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Membrane Topology of *Escherichia coli* Diacylglycerol Kinase

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The topology of *Escherichia coli* diacylglycerol kinase (DAGK) within the cytoplasmic membrane was elucidated by a combined approach involving both multiple aligned sequence analysis and fusion protein experiments. Hydropathy plots of the five prokaryotic DAGK sequences available were uniform in their prediction of three transmembrane segments. The hydropathy predictions were experimentally tested genetically by fusing C-terminal deletion derivatives of DAGK to β-lactamase and β-galactosidase. Following expression, the enzymatic activities of the chimeric proteins were measured and used to determine the cellular location of the fusion junction. These studies confirmed the hydropathy predictions for DAGK with respect to the number and approximate sequence locations of the transmembrane segments. Further analysis of the aligned DAGK sequences detected probable α-helical N-terminal capping motifs and two amphipathic α-helices within the enzyme. The combined fusion and sequence data indicate that DAGK is a polytopic integral membrane protein with three transmembrane segments with the N terminus of the protein in the cytoplasm, the C terminus in the periplasmic space, and two amphipathic helices near the cytoplasmic surface.

Diacylglycerol kinase (DAGK) from *Escherichia coli* is a small integral membrane protein which catalyzes the reversible formation of phosphatidic acid from MgATP and diacylglycerol. As an enzyme of lipid biosynthesis, DAGK represents a class of biocatalysts which have received relatively little structural or mechanistic attention.

DAGK is remarkable from several standpoints. While it is unclear whether it functions as a monomer or oligomer, its monomeric molecular mass is only 13.2 kDa, making it the smallest known kinase. Nevertheless, it catalyzes a topologically complex reaction: under normal conditions phosphatidic acid and diacylglycerol are membrane associated while MgADP and MgATP are highly water soluble. *E. coli* DAGK does not contain the sequence motifs typically found in kinases and related proteins (15). Indeed, with the exception of several microbial DAGK homologs, it exhibits no homology to any other known kinase, including several water-soluble mammalian DAGK isozymes which have been sequenced (25, 27).

The small size of DAGK (only 122 amino acid residues) and the unusual qualities summarized above make DAGK a particularly attractive candidate for detailed structural and mechanistic analysis. Previous studies by Bell and colleagues (19) have resulted in the cloning, overexpression, and reconstitution of the enzyme and in its kinetic characterization (34). A model for the membrane topology of *E. coli* DAGK based solely on hydropathy plots was proposed. In this study we employ the well-established gene fusion approach (3–5, 20, 21, 31, 37) to establish the membrane topology of DAGK experimentally. In addition, recent determinations of the sequences for several other prokaryotic DAGK homologs along with developments in primary sequence analysis allow identification of some additional structure-function information about DAGK.

