Rippe, K., Ramsing, N. B., Klement, R., & Jovin, T. M. (1990) *J. Biomol. Struct. Dyn. 7*, 1199–1209.
Sengupta, S. K., Tinter, S. K., Lazarus, H., Brown, B. L., & Modest, E. J. (1975) *J. Med. Chem. 18*, 1175–1180.
Sentenac, A., Simon, E. J., & Fromageot, P. (1968) *Biochim. Biophys. Acta 161*, 299–308.
Synder, J. G., Hartman, N. G., D'Estantoit, B. L., Kennard, O., Remeta, D. P., & Breslauer, K. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3968–3972.
Sobell, H. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5328–5331.
Straney, D. C., & Crothers, D. M. (1987) *Biochemistry 26*, 1987–1995.
Takusagawa, F., Dabrow, M., Neidle, S., & Berman, H. M. (1982) *Nature 296*, 466–469.
Waring, M. J. (1981) *Annu. Rev. Biochem. 50*, 159–192.
Wells, R. D., & Larson, J. E. (1970) *J. Mol. Biol. 49*, 319–342.
White, R. J., & Phillips, D. R. (1988) *Biochemistry 27*, 9122–9132.
White, R. J., & Phillips, D. R. (1989a) *Biochemistry 28*, 4277–4283.
White, R. J., & Phillips, D. R. (1989b) *Biochemistry 28*, 6259–6269.
Wilson, W. D., Jones, R. L., Zon, G., Scott, E. V., Banville, D. L., & Marzille, L. G. (1986) *J. Am. Chem. Soc. 108*, 7113–7114.
Winkle, S. A., & Krugh, T. R. (1981) *Nucleic Acids Res. 9*, 3175–3186.
Zhou, N., James, T. L., & Shafer, R. H. (1989) *Biochemistry 28*, 5231–5239.

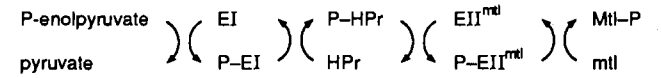
Cytoplasmic Phosphorylating Domain of the Mannitol-Specific Transport Protein of the Phosphoenolpyruvate-Dependent Phosphotransferase System in *Escherichia coli*: Overexpression, Purification, and Functional Complementation with the Mannitol Binding Domain†

Rob P. van Weeghel, Gert Meyer, Hendri H. Pas, Wolfgang Keck, and George T. Robillard*
BIOSON Research Institute, University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands
Received February 8, 1991; Revised Manuscript Received June 10, 1991

ABSTRACT: The cytoplasmic C-terminal domain, residues 348–637, and the membrane-bound N-terminal domain, residues 1–347, of EII^{mtl} have been subcloned and expressed in *Escherichia coli*. The N-terminal domain, IIC^{mtl}, contains the mannitol binding site, and the C-terminal domain, $IIBA^{mtl}$, contains the activity-linked phosphorylation sites, His-554 and Cys-384. Overexpression of the BA domain was achieved by a translational in-frame fusion of the gene with the *cro* ATG start codon, downstream of the strong P_R promoter of phage λ. The domain has been purified and characterized in in vitro complementation assays. It possessed no mannitol phosphorylation activity itself but was able to restore the phosphoenolpyruvate-dependent phosphorylation activity of two EII^{mtl} phosphorylation site mutants, lacking His-554 or Cys-384. The complementary N-terminal domain was also expressed. Membranes possessing IIC^{mtl} were unable to phosphorylate mannitol at the expense of phosphoenolpyruvate. However, when the membranes were combined with the purified C-terminal domain, mannitol phosphorylation activity was restored. Mannitol transport and phosphorylation were also restored in vivo when the two plasmids encoding the N- and C-terminal domains were expressed in the same cell. These data demonstrate the existence of structurally and functionally distinct domains in EII^{mtl}: a cytoplasmic domain with phosphorylating activity and a membrane-bound N-terminal domain which, in the presence of the cytoplasmic domain, is able to actively transport and phosphorylate mannitol. The ability to separate, overproduce, and purify structurally stable, enzymatically active domains opens the way for 3D structural studies as well as complete kinetic analysis of the activities of the individual domains and their interactions.

Mannitol transport in *Escherichia coli* occurs by the series of reactions shown in Scheme I [for reviews, see Postma and Lengeler (1985) and Robillard and Lolkema (1988)]. Mannitol-specific enzyme II (EII^{mtl})[1] has been extensively purified, and its gene, *mtlA*, has been cloned and sequenced (Jacobson et al., 1979; Lee & Saier, 1983). It consists of a membrane-bound N-terminal domain, which binds mannitol (Grisafi et al., 1989; Lolkema et al., 1991), and a phosphorylating C-terminal domain, located in the cytoplasm. The

Scheme I



C-terminal domain contains two activity-linked amino acid residues, His-554 and Cys-384, which are transiently phos-

†This research was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Scientific Research.
* To whom correspondence should be addressed.

[1] Abbreviations: PTS, phosphoenolpyruvate-dependent sugar phosphotransferase system; PEP, phosphoenolpyruvate; DTT, dithiothreitol; EII^{mtl}, mannitol-specific enzyme II; EII^{nag}, *N*-acetylglucosamine-specific enzyme II; EII^{bgl}, β-glucoside-specific enzyme II; IIA^{mtl}, cytoplasmic domain, residues 490–637; IIB^{mtl}, cytoplasmic domain, residues 348–489; $IIBA^{mtl}$, cytoplasmic domain, residues 348–637; IIC^{mtl}, N-terminal domain, residues 1–347; ISO, inside-out; IIA^{man}, P13 domain of $EIII^{man}$; IIB^{man}, P20 domain of $EIII^{man}$.

0006-2960/91/0430-9478$02.50/0

phorylated during mannitol transport (Pas et al., 1988; Pas & Robillard, 1988a,b; Grisafi et al., 1989; White & Jacobson, 1990; van Weeghel et al., 1991a; Stephan et al., 1989; Stephan & Jacobson, 1986).

Molecular weight and sequence comparisons between sugar-specific EII species lacking a soluble EIII and EII/EIII pairs led to the proposal that all EII species had a common evolutionary origin and followed the same sequence of phosphorylation steps (Saier, 1985; Saier et al., 1985). This implied, in the case of EII^{mtl}, the existence of a covalently attached EIII-like domain. Homology was observed between the C-terminal portion of several EII species lacking a soluble EIII and some of the separate EIII species (Saier et al., 1988). Fischer et al. (1989) recently showed 36% sequence homology between the *Staphylococcus carnosus* $EIII^{mtl}$ and the extreme C-terminal domain of *Escherichia coli* EII^{mtl}. The enzymatic activities of these EIII-like domains were first demonstrated by Vogler et al. (1988) and Vogler and Lengeler (1988) by showing that EII^{bgl} and EII^{nag}, with C-terminal domains homologous to $EIII^{glc}$, could replace $EIII^{glc}$ in $EIII^{glc}$-dependent transport and phosphorylation.

