Topological Analysis of the Membrane-localized Redox-responsive Sensor Kinase PrrB from *Rhodobacter sphaeroides* 2.4.1*

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Photosynthesis gene expression in *Rhodobacter sphaeroides* is controlled in part by the two-component (Prr) regulatory system composed of a membrane-bound sensor kinase (PrrB) and a response regulator (PrrA). Hydrophathy profile-based computer analysis predicted that the PrrB polypeptide could contain six membrane-spanning domains at its amino terminus and a hydrophilic, cytoplasmic carboxyl terminus. Both the localization and the topology of the PrrB sensor kinase have been studied by generating a series of gene fusions with the *Escherichia coli* periplasmically localized alkaline phosphatase and the cytoplasmic β-galactosidase. Eighteen prrB-phoA and five prrB-lacZ fusions were constructed and expressed in both *E. coli* and *R. sphaeroides*. Enzymatic activity assays and immunoblot analyses were performed to identify and to localize the different segments of PrrB in the membrane. The data obtained in *E. coli* generally correlated with the data obtained in *R. sphaeroides* and support the computer predictions. On the basis of the theoretical model and the results provided by these studies, a topological model for the membrane localization of the PrrB polypeptide is proposed.

*Rhodobacter sphaeroides* can grow either aerobically (aerobic respiration) or anaerobically (photosynthesis/anaerobic respiration), as do many other purple bacteria. To adapt to these diverse environmental conditions, this bacterium has to continuously monitor light quality and intensity as well as oxygen tension. To adapt to the photosynthetic life style from aerobic growth, *R. sphaeroides* uses, in part, a two-component signaling system composed of a sensor histidine kinase and a response regulator that are able to control photosynthesis gene expression in response to decreasing oxygen tensions (1, 2). In this system, the sensor PrrB is believed to detect changes in oxygen levels by responding to change in either the flow of reductant or a reduct carrier through the *cbb*3/RdxB “redox” centers, ultimately leading to the activation of the response regulator PrrA. Activated PrrA then serves to modulate photosynthesis gene expression and to allow cells to rapidly adapt to the new growth conditions (3). Additional regulatory factors involved in the control of photosynthesis gene expression in *R. sphaeroides* have been discovered, and their interaction with the Prr sensory transduction proteins is still under investigation (for review, see Refs. 4 and 5).

Studies in both *R. sphaeroides* and *Rhodobacter capsulatus* (2, 3, 6), where the two-component activation system is designated RegBA, reveal the singular importance of this two-component activation system. This system has been shown to be involved in the control of the expression of the photosynthesis genes *puf*, *puh*, and *pac* encoding the reaction center and the light-harvesting antenna subunits (2, 7) and the *cycA* gene encoding cytochrome *c*2 (3). This system also serves to control those genes involved in carbon dioxide assimilation and nitrogen fixation (8).

In general, sensor proteins are composed of two domains: the input domain (generally within the amino terminus), which is responsible for sensing the “signal,” and the transmitter domain (generally at the carboxyl terminus), which is responsible for communicating with the response regulator (for review, see Refs. 9 and 10). The transmitter domain from PrrB shows homologies to other histidine kinases (2, 6). The PrrB polypeptide is 462 amino acids; autophosphorylation of PrrB involves the conserved histidine residue at position 221; and PrrB has been proposed to have both kinase and phosphatase activities (3). As to the PrrB input domain, it is still unclear how PrrB monitors changes in either the flow of reducton through the *cbb*3/RdxB redox centers or the change in concentration of a critical redox intermediate. It has been proposed that PrrB senses changes in oxygen tension indirectly by responding to changes in the localized redox state/intermediate, which in turn will result from changes in oxygen levels (11, 12). It has also been shown (11) that the Prr system is the obligatory intermediate between *cbb*3/RdxB and activation of photosynthesis genes. Although the cytoplasmic domain of RegB from *R. capsulatus* has been expressed and purified (13), the intact protein from either *R. sphaeroides* (PrrB) or *R. capsulatus* (RegB) has not yet been purified.

