Folding and activity of circularly permuted forms of a polytopic membrane protein

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The transmembrane subunit of the Glc transporter (IICB^{Glc}), which mediates uptake and concomitant phosphorylation of glucose, spans the membrane eight times. Variants of IICB^{Glc} with the native N and C termini joined and new N and C termini in the periplasmic and cytoplasmic surface loops were expressed in *Escherichia coli*. *In vivo* transport/*in vitro* phosphotransferase activities of the circularly permuted variants with the termini in the periplasmic loops 1 to 4 were 35/58, 32/37, 0/3, and 0/0% of wild type, respectively. The activities of the variants with the termini in the cytoplasmic loops 1 to 3 were 0/25, 0/4 and 24/70, respectively. Fusion of alkaline phosphatase to the periplasmic C termini stabilized membrane integration and increased uptake and/or phosphorylation activities. These results suggest that internal signal anchor and stop transfer sequences can function as N-terminal signal sequences in a circularly permuted α-helical bundle protein and that the orientation of transmembrane segments is determined by the amino acid sequence and not by the sequential appearance during translation. Of the four IICB^{Glc} variants with new termini in periplasmic loops, only the one with the discontinuity in loop 4 is inactive. The sequences of loop 4 and of the adjacent TM7 and TM8 are conserved in all phospho*enol*pyruvate-dependent carbohydrate:phosphotransferase system transporters of the glucose family.

Escherichia coli | glucose transporter | membrane insertion | phosphotransferase system

Folding of multispanning (polytopic) membrane proteins is a sequential process, which according to the current model is coupled to the translation of the protein at the ribosome (1–4). Insertion of membrane proteins requires a special translocation apparatus (translocon), a heterotrimeric complex consisting of the SecY, SecG, and SecE protein subunits in bacteria and the Sec61$\alpha\beta\gamma$ subunits in eukaryotes (for a review, see ref. 5). In contrast, folding of globular proteins is a cooperative, posttranslational process whereby chaperones of the DnaK class keep the nascent chains in a folding-competent state until protein synthesis is complete (for a review, see refs. 6 and 7).

There are two types of transmembrane segments in polytopic membrane proteins. The signal anchor sequences, which initiate membrane insertion with retention of their N termini in the cytoplasm, and the stop transfer sequences, which terminate the translocation of extracytoplasmic hydrophilic loops and domains. Stop transfer sequences are followed by a high local concentration of positively charged residues, which may arrest further passage of the polypeptide through the translocon (positive inside rule; ref. 8). Transmembrane segments are hydrophobic and assumed to form α-helical structures, and they are retained in the translocon, from where they move into the lipid bilayer either sequentially or cooperatively after preassembly in the translocon (reviewed in ref. 9). The first, most N-terminal membrane-spanning sequence can have the orientation of either a signal anchor sequence or a stop transfer sequence and in the latter case is termed a reverse signal anchor sequence. Herein, we ask the question whether internal signal anchor and stop transfer sequences of a polytopic membrane protein also can function as N-terminal signal sequences. To answer this question, variants of the transmembrane subunit of the Glc transporter (IICB^{Glc}) subunit of the glucose transporter were constructed in which the native N and C termini were linked by a short peptide and new N and C termini were introduced elsewhere along the polypeptide chain. These circularly permuted variants were analyzed for membrane insertion and transport activity *in vivo*. Prompted by the analogy—albeit a superficial one—between the soluble 8-fold $\beta\alpha$ barrel proteins (TIM barrel), the 8-fold β barrel outer membrane OmpA porin (10), and a membrane protein with eight membrane-spanning helices, new N termini were created in the periplasmic and cytoplasmic surface loops of IICB^{Glc} affording proteins with internal signal anchor sequences and stop transfer sequences as new N-terminal signal sequences.

IICB^{Glc} (Fig. 1) is the membrane-spanning subunit of the Glc transporter of the bacterial phospho*enol*pyruvate-dependent carbohydrate:phosphotransferase system (PTS; for a review, see ref. 11). It mediates uptake with concomitant phosphorylation of Glc. Phospho*enol*pyruvate serves as phosphoryl donor, and the phosphoryl groups are transferred from phospho*enol*pyruvate to IICB^{Glc} through a cascade of three cytoplasmic phosphoproteins, termed enzyme I, HPr, and IIA^{Glc}. IICB^{Glc} consists of two domains, the N-terminal IIC^{Glc} domain and the C-terminal IIB^{Glc} domain (12). The IIC^{Glc} domain (residues 1–≈380), the structure of which is not yet known, is predicted to span the membrane eight times, based on the analyses of protein fusions to alkaline phosphatase (PhoA) and β-galactosidase (13), and this prediction is further substantiated by random linker insertion mutagenesis (R.B., M. Kaufmann, F.R., and B.E., unpublished work). IIC^{Glc} contains the substrate binding and translocation sites. The hydrophilic IIB^{Glc} domain (residues ≈390–477) has a split α/β sandwich fold composed of a four-stranded antiparallel β-sheet and three α-helices on one side (14). IIB^{Glc} contains the phosphorylation site Cys-421. Cys-421 receives the phosphoryl group from the IIA^{Glc} subunit and donates it to the translocated Glc (15, 16).

