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A Topological Model for the General Aromatic Amino Acid Permease, AroP, of *Escherichia coli*

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The general aromatic amino acid permease, AroP, of *Escherichia coli* is responsible for the active transport of phenylalanine, tyrosine, and tryptophan. A proposed topological model for the AroP permease, consisting of 12 hydrophobic transmembrane spans connected by hydrophilic loops, is very similar to that of the closely related phenylalanine-specific permease. The validity of this model and its similarity to that of the PheP permease were investigated by studying fusion proteins of AroP permease and alkaline phosphatase. Based on the results obtained from the AroP-alkaline phosphatase sandwich fusions, we have significantly revised the proposed topological model for AroP in two regions. In this modified AroP topological model, the three charged residues E151, E153, and K160 are repositioned within the membrane in span 5. These three residues are conserved in a large family of amino acid transport proteins, and site-directed mutagenesis identifies them as being essential for transport activity. It is postulated that these residues together with E110 in transmembrane span 3 may be involved in a proton relay system.

The general aromatic amino acid permease, AroP, of *Escherichia coli* is an integral cytoplasmic membrane protein involved in the active transport of phenylalanine, tyrosine, and tryptophan (8, 12, 46). AroP, together with the closely related phenylalanine-specific permease PheP, belongs to a superfamily of permeases involved in the transport of amino acids in bacteria and yeast (32, 33).

A possible model for the secondary structure of the AroP permease in the cytoplasmic membrane has been proposed based on the hydrophobicity profile and distribution of charged amino acid residues. In this model the hydrophobic nonpolar residues are arranged in 12 membrane-spanning regions of approximately 21 amino acids connected by hydrophilic loops of various lengths (14). This proposed topological model is very similar to that reported for the PheP permease (31), which has 61% sequence identity with the AroP permease (Fig. 1). Despite the high degree of sequence identity and similarity in hydrophobicity profiles, AroP and PheP differ in their range of substrate specificities and affinities. While PheP shows a specific activity for transporting phenylalanine, AroP is equally capable of transporting each of the three aromatic amino acids.

In order to validate the proposed topological model of AroP and to determine whether there are major differences between the membrane topologies of AroP and PheP, alkaline phosphatase was used as a reporter enzyme to analyze the membrane topology of the AroP permease. Alkaline phosphatase is enzymatically active only when translocated across the cytoplasmic membrane into the periplasm (28), where its intrachain disulfide bonds can form (15). In the absence of its leader peptide, this translocation can occur only if alkaline phosphatase is fused to a signal sequence or to a periplasmic domain of a membrane protein, with such fusions exhibiting high-level enzymatic activity. However, when the leaderless alkaline phosphatase is fused to a cytoplasmic domain of a membrane protein, only a low level of enzymatic activity is observed. The study of alkaline phosphatase activities of a variety of fusions in which alkaline phosphatase is joined to different regions of a membrane protein can help establish the disposition of the protein in the membrane.

As the final topology of the permease may be affected by interactions between the amino- and carboxyl-terminal sequences of the protein, we constructed AroP-PhoA sandwich fusions in which alkaline phosphatase is inserted into regions of the whole AroP protein (16). This approach enables the entire membrane protein to be present and has been shown to give a more accurate representation of membrane protein topology (3, 16, 23, 31, 39).

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The *E. coli* K-12 strains, plasmids, and phages used in this study are described in Table 1.

Growth media and reagents. The minimal media used were the half-strength buffer 56 of Monod et al. (29) and the 121-salts medium of Torriani (41), both supplemented with 0.2% glucose and the required growth factors. Kanamycin was added to nutrient and minimal media at a final concentration of 25 μg/ml. The chromogenic substrate 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (XP) from Sigma Chemical Co. was dissolved in dimethylformamide and used at 40 μg/ml in solid media. All enzymes were purchased from Amrad Pharmacia Biotechnology, Melbourne, Australia, unless otherwise indicated. α-^{35}S-dATP (1,200 Ci/mmol; 10 mCi/ml) for use in DNA sequencing, [^{35}S]methionine-cysteine (1,175 Ci/mmol; 7.9 mCi/ml) for use in pulse-labeling, and L-[^{14}C]tyrosine (497.7 mCi/mmol; 100 μCi/ml) and L-[^{14}C]phenylalanine (497.6 mCi/mmol; 100 μCi/ml) for use in transport assays were obtained from NEN/DuPont. ProtoGel 30% (wt/vol) acrylamide for protein gels was purchased from National Diagnostics. Oligonucleotides were synthesized on a Gene Assembler Plus (Pharmacia-LKB) or obtained commercially from Bresatec.