In the case of EII^{mtl}, we proved by chemical modification studies and site-directed mutagenesis of catalytic residues that both phosphorylation sites, His-554 and Cys-384, were located on the cytoplasmic domain (Pas & Robillard, 1988b; van Weeghel et al., 1991a,b). Results obtained with C-terminal deletion mutants were used to propose that both phosphorylation site residues, His-554 and Cys-384, were located on two smaller C-terminal domains and that the membrane-bound C domain could bind mannitol in the absence of the C-terminal domains (Grisafi et al., 1989). White and Jacobson (1990) demonstrated complementarity between the two C-terminal domains by subcloning a C-terminal fragment containing His-554 and showing an intermolecular phosphotransfer from this His-554 to a Cys-384 on a truncated EII^{mtl}.

Our approach to study EII^{mtl} structure/function has been to introduce restriction sites into the EII^{mtl} structural gene for easy subcloning of the proposed domains. Furthermore, we have generated mutant enzymes by site-directed mutagenesis to block individual steps in the overall reaction pathway catalyzed by EII^{mtl} and then restored the activity by complementing one mutant EII^{mtl} with another or with subcloned and purified domains (van Weeghel et al., 1991a,b). Data from Grisafi et al. (1989) plus sequence comparisons and a search for flexible regions (Karplus & Schulz, 1985) indicated three possible domains, residues 1–347, 348–489, and 490–637. Restriction sites were introduced at the corresponding nucleotide positions of the flexile loops by site-directed mutagenesis. Using this approach, we have achieved the overexpression, purification, and characterization of the A domain, residues 490–637 (van Weeghel et al., 1991b). The kinetic data showed that IIA^{mtl} [2] was a structurally stable domain which retained its catalytic activity to phosphorylate Cys-384 on the B domain, residues 348–489 of EII-H554A,[3] and to substitute for $EIII^{mtl}$ in the Gram-positive *S. carnosus* PTS. The overexpression, purification, and characterization of the smaller B domain will be described in a following paper.

[2] The nomenclature used in this paper to designate the individual domains has recently been introduced by Saier and Reizer (1991) to standardize the nomenclature for all PTS enzymes. The domains IIA, IIB, IIBA, and IIC correspond to what we previously referred to as CI, CII, CIII, and NIII (van Weeghel et al., 1991a,b).

[3] Amino acid replacements are designated as follows: The one-letter amino acid code is used followed by a number indicating the position of the residue in the wild-type enzyme and then by a second letter denoting the amino acid replacement at this position.

Table I: *E. coli* Strains and Plasmids

name	genotype or phenotype	source or reference
L146-1	[F⁻, *lac*Y1, *gal*T6, *xyl*-7, *thi*-1, *his*G1, *arg*G6, *met*B1, *rps*L104, *mtl*A2, *gut*A50, *gat*A50 (MAL⁺)]	Grisafi et al. (1989)
ASL-1	L146-1, recA	*a*
JM101	[Δ(*lac-pro*AB), *thi*; F′, *tra*D36, *pro*AB, *lac*I^{q}ZΔM15]	Yanish-Perron et al. (1985)
BMH71-18MutL	[Δ(*lac-pro*AB), *thi*, *sup*E; F′ *lac*I^{q}ZΔM15, *pro*A⁺B⁺]	Kramer et al. (1984)
HB2154	[Δ(*lac-pro*AB), *thi*, *ara*, *phx*, *mut*L::Tn10 (Tc, 10μg/mL); F′ *pro*A⁺B⁺, *lac*I^{q}ZΔM15]	Carter et al. (1985)
pWAMa	pMa5-8, *mtl*A	van Weeghel et al. (1990)
pJRD187	Ap^R, *c*I857-P_R	Davison et al. (1987)
pMc5-8	Ap^S, Cm^R, f1 ori	Stanssens et al. (1989)
pWAMc11	pWAMa, *Bam*HI site	*b*
pJRDCIII	pJRD187, P_R-*c*I857-CIII (=$IIBA^{mtl}$)	*b*
pMcCIII	pMc5-8, P_R-CIII ($IIBA^{mtl}$), Δ*c*I857	*b*
pMcCIIIi	pMc5-8, P_R-*c*I857-CIII ($IIBA^{mtl}$)	*b*
pMcNIII	pMc5-8, P_{mtl}-NIII (IIC^{mtl})	*b*

[a] Lengeler and Scholle (Department of Biology, University of Osnabruck, Osnabruck, FRG). [b] This study.

The present study deals with the complete cytoplasmic portion of EII^{mtl}, residues 348–637, and the membrane-bound N-terminal portion, residues 1–347. The cytoplasmic portion is called $IIBA^{mtl}$ and is composed of the two smaller domains A and B (see Figure 4A). Since this portion possesses both known phosphorylation sites, it should be functional in phosphorylating mannitol when combined with the N-terminal domain which possesses the mannitol binding site. The data presented in this paper will show that $IIBA^{mtl}$ and IIC^{mtl} are structurally stable domains which can be expressed separately and retain their catalytic activities. Combination of both domains restored the mannitol phosphorylation activity due to reconstitution of a functional EII^{mtl} in vivo.

Materials and Methods

Materials. Restriction endonucleases, DNA polymerase Klenow fragment, T4 DNA ligase, and T4 polynucleotide kinase were obtained from Boehringer Mannheim. M13K07 helper phage was purchased from Pharmacia. Q-Sepharose Fast Flow and a Mono-P/HPLC column were from Pharmacia, and hydroxylapatite was from Bio-Rad. Nitrocellulose filters were from Schleicher & Schuell. The oligonucleotide R11, 5′-pATACGACGAGGATCCGCTTCAATA-3′, obtained from Eurosequence BV Groningen, was prepared on an Applied Biosystems Model 380B DNA synthesizer, completely deprotected, and used unpurified. Enzyme I and HPr were purified as described (Dooijewaard et al., 1979; Van Dijk et al., 1990). D-[1-^{14}C]Mannitol (59 mCi/mmol; 1 mCi = 37 MBq) was purchased from the Radiochemical Centre Amersham. The nonionic detergent decyl-PEG was obtained

from B. Kwant (Department of Chemistry, University of Groningen). IgG–horseradish peroxidase conjugate and reagents for color development were purchased from Bio-Rad. Other biochemicals were of analytical grade.

Bacterial Strains, Plasmids, and Growth Medium. E. coli strains and plasmids used in this study are listed in Table I. ASL-1 and JM101 were used for plasmid propagation and as host strains for protein expression, while strains BMH71-18MutL and HB2154 were both used for oligonucleotide-directed mutagenesis. Phagemid pWAMa, containing the *mtlA* gene encoding EII^{mtl} under the control of its own promoter P_{mtl}, was used as the parental vector in the mutagenesis procedure in combination with the pMac5-8 phagemid system (van Weeghel et al., 1990; Stanssens et al., 1989). Strains harboring different plasmids or phagemids were grown in 2×TY broth (16 g of Bacto-tryptone, 10 g of yeast extract, and 5 g of NaCl in 1 L) supplemented with the appropriate antibiotics. McConkey indicator plates (Agar Base, Difco) containing 0.2% D-mannitol and appropriate antibiotics were prepared as indicated by the supplier.

