Protein Science (1996), 5:1093–1099. Cambridge University Press. Printed in the USA.

The proteins encoded by the *rbs* operon of *Escherichia coli*: I. Overproduction, purification, characterization, and functional analysis of RbsA

CHARLENE F. BARROGA,[1] HUIDE ZHANG,[2] NADEEM WAJIH,[3] JAMES H. BOUYER,[4] AND MARK A. HERMODSON

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-1153

(RECEIVED December 14, 1995; ACCEPTED March 27, 1996)

Abstract

The nucleotide-binding component of the high-affinity ribose transport system of *Escherichia coli*, RbsA, was overproduced from a T7-7 expression vector, and the protein was purified. Biochemical analyses of the purified protein indicated that the ATP analogues, 5′-FSBA and 8-azido ATP, covalently labeled the protein, a reaction that was inhibited by ATP, but not by GTP or CTP. The pure protein exhibited low-level ATPase activity with a K_m of about 140 μM. Analyses of bacterial strains carrying chromosomal deletions of *rbsA* and other *rbs* genes suggested that RbsA is important for the chemotaxis function, a surprising result that was not anticipated from previous studies. However, an inconsistency between the several results from deletion strains raises questions regarding the interpretations of the in vivo data.

Keywords: ABC transporters; *E. coli*; membrane transport proteins; RbsA; *rbs* operon

The product of the *rbsA* gene, RbsA, is predicted to be the nucleotide-binding component that couples ATP hydrolysis to the active transport of ribose across the cytoplasmic membrane in *Escherichia coli* (Buckel et al., 1986). It performs this function in association with the product of the *rbsC* gene, the hydrophobic component that is predicted to span the membrane several times (Bell et al., 1986), and the product of the *rbsB* gene, which encodes the periplasmic binding protein (Groarke et al., 1983). These genes are part of the *rbs* operon, *rbsDACBKR*, located at 84 min of the *E. coli* chromosome (Anderson & Cooper, 1970; Bell et al., 1986; Mauzy & Hermodson, 1992). Ribose diffuses through nonspecific pores of the outer membrane and is bound tightly by the binding protein in the periplasm. The binding causes a conformational change that allows the liganded binding protein to interact productively with the Trg receptor to initiate chemotaxis (Zukin et al., 1979) or with the membrane-associated components, which eventually transport ribose into the cell in an energy-dependent fashion. This general mechanism classifies the membrane transport complex encoded by the *rbs* operon as a member of a large family of periplasmic binding protein-dependent transport systems that function in the uptake of a variety of nutrients such as sugars, amino acids, and ions (reviewed in Ames, 1986; Higgins, 1992).

The overall organization and mechanism of the various binding protein-mediated transport systems appear to be similar, but there are many variations on specific features of the complexes. A soluble binding protein binds the substrate with high specificity and delivers it to the membrane-associated components. The transport complex consists of one or two types of hydrophobic, integral membrane proteins and a more hydrophilic protein containing one or two nucleotide-binding motifs. Current evidence suggests that two subunits of each type may be needed to form an active complex (reviewed in Higgins, 1992). Comparison of the predicted amino acid sequences of the hydrophobic components reveals little sequence homology. However, the ATP-binding components, RbsA and its counterparts, are

Reprint requests to: Mark Hermodson, Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-1153; hermodson@biochem.purdue.edu.

Abbreviations: 5′-FSBA, 5′-*p*-fluorosulfonylbenzoyl adenosine; DTT, dithiothreitol; IPTG, isopropylthio-β-galactoside; PMSF, phenylmethylsulfonyl fluoride.

[1] In partial fulfillment of the requirements of the Ph.D. degree. Dr. Barroga purified the RbsA, determined its ATPase activity, nucleotide binding, and FSBA labeling characteristics. She also tested the RbsA topography in the cell membrane by PhoA fusions.

[2] Huide Zhang constructed bacterial strains carrying chromosomal deletions of the *rbs* genes and tested them for the physiological effects on the chemotaxis and ribose transport functions. She also constructed and tested the single amino acid point mutations in the RbsA protein.

[3] Nadeem Wajih performed the azido-ATP labeling studies and assisted with developing the purification and affinity labeling protocols.

[4] In partial fulfillment of the requirements of the Ph.D. degree. Dr. Bouyer constructed the expression vector for *rbsA* and did the initial analyses of the overproduction of the RbsA protein and of its ATPase activity.

clearly homologous over the entire ATP-binding cassette (Higgins, 1992) of about 250 amino acids, well beyond the two short stretches implicated for ATP binding (Walker et al., 1982; Higgins et al., 1985, 1986). In these systems, the nucleotide-binding component may be a single protein, as in HisP and MalK of the histidine and maltose transport systems (Ames, 1986), or two proteins, as in OppD and OppF of the oligopeptide transport system (Ames, 1986), or a single protein having homologous N- and C-terminal halves, hence containing two ATP-binding sites, as in RbsA, MglA, and AraH of the ribose, galactose, and arabinose transport systems (Buckel et al., 1986; Scripture et al., 1987; Hogg et al., 1991). The absolute necessity of the nucleotide-binding component implicates ATP hydrolysis in the mode of energization of these transport systems.