Herein, we show that a polytopic inner membrane protein can be circularly permuted. Three of four circularly permuted forms with the N and C termini in periplasmic loops are stably expressed and retain up to 58% phosphotransferase activity. Similarly, three variants with new termini in cytoplasmic loops retain up to 70% phosphotransferase activity.

Materials and Methods

Bacterial Strains and Plasmids. *Escherichia coli* K12 ZSC112LΔG (*ptsG*::*cat manZ glk*) was used as host for all experiments (17).

Abbreviations: PTS, phospho*enol*pyruvate-dependent carbohydrate:phosphotransferase system; IICB^{Glc}, transmembrane subunit of the Glc transporter; CL1–3, cytoplasmic loops 1 to 3 of IICB^{Glc}; PL1–4, periplasmic loops 1 to 4 of IICB^{Glc}; PhoA, alkaline phosphatase; αMG; α-methyl-D-glucopyranoside.

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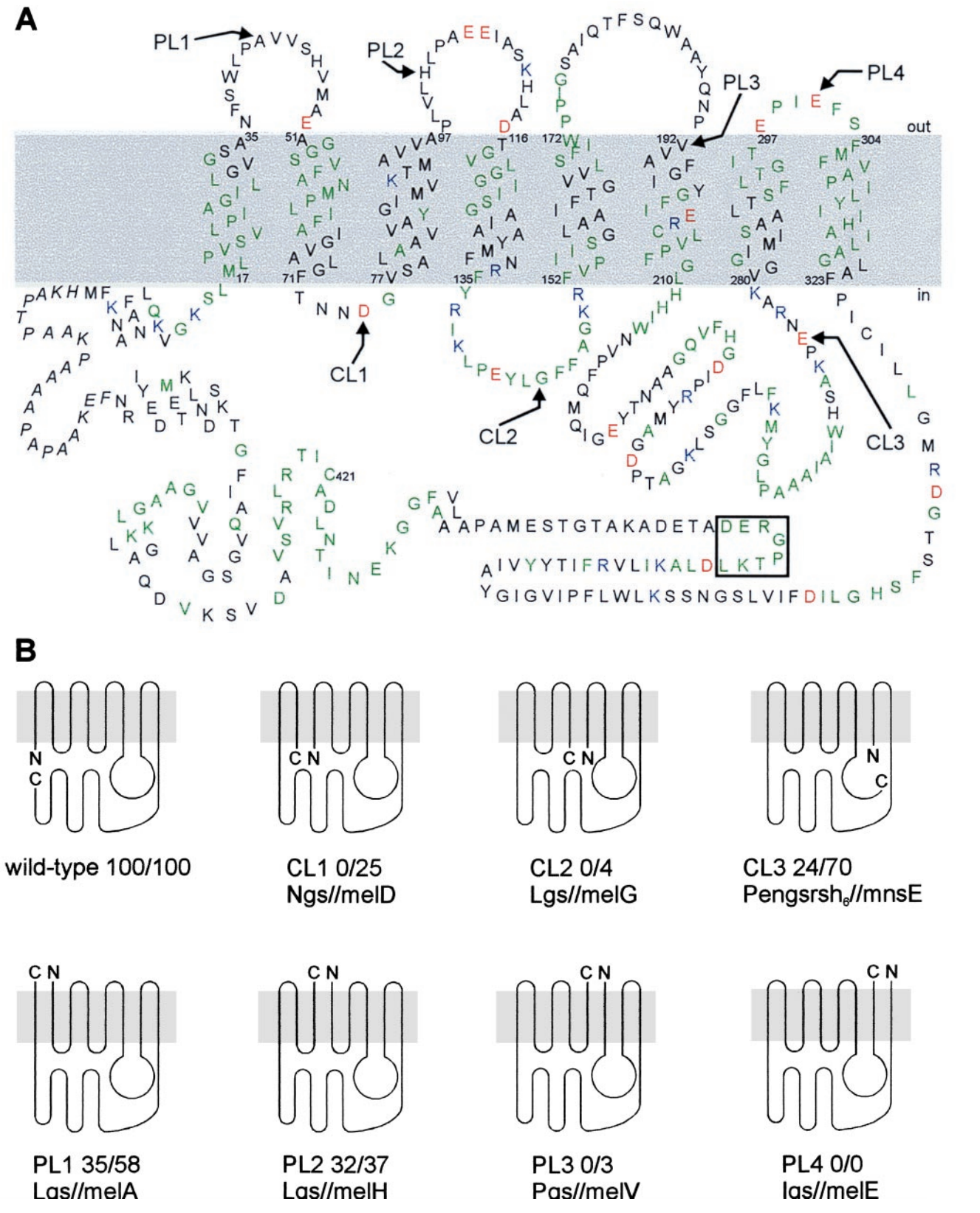

