Determinants of membrane protein topology

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Contributed by Jon Beckwith, August 7, 1987

ABSTRACT The topology of the integral membrane protein MalF, which is required for maltose transport in Escherichia coli, has been analyzed using fusions of alkaline phosphatase (EC 3.1.3.1). The properties of such fusion strains support a MalF structure previously proposed on theoretical grounds. Several transmembrane segments within MalF can act as signal sequences in exporting alkaline phosphatase. Other transmembrane sequences, in conjunction with cytoplasmic domains, can stably anchor alkaline phosphatase in the cytoplasm. Our results suggest that features of the amino acid sequence (possibly the positively charged amino acids) of the cytoplasmic domains of membrane proteins are important in anchoring these domains in the cytoplasm. These studies in conjunction with our earlier results show that alkaline phosphatase fusions to membrane proteins can be an important aid in analyzing membrane topology and its determinants.

We recently described a genetic approach to analyzing the topology of integral membrane proteins (1). Using a membrane protein with a relatively simple and well-defined structure (the tsr-encoded serine chemoreceptor of Escherichia coli), we showed that the properties of alkaline phosphatase (EC 3.1.3.1) fusions to the protein correlated with its topology. Alkaline phosphatase fused to the extracytoplasmic (periplasmic) domain of Tsr protein exhibited high alkaline phosphatase enzymatic activity. Alkaline phosphatase fused to the cytoplasmic domain of the protein showed very low enzymatic activity. This strict correlation of the enzymatic activities of hybrid proteins with the cellular location of the domain to which alkaline phosphatase was fused suggested that this fusion approach could be used to determine the topologies of proteins of unknown disposition in the membrane.

In this paper, we present experiments that extend this approach to a membrane protein of complex topology. This protein, the malF gene product, is required for maltose transport in E. coli (2). The DNA sequence of the malF gene revealed the existence of eight possible transmembrane sequences within the protein (3). These putative transmembrane segments are composed of stretches of 20 or more amino acids that are uncharged and have a high proportion of hydrophobic amino acids.

The amino acid sequence of MalF suggested the model (shown below) for the arrangement of the protein in the membrane (3).

The transmembrane segments of MalF protein in the model correspond to the hydrophobic sequences. The amino term minus of MalF was positioned in the cytoplasm because of a cluster of positively charged amino acids that precedes the first presumed transmembrane segment. von Heijne (4) has shown that the charge distribution around transmembrane segments is such that there is usually a net positive charge at the cytoplasmic end of such stretches. The representation of the fourth and seventh transmembrane segments implies nothing about their actual structure but simply reflects the longer lengths of the hydrophobic stretches in these two cases. Support for this structure for MalF comes from studies on fusions of β-galactosidase to the MalF protein (5).

In this paper, we describe experiments that show that fusions of alkaline phosphatase to the MalF protein have properties that are consistent with the proposed structures. These results indicate that the alkaline phosphatase fusion approach can be a useful aid in analyzing the membrane arrangement of proteins of complex as well as simple topologies.

MATERIALS AND METHODS

Bacteria, Phage, and Plasmids. E. coli strains used were CC118(F′lacIq pro) (ref. 6); DBH3, which is araD139 Δ ara-leu)7697 Δ lacX74 Δ phoA Pvu II (ref. 7) phoR Δ malF3 [a nonpolar deletion of the malF gene (ref. 3) from base pair –88 to base pair [404] galE galK thi rpsL ]; DBH4, which is DHB3 (F lacIq pro); DHB24, which is DHB4 pcnB zad::Tn10 (ref. 8); DBH5060, which is lacZam argEam rpoB rpsL araD139 Δ ara-leu)7697 galE galK thi hsr “hsm” λ CH616 [which expresses M13 gene II (ref. 9)] pOXGen [a derivative of the F factor pOX8] (ref. 10) carrying a gene for resistance to gentamicin (ref. 11) plQ [a derivative of pACYC184 (ref. 12) carrying lacIq]; and BW10724, which is (α recA Δ lacD169 proS10 thi rpsL , a gift of B. Wanner. The plasmid pSF691 is described in ref. 3; pHS17 (a gift of H. Shuman) contains the E. coli malG gene and part of the malG gene expressed under the tac promoter of pKK223-3 (ref. 13); and pDH32 is similar but contains the M13 intergenic region from pZ150 (ref. 14).