Genetic data from our laboratory suggest that PrrB is in close communication with other components of the cell, such as cytochrome oxidase *cbb*3, RdxB, and PrrC (11, 12). Because of their membrane localization and the nature of these proteins as reduct carriers, we believe that if there is to be a direct interaction between these proteins, then it will likely occur at or within the membrane space. This hypothesis is presently under genetic and biochemical investigation, but further understanding of these processes and the functional domains of the participating proteins requires a greater understanding and knowledge of their structural topology. For this purpose and as a important first step, we have begun an investigation of the membrane topology of the PrrB polypeptide. Until we are successful in purifying the intact PrrB protein, these indirect approaches should provide us with sufficient information to proceed with our functional analysis.

It was earlier suggested by Eraso and Kaplan (2) that PrrB might possess six transmembrane-spanning domains. The model developed for PrrB based on the hydrophathy profile of the amino acid sequence indicates six transmembrane helices.

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In this model, the amino and carboxyl termini of the polypeptide are predicted to be in the cytoplasm, with three loops in the periplasm and two loops in the cytoplasm. Gene fusion techniques with the *Escherichia coli* periplasmically localized alkaline phosphatase and the cytoplasmic β-galactosidase have been used as a genetic approach for the analysis of the topology of cytoplasmic membrane proteins from *Rhodobacter sphaeroides* and *R. capsulatus* (14–17). We therefore elected to investigate the topological model for *PrrB* by making phoA and lacZ fusions at numerous locations within the *prrB* gene. In this paper, we report the result of such topological analyses of *PrrB*, and we propose a model for the structure of the polypeptide in the cell membrane.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—*E. coli* strains were grown at 37 °C in LB medium. DH5α-phe (1) was used as a host for construction and expression of the *prrB-phoA* and *prrB-lacZ* fusions. *R. sphaeroides* strains were grown semi-aerobiically at 28 °C in SIS medium (18). Antibiotics were used at the following concentrations for *E. coli* and *R. sphaeroides*: spectinomycin, 50 μg/ml; streptomycin, 50 μg/ml; kanamycin, 25 μg/ml; and tetracycline, 10 and 1 μg/ml, respectively. Plasmids and phage DNA in this work are termed pSOP. These plasmids are replicative in *E. coli* and *R. sphaeroides* strains using the tri-parental conjugation system previously described by Davis et al. (19).

**Directed Mutations**—Alkaline phosphatase and β-galactosidase activities were detected as blue colonies on LB and SIS agar plates containing 5-bromo-4-chloro-3-indolyl phosphate and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, respectively, at a concentration of 40 μg/ml.

**Computer Hydropathy Analyses**—The *PrrB* protein sequence from *R. sphaeroides* was analyzed using the protein structure programs provided through the Internet by EMBL, Heidelberg; the Protein Sequence Analysis from the Biomolecular Engineering Research Center, Boston; the SosUI from Tokyo University of Agriculture and Technology; and the algorithm developed by Kyte and Doolittle (19). Enzymes and chemicals were purchased from New England Biolabs Inc. (Beverly, MA), Stratagene (La Jolla, CA), Promega (Madison, WI), and Sigma. Plasmid DNAs were purified using the Bio-Rad Quantum prep plasmid kit. DNA was treated with restriction enzymes and other nucleic acid-modifying enzymes (Klenow fragment, alkaline phosphatase, T4 DNA polymerase, and T4 DNA ligase) according to the manufacturer's specifications. DNA fragments were analyzed on agarose gels, and different restriction fragments were purified using the Gene Cleanup kit (Bio 101, Inc.). DNA sequencing was performed using an ABI 373A automatic DNA sequencer (Applied Biosystems Inc., Foster City, CA) at the DNA Core Facility of the Department of Microbiology and Molecular Genetics, University of Texas Health Science Center (Houston, TX). Site-directed mutations were constructed using the Stratagene Quick Site-directed mutagenesis kit according to manufacturer's instructions.