Homologous membrane transport systems have now been found in organisms from mycoplasma to higher eukaryotes (Higgins, 1992). These include the multidrug-resistance transporters of tumor cells (MDR) (Chen et al., 1986), the yeast protein STE6 responsible for the secretion of the a-mating factor (Kuchler et al., 1989), and the cystic fibrosis gene product (CFTR), a chloride ion channel (Riordan et al., 1989). Like RbsA, these proteins arose from duplication of the primordial gene and end-to-end fusion of the products. Unlike the bacterial systems in the above examples, the two hydrophobic components and the two nucleotide-binding components are all fused in a large multidomain polypeptide, and there is no known binding protein. A simpler two-domain organization, where one hydrophobic component is fused to one nucleotide-binding component, has also been identified; examples include the membrane transporters found in pathogenic bacteria, such as the *E. coli* haemolysin transporter, HlyB (Felmlee et al., 1985; Higgins, 1992), the *Bordetella pertussis* cyclolysin transporter, CyaB (Glaser et al., 1988), as well as in eukaryotic transporters such as the white-brown loci of *Drosophila melanogaster* involved in the export of eye pigments (Dreesen et al., 1988), and the peptide transporters of the major histocompatibility complex class I and II molecules (Monaco et al., 1990; Trowsdale et al., 1990).

In spite of the prevalence and physiological importance of these ATP-binding membrane transporters, almost nothing is known about their mechanism. This is partly due to the difficulties of working with membrane-associated proteins, including their low abundance and insolubility of the hydrophobic components, and also to the lack of good in vitro assays for individual components and the whole transport system. Here we describe the overproduction of RbsA, using the T7 overexpression system, and its rapid and simple purification in 40 mg quantities using anion exchange and dye-affinity ligand column chromatography. RbsA is functional in binding nucleotides, as shown by specific labeling with the ATP analogues, 5′-*p*-fluorosulfonylbenzoyladenosine and 8-azido ATP, and it exhibits low-level uncoupled ATPase activity.

Analysis of the involvement of RbsA in the chemotaxis function has produced results that are not explained readily. These data will also be presented and discussed.

Results

An *rbsA* overexpression vector, pT7AC, was constructed in which the ATG initiation codon of *rbsA* was fused to the T7 promoter and ribosome binding site of plasmid pT7-7. Upon induction of the promoter, high-level production of an M_r 55,000 protein, corresponding to the molecular weight of RbsA (Buckel et al., 1986), was observed (Fig. 1). After 3 h of induction, it was estimated that the 55-K band constituted 25% or more of the cell protein. Most of the protein was readily soluble in buffer containing 20% glycerol (Fig. 1). However, little or no increased expression of *rbsC* was observed (RbsC migrates on SDS gels with an apparent M_r of approximately 29,000), even though a complete copy of the gene and its native ribosome binding site were present on the plasmid (Fig. 1). This suggested that translational coupling of expression did not occur.

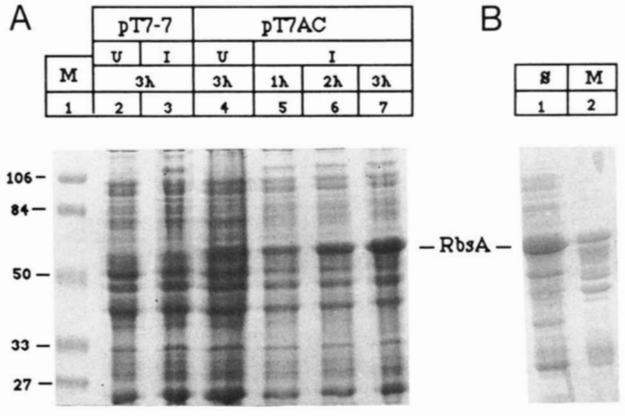

Fig. 1. Overproduction of RbsA. **A:** SDS-PAGE of total cell protein. *E. coli* strain BL21(DE3)/pLysS was transformed with the vector plasmid, pT7-7, or with the overexpression plasmid, pT7AC. Cells were grown at 37 °C in LB medium supplemented with ampicillin (50 μg/mL) for 3 h (up to $OD_{600} \approx 1.0$). IPTG was added to 0.5 mM, and incubation proceeded for the indicated time. Lane 1, molecular weight markers; lanes 2, 3, BL21(DE3)/pLyS/pT7-7, uninduced and induced for 3 h, respectively; lanes 4–7, BL21(DE3)/pLysS/pT7AC, uninduced and induced, 1, 2, and 3 h, respectively. **B:** SDS-PAGE of cell fractions obtained from cells induced for 3 h, passed though a French pressure cell and spun at 100,000 × *g*. Lane 1, soluble fraction; lane 2, membrane fraction. The induced band at 55 kDa is RbsA. The samples were boiled for 5 min before running on a 12% SDS-PAGE gel. Bands were visualized by Coomassie blue staining.

The soluble proteins from induced cells were subjected to DEAE Trisacryl M chromatography followed by affinity chromatography on Reactive Red® agarose (Fig. 2). RbsA eluted early in the gradient from the anion exchanger (at about 0.25 M NaCl), but bound very tightly to the red agarose, eluting in a broad peak at about 0.75 M NaCl. The protein appeared to be homogeneous by SDS gel analysis at this stage (Fig. 2) and was stable indefinitely in 20% glycerol at −70 °C. In the absence of glycerol, some degradation could be observed after a week at 4 °C as determined by western blotting (data not shown).

One liter of cell culture produced about 40 mg of pure RbsA. N-terminal sequence analysis gave the sequence Met-Glu-Ala-Leu-Leu-Gln-, confirming the identity of the protein as RbsA (Bell et al., 1986) and again indicating a high state of purity of the preparation.