Media and Enzymes. Media were made according to Miller (15). T4 DNA ligase was purchased from Pharmacia. T4 DNA polymerase and T4 gene 32 protein were obtained from Boehringer Mannheim. Both enzymes were obtained from New England BioLabs and were used according to the manufacturer’s suggestions.

Assay of Alkaline Phosphatase. Fresh stationary-phase cultures in LB broth were diluted 100-fold and were grown for 2 hr and 40 min at 37°C before assay as described (16). A background of 0.1 unit has been subtracted from each value.

Isolation of Transposon Insertions. Transposon insertions were isolated using two different protocols. The Flac– TnphoA method (6) was used to isolate fusions d2 and d44 in pSF691/CC118(F lacIq pro), fusions 17-1 and 17-3 in pHs17/CC118(F lacIq pro), and fusions 160, 121, 165, and 3-826 in pDH32/DHB4. Fusions 4.30c, 4.39b, and 4.29c in

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pDHB32/DHB24 were isolated using a TnphoA as described (17), except that 5 × 10^6 phage particles were used to infect 2 × 10^9 cells in 5 ml of LB broth. After 1 hr at 37°C, the culture was diluted into 100 ml of LB broth containing kanamycin (30 μg/ml) and ampicillin (100 μg/ml) and was divided into 50 aliquots, which were incubated overnight at 37°C. Phage f11R1 (18) lysates were prepared from 0.1 ml aliquots of each of the 50 independent, resistant cultures as described (15). These lysates have approximately equal numbers of phage particles and transducing particles that contain single-stranded plasmid DNA. The lysates were used to transduce DHB24 with selection for resistance to kanamycin (40 μg/ml) and ampicillin (200 μg/ml) to isolate transductant cells carrying plasmids with transposon insertions. Between 0.1% and 10% of the transductant colonies were blue on plates containing the chromogenic indicator of alkaline phosphatase activity, 5-bromo-4-chloro-3-indolyl phosphate. Prior to analysis, fusions not isolated in pDHB32 were cloned into this plasmid in vitro.

**DNA Sequence Analysis.** Plasmids coding for fusion proteins were reduced in size by deletion of the transposase and kanamycin resistance genes in vitro using Sau I and Xho I restriction enzymes (Fig. 1). Dideoxy sequence analysis (19) to locate function positions was carried out using single-stranded template DNA obtained from f11R1 phage lysates grown on plasmid-containing strains (14).

**Oligonucleotide-Directed Deletion Mutagenesis.** To construct phoA fusions to selected sites in malf such that the fusions are isogenic to those generated by TnphoA transposition, we synthesized 43- to 48-mer oligonucleotides with 23 residues complementary to the 5' end of TnphoA and the rest complementary to the target site in malf. [The oligonucleotides were prepared by Steve Lin on Applied Biosystems (Foster City, CA) apparatus.] Oligonucleotides were phosphorylated with T4 polynucleotide kinase, and 40 ng was annealed to 2 μg of single-stranded DNA prepared from an f11R1 phage lysate grown on a strain containing the parent fusion plasmid (Fig. 1). The oligonucleotide primer was extended by incubation for 2 hr at 37°C with T4 DNA polymerase, T4 gene 32 protein, and T4 DNA ligase (20). DNA remaining single stranded was digested with mung bean nuclease. Between 1% and 50% of the transformants obtained in DHB24 contained plasmids with the new fusion.

**Recombination of Fusions into the Chromosome.** A derivative of each of the pDHB32 fusion plasmids was constructed in which the plasmid replicative origin was deleted using restriction endonucleases BamHI and Ava I as shown in Fig. 1. DNA molecules that contain the M13 intergenic region but have no plasmid replicative origin can be maintained as plasmids in strains (such as DH5B060) that express the M13 gene II protein (9). Origin deletion plasmid DNA containing each fusion was prepared from a DH5B060 derivative and used to transform DHB4 by selecting resistance to ampicillin (25 μg/ml). The transformants, which were obtained at a frequency about 10^-8 of that expected for origin-containing plasmid DNA, appear to have one or more copies of the originless plasmid integrated by recombination at the phoA locus on the chromosome. Integrated plasmids were stabilized by transduction of recA::cat into the strains. The number of integrated plasmids in some of these strains was determined to be only one by testing f11R1 lysates prepared on them for transducing particles. Strains with two or more originless plasmids integrated at phoA package transducing particles efficiently, whereas those with only one originless plasmid package fewer (by a factor of 10^-5) transducing particles (unpublished results).