**Construction of pSOP Plasmids**—Plasmid DNA was mobilized into *E. coli* strains using the tri-parental conjugation system previously described by Davis et al. (21), with *E. coli* HB101 (pRK2013) as a helper strain. Exconjugants were selected on SIS plates supplemented with the appropriate antibiotics and the desired chromogenic substrate.

**Construction and Screening for prrB-phoA and prrB-lacZ Fusions**—The *Apal-KpnI* fragment from pUI132 (see Table I) containing three different reading frames coding for the mature alkaline phosphatase was cloned into the multiple cloning site of pBBR1MCS-2. The resulting plasmids are termed pSOP. These plasmids are replicative in both *E. coli* and *R. sphaeroides*. The *prrB-phoA* gene fusions were constructed as follows. The *prrB* gene was digested with the restriction enzymes cited in Table II, and purified *prrB* DNA fragments were treated with Klenow fragment or with T4 polymerase when appropriate to generate blunt-ended fragments, which were ligated into the Smal restriction site of the pSOP plasmids to generate in-frame *prrB-phoA* constructs. The orientations of the fusions were determined by restriction analyses, and the reading frame was confirmed by DNA sequencing using the phoA primer (5′-ACGCCGCGGTGTCAGTAATAT-3′).

To construct *prrB-lacZ* fusions, the *prrB* gene was digested with different restriction enzymes leading to individual blunt-ended fragments. The orientations and the in-frame reading of the fusions were analyzed by restriction analysis and then by DNA sequencing using the lacZ primer (5′-GGGATGGGCTGCAAGGCG-3′). All the *prrB-phoA* and *prrB-lacZ* constructs were made in a way that the expression of these fusions is under the control of the *prrB* promoter. The resulting plasmids (Table I) encode mature alkaline phosphatase and β-galactosidase, the former lacking its signal sequence fused to varying lengths of the N-terminal ends derived from *PrrB*.

**Site-directed Mutagenesis**—Since some regions at the 5′-end of the *prrB* gene did not contain any useful restriction sites, oligonucleotides were designed to introduce restriction sites following the linearized restriction sites (NaeI, SmaI, and Rsal). Six *prrB* mutations were generated using site-directed mutagenesis. The position and the sequence of each oligonucleotide as well as the residue substitutions are indicated in Table II. The constructed plasmids were confirmed by restriction analysis and then by DNA sequencing as described above.

**β-Galactosidase Assays**—Alkaline phosphatase and β-Galactosidase Assays—Alkaline phosphatase activity was determined as described by Brickman and Beckwith (22). *E. coli* or *R. sphaeroides* cells carrying *prrB-phoA* plasmids were grown in LB or SIS medium containing the appropriate antibiotic. When the culture reached A600nm = 0.5, 1 ml of each culture was centrifuged and washed once with 1 ml of ice-cold 1 mM Tris-HCl (pH 8.0). Pellets were reuspended in 1 ml of the same buffer, a drop of 0.01% SDS and chloroform was added; and the samples were incubated at 37 °C for 5 min to permeabilize the cells. 0.5 ml of the samples were then mixed with 0.5 ml of 0.8 μg/ml substrate for the alkaline phosphatase assay (p-nitrophenyl phosphate in 1 mM Tris-HCl (pH 8.0); Sigma) and incubated at 37 °C. When a yellow color started to appear, the time was noted, and the reaction was stopped by adding 200 μl of 0.5 M K2HPO4 (pH 8.0). Cell debris was removed by centrifugation, and the absorbance of the supernatants at 420 nm was recorded.

**Immunoblot Analyses**—Cells (*E. coli* and *R. sphaeroides*) harboring the different *prrB-phoA* encoding plasmids were grown to early logarithmic phase. 10 μl of each culture were resuspended in loading buffer (60 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 0.1% bromphenol blue, and 3% β-mercaptoethanol) and boiled for 3 min, after which the β-ml samples were loaded onto SDS-polyacrylamide gels (12%). After running of the gels, the proteins were transferred to nitrocellulose membranes by wet electrotransfer in transfer buffer (50 mM Tris, 380 mM glycine, 0.1% SDS, and 20% methanol). PhoA fusion proteins were detected with polyclonal antibody (at a dilution of 1:5000) directed against alkaline phosphatase and with goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase as the secondary antibody (at a dilution of 1:25000). *PrrB* PhoA polypeptides were detected on the protein blots by the alkaline phosphatase color detection system (Promega).

