Assembly of XcpR in the Cytoplasmic Membrane Is Required for Extracellular Protein Secretion in *Pseudomonas aeruginosa*

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A broad range of extracellular proteins secreted by *Pseudomonas aeruginosa* use the type II or general secretory pathway (GSP) to reach the medium. This pathway requires the expression of at least 12 xcp genes. XcpR, a putative nucleotide-binding protein, is essential for the secretion process across the outer membrane even though the protein contains no hydrophobic sequence that could target or anchor it to the bacterial envelope. For a better understanding of the relationship between XcpR and the other Xcp proteins which are located in the envelope, we have studied its subcellular localization. In a wild-type *P. aeruginosa* strain, XcpR was found associated with the cytoplasmic membrane. This association depends on the presence of the XcpY protein, which also appears to be necessary for XcpR stability. Functional complementation of an xcpY mutant required the XcpY protein to be expressed at a low level. Higher expression precluded the complementing activity of XcpY, although membrane association of XcpR was restored. This behavior suggested that an excess of free XcpY might interfere with the secretion by formation of inactive XcpR-XcpY complexes which cannot properly interact with their natural partners in the secretion machinery. These data show that a precise stoichiometric ratio between several components may be crucial for the functioning of the GSP.

*Pseudomonas aeruginosa* is an opportunistic pathogen causing chronic and acute infections in humans. Like many other bacterial pathogens of plants and animals, the virulence character of *P. aeruginosa* is multifactorial and associated with the elaboration of a large number of extracellular proteins with toxic or hydrolytic activities (25). During chronic infections in patients with cystic fibrosis, these enzymes have been implicated as important factors contributing directly or indirectly to the lung diseases (46). To be targeted to the surrounding environment, the secreted proteins must cross the two membranes enveloping this gram-negative bacterium. At least three distinct secretion pathways, each specialized for different substrate proteins, coexist in *P. aeruginosa* (14, 18, 48).

A majority of *P. aeruginosa* exoproteins are secreted by means of a two-step pathway (43), known as the type II or general secretory pathway (GSP) (34, 37). The first step of secretion, through the cytoplasmic membrane, is promoted by the presence of an N-terminal signal sequence and apparently occurs via a classical sec-dependent pathway (11). The second step, from the periplasm to the surrounding medium, requires the products of the xcp genes in *P. aeruginosa*. Genetic analysis of mutants defective for the secretion of proteases, lipase, and toxin A led to the identification of 12 genes (*xcpP* to *xcpZ* and *xcpA/pilD*) that are essential for protein translocation across the outer membrane (1, 5, 6, 14). Nucleotide sequence data revealed that these genes are homologous to the *pul* genes involved in pullulanase secretion by *Klebsiella oxytoca*, the first organism in which this pathway was identified (36). In recent years, similar sets of secretion genes have been found in a wide variety of gram-negative bacteria (20, 24, 31, 35).

The GSP mechanism involved in the outer membrane translocation step has not been determined, and there is little information about the structure or function of the majority of the secretion factors. XcpQ is an integral outer membrane protein that forms large ring-shaped homomultimers which may function as specialized pores (7). XcpT, -U, -V, and -W are structurally related to the type IV pilus subunits and are processed on the cytoplasmic face of the membrane by XcpA/PilD, a peptidase/methylase also required for pilus assembly (5, 30). An additional protein, XcpX, was recently shown to belong to the same family (the pseudopilins) and to also be processed by XcpA/PilD, although it contains an atypical N-terminal region (9). Four other proteins are located in the cytoplasmic membrane. XcpS is a polytopic protein with three transmembrane domains (3, 42); XcpP, -Y, and -Z span the membrane once, with their N termini in the cytoplasm (8). In XcpP and XcpZ, the transmembrane domain is close to the N terminus, so most of the protein is exposed to the periplasm. In contrast, the majority of XcpY is located in the cytoplasm, with a smaller domain extending into the periplasm. XcpR and its homologues have conserved sequences commonly found in nucleotide-binding proteins (Walker box A [45]). Mutations in these motifs result in defects in protein secretion (32, 33, 44), suggesting the involvement of these proteins in an energy-dependent step of the mechanism. Although one might expect such an activity to be performed by a membrane protein, a particularity of XcpR is an overall hydrophilic character suggesting a cytoplasmic location (5).