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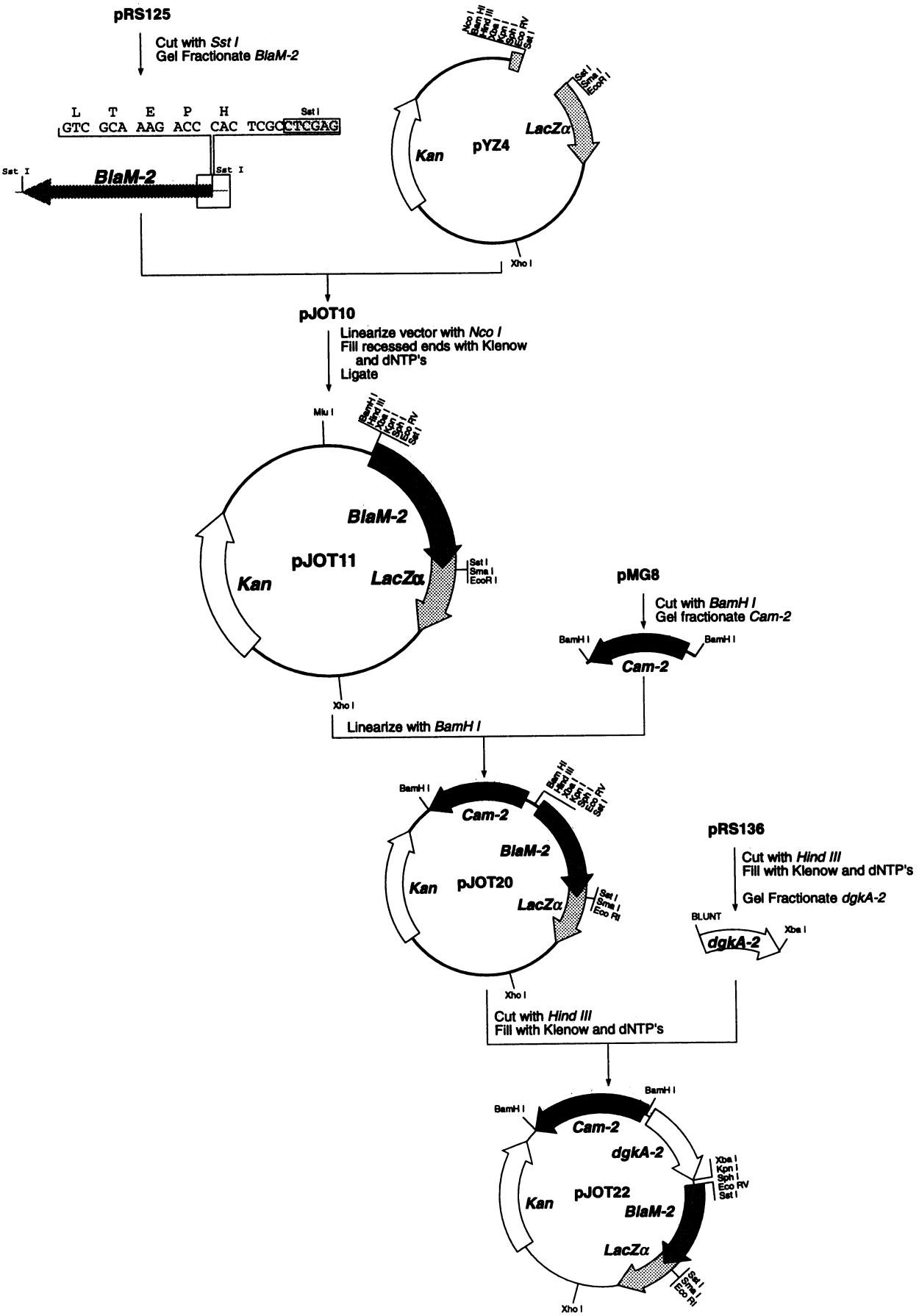
MATERIALS AND METHODS

Culture media and reagents. Luria-Bertani (LB) broth was routinely used as the complex growth medium. Antibiotics were added to the following concentrations: sodium ampicillin, 50 mg/liter; tetracycline, 20 mg/liter; kanamycin sulfate, 50 mg/liter; and chloramphenicol, 50 mg/liter. Restriction endonucleases, T4 DNA ligase, and calf intestinal alkaline phosphatase were supplied by GIBCO-BRL. Sequenase 2.0 and related biochemicals were obtained from U.S. Biochemicals, Inc. Magic Prep plasmid purification kits were obtained from Promega. Additional chemicals were obtained from standard suppliers.

DNA manipulations. Plasmid DNA was prepared from 50-ml cultures on modified silica gel columns obtained from Qiagen. Small-scale preparations were made with the Magic Prep plasmid purification kit. Conditions for restriction endonuclease digestion, DNA ligation, and transformation have been described (2, 11).

DNA sequencing and PCR. DNA sequencing was performed by the dideoxy chain termination method of Sanger (26) as modified by Tabor and Richardson (30), using Sequenase-modified T7 DNA polymerase. Reaction mixtures employed either synthetic oligodeoxynucleotide primers or commercially prepared T7, T3, and M13-20 promoter primers obtained from Promega. PCRs were performed with the Gene Amp Kit obtained from Perkin-Elmer and oligodeoxynucleotide primers obtained from Oligos, Etc. (Wilsonville, Oreg.) and Midland Certified Reagent (Midland, Tex.).