Recombinant DNA techniques. Standard recombinant DNA techniques were used essentially as described by Sambrook et al. (36).

Construction of *aroP′-phoA-′aroP* sandwich gene fusions. Oligonucleotide-mediated site-directed mutagenesis (44) was used to create individual unique *Bgl*II restriction sites at 25 specific positions within the *aroP* gene present on mpMU202. Restriction analysis and sequencing of the mutants confirmed the presence of the introduced *Bgl*II sites. The *′phoA* gene from pSWFII (16) was excised on a *Bam*HI fragment and inserted in frame into the *aroP* gene at each of the *Bgl*II sites. To minimize the presence of parental molecules, the ligation mixture was digested with *Bgl*II before transformation into JM101. After screening for the correct orientation by restriction analysis, the two junctions between *aroP* and *′phoA* were sequenced to ensure that the reading frame was maintained. The fusions were cloned into the low-copy-number vector pLG339 on a *Bam*HI-*Eco*RV fragment and transformed into the Δ*phoA* strain JP8442. Transformants were selected on minimal medium-kanamycin-XP plates. Colonies with high levels of alkaline phosphatase activity are blue, whereas those with low

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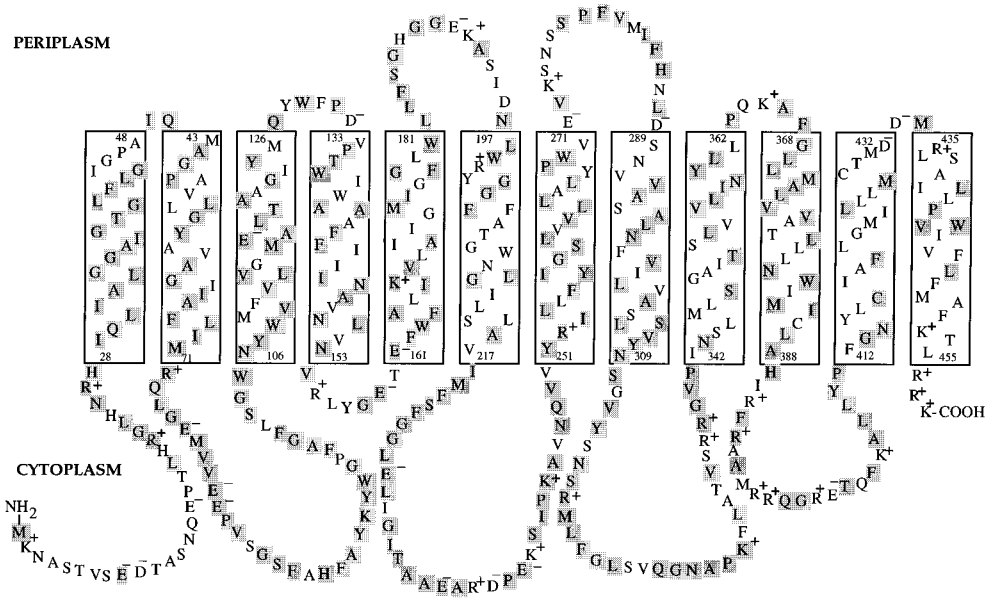

FIG. 1. Topological model of the PheP permease (31). The amino acid residues which are identical in the AroP and PheP permeases are shaded.

levels of alkaline phosphatase activity remain relatively white, and the latter were screened by restriction mapping.

Alkaline phosphatase assays. Cultures of JP8442 harboring *aroP′-phoA-′aroP* gene fusions were grown in half-strength buffer 56 containing 0.2% glucose and kanamycin at 37°C to an optical density at 600 nm of 0.45 to 0.55. Alkaline phosphatase activity was assayed as described by Manoil (26). Each assay was performed in duplicate on at least three separate occasions.

