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Reconstitution of Binding Protein Dependent Ribose Transport in Spheroplasts Derived From a Binding Protein Negative Escherichia coli K12 Mutant and From Salmonella typhimurium

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Highly purified ribose-binding protein from Escherichia coli has been used to reconstitute a binding-protein-dependent ribose transport in spheroplasts derived from a binding-protein-deficient mutant of E coli K12, and in spheroplasts derived from Salmonella typhimurium. The cross-species reconstitution was nearly as efficient as the reconstitution of the E coli strain from which the binding protein was derived. Antibody raised against the ribose binding protein completely prevented reconstitution, whereas it had no effect on whole cells. The reconstitution procedure has been improved by generating spheroplasts from cells grown in a rich medium and by reducing the background uptake in spheroplasts through a special washing procedure. Rapid purification of ribose binding protein by high pressure liquid chromatography is also described.

Key words: reconstitution, ribose, transport, Escherichia coli, Salmonella typhimurium, ribose-binding protein

Previous studies on the reconstitution of binding-protein-dependent ribose transport demonstrated that high levels of ribose-binding protein alone were not sufficient to reconstitute uptake in spheroplasts. Uptake could be reconstituted only in spheroplasts derived from cells which had been induced with ribose [1]. Reconstitution was unsuccessful in mutants which were defective for ribose-binding protein, presumably due to insufficient expression of the ancillary protein components required for the complete transport system. Another difficulty encountered in the reconstitution system was a variable background of transport in unsupplemented spheroplasts, probably caused by residual binding protein associated with the spheroplasts.

This report describes the reconstitution of the binding-protein-dependent ribose uptake in spheroplasts derived from a ribose-binding protein-negative mutant of E coli K12,

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as well as a cross-species reconstitution of spheroplasts derived from Salmonella typhimurium LT2. Reconstitution could be completely blocked with antiserum directed against the ribose-binding protein. The variable background uptake in unsupplemented spheroplasts has been largely eliminated through a modification of the spheroplasting procedure.

MATERIALS AND METHODS

Preparation of Spheroplasts

E coli K12 spheroplasts were prepared from cells grown to late log phase (A_{660} nm = 1.0) as described in detail by Galloway and Furlong [1]. Cells were grown in either nutrient broth (Difco) supplemented with 1% ribose and 1% glycerol, or minimal medium [2, 3] supplemented with 1% ribose and 1% glycerol. Salmonella typhimurium LT-2 was kindly provided by Giovanna Ames. Salmonella spheroplasts were prepared always in parallel with an E coli K12 preparation, with identical initial treatments. Partial spheroplasting was usually observed in the E coli preparation 5 minutes after the final addition of EDTA, which was always added gradually [1]. The E coli preparation was diluted with 10% sorbitol–10 mM Tris pH 8.0 at this point.

The Salmonella preparation, containing 10 mM EDTA, usually did not contain any spheroplasts at this stage. The EDTA concentration was then raised gradually (over a 2.5-min interval) to 20 mM in the Salmonella preparation, and incubation was continued for an additional 5–10 minutes. When spheroplast formation was observed, the culture was diluted with 1/3 volume of 10% sorbitol/10 mM Tris pH 8.0. Lysozyme was added to maintain a concentration of 100 μg/ml throughout the spheroplasting steps. Complete (> 99%) spheroplasting was observed within 10 minutes as judged by phase contrast microscopy, and was confirmed by a tenfold dilution and plating on nutrient agar [4]. The spheroplast solution was supplemented with $MgSO_4$ to a final concentration of 20 mM, and DNase and RNase were added to a final concentration of 10 μg/ml each. After 1–2 minutes further incubation, the spheroplasts were harvested by centrifugation at 12,000g for 6 minutes at 4°C and resuspended gently in 10 ml of cold 20% sucrose containing 1 mM $MgSO_4$ along with DNAse and RNase (10 μg/ml each). The spheroplasts were harvested immediately by centrifugation at 12,000g for 6 min at 4°C, and resuspended in 2 ml of 100 mM Tris-HCl, pH 6.9 +, 20% sucrose +, 1 mM $MgSO_4$ (per 45 ml of original culture, A_{660} = 1.0). The final wash produces preparations that remain stable for several hours at 4°C and do not exhibit the clumping characteristic of unwashed spheroplasts. In addition, the background transport in these preparations is much lower and more reproducible than observed earlier [1], probably due to more complete removal of periplasmic binding proteins. The preparation contains 3–4 mg/ml cell protein.

