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Mechanism and Hydrophobic Forces Driving Membrane Protein Insertion of Subunit II of Cytochrome bo_3 Oxidase

Nil Celebi, Ross E. Dalbey* and Jijun Yuan

Department of Chemistry
The Ohio State University
Columbus, OH 43210, USA

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Subunit II (CyoA) of cytochrome bo_3 oxidase, which spans the inner membrane twice in bacteria, has several unusual features in membrane biogenesis. It is synthesized with an amino-terminal cleavable signal peptide. In addition, distinct pathways are used to insert the two ends of the protein. The amino-terminal domain is inserted by the YidC pathway whereas the large carboxyl-terminal domain is translocated by the SecYEG pathway. Insertion of the protein is also proton motive force (pmf)-independent. Here we examined the topogenic sequence requirements and mechanism of insertion of CyoA in bacteria. We find that both the signal peptide and the first membrane-spanning region are required for insertion of the amino-terminal periplasmic loop. The pmf-independence of insertion of the first periplasmic loop is due to the loop's neutral net charge. We observe also that the introduction of negatively charged residues into the periplasmic loop makes insertion pmf dependent, whereas the addition of positively charged residues prevents insertion unless the pmf is abolished. Insertion of the carboxyl-terminal domain in the full-length CyoA occurs by a sequential mechanism even when the CyoA amino and carboxyl-terminal domains are swapped with other domains. However, when a long spacer peptide is added to increase the distance between the amino-terminal and carboxyl-terminal domains, insertion no longer occurs by a sequential mechanism.

Keywords: YidC; oxa1; membrane protein insertion; CyoA; membrane insertase

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Introduction

Membrane proteins comprise 30% of the total cellular proteins and have essential cell functions such as in bioenergetics, membrane transport, chemotaxis, cell division, and many other processes. The insertion and assembly of proteins into the membrane rarely occur spontaneously. Rather, there are translocation and insertion devices that facilitate this process. In bacteria, the SecYEG translocase catalyzes the insertion of the majority of proteins into the inner membrane.[1–3] The other membrane proteins are inserted and assembled into the membrane by the YidC membrane insertase.[4–6]

Like exported proteins, integral membrane proteins have hydrophilic domains that must be translocated across membrane bilayer. In addition, membrane proteins contain hydrophobic regions that reside in the membrane after the insertion process. To establish the membrane topology, membrane proteins are synthesized with topogenic sequences that specify for the translocation and membrane integration of the protein.[7] These topogenic elements function as cleavable signal peptides,[8] uncleaved signals,[9] reverse signal-anchors,[10] stop transfers,[11] and helical hairpins.[12] Cleavable signal peptides and uncleaved signals translocate C-terminal domains of proteins. Reverse signals, which have the opposite orientation of uncleaved signals, initiate translocation of N-terminal domains of membrane proteins. In contrast, stop–transfer domains do not play a role in translocation of hydrophilic domains but act as membrane anchors.[11]

*Corresponding author. E-mail address: dalbey@chemistry.ohio-state.edu.

Abbreviations used: PK, proteinase K; SRP, signal recognition particle; pmf, proton motive force; OmpA, outer membrane protein A; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Some membrane proteins have domains that insert as helical hairpins comprised of a signal peptide and a membrane anchor.[12]

Recently, the membrane biogenesis of subunit II (CyoA) of cytochrome bo_3 oxidase has been studied. CyoA is synthesized in a precursor form with an amino-terminal signal peptide (Figure 1). After processing by lipoprotein signal peptidase (also called signal peptidase II), CyoA spans the membrane two times with a short N-terminal tail and a large C-terminal domain.[13] Strikingly, the amino-terminal and carboxyl-terminal domains have different requirements for insertion.[14–16] The membrane insertion of the amino-terminal domain is carried out by the "YidC only" pathway while the carboxyl-terminal domain goes by the SecYEG pathway. Translocation of the amino and carboxyl-terminal domains of CyoA occurs primarily in a proton motive force (pmf)-independent manner.[14]

Here we investigated the mechanism by which CyoA inserts into the membrane. We show that both the signal peptide and the membrane anchor domain of CyoA are necessary for translocation of the short amino-terminal domain of CyoA. We find that translocation of the amino-terminal domain is pmf-independent due to the overall neutral charge of the periplasmic loop. When negatively charged residues are introduced into the loop, translocation becomes dependent on the pmf. Conversely, translocation of the loop is hindered by the pmf when positively charged residues are introduced. Additional evidence is presented to show that CyoA inserts by a strict sequential mechanism requiring the insertion of the amino-terminal domain prior to the insertion of the carboxyl-terminal domain.

Results

Both the signal peptide and the first transmembrane segment of CyoA drive membrane insertion of the amino-terminal domain of the protein

Pre-CyoA is synthesized with three hydrophobic regions [17] (Figure 1). The first, residues −16 to −1 is within the signal peptide[13] and is followed by a hydrophilic region (residues 1–26) that faces the periplasmic space. The second hydrophobic segment (residues 27–45) spans the membrane. The third hydrophobic segment (residues 69–87) spans the membrane with a large carboxyl-terminal domain (residues 88–291) exposed to the periplasmic space. Although the precise beginning and ending of the membrane-spanning regions is not known, the structure of the cytochrome bo_3 oxidase[18] suggests that membrane anchor domain 1 is much larger, beginning to span the membrane around residue 12. To examine the mechanism by which the amino-terminal domain of CyoA inserts into the membrane, we first used the pre-CyoA-N-P2 construct which has the leader peptidase P2 (lep P2) domain attached after the first transmembrane segment of CyoA. Previously we showed that using this construct the amino-terminal region of CyoA inserts by the YidC pathway.[14]

To assess the importance of the signal peptide and first hydrophobic domain of pre-CyoA for membrane protein insertion, we introduced positively charged arginine residues at various positions in these domains. It is well-established that the introduction of positively charged residues can perturb the function of topogenic sequences by disrupting the hydrophobic character of these sequences.[8–12] A single arginine was introduced at positions -14 and -9 of the signal peptide or at positions 32, 36 and 39 of the membrane anchor domain 1. We performed protease-accessibility studies to evaluate the effects of these mutations on translocation of the short periplasmic domain. Cells expressing pre-CyoA-N-P2 L(-14)R were pulse-labeled with [^{35}S]methionine for 2 min, then converted to spheroplasts, and subjected to protease mapping. As can be seen, the mature CyoA-N-P2 L(-14)R is observed in the pulse and it is digested to a shortened protected band by externally added proteinase K, indicating that the protein is inserted. Similar results were observed with the L(-9)R, I36R, and the V39R mutants (Figure 2(a)). It should be noted that there is a background lower molecular mass band that is observed in the "no protease" odd lanes in Figure 2(a). In pre-CyoA-N-P2 I32R with an arginine introduced at position 32 we see the

