Molecular interactions in ribose transport: the binding protein module symmetrically associates with the homodimeric membrane transporter

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The *Escherichia coli* high-affinity ribose transporter is composed of the periplasmic ribose-binding protein (RBP or RbsB), the membrane component (RbsC) and the ATP-binding protein (RbsA). In order to dissect the molecular interactions initiating the transport process, RbsC suppressors for transport-defective *rbsB* mutations were isolated. These suppressors are localized in two regions of RbsC, which are allele-specific to N- or C-terminal domain mutations of RBP, suggesting that there are two distinct regions of RbsC, each interacting with one of the two domains of RBP. To demonstrate that these two regions provide a homodimeric binding surface for RBP we constructed a dimeric *rbsC* in which two genes are joined tandemly from head to tail with the addition of a linker. The dimeric RbsC protein is stable and functional in growth and ribose uptake. By exploiting the allele specificity between the domain-specific mutations and their suppressors, we generated all mutation-suppressor combinations in a single *rbsB* plus the dimeric *rbsC* genes. Their phenotypes are consistent with the proposal that the binding protein module interacts symmetrically with homodimeric RbsC. The mode of association proposed here for the ribose transport components could be extended to other ABC transporters with similar structural organizations.

Keywords: ABC transporter/integral membrane protein/ribose-binding protein/transport mechanism

Introduction

The ABC (ATP-binding cassette) transporters are abundant and widely distributed in a variety of organisms. These include the binding protein-dependent transporter of bacteria, the STE6 protein of yeast, and the cystic fibrosis transmembrane conductance regulator (CFTR) and the P-glycoprotein of higher eukaryotes (Higgins, 1992; Doige and Ames, 1993; Borst and Schinkel, 1997). The modular architecture of the transporter is built up either from several proteins or from a single polypeptide. For example, the bacterial ABC transporter is composed of the periplasmic binding protein, one or two membrane proteins and two subunits of the peripheral ATP-binding protein. In contrast, P-glycoprotein, studied extensively as a model of a eukaryotic ABC transporter, consists of a single

polypeptide with two ATP-binding and two hydrophobic membrane domains (Gottesman and Pastan, 1993), which has been visualized by electron microscopy at 2.5 nm resolution (Rosenberg *et al.*, 1997).

The most studied examples of the bacterial transporters include the maltose and histidine permeases (Davidson and Nikaido, 1991; Kerppola *et al.*, 1991), which are not structurally homologous in terms of their transmembrane organization. However, their overall modular organizations are similar in that they comprise one binding protein (MalE, HisJ), two heterodimeric membrane components (MalF/G, HisQ/M) and two ATP-binding proteins (MalK, HisP). In the maltose system, MalF and MalG, both reported to interact with either one of the two domains of MalE (Hor and Shuman, 1993), have eight and six transmembrane (TM) peptides, respectively (Boyd *et al.*, 1987; Dassa and Muir, 1993). The heterodimeric HisQ/M transporter is composed of five TM subunits (Kerppola and Ames, 1992). In contrast, the RbsC transporter, a member of the structurally homologous AraH/RbsC superfamily, is a single membrane component with six TM peptides (Park and Park, 1999). Although their structures have not been analyzed experimentally, other AraH members, currently comprising the majority of permeases, appear to have similar transmembrane organizations to that of RbsC.

A large number of eukaryotic ABC transporters, classified as the CFTR/MDR families serving as a channel or an exporter, have a 'two-times-six' transmembrane architecture including an ATP-binding domain (Higgins, 1992). For instance, P-glycoprotein contains two homologous internal repeats of the 6-TM units and generates a pseudo 2-fold symmetry as viewed in the 2.5 nm electron microscopic structure (Rosenberg *et al.*, 1997). A bacterial homolog of P-glycoprotein, the LmrA protein of *Lactococcus lactis* with 6-TM peptides, is functional in eukaryotic cells (van Veen *et al.*, 1998). It is very likely that LmrA forms an oligomeric structure resembling P-glycoprotein in order to function in both prokaryotic and eukaryotic systems. Structural conservation must have been maintained in the majority of eukaryotic ABC transporters during evolution.

In a previous report (Eym *et al.*, 1996), we characterized regions that interact with the membrane permease on the three-dimensional structure of RBP (Bjorkman and Mowbray, 1998) by genetic and biochemical analyses. These are located on the surfaces of two domains around the binding cleft, suggesting that both domains are involved in the transport interaction. Recent determination of the topology of RbsC (Park and Park, 1999) using PhoA fusions may provide useful information about the regions associating with RBP as well as with the ATP-binding protein (RbsA). RbsC contains six transmembrane regions with the consensus EAA-G motif in the second

cytoplasmic loop (Saurin *et al.*, 1994). In order to investigate the molecular interactions involving sugar transport, approaches were taken to isolate suppressors of *rbsC* correcting the transport defect of *rbsB* mutations and to construct the chimeric transporter. Furthermore, an allelic suppression between the binding protein and membrane transporter mutations was exploited to analyze their interaction pattern by constructing various RbsC dimers with the suppressor mutations. The data suggest that the domains of RBP interact with different regions of RbsC and that the interaction occurs symmetrically with respect to the homodimeric binding surface of RbsC.