Transport Assays

The transport assays for whole cells and for spheroplasts have been described previously [1]. The energy source in the assays was 1% glycerol. Antiserum raised against purified ribose binding protein was added to the reaction mixtures at a final concentration of 2% (v/v) where indicated. This concentration of antiserum did not alter the rate of ribose uptake in whole cells assayed at a final ribose concentration of 80 mM. The filters used for transport assays were boiled for 15 min in two changes of distilled water, and soaked in sucrose containing transport buffer with 5 mg/ml of bovine serum albumin to eliminate interaction of binding protein–ligand complexes with the filter.

Purification of Ribose-Binding Protein

Ribose-binding protein was purified from shock fluids obtained from the ribose-binding protein constitutive E coli K12 strain DG 50.3 through the DEAE cellulose step as previously described [5]. The second column step involved fractionation on an SP–Sephadex column which was equilibrated with 10 mM ammonium acetate, pH 5.5, and eluted with an ammonium acetate (pH 5.5) gradient from 10 mM to 150 mM. The binding protein was judged pure by acrylamide gel electrophoresis in the acid–urea system of Panyim and Chalkley [6], and by analytical chromatography on a Waters I-125 column operated by a Spectra Physics 8000 high pressure liquid chromotograph (HPLC). An alternative procedure involved purification of the DEAE fraction by HPLC as shown in Figure 1. Material recovered from the major peak eluting from the Waters I-125 column was lyophilized and used in reconstitution. No binding activity was lost during high pressure liquid chromatography.

Protein concentrations were determined by the method of Lowry et al [7] using bovine serum albumin as a standard.

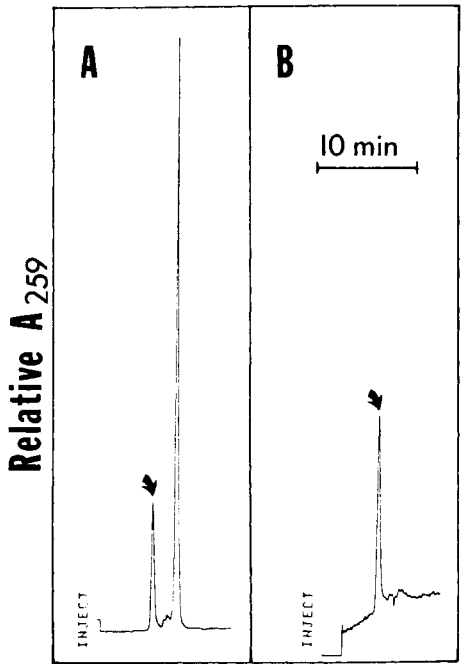

Fig. 1. A) High performance liquid chromatography of purified ribose-binding protein (DEAE-cellulose fraction) through a Waters I-125 gel filtration column attached to a Spectraphysics Model 8000 high pressure liquid chromatograph. The buffer used was 100 mM ammonium acetate pH 5.5, the flow rate was 1.5 ml/min, and absorbance was monitored at 254 nM. All of the ribose-binding activity was contained in the peak eluting at 5.6 min (marked with the arrow). B) Rechromatography of the ribose binding activity eluting at 5.6 min in Figure 1A. The conditions were the same as used in Figure 1A, except that the sensitivity of detection was increased.

RESULTS

Two-dimensional gel electrophoretic studies of whole cell protein preparations [Rosenblum and Furlong, unpublished results] suggested that one of the previously described E coli K12 ribose-transport-negative mutants DG1-9 [1] was deficient in only the ribose binding protein. Our earlier efforts to reconstitute ribose uptake in spheroplasts derived from DG 1–9 cells grown in Vogel-Bonner minimal medium were unsuccessful. We therefore decided to change several parameters of the reconstitution procedure. First, since the background levels of transport in spheroplasts derived from wild type cells were highly variable in our previous work [1], we included a wash step in the spheroplast preparation procedure with the aim of minimizing residual traces of endogenous binding protein. This was based on the rationale that the background was due to variable amounts of residual binding protein remaining with spheroplast preparations derived from binding-protein-positive strains. This seemed to be a reasonable assumption, since the spheroplasts used in earlier experiments [1, 4] were assayed directly after harvesting from the spheroplasting medium. Inclusion of DNase and RNase in the wash step provided spheroplasts which resuspended uniformly after centrifugation and remained active for several hours.