**Proteolysis and Fractionation.** Osmotic shock fractionation was carried out as described (1) except for the differences noted below. Exponential cultures were induced with 5 mM isopropyl-β-D-thiogalactoside (Bachem, Torrance, CA) for 30 min before washing. They were then pulse labeled with 60 μCi of [35S]methionine per ml (106 Ci/ml; Amersham; 1 Ci = 37 GBq) for 1 min and were incubated in the presence of unlabeled methionine for 10 min. Washed cells were resuspended in spheroplast buffer (0.1 M Tris-HCl, pH 8/0.5 M sucrose/0.5 mM EDTA), incubated 5 min on ice, and centrifuged. The pellet was warmed to room temperature, resuspended in ice-cold water with or without trypsin (type XIII, Sigma), incubated for 5 min on ice, and separated into periplasmic and cellular (membranes plus cytoplasm) fractions by centrifugation in the presence of 0.5 mM phenylmethylsulfonlfyl fluoride (Sigma). The two fractions were then precipitated with trichloroacetic acid and were analyzed by immunoprecipitation and sodium dodecyl sulfate polyacrylamide gel electrophoresis (NaDodSO4/PAGE) (6). Parallel unlabeled preparations were assayed to determine the fractionation of alkaline phosphatase activity (16) and a cytoplasmic marker, glucose-6-phosphatase dehydrogenase activity (21).

**RESULTS**

**In Vivo Fusions and Properties.** We have isolated malf-phoA gene fusions after transposition of the transposon TnphoA into plasmids carrying malf (see Materials and Methods). Transposition events resulting in significant alkaline phosphatase activity were identified by the formation of pale blue to dark blue colonies on media containing the
indicator dye 5-chloro-3-bromo-indolyl phosphate. To determine the sites of fusion of alkaline phosphatase to MalF, plasmids were analyzed by restriction mapping and by DNA sequencing. In addition, levels of alkaline phosphatase activity in permeabilized cells carrying the fusion plasmid were assayed. Finally, the amounts and stabilities of the hybrid proteins were analyzed by precipitation with anti-alkaline phosphatase antibody and NaDodSO₄/PAGE.

The results of these studies are summarized in Fig. 2. Fusions at nine different sites in MalF had similarly high levels of alkaline phosphatase activity. This group comprised fusions in each of the four proposed periplasmic domains of MalF. These fusion strains all made stable fusion proteins of the predicted size (data not shown). Two other fusion plasmids isolated from transformant colonies showing paler blue color had fusion junctions at the carboxyl end of a membrane-spanning stretch crossing from the periplasm to the cytoplasm according to our model for MalF (fusions 17-1 and d2b, see Fig. 2). These fusions are at sites predicted to be cytoplasmic in the model, yet they show 30–100% of the enzymatic activity of fusions to the periplasmic domains. In our previous studies with a membrane protein of simpler structure, alkaline phosphatase fusions to the cytoplasmic domain exhibited enzymatic activities only 2–5% of those seen with fusions to the periplasmic domain (1). We obtained no other fusions to putative cytoplasmic domains by this approach.

It seemed possible that the intermediate activity of fusions 17-1 and d2b was due to the absence of amino acid sequences more carboxyl terminal to the fusion junctions in the corresponding hybrid proteins. The missing sequences, which include the entire putative cytoplasmic domain following this transmembrane segment, could be important in anchoring this part of the protein in the cytoplasm. In this case, fusions to other portions of cytoplasmic domains might have alkaline phosphatase more stably localized to the cytoplasm and would show lower activity. These might be found among the white or very pale blue colonies in transposition selections. Nine independent colonies of these latter types were analyzed. In all cases, DNA sequencing of the hybrid genes showed that they were the result of out-of-frame TnphoA insertions rather than in-frame fusions giving low alkaline phosphatase activity. Given these findings, searching for the predicted low activity additional cytoplasmic domain fusions using this approach appeared impractical. Therefore, to test our explanation for the intermediate activity of fusions 17-1 and d2b, we made use of an in vitro technique to generate additional fusions at critical positions in the presumptive MalF cytoplasmic domains.