Results

Screening of permease suppressors for the transport-defective RBP mutations

In a previous study (Eym *et al.*, 1996), four residues of the periplasmic RBP were characterized as being involved in transport. The changes D52G, D52N, D52N/G72D (all in the N-terminal domain of RBP), G134D, R166H and R166C (in the C-terminal domain) were isolated by random mutational screening. Here we investigated the interaction between RBP and the membrane permease by isolating suppressors that restore transport function to the D52G, D52N/G72D, G134D and R166H mutations. In order to recover suppressors localized in *rbsC* encoding the membrane permease, the chromosomal region around *rbsC* was transduced using *ilv::Tn10* into a new background in which a screening was performed. Suppressors not localized in *rbsC* are those enhancing the activity of a low-affinity transporter as characterized previously (Kim *et al.*, 1997). Initial mutagenesis and enrichment were carried out with the strain ($\Delta rbsB$) containing the mutant *rbsB* gene on a plasmid (for details, see Materials and methods). The frequencies of suppressors were estimated to be $\sim 10^{-8}$. After confirming the location of suppressors, genes were cloned and sequenced; the A313T change of *rbsC* was obtained in three independent isolates for *rbsB*-D52G, and two independent *rbsC*-A120V changes for *rbsB*-R166H. To isolate suppressors for *rbsB*-D52N/G72D, a strain containing the *rbsC*-A313T suppressor was used, thereby creating double suppressors in *rbsC* with a secondary G296D (once) or S298F (twice) change (Table I). Several attempts to isolate suppressors for the G134D change were unsuccessful.

Allele specificity between the transport mutations and their suppressors

In order to test whether the RbsC suppressors affect other RBP mutations, plasmids with the *rbsB* mutations (D52G, D52N, D52N/G72D, G134D, R166H and R166C) were introduced into the CK251 background containing a suppressor (Table I). When there is no suppressor, the mutant with *rbsB* alleles exhibited <5% of the wild-type uptake rates (Eym *et al.*, 1996). The A313T suppressor, isolated for D52G (42%), also affects D52N (44%), while it is no longer compatible with the wild-type RBP (4%). Since the RBP mutations did not impair chemotactic function (Eym *et al.*, 1996), chemotactic phenotypes that can be assessed on swarm plates when transport function is active were also restored by the presence of a suppressor (Table I). However, the G134D, R166H and R166C alleles

of *rbsB* were not suppressed by *rbsC*-A313T, indicating that the A313T suppressor is specific for the Asp52 residue of RBP. The D52N/G72D double mutation was moderately suppressed (19%) by *rbsC*-A313T.

This incomplete suppression could be improved by the presence of an additional suppressor allele, G296D (49%) or S298F (61%), in CK251 *rbsC*-A313T. The presence of an additional suppressor does not impair the action of the original A313T suppressor for the *rbsB*-D52G or *rbsB*-D52N mutations, indicating that the S296D and S298F suppressors are specific for the G72 residue of RBP and are compatible with the wild-type residue at the same position. The RbsC-A120V suppressor, isolated for R166H, affects all the changes in R166 of RBP, e.g. R166H and R166C, but not the changes in D52, G72 and G134, although it is still compatible with the wild-type RBP (76%, Table I). Recoveries of swarm phenotypes were also observed for R166H and R166C in the presence of RbsC-A120V, suggesting the specificity between A120V and the R166 residue of RBP.

In order to test whether there is a functional dependence between the RbsC suppressors when present in a single molecule, we constructed a double RBP mutant (D52N/R166C) on a plasmid. When this plasmid was transformed into CK251 and its derivatives containing the RbsC suppressors, suppression was not observed with the single (A313T and A120V) or double (S298F/A313T and G296D/A313T) suppressor alleles (Table I). The result was more or less expected since only one suppressor mutation of RbsC exists that could restore either the D52 or R166 defect of RBP. Thus, we constructed the strain (YP42) with both A120V and A313T suppressors in a single *rbsC* (see Materials and methods for details). This strain exhibited a swarm ring and the recovery of ribose uptake for the D52N/R166C double mutant (Table I), indicating that each mutation of RBP is allele-specific for the suppressors of RbsC in accomplishing transport. For some reason, the combined RbsC suppressor exhibited a higher level of suppression for the single D52G, D52N, D52N/G72D and R166H mutations than the single RbsC suppressors.