In this work, we investigated the cellular localization of XcpR and found that the protein is associated with the cytoplasmic membrane through an interaction with XcpY. These
findings corroborate and expand those previously obtained with EpsE and EpsL, the XcpR and XcpY homologues, respectively, in Vibrio cholerae (38). In addition, we present data implicating a stoichiometric relationship between XcpR and one or several additional components of the secretion system. These results are consistent with the idea of a multiprotein secretion complex organized within the envelope of gram-negative bacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. The Escherichia coli K-12 strain TG1 was used as a host for cloning experiments and for expression of constructs carrying xcpR and xcpY. The xcp-deleted strain DZQ40 is a derivative of P. aeruginosa wild-type strain PAO1 constructed as follows. A 8.5-kb deletion between the ScaI-1 site in xcpZ and the ScaI-6 site in xcpQ was created by coligation of the 1.1-kb EcoRI-1-ScaI-1 and the 1.1-kb Sall-6-Sall-7 DNA fragments into pUC19 (Fig. 1). Cohesive ends between the two DNA fragments were generated by previous cloning in the pUC19 polynlinker. This construction was introduced into P. aeruginosa PAO1 by electroporation (39). Since pUC19 cannot replicate autonomously in P. aeruginosa, its maintenance is dependent solely on its ability to integrate into the chromosome. Clones with plasmid integration were selected for on plates containing carbenicillin. The second homologous recombination event resulting in loss of the integrated plasmid and the xcp chromosomal region was generated during serial subcultures in the absence of antibiotic. Several carbenicillin-sensitive clones were obtained and tested for their secretion phenotype on tryptic soy agar plates containing either 1.5% skim milk (Difco Laboratories) or elastine (15). The 8.5-kb chromosomal deletion in clone DZQ40 was verified by Southern blot analysis (not shown).

The conjugative properties of pRK2013 (13) were used to transfer recombinant plasmids from E. coli to P. aeruginosa by triparental mating. Bacterial cells were grown at 37°C in Luria-Bertani broth for E. coli and in tryptic soy broth (TSB; Difco) for P. aeruginosa. When necessary, isopropyl-β-D-thiogalactopyranosidase (IPTG) was added to the medium to a final concentration of 1 mM.

TABLE 1. Bacterial strains and plasmids used

<table>
<thead>
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<th>Strain or plasmid</th>
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<th>Reference or source</th>
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<tr>
<td><strong>E. coli strains</strong></td>
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<tr>
<td>TG1</td>
<td>supE Δ(lac-proA) thi hsdRΔ5 (F’ traD36 proA’ B’ lacI’ ΔM15)</td>
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<tr>
<td>pop2136</td>
<td>endA thi hsdR mfd::cI857</td>
<td>O. Raibaud, Institut Pasteur</td>
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<td>DZQ40</td>
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<td>This study</td>
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<tr>
<td>KS910-503</td>
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FIG. 1. Genetic organization and restriction map of the P. aeruginosa xcp region at 40 min. The 10.3-kb DNA fragment carrying the xcpP to -Z genes is shown by a double line; the 8.5-kb chromosomal sequence deleted in strain DZQ40 is shown by a dotted line; locations of plasmid subclones are indicated by bold lines. Restriction sites: A, AusII; B, BamHI; Ba, BstI; E, EcoRI; N, NotI; S, SalI; Sc, ScaI; Sp, SphI; P, PstI. Only the relevant positions are indicated for the AusII, PstI, and NotI restriction sites.
Subcellular localization of XcpR in *P. aeruginosa*. Among the 12 identified Xcp components (XcpP to -Z encoded by the **xcp** gene cluster), XcpR is the only protein that lacks a hydrophobic domain and exhibits the general characteristics of a cytoplasmic protein (5). To determine its cellular localization, soluble and membrane fractions of *P. aeruginosa* wild-type strain PA01 were analyzed by SDS-PAGE followed by immunodetection with anti-XcpR antibody. As shown in Fig. 2A, XcpR was confined to the membrane fraction (lane 4) and was not detected in the fraction containing cytoplasmic and periplasmic proteins (lane 3). Separation of membrane fractions by centrifugation through a sucrose gradient showed that XcpR is associated with the low-density fractions corresponding to the cytoplasmic membrane. The nature of the association was further examined by incubating corresponding fractions in the presence of various reagents and studying the susceptibility of XcpR to extraction. Alkali treatments (0.1 N NaOH, 0.1 M Na₂CO₃ [pH 11]), high salt (1 M NaCl), or protein denaturant (5 M urea) released almost all XcpR, suggesting that it is peripherally bound to the cytoplasmic membrane. Treatment of membranes with the nonionic detergent Triton X-100 resulted in complete solubilization (data not shown).