**RESULTS**

**Analyses of prrB-phoA Fusions**—To identify the hydrophobic segments of the *PrrB* polypeptide that could act as transmembrane-spanning regions, hydrophatic profile computer analysis of the amino acid sequence of *PrrB* was performed. This analysis suggested that *PrrB* is a membrane-bound protein and that the first 190 amino acids of a total length of 462 amino acids from the N-terminal of the protein should form the membrane-spanning domain as shown in Figs. 1 and 2. According to these analyses, both the amino and carboxyl termini of the protein should be located in the cytoplasm, and the protein should cross the membrane six times and should therefore possess three periplasmic and two cytoplasmic loops. To investigate these predictions, a series of 18 *prrB-phoA* fusion-derived proteins were constructed (Fig. 1). The design of the hybrid proteins was such that at least one fusion was placed in each loop facing either the periplasm or the cytoplasm. Additional fusions were placed in the putative transmembrane domains and in the C-terminal portion of the protein that was predicted to be cytoplasmic (Fig. 2). The *prrB-phoA* gene fu-
sions were made *in vitro* and encode hybrid proteins in which the amino terminus corresponded to the *R. sphaeroides* PrrB amino-terminal region and the carboxyl-terminal portion corresponded to the *E. coli* alkaline phosphatase lacking its signal peptide sequences. Plasmids containing the different fusions were expressed in *E. coli* DH5α-phe, and alkaline phosphatase activity was screened on 5-bromo-4-chloro-3-indolyl phosphate/LB plates.

Most of the fusions in *E. coli* displayed a white color, suggesting a cytoplasmic location of the PrrB junction region with the PhoA moiety. Only fusions 3, 9, and 12–14 produced pale blue colonies in *E. coli*, suggesting a periplasmic location for the PhoA moiety in these fusions. These results were confirmed by measuring the alkaline phosphatase activity in permobilized cells (see Table III). Only fusions 3, 9, and 12–14 displayed relatively high activities. It is well established that the *E. coli* alkaline phosphatase is active when it is exported to the periplasm, and inactivates in the cytoplasmic compartment (25). Based on these data, our results indicate that PrrB has three periplasmic loops. Additionally, fusions 1 and 15–18 were white and had low alkaline phosphatase activity, suggesting that both the N and C termini are located in the cytoplasm. According to these results in *E. coli*, the PrrB protein should possess six transmembrane domains; this is consistent with the theoretical model. However, the pale blue color observed in *E. coli* might indicate either a low level of expression or instability of the hybrid PrrB-PhoA proteins.

With these encouraging, but preliminary results in hand, we proceeded to follow the expression of these same constructs in *R. sphaeroides*. Plasmids were mobilized into *R. sphaeroides* strain PRRB1 from *E. coli*, and exconjugants were selected on plates containing the appropriate antibiotic plus 5-bromo-4-chloro-3-indolyl phosphate to directly screen for cells capable of expressing the fusion proteins. *R. sphaeroides* lacks significant endogenous alkaline phosphatase activity; therefore, alkaline phosphatase activities detected in the exconjugants should result from the expression of the hybrid proteins. Seven fusion strains displayed a blue color, suggesting a periplasmic location of the alkaline phosphatase moiety, and 11 were pink, suggesting cytoplasmic or membrane localization of the alkaline phosphatase. In general, the alkaline phosphatase activi-