Location of suppressors on the structure of RbsC

In Figure 1, the locations of the RbsC suppressors predicted previously are shown on the structure of RbsC (Park and Park, 1999). The suppressors (G296, S298 and A313) for the N-terminal domain mutations (D52 and G72) of RBP are all localized in the last transmembrane helix, while the suppressor (A120) for the C-terminal domain mutation (R166) is in the first cytoplasmic loop near TM III. Although the domain-specific suppressors are segregated into two distinct regions of the integral membrane transporter, their localizations imply an indirect mechanism of suppression instead of an initial surface contact in the periplasm. Interestingly, a recent report suggests that the periplasmic binding protein interacts not only with the periplasmic loop but also with the transmembrane region or cytoplasmic loop of the membrane transporter (Mademidis *et al.*, 1997). In this regard, the interaction between RBP and RbsC may not be simple. The specificity of allelic suppression and their regional segregation led us to investigate further the mode of interaction between the

Table I. Phenotypes of the RbsC suppressors for the RBP mutations

RbsB	RbsC ^a Wild-type Growth ^b	Uptake ^c (%)	A120V Growth	Uptake	A313T Growth	Uptake	G296D/ A313T Growth	Uptake	S298F/ A313T Growth	Uptake	A120V/ A313T Growth	Uptake
No	+	8.02 (2)	+	10.06 (2)	+	11.76 (2)	+	8.74 (2)	+	16.78 (3)	+	12.22 (3)
Wild-type	++++*	483.3 (100)	++++*	368.64 (76)	+	21.18 (4)	+	25.6 (5)	+	18.02 (4)	+	20.04 (4)
D52G	+	6.44 (1)	+	8.48 (2)	++++*	202.58 (42)	++++*	234.84 (49)	++++*	292.5 (61)	++++*	619.72 (128)
D52N	+	23.08 (5)	+	43.04 (9)	++++*	212.26 (44)	++++*	255.86 (53)	++++*	321.3 (66)	++++*	1090.64 (226)
D52N/G72D	+	6.18 (1)	+	12.16 (3)	++	89.56 (19)	++++*	238.36 (49)	++++*	293.86 (61)	++++*	470.52 (97)
G134D	+	6.22 (1)	+	13.18 (3)	++	46.54 (10)	+	29.46 (6)	+	36.64 (8)	+	15.9 (3)
R166H	+	13.26 (3)	++++*	58.96 (12)	+	13.28 (3)	++	11.02 (2)	+	11.54 (2)	++	297.08 (61)
R166C	+	0.84 (0.2)	++++*	234.78 (49)	+	24.9 (5)	+	32.98 (7)	+	0.52 (0.1)	++	90.18 (19)
D52N/R166C	+	24.84 (5)	+	52.24 (11)	+	66.48 (14)	+	87.54 (18)	++	14.14 (3)	++++*	216.94 (45)

^aThe suppressor *rbsC* tested was on the chromosome.

^bGrowth on 0.05% (3.3 mM) ribose minimal plates was scored after incubation for 3 days. The scale represents relative colony size. A positive swarm ring phenotype (*) was observed only when the growth scales are more than '++++'.

^cRibose uptake rate is in d.p.m./s per 10⁸ cells. The uptake assay was carried out with 15.7 μM [¹⁴C]ribose. Values in bold indicate suppression. The number in parentheses is a percentage, with the wild-type RBP/RbsC set to 100 for relative comparison.

Fig. 1. Locations of RbsC suppressor mutations. The membrane topology was drawn as proposed (Park and Park, 1999). The residue with a circle (120A) indicates the site of suppression for the R166H mutation of RBP (C-terminal domain). The rectangles indicate the suppressor residues (296G, 298S and 313A) in RbsC for the D52G or G72D mutations of RBP (N-terminal domain). Residues in the transmembrane helices are shown with an α -helical structure.

ligand-binding domains in transport by designing an experiment with a dimeric RbsC protein.

Construction of a dimeric RbsC transporter

The previous study (Park and Park, 1999) indicated that both the N- and C-terminal regions of RbsC are exposed to the cytoplasm (Figure 1), enabling us to create a fusion without causing a topological problem. The pYP119 plasmid harboring the dimeric *rbsC* gene was constructed by using the *NdeI* restriction enzyme. An *NdeI* site was created at the 3' end of the first *rbsC* gene immediately

after the last amino acid (Q321) and at the 5' end of the second *rbsC* gene before the initiator methionine by PCR mutagenesis (Materials and methods). The two genes were then ligated in-frame using the *NdeI* restriction sites with a four amino acid (LASH) linker. The fusion gene was placed under the control of the promoter of a tetracycline resistance gene derived from the parent plasmid. The fusion protein was functional and exhibited ribose transport activity (80%) similar to RbsC expressed from a single gene (Table II).