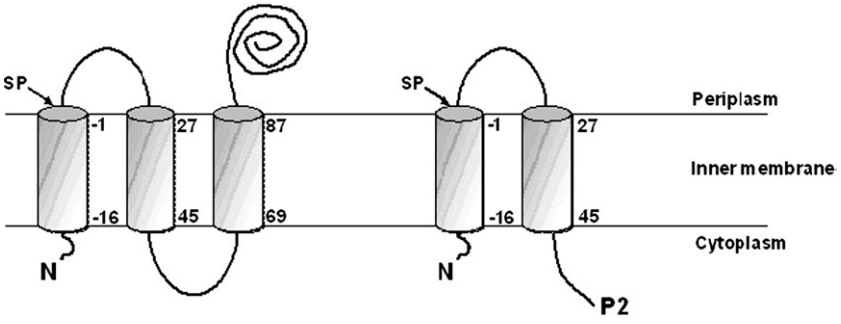

Figure 1. Membrane topology of full length pre-CyoA and the amino-terminal pre-CyoA domain. The full-length pre-CyoA is made with a cleavable signal peptide that is proteolytically removed by signal peptidase II processing. Pre-CyoA-N-P2 contains the Lep P2 domain added after transmembrane segment 1, allowing the construct to be immunoprecipitated using leader peptidase antiserum. SP represents the signal peptidase II cleavage site. The residues within transmembrane domain 1 and 2 were predicted from hydropathy plot and alkaline phosphatase fusions methods [17] although the structure of the *E. coli* cytochrome bo_3 oxidase [18] suggests the transmembrane segment 1 is much longer beginning around residue 12.

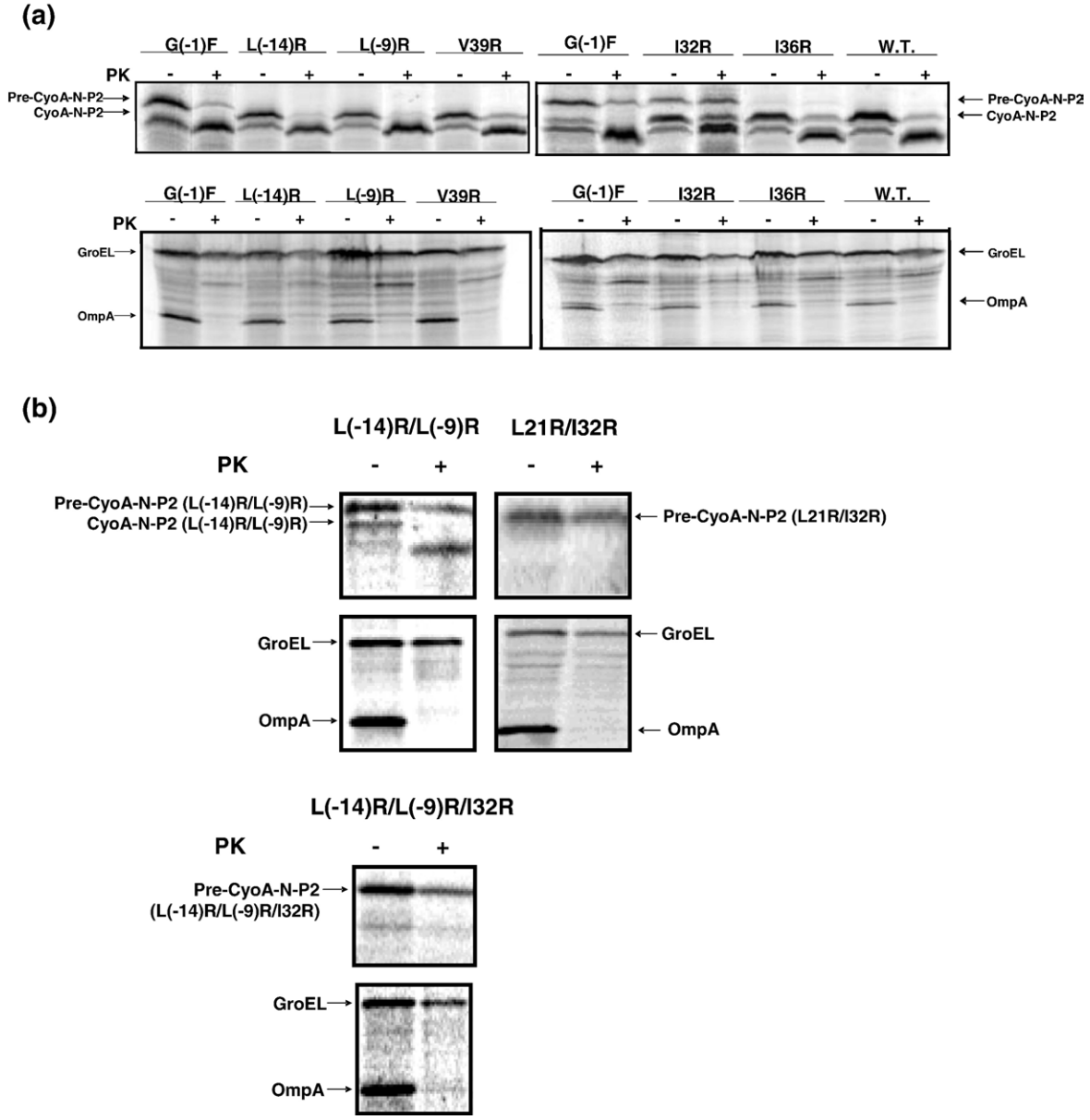

Figure 2. Membrane insertion of the amino-terminal domain of CyoA requires both the signal peptide and the membrane anchor domain 1. (a) Protease accessibility assay of the CyoA-N-P2 wild-type and single positively charged mutants. (b) Protease accessibility assay of CyoA-N-P2 double and triple positively charged mutants. DH5α cells expressing the indicated positively charged mutant of CyoA-N-P2 from plasmid pMS119 were grown to the mid-log phase and induced by the addition of 1 mM (final concentration) IPTG for 5 min. Samples were pulse-labeled with 50 μCi/ml of [^{35}S]methionine for 2 min, and analyzed by the protease accessibility assay as described in Materials and Methods. The protein samples were immunoprecipitated using leader peptidase antiserum (which recognizes the P2 domain) and analyzed by SDS–PAGE and phosphorimaging. GroEL, a cytoplasmic marker, and outer membrane protein A (OmpA), an outer membrane marker, were used as controls. G(-1)F pre-CyoA mutant was used as a precursor control. The lower band in the –PK lanes in (a) is a background band picked up with this batch of leader peptidase antiserum. A different batch of antiserum was used in (b).

