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THE PHOSPHATE-BINDING PROTEIN OF *ESCHERICHIA COLI*

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SUMMARY

1. *Escherichia coli* contains a phosphate-binding protein, in amounts exceeding $2 \cdot 10^4$ molecules per cell. Over 80% of this protein is released by osmotic shock.

2. The phosphate-binding protein has been purified and found to have a molecular weight of 42000. There is no evidence of subunits or aggregation.

3. Each molecule of the protein binds one molecule of phosphate. This activity is inhibited by a specific rabbit antiserum.

4. Cold-shocked *E. coli* lose the ability to transport phosphate. The purified binding protein when added to cold-shocked *E. coli* stimulates phosphate uptake. This effect is abolished by the specific antiserum.

5. The phosphate-binding protein also stimulates phosphate uptake in a mutant of *E. coli* with impaired phosphate transport and which also lacks this protein. It does not have any effect on phosphate uptake in another mutant which has the binding protein but is deficient in phosphate uptake through another lesion.

INTRODUCTION

Several binding proteins have been isolated from bacteria in recent years[1–5]. These proteins are believed to be concerned with the transport of the molecule which they bind specifically, but their exact function is not known. In a previous communication we described the isolation and some properties of a phosphate-binding protein from *Escherichia coli*[6]. The present paper describes the purification of the phosphate-binding protein and some of its physical properties. We also describe the effects of the phosphate-binding protein on the restoration of phosphate uptake in cold-shocked *E. coli* and in a mutant which lacks this protein, as well as the abolition of this effect by a specific anti-phosphate-binding protein serum.

MATERIALS AND METHODS

Chemicals

The *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was a product of K and K Laboratories, and Freund adjuvant (complete) of Difco. Tris was obtained from Sigma Chemicals. All other chemicals used were of the highest purity available.

Abbreviation: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

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Radioactive compounds

Radioactive orthophosphate ($^{32}P_i$) was obtained from the Australian Atomic Energy Commission. The carrier-free solutions were adjusted to a final concentration of 1.0 mM with respect to P_i by the addition of sterile nonlabelled phosphate and stored at 3° to avoid the formation of particle-absorbed $^{32}P_i$ which we and other workers[7] have encountered. Appropriate solutions were membrane-filtered before use.

Bacterial culture

The strain of *E. coli* used in this study was AB3311 (Reeves *met*$^-$) and the conditions for its propagation have been described previously[6]. In addition, *E. coli* K-10 and two mutants strains (C-93 and C-85a) originating from this parent were used. These were the gift of Dr. A. Garen. For large-scale cultures the medium consisted of: 10 mM potassium phosphate buffer (pH 7.0), 0.2 % $(NH_4)_2SO_4$, 0.02 % $MgCl_2$, 0.02 % $CaCl_2$, 0.02 % NaCl, 0.1 % yeast extract, 1 mM methionine and 0.4 % glucose.

For the study of phosphate uptake, cells were grown in TMY medium (50 mM Tris–HCl buffer (pH 7.4), 10 mM KCl, 0.2 % $(NH_4)_2SO_4$, 0.02 % $MgSO_4 \cdot 7H_2O$, 0.1 % yeast extract, 1 mM methionine and 0.4% glucose) in a gyratory water bath (New Brunswick Scientific) at 37°. They were deprived of phosphate for 4–6 h in TM medium (TMY, less yeast extract).

Cold shock of the bacteria and preparation of spheroplasts

For reasons described below (see RESULTS), a modified cold-shock procedure was used in experiments on the restoration of phosphate uptake: The cells were harvested, resuspended in a minimal volume of distilled water at room temperature and rapidly dispersed into 80 vol. of water at 3°. After 5 min, the cells were centrifuged and used immediately for the uptake experiments.

