JOURNAL OF BACTERIOLOGY, Jan. 1993, p. 565–567
0021-9193/93/020565-03$02.00/0

Vol. 175, No. 2

Membrane Topology Model of *Escherichia coli* α-Ketoglutarate Permease by PhoA Fusion Analysis

WONGI SEOL† AND AARON J. SHATKIN*

Center for Advanced Biotechnology and Medicine, 679 Hoes Lane, Piscataway, New Jersey 08854-5638

Received 27 August 1992/Accepted 9 November 1992

***Escherichia coli* α-ketoglutarate permease (KgtP) is a 432-amino-acid protein that symports α-ketoglutarate and protons. KgtP was predicted to contain 12 membrane-spanning domains on the basis of a calculated hydropathy profile. The membrane topology model of KgtP was analyzed by using *kgtP-phoA* gene fusions and measuring alkaline phosphatase activities in cells expressing the chimeric proteins. Comparisons of the phosphatase activity levels and the locations of the KgtP-PhoA junctions are consistent with the predicted membrane topology model of KgtP.**

α-Ketoglutarate permease (KgtP) is a hydrophobic membrane protein that cotransports α-ketoglutarate and protons into *Escherichia coli* (13–16). The deduced amino acid sequence of KgtP indicates that it contains 12 hydrophobic regions that may correspond to membrane-spanning domains as in the prototypic membrane carrier protein, *lac* permease (LacY [8]). KgtP has sequence homology to several membrane transporters, including *E. coli* citrate (CitA), arabinose (AraE), and galactose (GalP) permeases as well as yeast and mammalian glucose carriers (6, 15). More specifically, KgtP belongs to the subfamily designated group V (5). In addition, active-site analyses with mutants containing point and nonsense mutations in *kgtP* confirmed that it belongs to the transporter superfamily (17).

A membrane topology model of KgtP has been derived on the basis of computer calculations of hydropathy values. As documented for other members of the transporter superfamily (5), KgtP was predicted to contain 12 transmembrane domains. To test this model, we have constructed a number of gene fusions between *kgtP* and *phoA*. The resulting hybrid proteins consist of alkaline phosphatase (PhoA) with its signal sequence replaced by various N-terminal portions of KgtP. Since the phosphatase activity of PhoA in intact cells depends on its extrusion to the periplasmic space, fusion proteins in which the KgtP-PhoA junction is predicted to be near the periplasmic regions of KgtP should have high levels of alkaline phosphatase activity. Conversely, PhoA fusions with a junction at or near cytoplasmic domains of KgtP are expected to be relatively inactive. This experimental approach has been used previously to confirm the membrane topologies of *E. coli lacY* permease (1) and mannitol permease (19) and the L subunit of the *Rhodobacter sphaeroides* photosynthetic reaction center (20).

Construction of *kgtP-phoA* gene fusions in vivo and in vitro. *E. coli* CC118(DE3) was made by infecting strain CC118 [*ΔlacX74 galE galK araD139 Δ(ara leu)7697 phoAΔ20 thi rpsE rpoB argE*(Am) *recA1* Spc^r] (10) with phage DE3 containing the T7 RNA polymerase gene and selecting lysogens as described elsewhere (18). To obtain KgtP-PhoA fusion plasmids, *E. coli* CC118 that had been transformed with *kgtP*-containing pES16 (16) was infected with λTn*phoA* (3). Candidate plasmids for *kgtP-phoA* fusions were used for dideoxynucleotide sequencing (12) to determine whether *phoA* was fused in the correct open reading frame and orientation and to identify fusion sites. Correct plasmids were selected and named the pETP series.