Plasmid construction. *E. coli* DH5α was used as the host strain for all plasmids described in this study. Plasmid constructs are shown in Fig. 1. The wild-type allele of *dgkA* was liberated as a 500-bp *Eco*RI fragment from plasmid pJW10, provided by R. M. Bell (19). This cassette was ligated into the *Eco*RI site of pBC KS+ (Stratagene) to create plasmid pRS136. Plasmid pJOT10 was constructed by ligating a *blaM-2* cassette into the *Sst*I site of plasmid pYZ4, obtained from J. K. Broome-Smith (4). The *blaM-2* cassette encoded by pRS125 (28) was amplified by PCR from pIBI31 (International Biotechnologies, Inc.) and encodes a mature form of β-lactamase which lacks the ATG initiation codon and membrane export


pRS125
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Gel Fractionate BlaM-2
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GTC GCA AAG ACC CAC TCGCTCGAG
Sst I
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BlaM-2
pYZ4
Kan
LacZα
Xho I
pJOT10
Linearize vector with Nco I
Fill recessed ends with Klenow
and dNTP's
Ligate
Mlu I
BlaM-2
pJOT11
Kan
LacZα
pMG8
Cut with BamH I
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BamH I
BamH I
Cam-2
Linearize with BamH I
Cam-2
BlaM-2
pJOT20
Kan
LacZα
pRS136
Cut with Hind III
Fill with Klenow and dNTP's
Gel Fractionate dgkA-2
BLUNT
Xba I
dgkA-2
Cut with Hind III
Fill with Klenow and dNTP's
Cam-2
dgkA-2
pJOT22
Kan
BlaM-2
LacZα

FIG. 1. Plasmid construction. The *blaM-2* cassette encoded by pRS125 (28) was amplified by PCR from pIBI31 (International Biotechnologies, Inc.). Plasmid pYZ4 was obtained from J. K. Broome-Smith (4), and plasmid pJW10 was provided by R. M. Bell (19). Plasmid pMG8 was constructed by M. Gibson (10). Plasmids pJOT11 and pJOT22 were routinely used to construct in-frame fusions to both *blaM* and *lacZα* as described in Materials and Methods.

sequences. To prevent translation of a *lacZ–blaM-2* fusion product, the *lacZ* initiation codon contained within the *Nco*I restriction site 5′ to the multiple cloning region of pJOT10 was destroyed (Fig. 1). This plasmid was designated pJOT11. Plasmid pJOT20 was constructed by ligating a 1.3-kbp *cam-2* cassette, liberated from plasmid pMG8 (10), into the *Bam*HI site of pJOT11. A *dgk2* allele from pRS136 was ligated into the *Xba*I and modified *Hin*dIII sites of pJOT20 to create pJOT22 (Fig. 1).

Isolation of *blaM* and *lacZ* fusions to DAGK. Deletion derivatives of *dgkA* were amplified by PCR from either pRS136 or pJOT22 by using oligodeoxynucleotide primers that generated unique restriction sites at the 5′ (*Kpn*I or *Hin*dIII) and 3′ (*Xba*I or *Not*I) ends. The 3′ sites were situated as to create an in-frame fusion to either *blaM-2* or *lacZα* cassettes upon ligation into pRS138 (28), pJOT11, or pBC KS+. *dgkA-blaM* chimeras derived from pJOT11 could be readily converted to *lacZα* fusions by liberating *blaM-2* as an *Sst*I cassette and religating the plasmid vector.

Assay of enzymatic activity. The MICs of ampicillin for strains encoding *DAGK-blaM* fusions were determined by spotting 10 μl of a 10^{-6} dilution of an overnight culture onto LB agar plates containing ampicillin at concentrations ranging from 0 to 450 μg/ml (35). β-Galactosidase specific activity was qualitatively scored by the ability to cleave the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside contained in LB agar plates.