In order to ensure that a monomeric protein had not

Table II.Suppressions of the RBP mutations by RbsC fusion dimers

RBP	RbsC ^a Wild-type Growth ^b	Uptake ^c (%)	D52N Growth	Uptake	R166C Growth	Uptake	D52N/R166C Growth	Uptake
WT	++++	575.13 (100)						
WT-WT	++++	462.07 (80)	+	43.67 (8)	+++	411.65 (72)	+	4.71 (1)
WT-A313T			+++	468.02 (81)	+++	268.9 (47)	++	31.81 (6)
A313T-WT			+++	314.79 (55)	+++	462.21 (80)	++	62.23 (11)
WT-A120V			+	37 (6)	+++++	764.4 (133)	+	36.03 (6)
A120V-WT			+	42.07(7)	+++++	621.67 (108)	+	27.65 (5)
A313T-A120V							+++	165.94 (29)
A120V-A313T							+++	121.24 (21)

^aSuppression locations are designated in either the first or second gene of the dimeric *rbsC* on plasmid with ‘-’ in between.
^bGrowth on 0.05% (3.3 mM) ribose minimal plates was scored after incubation for 3 days. The scale represents relative colony size.
^cRibose uptake rate is in d.p.m./s per 10⁸ cells. The uptake assay was carried out with 15.7 μM [¹⁴C]ribose. Values in bold indicate suppressions. The numbers in parentheses are percentages, with the wild-type RBP/RbsC set to 100 for relative comparison.

Fig. 2. Immunoblotting of the monomeric and dimeric RbsC-His-tagged proteins. Equal amounts of solubilized cells containing His-tagged proteins were resolved on SDS-PAGE (12%) and immunoblotted with Ni-NTA-horseradish peroxidase conjugate. The positions of molecular weight standards (kDa) are indicated. Lane 1 is the carbonic anhydrase (31 kDa) marker (Amersham); lane 2, BL21/pOH3 (*rbsC*); lane 3, BL21/pYP118 (*rbsC-rbsC*).

been generated by proteolysis, we confirmed the presence of the fusion protein by immunoblotting using anti-His tag conjugate. To do so, His tags were attached to the C-terminal regions of the monomeric and dimeric RbsC proteins. As shown in Figure 2, the dimeric fusion protein was detected as a prominent band at 55 kDa. Minor bands appearing around that region depended on the SDS solubilization conditions, and seem to be associated with the RbsC protein. These extra bands were not detected in the vector control (data not shown). Interestingly, the same 55 kDa band was also found in the monomeric RbsC (Figure 2). In the case of lactose permease (Sahin-Toth *et al.*, 1994), dimeric aggregates were also detected on SDS-PAGE.

Dimeric interaction of the RbsC transporter demonstrated by allelic suppression

The functionality of the RbsC fusion does not necessarily imply that a homodimeric interaction is required for its activity because each monomer may still exert its activity. Therefore, it is necessary to demonstrate that both units are functional in transport. When we combined RbsB-D52N and its allelic suppressor RbsC-A313T, suppression was observed (Table II, Figure 3) regardless of the suppressor location, either in the first (55%) or in the second unit (81%) of RbsC. The same is true for RbsB-R166C and RbsC-A120V, although here the wild-type RbsC dimer exerts some residual activity (72%). The

Fig. 3. Schematic illustration of the interaction between the dimeric RbsC fusion and RBP. The D52N and R166C mutations of the N- and C-terminal domains of RBP are designated ‘NM’ and ‘CM’, respectively. The ‘NS’ and ‘CS’ abbreviations for RbsC represent the A313T (shaded triangle) and A120V (shaded half-circle) suppressors specific for the N- and C-terminal domain mutations, respectively. The interactions are shown symbolically as a direct contact in order to emphasize allele specificity, but they could be either indirect or secondary.

Fig. 4. A model for the interaction between RBP and the homodimeric RbsC. The homodimeric RbsC is shown to have 2-fold symmetry with a head-to-tail organization, similar to the MalF/G transporter (Ehrmann *et al.*, 1998). The larger open triangle and circle at the top represent the 52D (N) and 166R (C) sites of RBP. The smaller shaded triangle and circle represent the A313T and A120V suppressors of RbsC interacting with RBP. The small open triangle and circle indicate the locations of residues (313A and 120A) in RbsC. The interactions are again shown as a direct contact to emphasize their allele specificity.

suppressions by RbsC-A120V were certainly higher, 108 and 133% compared with the wild-type combination. These results indicate that both monomers in the fusion are active.