precursor form accumulate. The precursor form runs at a similar position as the G(-1)F CyoA mutant which accumulates in the precursor form as processing by signal peptidase II is inhibited. As expected, the precursor form of CyoA-N-P2 I32R was resistant to proteinase K (PK) digestion while the mature form was mostly digested. In these studies, outer membrane protein A (OmpA) served as a positive control. Degradation of OmpA shows the efficiency of spheroplast formation. The cytoplasmic protein GroEL serves as a negative control. We use GroEL to show that the spheroplasts are intact.

Next, we introduced two arginine residues either into the signal peptide or membrane anchor domain 1 (Figure 2(b)). As can be seen, the precursor form of the L(-14)R/L(-9)R mutant with two arginine residues introduced into the signal peptide accumulates and is resistant to PK proteolysis. A complete block in translocation is seen with the L21R/I32R mutant where two arginine residues are

introduced into the membrane anchor domain 1. Note that L21 is predicted to be within the membrane-spanning region according to the structure of cytochrome bo_3 oxidase.[18] Finally, the introduction of three arginine residues completely blocks translocation for the L(-14)R/L(-9)R/I32R mutant. These results indicate that both the signal peptide and the membrane anchor domain are important for translocation of the central loop, consistent with this region of CyoA inserting as a helical hairpin.

pmf-independence of membrane insertion of CyoA is due to the neutral charge of the periplasmic tail

Previously, the insertion of the amino-terminal domain of CyoA was shown to be pmf-independent[14] unlike the insertion of the amino-terminal domain of the mitochondrial subunit II of cytochrome *c* oxidase which was absolutely dependent on the pmf.[19,20] We hypothesized that this difference is due to the negative charge of the N-terminal domain of the mitochondrial subunit II in contrast to the overall neutral charge of the same domain in CyoA with two acidic and two basic residues.[14] We tested this hypothesis by mutating the two positively charged residues at positions 9 and 17 to negatively charged residues and studying the insertion of this mutant by PK mapping. Cells expressing pre-CyoA-N-P2 K9D/R17D were treated for 45 s with or without carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), prior to labeling cells with [^{35}S]methionine. CCCP is a protonophore that dissipates the proton motive force.[21] Figure 3(a) shows that the radiolabeled