Polyacrylamide gel electrophoresis and Western immunoblotting. Cells were fractionated into periplasmic, cytosolic, and membrane components by the method of Harayama et al. (12). Sample preparation and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were carried out as described by Laemmli (17). Proteins from unstained SDS gels were electrophoretically transferred to nitrocellulose membranes obtained from Schleicher & Schuell (Keene, N.H.). Unused protein binding sites were blocked by incubating the membrane overnight at 4°C in a blocking buffer consisting of 5.0% (wt/vol) commercial, powdered, nonfat milk, 0.9% NaCl (14), and 1.0% goat serum in distilled water. Subsequent incubation and wash procedures were performed in blocking buffer without goat serum supplementation. Anti-β-lactamase, anti-rabbit immunoglobulin G, and horseradish peroxidase conjugated to avidin were purchased from 5 Prime-3 Prime, Inc., and used at dilutions of 1:1,000. Western blots (immunoblots) were developed by using an Amersham ECL detection kit in accordance with the manufacturer's specifications.

RESULTS AND DISCUSSION

Sequence alignment and homology analysis. The membrane topology previously proposed by Bell and coworkers (19) was based on analysis of the single DAGK sequence available at the time (1985) of their study. Protein and oligonucleotide sequence searches made by using the BLAST algorithm (1) uncovered four new known or probable prokaryotic DAGK sequences, which are listed in Fig. 2 (6, 18, 22, 29, 36). Examination of the manually aligned sequences shows that four of the five exhibit fairly divergent sequences (e.g., <40% identity between *E. coli* DAGK and the DAGK from the other organisms). This is welcome from an analytical standpoint because it means that similarities in overall sequence patterns (such as the location of hydrophobic stretches and putative amphipathic helices) which truly relate to the actual membrane topology should be conserved even though a majority of individual residues diverge.

Kyte-Doolittle hydropathy analysis (16) with a sliding 15-

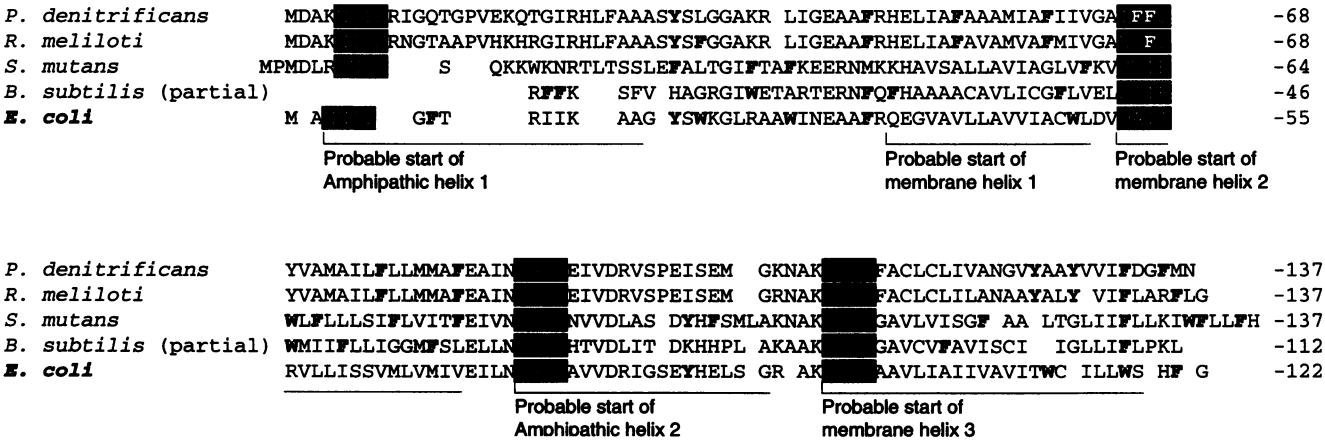

FIG. 2. Sequence alignment. Five prokaryotic DAGK sequences were manually aligned to identify conserved structural features. Sequences contained in black boxes indicate conserved N-terminal helix capping motifs. Sequences contained in the cross-hatched boxes represent a potential N-terminal capping motif that is not as well conserved. Aromatic amino acid residues are indicated by boldface text. Probable positions of the transmembrane segments and amphipathic helices are bracketed. Sequences of the following organisms were obtained from the GenBank database and have been previously reported: *Pseudomonas denitrificans* (accession number M62868 [6]), *Rhizobium meliloti* (accession number M94058 [22]), *Streptococcus mutans* (accession number L12211 [36]), *Bacillus subtilis* (accession number X17430 [29]), and *E. coli* (accession number K00127 [18]). The *B. subtilis* DAGK is most likely a partial sequence.