Fig. 1. (*A*) Topology model of IICB^{Glc}. Arrows indicate the position at which the new N and C termini were introduced in the circularly permuted variants PL1–PL4 and CL1–CL3. The Ala-Pro-rich linker connecting the original C and N terminus is shown in italics. Positively charged residues of the IIC^{Glc} domain are blue; negatively charged residues are red. The LKTPGRED linker between the IIC^{Glc} and the IIB^{Glc} domain is framed. Amino acids that are conserved in six homologous sequences and/or are within amino acid sequences with a similarity score of more than five are green (average similarity 4.3). (*B*) Topology models of the circularly permuted variants PL1–PL4 and CL1–CL3. The *in vivo* transport activity and the *in vitro* phosphotransferase activity in percentage of the wild-type control are given together with the exact amino acid sequences of the new C and N termini. Upper case letters indicate the last and first residues of the native IICB^{Glc} sequence; lower case letters indicate residues added to generate the translation start and termination signals.

XL1-Blue (Stratagene) was used for cloning and plasmid amplification. pTSGH11 (18) contains *lacI* and encodes, under the control of Ptac, a IICB^{Glc} with a C-terminal His tag.

Construction of Circularly Permuted Variants of ptsG. Plasmids pJFGPL1, pJFGCL1, pJFGPL2, pJFGCL2, pJFGPL3, pJFGCL3, and pJFGPL4 encoding circularly permuted variants of IICB^{Glc} with termini in periplasmic and cytoplasmic loops (PL1–PL4 and CL1–CL3) were constructed by PCR amplification of the desired coding region from a template containing two *ptsG* genes in tandem linked by a sequence encoding an Ala-Pro-rich linker peptide (19). The 5′ PCR primers encoded the translation start and a *Sac*I restriction site; the 3′ primers encoded a translation stop and a *Hin*dIII restriction site. The PCR products and the expression vector pMSEH2 (a derivative of pJF119EH; ref. 20) were digested with *Sac*I/*Hin*dIII and ligated. Plasmid pJFGCL3 with termini in the CL3 contains a His tag at the C terminus.

Construction of ptsG-phoA Fusions. Plasmids pJFGPL1phoA to pJFGPL4phoA code for protein fusions between circularly permuted forms of IICB^{Glc} and PhoA (PL1-PhoA–PL4-PhoA). They were constructed as follows. Plasmids pJFGPL1–4 were digested with *Nsi*I and *Hin*dIII, and the insert fragments were replaced by DNA fragments encoding the same sequences extended at the 3′ end by translational fusion to *phoA* without a signal sequence. These DNA fragments were generated by PCR amplification from template plasmid DNA encoding the previously characterized IICB^{Glc}-PhoA fusions V47, I108, P191, and F302 (13).

Purification of the CL3 Variant of IICB^{Glc}. Membranes from ZSC112LΔG(pJFGCL3) were prepared, and the proteins were purified as described (19) with the following modifications. Membranes from a 1-liter cell culture were suspended in 4 ml of buffer A (10 mM Tris·HCl, pH 9.3/10 mM β-mercaptoethanol) and solubilized by the addition of *N*-dodecyl-β-D-maltopyranoside (Anatrace, Maumee, OH) to a final concentration of 40 mM, vortexed, incubated on ice for 15 min, sonicated in a bath-type sonicator for 1 min, and incubated on ice for another 15 min. Insoluble material was removed by ultracentrifugation, and the supernatant was mixed with 4 ml of Ni^{2+}-nitrilotriacetic acid agarose (Qiagen, Chatsworth, CA), equilibrated with buffer B, pH 8 (50 mM NaP_i/500 mM NaCl/10 mM β-mercaptoethanol/0.4 mM *N*-dodecyl-β-D-maltopyranoside), and incubated for 30 min at room temperature. The slurry was transferred into a chromatography column at 4°C, and excess solution was drained off. The column was washed at 4°C with buffer B, pH 8 and pH 6, and with buffer C25 (25 mM imidazole/50 mM NaP_i, pH 7.5/500 mM NaCl/10 mM β-mercaptoethanol/0.4 mM *N*-dodecyl-β-D-maltopyranoside), and the bound protein was eluted with buffer C80 (80 mM imidazole/50 mM NaP_i, pH 7.5/500 mM NaCl/10 mM β-mercaptoethanol/0.4 mM *N*-dodecyl-β-D-maltopyranoside).