Spheroplasts were prepared by the method of ABRAMS[8] in 0.4 M glycylglycine buffer at pH 7.4 containing either 1.0 mM $MgCl_2$ or 1.0 mM EDTA. The completeness of the conversion was checked by phase-contrast microscopy. Protein solutions were concentrated by ultrafiltration in a Diaflo cell (Amicon Co.), under a pressure of 4 atm of He, using the UM-10 membrane (retention 10000 daltons). Protein was estimated by the method of LOWRY *et al.*[9].

Uptake studies

Membrane filters were soaked in 0.5% KH_2PO_4 and washed once with water before use. Cells were suspended in TM medium at a density of 100 Klett units (about 10^9 cells per ml) at 37°. Labelled P_i was added and 0.5-ml samples were removed at intervals. These were either filtered immediately on membrane filters or placed into tubes chilled on ice (we have found that this treatment did not result in any loss or gain of label by the cells). The cells were then filtered at the end of the experiment, usually within 20 min, and the membranes were washed with 0.9 % NaCl containing 0.1 % KH_2PO_4, except in the case of uptake measurement with spheroplasts, when the wash solution was osmotically balanced with either sucrose or glycylglycine (see above). The intracellular distribution of the label was examined as described elsewhere[10].

Preparation of rabbit antiserum to phosphate-binding protein

Each of two rabbits was injected subcutaneously with phosphate-binding protein (1 mg/kg) mixed with Freund adjuvant (0.5 ml). 4 weeks later each rabbit was challenged with a further injection of phosphate-binding protein (1 mg/kg) alone. The animals were bled through the ear vein 1 week later. The challenge and subsequent bleeding procedures were repeated at 5-week intervals.

The selection of mutants with reduced ability to transport phosphate

TMY-grown *E. coli* were starved of phosphate and treated with MNNG[11]. Mutants were selected on meat infusion agar containing 0.5 % arsenate, because previous results with *Bacillus cereus*[12] showed that such mutants were cryptic to both arsenate and phosphate. Resistant *E. coli* mutants thus selected were checked for their ability to transport phosphate. Mutants lacking phosphate-binding protein were selected by plating the MNNG-treated cells on TM plates containing 1% phosphate and minute colonies from this plate were transferred to TM plates containing 10 mM phosphate and to TMY plates. The cells that grew on the TMY, but not on the TM *plus* 10 mM phosphate, were selected, grown in batches on TMY and osmotically shocked. The concentrated shock fluid was then assayed for phosphate-binding activity.

RESULTS

Purification of phosphate-binding protein

The details of the procedure, briefly described elsewhere[6], were as follows (all procedures, except growth, were carried out at 3°):

Cells were grown at 37° in large-scale culture (see MATERIALS AND METHODS) and harvested in the stationary phase. They were osmotically shocked according to the method of NEU AND CHOU[13] and the shock fluid was concentrated by ultrafiltration to a protein content of about 4 mg/ml. $(NH_4)_2SO_4$ was added to this solution at 3° and the fraction precipitating between 0.8 and 0.9 saturation was collected. The precipitate was dissolved in water, dialysed, concentrated and applied to a Sephadex G-150 column (100 cm × 2.5 cm). The column was eluted with 10 mM Tris–HCl buffer (pH 8.5) containing 10 mM KCl and 1 mM $MgCl_2$ (Tris–KCl–$MgCl_2$ buffer). Fractions containing phosphate-binding protein were pooled, concentrated and applied to a Sephadex G-100 column which was again eluted with Tris–KCl–$MgCl_2$ buffer. The progress of purification was followed by polyacrylamide-gel electrophoresis (Fig. 1) and by determination of specific activity of the phosphate-binding protein (Table I).

The active fraction eluted from Sephadex G-100 appears to be homogeneous by gel electrophoresis.

Determination of the molecular weight of the phosphate-binding protein

The molecular weight of the phosphate-binding protein was found to be 42000 ± 1000 by the measurement of elution volume from Sephadex[16] (Fig. 2) and by the relative migration rate in polyacrylamide gels[19] (Fig. 3).