DNA Manipulations. Transformation of *E. coli* strains, plasmid isolation procedures, and other recombinant DNA techniques were carried out according to Maniatis et al. (1982). The mutant *mtlA* gene containing the mutation was sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977).

Oligonucleotide-Directed Mutagenesis and Construction of IIC^{mtl} and $IIBA^{mtl}$ Expression Vectors. Isolation of single-stranded DNA and oligonucleotide-directed mutagenesis were carried out according to the gapped-duplex method with amber selection using the pMac5-8 vector system as described by Stanssens et al. (1989). To identify putative mutants with a newly generated *Bam*HI restriction site, double-stranded plasmid DNA from randomly picked transformants was prepared and cut with *Bam*HI restriction enzyme. The gene fragment encoding the C-terminal BA domain, amino acids 347–637, was excised from plasmid pWAMc11 DNA by using the restriction enzyme *Bam*HI and ligated in the correct orientation into pJRD187 plasmid DNA that had been linearized with the same enzyme (Figure 1). The vector containing CIII in frame behind the strong λ P_R promoter was designated pJRDCIII. The remaining fragments of plasmid pWAMc11, vector DNA and NIII with its own promoter P_{mtl}, were religated. The resulting plasmid was designated as pMcNIII (not shown in Figure 1). The NIII structural gene does not possess its own termination codon. Consequently, IIC^{mtl} produced from this plasmid will probably possess an extra C-terminal 22 amino acids arising from plasmid DNA prior to a natural termination codon. The vector pMcNIII was transformed into ASL-1 cells to construct a IIC^{mtl} production strain or into ASL-1 harboring plasmid pJRDCIII for complementation of $IIBA^{mtl}$. ASL-1/pJRDCIII cells transformed with pMcNIII were selected on McConkey plates containing ampicillin (100 μg/mL) and chloramphenicol (25 μg/mL) to ensure that both plasmids were present in the same cells for expression. Because both expression vectors were compatible, it was possible to express the C domain and the BA domain simultaneously in ASL-1 cells for complementation analysis. pJRDCIII was digested either with *Eco*RI and *Sal*I or with *Hind*III, generating two different DNA inserts encoding the promoter–$IIBA^{mtl}$ fusion. Both fragments were recloned into phagemid pMc5-8 digested with the same restriction enzymes. The resulting vectors, pMcCIIIi and pMcCIII, were transformed into competent *E. coli* JM101 cells to construct the corresponding overproduction strains.

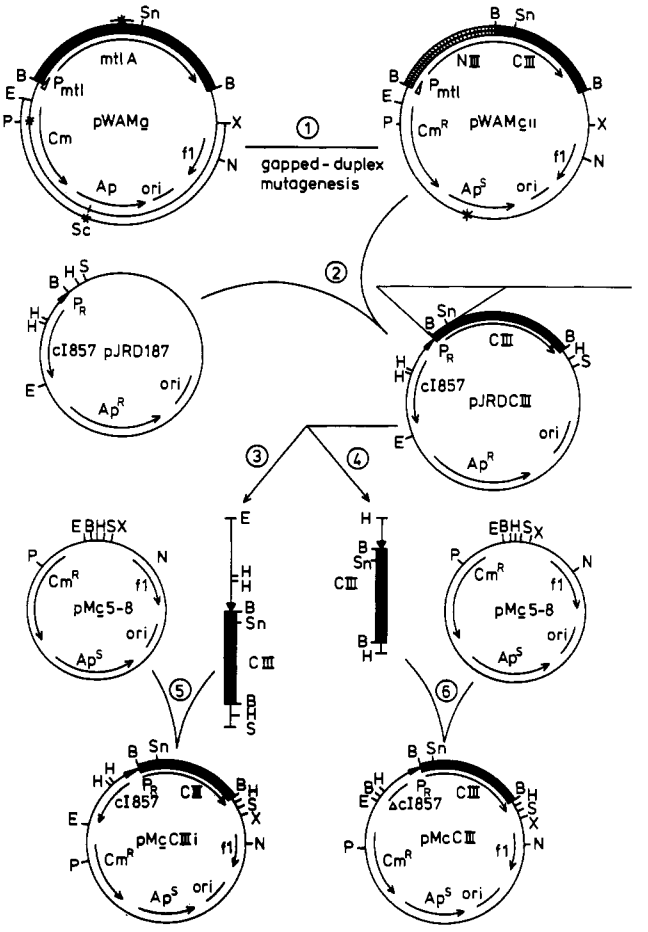


FIGURE 1: Construction of mutagenesis/expression plasmids pMcCIII and pMcCIIIi. Plasmids are not drawn to scale, and only relevant restriction sites are shown. The black areas correspond to the *mtlA* gene and its specific gene fragments NIII and CIII. (1) A new *Bam*HI site was introduced in the vector pWAMa after a round of site-directed mutagenesis, yield pWAMc11. (2) The latter plasmid was digested with *Bam*HI. The small *Bam*HI–*Bam*HI fragment, encoding CIII, was purified and ligated into the unique *Bam*HI site of pJRD187, yielding pJRDCIII. The correct orientation of CIII lead to a translational in-frame fusion with the *cro* ATG start codon downstream of the λ P_R promoter. (3) Cleavage of pJRDCIII with *Eco*RI and *Sal*I gives a DNA fragment containing the *c*I857 gene, the P_R promoter, and the *cro*-CIII fusion. (4) Cleavage of the same plasmid with *Hind*III resulted in a promoter P_R and a *Cro*-CIII fragment without an intact *c*I857 gene. (5) Purified *Eco*RI–*Sal*I fragment was inserted into the multiple cloning site of the mutagenesis vector pMc5-8 digested with identical enzymes, resulting in pMcCIIIi (i, thermo*i*nduction). (6) Plasmid pMcCIII was generated after ligation of the *Hind*III promoter–fusion fragment into the *Hind*III site of pMc5-8. Abbreviations: *a*, ampicillin resistance; *c*, chloramphenicol resistance; B, *Bam*HI; E. *Eco*RI; H, *Hind*III; N, *Nae*I; P, *Pvu*II; S, *Sal*I; Sc, *Sca*I; Sn, *Sna*BI; X, *Xba*I; asterisk, amber mutation. The direction of the genes is indicated by an arrow. CIII = IIBA domain, NIII = IIC domain.

Preparation of ISO Vesicles. E. coli ASL-1 cells were transformed with the expression plasmids pWAMa, pWAMc7, and pWAMc5 [encoding wild-type EII^{mtl} and the phosphorylation site mutants EII-H554A and EII-C384S as previously described (van Weeghel et al., 1991a)], and with pMcNIII encoding IIC^{mtl}. Cells were grown to an OD_{600} of 1.5, collected by centrifugation, and disrupted by passage through a French pressure cell. The membrane fraction (ISO vesicles) was prepared as previously described (van Weeghel et al., 1990). Aliquots of ISO vesicles were stored at −80 °C until used.