CD Spectroscopy. CD spectra of purified wild-type IICB^{Glc} (0.3 mg/ml) and CL3 (0.4 mg/ml) were recorded in 5 mM Hepes, pH 7.0/0.4 mM *N*-dodecyl-β-D-maltopyranoside. Measurements were performed with a Jasco J-715 spectropolarimeter at room temperature in a 0.05-cm path-length cuvette. Spectra were noise reduced and corrected for the buffer contributions.

Other Methods. Sugar phosphotransferase activity in membrane fractions was assayed by the ion exchange method as described (21, 22). [^{14}C]Glc and [^{14}C]α-methyl-D-glucopyranoside (αMG) were used as substrates. Sugar uptake activity was assayed as described (28). PhoA activity was assayed in permeabilized CC118 cells [*ara*D139 Δ(*ara leu*)7697 Δ*lac*X74 Δ*pho*A20 *gal*E *gal*K *thi rps*E *rpo*B *arg*E(am) *rec*A1] (23) as described (24).

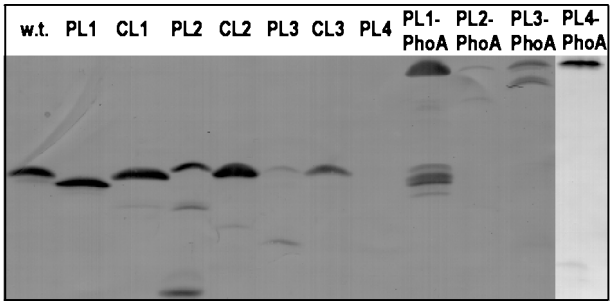

Fig. 2. Protein expression and stability. Circularly permuted variants were visualized with mAbs against the IIB^{Glc} domain on a Western blot of membrane preparations. PL2, PL3, PL1-PhoA, PL2-PhoA, and PL3-PhoA are partially degraded. A total of 100 μg of membrane protein was loaded per lane. w.t., wild-type.

CC118 cells expressing PhoA fusion proteins were grown in LB, and protein expression was induced with 10 μM isopropyl-1-thio-β-D-galactopyranoside. Protein samples were not boiled in sample buffer before electrophoresis on standard 15–20% polyacrylamide gels (22). IICB^{Glc} was visualized on Western blots with monoclonal mouse anti-IICB^{Glc} and horseradish-peroxidase-coupled rabbit anti-mouse IgG (25, 26). Protein concentrations were determined by a modified Lowry assay (27) with BSA as the standard. The similarity plot was generated with sequences ptgb_ecoli, ptgb_salty, ptaa_ecoli, ptaa_klepn, ptga_bacsu, and ptoa_ecoli from the SWISS-PROT database release 37.0 with a window of 10 residues by using the program GCG GENETICS COMPUTER GROUP (version 10.0).

Results

Construction of Circularly Permuted IICB^{Glc} Variants and Circularly Permuted IICB^{Glc}-PhoA Fusions. Variants of IICB^{Glc} were constructed with new N and C termini in the four periplasmic loops (PL1, PL2, PL3, and PL4) and in the three cytoplasmic loops (CL1, CL2, and CL3). The respective DNA sequences were synthesized by PCR amplification from a template containing two copies of the *ptsG* coding region separated by a DNA sequence encoding a 27-residue Ala-Pro-rich linker peptide. To confirm the location of the new C termini in the periplasm, four fusion proteins were constructed between circularly permuted variants ending at residues Val-47, Ala-109, Val-192, and Ser-303, respectively, and PhoA without a leader sequence (abbreviated PL1-PhoA, PL2-PhoA, PL3-PhoA, and PL4-PhoA). *E. coli* strain ZSC112LΔG (*ptsG*::*cat manZ glk*) was transformed with the recombinant plasmids and streaked on McConkey indicator plates supplemented with 0.4% Glc and 10 μM isopropyl β-D-thiogalactoside to induce approximately wild-type level of protein expression. Of 11 transformants, 5 (PL1, PL2, CL3, PL1-PhoA, and PL2-PhoA) formed red colonies indicative of Glc uptake and phosphorylation. CL1 formed red centered colonies; CL2 formed weakly red centered colonies; PL3 and PL3-PhoA formed faintly red colonies; and PL4 and PL4-PhoA formed yellow colonies. Only the variant PL4 with the new termini in the fourth periplasmic loop was so unstable (see below) that no activity could be detected.