Localization of XcpR in the absence of other Xcp proteins. Anticipating that XcpR might be membrane associated in *P. aeruginosa* through interaction with one or several Xcp proteins, we sought to determine its localization in the absence of the components encoded by the **xcp**P to -Z gene cluster. The 1.8-kb **PstI-AsuII** DNA fragment that carries the **xcp**P gene was cloned into pMMB67HE to give pMR1 (Fig. 1). In this construction, xcpR is expressed under the control of the **p lac** promoter. Plasmid pMR1 was introduced by triparental mating into strain DZQ40, a PAO1 derivative which is chromosomally...
deleted for the xcpP to -Z genes (see Fig. 1 and Materials and Methods for details on strain construction). Following DZQ40/pMR1 cell fractionation and immunodetection, the XcpR protein was detected mainly in the soluble fraction (Fig. 2B, lanes 2 and 3). The low amount of protein found in the particulate fraction was not membrane associated because it was only weakly soluble in 2% Triton X-100 (Fig. 2B, lanes 4 and 5). Such behavior suggested that upon overproduction, a minor fraction of XcpR could form aggregates segregating with membrane vesicles, as suggested earlier for the homologous PsLE protein of K. oxytoca (32). These results indicate that the association of XcpR to the cytoplasmic membrane of P. aeruginosa requires the expression of other Xcp proteins encoded by the 40-min locus.

Role of other Xcp proteins in the association of XcpR to inner membrane. The previous identification of an interaction between EpsL and EpsE, the XcpY and XcpR homologues in V. cholerae (38), was suggestive of a possible role of the XcpY protein in the membrane localization of XcpR. To test this hypothesis, the cloned xcpR and xcpY genes were expressed in the genetic background of the deletion strain DZQ40. Plasmid pLFR4, which carries xcpR under control of the native promoter, was mobilized into DZQ40. Figure 3A shows that XcpR was detected in cells of DZQ40/pLFR4 but was not recovered after the cell fractionation procedure (lane set 1). In contrast, the results in Fig. 2B show that XcpR overexpressed under p_tac promoter control in DZQ40 was stable during cell fractionation and recovered mainly in the soluble fraction. Possibly, a high expression level causes the accumulation of XcpR under a state, different from the native one, that increases the stability of the protein. Introduction of a second plasmid (pSB31, carrying xcpY) into DZQ40/pLFR4 led to increase in the level of XcpR. Under these conditions, XcpR was found to cofractionate mainly with the pellet (Fig. 3A, lane set 2), showing that XcpY alone can promote its association with the membrane of P. aeruginosa.

Although it seems clear that XcpY is required for the subcellular location of XcpR, the effect of XcpY could still be indirect, with some other, unidentified component providing a link between XcpY and XcpR. To further address this question, we examined the localization of XcpR in the heterologous genetic background of E. coli. When expressed alone in strain TG1/pMR1, XcpR was detected exclusively in the soluble fraction (Fig. 3B, lane set 1). The xcpR gene cloned on the compatible plasmid pSB34 was introduced into TG1/pMR1. Immunodetection after cell fractionation showed that approximately half of XcpR was membrane associated (Fig. 3B, lane set 2). The dual localization of XcpR in membrane and soluble fractions observed here might be related to some limitation in the number of XcpR molecules inserted into the cytoplasmic membrane.