Protein Characterization. Crude cell lysates and column fractions were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), in 15% gels which were stained either

with Coomassie brilliant blue or with silver (Laemmli, 1970; Wray et al., 1981), or were used for transfer of proteins to nitrocellulose filters (Western blots) as described by Towbin et al. (1979). $IIBA^{mtl}$ was detected immunochemically on the blots by polyclonal antiserum raised in rabbit against purified EII^{mtl}, in combination with an IgG–horseradish peroxidase conjugate (Pas et al., 1987). The N-terminal amino acid sequence was determined with an Applied Biosystems Model 477A protein sequencer (pulse-liquid sequenator) connected on-line with a 120A PTH-analyzer (Eurosequence BV, University of Groningen).

Protein Determination. The $IIBA^{mtl}$ concentration was determined by the method of Bradford (1976) with bovine serum albumin as the standard, in combination with a pyruvate burst (Robillard & Blaauw, 1986; Pas et al., 1988). The concentration of EII-H554A (2.8 μM), EII-C384S (0.68 μM), and IIC^{mtl} (3 μM) in the ISO vesicle preparations was calculated from mannitol binding data measured with flow dialysis (Lolkema et al., 1991), assuming one high-affinity binding site per EII dimer (Pas et al., 1988).

$IIBA^{mtl}$ Assay: Complementation of Phosphoenolpyruvate-Dependent Phosphorylation Activity. PEP-dependent mannitol phosphorylation activity was assayed by using the mutant complementation assay as described (van Weeghel et al., 1991b), to characterize the enzymatic activity of $IIBA^{mtl}$ in vitro and to calculate the specific activities. The EII^{mtl} mutants, EII-H554A and EII-C384S or IIC^{mtl}, lacking one or both phosphorylation sites, were complemented by mixing different amounts of purified $IIBA^{mtl}$ with solubilized ISO vesicles containing EII-H554A, EII-C384S, or IIC^{mtl}, as schematically depicted in Figure 4. Mannitol phosphorylation activity was measured as the amount of [^{14}C[mannitol 1-phosphate formed per minute per mole of EII^{mtl}. A typical 100-μL assay mixture consisted of 25 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 0.35% decyl-PEG, 3 μM HPr, 0.22 μM EI, 20 μM [^{14}C]mannitol, and ISO vesicles containing 1.7 nM wild-type EII or 5.6 nM EII-H554A or 34.4 nM EII-C384S, or 15 nM IIC^{mtl}. Purified $IIBA^{mtl}$ was added to the mixture in different amounts. The assay mixture was preincubated at 30 °C for 5–10 min, after which 5 mM PEP was added to initiate the reaction. Activities were measured as a function of time at various enzyme concentrations and were linear with respect to both parameters. K_m and V_{max} constants were derived by plotting the data in a double-reciprocal plot.

Results

Vector Constructions and Subcloning of $IIBA^{mtl}$ and IIC^{mtl}. An oligonucleotide primer was designed to introduce a convenient *Bam*HI restriction site at position 1179 of the *mtl*A nucleotide sequence, which also resulted in the replacement of two amino acids, Ala-348 and Thr-349, with Asp and Pro, but not in inactivation of the protein. Figure 1 shows the further subcloning of the gene fragment encoding the $IIBA^{mtl}$ domain into plasmid pJRD187, resulting in translational *cro*ATG-$IIBA^{mtl}$ gene fusion, which was confirmed by DNA sequencing. The plasmids pMcCIIIi and pMcCIII, with the expression under the control of the strong, temperature-inducible P_R promoter, were constructed to maintain a coupled expression/mutagenesis system for the IIBA domain. Plasmid pMcNIII directs the expression of the remaining C domain, with a probable extra 22 amino acids, from its own promoter P_{mtl} and the promoter of the tetracycline resistance gene (P_{tet}).

$IIBA^{mtl}$ Domain Expression and Purification. Overproduction strains harboring pMcCIIIi for thermoinducible expression or pMcCIII for constitutive expression, due to *c*I857 gene deletion (Figure 1), showed the same amount of overproduction of $IIBA^{mtl}$ without any severe problems for the cells. Therefore, the constitutive expression plasmid pMcCIII was used for the overproduction of $IIBA^{mtl}$. Figure 2 examines the production of the BA domain in cells harboring the plasmid pMcCIII (lane 1) versus host cells lacking the plasmid (lane 3) or harboring the parental plasmid pMc5-8 (lane 2). The 31.6-kDa protein in lane 1 is that expected for $IIBA^{mtl}$. Lane 2 shows a 25-kDa protein, which is most likely the chloramphenicol acetyltransferase (CAT) involved in conferring chloramphenicol resistance to cells harboring the plasmid pMc5-8 and its derivatives.

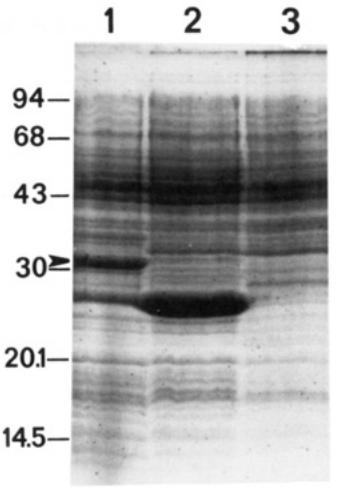


FIGURE 2: Overproduction of the BA domain of EII^{mtl}. Lanes 1–3 show analysis of whole cell lysates on a 15% SDS–polyacrylamide gel of samples taken after the growth of strain JM101. Lane 1, JM101 containing pMcCIII; lane 2, JM101 containing the parental plasmid pMc5-8; Lane 3, control lysate of JM101 without plasmid. The positions of marker proteins and their molecular weights ($\times 10^{-3}$) are indicated on the left. The arrow indicates the expressed BA domain of 31 685 daltons.

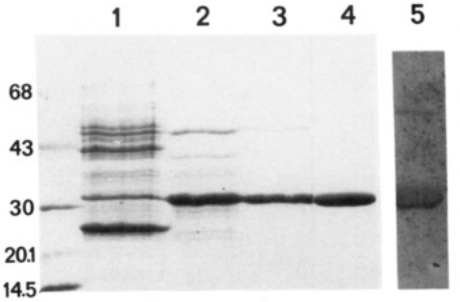


FIGURE 3: Purification of $IIBA^{mtl}$ as illustrated by Western blotting and SDS–polyacrylamide gel electrophoresis (15%) of fractions obtained during successive chromatography steps. Lane 1, crude cell extract of JM101/pMcCIII; lane 2, pooled fractions after chromatography on Q-Sepharose Fast Flow; lane 3, pooled fractions after chromatography on hydroxylapatite; lane 4, peak fraction of the HPLC/Mono-P column; lane 5, immunodetection of purified $IIBA^{mtl}$. The positions of the protein markers and their molecular weights ($\times 10^{-3}$) are indicated on the left.