### Table I

**Bacterial strains and plasmids**

| Strains and plasmids | Comment | Ref./source |
|----------------------|---------|-------------|
| **E. coli** DH5α-phe | F′80dlaczZAM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdRIr.imK≥ rKm' supE44 λ thi-1 gyrA relA1 phe-8 dcm | 1 |
| XL-1Blue             | supE44, hsdRIr, recA1, endA1, gyrA46, thi-1, relA1, lacF [proAB, lacIq, lacX74, Tn10(Tc')] lacY1 galK2 supE44 ara14 proA2 rpsL20 recA13 xyl-5 mtl-1 hisd20 mcrB mrr | Stratagene |
| HB101                | Wild type | W. Sistrom |
| **R. sphaeroides**   |         | 2 |
| 2.4.1                | pPrrB1   | 41 |
| Plasmids             | pSO1028  | This work |
| pBBR1MCS-2           | Expression vector (bom-, Km') | 39 |
| pBlueScript II       | pBSII cloning vector Ap', with T3 and T7 promoters | Stratagene |
| pUI1156              | pBSII containing phoA gene | a |
| pUI1158              | pBSII containing phoA gene | a |
| pUI1160              | pBSII containing phoA gene | a |
| pUI1265              | pBSII containing a ClaI-SmaI prrB fragment | b |
| pUI23A               | Translational lacZ fusion vector (bom-, Tc') | 24 |
| pSO1                   | PhoA from pUI1160 in pBBR1MCS-2 | This work |
| pSO2                   | PhoA from pUI1158 in pBBR1MCS-2 | This work |
| pSO3                   | PhoA from pUI1156 in pBBR1MCS-2 | This work |
| pSO1010               | prrB BsmI fragment in pSO1000 | This work |
| pSO1011               | prrB MsdI fragment in pSO1002 | This work |
| pSO1012               | prrB BglI fragment in pSO1002 | This work |
| pSO1013               | prrB NruI fragment in pSO1002 | This work |
| pSO1014               | prrB BstNI fragment in pSO1001 | This work |
| pSO1015               | prrB PvuII fragment in pSO1002 | This work |
| pSO1017               | prrB Xhol fragment in pSO1002 | This work |
| pSO1018               | prrB EcoRI fragment in pSO1001 | This work |
| pSO1020               | prrB ScaI fragment in pSO1002 | This work |
| pSO1021               | prrB StyI fragment in pSO1000 | This work |
| pSO1023               | prrB NaeI fragment in pSO1002 | This work |
| pSO1025               | prrB HincII fragment in pSO1000 | This work |
| pSO1028               | prrB NaeI fragment in pSO1002 | This work |
| pSO1029               | prrB SmaI fragment in pSO1001 | This work |
| pSO1030               | prrB RsaI fragment in pSO1002 | This work |
| pSO1031               | prrB RsaI fragment in pSO1000 | This work |
| pSO1032               | prrB SmaI fragment in pSO1000 | This work |
| pSO1033               | prrB SmaI fragment in pUI523A | This work |
| pSO1044               | prrB NruI fragment in pUI523A | This work |
| pSO1045               | prrB NruI fragment in pUI523A | This work |
| pSO1046               | prrB SmaI fragment in pUI523A | This work |
| pSO1047               | prrB PvuII fragment in pUI523A | This work |
| pSO1048               | prrB RsaI fragment in pUI523A | This work |

* M. Wood, J. M. Eraso, and S. Kaplan, unpublished data.

b J. M. Eraso and S. Kaplan, unpublished data.

c Sites that were introduced in the pprB gene following site-directed mutagenesis.
exhibited very low β-galactosidase activity, whereas fusion 6# exhibited the highest activity, consistent with a cytoplasmic exposure of the fusion. Fusion 5# exhibited a low but more intermediate activity. These results, taken together with the alkaline phosphatase activity for the same fusions, support the model proposed in Fig. 2. The slightly high alkaline phosphatase and β-galactosidase activities obtained with fusion 5 can possibly be explained by the inability of the fusions to completely reenter the cytoplasm. This problem has been reported in other topological studies using these approaches (26–29).

Furthermore, we analyzed the structure of each transmembrane-spanning domain using a computer program (30) to predict their secondary structure. According to this analysis, five transmembrane-spanning domains (I and III–VI) should have a pronounced α-helical structure. However, the program was unable to predict the structure for the second transmembrane-spanning domain (II; containing fusion 5). This transmembrane-spanning domain may possess a structure that permits this region to “move in or out” of the membrane depending upon the nature of the fusion protein, and this may explain the relatively high enzymatic activities observed with fusions in this region (see “Discussion”). In conclusion, results obtained with PrrB-PhoA and PrrB-LacZ fusion proteins expressed in either E. coli or R. sphaeroides are in accordance with the computer-predicted topology, are generally consistent, and suggest the same relative model for PrrB polypeptide topology.