RbsA was treated with differing concentrations of 5′-FSBA, an ATP analogue that can modify ATP-binding sites covalently in proteins (Colman, 1983). The label was attached covalently as demonstrated by co-migration with the protein on SDS gels (Fig. 3). Increasing amounts of ATP inhibited the labeling. GTP

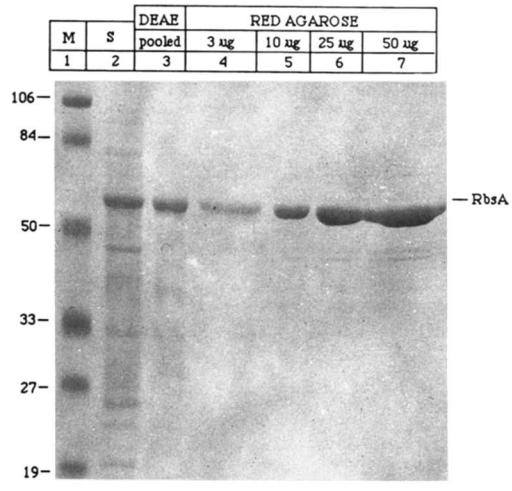

Fig. 2. Purification of the RbsA protein. RbsA protein was purified by the chromatographic steps outlined in the Materials and methods. Lane 1, molecular weight markers; lane 2, soluble fraction of induced cells after two passages through a French pressure cell and a 100,000 × *g* spin (25 μg protein); lane 3, pooled fractions from the DEAE Trisacryl M column (6 μg protein); lanes 4–7, 3, 10, 25, and 50 μg of RbsA from the red agarose column. Samples were run on a 12% SDS-PAGE gel and stained with Coomassie blue.

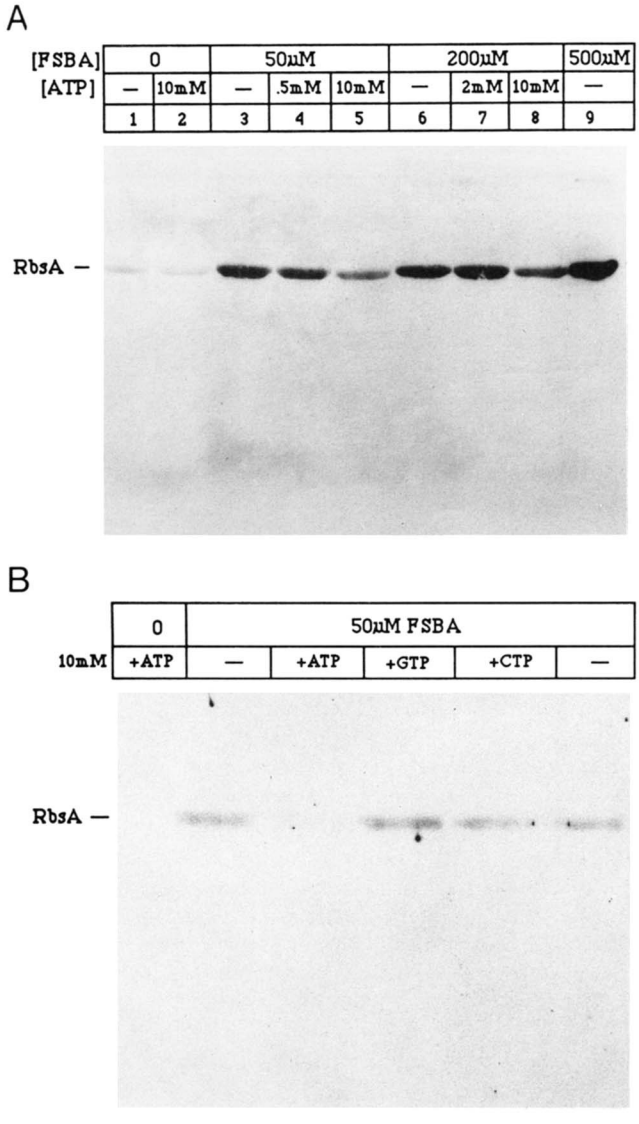

Fig. 3. FSBA labeling of the RbsA protein. 5′-FSBA labeling and detection were performed as described in the Materials and methods. RbsA (2 μg) was incubated with different concentrations of 5′-FSBA for 1 h at 37 °C in the absence or presence of different concentrations of ATP. **A:** Specific labeling with different concentrations of 5′-FSBA. **B:** Competition with different nucleotides at concentrations of 10 mM. After labeling, samples were run on a 12% SDS-PAGE gel, transferred to nitrocellulose, and probed with anti-FSBA antibodies (1:1,000 dilution) followed by an HRP-conjugated anti-rabbit IgG (1:5,000 dilution). The signal was detected by autoradiography using the ECL kit.

and CTP, even at 10 mM concentrations, did not protect RbsA from 5′-FSBA modification (Fig. 3).

Similar labeling is also possible using the photoactivatable label, 8-azido-[γ-^{32}P] ATP (Fig. 4). The label remains attached covalently to the RbsA through SDS gel electrophoresis (Fig. 4), but the bond(s) to the protein are highly labile, making isolation of labeled peptides very difficult (N. Wajih, unpubl. obs.).

Purified RbsA exhibited low-level ATPase activity (Fig. 5). The K_m for ATP with RbsA was found to be 140 μM. The significance of the ATPase activity in an uncoupled system is somewhat questionable, but the K_m value is reasonable in a physiological sense.

A phagemid (p4B1) was originally constructed in our lab (Binnie et al., 1992) for in vivo functional studies on RbsB, the ribose binding protein, in which the levels of expression of *rbsB* were critical (overexpression and underexpression were both deleterious). Phagemid p4B1 was modified by removing some plasmid DNA between the pBR322 promoter and the translation start site and replacing the *rbsB* gene with *rbsAC* to create phagemid pAC (Binnie, 1992). This phagemid complemented the strain carrying a chromosomal deletion of *rbsAC*. Phagemid pAC was modified subsequently by replacing the *rbsAC* genes with various *rbs* genes. In every case, the gene(s) supplied on this plasmid (which are constitutively expressed from the pBR322 promoter upstream of the cloning site) complemented bacterial strains in which the particular gene(s) was deleted from the chromosome for both the chemotaxis and ribose transport function in vivo (Table 1). Absence of either RbsA or RbsC clearly eliminated ribose transport by the high-affinity transport system, as expected (Table 1). Surprisingly, absence of RbsA severely depressed chemotaxis also, except for two cases (last two lines, Table 1). The various plasmids were made independently more than three times each, with consistent results.