In addition, we prepared spheroplasts from wild type and DG 1–9 cells grown in different media. Figure 2 shows that it is possible to achieve a slight reconstitution of ribose uptake in spheroplasts prepared from cells of strain DG 1–9 grown in minimal medium M9 supplemented with glycerol and ribose. Figure 3 shows that a much higher level of reconstitution was achieved when the experiment was repeated with spheroplasts derived from DG 1–9 cells grown in nutrient broth containing ribose. (The time lag was seen in most of our experiments.) Reconstitution was prevented when antiserum raised against pure ribose binding protein was included in the assay mixture (Fig. 3).

Spheroplasts derived from the parent E coli K12 cells grown in nutrient broth plus ribose showed high levels of reconstitution (Fig. 4). The background levels were much lower than those observed on our earlier work, indicating that the wash step following the spheroplast preparation is worthwhile.

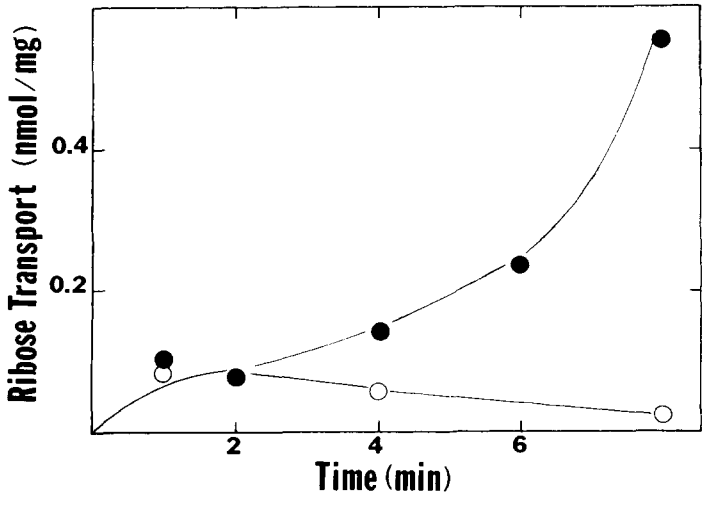

Fig. 2. Reconstitution of ribose uptake in spheroplasts derived from E coli ribose-negative mutant DG 1–9, grown in M9 minimal medium containing 1% glycerol and 1% D-ribose. Open circles, spheroplasts without added ribose protein. Closed circles, spheroplasts supplemented with 0.23 mg/ml ribose-binding protein.

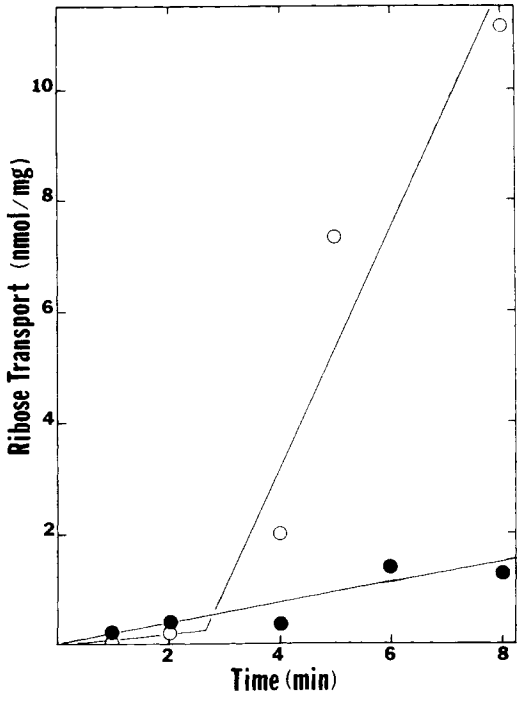

Fig. 3. Reconstitution of spheroplasts derived from cells of E coli DG 1–9 grown in nutrient broth containing 1% D-ribose. Open circles, spheroplasts supplemented with 0.27 mg/ml ribose binding protein. Closed circles, spheroplasts assayed in the presence of 0.27 mg/ml ribose-binding protein and 2% (v/v) antiserum raised againse pure ribose-binding protein.