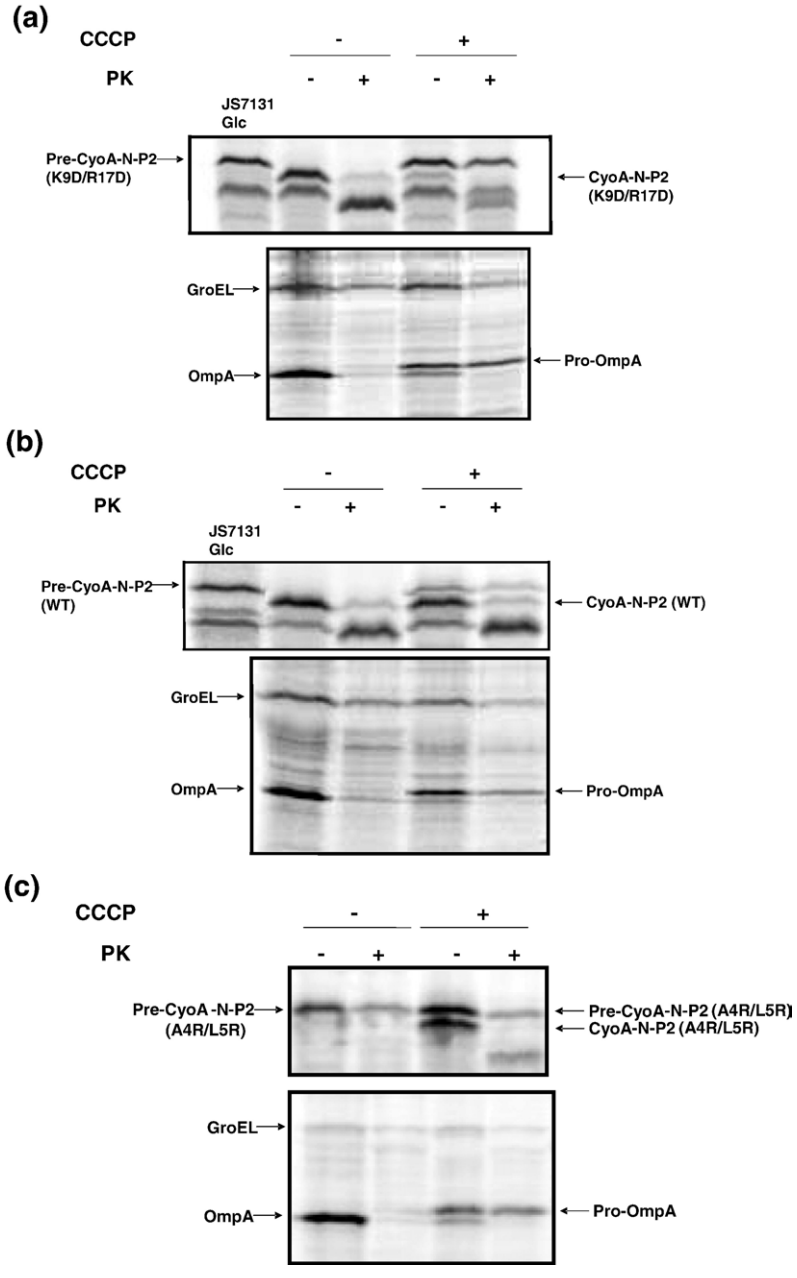

Figure 3. The pmf-dependence of membrane insertion of the periplasmic domain is determined by its net charge. (a) The pmf stimulates membrane insertion of a negatively charged CyoA-N-P2 (K9D/R17D) mutant. (b) pmf-independent translocation of periplasmic loop in the pre-CyoA-N-P2 (WT). (c) The pmf impedes the membrane insertion of a positively charged CyoA-N-P2 (A4R/L5R) mutant. DH5α cells expressing the indicated mutant of CyoA-N-P2 from plasmid pMS119 were grown to mid-log phase and induced with 1 mM (final concentration) IPTG for 5 min. Cultures were then labeled with 50 μCi/ml of [^{35}S]methionine for 2 min and analyzed by the protease accessibility assay. Where indicated, the pmf was abolished by treatment with CCCP (50 μM final concentration) for 45 s after IPTG induction. The protein samples were immunoprecipitated and analyzed as for Figure 2. The lower band in the –PK lanes (Figure 2(a) and (b)) is a background band picked up with the batch of leader peptidase antiserum. The position of the precursor form of CyoA was determined by accumulation of the precursor by growth of YidC-depletion strain, JS7131, bearing either the CyoA-N-P2 (K9D/R17D) or CyoA-N-P2 (WT) in glucose.

CyoA-N-P2 K9D/R17D mutant was strictly dependent on the pmf for signal peptidase processing and insertion. In the presence of a pmf, the mature form of CyoA-N-P2 K9D/R17D is observed and this is completely digested to a shifted fragment by PK treatment. On the other hand, in the absence of a pmf, the precursor form of pre-CyoA-N-P2 K9D/R17D is detected which is resistant to PK digestion. The bottom band in the "no protease" lanes is a background band recognized by the batch of leader peptidase antibody used in this study. We show that the pmf-dependent OmpA accumulates in a precursor form that is also protease-resistant. As a control, we confirmed that the wild-type Pre-CyoA-N-P2 inserts largely in a pmf-independent manner with only a trace of precursor detected when CCCP is added to abolish the pmf (Figure 3(b)). The periplasmic loop of pre-CyoA-N-P2 is neutral in charge with Asp, Lys, Glu and Arg residues at positions 7, 9, 15 and 17, respectively. Also, the position of the precursor form of CyoA-N-P2 WT and K9D/R17D was determined by accumulation of the precursor proteins by growth of the YidC-depletion strain JS7131 expressing these proteins in glucose (Glc)(see Figure 3(a), left lane, top panel).

The pmf-dependent insertion of the mutant preCyoA-N-P2 with four negatively charged residues in the periplasmic tail is consistent with the notion that pmf drives the translocation of the acidic tail segment. The positively charged periplasmic side of the membrane created by the pmf would promote the movement of a negatively charged region across the bilayer. Conversely, if an electrophoresis mechanism is operational here, then it is expected that the pmf would inhibit the membrane translocation of a positively charged domain. Figure 2(c) shows that the precursor form is detected for CyoA-N-P2 A4R/L5R where two positively charged residues were introduced into the periplasmic domain. However, when the pmf is abolished by the addition of CCCP, equal amounts of the mature and precursor form are observed. The addition of PK digests the mature form of the protein while the precursor form is largely resistant to proteolysis.

CyoA inserts by a sequential mechanism

Recently, the amino-terminal domain of CyoA was shown to insert by the YidC pathway, while the carboxyl-terminal domain inserted by the SecYEG pathway.[14–16] Surprisingly, the insertion of the large carboxyl-terminal domain of CyoA in the intact protein was dependent on YidC, but was not dependent on YidC when the carboxyl-terminal portion of CyoA was studied separately.[14] This is consistent with a sequential insertion mechanism where the amino-terminal domain must insert first in order for the carboxyl-terminal domain of the full-length CyoA to insert. To study the insertion mechanism, we introduced two positively charged residues into the signal peptide or membrane anchor domain 1 of the full-length CyoA and tested whether conditions that prevent insertion of the amino-terminal domain block carboxyl-terminal translocation of the CyoA protein. First, to identify the plasmid-encoded pre-CyoA-10His-L21R/I32R protein on the SDS–PAGE gel, we performed a −/+ IPTG induction experiment followed by pull-downs using metal affinity resins. In this experiment, we used the JS7131 strain to be able to analyze membrane insertion in the presence or absence of YidC. It is known that the precursor form of CyoA accumulates when YidC is depleted by growth of JS7131 in glucose media allowing us to determine which band is the precursor (see Figure 4(a), right two lanes). The addition of IPTG results in two induced bands (compare −/+ induction lanes), one of which runs at the same position as when YidC is depleted (see Glc lanes). Therefore, we assign the higher molecular band as pre-CyoA-10His-L21R/I32R. The lower induced band is assigned as the mature CyoA-10His-L21R/I32R. The PK data show that the mature form of the 10His-L21R/I32R is completely digested by PK while the precursor form is resistant to digestion by externally added PK (Figure 4(a)). Therefore, when the amino-terminal domain of CyoA does not insert across the membrane, the carboxyl-terminal domain does not insert.

The effects of the positively charged residues introduced into membrane anchor domain 1 on insertion may have to do with targeting since CyoA requires signal recognition particle (SRP) for insertion,[14–16] and the SRP was shown to interact specifically with membrane anchor domain 1.[15] To avoid this potential problem, we altered the signal peptide which has been shown not to interact with the SRP.[15] The hydrophobic character of the signal peptide was modified by introducing either two positively charged residues into the hydrophobic core or by deleting its hydrophobic domain. We then tested whether the translocation of the carboxyl-terminal domains of the accumulated precursor forms of CyoA is blocked. These mutations in the signal peptide are unlikely to perturb the SRP targeting, since the SRP only binds the membrane anchor domain of CyoA.[15] As can be seen in Figure 4(b), membrane insertion of ^{35}S-labeled CyoA L(-14)R/L(-9)R is inhibited and the precursor form can be detected (see the pre-CyoA-10His-L14R/I-9R band) upon IPTG induction (compare - + IPTG lanes) and pulled down with the nickel affinity beads. The precursor form that accumulates is not digested by PK, indicating that the C terminus of the protein is not inserted into the membrane. Similar results were obtained with the CyoA Δ (-15-(-7)) mutant that is seen by the IPTG induction. We confirmed that the positive control OmpA is digested.

