ}$ C until an A_{600} of 0.6 at which point the cultures were induced by addition of subtilin-containing supernatant from *B. subtilis* ATCC 6633 prepared as described by Bongers *et al.* (57). After 3 h of induction, cells were harvested and subjected to subcellular fractionation, NuPAGE, and Western blotting.

AMS Cross-linking—Cross-linking experiments with 4-acetamido-4'-maleimidyl-stilbene-2,2'-disulfonate (AMS)² were performed as described previously (58). Samples were analyzed by non-reducing NuPAGE and Western blotting with specific antibodies against QcrA and TrxA.

Gel Electrophoresis and Western Blotting—Proteins were separated using NuPAGE gels (Invitrogen) and transferred onto nitrocellulose membranes (Protran, Schleicher & Schuell) by semi-dry blotting. Polyclonal antibodies specific for BdbD, LipA, QcrA, and TrxA have been described previously (28, 59, 60). Bound antibodies were detected with fluorescent IgG secondary antibodies (IRDye 800 CW goat anti-rabbit from LiCor Biosciences) and visualized at 700 or 800 nm with the Odyssey Infrared Imaging System (LiCor Biosciences).

RESULTS

Proofreading Hierarchy with Regard to Co-factor Binding and Disulfide Bond Formation—The Rieske domain is highly conserved in all known Rieske iron-sulfur proteins and, therefore, the residues responsible for both the formation of the disulfide bond and those associated with co-factor attachment are well defined (Fig. 1) (32, 33, 40–42). To assess the roles of co-factor binding and disulfide bonding in QcrA export in *B. subtilis*, site-specific mutations were introduced into the Rieske domain of QcrA. These involved single amino acid changes altering the co-factor insertion site (H102L or H124L), or a cysteine residue

²The abbreviation used is: AMS, 4-acetamido-4'-maleimidyl-stilbene-2,2'-disulfonate.