TABLE 1. Characteristics of DAGK chimeric proteins

Fusion plasmid	Residue	Reporter	Junction sequence			β-Lactamase activity[a]	β-Galactosidase activity[b]	Cellular disposition[c]
			DAGK	Spacer	Reporter			
pJOT24	Gly-21	BlaM	SYKG	LEVPHAISSSA	HPETLV	0		Cytoplasm
pJOT25	Gly-21	LacZα	SYKG		LEVPHA	0	+	
pJOT26	Arg-33	BlaM	AAFR	LEVPHAISSSA	HPETLV	0		Cytoplasm
pJOT27	Arg-33	LacZα	AAFR		LEVPHA	0	+	
pRS211	Leu-49	LacZα	ACWL		SSNSPY	0	−	
pJOT5	Asp-52	BlaM	LDVD	GGR	HPETLV	450		Membrane
pRS212	Leu-58	LacZα	TRVL		SSNSPY	0	−	
pRS213	Val-69	LacZα	VMIV		SSNSPY	0	+	
pJOT3	Met-97	LacZα	AKDM		RPPP	0	+	
pJOT1	Phe-121	BlaM	YSHF	LEVPHAISSSA	HPETLV	450		Membrane
pJOT2	Phe-121	LacZα	YSHF		LEVPHA	0	−	

[a] β-Lactamase activity is expressed as the MIC (in micrograms per milliliter) and represents the mean value of five independent trials.
[b] β-Galactosidase activity is scored as the ability to cleave the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in Luria agar plates.
[c] Cellular disposition was determined by Western blot analysis of cellular fractions with anti-BlaM antibody (Fig. 5).

residue window was performed on all five DAGKs. Identical topologies for all of the sequences were predicted. The suggested topology contained three transmembrane segments.

Analysis of *DAGK-blaM* and *DAGK-lacZ* chimeras. In order to validate the predictions of the Kyte-Doolittle hydropathy plots, a series of genetic fusions that created *DAGK-blaM* and *DAGK-lacZα* hybrid proteins were constructed. The compartment-specific activities of these reporter proteins have been well established and are routinely used to investigate the topology of integral membrane proteins (3–5, 20, 21, 31, 37). Eleven such fusions were constructed (Table 1). All of the plasmids were sequenced to verify the fusion junctions. Western immunoblots with anti-BlaM antibodies were performed on critical fusions to determine the cellular disposition of the chimeric proteins (Fig. 3).

A BlaM fusion at Phe-121 conferred resistance to ampicillin at concentrations of at least 450 μg/ml. This activity indicates that the C terminus of DAGK is localized within the periplasmic space. This interpretation is supported by the lack of detectable β-galactosidase activity from a LacZα fusion to the same residue. The Phe-121–BlaM chimera was found to localize to the cell membrane by Western blot hybridization with anti-BlaM antibody, confirming that the fusion protein remains membrane bound (Fig. 3). The cellular location of the N terminus was determined by LacZα fusions at Gly-21 and Arg-33. These chimeras exhibit strong β-galactosidase activity while BlaM fusions to the same residues fail to confer ampicillin resistance to the host strain. These combined activities indicate that the N terminus of DAGK is arrayed within the cytoplasm.