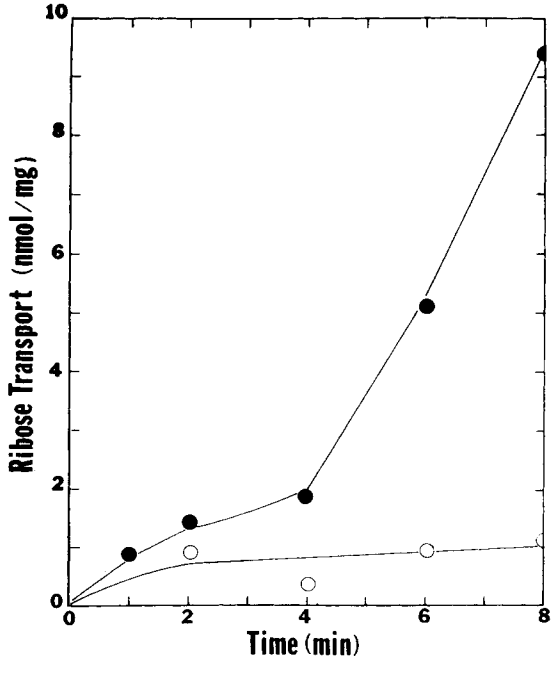

Fig. 4. Reconstitution of ribose uptake in E coli K12 spheroplasts prepared from cells grown in nutrient broth containing 1% ribose. Open circles, spheroplasts without added ribose binding protein. Closed circles, spheroplasts supplemented with 0.23 mg/ml ribose binding protein. Cells were grown in nutrient broth containing 1% D-ribose. Transport (also for Figs. 3–5) is expressed as nmol ribose transported per mg cell protein.

In view of the results indicating good reconstitution of cells grown in nutrient broth, and in view of decreased background uptake in unsupplemented spheroplasts, we attempted a cross-species reconstitution. We used pure ribose protein prepared from E coli K12 to reconstitute ribose uptake in spheroplasts prepared from Salmonella typhimurium LT2. Figure 5 shows that unsupplemented spheroplasts prepared from Salmonella cells grown in nutrient broth also gave low backgrounds of ribose uptake. Spheroplasts supplemented with pure E coli ribose binding protein reconstitutioned nearly as well as E coli spheroplasts assayed under the same conditions. Growth of Salmonella in minimal ribose medium resulted in poor reconstitution and high backgrounds (Fig. 5). The maximum level of reconstitution was achieved with 0.25 to 0.5 mg/ml of ribose binding protein (Fig. 6). These results are similar to those reported by Galloway and Furlong for reconstitution of the E coli ribose transport system [1].

DISCUSSION

The water soluble periplasmic binding proteins are essential components of one class of bacterial active transport systems, and possess substrate recognition and regulatory properties which are similar to those found for the corresponding complete transport systems. This implies that the proteins function in the initial recognition of substrates, and that the additional components of these systems cannot function without interacting with the cor-

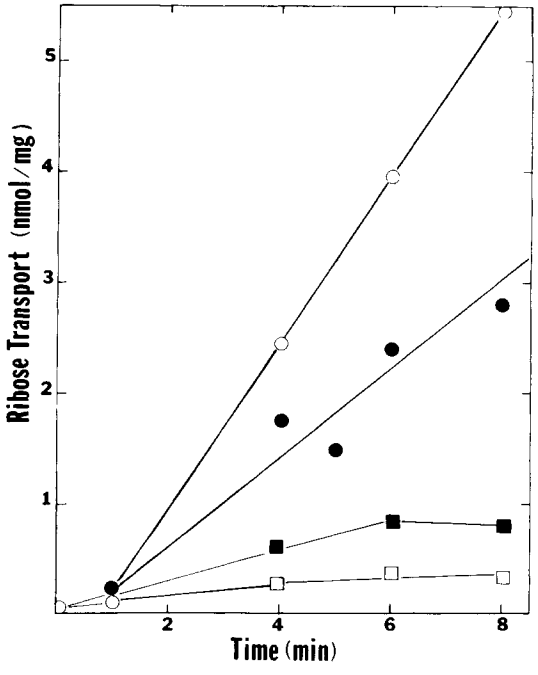

Fig. 5. Reconstitution of ribose transport in spheroplasts of Salmonella typhimurium LT2 derived from cells grown in nutrient broth (open symbols) and minimal medium (closed symbols) supplemented with 1% ribose and 1% glycerol. Circles represent spheroplasts supplemented with 0.5 mg/ml E coli ribose-binding protein. Squares represent controls assayed without added ribose-binding protein.