Dosage-dependent complementation and interference in the xcpY51 mutant. Previous studies have shown that introduction of a wild-type xcpY gene in the xcpY51 mutant strain restores the secretion phenotype (14). To analyze the effect of the xcpY51 complementation on the localization of XcpR, we mobilized plasmid pSB31, carrying xcpY under p_tac control, into strain KS910-503 (xcpY51). As shown in Fig. 4B, XcpY was not detected in extracts of the control strain KS910-503/pMMB67HE (lane set 1). Sequencing of chromosomal DNA from xcpY51 identified a frameshift mutation that is responsible for the synthesis of a modified protein with a basic C-terminal domain that probably impairs membrane insertion and causes a rapid degradation (28). The presence of the wild-type xcpY gene in trans allowed production of a detectable amount of XcpY in KS910-503/pSB31 without IPTG induction (Fig. 4B, lanes 2). Protease plate assay (not shown) and im-
mumodetection of LasB elastase, the major protease secreted by the Xcp pathway (43), showed that the secretion defect of xcpY51 was complemented (Fig. 4A, lane set 2). It is known that LacI repression of the p_tac promoter is incomplete in P. aeruginosa (2), and the basal expression level of xcpY from uninduced p_tac on pSB31 thus appears sufficient for phenotypic complementation of the xcpY51 mutation. This complementation was correlated with the presence of XcpR in the membrane fraction, whereas trace amounts of XcpR were detected only in the soluble compartment of the control strain (Fig. 4B, lane sets 1 and 2).

To examine the effects of an increased expression of xcpY, we repeated the experiment in the presence of inducer. Addition of 0.1 mM IPTG during the growth of KS910-503/pSB31 resulted in a higher level of XcpY (Fig. 4B, lane set 3) and in a partial secretion defect, as shown by the accumulation of mature-sized LasB in total cell fraction (Fig. 4A, lane set 3). This result suggested that the overproduced XcpY might interfere with normal extracellular protein secretion. A small amount of XcpY, possibly representing molecules that failed to enter the export pathway to be inserted into the cytoplasmic membrane, was detected in the soluble fraction of induced cells. Immunodetection of XcpR showed that induced cells contained reduced amounts of the protein but did not reveal a marked difference in its subcellular distribution, indicating that the overexpressed XcpY efficiently binds XcpR to the membrane. The secretion defect was not the result merely of a decreased level of XcpR, because a stronger induction by 2 mM IPTG resulted in a complete block of elastase secretion (Fig. 4A, lane set 4) whereas the amount of XcpR was unchanged (Fig. 4B, lane set 4).

Since the XcpR-XcpY complexes were formed, we assumed that one or several other components of the secretion machinery were becoming limiting for interaction or coassembly with XcpY, XcpR, or both, so that the chromosomally encoded XcpR was displaced from the functional machinery by the excess of membrane-bound XcpY located outside the active secretion complexes. To test this hypothesis, we reasoned that if the negative effect is brought about by sequestration of XcpR from functional complexes, it should be overcome by the concomitant overexpression of XcpR. To this end, the xcpR and xcpY genes were cloned together under p_tac from pMMB67, to give pMYR. This construct was introduced into PAO1, and the effects of IPTG induction were analyzed in comparison to PAO1 carrying xcpY alone on pSB31. In the absence of induction, both strains secreted LasB into the extracellular medium (Fig. 5A, lane sets 1 and 3). In the presence of 2 mM IPTG, the LasB elastase produced by PAO1/pSB31 was accumulated inside the cells (lane set 2), while the protein was secreted by PAO1/pMYR and not detected in the cellular compartment (lane set 4). Immunodetection of XcpY and XcpR showed that the two proteins were efficiently overproduced from pMYR (Fig. 5B). We thus concluded that the increased amount of XcpR allows the restoration of a number of active secretion complexes because it can saturate the excess of XcpY responsible for the secretion interference.