Stability of the Circularly Permuted IICB^{Glc} Variants. Membranes were prepared from *E. coli* ZSC112LΔG induced with 100 μM isopropyl β-D-thiogalactoside to overexpress the circularly permuted variants, and the proteins were visualized on a Western blot with a mAb against the IIB^{Glc} domain (Fig. 2). In spite of their almost identical molecular mass, the variants have slightly different electrophoretic mobilities, most likely because of differences in binding of SDS. The variants are also expressed in

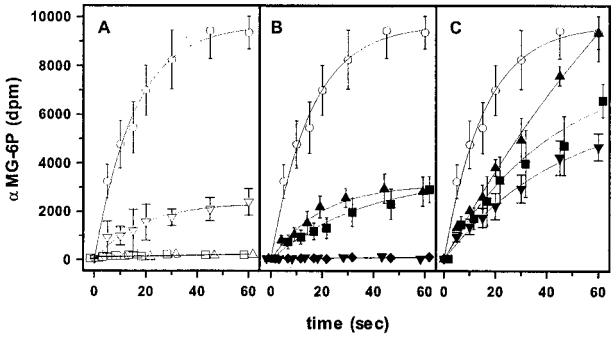

Fig. 3. Uptake of αMG by intact cells expressing circularly permuted variants of IICB^{Glc}. (*A*) Wild-type (open circle) and variants with N and C termini in CL1 (open square), CL2 (open triangle up), and CL3 (open triangle down). (*B*) Wild-type (open circle) and variants with N and C termini in PL1 (solid square), PL2 (solid triangle up), PL3 (solid triangle down), and PL4 (solid diamond). (*C*) Wild-type (open circle) and PhoA fusion proteins PL1-PhoA (solid square), PL2-PhoA (solid triangle up), and PL3-PhoA (solid triangle down). The uptake reaction was started by the addition of 12 μl of 10 mM [^{14}C]αMG (6,000 dpm/nmol) to 1 ml of the cell suspensions diluted to OD_{550} = 13 at room temperature, and 100-μl aliquots were withdrawn at the indicated time points, filtered through glass fiber filters, and counted.

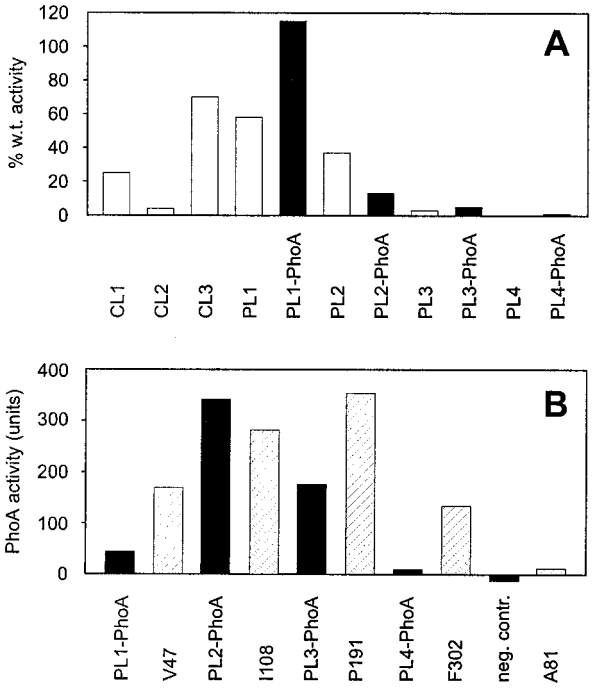

Fig. 4. Enzymatic activities of circularly permuted variants. (*A*) Glc phosphotransferase activities of membrane preparations. Activities of the circularly permuted variants (open bars) and the fusion proteins with PhoA (solid bars) are given in percentage of wild-type (w.t.) IICB^{Glc}; 100% activity corresponds to 31,000 nmol Glc-6-phosphate per mg of membrane protein per 30 min for Glc and to 15,000 nmol α-Me-Glc-6-phosphate per mg of membrane protein per 30 min for αMG. (*B*) PhoA activities of fusion proteins between circularly permuted variants and PhoA (solid bars) and between truncated IICB^{Glc} and PhoA (hatched bars). PhoA activities were measured in permeabilized cells.

different amounts. CL1 and CL2 with the new termini in the cytoplasmic loops are expressed in amounts similar to wild-type IICB^{Glc}, and CL3 is expressed in slightly lower amounts, suggesting that the internal signal sequences (N terminus in the cytoplasm) are competent to initiate membrane insertion. In contrast, the variants PL2 and PL3 with new termini in PL2 and PL3 are present in significantly smaller amounts and prone to proteolytic breakdown, as indicated by the presence of high-mobility protein fragments in the gel. PL4 is not detectable at all.