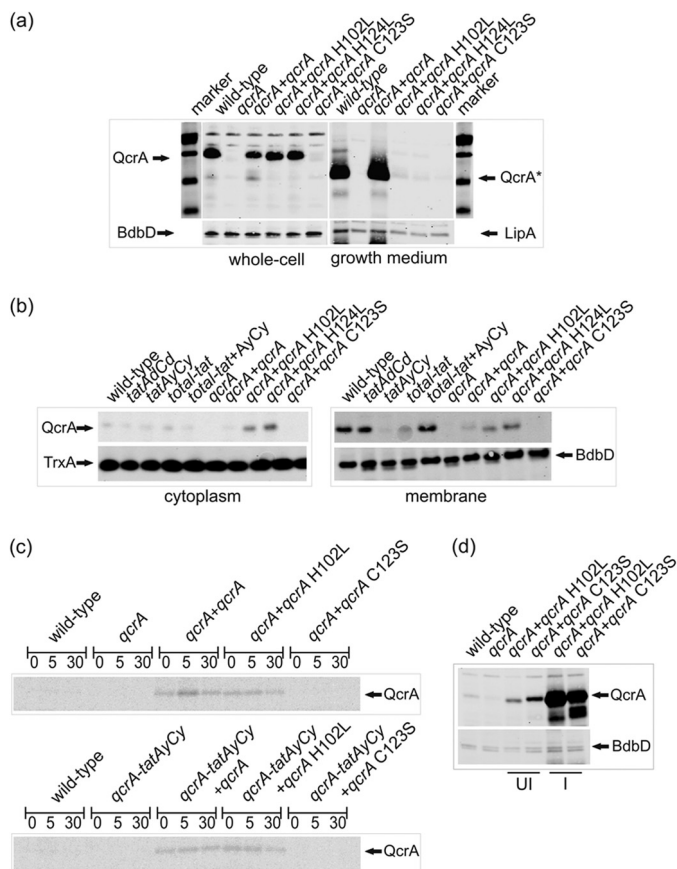


FIGURE 2. Translocation and quality control of QcrA mutant proteins defective in co-factor binding or disulfide bond formation. *a*, translocation of QcrA in strains with a deleted chromosomal *qcrA* gene that ectopically express wild-type *qcrA* or site specifically mutated *qcrA* genes from pHB-201. The mutant *qcrA* genes specify the H102L or H124L mutant QcrA proteins impaired in co-factor binding, or the C123S mutant QcrA protein defective in disulfide bond formation. All strains were grown in LB with 1% NaCl. Cells were separated from the growth medium, and crude whole cell extracts and growth medium fractions were analyzed by Western blotting using specific antibodies against QcrA, BdbD, or LipA. The positions of the 18-kDa full-size QcrA protein in the whole cell fraction and the 14-kDa processed QcrA protein (QcrA*) in the growth medium fraction, both separated on the same gel, are marked with *arrows*. The membrane protein BdbD and the secreted protein LipA were used as positive controls. *b*, subcellular fractionation was performed to separate the cytoplasmic and membrane proteins of *tat* mutant cells and *qcrA* mutant cells producing site specifically mutated QcrA proteins as indicated. The positions of QcrA, the cytoplasmic marker protein TrxA, and the membrane protein BdbD are marked with *arrows*. *c*, pulse-chase labeling of wild-type QcrA and QcrA-H102L or QcrA-C123S mutant proteins in *tat*-proficient cells (*upper panel*) or *tatAy-tatCy*-deficient cells (*lower panel*). Cells were labeled for 2 min with a [³⁵S]methionine/cysteine mixture prior to chase with an excess of non-radioactive methionine/cysteine. At the time of chase (*t* = 0) and 5 and 30 min after the chase, samples were collected in which the presence of labeled QcrA was assessed by immunoprecipitation, NuPAGE, and autoradiography. The position of QcrA is marked with an *arrow*. *d*, the possibility to severely overexpress QcrA-C123S was assessed with the subtilin-inducible "SURE" expression plasmid pNZ8910. In this case, wild-type cells and cells over-expressing QcrA-H102L were used as controls. The positions of QcrA and the control protein BdbD are marked with *arrows*. UI, uninduced; I, subtilin-induced expression.

involved in disulfide bond formation (C123S). The *qcrA* genes specifying these amino acid mutations were cloned into the pHB-201 expression plasmid and introduced into a *B. subtilis* strain with a mutated chromosomal *qcrA* gene. As illustrated in Fig. 2*a*, expression of the wild-type *qcrA* gene from pHB-201 in the *qcrA* mutant strain (lanes labeled *qcrA* + *qcrA*) resulted in the production of cellular and extracellular QcrA levels that

are comparable with those of the parental strain 168 (lanes labeled wild-type).

As previously shown by subcellular fractionation, the intact QcrA is an 18-kDa membrane-associated protein with N_{in} - C_{out} topology (28). Importantly, the translocated QcrA is exposed to signal peptidase activity, resulting in the release of a 14-kDa processed form (QcrA*) into the growth medium (Fig. 1*a*). In fact, the processing of QcrA was only abolished when four of the five type I signal peptidase-encoding genes of *B. subtilis* were deleted (28). Because the catalytic site of signal peptidase is positioned on the extracytoplasmic side of the membrane, QcrA processing and release of QcrA* into the medium can be used as a read-out for the translocation of this protein. Hence, membrane translocation of QcrA was visualized by Western blotting of crude whole cell extracts and growth medium fractions. As shown in Fig. 2*a*, QcrA* was absent from the growth media of all strains expressing mutated forms of QcrA (C123S, H102L, or H124L). Interestingly, in the crude whole cell extract, the presence of full-size QcrA was observed only for the co-factor-binding site mutants (H102L and H124L) and the wild-type protein, but not for the mutant impaired in disulfide bonding (C123S).