Alternatively, gene fusions were constructed in vitro (11). pES16 was digested with *Nsi*I, which cleaves at the unique site corresponding to the position coding for amino acid 275 in KgtP. The cleaved plasmids were treated for different times with *Bal* 31, and samples were removed at regular intervals (including time zero). After the ends were filled in by incubation with the large fragment of DNA polymerase I, the DNAs were used for ligation with the smaller of the two *Pst*I fragments, which were treated with T4 DNA polymerase to make blunt ends after excision from pCH2, pCH39, and pCH40 (7). The small *Pst*I fragments derived from the three plasmids contain the PhoA-coding region, minus the promoter and signal sequence, in three different reading frames to increase the efficiency of selection of correct constructs. After incubation of the ligation mixture with CC118(DE3), plasmids isolated from the transformants were screened by restriction endonuclease analysis and sequenced. The correct plasmids were designated the pEP series.

Alkaline phosphatase activities of KgtP-PhoA fusion proteins. To test the KgtP topology model, 21 *kgtP-phoA* fusion genes, including 10 pETP and 11 pEP constructs, were selected for expression studies on the basis of their fusion sites relative to the model shown in Fig. 1. The constructs included fusions located within hydrophilic loops as well as at the ends of or within hydrophobic domains. The plasmids were used to transform *E. coli* CC118 (*phoA*) cells, and the resulting transformants were assayed for alkaline phosphatase activity after isopropylthiogalactoside (IPTG) induction (Table 1). When the activities were correlated with the corresponding fusion sites, the phosphatase levels were generally consistent with the membrane topology model (with the exception of pETP49 [described below]). Chimeric protein fusion sites closer to the outer side of the cell membrane or periplasmic space were correlated to higher phosphatase activities. Cells with high alkaline phosphatase activity (>200 U) were obtained with KgtP-PhoA chimeric proteins expressed from plasmids pEP111, pEP122, pEP127, pEP183, pEP187, pEP193, pEP275, pETP258, pETP326,

* Corresponding author. Electronic mail address: Shatkin@biovax.bitnet.

† Present address: Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114.