We further extended this approach to demonstrate that formation of a dimer is required for the function of RbsC. To do so, the double RBP mutant (D52N/R166C) was combined with various RbsC suppressors containing either one or both of the A120V and A313T changes (Table II and Figure 3). The combinations with A120V or A313T alone, i.e. A120V-WT, WT-A120V, A313T-WT and WT-A313T, did not exhibit suppression of RbsB-D52N/R166C. However, fusions with both A120V and A313T, i.e. A120V-A313T and A313T-A120V, exhibited suppressions of 21 and 29% efficiency, respectively. This indicates that as well as the requirement for dimeric association, the domains of RBP interact symmetrically with the RbsC dimer (Figures 3 and 4).

Dimeric interaction of RbsC in a reconstituted vesicle

In order to demonstrate further that RbsC exists as a dimer in the membrane, a single cysteine residue at 217 of RbsC was labeled with either a pyrene maleimide or BODIPY fluorescent probe in a proteoliposome (Ying-Ming and Franklin, 1992) after purifying and solubilizing the protein (Materials and methods; see Cheng and Giovana, 1997). Since the emission fluorescence (λ_{max} at 475) of the excited state dimer (excimer) for pyrene overlaps with the excitation fluorescence (λ_{max} at 500) of BODIPY, energy transfer can occur between the two fluorescent probes if they are in close proximity, which can be expressed quantitatively as the excimer to monomer (E/M) ratio of pyrene fluorescence. The E/M (475/385 nm) ratio decreases when molecules labeled with these fluorescent probes form a heterodimer. The differentially labeled RbsC

Fig. 5. Fluorescence emission of labeled RbsCs reconstituted into a proteoliposome. The RbsC protein was labeled with two different fluorescent probes and reconstituted into proteoliposomes containing *E. coli* total phospholipid (see Materials and methods for details); (a) pyrene homodimer, (b) 1:1 mixture of pyrene and BODIPY heterodimer, (c) BODIPY-labeled homodimer and (d) pyrene and BODIPY heterodimer with Triton X-100. The sample (d) was treated with Triton X-100 to disrupt the membrane vesicle. The fluorescence emission of each sample was measured in the range of 360–560 nm after excitation at 342 nm. A control without protein was used for background subtraction. Wavelengths for calculating the E/M ratio of pyrene are 385 and 475 nm (▼), while the λ_{max} of BODIPY is 511 nm (◻). The E/M ratios were 0.261 ± 0.013 (a), 0.175 ± 0.015 (b), 0.183 ± 0.009 (d). The efficiency of energy transfer obtained from the steady-state fluorescence was calculated as 0.329 from the relative decrease of the E/M ratios.

species in a 1:1 ratio were reconstituted into liposomes containing *Escherichia coli* total phospholipids with the pyrene or BODIPY homodimer as a control. The pyrene- and BODIPY-labeled heterodimers showed a considerable amount of energy transfer as indicated by a decrease in fluorescence of pyrene at 475 nm relative to that of pyrene in a homodimer, with an accompanying increase in BODIPY fluorescence of the heterodimer at 511 nm (Figure 5). The E/M ratio for the pyrene homodimer was calculated as 0.261 ± 0.013 , while it was 0.175 ± 0.015 for the pyrene-BODIPY heterodimer. The efficiency of energy transfer (0.329) was calculated from the relative decrease, i.e. the proportion of the difference in E/M ratio (0.086) to that of the pyrene homodimer (0.261). When Triton X-100 was added to disrupt the membrane vesicle, both pyrene excimer (475 nm) and BODIPY emission (511 nm) fluorescence were decreased, resulting in a decrease in the E/M ratio. A similar reduction in the E/M ratio was observed with addition of Triton X-100 to the pyrene homodimer (data not shown). These data indicate that RbsC forms a dimer in the membrane, consistent with the genetic data described in the previous section.

Discussion

The domain segregation of transport mutations on the surface of RBP (Eym *et al.*, 1996) suggests the presence of at least two interacting regions in the membrane permease. We investigated these regions by isolating a specific suppressor mapped in *rbsC* (Figure 1). They are

concentrated in two different regions, each of which is specific for a particular site, not for a mutated residue, of RBP; A313T for any changes in RbsB-D52, and A120V for the changes of R166. Cross-specificity was not observed, and thus the pattern of suppression more strictly may be termed 'site-specific'. The suppression was also observed with the D52N/R166C double mutant and the double suppressors (Table I), further indicating that each domain of RBP interacts with different regions of RbsC. On the other hand, more residues were found to be involved in the interaction of RbsC with the N-terminal domain of RBP than was suggested by the earlier observation that the functional surface is much larger in the N-terminal domain than in the C-terminal domain of RBP (Binnie *et al.*, 1992).