The first of the three predicted transmembrane segments was confirmed by the β-lactamase activity of a BlaM fusion to Asp-52, which conferred resistance to ampicillin at concentrations greater than 450 μg/ml, indicating a periplasmic disposition of the fusion junction. The Asp-52–BlaM chimera was also identified in membrane fractions by Western blot hybridization (Fig. 3). This interpretation is supported by the enzymatic activity of a LacZα fusion to Leu-49, which had no detectable β-galactosidase activity. The remaining two transmembrane segments were confirmed by the enzymatic activities of a LacZα fusions to Leu-58, Val-69, and Met-97. The Leu-58 chimera did not exhibit any β-galactosidase activity, indicating that this residue is either periplasmic or, more likely, buried within the membrane. LacZα fusions to both Val-69 and Met-97 exhibited strong β-galactosidase activity consistent with cytoplasmic dispositions. Therefore, a transmembrane segment must reside in the sequence between the periplasmic Leu-49 and Asp-52 and the cytoplasmic Val-69. Moreover, the enzymatic activities of BlaM and LacZα fusions to Met-97 and Phe-121 indicate the presence of a third transmembrane segment which orients the C terminus of DAGK within the periplasm.

Additional structural properties. The Eisenberg hydrophobic moment (9) and DeLisi algorithms (7) were used to identify possible amphipathic helices within the five published DAGK sequences (Fig. 4). Both algorithms predict two such helices for *E. coli* DAGK. The first amphipathic helix is located at the N-terminal region of the protein between residues 5 and 25. The second putative amphipathic helix is situated between the second and third transmembrane segments in the central

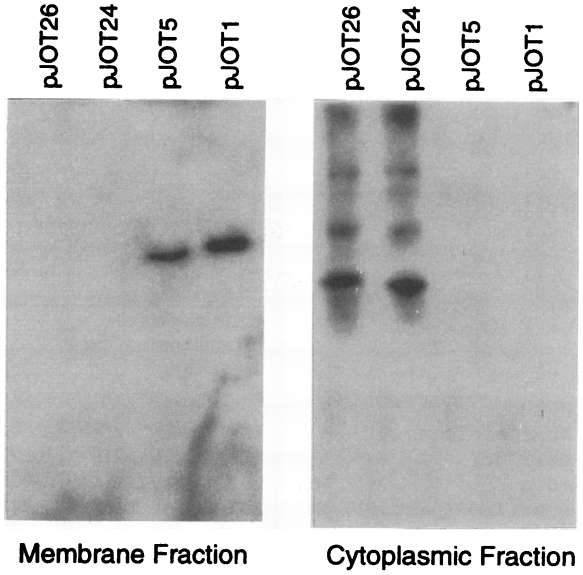

FIG. 3. Western blot analysis of DAGK-BlaM chimeras. Strains of DH5α containing the indicated plasmids (Table 1) encoding *DAGK-blaM* fusions were grown overnight in LB broth and prepared for Western blot analysis as described in Materials and Methods. Membrane and cytoplasmic fractions were prepared from each strain and were probed with anti-BlaM polyclonal antibodies. No bands were identified in experiments using periplasmic and inclusion body fractions (data not shown).