Determination of the number of phosphate-binding sites

The stoichiometry of phosphate binding by phosphate-binding protein was

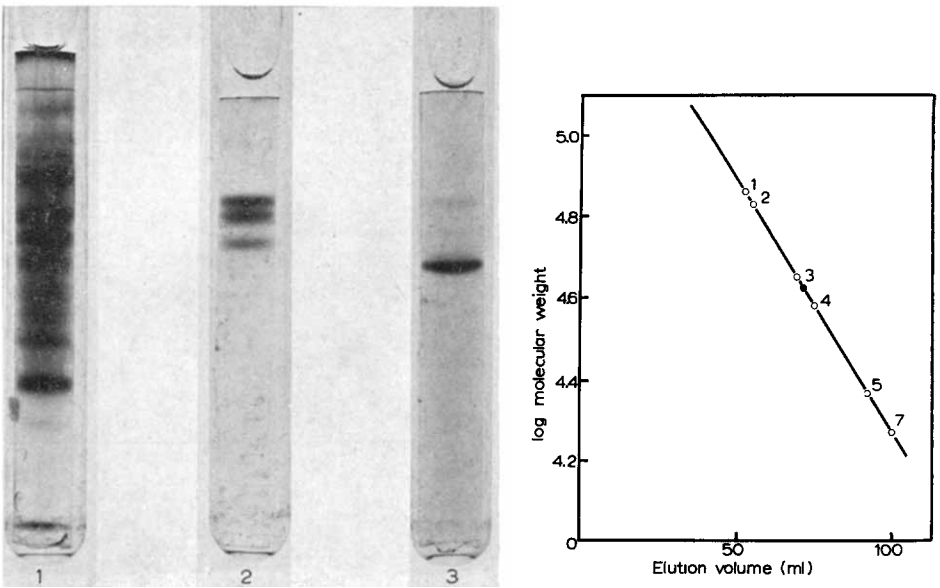


Fig. 1. Polyacrylamide-gel electrophoresis, by the method of DAVIS[14], of the concentrated shock protein[1], and of the purified phosphate-binding protein from the Sepahadex G-150 fraction[2] and the Sephadex G-100 fraction[3].

Fig. 2. The determination of the molecular weight of phosphate-binding protein by elution volume from Sephadex G-100. The column (100 cm × 1.5 cm) was eluted with Tris–KCl–$MgCl_2$ buffer and the eluate assayed for protein by absorbance at 280 nm and for phosphate-binding activity by the resin method[15]. The following standard proteins (○) were used for the molecular weight determination of phosphate-binding protein (●): 1, pepsin dimer (70000); 2, bovine serum albumin (68000); 3, ovalbumin (43000); 4, arginine kinase (38000); 5, α-chymotrypsinogen (25700); 6, trypsin (23300); 7, myoglobin (17200); and 8, ribonuclease (13700). The molecular weight of arginine kinase was obtained from SMITH[17]; the others, from WEBER AND OSBORN[18].

TABLE I

THE PURIFICATION OF PHOSPHATE-BINDING PROTEIN FROM *E. coli* AB 3311

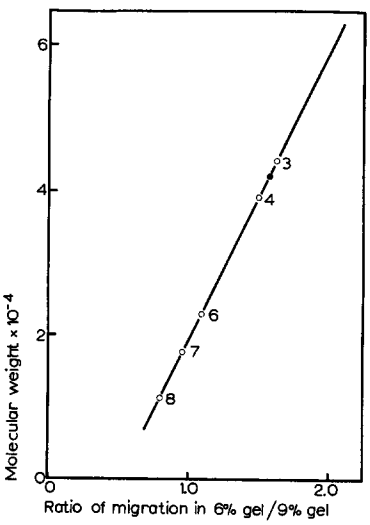
Phosphate-binding activity was measured by the resin method[15]. All fractions were adjusted to the same ionic strength (0.2 M Tris–HCl buffer (pH 8.5), 0.2 M KCl–20 mM $MgCl_2$ buffer). A sample of each fraction was shaken for 5 min with 0.1 g of the Dowex-1 acetate–[$^{32}P_i$] in a total volume of 1.0 ml. The resin was filtered off and the phosphate released was estimated by counting a sample of the filtrate. All values below have been corrected for blank values, *i.e.* release of phosphate due to ionic effect of the buffer. The blank value in the present assay was 2.4 nmoles [$^{32}P_i$] per ml, or 3 times the value of the K_D (0.8 μM).