A second way to show that the membrane insertion of CyoA occurs in an obligatory sequential mechanism is to make insertion of the amino-terminal domain pmf-dependent by introducing negatively charged residues into the periplasmic

tail. A strict sequential mechanism would therefore require the insertion of the carboxyl-terminal domain to occur in a pmf-dependent manner. Previously, we showed for the wild-type protein that insertion of the amino-terminal domain is pmf-independent while the translocation of the C-terminal domain is only slightly pmf-dependent.[14] Figure 4(e) shows almost no precursor form of pre-CyoA (WT) is detected when CCCP is added to collapse the pmf while the export of proOmpA is strongly inhibited and accumulates in a precursor form. Under these conditions, translocation of the carboxyl-terminal domain of the mature CyoA is inhibited to some extent as indicated by the shifted band upon adding protease (see *). Pre-CyoA-(-5) with L5E, K9D and R17D mutations in the periplasmic loop, inserts efficiently in the presence of a pmf (Figure 4(d)). However, abolishing the pmf by the addition of CCCP, prior to [^{35}S]methionine labeling, results in the accumulation of the precursor form of the pre-CyoA (-5). This indicates that the amino-terminal domain of CyoA did not translocate across the membrane when the pmf was dissipated. However, the precursor form is largely protease resistant, indicating the carboxyl-terminal domain has not translocated across the membrane. Only about 30% of pre-CyoA (−5) is digested by externally added PK (Figure 4(d)), compared to 70% of the CyoA in the wild-type situation when CCCP is added (Figure 4(e)). Taken together, these results reinforce a co-translational mechanism where the export of the amino-terminal domain must precede the export of the carboxyl-terminal domain.

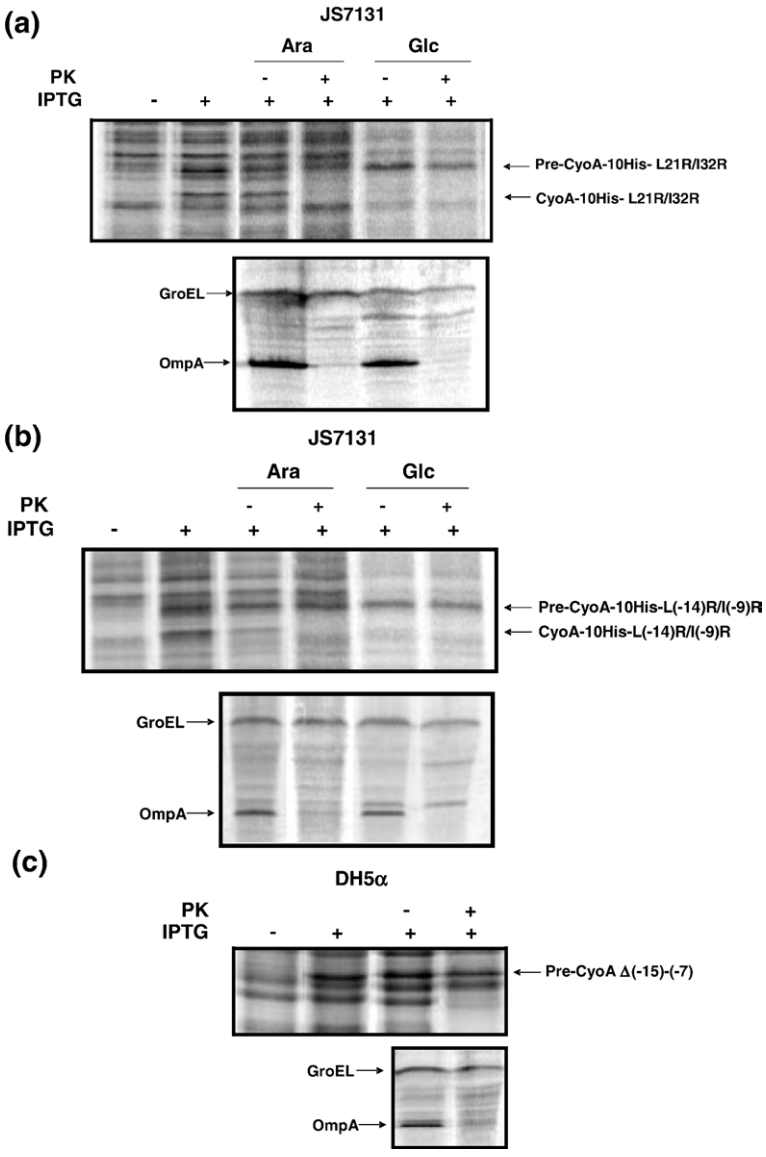

Figure 4. Translocation of the large carboxyl-terminal periplasmic domain of CyoA requires insertion of the amino-terminal domain. (a) Introduction of two positively charged residues into membrane anchor domain I inhibits membrane insertion. (b) Introduction of two positively charged residues into the signal peptide inhibits membrane insertion. (c) A deletion within the signal peptide blocks membrane insertion of CyoA. (d) pmf-dependent insertion of the carboxyl-terminal domain of CyoA containing acidic residues in the amino-terminal domain. (e) Slight pmf-dependent insertion of the CyoA carboxyl-terminal domain and pmf-independent insertion of the amino-terminal domain of the wild-type CyoA. JS7131 cells bearing the plasmid pMS119 containing the indicated positively charged CyoA-His$_{10}$ construct were grown under YidC expression (Ara) or depletion (Glc) conditions, the protein was induced with 1 mM IPTG for 5 min, pulse-labeled with 50 μCi/ml of [^{35}S] methionine for 2 min and analyzed for membrane insertion by the protease accessibility assay, as described in Materials and Methods. One JS7131 culture was treated without IPTG induction. DH5α cells expressing the indicated signal peptide deletion mutant of CyoA, pre-CyoA (-5), or pre-CyoA (WT) from plasmid pMS119 were grown to the mid-log phase and induced by the addition of 1 mM (final concentration) IPTG for 5 min. Samples were pulse-labeled with 50 μCi/ml of [^{35}S]methionine for 2 min, and analyzed by the protease accessibility assay as described in Materials and Methods. Where indicated, the pmf was abolished in DH5α cells by treatment with CCCP (50 μM) for 45 s after IPTG induction. The protein samples were then either acid-precipitated or pulled down using BD TALON™ metal affinity resin when a His-tag was present, as described in Materials and Methods, and analyzed by SDS–PAGE and phosphorimaging.