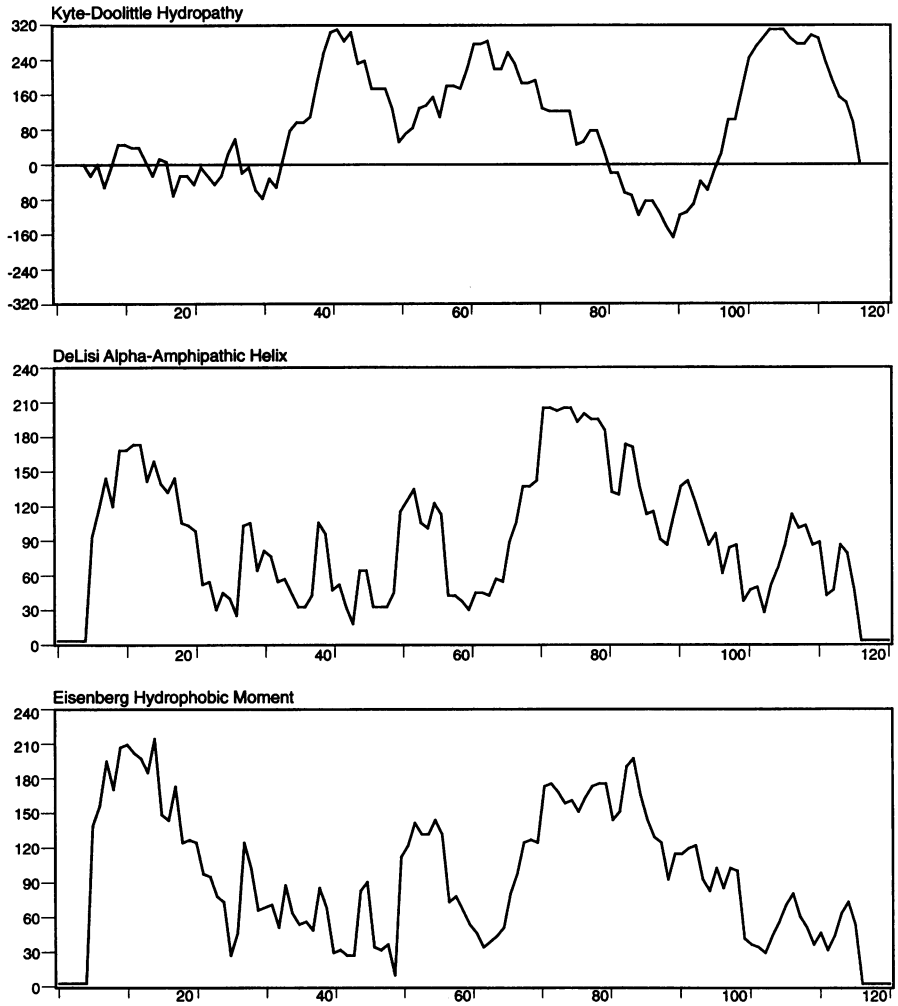

FIG. 4. Hydropathy profiles of the *E. coli* DAGK sequence. The top panel shows the hydropathy of DAGK as determined by the sequence algorithm of Kyte and Doolittle (16), using a sliding window of 15 amino acids. The middle and bottom panels illustrate structural predictions based on the Eisenberg hydrophobic moment (9) and DeLisi α-amphipathic algorithms (7) which identify potential amphipathic helices.

region of the protein at residues 74 to 91. These structures are conserved among the other four DAGKs (Fig. 2).

The five sequences were also examined for the presence of N-terminal helix capping motifs. Such N-cap box motifs, described by Harper and Rose (13), can be composed of a subset of specific combinations of residue pairs with hydrogen-bonding side chains and an intervening -X-X- sequence (e.g., S-X-X-E). We observed that capping boxes are conserved within all of the sequences at three locations (Fig. 2). It is interesting to note that while the capping boxes are conserved, the exact residues composing the key first and fourth residues sometimes vary. The presence of these conserved motifs can be used to increase the probability of correctly locating the initiation of α-helices within structural models of DAGK.

The combined sequence and protein fusion data can be used to generate a new model for the membrane topology of DAGK. The precise positions of the helix boundaries cannot be resolved from the fusion techniques alone; however, their approximate positions can be estimated on the basis of the activities of the fusions and on the distribution of charged residues and the presence of N-terminal helix capping motifs. The enzymatic activities of the chimeric proteins and their cellular dispositions identified three transmembrane segments, in agreement with initial predictions based solely on hydropathy analysis of the *E. coli* sequence (19), and further indicate that the N terminus of DAGK is situated in the cytosol while the C terminus is arrayed within the periplasmic space.

The N-terminal region of DAGK is characterized by a putative amphipathic α-helix which is predicted to begin at Asn-3 with a conserved helix capping motif. The C-terminal boundary of this structure is more ambiguous, although it is likely to terminate at or around Gly-16. The first transmembrane segment is predicted to be formed by the residues between Gly-36 and Asp-50. The C-terminal end of this helix appears to be constrained by the presence of a helix N-cap box which likely initiates a second transmembrane helix of about 20 residues. The first transmembrane segment contains only 14 hydrophobic residues in a row, quite short for a transmembrane α-helix. Extension of this helix to more than 14 residues, and certainly to the 20 to 25 residues expected for a transmembrane α-helix, would require incorporation of at least two charged residues within the hydrophobic membrane environment without apparent possibilities for charge compensation in the other transmembrane segments. One possibility is that the