Immunoblot Analyses—To ensure that the prrB-phaA fusions expressed in E. coli and R. sphaeroides were of relatively equal abundance, we performed Western blot analyses with an anti-alkaline phosphatase antibody on all of the strains described here. In E. coli, for all the fusion proteins constructed, only an immunoband corresponding to the mature alkaline phosphatase (43 kDa) was revealed, but no bands of the predicted size corresponding to the hybrid proteins were observed (data not shown). These results show that although the fusions were expressed in E. coli, the hybrid proteins are unstable and rapidly degraded. This could explain the very pale blue color of the colonies and the low alkaline phosphatase activities observed in E. coli. In R. sphaeroides, immunoblot analyses showed that the hybrid proteins were expressed, but only PhoA fusions that were predicted to be in the periplasm (fusions 3, 4, 9, and 12–14) and fusion 5 could be detected (Fig. 3). Its seems that the periplasmic fusions are more stable, probably because of the dimerization of the alkaline phosphatase that occurs only in the periplasm. These results correlated well with the high alkaline phosphatase activity detected in these periplasmic fusions (Table III). Hybrid proteins from fusions that are proposed to be in the cytoplasm could not be detected; probably because of the improper folding of the alkaline phosphatase in the cytoplasm, they were degraded, yielding free alkaline phosphatase and giving a low alkaline phosphatase activity. Other cross-reacting bands were detected, and they could correspond to degradation products; the band at 43 kDa could correspond to the free alkaline phosphatase. Hybrid PhoA proteins are often unstable and rapidly degraded; such instability of fusion proteins has been observed previously for different phoA fusions (28, 29, 31–34).

Topological Model for PrrB—Based upon the results presented in this study together with the theoretical predictions, the PrrB protein can be divided into two distinct domains: the amino-terminal domain from residues 1 to 183 comprises the membrane-spanning domain probably containing the as yet unknown input module responsible for sensing the hypotheti-cal redox signal, and the carboxyl-terminal domain from residues 184 to 462 forms the cytoplasmic domain comprising the transmitter module responsible for communicating with the response regulator PrrA. In the membrane-spanning domain,
the data best fit six transmembrane helices (I-VI) that are well defined. With both the amino-terminal end and the carboxy-terminal portion of the protein located in the cytoplasm (Fig. 2), the protein possesses three periplasmic loops and two cytoplasmic loops. In addition, computer analysis suggests the presence of an amphiphilic α-helix (Ala 251–Ala292) in proximity to the His221 presumed to be the phosphorylation site (2, 6).

DISCUSSION

The process of transmembrane signaling is of major importance in understanding signal transduction pathways in biological systems, specifically bacteria. Many studies have reported that the structural features of the proteins are implicated in membrane signaling and that alterations in signaling can induce conformational changes in response to environmental stimuli that can serve to transmit the signal intracellularly (10). Conformational and topological information for membrane-localized signal transduction proteins should be very helpful in targeting protein domains and/or segments that might play some role in the signaling process. Therefore, we have chosen to begin an examination of the topology of the PrrB protein, a sensor kinase from R. sphaeroides involved in signaling and gene regulation in response to changes in oxygen tension (1, 2). In the oxygen signal transduction pathway of R. sphaeroides, other membrane proteins such as cytochrome oxidase cbb₃, RdxB, and perhaps the PrrC polypeptide appear to be part of the signal transduction pathway that might interact directly or indirectly with PrrB (2, 12). We have recently shown that within the cbb₃ terminal oxidase, it is likely that the Q polypeptide is responsible for transmitting the signal from cbb₃ to PrrB (35). In this initial study, we have begun to investigate the membrane topology of the PrrB protein, which we believe will help in a more precise understanding of the oxygen signal transduction pathway involving this protein.