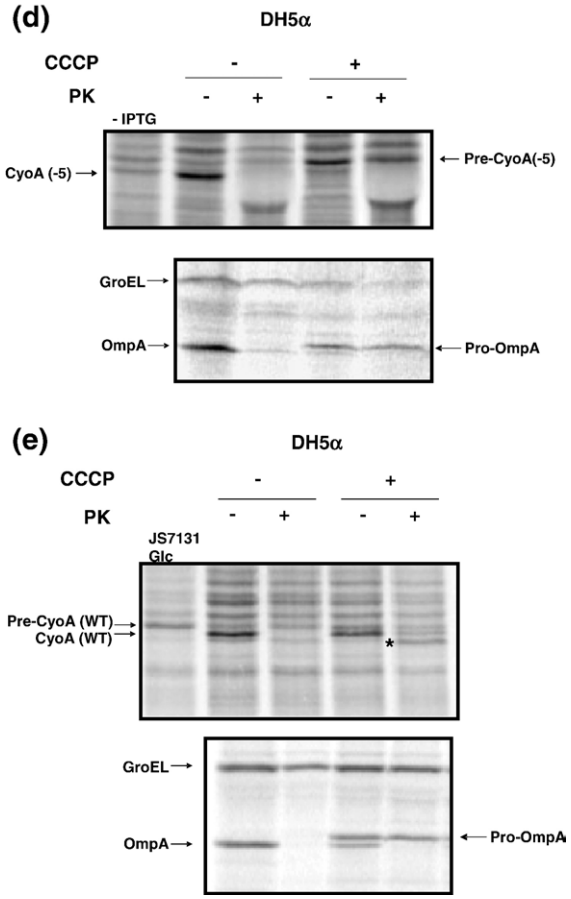

Figure 4 (*legend on previous page*)

Factors determining whether insertion goes by a strict cotranslational mechanism

Exactly how the failure of the amino-terminal domain to insert perturbs the translocation of the carboxyl-terminal hydrophilic domain *via* the SecYEG pathway is not known. However, it may have to do with the proximity of the domains since the CyoA carboxyl-terminal domain can insert when expressed separately,[14] or it may have to do with the specific nature of the amino-terminal or carboxyl-terminal domain, or both. To test whether distance that separates the two domains is the critical factor for a sequential mechanism, we separated the amino-terminal and carboxyl-terminal domains by the addition of ten proline residues. Ten proline residues would form an extended β-strand with a predicted distance of approximately 36 Å separating the two ends.[22,23] Figure 5(a) shows that most of pre-

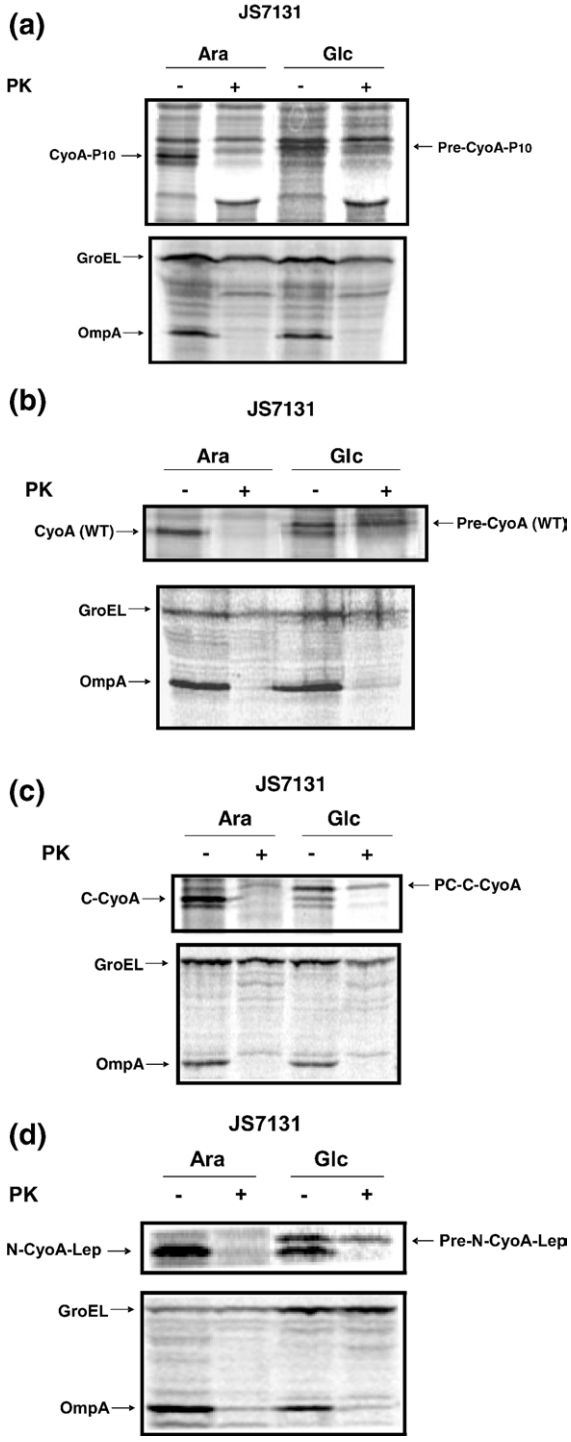

Figure 5. Addition of a long linker after membrane anchor 1 of pre-CyoA allows insertion to proceed by a non-sequential mechanism, but a sequential mechanism still occurs with different amino-terminal and carboxyl-terminal domains. YidC depletion studies with constructs CyoA-P_{10} (a), CyoA (WT) (b), PC-C-CyoA (c) and N-CyoA-Lep (d). JS7131 cells bearing the plasmid pMS119 with the indicated construct were grown under YidC expression (Ara) or depletion (Glc) conditions, the protein was induced with 1 mM IPTG for 5 min, pulse-labeled with 50 μCi/ml of [^{35}S]methionine for 2 min and analyzed for membrane insertion by the protease accessibility assay, as described in Materials and Methods. The protein samples were then either acid-precipitated (CyoA, top panel) or immunoprecipitated using GroEL and OmpA antisera (bottom panel) and analyzed by SDS–PAGE and phosphorimaging.