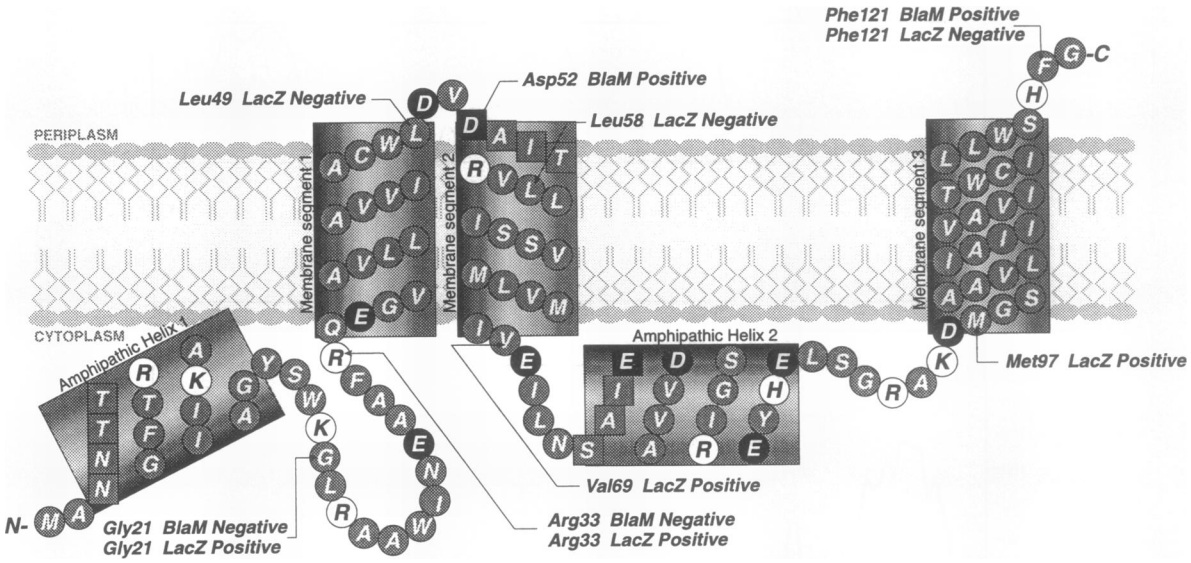

FIG. 5. Model of the membrane topology of DAGK. The model was derived from the protein fusion data and sequence analysis (see Discussion). Individual amino acids are shown in single-letter code. Positively charged residues are enclosed in white circles, negatively charged residues are enclosed in black circles or boxes, and hydrophobic amino acids are enclosed in cross-hatched circles or boxes. Residues that compose putative N-terminal helix capping motifs are contained in boxes. Locations of *blaM* and *lacZ*α fusions are labeled. The text following each fusion indicates the phenotype of strains containing the plasmid encoding the chimera. BlaM refers to β-lactamase activity, while LacZα refers to β-galactosidase activity. The fusion data cannot establish whether the putative amphipathic helices clear the membrane surface, lie on the membrane surface, or are partially buried within the membrane, as recently proposed for prostaglandin synthase (24).

first transmembrane segment could be a 3_{10} helix, which would only require about 15 residues to span a membrane.

A second amphipathic α-helix is predicted to reside in the cytoplasmic loop between the second and third transmembrane helices. This structure is predicted to begin with a helix N-cap box motif that is conserved in all five DAGK sequences. The third transmembrane segment is also predicted to be initiated by a potential α-helical N-cap (D-M-G-S), although this box is not as well conserved among the five DAGK sequences as the other N-capping boxes. In contrast to the first transmembrane segment, this segment contains about 23 uncharged residues in a row, fully consistent with an α-helical secondary structure.

The model described above is depicted in Fig. 5. In general, this model satisfies the positive inside rule in that most of the lysine and arginine residues are located on the cytoplasmic side of the membrane (23, 32, 33). It is also interesting to note that many of the aromatic residues predicted to be within the transmembrane segments lie near the water-lipid interface, a result consistent with their location in the available crystal structures of transmembrane proteins (8). The derived membrane topology will provide a foundation for future studies to determine the functional oligomeric state and tertiary structure of DAGK.

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