**In Vitro Fusions.** Our further analysis of the model for MalF protein is designed to test whether cytoplasmic loops between membrane-spanning stretches of integral membrane proteins contain information that contributes to the topological arrangement of the proteins. We constructed fusions to each of the MalF protein cytoplasmic loops, which differ only in whether they are positioned at the very beginning or very end of the loop. If sequences within the loop contribute to the loop's stable cytoplasmic localization, fusions at the ends of the loops in which the loop is present should show less activity than those at the beginning of the loops in which the loop is deleted.

We have used oligonucleotide-directed deletion mutagenesis to construct the set of fusions. This was done in the following way (see Fig. 1). We began with either a late malF-phoA fusion or a malG-phoA fusion (unpublished results). (The malG gene is distal to malF in an operon, and part of it is present in the plasmid used.) Oligonucleotides were synthesized that contained 23 nucleotides of the end of TnphoA that generates the fusion and 20–25 nucleotides of the target sequence in malF. These oligonucleotides were used to prime second-strand synthesis with a single-stranded parental template. Such fusions are isogenic to those obtained by transposition of TnphoA in the sense that they substitute exactly the same fusion joint sequence at different points in malF. This technique was used to generate fusions 0-55, II-175, IV-958, VI-1177, VI-1249, and VIII-1548 (see Fig. 2).

We determined the alkaline phosphatase activity of these fusions. Because the particular plasmid we used was unstable and this instability interfered with accurate comparison of the activity of different fusions, we recombined all of the in-frame fusions onto the chromosome. The results of assays of these fusions are summarized in Fig. 2. They show the following: (i) Fusions to periplasmic domains all show 20–30 units of alkaline phosphatase activity. (ii) Fusions to cytoplasmic domains show up to 200 times lower activity, but they vary greatly. (iii) Fusions to the carboxyl ends of cytoplasmic loops have 7–20 times lower activity than fusions to the amino-terminal ends of the same loops. (iv) The fusions following the second membrane-spanning stretch have at least ten times lower levels of activity than the corresponding fusions following the fourth such stretch, whereas the fusions following the sixth stretch are intermediate. (v) The fusion 0-55, which deletes all hydrophobic regions of MalF, has no detectable alkaline phosphatase activity. This fusion protein is stable (data not shown). (vi) The VIII-1543 fusion, which

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**Fig. 2.** Alkaline phosphatase activity of malF-phoA fusion strains. The alkaline phosphatase activity of each malF-phoA fusion is shown with an arrow pointing to the site at which the fusion joint is located in the topological model of malF. Assays were carried out using strains with a single copy of the fusion stably integrated into the chromosomes. Assays were performed using strains at 28°C. The fusion and the values shown are averages. A is fusion 0-55, fused at base 55; B, 17-3 at 94; C, II-175 at 175; D, II-205 at 205; E, 160 at 295; F, 4,30c at 607; G, 121 at 649; H, 165 at 682; I, d4a at 712; J, 3-826 at 826; K, 17-1 at 907; L, d2b at 920; M, IV-958 at 958; N, 4,39b at 1051; O, VI-1177 at 1177; P, VI-1249 at 1249; Q, 4,29c at 1417; R, VIII-1548 at 1548.
has alkaline phosphatase fused to the carboxyl-terminal amino acid of MalF has a MalF$^+$ phenotype, whereas all other fusions are MalF$^-$.

**Proteolysis and Fractionation.** The similar alkaline phosphatase activities of different fusions to presumed periplasmic domains of MalF suggest that the active moieties of these fusion proteins have the same specific activity and that they are positioned in the periplasm, presumably on the periplasmic face of the inner membrane. To test this supposition, labeled cells of one fusion strain were subjected to osmotic shock in the presence of trypsin. This treatment releases the periplasm and makes the outer face of the cytoplasmic membrane accessible to trypsin. Wild-type alkaline phosphatase activity is resistant to trypsin, although the protein is cleaved at a site near the amino terminus (22). Mild trypsin treatment released 86% of the alkaline phosphatase activity of fusion 3-826 from shocked cells. At the same time, less than 15% of the activity of glucose-6-phosphate dehydrogenase, a cytoplasmic protein, was lost from the cells. Analysis of the labeled protein by immunoprecipitation with anti-alkaline phosphatase antiserum and NaDodSO$_4$/PAGE shows a prominent fusion-protein-sized band in the cellular (cytoplasm plus membrane) fraction from untreated cells (Fig. 3). This band is also present in cells treated with trypsin in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (not shown). This band is missing from the cellular fraction of cells treated with trypsin, which instead shows a prominent alkaline-phosphatase-sized band in the periplasmic fraction. $\beta$-Galactosidase was also identified by immunoprecipitation and recovered in the cellular fractions of both treated and untreated cultures (Fig. 3). These results indicate that the alkaline moiety of the fusion protein is predominantly localized at the outer face of the inner membrane.