CyoA-P10 is processed to the mature protein by signal peptidase II and both termini of the protein are inserted (see Ara lanes). As can be seen, the mature form of [^{35}S]methionine pre-CyoA-P$_{10}$ is observed in the pulse and this protein is digested by externally added PK. Interestingly, if insertion of the amino-terminal domain is prevented by YidC depletion in the JS7131 strain by growth in glucose (Glc), the pre-CyoA-P10 mutant is digested by PK, showing that there is efficient translocation of the carboxyl-terminal domain (Figure 5(a)). This is very different than what happens with the wild-type CyoA. The precursor form of CyoA (WT) that accumulates under YidC depletion condition is resistant to PK digestion, as the carboxyl-terminal domain cannot translocate across the membrane if the preceding amino-terminal domain is not inserted (Figure 5(b)).

We next tested whether the sequential nature of the membrane insertion mechanism also had to do with the characteristics of the CyoA amino-terminal domain or the carboxyl-terminal domain. To test this, we replaced the amino-terminal domain of CyoA with the pro-coat region to make PC-C-CyoA or the carboxyl-terminal domain of leader peptidase to make N-CyoA-Lep. Figure 5(c) shows that the pre-CyoA derivative still largely inserts by a sequential mechanism even with pro-coat replacing the amino-terminal domain. Pro-coat, like the pre-CyoA amino-terminal domain, contains a cleavable signal peptide and has one transmembrane segment. Radiolabeled PC-C-CyoA is processed efficiently by signal peptidase and the mature protein is digested by added PK, indicating that the carboxyl-terminal domain translocated across the membrane (Figure 5(c)). However, when insertion of the amino-terminal domain of PC-C-CyoA is blocked by YidC depletion in JS7131, then the precursor form of PC-C-CyoA accumulates and approximately 45% of the protein is resistant to proteolysis. Similar results were found with the N-CyoA-Lep construct. The ^{35}S-labeled N-CyoA-Lep is rapidly processed and inserted across the membrane when YidC is present at a normal level with the large C-terminal Lep domain translocated across the membrane (Figure 5(d)). However, when the amino-terminal domain is blocked by YidC depletion, the carboxyl-terminal domain of *N*-CyoA-Lep is inhibited in membrane translocation and about 65% of the protein is resistant to proteolysis by PK digestion.

Discussion

CyoA, a two membrane-spanning protein, has sparked excitement recently because it uses two distinct pathways for the insertion of the two ends of the protein. The YidC pathway catalyzes the insertion of the amino-terminal domain and the SecYEG pathway facilitates the export of the carboxyl-terminal domains of the protein. In mitochondria, subunit II of cytochrome *c* oxidase requires Oxa1, the YidC homolog, for insertion of the amino-terminal domain.[24,25] Interestingly, both the mitochondrial and bacterial subunit II homologs are synthesized with a cleavable signal peptide that gets processed during export.

Here we found that the amino-terminal domain of CyoA requires both the signal peptide and membrane anchor domain 1 for insertion (Figure 2). The simplest interpretation of the data is that this region inserts as a helical hairpin with both hydrophobic regions driving insertion, although other mechanisms are possible. This helical hairpin insertion mechanism is reminiscent of the single-membrane spanning M13 pro-coat protein, which is also synthesized with a signal peptide.[12] Helical hairpins are involved in the pair-wise insertion of hydrophobic segments in several polytopic membrane proteins. This includes the tetracycline resistance protein,[26] the anion-exchanger domain of erythrocyte band 3 protein,[27] lac permease,[28] and yeast Sec61p.[29] It has been shown that hydrogen bonding between certain polar residues on different hydrophobic segments can promote the formation of helical hairpins during Sec-assisted translocation.[30,31]

A difference between the insertion of subunit II of the mitochondrial and bacterial cytochrome oxidase is that the mitochondrial subunit II absolutely requires the pmf for insertion,[19,20] whereas the bacterial subunit II inserts predominantly independent of the pmf.[14] We show here that this different pmf requirement is due to the fact that the mitochondrial subunit II has a highly negatively charged N-terminal luminal domain whereas the periplasmic domain of subunit II in bacteria has a neutral charge. In both bacteria and mitochondria, the pmf is such that it is negatively charged on the inside surface of the inner membrane and positively charged on the outside surface of the inner membrane. Therefore the pmf could promote the export of negatively charged residues by an electrophoretic mechanism. Indeed, when we introduce negatively charged residues into the periplasmic loop of pre-CyoA-N-P2, then insertion of this domain becomes pmf dependent (Figure 3). In contrast, when we add basic residues into the periplasmic loop then insertion is inhibited by the pmf. The hindrance of the transport of positively charged residues is expected because the positively charged residues are being moved to the positively charged side of the membrane.[32] However, in our study we did not determine whether it is the electrical component of the pmf that has its effects on the membrane translocation of charged residues. Nor can we distinguish whether the pmf has its translocation effects on the substrate directly or on the YidC insertase.

In bacteria, there is abundant data that show a correlation between the requirement of pmf and the translocation of negatively charged residues within the Pf3 coat and the M13 pro-coat proteins.[33,34] In addition, the introduction of additional positively charged residues into the translocated domain of the M13 pro-coat protein has been shown to impede the translocation across the membrane when a pmf is present.[35] The reason for this inhibition is partly due to the pmf. We have shown with the M13 pro-coat

protein that there is increased translocation of positively charged residues when the pmf is abolished.[32] Clearly, this negative effect of the pmf on the translocation of positively charged residues is a contributor to the positive inside rule which predicts positively charged residues flanking the hydrophobic segments of membrane proteins face the cytoplasmic side of the membrane.[36] However, it is not the sole determinant of this topological rule.[37,38]

In mitochondria, subunit II of cytochrome *c* oxidase inserts by a sequential mechanism, requiring export of the amino-terminal domain for the export of the carboxyl-terminal domain to proceed. Three pieces of evidence suggest that subunit II of cytochrome bo_3 oxidase in bacteria inserts also by a strict sequential mechanism. First, YidC depletion which blocks insertion of the amino-terminal domain also prevents insertion of the carboxyl-terminal domain.[14] Second, the introduction of positively charged residues either in the signal peptide or the membrane anchor domain 1, which block translocation of the amino-terminal tail, also block the export of the carboxyl-terminal domain (Figure 4). Third, blocking the export of the pmf-dependent amino-terminal domain by the dissipation of the pmf, also blocks the translocation of the pmf-independent carboxyl-terminal domain. Sequential insertion mechanisms for polytopic membrane proteins have been reported.[39,40]

A strict sequential mechanism as seen here with CyoA is believed to be unusual for membrane insertion of proteins. There are a number of examples where membrane proteins insert in a non-sequential manner and blocking of insertion of an upstream domain does not disturb the insertion of a downstream region.[41,42] Moreover, in some cases, membrane insertion of downstream regions of a protein can control the insertion of upstream domains.[43–45] These later studies suggest that there are topological determinants that are localized throughout the membrane protein and that long-range cooperation between hydrophobic domains can dictate the insertion and topology of a membrane protein.