Discussion

The *rbsA* gene was cloned originally in a lambda P_L expression vector and expressed in temperature-inducible (cI857) strains (Buckel et al., 1986). The standard induction protocol, which involved a shift in temperature to 42 °C, resulted in high levels of production of RbsA, which was mostly in insoluble protein bodies. When the induction temperature was lowered to 38 °C, most of the RbsA was soluble. The soluble protein could be isolated to >95% purity by a combination of ammonium sulfate fractionation, gel filtration, and anion exchange chromatogra-

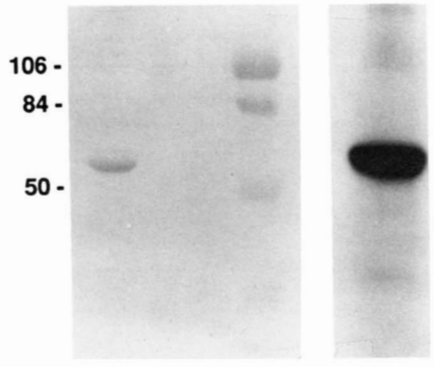

Fig. 4. 8-Azido ATP labeling of RbsA. Purified RbsA was radiolabeled with 8-azido-[γ-^{32}P] ATP by photolysis as described in the Materials and methods. Left: Coomassie-stained SDS gel of the product of the labeling (left lane) and molecular weight standards (right lane). Numbers on the left refer to the molecular masses of the standards in kilodaltons. Right: Autoradiogram of the gel.

Table 1. *Transport and chemotaxis analysis of bacterial strains carrying chromosomal deletions of rbs genes and various plasmids*[a]

Plasmid/Strain	Consequence	Transport	Capillary
pA/MHK-7[b]	Complete	(100)	(100)
pAC/MH-11	Complete	100	100
pC/MH-17	Complete	100	100
pA-(K43R)/MHK-7	RbsA-(K43R)[c]	<5	<5
pBR322/MHK-7	No RbsA	<5	<5
pC/MH-11	No RbsA	<5	<5
pA/MH-11	No RbsC	<5	55
pBR322/MH-17	No RbsC	<5	45
pBR322/MH-11	No RbsA or RbsC	<5	<5
pBR322/MH-15	No RbsD, RbsA, or RbsC	<5	<5
No plasmid/MH-11	No RbsA or RbsC	<5	30
pT7D/MH-15	No RbsA or RbsC	<5	30

[a] The numbers are percentages of the values obtained for the complete systems and are accurate to approximately ±10%.
[b] MHK-7 lacks the *rbsA* gene; MH-11 has a deletion of *rbsAC*; MH-17 has a deletion of *rbsC*; MH-15 has the *rbsDAC* genes deleted.
[c] RbsA (K43R) is a site-directed mutation of the Walker A sequence (Walker et al., 1982) in the first ATP-binding cassette of the protein.

phy. However, proteolysis of the protein occurred during storage and during attempts to crystallize it. Considerable effort was also required to produce milligram quantities of RbsA (S.D. Buckel, C.A. Mauzy, & M.A. Hermodson, unpubl. obs.).

The current approach for production and purification of RbsA is a considerable improvement over the earlier methods. The induction of *rbsA* under control of the T7 promoter takes place at lower temperature, resulting in a high yield of soluble, properly folded product. The purification protocol, adapted from Rosen et al. (1988), employs dye affinity columns that have been effective in the purification of numerous ATP-binding proteins (Thompson et al., 1975; Siebers et al., 1992; Walter et al., 1992). The affinity of RbsA for the dye column clearly suggests that the RbsA has a properly configured binding site.

The most convincing evidence that RbsA has a properly configured nucleotide binding site comes from ATP-inhibitable labeling with 5′-FSBA. The label is covalently bound, because the product can be detected after SDS electrophoresis, but we have had considerable difficulty in isolating labeled polypeptides after proteolysis of the labeled protein. Apparently, a very labile

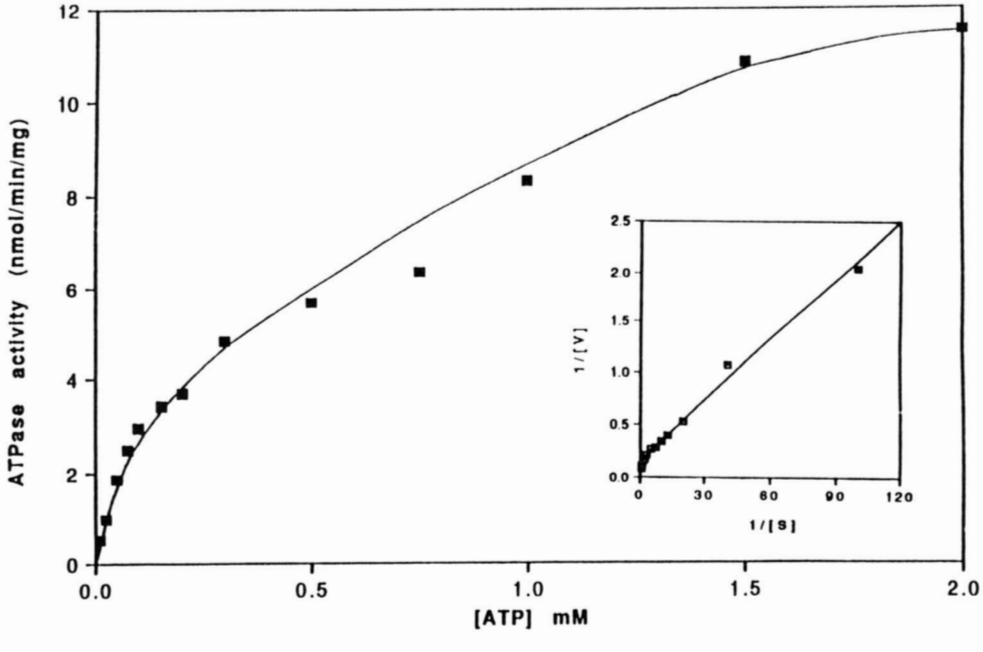

Fig. 5. ATPase activity of RbsA. Activity of purified RbsA (5 μg) was measured over a 200-fold range of ATP concentration in the presence of 10 mM $MgCl_2$ for 20 min at 37 °C.