$IIBA^{mtl}$ Purification. A 7.5-L culture of JM101/pMcCIII was grown at 37 °C in 2×TY supplemented with chloramphenicol (25 μg/mL) for 20 h. Samples of 1 mL were taken at different times to control the expression level during the fermentation. The cells were harvested (20 g of cells, wet weight) and resuspended in 180 mL of buffer A (20 mM Tris-HCl, pH 9.5, 1 mM DTT, 1 mM EDTA, and 1 mM PMSF). This and all subsequent steps were carried out at 4 °C. The resuspended cells were lysed by passage through a French pressure cell at 10000 psi (1 psi = 6.89 kPa) followed by centrifugation at 200000*g* for 60 min to remove cell debris. The supernatant (Figure 3, lane 1) was dialyzed overnight

Table II: In Vitro Complementation of EII^{mtl} Phosphorylation Site Mutants or the N-Terminal Domain with Purified $IIBA^{mtl}$

	ISO vesicles[a]	$IIBA^{mtl}$ concentration[b] (μM)	PEP-dependent phosphorylation activity[c] [nmol of Mtl-P min^{-1} (nmol of EII)$^{-1}$]
	no EII^{mtl}		0
	no EII^{mtl}	80	0
	EII^{mtl}		855
A	EII-H554A		0
	EII-H554A	2	49.0
	EII-H554A	10	169.4
	EII-H554A	20	272.3
	EII-H554A	40	348.0
	EII-H554A	80	428.7
B	IIC		0
	IIC	10	14.6
	IIC	20	25.0
	IIC	30	35.0
	IIC	40	48.4
	IIC	80	79.4
C	EII-C384S		0
	EII-C384S	20	12.8
	EII-C384S	40	22.4
	EII-C384S	80	41.6

[a] ISO vesicles were prepared as described under Materials and Methods by using ASL-1 as host strain, harboring the expression plasmids pWAMa (EII^{mtl}), pWAMc7 (EII-H554A), pMcNIII (IIC), and pWAMc5 (EII-C384S). [b] Purified BA domain from *E. coli* was used in the assays. [c] Assay conditions and protein concentrations used are specified under Materials and Methods. All activities were corrected for background activity. Background activity is defined as the mannitol phosphorylation activity measured in the absence of IIBA but in the presence of all other components of the normal complementation assay. In the case of EII-H554A, it was 0.12 nmol of Mtl-P min^{-1} (nmol of EII-H554A)$^{-1}$; for EII-C384S, 0.7 nmol of Mtl-P min^{-1} (nmol of EII-C384S)$^{-1}$; and for IIC, 0.10 nmol of Mtl-P min^{-1} (nmol of IIC)$^{-1}$.

against buffer B (20 mM Tris-HCl, pH 9.5, 1 mM DTT, and 150 mM NaCl), after which it was loaded onto a Q-Sepharose Fast Flow column (2.5 × 20 cm, 60 mL/h) equilibrated with buffer B. After the column was washed with 100 mL of buffer, it was eluted with a linear gradient of 150–350 mM NaCl in buffer B (400 mL, 60 mL/h). SDS–polyacrylamide gels and activity measurements in a complementation assay with EII-H554A, as described under Materials and Methods and by van Weeghel et al. (1991b), were used to identify fractions containing $IIBA^{mtl}$. These were pooled (Figure 3, lane 2), dialyzed against buffer C (50 mM Tris-HCl, pH 6.8, 25 mM sodium phosphate, and 1 mM DTT), and loaded onto a hydroxylapatite column (1 × 10 cm, 20 mL/h) equilibrated with buffer C. Fractions containing $IIBA^{mtl}$ were obtained after the column was washed with 100 mL of buffer and eluted with a linear gradient of 25–100 mM sodium phosphate (200 mL, 15 mL/h). The hydroxylapatite pool (Figure 3, lane 3) was dialyzed against buffer D (25 mM Bis-Tris-HCl, pH 6.3, and 1 mM DTT) and loaded onto a Mono-P/HPLC column equilibrated with the same buffer, at a rate of 60 mL/h. The column was washed with 10 mL of buffer D and eluted with a pH gradient of 10% polybuffer (7-4) at a rate of 60 mL/h. $IIBA^{mtl}$ eluted at pH 4.5. Figure 3, lane 4, shows the pool of the peak fractions containing pure $IIBA^{mtl}$. An immunoblot of purified $IIBA^{mtl}$ with antibodies raised against pure EII^{mtl} is shown in lane 5. The activity of the purified $IIBA^{mtl}$ was measured, and aliquots of $IIBA^{mtl}$ were stored in liquid nitrogen until used. The procedure resulted in 99% pure $IIBA^{mtl}$ with a typical yield of 50–75 mg from 20 g of cells wet weight. N-Terminal sequence analysis of the purified BA domain confirmed the primary amino acid sequence, Met-Asp-Pro-Arg-Arg-..., as predicted from the sequence DNA fusion.

SDS–polyacrylamide gels of $IIBA^{mtl}$ fractions during various isolations indicated that the band corresponding to $IIBA^{mtl}$ often consisted of two components with slightly different mobilities. These represent phosphorylated and dephosphorylated forms of $IIBA^{mtl}$. This has been demonstrated by showing the interconversions of the forms by (a) preincubation with mannitol to dephosphorylate the enzyme or (b) preincubation with PEP, EI, and HPr to phosphorylate the enzyme. Phosphorylation results in the form with a lower mobility on SDS gels.

Complementation of the PEP-Dependent Phosphorylation Activity of EII-H554A. Figure 4A shows the current ideas on the domain structure and phosphorylation sites of EII^{mtl}. $IIBA^{mtl}$ consists of two domains, A and B. The function of the IIA^{mtl} is to catalyze the phosphorylation of the active-site Cys-384. We have shown that IIA^{mtl} can restore the activity of an EII^{mtl} mutant (EII-H554A) by passing the phosphoryl group from its own His-554 to the Cys-384 of the mutant (van Weeghel et al., 1991a,b). $IIBA^{mtl}$ should also possess a functional A domain and be able to catalyze the same intermolecular phosphotransfer. The ability of $IIBA^{mtl}$ to restore the mannitol phosphorylation activity of EII-H554A is reported in Table IIA. There is a clear Michaelis–Menten $IIBA^{mtl}$ concentration dependence of mannitol phosphorylation in the presence of PEP, EI, and HPr. These data yield a K_m for phospho-$IIBA^{mtl}$ on EII-H554A of 22 μM and a V_{max} of 567 nmol min^{-1} (nmol of EII-H554A)$^{-1}$. The K_m is very similar to the 26.4 μM K_m of purified IIA^{mtl} for EII-H554A in the same complementation assay. The V_{max}, on the other hand, was 2–3 times higher than the V_{max} of IIA^{mtl}, 212 nmol min^{-1} (nmol of EII-H554A)$^{-1}$, presumably due to the presence of the IIB^{mtl} domain in $IIBA^{mtl}$. The V_{max} is more than half of that of the mannitol phosphorylation rate of wild-type EII^{mtl}, 855 nmol min^{-1} (nmol of EII^{mtl})$^{-1}$, measured under the same conditions and HPr, EI, and mannitol concentrations, but in the absence of $IIBA^{mtl}$. Therefore, 67% of the phosphorylation activity of EII-H554A could be restored due to the presence of saturating $IIBA^{mtl}$.