Strains producing mutated QcrA proteins were further investigated by subcellular fractionation. Samples were taken from cultures in the early stationary phase. Mutant proteins defective in the co-factor-binding site (QcrA-H102L or -H124L) were detectable in the membrane fraction (Fig. 2*b*). As signal peptidase-processed forms of QcrA-H102L and QcrA-H124L were completely absent from the growth medium (Fig. 2*a*), these findings imply that correct co-factor binding by QcrA is a prerequisite for productive membrane translocation. In contrast, the QcrA-C123S mutant protein defective in disulfide bond formation was neither observed in the cytoplasmic, nor the membrane or extracytoplasmic fractions (Fig. 2, *a* and *b*). Because QcrA-C123 was produced from exactly the same expression plasmid (pHB-201) as QcrA-H102L and QcrA-H124L, we performed pulse-chase labeling analyses with *tat*-proficient and *tatAy-tatCy* mutant cells to detect any short-lived intermediate forms of QcrA-C123. However, such short-lived forms remained undetectable, whereas the full-size forms of wild-type QcrA and QcrA-H102L were readily detectable, albeit only after labeling for 2 min (Fig. 2*c*). Since the C terminally truncated QcrA protein encoded by the *qcrA* mutant strain (29) remained undetectable in both our Western blotting and pulse-chase labeling analyses (Fig. 2), we conclude that the QcrA-C123 mutant protein with the inability to form the correct disulfide bond is degraded shortly after synthesis and does neither build up in the cytoplasm nor reach the membrane. Nevertheless, this protein can accumulate in *B. subtilis* when heavily overexpressed from the very strong subtilin-inducible promoter on pNZ8910 (Fig. 2*d*), confirming our sequencing data that the *qcrA*-C123 mutant gene is intact. Notably, this promoter is leaky, which is also why uninduced cells overproduced QcrA-C123S. Together, our observations on the behavior of the QcrA mutant proteins imply that there is a proofreading hierarchy where non-disulfide bonded QcrA is rapidly degraded, whereas QcrA defective in co-factor binding is stable.

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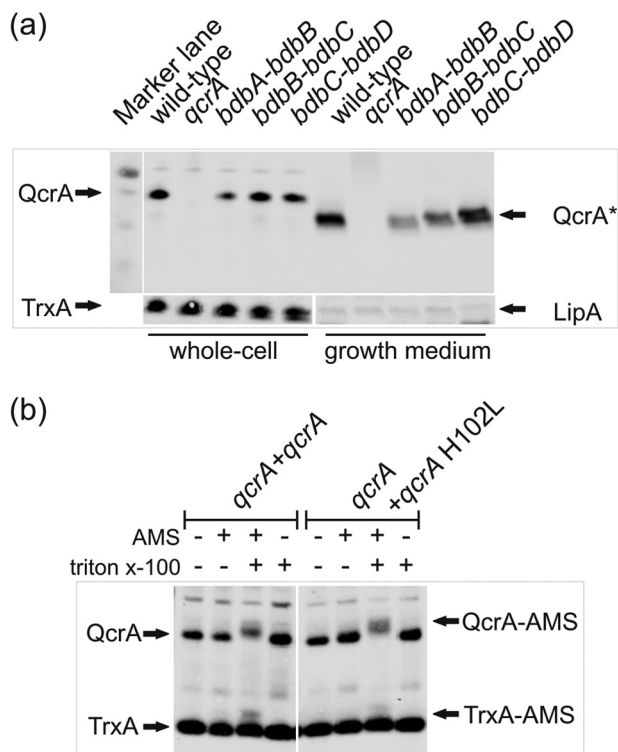


FIGURE 3. Bdb-independent biogenesis of QcrA and cross-linking with AMS. *a*, translocation of QcrA by *bdbA-bdbB*, *bdbB-bdbC*, or *bdbC-bdbD* mutant cells was assessed by Western blotting as described in the legend to Fig. 2*a*. All protein samples were loaded on the same gel. The positions of QcrA, QcrA*, TrxA, and LipA are marked with arrows. *b*, AMS cross-linking of QcrA in protoplasts of *B. subtilis* 168. AMS cross-linking was detectable by an up-shift of the QcrA band upon NuPAGE and Western blotting only if Triton X-100 was present during the incubation of protoplasts with AMS. TrxA was used as a cytoplasmic control protein. Note that under the tested conditions only a minor fraction of TrxA was cross-linkable with AMS upon protoplast lysis with Triton X-100. The positions of QcrA, QcrA-AMS, TrxA, and TrxA-AMS are indicated.