Transport assays. Cultures of JP7910 harboring the *aroP′-phoA-′aroP* gene fusions were grown under the same conditions as those described for the alkaline phosphatase assay, and transport activity was assayed as previously described (48), in the presence of 10 μM L-[^{14}C]phenylalanine or L-[^{14}C]tyrosine.

Western immunoblotting analysis. Cultures were grown under the same conditions as those described for alkaline phosphatase assays to an optical density at 600 nm of 0.5. Strain W3110 was grown in 121-salts medium containing 0.2% glucose and 0.1 mM KH_2PO_4, under which conditions the expression of the chromosomal *phoA* gene is derepressed (7). Cells were concentrated 50-fold, and 20-μl samples were electrophoresed on sodium dodecyl sulfate–8% polyacrylamide gels (24), followed by electrophoretic transfer to a nitrocellulose membrane (BA85; Schleicher and Schuell). Immunoblotting was performed as previously described (9), with antisera against bacterial alkaline phosphatase (5 Prime-3 Prime, Inc.). Prestained protein molecular weight standards were purchased from Novex. Bound antibody was visualized by using the enhanced chemiluminescence Western blotting detection reagents (Amersham).

Pulse-labeling and immunoprecipitation. Cultures were grown under the same conditions as those described for Western immunoblotting experiments to an optical density at 600 nm of 0.5. Aliquots of 0.5 ml of cell culture were pulse-labeled with 50 μCi of [^{35}S]methionine-cysteine for 1 min at 37°C. Cell extracts were prepared and the immunoprecipitation conditions were set up according to the method specific for integral membrane proteins described by Ito and Akiyama (21). Specific proteins were precipitated with antisera against bacterial alkaline phosphatase, and samples were electrophoresed on sodium dodecyl sulfate–8% polyacrylamide gels. The dried gel was exposed to X-ray film for at least 48 h.

Nucleotide sequence accession number. The nucleotide sequence of the *aroP* gene has been submitted to GenBank (accession no. U87285).

RESULTS

Construction of AroP permease-alkaline phosphatase sandwich fusions. A total of 25 AroP-PhoA sandwich fusions were generated by introducing unique *Bgl*II sites throughout the

TABLE 1. *E. coli* K-12 strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant characteristic(s)[a]	Source or reference
Strains		
JM101	Δ(*lac-pro*) *supE thi* (F′ *traD36 proA*$^+$*B*$^+$*lacI*^{q} Δ*lacZM15*)	27
JP777	F$^-$ *thr-1 leuB6 supE44 tonA21 gal351 thi-1 rpoB365*	4
JP7910	JP777 *aroP1000 pheP367 tyrP571 mtr-24 tnaA2 tyrR366*	13
JP8442	W3110 (Δ*phoA8 tsx*)	38
W3110	Prototroph	1
Plasmids		
pLG339	Tc^r Km^r, low-copy-number vector, pSC101 *ori*	40
pMU2195	Km^r, 3.2-kb *Sal*I-*Eco*RI *aroP* fragment cloned in pLG339	30
pSWFII	Ap^r, *phoA* sandwich fusion construction vector	16
Phages		
mpMU555	M13tg130 with *Bgl*II site removed	47
mpMU202	3.2-kb *Hin*dIII-*Sph*I *aroP* fragment cloned in mpMU555	This study

[a] The genetic nomenclature is that described by Bachmann (2). Allele numbers are indicated where known.