The R. capsulatus PrrB homologue (RegB) shows a high degree of similarity to the R. sphaeroides PrrB protein (2, 6); thus, one can anticipate that conserved structural features for both proteins should be informative as to their structure/function interrelationship. Mesley et al. (6) proposed a model in which RegB has five hydrophobic segments that could constitute a membrane-spanning domain. In such a model, either the N or C terminus should be periplasmic. The hydropathy computer analyses of PrrB revealed six hydrophobic segments with lengths between 18 and 20 residues; all of these were located in the first 190 amino acids of the amino terminus of the protein. However, experimental evidence in support of either model was lacking. The RegS protein from Bradyrhizobium japonicum, a
homologue of PrrB/RegB, does not show clear evidence of membrane-spanning domains (29).

To determine a structural model of PrrB, the topology of this protein was studied by generating fusions to the alkaline phosphatase PhoA and β-galactosidase LacZ moieties derived from E. coli. The topology of the fusion proteins was inferred from the level of alkaline phosphatase and β-galactosidase activities and was more strictly refined through the results in R. sphaeroides. The experimental data obtained from the characterization of the 18 PrrB-PhoA and five PrrB-LacZ fusion proteins gave a topological model that fits well with our hydropathy computer analyses. According to our results, both the N and C termini are located in the cytoplasm, supporting the presence of six transmembrane segments. Using this model, we identified three loops facing the periplasm, which is inconsistent with a model of five transmembrane segments (6). According to the model for PrrB presented here, the PrrB protein comprises two distinct domains with both amino and carboxyl termini located in the cytoplasm: (i) the anchor containing the membrane-spanning domain, is composed of six transmembrane helices, three periplasm loops, and two cytoplasmic loops and (ii) the catalytic region forming the cytoplasmic domain. This topology is also in general agreement with the “positive inside” rule, as most of the positively charged residues are located in the cytoplasmic loops (36).

### Table III

| Fusion | Plasmid | Activity in E. coli | Activity in R. sphaeroides |
|--------|---------|---------------------|---------------------------|
| A. Control | pSOP1 | 0.0 | 0.0 |
| 1 | pS01010 | 2.6 | 41 |
| 2 | pS01011 | 2.0 | 59 |
| 3 | pS01028 | 9.3 | 391 |
| 4 | pS01012 | 2.2 | 434 |
| 5 | pS01013 | 1.2 | 394 |
| 6 | pS01029 | 3.5 | 151 |
| 7 | pS01014 | 4.3 | 59 |
| 8 | pS01015 | 0.0 | 39 |
| 9 | pS01030 | 15.4 | 332 |
| 10 | pS01017 | 0.2 | 65 |
| 11 | pS01031 | 4.3 | 36 |
| 12 | pS01032 | 15.6 | 589 |
| 13 | pS01033 | 23.9 | 653 |
| 14 | pS01018 | 7.5 | 504 |
| 15 | pS01020 | 0.3 | 31 |
| 16 | pS01021 | 0.9 | 30 |
| 17 | pS01023 | 1.7 | 36 |
| 18 | pS01025 | 2.3 | 56 |
| B. C# | pUI1523A | 0.0 | 0.0 |
| 3# | pS01044 | 0.04 | 0.10 |
| 5# | pS01045 | 2.60 | 4.0 |
| 6# | pS01046 | 13.50 | 24.0 |
| 8# | pS01047 | 0.05 | 0.10 |
| 9# | pS01048 | 0.02 | 0.08 |

Computer analyses showed that the C terminus contains an amphipathic α-helix located in proximity to His221. This α-helix could serve either as a transmembrane span or as a site for interaction with other proteins. In the first case, we would expect that the C terminus should be on the periplasmic side of the membrane, but our PhoA fusion analyses strongly support a cytoplasmic C-terminal location. Thus, this amphipathic domain could serve other structural functions such as interaction with other proteins.