bond is formed with the FSBA. The nucleotide specificity of the 5′-FSBA reaction appears to be quite high; only ATP competes well. In other studies of related transporters, results vary (Hobson et al., 1984; Higgins et al., 1985; Walter et al., 1992). Binding of labeled ATP to MalK has been shown to be inhibited effectively by ATP, ADP, and GTP, but not CTP (Walter et al., 1992). CTP cannot remove OppD from a dye affinity column, whereas ATP can (Higgins et al., 1985). Labeling of HisP by 8-azidoATP is inhibited by ATP, GTP, UTP, CTP, and ADP, with ATP being a bit more effective (Hobson et al., 1984). In order for this question to be resolved, reconstitution of transport in vitro with pure constituents under appropriately controlled conditions is needed to measure inhibition of transport by the various nucleotides and nucleosides directly.

Uncoupled ATPase activity has been observed for proteins of this superfamily before, and the extent of the activity varies considerably with the protein and the reaction conditions. MalK that has been refolded from urea-solubilized protein bodies binds ATP with a K_D of 150 μM (Walter et al., 1992) and hydrolyzes both ATP and GTP with a K_m of about 70 μM (Morbach et al., 1993). It also hydrolyzes CTP, but with a much higher K_m, consistent with the competitive binding studies (above). The N- and C-terminal half molecules of P-glycoprotein (the product of the MDR1 gene), as well as the whole molecule, exhibit low-level ATPase activity in the absence of transportable drug (Loo & Clarke, 1994). The intact P-glycoprotein and the co-expressed N- and C-terminal halves exhibit drug-stimulated increases in the ATPase activity (Loo & Clarke, 1994). This protein is a complete transporter, however, so the results are not directly comparable to studies on the isolated ATPase component. The first nucleotide-binding domain of the cystic fibrosis transmembrane conductance regulator (CFTR) has been found recently to hydrolyze ATP slowly when it is expressed as a chimeric protein with maltose-binding protein (Ko & Pedersen, 1995). ATP analogues inhibited the hydrolysis, and maltose-binding protein alone showed no ATPase activity, so the first ATP-binding cassette of CFTR has similar ATPase properties to RbsA in an uncoupled system. Of course, the ATPase activity of biochemical relevance is that associated with productive membrane transport, and it can only be measured with confidence in a defined, catalytically complete system. Steps toward accomplishing that goal are described in the companion paper (Zaitseva et al., 1996).

Binding protein-mediated transport requires coupling of energy first to the opening of the binding protein, which, with a K_D of 10^{-7} M, requires almost 10 kcal/mol. Then transport of the ribose through the inner membrane via the transport complex to the cytosol must be energized. The RbsA protein is not at all hydrophobic nor does it have any segments of sequence that would be predicted to span a membrane (Bell et al., 1986). Genetic evidence in the histidine transport system indicates direct coupling between the histidine-binding protein and HisP, the RbsA analogue (Ames, 1986; Kerppola et al., 1991), which would imply that HisP crosses the membrane. It is attractive to consider the possibility that RbsA may extend through the membrane and interact directly with the binding protein, because that would not require coupling of the energy of hydrolysis of ATP to a third protein, RbsC, in the transport process. More detailed structural information is needed before a reasonable mechanism can be proposed, however.

We cannot fully explain the chemotaxis analyses (Table 1) by current understanding of the system. The ribose transport data indicate that RbsA and RbsC are required (Table 1), but RbsD is not (Bouyer, 1991). That is in accord with all other analyses of related systems. However, the periplasmic binding protein is theoretically the only common element to the transport and chemotaxis systems, yet we observe severe effects on taxis when RbsA is absent but RbsC is present, with two exceptions (Table 1). The data in Table 1 are internally inconsistent, however, in that the last four lines should all be alike. Because each of the experiments was performed several times with new transformations of the strains with the indicated plasmids, the inconsistency is not due to incorrect strain constructions. Two interpretations of the data are possible, each with an exception. First, and most likely, the presence of RbsC in the absence of a functional transporter interferes with taxis, possibly by binding holo-binding protein and interfering with the sensing of the ratios of holo- versus apo-binding protein by the chemotaxis receptor. This would be consistent with our earlier observations (Binnie et al., 1992) that alteration of binding protein levels, either by overexpression or underexpression, interfered with chemotaxis assays. The pBR322 controls in strains MH-11 and MH-15 are not consistent with this interpretation, unless there is some unknown deleterious effect of the pBR322 plasmid on chemotaxis. We have some evidence that pBR322 may, indeed, suppress the chemotactic response in that wild-type cells (strain OW1) carrying pBR322 show only 75% of the swarm ring values of the same strain carrying no plasmid (H. Zhang, unpubl. obs.). Although easily measurable, that degree of suppression is relatively small compared to the effects seen in deletion strains MH-11 and MH-15 carrying pBR322 (Table 1). The alternative explanation is that RbsA is somehow involved in chemotaxis. Then the last two lines of Table 1 are inconsistent, but all other data would fit the hypothesis. What the connection between the transport and chemotaxis systems through RbsA may be is unknown. The first hypothesis appears more likely to be correct.