Complementation of the PEP-Dependent Phosphorylation Activity of IIC^{mtl}. Figure 4B shows the possible routes of phosphoryl transfer in the complementation assay of $IIBA^{mtl}$ with EII-H554A. Route 1 is the same process catalyzed by IIA^{mtl} (van Weeghel et al., 1991b) and demonstrated by Stephen et al. (1989) and White and Jacobson (1990). $IIBA^{mtl}$ could also catalyze transfer via routes 1+2 and 1+3. The ability of $IIBA^{mtl}$ to phosphorylate mannitol bound to IIC^{mtl}

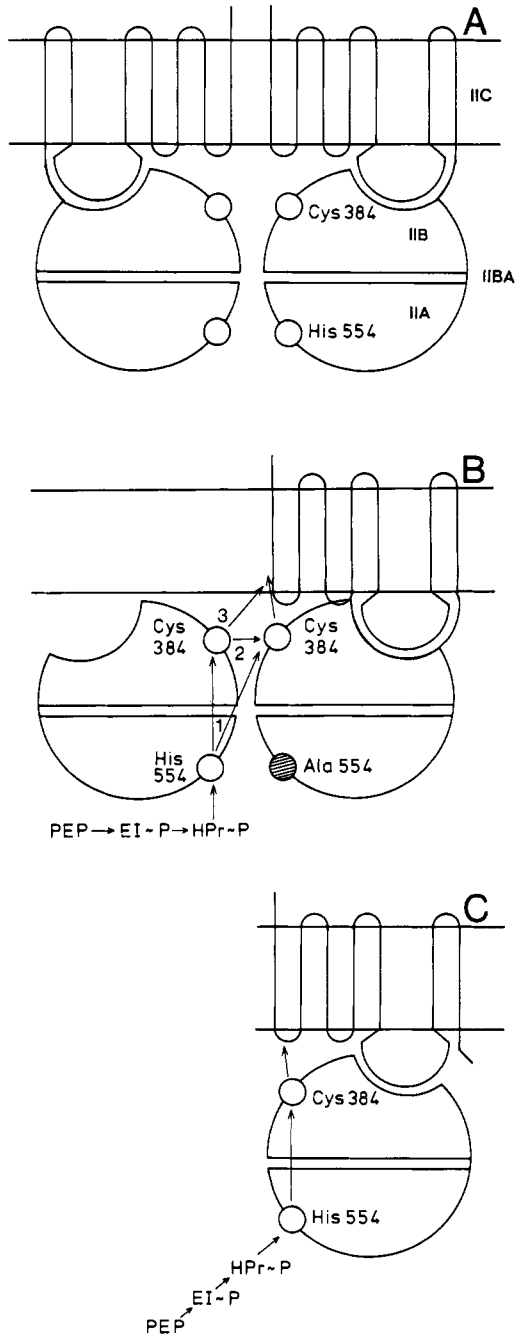


FIGURE 4: (A) Schematic representation of the domain structure of EII^{mtl} and its phosphorylation sites His-554 and Cys-384. (B) Schematic representation of the interaction of $IIBA^{mtl}$ with the EII-H554A mutant missing the first phosphorylation site, His-554. (C) Schematic representation of the C-terminal and N-terminal domain interaction resulting in mannitol phosphorylation activity. The binding of $IIBA^{mtl}$ to the N-terminal C domain should occur in such a way that phospho-Cys-384 is in the position to transfer the phosphoryl group to mannitol. The flow of the phosphoryl group in all three cases is from PEP via the general phosphocarriers EI and HPr to His-554 located on $IIBA^{mtl}$. Three possible routes of phosphoryl group flow to mannitol are indicated by the arrows: (1) from His-554 on one subunit to Cys-384 on an adjacent subunit; (2) from His-554 to Cys-384 on the same subunit and then to Cys-384 on an adjacent subunit before transfer to mannitol; (3) from His-554 to Cys-384 on the same subunit and then directly to mannitol on an adjacent subunit.

via its phosphorylation sites, His-554 and Cys-384, was examined by using membranes containing the expressed C domain and no other form of EII^{mtl} (Figure 4C). Membranes possessing IIC^{mtl} alone are incapable of phosphorylating mannitol in the presence of PEP, EI, and HPr. This has been determined by using IIC^{mtl} concentrations between 30 and 300 nM in the absence of $IIBA^{mtl}$ (Table IIB). $IIBA^{mtl}$ alone or in combination with membranes lacking EII^{mtl} or the IIC^{mtl} domain also shows no mannitol phosphorylation activity. The data in Table IIB demonstrate that $IIBA^{mtl}$ is active in complementation with IIC^{mtl}, in that it can phosphorylate mannitol to bound IIC^{mtl}. The results prove that the two phosphorylation sites on $IIBA^{mtl}$ are functional in the PEP-dependent phosphorylation in combination with IIC^{mtl} and they establish the engineered C domain as a structural and functional entity which, after expression, is inserted in an active conformation into the membrane. Nonlinearity shows up in the Lineweaver–Burk plot of these data at the three highest $IIBA^{mtl}$ concentrations, suggesting a complex dependence of IIC^{mtl} kinetics on the $IIBA^{mtl}$ concentration. As a result, estimates of K_m and V_{max} refer only to the process catalyzed by low $IIBA^{mtl}$ concentrations.

As stated earlier under Results, there are an extra 22 amino acids at the C terminus of IIC^{mtl}. A termination codon is being placed in the gene at the position corresponding to residue 348 to examine whether this extension influences the IIC^{mtl} kinetic behavior.

Complementation of the PEP-Dependent Phosphorylation of EII-C384S. The most straightforward explanation of the above data is that there is a direct complementation of the two domains as depicted in Figure 4C. Whether or not $IIBA^{mtl}$ can complement activity when "inactive" $IIBA^{mtl}$ is also present was examined with membranes containing EII-C384S. This enzyme is completely inactive in mannitol phosphorylation, because it lacks its second phosphorylation site, Cys-384. Nevertheless, the C domain is still present and functional in binding mannitol (van Weeghel et al., 1991a). Mannitol phosphorylation has been assayed with different amounts of $IIBA^{mtl}$ in the presence of saturating concentrations of PEP, HPr, and EI. The data shown in Table IIC demonstrate that EII-C384S alone is completely inactive but that $IIBA^{mtl}$ could restore phosphorylation activities up to rates comparable to those observed in Table IIB with IIC^{mtl}. The presence of an inactive BA domain attached to IIC^{mtl} does not substantially inhibit phosphoryl group transfer between mannitol bound to IIC^{mtl} and a separate BA domain; however, since measurements could not be done at $IIBA^{mtl}$ concentrations in the range of the apparent K_m, no quantitative statements can be made concerning the K_m and V_{max} of $IIBA^{mtl}$ with EII-C384S. These data indicate that the flexible peptide region between the two domains allows for considerable independence so that the inactive domain can move out of the way and allow proper binding of the active cytoplasmic domain to the N-terminal domain (Figure 4C). On the other hand, it could indicate that there is a transfer from Cys-384 across the dimer interface to mannitol bound to IIC^{mtl} (see route 3 in Figure 4B).