Fraction	*Total activity* (%)	*Phosphate-binding protein activity* (*nmoles P_i per mg protein*)	*Purification*
Crude shock fluid	100	0.8	1
$(NH_4)_2SO_4$ fraction	80	5.8	7
Sephadex G-150	70	15	19
Sephadex G-100	55	22	28

determined by two different methods. In the first, the value was obtained from a kinetic plot (Fig. 4). In the second method, the value was calculated directly from the amount of phosphate released from Dowex-1 with near-saturating concentrations of free phosphate (Table II). As determined by either of the two methods, each molecule of phosphate-binding protein binds one molecule of phosphate. In this respect the phosphate-binding protein resembles the other bacterial binding proteins[1,3].

Determination of the number of molecules of phosphate-binding protein per cell

The method described in the previous section has been adapted to calculate both the total number of molecules of phosphate-binding protein per cell and the



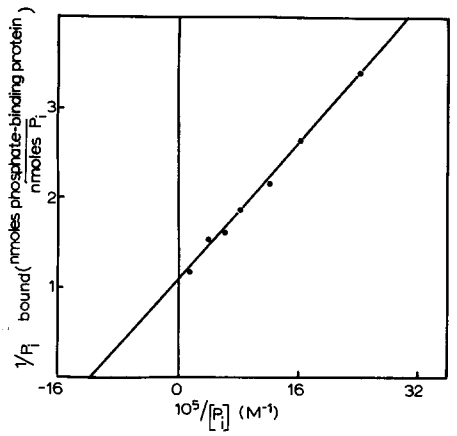


Fig. 3. The determination of the molecular weight of phosphate-binding protein by relative migration in polyacrylamide gel. Electrophoresis in 6 and 9% gels was carried out by the method of DAVIS[14]. The standard proteins were as for Fig. 2. Electrophoresis in the presence of 8 M urea yielded essentially the same results.

Fig. 4. The calculation of the molar ratio of phosphate bound by phosphate-binding protein. Phosphate-binding activity was measured by the method of PEARLMAN *et al.*[20]. The inverse plot of phosphate bound per mg phosphate-binding protein *versus* phosphate concentration was used to determine binding at infinite P_i concentration. 1 mg phosphate-binding protein (at 42 000 daltons) is equivalent to 24 nmoles.

TABLE II

THE DETERMINATION OF THE MOLAR RATIO OF PHOSPHATE BOUND PER MOLECULE OF PHOSPHATE-BINDING PROTEIN

Phosphate-binding activity was assayed by the resin method[15] as described in Table I.

nmoles P_i in blank	2.4
nmoles P_i after addition of 50 μg (1.2 nmoles) phosphate-binding protein	3.5
nmoles P_i released from resin by 1.2 nmoles phosphate-binding protein	1.1
nmoles P_i per nmole phosphate-binding protein	0.9

relative proportion of phosphate-binding protein released by osmotic shock. We found that cells which had been subjected to osmotic shock according to NEU AND CHOU[18] released about 85% of the total binding activity (Table III). On the other hand, when the treatment with sucrose–Tris–EDTA was omitted, the release of phosphate-binding protein fell to about one-half the previous value. The total number of molecules of phosphate-binding protein per cell, as determined in these experiments, was in the range $2 \cdot 10^4$–$2.5 \cdot 10^4$. This was calculated using the Avogadro number, the known binding ratio of 1 and the fact that the phosphate-binding protein was saturated with phosphate since the concentration of free P_i in the assay system (2 μM) was 3 times the K_D value (0.8 μM).