It is interesting to observe that the RbsA (K43R) mutant is nonfunctional. RbsA, being an internally duplicated molecule with homologous N- and C-terminal halves, has two potential ATP-binding sites with clearly identifiable Walker A sequences: G-E-N-G-A-G-K-S-T (residues 37–45) and G-L-M-G-A-G-R-T-E (residues 285–293). The first of these is more consistent with the consensus Walker A sequence, having a lysyl residue and two hydroxy-amino acid residues at its C-terminal end. Because changing the lysyl residue to an arginyl residue impairs the function of the protein severely, it is tempting to speculate that the ATP-binding cassette that energizes the active transport is the one in RbsA nearer the amino terminus. Again, better structural information is needed to construct a more detailed mechanistic hypothesis.

Materials and methods

Materials

[γ-^{32}P]-ATP (30 Ci/mmol) and the ECL kit for western blotting were purchased from Amersham. DEAE Trisacryl M, reactive red agarose 120 (type 3000-CL), blue agarose (Cibacron Blue 3GA), and ATP-agarose were purchased from Sigma. 5′-FSBA, ATP, GTP, and CTP were obtained from Sigma. Enzymes and other materials for recombinant DNA techniques were obtained from Boehringer-Mannheim and New England Biolabs. pT7-7 vector was generously provided by Dr. Stanley

Tabor. Oligonucleotides were synthesized by the Laboratory for Macromolecular Structure, Purdue University. Protein standards and nitrocellulose were from Bio-Rad. Crude anti-FSBA antibodies were generously provided by Dr. Robert Geahlen of the Department of Medicinal Chemistry and Pharmacognosy of Purdue University and were affinity purified using an ATP-agarose column.

Construction of the overexpression plasmid, pT7AC

An *Nde* I/*Pst* I fragment containing the *rbsD*, *rbsA*, and *rbsC* genes (≈3 kb) was isolated from a pUC-based plasmid pDAC using low-melting agarose gel electrophoresis (Sea plaque, Intermountain Sci.) and phenol extraction. This fragment was ligated into the *Nde* I/*Pst* I sites of the bacteriophage T7 overexpression vector, pT7-7, to yield plasmid pT7DAC1. To fuse the start codon of *rbsA* gene to the Shine–Dalgarno sequence of the T7 vector, the *Nde* I/*Sma* I fragment containing *rbsD* and 44 bp of *rbsA* coding sequence was deleted. This was replaced by an adaptor,

5′TATGGAAGCATTACTTCAGCTTAAAGGCATCGATAAAGCCTCCCC3′

3′ ACCTTCGTAATGAAGTCGAATTTCCGTAGCTATTTCGGAGGGG5′,

containing an *Nde* I half-site and the ATG start site of RbsA at the 5′ end, the missing 44 bp of *rbsA* coding sequence, and a *Sma* I half-site at the 3′ end, to yield pT7AC. pT7AC only overproduces native RbsA, but not RbsC, which is expressed from its native ribosome binding site.

Overproduction and fractionation of cells

E. coli strain BL21(DE3)/pLysS was transformed with the plasmid pT7AC. Single colonies were picked and grown overnight at 37 °C in 10-mL LB medium containing 100 μg/mL ampicillin. The overnight cultures were subcultured in 1 L LB medium in 4-L flasks containing ampicillin and allowed to grow until they reached an $OD_{600} \approx 1.0$. Cells were induced by the addition of IPTG to a final concentration of 0.5 mM and allowed to grow for another 3 h. All subsequent manipulations were at 4 °C. Cells were harvested by centrifugation at 6,000 × *g* for 20 min, and the cell pellet (3.5 g wet cells/L) was resuspended in 35 mL of buffer A (25 mM Tris-HCl, pH 7.5, 2 mM Na_2EDTA, 1 mM DTT, and 20% (v/v) glycerol). One millimolar PMSF and 5 mM 1,10 phenanthroline (Sigma) were added prior to cell lysis. Cells were broken by two passages through a French pressure cell at 12,000 psi. The soluble fraction was collected by centrifugation at 100,000 × *g* for 1 h. Prepared in this manner, RbsA was found mainly in the soluble fraction.

Purification of RbsA protein

The cytosol was applied to a 2.5 × 20-cm diameter column packed with 100 mL of DEAE Trisacryl M anion exchanger (Sigma) equilibrated with buffer A. The column was washed with 300 mL of the same buffer and eluted with a linear gradient of 0–0.5 M NaCl in 300 mL of buffer A at a flow rate of 1 mL/min. Fractions of 5 mL were collected and analyzed by SDS-PAGE according to the method of Laemmli (1970). RbsA eluted at about 0.25 M NaCl. The fractions containing RbsA protein were pooled and applied to a 1.5-cm diameter column packed with 35 mL of red agarose (Reactive Red 120-Agarose, type 3000-CL, Sigma) equilibrated with buffer A. The column was washed with 300 mL of the same buffer followed by a linear gradient of 0–1.0 M NaCl in 300 mL of buffer A at a flow rate of 0.5 mL/min. Fractions (2.5 mL) were collected and analyzed by SDS-PAGE. RbsA eluted at 0.75 M NaCl. Fractions containing RbsA protein were analyzed immediately for ATPase activity and then stored at −20 °C.

ATPase assay

Pure RbsA from the dye columns was assayed for ATP hydrolysis. Assay mixtures (100 μL) contained 50 mM HEPES-KOH, pH 7.5, 100 mM KCl, 5–10 μg RbsA, and 1 mM ATP (labeled with [γ-^{32}P]ATP at 10–30 cpm/pmol). RbsA was pre-incubated with the reagents at 37 °C for 10 min. The reaction was initiated by the addition of 100 mM $MgCl_2$, followed by further incubation at 37 °C for the indicated amount of time. The released [γ-^{32}P]-PO_4 was extracted according to the method of Conway and Lipman (1964) and was counted in a scintillation counter.