The suppressors for the N-terminal domain mutations of RBP in RbsC were found in the G296, S298 and A313 residues, all in the last transmembrane region (Figure 1), suggesting the possibility that the residues may be involved in transmembrane communication modulating the ATPase (RbsA) activity. The MBP-independent transport mutations localized in the transmembrane region of the maltose transporter were shown to activate the ATPase constitutively (Covitz *et al.*, 1994). This indicates that the transmembrane region of the membrane transporter mediates signaling in energy coupling. Such a function might be achieved dynamically by an interaction between the domains of RBP and the transmembrane region of RbsC. Recent identification of the peptide regions involved in interaction between the periplasmic binding protein and the membrane component in the ATP-dependent ferric hydroxamate transport system (Mademidis *et al.*, 1997) revealed that there are two regions in the membrane component (FhuB), the periplasmic loop and a transmembrane segment with an adjacent cytoplasmic loop, responsible for such an interaction.

The suppressor (A120V) of RbsC for the C-terminal domain mutation of RBP is located in the first cytoplasmic loop near TM III (Figure 1). Unlike the second cytoplasmic loop containing the EAA-G region, known to interact with the conserved helical domain of ATPase (Mourez *et al.*, 1997), the function of the first cytoplasmic loop has not been assigned. In RbsC, this region is hydrophobic and larger (48 amino acids) than the second cytoplasmic loop (30 amino acids). We speculate that the change in this region may function as a conformational suppressor, for example facilitating a shift towards an open form after releasing the sugar.

It is a general tendency of integral membrane permeases of the binding protein-dependent ABC transporters to form oligomers if the subunits are small and have modular architectures; MalF/G, HisQ/M and OppB/C are known to form heterodimers (Davidson and Nikaido, 1991; Kerppola *et al.*, 1991; Pearce *et al.*, 1992). Although the FhuB protein in the hydroxamate transport system functions as a single polypeptide with 20 transmembrane regions, it consists of two homologous repeats. The transporter is functional when the repeats are divided (Koster and Braun, 1990). However, a group of transporters including the AraH/RbsC superfamily and the glycine betaine/proline permease (Haardt and Bremer, 1996) have only one membrane component, suggesting the possibility that they

form homodimers. Our data clearly prove that this is the case.

Regardless of their quaternary structures, most ABC transporters have fairly constant transmembrane units which number 12 or 'two-times-six'. From bacteria to mammals, these numbers are preserved (Higgins, 1992), e.g. in the OppB/C oligopeptide permease of *Salmonella typhimurium* (Pearce *et al.*, 1992), and in the P-glycoprotein and the CFTR in mammals (Higgins, 1992; Doige and Ames, 1993). In some eukaryotic transporters, the six transmembrane units are separated by an ATP-binding domain. Recently, the structure of P-glycoprotein was revealed by electron microscopy at 2.5 nm resolution, showing the 12 TM core structure with a 2-fold pseudo symmetry (Rosenberg *et al.*, 1997). It is highly probable that the dimeric RbsC proteins are arranged to have 2-fold symmetry (Figure 4). This symmetrical organization is consistent with the data predicting the presence of a symmetrical binding surface for RBP. Even in the heterodimeric model of the MalF/G complex, an internal symmetry was implied from the mutation suppressor data (Ehrmann *et al.*, 1998). The structural organization and the mode of interaction uncovered by the study of the ribose transporter may provide an evolutionary prototype for the structural conservation of the modular nature of the ABC transporter family.

Materials and methods

Bacterial strains, media and reagents

Strains used in this study are derivatives of *E. coli* K-12 and are listed in Table III. Genetic techniques were performed as described (Miller, 1972). The CK244 strain was used to isolate strains having permease suppressors for the mutant RBPs (Table III). In order to prevent a recombinational event, the YP111 strain (*recA*) was used as a host for the derivatives of pYP119 harboring the dimeric *rbsC* gene. The BL21 strain was used in immunoblotting to detect the monomeric or dimeric RbsC-His-tagged proteins. The derivatives of pEY13 (Table III) were used to screen permease suppressors and to test their specificity. [^{14}C]D-Ribose (0.08 mCi/ml) was purchased from New England Nuclear (NEN, DuPont). H1 minimal salts were as described (Eym *et al.*, 1996), and semi-solid agar swarm plates contained 0.25% agar, H1 minimal salts, supplements for amino acids (1 mM) or 0.01% thiamine, and D-ribose (0.1 mM). All media and agar were purchased from Difco. D-Ribose, chloramphenicol, ampicillin and other reagents were purchased from Sigma Chemical Co.