In Vivo Complementation of IIC^{mtl} with $IIBA^{mtl}$. ASL-1 cells containing the plasmid pMcNIII (=IIC^{mtl}) or pJRDCIII (=$IIBA^{mtl}$) form white colonies when grown on mannitol-containing McConkey indicator plates, indicating a negative mannitol fermentation phenotype. Transformation of both plasmids into the same cell and growth at 30 °C still gave white colonies. Only incubation of the indicator plates at 42 °C, which leads to thermoinduction of the P_R promotor and consequent expression of $IIBA^{mtl}$, resulted in dark red colonies, a positive fermentation phenotype. The most important conclusion of this in vivo experiment is that the two domains also complement each other in restoring transport activity. The N-terminal domain is able to bind mannitol located in the periplasm and to transport it across the inner membrane to

the cytoplasm where it is phosphorylated by phospho-IIBA^{mtl}.

Discussion

The present data prove that the C- and N-terminal halves of EII^{mtl}, IIBA^{mtl} and IIC^{mtl}, can exist as structurally stable entities and that they retain their function in the separated state; when recombined in vivo and in vitro, transport and phosphorylating activity are restored. Previous reports indicated that the N-terminal half, produced by trypsin removal of the C-terminal half or obtained from deletion mutants, retained the ability to bind mannitol (Stephan et al., 1989; Grisafi et al., 1989). Lolkema et al. (1990) have recently shown that this domain appears to catalyze facilitated diffusion of mannitol over the membrane. Thus, EII^{mtl} consists, functionally, of a phosphorylating domain and a mannitol binding/translocating domain.

The proposed organization of IIBA^{mtl} with each phosphorylation site on a separate domain is analogous to the subunit structure of *E. coli* IIBA^{man} (=EIII^{man}) in which the two sites are located on IIA^{man} and IIB^{man} (Erni et al., 1989). One domain, IIA^{man}, is necessary for binding to phospho-HPr and passing on the phosphoryl group to IIB^{man}, which, in turn, is important for binding to the membrane-bound IIDC^{man} complex and phosphorylation of the sugar. IIA^{mtl} has been isolated as a stable domain which bound to a saturatable binding site on EII-H554A to phosphorylate Cys-384 (van Weeghel et al., 1991b). The same activity has been reported here with IIBA^{mtl}, indicating that it also possesses a functional A domain. The similar K_m value measured for the IIA^{mtl}-catalyzed reaction (route 1), with isolated IIA^{mtl} or with IIBA^{mtl}, suggests considerable independence of the A and B domains in IIBA^{mtl} and implies that IIB^{mtl} is also a structurally stable domain. Work is in progress to purify and characterize this domain.

Computer predictions (Karplus & Schultz, 1985) indicated flexible peptide segments between the various domains as has been reported for the mannose system between IIA^{man} and IIB^{man}. They may confer considerable motional independence of the domains. This is almost certainly the case between IIA^{mtl} and IIB^{mtl}, since we have shown that IIB^{mtl} can be phosphorylated at high rates by IIA^{mtl} of intact enzymes, even when its own inactive A domain is still attached.

Relevance to the Mechanism of Mannitol Transport and Phosphorylation. Lolkema et al. (1990) measured mannitol binding to inside-out and right-side-out cytoplasmic membrane vesicles using flow dialysis and showed that mannitol binds rapidly and with high affinity to EII^{mtl} at either side of the membrane. At equilibrium, the loaded site appears to be situated at the periplasmic face. Displacement studies were consistent with a slow movement of the loaded site over the membrane, thus indicating that EII^{mtl}, in the unphosphorylated state, catalyzes a slow facilitated diffusion. A similar phenomenon was observed if the membranes were treated with trypsin, which removes the C-terminal hydrophilic domain. Facilitated diffusion catalyzed by EII^{mtl} has also been observed using purified enzyme reconstituted in liposomes (Elferink et al., 1990). Since the unphosphorylated, intact enzyme and the separated N-terminal domain are capable of binding and translocating mannitol, the phosphorylation event cannot be considered as a mechanistic requirement for transport in the sense of "no transport without phosphorylation". This has also been confirmed in the II^{glc} system by finding mutant forms of II^{glc} which catalyze facilitated diffusion (Postma, 1981). Our data and those of Erni et al. (1989) for the II^{man} system show that the membrane domain does not become phosphorylated. The phosphoryl group appears to be transferred directly from the IIB domain to the carbohydrate. Since translocation of mannitol without phosphorylation is slow whereas transport coupled to phosphorylation is about 1000 times faster, Lolkema et al. (1991) have proposed that phosphorylation of the B domain must increase the rate at which the C domain translocates the mannitol by a conformational coupling of the two domains. Studies of the structures and activities of the individual domains should provide important insights into how the individual domains modulate the structure and activities of their neighbors.

Acknowledgments

We thank H. Enequist for the DNA sequencing work.

Registry No. PTS, 56941-29-8; EII^{mtl}, 37278-09-4; mannitol, 69-65-8.

References

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Carter, P., Bedouelle, H., & Winter, G. (1985) *Nucleic Acids Res. 13*, 4431–4443.
Davison, J., Heusterspreute, M., Chevalier, N., & Brunel, F. (1987) *Gene 60*, 227–235.
Dooijewaard, G., Roossien, F. F., & Robillard, G. T. (1979) *Biochemistry 18*, 2990–2996.
Elferink, M. G. L., Driessen, A. J. M., & Robillard, G. T. (1990) *J. Bacteriol. 172*, 7119–7125.
Erni, B., Zanolari, B., Graff, P., & Kocher, H. P. (1989) *J. Biol. Chem. 31*, 18733–18741.
Fischer, R., Eisermann, R., Reiche, B., & Hengstenberg, W. (1989) *Gene 82*, 249–257.
Grisafi, P. L., Scholle, A., Sugayama, J., Briggs, L., Jacobson, G. R., & Lengeler, J. W. (1989) *J. Bacteriol. 171*, 2719–2727.
Jacobson, G. R., Lee, C. A., & Saier, M. H., Jr. (1979) *J. Biol. Chem. 254*, 249–252.
Karplus, P. A., & Schulz, G. E. (1985) *Naturwissenschaften 72*, 212–216.
Kramer, B., Kramer, W., & Fritz, H. J. (1984) *Cell 38*, 879–887.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lee, C. A., & Saier, M. H., Jr. (1983) *J. Biol. Chem. 258*, 10761–10767.
Lolkema, J. S., Swaving-Dijkstra, D., & Robillard, G. T. (1990) *Biochemistry 29*, 10659–10663.
Lolkema, J. S., Ten Hoeve-Duurkens, R. H., Swaving Dijkstra, D., & Robillard, G. T. (1991) *Biochemistry* (submitted for publication).
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Pas, H. H., & Robillard, G. T. (1988a) *Biochemistry 27*, 5515–5519.
Pas, H. H., & Robillard, G. T. (1988b) *Biochemistry 27*, 5835–5839.
Pas, H. H., Ellory, J. C., & Robillard, G. T. (1987) *Biochemistry 26*, 6689–6696.
Pas, H. H., Ten Hoeve-Duurkens, R. H., & Robillard, G. T. (1988) *Biochemistry 27*, 5520–5525.
Postma, P. W. (1981) *J. Bacteriol. 147*, 382–388.
Postma, P. W., & Lengeler, J. W. (1985) *Microbiol. Rev. 49*, 232–269.
Robillard, G. T., & Blaauw, M. (1987) *Biochemistry 26*, 5796–5803.
Robillard, G. T., & Lolkema, J. S. (1988) *Biochim. Biophys. Acta 947*, 493–519.
Saier, M. H., Jr., & Reizer, J. (1991) *Res. Microbiol.* (in press).