rect binding protein. At present, although the binding proteins are well characterized and additional components have been identified in the cytoplasmic membrane [8, 9], the nature of the protein interactions and ligand transfer steps remains obscure. Reconstitution of binding-protein-dependent phosphate transport in E coli spheroplasts, reported by Gerdes et al [4], provided the first reliable biochemical assay for the interaction of the binding protein with its membrane receptor(s). Earlier reports of reconstitution in cells depleted of binding protein by osmotic shock proved intractable during repeated studies in other laboratories [10, 11]. The procedure of Gerdes et al [4] has proven to be reproducible in the case of the reconstitution of ribose uptake in spheroplasts derived from E coli K12 [1]. This system is a favorable general model for binding-protein-dependent systems, since ribose-binding protein-deficient strains are devoid of other systems for ribose uptake. This is in contrast with glutamate and aspartate transport where, in addition to the binding-protein-dependent system, at least four other systems transport these two amino acids [12]. In addition, reconstitution of ribose uptake has been unsuccessful in spheroplasts derived from uninduced cells and in mutants defective for ribose uptake, implying that the non-binding protein components are also ribose inducible [1]. During attempts to identify the ancillary protein components of the ribose uptake system, comparison of two-dimensional gel electrophoretograms of whole cell proteins from wild type and ribose-transport-deficient strains (Rosenblum and Furlong, unpublished results) suggested that one of the binding-protein-deficient strains (DG 1–9) appeared to have all of the proteins observed in induced wild type cells, except for mature ribose-binding protein. This mutant appeared to be a good candidate for reconstitution studies. Several different growth-spheroplasting protocols were attempted with E coli K12 and the mutant DG 1–9. The results in Figure 2 show that growth of DG 1–9 in M9 minimal medium provided spheroplasts in which reconstitution was significantly improved relative to spheroplasts derived from the minimal medium grown cells described previously [1]. Reconstitution of E coli DG 1–9 spheroplasts was low, however, when compared to the level of recon-

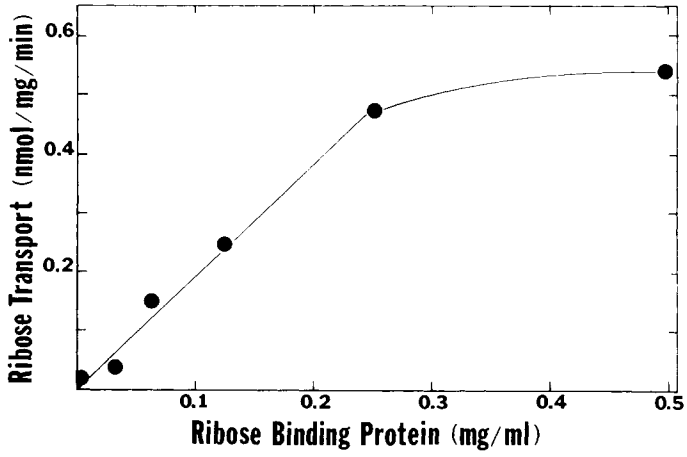

Fig. 6. Effect of increasing concentrations of E coli ribose-binding protein on the reconstitution of ribose uptake in spheroplasts of Salmonella typhimurium LT2 prepared from cells grown in nutrient broth containing 1% D-ribose.

stitution observed with spheroplasts derived from the parent strain (Fig. 4). A much higher level of reconstitution was observed when spheroplasts were prepared from the mutant DG 1–9 grown in nutrient broth supplemented with ribose and glycerol (Fig. 3). Apparently one or more components of the rich medium either induce the production of the ancillary protein components required for ribose uptake, or the richer medium allows the entry of sufficient ribose for induction. Intact E coli DG 1–9 cells grown under these conditions do not have detectable ribose uptake, and this provides for a very low background uptake of ribose during the assay of unsupplemented strain DG 1–9 spheroplasts (Fig. 2).

The effect of anti-ribose binding protein antiserum in virtually abolishing reconstitution (Fig. 3) indicates that all of the ribose-binding protein which is functional in reconstitution is accessible to large molecules. Our early attempts at reconstituting the ribose transport system of Salmonella typhimurium LT2 were ineffectual, due to the high EDTA concentrations (50–60 mM) required to obtain complete spheroplast formation with cells grown in minimal medium. Spheroplasts obtained under such conditions have very high background counts and reconstitute poorly (Fig. 5). The growth of Salmonella in a rich medium results in cells which form spheroplasts more readily, and which reconstitute almost as effectively as E coli K12 (Fig. 5). Thus, despite a difference of about 0.7 pI units in the isoelectric points of the ribose-binding proteins from E coli (pI = 6.6 [13]) and S typhimurium (pI = 7.3 [14]), the important structural features of the two proteins are sufficiently similar to provide efficient recognition of the E coli ribose binding protein by the Salmonella ribose transport system membrane sites. Apparently the interaction between the binding protein and the ancillary protein components has favored the conservation of the important regions of the respective proteins. Protein sequence work presently underway should provide more detailed information on the regions of difference between the ribose binding proteins of E coli and Salmonella typhimurium.

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