TABLE III

COMPARISON OF PHOSPHATE-BINDING ACTIVITY RELEASED FROM *E. coli* BY OSMOTIC SHOCK WITH RESIDUAL ACTIVITY OF OSMOTICALLY SHOCKED CELLS

Phosphate-binding activities were determined as described in Table I and are expressed as molecules of phosphate-binding protein released (or retained) per cell. The residual activity was assayed after disruption of the osmotically shocked cells.

Expt. No.	*Molecules phosphate-binding protein*		*Release by osmotic shock* (%)
	Released per cell	*Retained per cell*	
1	$1.8 \cdot 10^4$	$3 \cdot 10^3$	86
2	$2.1 \cdot 10^4$	$3.5 \cdot 10^3$	87

Restitution by phosphate-binding protein of phosphate uptake in cold-shocked cells, in spheroplasts and in mutants with impaired phosphate transport

Preliminary experiments showed that, when phosphate-starved cells were subjected to osmotic shock preceded by sucrose–Tris–EDTA treatment, or converted to spheroplasts by the lysozyme–EDTA method[8], phosphate transport in these cells was severely inhibited. Furthermore, attempts to restore this function by the addition of phosphate-binding protein met with varying degrees of success. However, since we found that the sucrose–Tris–EDTA treatment in the osmotic shock procedure was not obligatory for the release of the phosphate-binding protein, we examined preparations obtained by direct dilution of the cells in cold water (see MATERIALS AND METHODS). When cells were treated in this way, the phosphate uptake was still markedly reduced, but now treatment with phosphate-binding protein resulted in a significant increase in the rate of total phosphate uptake (Fig. 5). Spheroplasts, which have severely decreased phosphate uptake also responded to treatment with phosphate-binding protein by showing both increased total uptake and increased phosphate incorporation into acid-insoluble material (Fig. 6).

Control experiments were carried out with two mutants defective in phosphate transport: One of these, 10-1, lacked the phosphate-binding protein and would therefore be expected to respond to added phosphate-binding protein. The other, B-14, possessed functional phosphate-binding protein but lacked another (unknown) essential portion of the transport system. Stimulation of phosphate uptake by phosphate-binding protein in this mutant would not be expected and thus it would provide an estimate of possible artifacts in the experiment. We found that the addition