FSBA labeling

Purified RbsA protein (2 μg) was incubated with different concentrations of 5′-FSBA with or without ATP at 37 °C for 1 h. Reaction mixtures (20 μL) contained 50 mM HEPES-KOH buffer, pH 7.5, 0.25 mM Na_2EDTA, and 10 mM $MgCl_2$. Because 5′-FSBA has limited solubility in water, it was added in dimethylsulfoxide, with the final dimethylsulfoxide concentration adjusted to 10% (v/v) in all assays. Control reactions were incubated in the same manner except for the inclusion of the RbsA protein. The reaction was terminated by adding SDS gel loading buffer and boiling for 5 min. The 5′-FSBA-modified RbsA was immunodetected employing a modification of the procedure described by Zeitlin et al. (1992). Samples were electrophoresed on a 12% SDS-PAGE gel and transferred onto nitrocellulose in transfer buffer (0.04% SDS, 125 mM Tris base, pH 8.4, 192 mM glycine, 20% methanol) for 1 h at 100 V and 4 °C. All subsequent steps were performed at room temperature. The nitrocellulose was blocked with 2.5% gelatin in wash buffer (0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, 2% (v/v) Triton X-100) for 1 h. The blots were rinsed twice for 15 min in wash buffer. Anti-FSBA antibody was diluted 1:1,000 in 3% bovine serum albumin in wash buffer and applied with shaking for 1 h. The blots were rinsed twice (15 min) with wash buffer and exposed to the secondary antibody (horseradish peroxidase-linked donkey anti-rabbit IgG; Amersham) at a 1:5,000 dilution for 1 h. The blots were rinsed twice (15 min), drained, and exposed to enhanced chemiluminescence detection solution (ECL; Amersham). Autoradiographs were prepared with Hyperfilm after 1–2 min exposure.

Photolabeling with 8-azidoATP

8-Azido-[γ-^{32}P]ATP was purchased from ICN Biochemicals. Labeling was conducted on ice with 0.18 nmol RbsA in 20 mM Tris·HCl, pH 7.5, 2 mM EDTA, and 45 μM [^{32}P] 8-azidoATP. The reaction was initiated by insertion of the photoprobe with 60 s of photolysis at 254 nm. The reaction was terminated by bringing the solution to 50 mM DTT. Twelve percent SDS gels

of the product were autoradiographed to determine ^{32}P incorporation into RbsA.

Analytical procedures

Protein concentration was assayed with the micro-BCA protein assay kit from Pierce Chemical Co. The sequence of the N-terminal six amino acids of the purified protein was determined by the automated Edman degradation on an Applied Biosystems 470A protein sequencer.

Deletion strains

Deletion strain MH-17 (δC) lacks the portion of *rbsC* from the *Nco* I site through the *Acc* I site, removing the C-terminal 180 residues of RbsC. The CAT gene has been inserted into the deletion site for selection.

Deletion strain MHK-7 (δA) has the portion of the *rbsA* gene from codons 193–408 (the two *Sac* II sites) replaced by the kanamycin-resistance gene.

Acknowledgments

This is journal paper number 15036 from the Purdue University Agricultural Experiment Station. This work was supported in part by grant 9003012-DMB to Dr. Mark Hermodson from the National Science Foundation. The support of the Diabetes Research and Training Center, DK 20542, of the Public Health Service is acknowledged. We thank Dr. R. Alan Binnie for the cloning of pAC and Jelena Zaitseva for constructing deletion strain MHK-7 (δA). We thank Alison J. Baldwin for preparation of the RbsA antibodies and for assistance with several of the experiments in this work.

References

Ames GFL. 1986. Bacterial periplasmic transport systems: Structure, mechanism, and evolution. *Annu Rev Biochem 55*:397–425.

Anderson A, Cooper RA. 1970. Biochemical and genetical studies on ribose catabolism in *Escherichia coli* K12. *J Gen Microbiol 62*:335–339.

Bell AW, Buckel SD, Groarke JM, Hope JN, Kingsley DH, Hermodson MA. 1986. The nucleotide sequences of the *rbsD*, *rbsA*, and *rbsC* genes of *Escherichia coli* K12. *J Biol Chem 261*:7652–7658.

Binnie RA. 1992. Structure and function of the ribose-binding protein of *Escherichia coli*: Surfaces which mediate transport and chemotaxis [thesis]. West Lafayette, Indiana: Purdue University.

Binnie RA, Zhang H, Mowbray S, Hermodson MA. 1992. Functional mapping of the surface of *Escherichia coli* ribose-binding protein: Mutations that affect chemotaxis and transport. *Protein Sci 1*:1642–1651.

Bouyer JH. 1991. Characterization of RbsD of the D-ribose high-affinity transport system in *Escherichia coli* K12 [thesis]. West Lafayette, Indiana: Purdue University.

Buckel SD, Bell AW, Mohana Rao JK, Hermodson MA. 1986. An analysis of the structure of the product of the *rbsA* gene of *Escherichia coli* K12. *J Biol Chem 261*:7659–7662.

Chen CJ, Chin JE, Ueda K, Clark DP, Pasten I, Gottesman MM, Roninson IB. 1986. Internal duplication and homology with bacterial transport proteins in the *mdr1* (P-glycoprotein) gene from multidrug-resistant human cells. *Cell 47*:381–389.

Colman RF. 1983. Affinity labeling of purine nucleotide sites in proteins. *Annu Rev Biochem 52*:67–91.

Conway TW, Lipmann F. 1964. Characterization of a ribosome-linked guanosine triphosphatase in *Escherichia coli* extracts. *Proc Natl Acad Sci USA 52*:1462–1469.