**DISCUSSION**

In this paper we have extended the alkaline phosphatase fusion approach for analyzing membrane protein topology to a complex membrane protein, MalF. The model for MalF topology proposed in Fig. 2 was originally based on the amino acid sequence of the protein (3). The properties of $\beta$-galactosidase fusions to MalF are consistent with this model (5). Properties of alkaline phosphatase fusions to MalF follow a relatively simple pattern, which is also consistent with the proposed structure. All fusions to proposed periplasmic domains show high alkaline phosphatase activity with about the same activity for different fusions. In contrast, fusions to proposed cytoplasmic domains show activities that are slightly lower to 200 times lower than those of the periplasmic domain fusions.

The high activity of all fusions in which alkaline phosphatase is attached to a presumed periplasmic domain of malF indicates that each of the transmembrane segments preceding these domains is competent to initiate export of alkaline phosphatase. In this regard, these sequences act like the cleavable signal sequences of secretory proteins. Similar suggestions have been made for other integral membrane proteins (23–25). Proteolysis and cell fractionation studies of one fusion strain in this class, 3-826, confirm the assumption that high enzymatic activity of the hybrid correlates with efficient export of the alkaline phosphatase moiety. We presume that the transmembrane sequences promoting export of alkaline phosphatase in the fusion proteins play a similar role in determining the topological structure of the native protein.

The alkaline phosphatase activity of a fusion to a MalF protein cytoplasmic domain depends on the position of the fusion joint within that domain. Fusions of alkaline phosphatase to the very amino terminus of a cytoplasmic domain (i.e., in which none of that cytoplasmic domain is included in the hybrid protein) show 7- to 20-fold higher activity than those more carboxyl terminal in the same cytoplasmic domain. This pattern suggests that cytoplasmic domain sequences help to anchor alkaline phosphatase in the cytoplasm in such hybrid proteins. In other words, the hydrophobic transmembrane segment itself is not the sole determinant of stable anchoring. We presume that these same cytoplasmic sequences that stabilize alkaline phosphatase in the cytoplasm in such hybrids contribute to determining the topology of the unfused MalF protein in an analogous way. We suspect that positively charged amino acid residues near the cytoplasmic ends of membrane-spanning stretches may play a primary role in stabilizing such stretches. An analysis of many integral membrane proteins by von Heijne (4) suggests that these charged residues are a common structural feature of such domains and that they may play a role in determining the orientation of the membrane-spanning segments.

Other features of the determinants of membrane protein topology are suggested by a comparison of fusions in which alkaline phosphatase is fused to cytoplasmic domains of MalF. In particular, the fusions to the cytoplasmic domain following the fourth membrane-spanning segment have 10- to 20-fold higher activities than the corresponding fusions following the second such sequence. We can imagine various factors that might contribute to this dramatic difference. First, it is possible that properties of individual membrane-spanning segments or the hydrophilic domains themselves determine the efficiency with which alkaline phosphatase is retained in the cytoplasm of the fusion strains. For instance, the fourth hydrophobic sequence is quite long (32 amino acids) compared to others oriented in the same direction. Conceivably, it is long enough so that an amino-terminal portion of it could span the membrane and a carboxyl-terminal portion act as a “signal sequence” in the cytoplasm allowing efficient export of alkaline phosphatase. Alternatively, the large periplasmic hydrophilic domain that precedes the fourth membrane-spanning segment may destabilize the membrane interactions at its carboxyl terminus. In both of these cases, it seems unlikely to us that these sequences would behave this way in an intact MalF protein.