The fast degradation of the non-disulfide bonded QcrA-C123S protein is in line with the previously documented degradation of other extracytoplasmic proteins, such as ComEC and ComGC, lacking an essential disulfide bond (60, 61). It should, however, be noted that, unlike ComEC or ComGC, the presence of QcrA was not affected by any of the *bdb* mutations that are known to interfere with oxidative protein folding in *B. subtilis* (51, 61–63). Specifically, we tested QcrA production directed from the chromosomal *qcrA* gene in previously analyzed *bdbA-bdbB*, *bdbB-bdbC*, or *bdbC-bdbD* mutant strains (Fig. 3*a*). Because none of these mutations had a reproducible impact on the cellular and extracellular QcrA levels, we conclude that oxidative folding of QcrA is independent of the BdbABCD thiol-disulfide oxidoreductases of *B. subtilis*. Notably, the Bdb proteins are active at the extracytoplasmic side of the membrane, which could mean that disulfide bonding in QcrA takes place in a membrane-protected environment. We therefore investigated the localization of potentially cross-linkable Cys residues in QcrA-H102L and wild-type QcrA with the membrane-impermeable thiol-specific cross-linking reagent AMS. This analysis was aided by the fact that QcrA contains only four Cys residues, all of which are located at the 2Fe-2S co-factor binding site (Fig. 1). Therefore, protoplasts of cells producing either QcrA-H102L or wild-type QcrA were treated

with AMS. As shown in Fig. 3*b*, AMS cross-linking of QcrA-H102L and wild-type QcrA, which is reflected by a QcrA band up-shift upon NuPAGE and Western blotting, was only observed when the protoplast membrane was disrupted with 1% Triton X-100. This would suggest that the AMS cross-linkable Cys-100 and Cys-121 in QcrA-H102L (Fig. 1*b*), which lacks the co-factor, are located in a membrane-protected environment. Accordingly the adjacent disulfide-bonded residues Cys-105 and Cys-123 could also be located in this membrane-protected environment. The observed AMS cross-linking of wild-type QcrA would imply that AMS competes with the 2Fe-2S cofactor for binding to Cys-100 and Cys-121, eventually resulting in covalent AMS binding to these residues. Together, these findings would thus be consistent with the proposed proofreading hierarchy in disulfide bonding and co-factor binding.

DISCUSSION

The Tat system is unique in that it can translocate folded and co-factor-containing cargo proteins over the cytoplasmic membrane (8, 9, 64). There are a number of known requirements for successful Tat-dependent translocation of cargo proteins. These include the presence of an N-terminal twin-arginine signal peptide, a properly folded state of the cargo protein, and a functional Tat pathway (8, 9, 13, 64). In the present studies, the latter two requirements were investigated for the Tat-dependent membrane translocation of the Rieske protein QcrA of *B. subtilis*. Single amino acid substitutions were introduced into QcrA, which either precludes the oxidative folding of this protein or the insertion of its 2Fe-2S co-factor. In this manner, the importance of folding and co-factor insertion for the productive translocation of QcrA was shown.

Several previous studies have shown that, if cargo proteins of the Tat pathway are not correctly folded or if co-factors are inserted incorrectly, the membrane translocation process is terminated and the cargo degraded (4, 66–69). However, it is noteworthy that studies investigating disulfide bond-assisted folding in the Tat system have, thus far, only been performed on heterologous proteins or with *Escherichia coli* fusion proteins (1–3, 70, 71). Furthermore, spinach-derived Rieske proteins required a partially folded state to allow for co-factor insertion when expressed in *E. coli* (72). Our present study is the first to examine the Tat-dependent export of a single native protein, the Rieske protein QcrA of *B. subtilis*, which requires both co-factor binding and disulfide bond formation for its biological function. Importantly, the Rieske protein domain is highly conserved and residues involved in disulfide bond formation and co-factor insertion are well defined by high-resolution structural analyses (33, 40–42). On this basis, a total of three site-specific mutations in QcrA were generated; in two mutant proteins the His residues involved in co-factor binding were individually replaced (*i.e.* QcrA-H102L and QcrA-H124L), and in a third mutant protein a Cys residue involved in disulfide bond formation was replaced (*i.e.* QcrA-C123S). Membrane insertion and translocation of these mutant QcrA forms was assessed by Western blotting, taking advantage of the fact that translocated QcrA is to some extent processed by signal peptidase, which results in the release of a smaller form (QcrA*) into