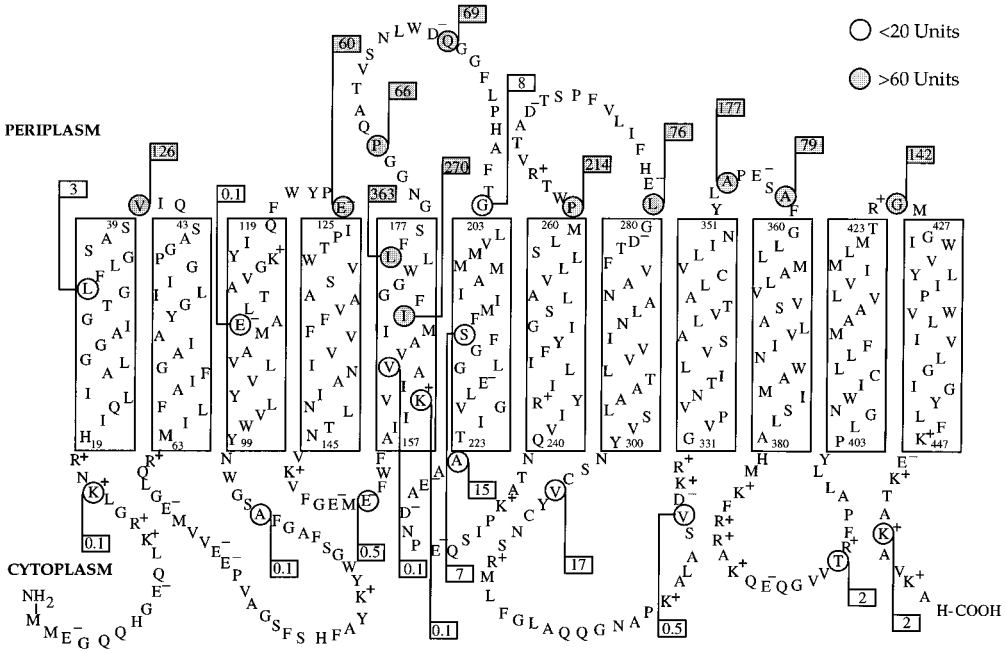

FIG. 2. Proposed topological model of the AroP permease. The fusion sites and alkaline phosphatase activities of the AroP-PhoA sandwich fusions are shown. Nucleotide sequencing of the *aroP* gene in this study identified a number of differences from the sequence reported by Honoré and Cole (19). The nucleotide at position 82 in the *aroP* structural gene was determined to be a G rather than a T, thus converting the amino acid residue at position 28 from serine to alanine. An alanine residue is present at the same position in the PheP permease. The insertion of 1 nucleotide (C) at position 1193 and 2 nucleotides (CT) at position 1198 was also observed. The addition of these 3 nucleotides changes the amino acid sequence at positions 397 to 402 from the reported FLLLY to FPALLY. The nucleotide and respective codon changes in *aroP* increase the amino acid identity between AroP and PheP from 59.6 to 61.0%.

aroP gene via site-directed mutagenesis, followed by in-frame insertion of the leaderless *'phoA* gene from pSWFII on a *Bam*HI fragment. The fusion sites were specifically positioned throughout the *aroP* gene so that at least one fusion was present in every putative periplasmic and cytoplasmic loop of the permease. To minimize the disruption of known topological signals, such as positively charged residues within the cytoplasmic region, the fusions were generally positioned in the C-terminal portion of each hydrophilic loop (6, 42). Fusions were also created within transmembrane spans 1, 3, 5, and 6. Based on the results obtained with some of these initial fusions (I169 and G202), which were inconsistent with the previously proposed model, additional fusions were constructed within putative transmembrane span 5 and within the proposed periplasmic loop between spans 5 and 6. The *aroP'-phoA-'aroP* fusions were cloned into the low-copy-number vector pLG339, transformed into the Δ*phoA* strain JP8442, and assayed for alkaline phosphatase activity.

Alkaline phosphatase activities of AroP-PhoA sandwich fusion proteins. The alkaline phosphatase activities of the AroP-PhoA sandwich fusion constructs and their positions in the proposed model of the AroP permease are presented in Fig. 2. Alkaline phosphatase activities were divided into two general categories of <20 U, reflecting a cytoplasmic or membrane location, and >60 U, indicating a periplasmic location. Most of the alkaline phosphatase fusions give results which are in general agreement with the proposed model of the AroP permease, with high-level enzymatic activity exhibited by fusion proteins containing alkaline phosphatase in a proposed periplasmic location and low-level activity shown in cases when the reporter enzyme is situated in a cytoplasmically located domain of the permease. However, there are inconsistencies with the proposed model in two distinct regions.