Functional Characterization of Circularly Permuted IICB^{Glc} Variants. Two assays were used to compare the function of the variant IICB^{Glc}: uptake of αMG by intact cells expressing the circularly permuted forms and Glc phosphotransferase activity in membrane fractions prepared from these cells. The former assay measures substrate translocation; the latter measures substrate phosphorylation only. Translocation and phosphorylation, which are tightly coupled in wild-type IICB^{Glc}, can be differently affected by different mutations (18, 28).

Of the variants with the new termini in the cytoplasmic loops, only CL3 has 24% of control uptake activity, whereas CL1 and CL2 are inactive (Fig. 3*A*), notwithstanding that all three mutants are expressed in similar amounts. The variants with new termini in PL1 and PL2 have 35% and 32% uptake activity, whereas the variants PL3 and PL4 are inactive (Fig. 3*B*). The loss of activity is reflected by a correspondingly weak and missing expression of PL3 and PL4, respectively.

Glc phosphotransferase activity was assayed with membranes in the presence of phospho*enol*pyruvate and the soluble phosphotransferase proteins EI, HPr, and IIA^{Glc}. The phosphotransferase activities (Fig. 4*A*) of the variants PL1, PL2, and PL3 with termini in the periplasmic loops are 58%, 37%, and 3%, respectively, and proportional to their *in vivo* uptake activities. The three variants with termini in cytoplasmic loops, CL1, CL2 and CL3, retain 25%, 4%, and 70% phosphotransferase activity, respectively. It is noteworthy that CL1, which has no transport activity, retains 25% phosphotransferase activity.

The circularly permuted version CL3 was solubilized with dodecylmaltoside and purified to homogeneity by metal chelate affinity chromatography (Fig. 5*A*). Purified CL3 had a specific Glc phosphotransferase activity (determined with [^{14}C]αMG) of 537,000 nmol α-methyl-glucopyranoside-6-phosphate per mg per 30 min, which is 43% of the IICB^{Glc} wild-type control. The near UV CD spectra of CL3 and IICB^{Glc} are similar, indicating that the two proteins also have similar secondary structures (Fig. 5*B*).

Activity of Fusion Proteins Between Circularly Permuted Variants and PhoA. All four periplasmic segments of IICB^{Glc} seem to be less than 20 residues long. Because of the possibility that the signal and stop transfer sequences flanking these short loops are not inserted sequentially but in a concerted process as helical hairpins, interruption of such a segment could have had unforeseen consequences. For instance, two transmembrane segments that are adjacent in the wild-type sequence might no longer be inserted into the membrane if located at the very N and C termini of a permuted variant. Therefore, the fusion proteins with PhoA, PL1-PhoA, PL2-PhoA, PL3-PhoA, and PL4-PhoA were constructed to assay directly for functional membrane insertion of the most C-terminal signal sequence. PhoA is active only if exported into the periplasmic space but remains inactive if retained in the cytoplasmic compartment (29). The fusion proteins have increased transport activities, from 35% to 84% for PL1-PhoA, from 32% to 100% for PL2-PhoA, and from 0% to 66% for PL3-PhoA (Fig. 3 *B* and *C*). The Glc phosphotransferase activity is increased 2-fold for PL1-PhoA but reduced for PL2-PhoA (Fig. 4*A*). The three fusion proteins are partially degraded as are two of their parents (Fig. 2). These results, taken together, suggest that PhoA has an overall stabilizing effect. The phosphatase activities of PL1-PhoA, PL2-PhoA, PL3-PhoA, and PL4-PhoA were assayed in permeabilized bacteria (Fig. 4*B*) and compared with the activities of the IIC^{Glc}-PhoA hybrid proteins