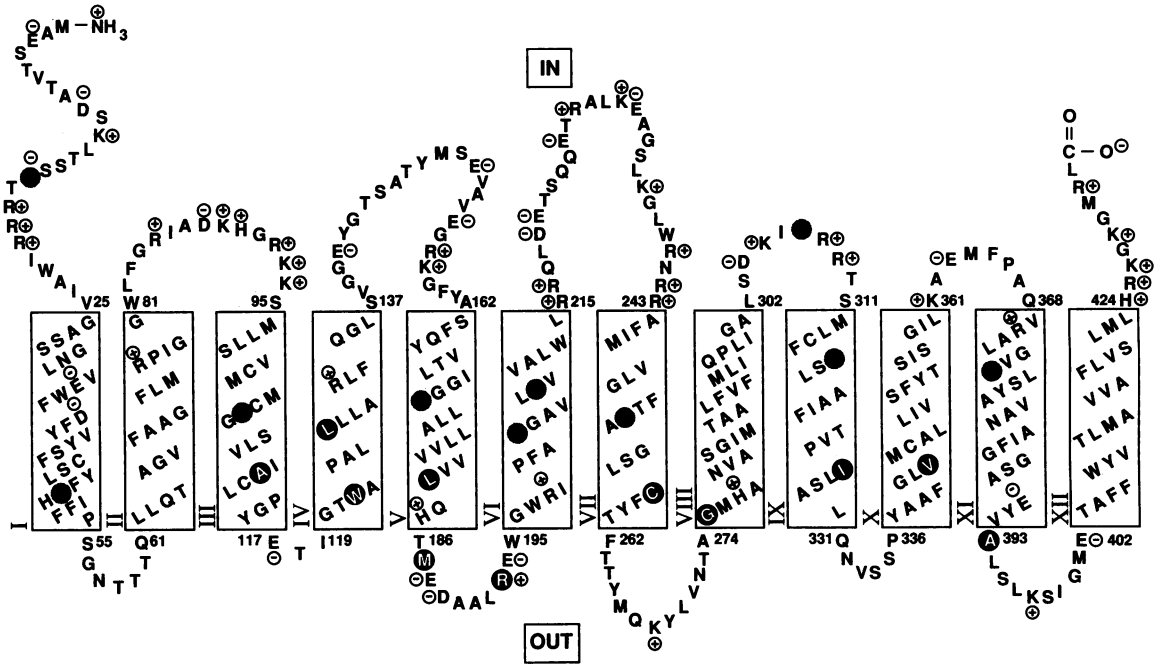

FIG. 1. Proposed model of KgtP in the cell membrane. The model is based on hydropathy calculations done by the PEPTIDESTRUCTURE program of the Genetics Computer Group sequence analysis software package (2). Junction sites of KgtP-PhoA fusion proteins are indicated by circles around the last KgtP residues. Filled circles correspond to high alkaline phosphatase activities (>200 U), and shaded circles correspond to low activities (<115 U).

pETP343, and pETP393 (Table 1). They contain *kgtP-phoA* junctions predicted to place the PhoA sequences within eight amino acids of, or outside, the cell membrane (Fig. 1). Chimeric junctions predicted to lie near or at the cytoplasmic side in the KgtP model, i.e., pETP16, pETP173, pETP307, pETP316, pETP375, pEP105, pEP203, pEP208, and pEP253, yielded fusion proteins with low (<115 U) activity (Table 1 and Fig. 1). The results suggest that the 12-transmembrane-domain model of transporter superfamily members also applies for KgtP.

TABLE 1. Alkaline phosphatase activity in *E. coli* CC118(DE3) expressing KgtP-PhoA fusion proteins

Plasmid[a]	Sequence of junction (5′ to 3′)[b]	Activity[c]
pETP16	AGT AGT GAT A/CT GAC	13 ± 9
pETP49	TAC TTT GCC C/CT GAC	5 ± 1
pEP105	ATG TGT TTC GG/G CCT	13 ± 11
pEP111	GTT ATC GCC / GCT CAG	591 ± 181
pEP122	GGT ACG TGG / GCT CAG	268 ± 106
pEP127	GCA TTA TTG C/GC CCT	996 ± 97
pETP173	GGC GGA CAA C/CT GAC	6 ± 4
pEP183	GTG GTT TTA CA/G CCT	216 ± 106
pEP187	CAC ACC ATG / GCT CAG	353 ± 155
pEP193	GCA CTC AGA GA/G CCT	264 ± 94
pEP203	TTC GCG TTA / GCT CAG	20 ± 17
pEP208	GTG TTA GCT / GCT CAG	33 ± 19
pEP253	TTT ACC GCT / GCT CAG	4 ± 2
pETP258	TCC CTT TGT T/CT GAC	426 ± 92
pEP275	ACT GCG GGA A/GC CCT	621 ± 161
pETP307	AAG ATT GGT C/CT GAC	111 ± 29
pETP316	TGT TTC GGT T/CT GAC	69 ± 7
pETP326	GTT CCT ATT C/CT GAC	458 ± 122
pETP343	GGT CTG GTG A/CT GAC	468 ± 74
pETP375	GGC GTT GGT C/CT GAC	66 ± 7
pETP393	TAC GTA GCG T/CT GAC	245 ± 53

[a] The number in each plasmid designation indicates the position of the last amino acid derived from KgtP at the junction in the KgtP-PhoA fusion.

[b] The slash in each sequence indicates the fusion site of each gene.

[c] Cell lysates prepared from CC118 cultures expressing fusion proteins (13) were assayed for alkaline phosphatase activity as described elsewhere (20). Activity was calculated as 1,000 × the linear A_{420} change per minute per unit of optical density at 600 nm of cell culture.

Expression levels of fusion proteins. Since expression of the KgtP-PhoA fusion proteins requires IPTG induction, immunoprecipitation with antibody against PhoA was done to compare the levels of the different hybrids. The results in Fig. 2 demonstrate that as the fusion sites approach the C terminus of KgtP, the molecular weights of the fusion proteins increase. The protein bands also become broader, as expected because of the increased hydrophobicity contributed by KgtP. The hydrophobicity of KgtP may also account for the fact that hybrid polypeptides migrated faster than predicted from their molecular weights.