The first region involves fusions within putative transmembrane span 5. Fusion of alkaline phosphatase at I169, which is predicted to be located centrally within transmembrane span 5, shows exceptionally high-level alkaline phosphatase activity, as does the fusion at L175. Fusions at V164, K160, and E153, on the other hand, give low values consistent with a proposed location within the membrane or on the cytoplasmic side. These results suggest that the amino acid residues I169 and L175 are actually located in the periplasmic loop between spans 5 and 6. A direct consequence of this change is that the charged residues E151 and E153 are moved from cytoplasmic loop 3 to within the membrane of span 5 and the charged residue K160 is moved further into the middle of that membrane span.

The second region of anomaly involves fusions in the periplasmic loop between spans 5 and 6. When alkaline phosphatase is fused at G202 in AroP, which is a proposed periplasmic location, unexpectedly low levels of alkaline phosphatase activity are observed. Other fusions in this putative periplasmic loop at positions Q192 and P182 result in high-level enzymatic activities, as predicted by the model. This result suggests that transmembrane span 6 actually begins somewhere between Q192 and G202.

Synthesis and stability of fusion proteins. The steady-state levels of the AroP-PhoA fusion products were analyzed by Western immunoblotting of proteins from whole-cell extracts with antibodies against alkaline phosphatase (Fig. 3). The majority of isolates produced a polypeptide band of the size expected for a fused AroP permease-alkaline phosphatase construct. Other workers have shown that those fusions which do not show any immunoreactivity are generally located in the cytoplasm or membrane where they are unable to fold correctly and are rapidly degraded and so not available for anti-

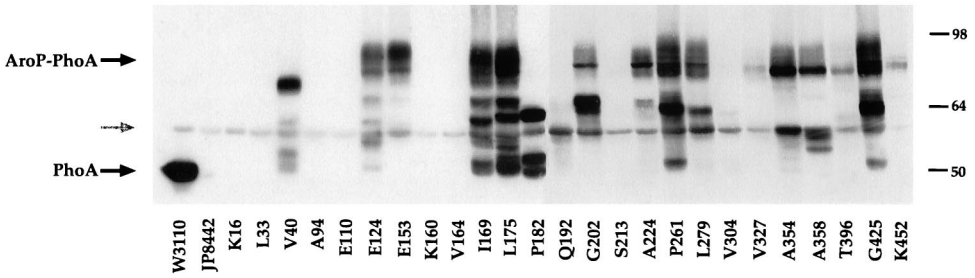

FIG. 3. Immunoblotting of AroP permease-alkaline phosphatase fusion proteins with anti-alkaline phosphatase serum. The position of native alkaline phosphatase encoded by the chromosomal *phoA* gene of strain W3110 is shown. Strain JP8442, which does not contain a gene fusion plasmid, was used as a negative control. A nonspecific band present in all lanes is marked by the unlabeled arrow. The positions of molecular weight standards (in thousands) are shown.

body binding (5, 37, 39). Proteolytic degradation products corresponding in size to that of the native alkaline phosphatase can be seen for a number of fusions. Other proteolytic degradation products of sizes intermediate between that of alkaline phosphatase and that of full-length fusion protein are also observed.

Fusion of alkaline phosphatase at P182 results in a termination codon immediately preceding the *′phoA* gene, thus generating an AroP′-PhoA fusion in which alkaline phosphatase replaces the carboxyl-terminal portion of the AroP permease rather than generating an AroP′-PhoA-′AroP sandwich fusion. As such, the full-length polypeptide band for this fusion is of a reduced size compared to the other sandwich fusions. Importantly, it can be seen that the AroP-alkaline phosphatase fusion at position G202, which was predicted to be located in a periplasmic loop but showed low-level alkaline phosphatase activity, is quite stable, and the low-level alkaline phosphatase activity does not appear to be due to instability.

As the majority of cytoplasmically located alkaline phosphatase fusions show no polypeptide band by Western immunoblotting analysis, the rate of synthesis of each AroP-alkaline phosphatase fusion protein was determined to ensure that all fusion proteins are synthesized at approximately the same rate. Fusion proteins were analyzed by pulse-labeling of strains carrying *aroP′-phoA-aroP* fusion constructs with [^{35}S]methionine-cysteine followed by immunoprecipitating the fusion products with antibody to alkaline phosphatase. The results of such analyses are shown in Fig. 4. It can be seen that the AroP-alkaline phosphatase fusion proteins are synthesized at comparable rates, and low-level alkaline phosphatase activity can be attributed to the cytoplasmic location of such fusions and not to the low rates of synthesis of the fusion proteins.