Mutagenesis and mapping of the RbsC suppressors

After the derivatives of pEY13 containing mutant *rbsB* genes were transformed into CK251 ($\Delta rbsB$), the strains were mutagenized by nitrosoguanidine (Miller, 1972). When 10^8 mutagenized cells were spread on a 0.05% ribose minimal plate, $\sim 10^3$ colonies restored ribose growth (first screening). However, this number includes mutations unrelated to the permease suppressor (Kim *et al.*, 1997; Song and Park, 1998; Oh *et al.*, 1999), and thus a second screening was performed. After collecting the 10^3 mutant colonies on a ribose plate, P1 pools were made following cell lysis. By using *ilv::Tn10* co-transducing the *rbs* gene with 80–90% frequency, the CK244 strain with the mutant *rbsB* plasmids was transduced with the P1 lysates (second screening). The transductants restoring growth on a 0.05% ribose minimal plate were all *rbs* permease suppressors.

In order to clone suppressor genes, they were transferred to YP13 (*polA^{ts}*) using *ilv::Tn10*. The suppressors in *rbsC* were picked up by double homologous recombination in the *polA^{ts}* strain (Kim *et al.*, 1997) onto the pYP100 plasmid containing the flanking regions of the *rbsC* gene (Table III). The suppressor genes were expressed from derivatives of pBR322 under the control of the promoter of the tetracycline resistance gene. The plasmid clones were transformed into the CK251 strain with the derivatives of pEY13 (Table III) and tested for their complementation

Table III. Strains and plasmids used in this study

Strains and plasmids	Genotype	Source
Strains		
BL21(DE3)	<i>hsdS gal(λcIts857 ind1 S7 nin5 lacUV5-T7 geneI)</i>	Sambrook <i>et al.</i> (1989)
OW1	<i>leu thr his thi rpsL lacY xyl ara tonA tsx mlc-101</i>	J.Adler
CK251	OW1 Δ <i>rbsB</i>	Eym <i>et al.</i> (1996)
CK244	CK251 <i>ilv::Tn10</i>	laboratory collection
YP13	OW1 <i>ilv::Tn10 polA^{ts}</i>	this work
YP42	CK251 <i>rbsC</i> 120AV 313AT	this work
YP111	OW1 Δ <i>rbs(D-R)::spc srl::Tn10 recA</i>	this work
YP211	CK251 <i>rbsC</i> 313AT	this work
YP301	CK251 <i>rbsC</i> 120AV	this work
YP401	CK251 <i>rbsC</i> 298SF 313AT	this work
YP411	CK251 <i>rbsC</i> 296GD 313AT	this work
Plasmids		
pAI12	pACYC184 <i>rbsCBK</i>	Iida <i>et al.</i> (1984)
pET21b	Ap ^r	Novagen
pEY13	pACYC184 <i>rbsB</i>	Eym <i>et al.</i> (1996)
pOH3	pET21b <i>rbsC</i> -His	Park and Park (1999)
pYP1	pEY13 <i>rbsB</i> 52DN 72GD	Eym <i>et al.</i> (1996)
pYP2	pEY13 <i>rbsB</i> 52DG	Eym <i>et al.</i> (1996)
pYP3	pEY13 <i>rbsB</i> 166RH	Eym <i>et al.</i> (1996)
pYP4	pEY13 <i>rbsB</i> 166RC	Eym <i>et al.</i> (1996)
pYP5	pEY13 <i>rbsB</i> 52DN	Eym <i>et al.</i> (1996)
pYP6	pEY13 <i>rbsB</i> 134GD	Eym <i>et al.</i> (1996)
pYP8	pEY13 <i>rbsB</i> 52DN 166RC	this work
pYP14	pBR322 <i>rbsC</i> 313AT	this work
pYP15	pBR322 <i>rbsC</i> 120AV	this work
pYP22A	pBR322 <i>rbsA</i>	this work
pYP100	pBluescript II SK(+) <i>rbsK</i> (758bp)	this work
pYP106	pACYC184 <i>rbsC</i>	Park and Park (1999)
pYP118	pET21b <i>rbsC</i> - <i>C</i> -His	this work
pYP119	pACYC184 <i>rbsC</i> - <i>CBK</i>	this work

activities for ribose transport. These suppressors mapped in *rbsC* were characterized further by DNA sequencing.

Construction of the double *rbsB* mutant and the double *rbsC* suppressor

The pYP8 plasmid (5.9 kb) harboring the *rbsB*-52DN/166RC double mutation was constructed by ligating a 4.7 kb fragment of pYP4 (*rbsB*-166RC) with a 1.2 kb fragment of pYP5 (*rbsB*-52DN), both digested with *Nru*I. The *rbsC*-120AV/313AT double suppressor was obtained by recombining the two single mutations, one (120AV) on the pYP15 plasmid and the other (313AT) on the chromosome, in a *polA^{ts}* strain. The double suppressor gene was confirmed by DNA sequencing.