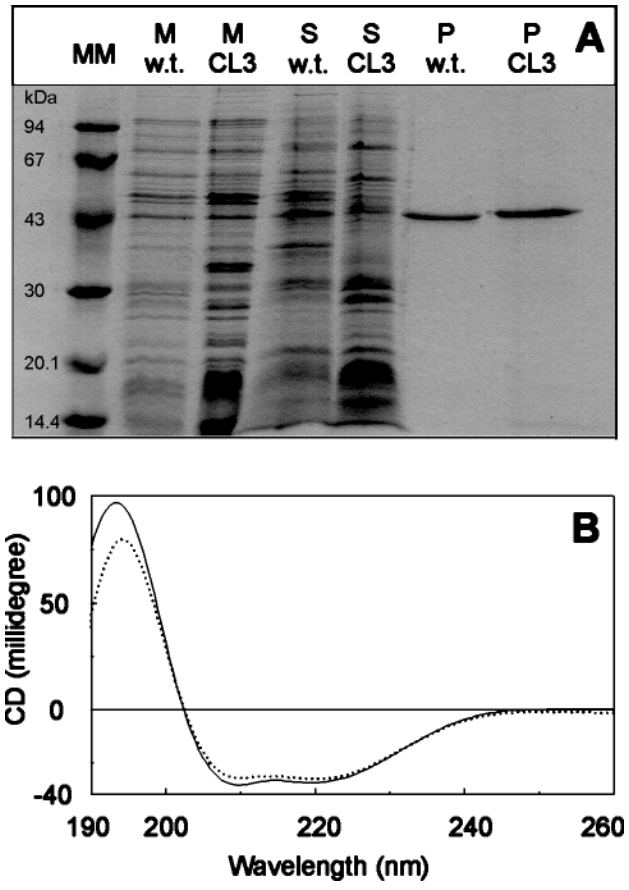

Fig. 5. Purification and CD spectroscopy of wild-type (w.t.) IICB^{Glc} and CL3. (*A*) Coomassie-stained SDS/PAGE of the purification of wild-type IICB^{Glc} and CL3 by metal chelate affinity chromatography. MM, molecular mass; M, membranes; S, solubilized membranes; P, purified proteins. (*B*) CD spectra of wild-type IICB^{Glc} (0.3 mg/ml) (solid line) and CL3 (0.4 mg/ml) (dashed line). Shown spectra were noise reduced and corrected for buffer contributions.

V47, I108, P191, and F302, which have PhoA fused to the IIC^{Glc} domain truncated after PL1, PL2, PL3, and PL4 (PhoA fused behind residues 1–47, 1–109, 1–192, and 1–303 of IICB^{Glc}; ref. 13). PL1-PhoA has 25% of V47; PL2-PhoA has 120% of I108; and PL3-PhoA has 50% of P191 activity. These activities are all significantly higher than the activity of the reference fusion protein A81, which has PhoA fused to a residue in a presumably transmembrane segment of IICB^{Glc} (13). These results indicate that the new C termini of the circularly permuted variants PL1–PL3 can export PhoA and therefore are likely to be located on the periplasmic surface of the inner membrane. PL4-PhoA has very low activity (7% of F302; Fig. 4*B*), but unlike the parent, PL4 is stable (Fig. 2).

Discussion

Circularly permuted variants of IICB^{Glc} with new N and C termini in three of the four periplasmic and in all three cytoplasmic loops are stably expressed, some retaining significant Glc transport and phosphorylation activity. Variants PL1, PL2, and CL3 retain both *in vivo* transport and *in vitro* phosphorylation activity. PL3, CL1, and CL2 retain between 3% and 25% *in vitro* phosphotransferase activity but have lost transport activity. Only PL4 is not stably expressed but can be stabilized when fused to PhoA. Fusion of PhoA to the periplasmic C termini enhances transport activity of PL1 and PL2 and restores transport activity in PL3. These results will be discussed with respect to three aspects: (*i*) membrane insertion of polytopic proteins, (*ii*) comparison with circular permutation and folding of globular proteins, and (*iii*) molecular function of IICB^{Glc}.

Recent work has given experimental evidence that most if not all inner membrane proteins are integrated by a SecA-, SecY-, and SRP (Ffh)-dependent mechanism and that Sec-independent integration is the exception (4). It therefore can be assumed that membrane integration of the IICB^{Glc} subunit of the Glc transporter is Sec-dependent, because such dependence has already been shown for the related PTS transporter for mannitol (1, 30).