Transport activities of AroP-PhoA fusions. The AroP-alkaline phosphatase fusion constructs were analyzed for the ability to transport tyrosine. Only three of the fusions, P261, A354, and G425, retained any ability to transport tyrosine, with transport activities of 24, 10, and 16% of the wild-type permease activity, respectively. These three fusions with some transport activity are all localized in the periplasm.

The importance of charged residues E151, E153, and K160. Based on the results of the AroP-PhoA fusions, we have significantly revised the topological model of AroP such that amino acid residues after V148 are moved from the cytoplasm into transmembrane span 5, and those residues after S213 are moved out of span 6 into the cytoplasm (Fig. 5). In the new model, the three charged residues E151, E153, and K160 are now embedded within transmembrane span 5. The insertion of charged residues within the hydrophobic environment of the membrane is energetically unfavorable, suggesting that such amino acids have an important role in the function or structure of the permease. To determine the potential importance of the charged residues E151, E153, and K160, site-directed mutagenesis was used to individually change each charged residue in AroP to alanine. Such substitutions totally abolish transport activity of the AroP permease (data not shown), indicating they are essential for transport function.

Characterization of truncated AroP proteins. The identity between AroP and PheP is dramatically reduced in the last two transmembrane spans (Fig. 1). In order to investigate the importance of these spans to overall transport activity, site-directed mutagenesis was used to introduce an in-frame termination codon within the structural region of the *aroP* gene, resulting in premature termination of the AroP polypeptide. Termination codons were introduced at positions G425, Y402, and F359, resulting in polypeptides of 425, 402, and 359 amino acid residues, respectively. Each termination codon results in the progressive removal, from the carboxyl terminus of the protein, of a membrane-spanning segment. Transport studies of strains with these truncated AroP proteins, composed of 11, 10, and 9 membrane-spanning segments, show that they have no uptake ability for tyrosine or phenylalanine.

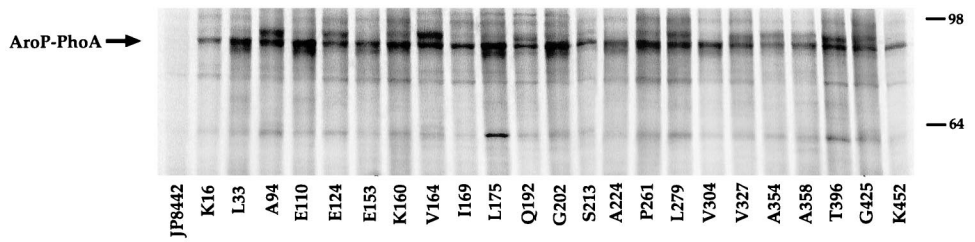

FIG. 4. Synthesis of AroP-alkaline phosphatase sandwich fusion proteins. JP8442 cells containing the gene fusion plasmids were pulse-labeled for 1 min with [^{35}S]methionine-cysteine and immunoprecipitated with alkaline phosphatase antibody. Strain JP8442, which does not contain a gene fusion plasmid, was used as a negative control. The positions of molecular weight standards (in thousands) are shown.