the growth medium (28). It is presently not known why QcrA is processed by signal peptidase and why QcrA* is released into the medium, especially because the known QcrA biological function is electron transfer via the cytochrome *bc*₁ complex the cytoplasmic membrane. Nevertheless, by analyzing QcrA processing and QcrA* release into the medium we were able to show that all three QcrA mutant proteins were defective in complete translocation. Therefore, both the correct folding and co-factor insertion are critical for Tat-dependent translocation of QcrA. Interestingly, QcrA-C123S or fragments thereof remained undetectable by Western blotting or even in pulse-chase labeling analyses, indicating that this mutant protein was degraded within the 2 min needed to sufficiently label the QcrA protein with [³⁵S]methionine/cysteine. Here it should be noted that 2 min is a relatively long period of time when it comes to proteolysis which, depending on the substrate, can happen within seconds. In contrast, QcrA-H102L and QcrA-H124L produced from the same expression plasmid as QcrA-C123S were observed in the cytoplasmic and membrane fractions. These findings suggest a sequential and hierarchical quality control process with regard to the Tat-dependent cargo protein QcrA, where oxidative folding undergoes quality control before co-factor insertion. The subsequent membrane translocation and/or processing of QcrA are rather slow as we did not observe any QcrA* in our pulse-chase labeling experiments even after a 30-min chase period.

The idea of a sequential and hierarchical quality control process would be supported by the finding that disulfide bond formation in QcrA is independent of the extracytoplasmic thiol-disulfide oxidoreductases BdbA, BdbB, BdbC, and BdbD, and it would be consistent with the view that insertion of the 2Fe-2S co-factor takes place in the cytoplasm. In addition, our AMS cross-linking analyses indicate that the four Cys residues of QcrA, two of which form a disulfide bond and the other two coordinating the 2Fe-2S cofactor, are located in a membrane-protected environment. If so, this is the first illustration of a cytoplasmic proofreading or quality control mechanism for a Tat-dependent cargo protein in *B. subtilis*. In this respect it is noteworthy that, although the majority of cytoplasmic proteins are in a reduced state, some cytoplasmic proteins of *B. subtilis* have been shown to contain disulfide bonds under normal physiological growth conditions (73). It is thus possible that disulfide bond formation in QcrA can take place in the cytoplasm or, perhaps more likely, at the interface between the cytoplasm and the membrane.

It is conceivable that disruption of the disulfide bond in the QcrA-C123S mutant protein causes a structural change that also affects cofactor insertion even though the side chains of Cys and Ser are relatively similar in size. Therefore, the apparently high instability of the QcrA-C123S protein could relate to defects in both oxidative folding and co-factor binding. Here it is relevant to bear in mind that Rieske proteins are defined by their 2Fe-2S co-factor and the conserved co-factor binding motif. In contrast, not all Rieske proteins have or require the disulfide bond within the co-factor binding motif (33). This suggests that the 2Fe-2S-binding motif may not be dependent on the disulfide bond for co-factor insertion. Unfortunately, it will be extremely hard to further separate the requirements for

oxidative folding and co-factor insertion in QcrA of *B. subtilis*, as these processes are interlinked and both important. Moreover, such studies are further complicated by the production of many proteases in *B. subtilis*, which will rapidly degrade folding intermediates of QcrA (74, 75). In this respect, it was interesting to observe that the quality control system leading to the degradation of QcrA-C123S can be saturated by high level production of this mutant protein. This finding may eventually allow us to unravel the nature of the respective quality control pathway, which may be localized in the cytoplasm, the membrane, or the cell wall, or even extracellularly.

In summary, our present studies show that residues involved in 2Fe-2S co-factor binding and oxidative folding are essential for productive membrane translocation of the QcrA protein of *B. subtilis*. Interestingly, a proofreading hierarchy with regard to mutated QcrA was uncovered. The inability to form a specific disulfide bond within its Rieske domain caused the immediate degradation of QcrA, most likely in the cytoplasm, whereas mutant proteins lacking the residues needed for co-factor binding were apparently proofread at the interface of the cytoplasm and membrane. Although stably produced, the latter QcrA mutants were not detectably translocated. Last, these are the first studies in the Tat field where both folding and co-factor attachment have been addressed in a single native molecule. The relevance of these studies on QcrA, the Rieske protein of *B. subtilis*, is underpinned by the important roles of the cytochrome *bc*₁ complex in oxidative phosphorylation in all three domains of life.

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