Most of the hybrid PrrB-PhoA proteins were unstable and probably degraded in E. coli. This phenomenon has been reported previously for different phoA fusions (28, 34, 37). Expression levels and/or stability of the PrrB-PhoA fusion proteins in R. sphaeroides was substantially higher than in E. coli, perhaps because of codon usage and/or the correct folding of PrrB within the membrane environment of R. sphaeroides; this was previously reported for PhoA fusions with other proteins from R. sphaeroides and R. capsulatus (14, 17). On the other hand, the cytoplasmic fusions were more unstable than the periplasmic fusions, probably for the reason that in the cytoplasm, these are unable to fold correctly and are degraded, whereas in the periplasm, dimerization of PhoA prevents its rapid degradation. The alkaline phosphatase activities obtained in E. coli correlated mostly with the data obtained in R. sphaeroides, allowing a clearer assignment as to the periplasmic location of the fusion junction of the hybrid proteins. The only exception to this topological assignment was fusion 5 and, to a much smaller extent, fusion 6; although these junctions were predicted to be in the transmembrane domain and the cytoplasm, respectively, we recorded high alkaline phosphatase activity in R. sphaeroides. A similar effect was obtained for fusion 5 when the phoA gene was replaced by the lacZ gene to create a LacZ fusion. We believe that because this fusion junction occurs near the middle of the second transmembrane domain where reentry of the chimeric protein from the periplasm takes place, improper folding of the chimera results in exposure of the PhoA moiety to the periplasm, where PhoA dimerization stabilizes the protein, and hence, activity is observed. The same situation could prevail with the β-galactosidase fusion; the protein should be retained within the membrane and not completely exposed to the cytoplasm, giving rise to low but discernible β-galactosidase activity. We should point out that in this

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**Fig. 3. Western blot analysis of the PrrB-PhoA fusion proteins from the constructs listed in Fig. 1.** Identical amounts of total protein (40 μg) were run on a 12% SDS-polyacrylamide gel, and the hybrid proteins were detected with the anti-PhoA antibody (see "Experimental Procedures"). The first lane corresponds to the strain containing the pSOP1 plasmid; the numbers at the top of each lane correspond to the fusions listed in Table I and in Fig. 2. Arrows show the bands that could be detected only in the periplasmic fusions. Molecular mass markers (in kilodaltons) are indicated to the right of the blots.
transmembrane-spanning domain, a cysteine residue is present, and this residue may alter the protein structure of the chimera in case of dimerization. However when considered in toto, the LacZ fusion results confirm and strengthen the PhoA fusion studies.

With regard to the general structural features of the PrrB protein, sequence analyses revealed homologies to other histidine kinases only in its cytoplasmic region containing the previously designated H, N, G1, F, and G2 boxes (2, 6), which are involved in phosphorylation of the response regulator. In addition, a sequence presenting an intriguing homology to the EAA conserved motif can be identified. Such a motif seems to play an important role in periplasmic transporters by interacting with hydrophilic components like ATPases (38). The role of such a motif (if any) in PrrB is still unknown; its location at the end of the amphiphilic α-helix may suggest a possible interaction of this portion of the protein with other cytoplasmic factors such as PrrA. This hypothesis will be tested by site-directed mutagenesis. The amino terminus does not show any homology to any other protein sequences to date. One can ask what the function of this portion of the protein is.

Based on the membrane topological model of PrrB presented in this paper and that best fits all of the data and on previous results presented by Eraso and Kaplan (2) on the analysis of the PRRB78 mutant (in which a substitution of leucine 78 with a proline in the first cytoplasmic loop dramatically affects phosphorylation), we can assume that the amino terminus of the protein responds to a redox signal by altering conformation, together with the L78P substitution in the cytoplasmic loop following this membrane span may suggest that this region of the protein interacts with a redox signal by altering conformation, which is an important function of the protein. Since PrrB seems to receive a redox signal of some sort involving electron transfer complexes such cytochrome oxidase cbb₃ (11, 12), it is thus tempting to speculate that PrrB may interact with membrane and periplasmic electron carriers or other proteins via this transmembrane portion of the polypeptide. Site-directed mutagenesis of the PrrB amino terminus is currently being carried out to test these assumptions, and our preliminary results support the conclusions reached here.

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