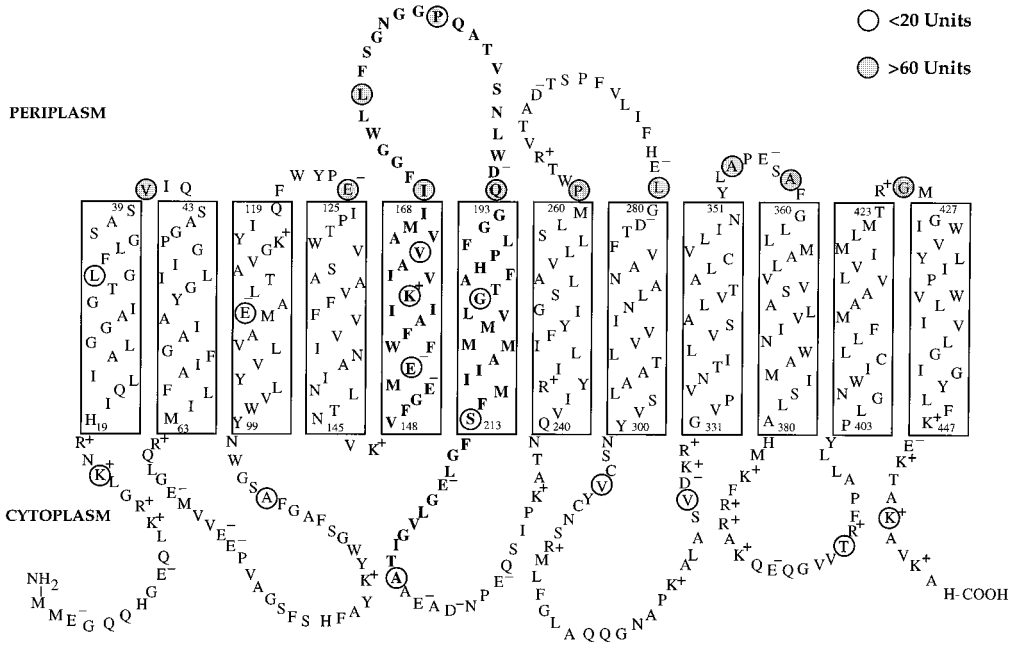

FIG. 5. Modified topological model of the AroP permease based on AroP-alkaline phosphatase fusion analysis. The modified regions of spans 5 and 6 are shown in boldface type.

DISCUSSION

In this study, we have characterized sandwich fusions between AroP permease and alkaline phosphatase in order to obtain information about the transmembrane topology of the permease. The pattern of activity of most of the fusions was consistent with the proposed topological model of the AroP permease, with the exception of two regions, involving fusions within the previously proposed transmembrane span 5 and in the putative periplasmic loop between spans 5 and 6.

The high-level alkaline phosphatase activities exhibited by fusions at I169 (270 U) and L175 (363 U) suggest that this region of the permease is actually located in the periplasm, rather than within transmembrane span 5 as predicted in the original model. Modification of the model in this area involves repositioning I169 to the junction between span 5 and periplasmic loop 3 and moving residues from V148 out of cytoplasmic loop 3 into transmembrane span 5 to ensure that a membrane span length of 21 amino acids is maintained. A consequence of such an alteration is the positioning of the three charged residues E151, E153, and K160 within the membrane. These three residues are absolutely conserved within all 22 members of the superfamily of bacterial and yeast amino acid transporters (32, 33), and investigation of the importance of these residues in AroP by site-directed mutagenesis showed they were essential for transport function. Similar mutagenic studies on these residues in the PheP permease, a protein very closely related to AroP, showed that they had an essential role to play in the transport of phenylalanine (32).

The use of hydropathy profiles and algorithms to predict transmembrane segments of a membrane protein have proved very useful in the generation of putative topological models (18, 22, 45). However, such models are based on the most energetically favorable configuration, which may not always reflect the actual arrangement of certain regions of the protein. Such techniques may assign a charged residue to an extramembranous environment due to the increased hydrophilicity of the region surrounding it, when in fact the charged residue is actually located within the membrane. As shown by this and other studies (11, 20, 32, 34), the presence of some membrane-buried charged residues is necessary for proper functioning of certain transport proteins. In the lactose permease for example, such charged residues located in the membrane have been implicated to contribute to a charge relay mechanism involved in lactose/H^+ symport (34). We postulate that the essential nature of the conserved charged residues E151, E153, and K160, together with the results from this study which position them within the membrane of the AroP permease, may similarly be involved in a proton relay system. It is possible that one or more of these charged residues may interact or function with the glutamate residue at position 110 in transmembrane span 3 of AroP. This residue has been shown to be essential in the PheP permease (32), and all members of the superfamily of bacterial and yeast amino acid transporters contain a negatively charged residue, either a glutamate or an aspartate, at this position.