Saier, M. H., Jr., Yamada, M., Erni, B., Suda, K., Lengeler, J., Ebner, R., Argos, P., Rak, B., Schnetz, K., Lee, C. A., Stewart, G. C., Breidt, F., Waygood, E. B., Peri, K. G., & Doolittle, R. F. (1988) *FASEB J. 2*, 199–208.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Stanssens, P., Opsomer, C., McKeown, Y. M., Kramer, W., Zabeau, M., & Fritz, H. J. (1989) *Nucleic Acids Res. 17*, 4441–4454.
Stephan, M. M., & Jacobson, G. R. (1986) *Biochemistry 25*, 8230–8234.
Stephan, M. M., Khandekar, S. S., & Jacobson, G. R. (1989) *Biochemistry 28*, 7941–7946.
Towbin, H., Staechelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
van Dijk, A. A., de Lange, L. C. M., Bachovchin, W. W., & Robillard, G. T. (1990) *Biochemistry 29*, 8164–8171.
van Weeghel, R. P., Keck, W., & Robillard, G. T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2613.
van Weeghel, R. P., van der Hoek, Y. Y., Pas, H. H., Elferink, M., Keck, W., & Robillard, G. T. (1991a) *Biochemistry 30*, 1768–1773.
van Weeghel, R. P., Meyer, G. H., Keck, W., & Robillard, G. T. (1991b) *Biochemistry 30*, 1774–1779.
Vogler, A. P., & Lengeler, J. W. (1988) *Mol. Gen. Genet. 213*, 719–726.
Vogler, A. P., Broekhuizen, C. P., Schuitema, A., Lengeler, J. W., & Postma, P. W. (1988) *Mol. Microbiol. 2*, 175–178.
White, D. W., & Jacobson, G. R. (1990) *J. Bacteriol. 172*, 1509–1515.
Wray, W., Boulikas, T., Wray, V. P., & Hanock, R. (1981) *Anal. Biochem. 118*, 197–203.
Yanish-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103–119.

Interactions between Pressure and Ethanol on the Formation of Interdigitated DPPC Liposomes: A Study with Prodan Fluorescence†

Junwen Zeng and Parkson Lee-Gau Chong*
Department of Biochemistry, Meharry Medical College, Nashville, Tennessee 37208
Received February 5, 1991; Revised Manuscript Received July 15, 1991

ABSTRACT: Steady-state fluorescence of 6-propionyl-2-(dimethylamino)naphthalene (Prodan) has been employed to study the interacting effects between ethanol and pressure on the formation of the fully interdigitated dipalmitoylphosphatidylcholine (DPPC). At 1 atm and 20 °C, a dramatic change in the emission spectrum of Prodan fluorescence is observed at about 1.1–1.3 M ethanol. The emission maximum shifts to longer wavelengths, and the intensity ratio of Prodan fluorescence at 435 nm to that at 510 nm, F_{435}/F_{510}, decreases abruptly with increasing ethanol content. The spectral changes are correlated to the ethanol-induced phase transition of DPPC from the noninterdigitated gel state to the fully interdigitated gel state [Rowe, E. S. (1983) *Biochemistry 22*, 3299–3305; Simon, S. A., & McIntosh, T. J. (1984) *Biochim. Biophys. Acta 773*, 169–172]. The spectral changes are attributed to the probe relocation from a less polar environment to a more polar environment due to lipid interdigitation. This relocation is either due to the bulky terminal methyl group of the lipids or due to the partition of Prodan into the bulk solution or both. The present study demonstrates that Prodan is a useful probe in monitoring the formation of the ethanol-induced fully interdigitated DPPC gel phase. Pressure is found to produce spectral changes similar to those induced by ethanol when the ethanol content amounts to 0.8–1.1 M. At lower (e.g., <0.4 M) and higher ethanol (e.g., >2.4 M) concentrations, pressure is unable to induce such spectral changes. The critical ethanol concentrations for the formation of the fully interdigitated DPPC gel phase (Cr) have been determined. Cr decreases with increasing pressure in a nonlinear manner, and the Cr's at 50 °C are less than those at 20 °C. These results suggest that high pressure and high temperature assist ethanol in forming the fully interdigitated gel phase in DPPC. The results imply that ethanol "toxicity" as a result of lipid interdigitation can be enhanced under pressure.

Ethanol produces pronounced effects on membranes. The main phase transition temperature (T_m)[1] of dipalmitoylphosphatidylcholine (DPPC) is reduced at low concentrations (<0.87 M) of ethanol but increased at high concentrations (>1.10 M) (Rowe, 1983, 1985). Above a critical ethanol concentration (0.98 M), the main phase transition of DPPC shows marked hysteresis (Rowe, 1985), and the pretransition of DPPC is completely abolished (Veiro et al., 1987). These ethanol-induced effects have been attributed to a transition from the bilayer phase to a gel phase in which the lipid acyl chains from opposing leaflets are fully interdigitated (designated as $L_{\beta}I$) (Simon & McIntosh, 1984). Using X-ray

†This research was supported by the NSF-MRCE (R11-8714805) and the U.S. Army Research Office. This work was done during the tenure of an Established Investigatorship (to P.L.-G.C.) from the American Heart Association and CIBA-GEIGY.
* Address correspondence to this author.

[1] Abbreviations: Cr, critical ethanol concentration; DMPC, dimyristoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPHPC, 1-palmitoyl-2-[3-(1,3,5-hexatrienyl)propanoyl]phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; Laurdan, 6-lauroyl-2-(dimethylamino)-naphthalene; L_{α}, liquid-crystalline phase; $L_{\beta'}$, tilted bilayer gel phase; $L_{\beta}I$, interdigitated gel phase; MLV, multilamellar vesicles; $P_{\beta'}$, rippled gel phase; Prodan, 6-propionyl-2-(dimethylamino)naphthalene; POPOP, *p*-bis[2-(5-phenyloxazolyl)]benzene; T_m, main phase transition temperature.

0006-2960/91/0430-9485$02.50/0