While we do not know what determines the requirement for a strict co-translational mechanism of CyoA it is determined at least partially by the proximity of the amino and carboxyl-terminal domains. We found that moving the amino and carboxyl-terminal domains of CyoA further apart by inserting a rigid ten proline spacer (predicted to be 36 Å from the beginning and ending of the linker[22,23]) did change the insertion mechanism. Translocation of the carboxyl-terminal domain of the CyoA proline construct can occur even without the membrane insertion of the amino-terminal domain when YidC was depleted (see Figure 5(a)). However, the nature of the domains that insert by the YidC only pathway or the Sec pathway does not change this sequential mechanism radically. Replacement of either the amino-terminal CyoA domain with the M13 pro-coat, known to insert by the YidC only pathway, did not alter the insertion mechanism significantly and neither did swapping the carboxyl-terminal domain of CyoA with the Sec-dependent leader peptidase domain. Efficient insertion of the carboxyl-terminal domain still required the amino-terminal domain to insert into the membrane first. However, carboxyl-terminal translocation of the preCyoA constructs with the swapped domains was not completely blocked as it was for the wild-type pre-CyoA when the insertion of the amino-terminal domain was blocked. Future studies will investigate the molecular mechanism by which CyoA is recognized and inserted by the YidC and Sec machineries. These studies may provide insight into why CyoA inserts in an obligatory amino- to carboxy-terminal directionality.

Materials and Methods

Materials

Amino acids, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and lysozyme were obtained from Sigma. Proteinase K was purchased from Qiagen. Trans [^{35}S]-label, a mixture of 85% [^{35}S]methionine and 15% [^{35}S] cysteine (1000 Ci/mmol), was from ICN. Anti-GroEL antiserum was purchased from Sigma. Anti-Leader peptidase (anti-Lep) and anti-OmpA antiserum were from our laboratory collection. BD TALON™ metal affinity resin was purchased from BD Bioscience.

Strains, plasmids and growth conditions

The *Escherichia coli* strain JS7131, YidC depletion strain,[46] was from our laboratory collection. The constructs pMS119-PC-Lep and pMS119-pf3-Lep were from our laboratory collection. The constructs pMS119-CyoA, pMS119-CyoA-His_{10}, and pMS119-CyoA-N-P2 were from our laboratory collection.[14] Their positively and negatively charged derivatives and a signal peptide deletion mutant, CyoA-$His_{10}\Delta$((-15)-(-7)), were made by PCR mutagenesis using the appropriate construct as a template. The construct pMS119-CyoA-P_{10} was made by adding ten proline residues after the 50th residue of CyoA by mutagenic PCR. The construct pMS119-PC-C-CyoA was made by ligating the DNA sequence encoding amino acids 50–291 of CyoA to the end of the procoat gene in the construct pMS119-PC-Lep. Specifically, the gene encoding CyoA residues 50 through 291 (with EcoRI sites at the beginning and end of the CyoA region) was isolated after EcoRI digestion and ligated into the opened EcoRI site at the end of the procoat gene generated by EcoRI digestion of the pMS119-PC-Lep. The orientation of the ligated *cyoA* portion was confirmed by DNA sequencing, giving rise to pMS119-PC-C-CyoA. The construct pMS119-N-CyoA-Lep was made by isolating the BamHI fragment (encoding pf3-Lep) from pMS119-pf3-Lep and ligating it to the opened BamH1 site introduced immediately 3′ of the DNA encoding amino acid 50 of CyoA in pMS119-CyoA of pMS119-CyoA. After confirming the orientation of the insert, all residues between amino acid 50 of CyoA and amino acid 46 of Lep were deleted by mutagenic PCR giving rise to pMS119-N-CyoA-Lep. For YidC depletion,

JS7131 cells were grown to mid-log phase in LB supplemented with glucose (0.2%(w/v) final concentration) for 2 h and 30 min. The cells were switched to M9 medium containing glucose and grown for 30 min before labeling. The growth conditions used in expressing YidC were exactly the same except for the addition of arabinose (0.2%(w/v) final concentration) instead of glucose. The membrane insertion studies were done using the *E. coli* strain DH5α and JS7131.

Protease-accessibility studies

Cells were grown to mid-log phase as described above under various growth conditions. Expression of CyoA constructs was induced for 5 min by the addition of IPTG at a final concentration of 1 mM. For the pmf depletion studies, the cells were treated with a final concentration of 50 mM CCCP for 45 s prior to labeling. Cells were labeled with [^{35}S]methionine (50 μCi/ml) for 2 min and then converted to spheroplasts. The labeled cells were chilled on ice, pelleted in a microcentrifuge and subjected to lysozyme treatment in sucrose, Tris–HCl (pH 8.0) and EDTA buffer, as described.[10] Unless indicated, aliquots of the spheroplasts were treated either with or without PK (1 mg/ml) for 1 h on ice. After inactivating the protease by the addition of ice-cold 10% (w/v) trichloroacetic acid (TCA), the samples were immunoprecipitated with antibody against leader peptidase (Lep) (which precipitates P2), GroEL (cytoplasmic protein marker), and outer membrane protein A (outer membrane protein marker). Samples were then analyzed by SDS–PAGE using a 15% (w/v) polyacrylamide gel and phosphorimaging.[47] Where indicated, the His_{10}-tagged construct CyoA-C-His_{10} mutants were isolated following the PK accessibility analysis using a BD TALON™ metal affinity resin from BD Bioscience. After inhibiting the protease, samples were TCA-precipitated and acetone-washed, and the pellet was dissolved in 600 μl of 8 M urea buffer (8 M urea, 10 mM Tris–HCl, 100 mM NaH_2PO_4 (pH 8.0)). The samples were then incubated and shaken with resin (30 μl) at 4 °C overnight. The resin was washed twice with urea buffer, and then the His_{10}-tagged constructs were eluted from the resin with urea buffer (40 μl) containing 0.5 M imidazole.

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