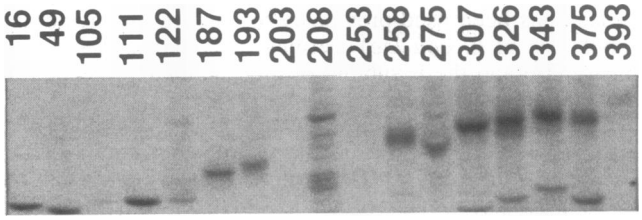

FIG. 2. Immunoprecipitation of KgtP-PhoA fusion proteins. Fusion proteins encoded by pEP or pETP plasmids and labeled with [^{35}S]methionine (16) were immunoprecipitated (4) by rabbit antibody against bacterial alkaline phosphatase (5 Prime-3 Prime, Inc.). Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (9), except that the samples were heated at 50°C for 15 min (not boiled) to avoid aggregation (16). Numbers at the top indicate junction sites (KgtP amino acid residues) of pEP or pETP plasmids.

The expression levels of pEP111, pEP127 (not shown), pEP187, and pEP193 as well as pETP258, pETP275, pETP326, and pETP343 generally reflected their high phosphatase activities (Fig. 2). pETP16, pETP307, and pETP375 were also expressed well but had relatively low enzymatic activities, consistent with the location of their respective fusion sites. The pETP49 hybrid protein was expressed to a degree similar to that of the other highly expressed proteins, and its apparent molecular weight was lower than that of fusion protein 16 (Fig. 2), suggesting that the first hydrophobic domains begins after position 16. Despite a fusion junction close to the periplasmic region in the topology model (Fig. 1), pETP49 expression yielded low phosphatase activity (Table 1). When assayed by [^{35}S]methionine labeling and immunoprecipitation, the pETP49 fusion protein fractionated with the soluble and pellet fractions and not with the periplasmic fraction; the same results were obtained with a position 46 fusion product which also showed low phosphatase activity (data not shown). The negatively charged Glu-34 and Asp-37 in putative helix I may require neutralization by positively charged amino acids in other transmembrane domains, e.g., Arg-76 in helix II and Arg-131 in helix IV, for correct insertion into the membrane, a suggestion supported by the cellular locations and low phosphatase activities of pETP49 and pETP46.

Hybrid proteins from pEP105, pEP203, and pEP253 were present in small amounts (Fig. 2). However, in other experiments (not shown), pEP105 and pEP203 were present in amounts similar to the amounts of pETP49 and pEP111 fusion proteins, suggesting that their reproducibly low activities were due to the positions of their fusion junctions. In addition, pEP122 and pETP393 were weakly expressed (Fig. 2) but yielded high phosphatase activities (Table 1), also in agreement with the topology model shown in Fig. 1.

Fusion sites: membrane locations and phosphatase activities. Calamia and Manoil (1) reported that when PhoA was fused to a putative membrane-spanning region of *lac* permease, the enzymatic activities differed depending on whether the hydrophobic domain was oriented with its amino terminus in the periplasm (incoming; even-numbered helices in Fig. 1) or in the cytoplasm (outgoing; odd-numbered helices in Fig. 1). In their experiments, hybrids fused to an incoming segment and containing as many as 10 or 11 amino acids showed high PhoA activity. An incoming segment fusion containing 22 residues showed reduced activity relative to fusions of the preceding periplasmic segment (1); however, the activity was still higher than those of most cytoplasmic segment fusions. It also appeared that the export function of outgoing segments required at least 9 to 11 residues in the segment. The data obtained for KgtP generally agree with these observations on *lac* permease.