DNA manipulations for the experiments with the dimeric *rbsC* gene

The pYP22A plasmid harboring the *rbsA* gene was constructed by inserting the *Pvu*II-*Xmn*I fragment (1.8 kb) of pAI13 (Iida *et al.*, 1984) into the *Eco*RV site of plasmid pACYC184. The *rbsA* gene is expressed under the control of the Tc^r promoter of pACYC184. The pYP119 plasmid containing the dimeric *rbsC* gene was constructed by inserting the PCR fragment amplified for the *rbsC* gene of pYP106 into pAI12 using *Xba*I and *Nde*I. The D52N and R166C mutations of *rbsB* in the pEY13 derivatives were transferred to pYP119 using *Cel*III, and the A120V and A313T mutations of the *rbsC* gene were transferred to pYP119 from pYP14 and pYP15 using *Xba*I, *Nde*I and *Bst*XI.

Ribose uptake assay

Cells were inoculated into 5 ml of medium containing H1 minimal salts with 0.4% glycerol and 0.05% ribose, and grown to an $A_{600} = 1.0$ (Iida *et al.*, 1984). Cells were then harvested, washed three times with ice-cold taxis buffer, and adjusted to $A_{600} = 1.0$ in a 450 μ l aliquot. After the sample was incubated for 10 min at 30°C, [1-¹⁴C]D-ribose was added to a final concentration of 15.7 μ M. At 10 s intervals, 100 μ l samples were withdrawn, filtered through membranes, washed and dried. The radioactivity was then measured in a scintillation cocktail.

Western blotting of His-tagged proteins

The pYP118 plasmid containing the dimeric *rbsC*-His-tagged gene was constructed by inserting the PCR fragment amplified for the *rbsC* gene

in pYP106 into pOH3 using *Xba*I and *Nde*I. After the BL21/pOH3 and BL21/pYP118 strains were grown in LB broth to $A_{600} = 0.4$, 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) (final concentration) was added to the culture and grown for 3 h to express RbsC-His- and RbsC-RbsC-His-tagged proteins. Aliquots of the samples were adjusted to $A_{600} = 1.0$ and 10-fold concentrated. After 5 $\times$ Laemmli sample buffer was added, the samples were boiled for 3 min. Samples (20 μ l) were electrophoresed on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher and Schuell). The His-tagged proteins were detected using the Ni-NTA-horseradish peroxidase conjugate (Qiagen) and ECL Western blotting detection reagents (Amersham).

Fluorescence energy transfer experiment with reconstituted RbsC proteins

The RbsC protein was induced from BL21/pOH3 with 0.5 mM IPTG, which was lysed by adding CHAPS to a final concentration of 2% in 30 ml of binding buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 5 mM imidazole) with 0.5 mM phenylmethylsulfonyl fluoride (PMSF). After removing cell debris by centrifugation, the supernatant was applied to a 3 ml His-binding column (Novagen, WI). The protein eluted was used for labeling fluorescent probes, *N*-(1-pyrene)maleimide and *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl)-*N'*-iodoacetyl ethylenediamine [BODIPY(R)FL IA] purchased from Molecular Probes (Eugene, OR). Stock solutions of 10 mM pyrene maleimide and 10 mM BODIPY(R)FL IA were prepared and added to 0.15–0.2 mg of protein solution to achieve a molar ratio of up to 20 M of dye per 1 M of protein. After a 1 h incubation at 37°C, excess mercaptoethanol was added to stop the labeling. Residual salt and probe were removed by passing through the D-Salt Dextran desalting column (Pierce, IL).

Reconstitution was performed with the labeled protein (0.1–0.15 mg/ml) in buffer A (20 mM Tris-HCl pH 7.5, 0.1 M NaCl, 1% CHAPS) and 3.7% *E. coli* total phospholipids (Avanti Polar Lipids Inc., AL) with additional CHAPS to a final concentration of 2%. The mixture was vortexed vigorously, sonicated briefly and dialyzed at 4°C for ~12 h against a 2000 $\times$ volume of buffer B (20 mM Tris-HCl pH 7.5, 0.1 M NaCl, 0.1 mM EDTA). Fluorescence measurement was made with the Shimadzu RF-5301 PC spectrofluorometer with excitation at 342 nm

and emission at 360–560 nm. Fluorescence intensities at 385 nm (for monomer) and 475 nm (for excimer) were chosen to calculate the excimer to monomer (E/M) ratio. Background fluorescence of a control without protein was subtracted from each sample.

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