Dreesen TD, Johnson DH, Henikoff S. 1988. The brown protein of *Drosophila melanogaster* is similar to the white protein and to components of active transport complexes. *Mol Cell Biol 8*:5206–5215.

Felmlee T, Pellett S, Welch RA. 1985. Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. *J Bacteriol 163*:94–105.

Glaser P, Sakamoto H, Bellalou J, Ullman A, Danchin A. 1988. Secretion of cyclolysin, the calmodulin-sensitive adenylate cyclase-haemolysin bifunctional protein of *Bordetella pertussis*. *EMBO J 7*:3997–4004.

Groarke JM, Mahoney WC, Hope JN, Furlong CE, Robb FT, Zalkin H, Hermodson MA. 1983. The amino acid sequence of the D-ribose-binding protein from *Escherichia coli* K12. *J Biol Chem 258*:12952–12956.

Higgins CF. 1992. ABC transporters: From microorganisms to man. *Annu Rev Cell Biol 8*:67–113.

Higgins CF, Hiles ID, Salmond GPC, Gill DR, Downie JA, Evans IJ, Holland IB, Gray L, Buckel SD, Bell AW, Hermodson MA. 1986. A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. *Nature 323*:448–450.

Higgins CF, Hiles ID, Whalley K, Jamieson DJ. 1985. Nucleotide binding by membrane components of bacterial periplasmic binding protein-dependent transport systems. *EMBO J 4*:1033–1040.

Hobson AC, Weatherwax R, Ames GFL. 1984. ATP-binding sites in the membrane components of histidine permease, a periplasmic transport system. *Proc Natl Acad Sci USA 81*:7333–7337.

Hogg RW, Voelker C, Von Carlowitz I. 1991. Nucleotide sequence and analysis of the *mgl* operon of *Escherichia coli* K12. *Mol Gen Genet 229*:453–459.

Kerppola RE, Shyamala VK, Klebba P, Ames GFL. 1991. The membrane-bound proteins of periplasmic permeases form a complex. *J Biol Chem 266*:9857–9865.

Ko YH, Pedersen PL. 1995. The first nucleotide binding fold of the cystic fibrosis transmembrane conductance regulator can function as an active ATPase. *J Biol Chem 270*:22093–77096.

Kuchler K, Sterne RE, Thorner J. 1989. *Saccharomyces cerevisiae* STE6 gene product: A novel pathway for protein export in eukaryotic cells. *EMBO J 8*:3973–3984.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*:680–685.

Loo TW, Clarke DM. 1994. Reconstitution of drug-stimulated ATPase activity following co-expression of each half of human P-glycoprotein as separate polypeptides. *J Biol Chem 269*:7750–7755.

Mauzy CA, Hermodson MA. 1992. Structural and functional analyses of the repressor, RbsR, of the ribose operon of *Escherichia coli*. *Protein Sci 1*:831–842.

Monaco JJ, Cho S, Attaya M. 1990. Transport genes in the murine MHC: Possible implications for antigen processing. *Science 250*:1723–1726.

Morbach S, Tebbe S, Schneider E. 1993. The ATP-binding cassette (ABC) transporter for maltose/maltodextrins of *Salmonella typhimurium*. *J Biol Chem 268*:18617–18621.

Riordan JR, Rommens JM, Kerem BS, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL, Drumm ML, Iannuzzi MC, Collins FS, Tsui LC. 1989. Identification of the cystic fibrosis gene: Cloning and characterization of the complementary DNA. *Science 245*:1066–1073.

Rosen BP, Weigel U, Karkaria C, Gangola P. 1988. Molecular characterization of an anion pump. *J Biol Chem 263*:3067–3070.

Scripture JB, Voelker C, Miller S, O'Donnell RT, Polgar L, Rade J, Horazdovsky BF, Hogg RW. 1987. High-affinity L-arabinose transport operon: Nucleotide sequence and analysis of gene products. *J Mol Biol 197*:37–46.

Siebers A, Kollmann R, Dirkes G, Altendorf K. 1992. Rapid, high-yield purification and characterization of the K^+-translocating Kdp-ATPase from *Escherichia coli*. *J Biol Chem 267*:12717–12721.

Thompson ST, Cass KH, Stellwagen E. 1975. Blue dextran-Sepharose: An affinity column for the dinucleotide fold in proteins. *Proc Natl Acad Sci USA 72*:669–672.

Trowsdale J, Hanson I, Mockridge I, Beck S, Townsend A, Kelly A. 1990. Sequences encoded in the class II region of the MHC related to the "ABC" superfamily of transporters. *Nature 348*:741–744.

Walker JE, Saraste M, Runswick MJ, Gay NJ. 1982. Distantly related sequences in the α- and β-subunits of ATP-synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J 1*:945–951.

Walter C, zu Bentrup KH, Schneider E. 1992. Large scale purification, nucleotide binding properties, and ATPase activity of the MalK subunit of the *Salmonella typhimurium* maltose transport complex. *J Biol Chem 267*:8863–8869.

Zaitseva J, Zhang H, Binnie RA, Hermodson M. 1996. The proteins encoded by the *rbs* operon of *Escherichia coli*: II. Use of chimeric protein constructs to isolate and characterize RbsC. *Protein Sci 5*:1100–1107.

Zeitlin PL, Crawford I, Lu L, Woel S, Cohen ME, Donowitz M, Montrose MH, Hamosh A, Cutting GR, Gruenert D, Huganir R, Maloney P, Guggino WB. 1992. CFTR expression in primary and cultured epithelia. *Proc Natl Acad Sci USA 89*:344–347.

Zukin RS, Hartig PR, Koshland DE. 1979. Effect of an induced conformational change on the physical properties of two chemotactic receptor molecules. *Biochemistry 18*:5599–5605.