The PhoA fusion method is a powerful means to predict the topology of membrane-associated proteins, including KgtP. A more accurate topology model of KgtP will require analyses of additional fusion mutants, particularly in the C-terminal region, in helix II, and in the second and sixth hydrophilic loops. Efforts to obtain additional fusions by transposition of Tn*phoA*, by the nested deletion method with *Bal* 31 and exonuclease III in vitro (11), or by polymerase chain reaction were unsuccessful because they yielded previously obtained fusions or no transformants. This may be due to the presence of "hot spots" or the formation of highly toxic proteins when other regions of KgtP are connected to PhoA. An unequivocal verification of the KgtP membrane topology model awaits determination of the three-dimensional structure of the protein.

Bacteriophage DE3 lysogen was provided by A. Rosenberg (Brookhaven National Laboratory, Upton, N.Y.); phage λTn*phoA*, plasmids pCH2, pCH39, and pCH40 and the *E. coli phoA* strain CC118 were from C. Manoil (Seattle, Wash.).

REFERENCES

1. **Calamia, J., and C. Manoil.** 1990. *lac* permease of *Escherichia coli*: topology and sequence elements promoting membrane insertion. Proc. Natl. Acad. Sci. USA **87**:4937–4941.
2. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
3. **Gutierrez, C., J. Barondess, C. Manoil, and J. Beckwith.** 1987. The transposon Tn*phoA* to detect genes for cell envelope proteins subject to a common regulatory stimulus. J. Mol. Biol. **195**:289–297.
4. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual, p. 423–470. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Henderson, P. J. F.** 1991. Sugar transport proteins. Curr. Opin. Struct. Biol. **1**:590–601.
6. **Henderson, P. J. F., and M. C. Maiden.** 1990. Homologous sugar transport proteins in *Escherichia coli* and their relatives in both prokaryotes and eukaryotes. Philos. Trans. R. Soc. Lond. B **326**:391–410.
7. **Hoffman, C. S., and A. Wright.** 1985. Fusions of secreted proteins to alkaline phosphatase: an approach for studying protein secretion. Proc. Natl. Acad. Sci. USA **82**:5107–5111.
8. **Kaback, H. R., E. Bibi, and P. D. Roepe.** 1990. β-Galactoside transport in *E. coli*: a functional dissection of *lac* permease. Trends Biochem. Sci. **15**:309–314.
9. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
10. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82**:8129–8133.
11. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 13.34–13.41, 15.14–15.26. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. J. Mol. Biol. **143**:161–178.
13. **Seol, W.** 1992. Ph.D. thesis. Rutgers University, New Brunswick, N.J.
14. **Seol, W., and A. J. Shatkin.** 1990. A new gene located between *pss* and *rrnG* on the *Escherichia coli* chromosome. J. Bacteriol. **172**:4745.
15. **Seol, W., and A. J. Shatkin.** 1991. *Escherichia coli kgtp* encodes an α-ketoglutarate transporter. Proc. Natl. Acad. Sci. USA **88**:3802–3806.
16. **Seol, W., and A. J. Shatkin.** 1992. *E. coli* α-ketoglutarate permease (KgtP) is a constitutively expressed proton symporter. J. Biol. Chem. **267**:6409–6413.
17. **Seol, W., and A. J. Shatkin.** 1992. Characterization of site-directed mutants of α-ketoglutarate permease. Biochemistry **31**:3550–3554.
18. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189**:113–130.
19. **Sugiyama, J. E., S. Mahmoodian, and G. R. Jacobson.** 1991. Membrane topology analysis of *Escherichia coli* mannitol permease by using a nested-deletion method to create mtlA-phoA fusions. Proc. Natl. Acad. Sci. USA **88**:9603–9607.
20. **Yun, C., S. R. Van Doren, A. R. Crofts, and R. B. Gennis.** 1991. The use of gene fusions to examine the membrane topology of the L-subunit of the photosynthetic reaction center and of the cytochrome b subunit of the bc1 complex from *Rhodobacter sphaeroides*. J. Biol. Chem. **266**:10967–10973.