Competitive inhibition by the soluble domains of XcpY. XcpY is an inner membrane protein with a single transmembrane segment which connects two hydrophilic domains (8). This topology is consistent with an interaction between XcpR and the N-terminal cytoplasmic domain of XcpY. To determine if this was the case, we constructed a 3’-truncated xcpY gene that lacks the sequences for the transmembrane and the C-terminal periplasmic regions. We introduced the truncated allele carried on pMYS in PAO1 and examined whether production of the cytoplasmic portion of XcpY (XcpYN) could compete for the XcpR-XcpY interaction. Data in Fig. 6B show that upon increasing synthesis of the XcpY_N product by induction of p_tac on pMYS, the majority (lane set 2) or the totality (lane set 3) of XcpR partitioned with the soluble fraction. Correlated with the displacement of XcpR from the membrane, secretion of extracellular LasB was impaired or abolished (Fig. 6A, lane sets 2 and 3). These results strongly indi-

![Image](http://jb.asm.org/16/11/figs/5a.png)  
**FIG. 5.** Phenotypic suppression of interference by XcpR overexpression. Wild-type strain PAO1 containing plasmid pSB31 (xcpY; lane sets 1 and 2) or pMYR (xcpR xcpY; lane sets 3 and 4) was grown in the absence (lane sets 1 and 3) or presence (lane sets 2 and 4) of 2 mM IPTG. (A) Proteins in cell lysates (i) or culture supernatants (o) were separated by SDS-PAGE, blotted, and probed with anti-LasB serum. (B) Equivalent amounts of cellular proteins were analyzed by SDS-PAGE and detected with anti-XcpY (left) or anti-XcpR (right) serum. Positions of molecular mass markers are indicated on the left in kilodaltons.

![Image](http://jb.asm.org/16/11/figs/6a.png)  
**FIG. 6.** Dominant negative effect of truncated XcpY protein. Strain PAO1 carrying pMYS (xcpY_N) was grown in TSB in the absence (lanes 1) or presence of 10 (lane set 2) or 100 μM (lane set 3) IPTG. (A) Same as for Fig. 5A. (B) Cellular proteins were separated by SDS-PAGE and immunodetected with anti-XcpR (top) or anti-XcpY (bottom) serum. The truncated form of XcpY (XcpYN) is indicated. Positions of molecular mass markers are indicated on the left in kilodaltons.
cate that the truncated XcpY exerts a dominant negative effect on secretion by competing with chromosomally encoded XcpY for the formation of XcpR-XcpY active complexes. Remarkably, the low level of XcpY\(^S\) produced in the absence of inducer was nevertheless sufficient to displace approximately half of XcpR from the membrane, while secretion was apparently not impaired (Fig. 6A, lane set 1). Thus, the secretory pathway can tolerate a large decrease in the number of functional XcpR molecules without effect on the efficiency of extracellular release, suggesting that the step involving XcpR is not limiting under the conditions used.

The accumulation of soluble forms of XcpR in the presence of XcpY\(^S\) indicates that XcpR has no permanent interaction with another membrane protein than XcpY. However, as the effects of XcpY overproduction (Fig. 4) support the existence of at least one additional partner interacting with the XcpR-XcpY complex, we considered the possibility that the C-terminal domain of XcpY is involved in interactions with some other component(s) in the periplasm. Plasmid pSB72 carries an in-frame gene fusion between the sequences encoding the LasB signal peptide (LasB\(^{28}\)) and those corresponding to the C-terminal periplasmic domain of XcpY (XcpY\(^S\); residues 259 to 382) (28). When the LasB\(^{28}\)-XcpY\(^S\) hybrid was expressed in PA01, the LasB signal peptide was cleaved off and the XcpY\(^S\) domain was immunodetected in the periplasm. However, LasB secretion was unaffected by the accumulation of this periplasmic form of XcpY (not shown).

**DISCUSSION**

In this report we show that XcpR, despite its hydrophilic character, is associated with the cytoplasmic membrane in *P. aeruginosa*. Membrane association is not an intrinsic property of XcpR but requires the xcpY gene product. XcpY spans the membrane once (8) and has a large N-terminal domain that faces the cytoplasm and likely interacts with XcpR. Consistent with this inference, the expression in *trans* of the soluble N-terminal region of XcpY resulted in a cytoplasmic location of XcpR and was inhibitory to protein secretion.