Recent work with lactose permease-alkaline phosphatase fusions demonstrates that approximately half of a transmembrane domain is sufficient to promote alkaline phosphatase translocation to the periplasmic surface of the membrane (10, 43). We have modified transmembrane span 5 of AroP based on the exceptionally high-level alkaline phosphatase activities of fusions I169 and L175, originally positioned within the central and carboxyl half of transmembrane span 5 but now localized in the periplasmic loop between spans 5 and 6. In support of this modification are the observed low-level activities for the equivalently positioned "outgoing" AroP-PhoA fusions L33 and E110, located within the carboxyl half of transmembrane spans 1 and 3, which do not translocate alkaline phosphatase to the periplasm. Furthermore, the LacY-PhoA fusions are constructed such that alkaline phosphatase replaces the carboxyl-terminal portion of the lactose permease, whereas the AroP-PhoA sandwich fusions contain alkaline phosphatase inserted

into regions of the whole AroP protein. The presence of the entire membrane protein in the AroP-PhoA sandwich fusions may contribute to the differences observed between the length of the transmembrane domain required for translocation in this study and that in the LacY-PhoA study. It has also been claimed that alkaline phosphatase sandwich fusions generally give a more-accurate representation of membrane protein topology (3, 16, 23, 31, 39). The precise junction sites of transmembrane span 5 with the cytoplasmic and periplasmic loops are not known; however, the summed hydrophobicity of this modified transmembrane segment as it is represented in this model is within the limits proposed by Lee and Manoil for a transmembrane domain (25), thus supporting the positioning of the three charged residues within the transmembrane domain.

The low level of enzymatic activity observed when alkaline phosphatase is fused at G202 in AroP (8 U), a proposed periplasmic location preceding span 6, necessitates the repositioning of the residues in the distal part of periplasmic loop 3 to within the membrane of span 6. Immunoblotting analysis shows this fusion to be quite stable, and the low level of alkaline phosphatase activity does not appear to be due to instability. Similar results have been obtained with PheP-PhoA fusions in this region (31). As observed with the PheP permease, an unexpected consequence of this modification is the removal of E218 from within the membrane in the old AroP model to within the cytoplasm. This glutamate residue is conserved within the superfamily of homologous membrane proteins, and it has been shown that a negatively charged residue is essential at this position in the PheP permease (32).

The analysis of AroP-alkaline phosphatase fusions supports the hydropathy data, which predicts that AroP and PheP permeases have very similar topologies. In both permeases, alkaline phosphatase fusions to the distal portion of putative periplasmic loop 3 indicate that this region is actually located within transmembrane span 6. Based on the high level of identity between the AroP and PheP permeases, the essential nature of the conserved charged residues in span 5, and the similarity in results of alkaline phosphatase fusions thus far, we predict that alkaline phosphatase fusions within putative transmembrane span 5 of PheP will result in a modification to the PheP model similar to that seen for AroP. To date, the lysine-specific permease of *E. coli* is the only other membrane protein within the superfamily of amino acid transporters whose membrane topology has been examined by using gene fusions (17). The LysP topological model generally resembles that of the AroP and PheP permeases, although fusions have not been studied within putative transmembrane span 5 nor in the distal portion of periplasmic loop 3 of the LysP permease. Ellis and coworkers propose a topological model for LysP in which transmembrane span 6 shows a closer resemblance to the modified AroP and PheP topological models, with the conserved glutamate residue (equivalent to E218 in AroP) localized in the cytoplasm rather than within transmembrane span 6 (17).

Fusions P261, A354, and G425 retained a low level of transport activity (24, 10, and 16% of the wild-type permease activity, respectively) despite the insertion of the large alkaline phosphatase moiety. As observed in the PheP and Mtr permeases, these fusions with some transport activity involve insertion of alkaline phosphatase into predominantly periplasmic domains of the protein (31, 39).

The proposed model which predicts AroP to be composed of 12 membrane-spanning segments is well substantiated by the alkaline phosphatase fusion results. Truncated forms of the AroP permease containing 9, 10, and 11 transmembrane segments exhibit no transport activity, suggesting that a full-length AroP protein consisting of all 12 transmembrane spans is required for transport activity. Similar studies with the lactose permease of *E. coli* have shown that truncation mutants in the lactose permease are defective with respective to lactose/H^+ symport (35). The loss of activity observed in these truncated proteins is suggested to be related to a defect in folding, which also results in increased proteolysis.

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