**FIG. 3.** Proteolysis and fractionation of an active MalF-PhoA fusion protein. Cells producing the 3-826 fusion protein were fractionated after incubation in the presence or absence of trypsin. Proteins precipitated by a mixture of antibodies to alkaline phosphatase and $\beta$-galactosidase were separated by NaDodSO$_4$/PAGE. Lanes: 1 and 2, immunoprecipitate from the cellular fraction (membrane plus cytoplasm); 3 and 4, immunoprecipitate from periplasmic fraction. Lanes 1 and 3 have been subjected to osmotic shock in the absence of trypsin. In lanes 2 and 4 the osmotic shock was carried out in the presence of trypsin (5 $\mu$g/ml) at 0°C for 5 min. The molecular weights of markers (prestained; Bethesda Research Laboratories) indicated by the arrows at right are from top to bottom: 200, 97, 4, 68, and 43 kDa. Band A is $\beta$-galactosidase, B is the fusion protein 3-826, and C is alkaline-phosphatase-sized.

but rather function aberrantly in the absence of the remaining carboxyl terminus of the protein.

Second, it may be that interactions between domains are normally important in determining the topology of the protein. For example, close packing in the membrane of the first two transmembrane stretches could contribute to the stabilization of the fused alkaline phosphatase in the cytoplasm, whereas the fourth transmembrane stretch may have no such interactions available to it in the relevant fusion proteins. We expect that these alternatives will be distinguishable by the engineering of further derivatives of the fusion strains.

The alkaline phosphatase moiety of hybrid protein 3-826, a high activity fusion, is fully exported to the periplasm (Fig. 3). Proteolysis and fractionation experiments with fusions having intermediate activity (such as 17-1) to determine the disposition of their alkaline phosphatase moieties have not been done. Two explanations for the alkaline phosphatase activity of these latter fusions are possible. The proteins may be efficiently localized to one compartment, periplasm or cytoplasm, and may have a specific activity lower than fusions such as 3-826. Alternatively, they may be partially exported and have a periplasmic fraction of high specific activity and a cytoplasmic fraction of low specific activity. Experiments with alkaline phosphatase signal sequence mutants (7, 26) suggest that the second alternative is likely to be correct. Cytoplasmic alkaline phosphatase is inactive and unstable. Stabilization of cytoplasmic alkaline phosphatase does not lead to increased activity or export. This finding suggests that cytoplasmic alkaline phosphatase may enter a nonexportable pool. The relative rates of export and entry into this pool may determine the level of activity of fusions with intermediate activity.

Our results suggest that isolating TnPhoA fusions by transposition alone may not provide a complete analysis of complex membrane proteins that have small, but critical, cytoplasmic domains. Our in vitro oligonucleotide deletion mutagenesis approach may be a necessary adjunct to in vivo methods for studying such proteins. The properties of fusions generated in vitro can be directly compared to those generated by TnPhoA transposition because the fusion joint segments are the same. As a general experimental approach, we believe that initial results with TnPhoA fusion proteins generated by transposition will allow construction of a working model, which can be tested with specific fusions at critical points generated by the in vitro method.

To use the oligonucleotide deletion method, one needs to begin with either a very late fusion or a fusion to a distal gene obtained in vivo. By using 40- to 50-mer oligonucleotides with one part complementary to the end of TnphoA and the other complementary to the target sequence, it is possible to recover the desired new fusions with high efficiency.

Fusions of the type described here replace the carboxyl-terminal part of the protein being studied with the probe alkaline phosphatase. The properties of such fusions are thus determined solely by the amino-terminal portion of the protein. In cases in which the arrangement of a membrane protein is determined by interactions between its amino-terminal and carboxyl-terminal portions, a fusion analysis may not give a valid picture of the final topology. In the case of the Male protein, the finding that the longest Male-phoA fusion retains Male activity indicates that the Male protein moiety has assumed its proper conformation in the membrane. The low alkaline phosphatase activity of this fusion indicates that the carboxyl-terminal domain of Male is normally cytoplasmic as was originally predicted from the orientation of the rest of the protein. The results suggest, then, that alkaline phosphatase fusions are not, in general, disrupting the orientation of segments of the protein, since we obtain an internally consistent picture from this analysis.

The consistency of the results presented here and their coherence with results obtained from lacZ fusions to malf, in addition to our earlier results with a simpler membrane protein, indicate that the alkaline phosphatase fusion approach can be generally used to analyze membrane protein topology.

We thank Ann McIntosh for assistance in preparation of the manuscript, Howie Shuman for providing malf plasmids, and Barry Wanner and Michael Berman for providing strains. This work was supported by American Cancer Society and National Institutes of Health grants to J.B. and a fellowship from the Arthritis Foundation to C.M.