Previous studies by Sandkvist et al. (38) established that EpsE, the *V. cholerae* XcpR homologue, is associated to the cytoplasmic membrane through interaction with EpsL, the XcpY homologue in this bacteria. Our data obtained with the *P. aeruginosa* system support the prediction that the pattern of interactions taking place during the secretion process must be conserved in the GSP from different bacteria. However, it is notable that the *K. oxytoca* XcpR homologue, PulE, has been found associated with the particulate fraction in a reconstituted system in *E. coli* regardless of whether the other Pul functions were expressed. As suggested by Possot and Pugsley (32), this discrepancy could be related to a peculiar propensity of PulE to form small aggregates that associate with membrane vesicles when the protein is produced in the absence of the complete set of Pul secretion proteins.

Beside anchoring the XcpR protein to the cytoplasmic membrane, XcpY also appears to be required for its accumulation in *P. aeruginosa*. The cellular level of plasmid-expressed XcpR in the *xcpP* to -Z chromosomal deletion strain is considerably increased by the expression of a wild-type xcpY allele in *trans*. Similarly, the level of XcpR is very low in the *xcpY*-defective KS910-503 strain but restored to a wild-type level in the presence of XcpY. The reason for the apparent instability is unclear but may be due to an altered conformation in the absence of XcpY resulting in susceptibility to cellular proteases. In the *V. cholerae* system, the expression of EpsL also resulted in stabilization of the membrane-associated EpsE (38).

The relative stoichiometry between XcpR and XcpY seems to be crucial for the functioning of the secretion machinery. Overexpression of XcpY causes a negative secretion phenotype, although the membrane localization of XcpR is apparently not altered. Our interpretation of these observations is that the amount of chromosomally encoded XcpR is sequestered by the large majority of XcpY molecules that have failed to appropriately interact with the other component(s) of the secretory apparatus. The excess of XcpY might thus interfere with the assembly of the system because limiting amounts of other interacting Xcp proteins may not be able to provide final active secretion complexes. Concomitant overexpression of XcpY can suppress the inhibitory effect very likely by reestablishing a stoichiometric equilibrium with XcpY, so that the population of XcpY molecules correctly located can be saturated by XcpR. Consistent with the notion of at least a second protein-protein interaction involving XcpY, recent work of this laboratory indicates that XcpY has additional interactions with XcpZ (28). Furthermore, interference by XcpY overproduction appears to be partially relieved by an increased level of XcpZ, suggesting that this protein could be required for activity of the XcpR-XcpY binary complexes or their targeting to secretion sites. Of course, the observed relief of interference could be related to more indirect events; further work is needed to determine the primary effect of XcpZ overexpression that might lead to the phenotypic suppression effect.

Current data are insufficient to propose a definitive model for the function of XcpR family members in GSP. These proteins have a typical Walker box A which is essential for their activity (32, 38, 44). Although attempts to demonstrate ATPase activity were not successful (27, 38), these proteins could carry out functions related to an energetic step of the secretory pathway. By analogy with the role of the homologous protein Piib in type IV pilus assembly, it has been proposed that XcpR participates in the membrane organization of the five so-called pseudopilins XcpT, -U, -V, -W, and -X into a polymeric structure spanning the periplasm (5, 19, 34). The recent isolation of a mutation of xcpR that is capable of suppressing a temperature-sensitive allele of xcpT supports the possibility of an interaction between XcpR and pseudopilins (22). It might be also that XcpR participates in a gatekeeping function and somehow regulates the opening of the outer membrane pore constituted by XcpQ (7), possibly by modulating the polymeric state of the pseudopilins that could act as a plug in the absence of exoprotein movement through the channel. Whether it is required for steps in the assembly of a macromolecular Xcp complex or for the regulated activity of the secretory apparatus, XcpR probably does not act directly in the cytoplasm. On the basis of the association between XcpR and XcpY reported here, one appealing possibility is that XcpY contributes to coupling an energy-dependent activity of XcpR to the process of outer membrane translocation, maybe by transmitting a conformational change to upper components of the system. Further understanding of XcpR and XcpY interactions is required before such functional aspects can be addressed.

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