Correct membrane localization (as opposed to inclusion body formation) and the moderate to excellent stability against proteolytic degradation of all but one of the circularly permuted IICB^{Glc} variants strongly indicate that the proteins were properly targeted and assembled in the membrane. Either any of the four signal anchor and stop transfer sequences can serve as N-terminal signal sequences in a cotranslational process, or insertion is initiated by an internal sequence (for instance at the original N-terminal signal anchor sequence) and is then passively followed by the insertion of the preceding as well as following membrane-spanning elements. The former mechanism is more likely in view of several facts. (*i*) The polytopic MalF subunit of the maltose transporter properly inserts in the complete absence of its N-terminal signal anchor sequence (31). (*ii*) The C-terminal periplasmic domain of leader peptidase integrates properly even when the insertion of the preceding N terminus is blocked (32). (*iii*) Charge inversion across the N-terminal signal anchor sequence compromises its insertion but does not affect the topology of the downstream regions of the polytopic Glc transporter in yeast (33). (*iv*) Individual membrane-spanning sequences can act as protein export signals (34). (*v*) Split constructs of the lactose transporter and other polytopic membrane proteins insert into the membrane and are functional (35–39). Our results with the periplasmic loop variants further suggest that internal stop transfer sequences with their N termini facing the periplasm can function as reverse signal anchor sequences. It seems unlikely that this N-terminal sequence would wait in a soluble and/or chaperoned state until after translation of the most C-terminal signal anchor sequence and that thereafter the two would form a helical hairpin ready for membrane integration. The membrane-spanning sequences that are adjacent in the native protein are ≈400 residues apart in the periplasmic loop variants and in addition are separated by the independently folding cytoplasmic IIB^{Glc} domain. All four periplasmic loop variants can be stabilized by fusing PhoA to their C termini. This beneficial effect can be caused by two things: (*i*) the C-terminal signal anchor sequences are a better substrate for the Sec machinery, because a longer C-terminal domain is being translocated (40); (*ii*) a stably folded PhoA may prevent the preceding signal anchor sequence from slipping back.

Circular permutation of soluble proteins has been taken as one piece of evidence that protein folding is not a sequential but rather a highly cooperative process that is thought to be initiated by the formation of secondary structure elements with concomitant hydrophobic collapse and is then followed by tight packing of the hydrophobic core. Examples are circularly permuted versions of the cytoplasmic phosphoribosyl anthranilate isomerase (PRAI; ref. 41), of the periplasmic disulfide isomerase DsbA (42), and of the outer membrane protein OmpA (10). In PRAI, new termini were placed in surface loops between β-strands and α-helices on both faces of the 8-fold $\beta\alpha$ barrel, and active variants were recovered. In DsbA, active versions were selected from a library of randomly permuted variants. New termini were found not only in aperiodic structures but also in the antiparallel β-sheet and in some α-helices. Only four α-helices were completely refractive to the insertion of new termini. In OmpA, new termini were placed in the three periplasmic loops of the 8-fold β-barrel. Assembly of all three variants was retarded, but two of

the three variants retained bacteriophage receptor activity. In the case of $IICB^{Glc}$, new termini were inserted in both cytoplasmic and periplasmic loops of a protein with eight membrane-spanning segments.

The periplasmic loops are short (6–19 residues) as in many multispanning membrane proteins and except for loop 4 do not seem to be essential for function. Fusion to PhoA stabilizes PL3 and restores transport activity. PL4 is also stabilized but does not recover any activity. The amino acid sequence of PL4 and the two flanking transmembrane segments is strongly conserved in all PTS transporters homologous to $IICB^{Glc}$. Moreover, the majority of mutations that result in facilitated diffusion without phosphorylation of Glc (43) in uptake of ribose (44) or in relaxed substrate specificity (45) are located in this region. If translocation of a substrate is mediated by a localized isomerization of large amplitude, the region including TM7, PL4, and TM8 is the most likely candidate for such a process. Its interruption would compromise the cooperative process, and fusion to PhoA might block it completely, resulting in a stable but inactive protein.

All the three versions with new termini in cytoplasmic loops have reduced Glc phosphorylation activity *in vitro* and CL1 and CL2 have lost translocation activity. CL2 contains a stretch of residues that are conserved in homologous proteins. Two mutations in this region impair translocation without affecting phosphorylation of Glc (28). CL3 is the most active of all permuted versions. Elongation of the C terminus of CL3 with a His tag does not interfere with function, and this version of CL3 could be purified to homogeneity in a single step. The amino acid sequence of CL3 is highly variable in homologous proteins. Only one functional mutation has been found in this region thus far (43). It is likely that the 70 residues form an independently folding domain. In *Mycoplasma genitalium*, this domain contains an additional 150 residues inserted between residues 225 and 240 of *E. coli* $IICB^{Glc}$.

In conclusion, $IICB^{Glc}$, a multispanning membrane protein, can be circularly permuted with retention of enzymatic activity. Circular permutation allows stabilization of the N and/or C termini of the wild-type form. It is conceivable that membrane proteins with their N and C termini localized in more ''constrained'' positions might crystallize differently and eventually achieve better ordering than proteins with loose ends.

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