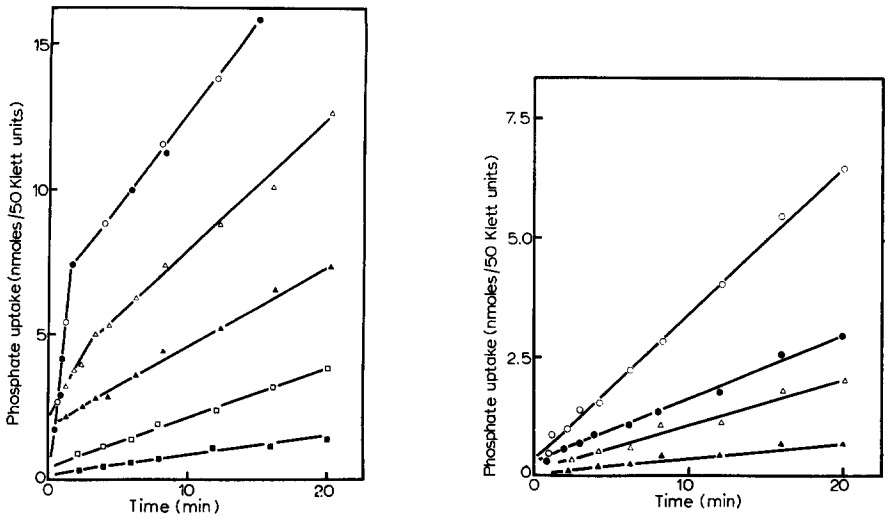


Fig. 5. The stimulation by phosphate-binding protein of phosphate uptake in cold-shocked *E. coli*. Cold-shocked cells were incubated at 500 Klett units with 250 μg phosphate-binding protein for 2 min at 0° and then diluted with 9 vol. of uptake medium. Phosphate uptake was measured at 50 μM phosphate. Total uptake was measured as described in MATERIALS AND METHODS; the incorporation of $^{32}P_i$ into acid-insoluble material was measured after electrophoretic removal of the soluble $^{32}P_i$ as described elsewhere[10]. ●—●, control cells alone; ○—○, control cells with phosphate-binding protein. Cold-shocked cells alone: ▲—▲, total uptake; ■—■, $^{32}P_i$ incorporated into acid-insoluble material. Cold-shocked cells with phosphate-binding protein: △—△, total uptake; □—□, $^{32}P_i$ incorporated into acid-insoluble material.

Fig. 6. The stimulation by phosphate-binding protein of phosphate uptake in *E. coli* spheroplasts. Total uptake and $^{32}P_i$ incorporation into acid-insoluble material was measured as described (see legend for Fig. 5). Untreated spheroplasts: ●—●, total uptake; ▲—▲, $^{32}P_i$ incorporated into acid-insoluble material. Spheroplasts treated with 50 μg phosphate-binding protein: ○—○, total uptake; △—△, $^{32}P_i$ incorporated into acid-insoluble material.

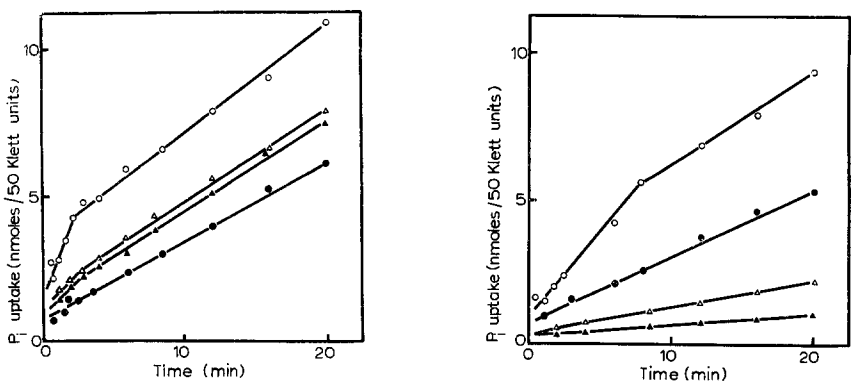


Fig. 7. The failure of phosphate-binding protein to stimulate phosphate uptake in cold-shocked B-14 cells. Conditions for uptake are as described for Fig. 5. ●—●, cold-shocked parent cells alone; ○—○, cold-shocked parent cells with phosphate-binding protein; ▲—▲, cold-shocked B-14 cells alone; △—△ cold-shocked B-14 cells with phosphate-binding protein.

Fig. 8. The increase by phosphate-binding protein of the rate of phosphate uptake in cold-shocked 10-1 cells. Total uptake and the $^{32}P_i$ incorporation into acid-insoluble material was measured as described (see legend for Fig. 5). Cold-shocked cells alone: ●—●, total uptake; ▲—▲, $^{32}P_i$ incorporated into acid-insoluble material. Cold-shocked cells with phosphate-binding protein: ○—○, total uptake; △—△, $^{32}P_i$ incorporated into acid-insoluble material.

of phosphate-binding protein to cold-shocked B-14 cells did not increase phosphate accumulation (Fig. 7). On the other hand, the phosphate-binding protein significantly enhanced phosphate uptake by the cold-shocked 10-1 mutants but had no effect when added to nonshocked cells. Again, the incorporation of label into the acid-insoluble fraction was similarly stimulated (Fig. 8) in these cells.

These experiments show that, under specific conditions, the addition of phosphate-binding protein restores to a significant degree the ability to transport phosphate in cells which have lost this function by diverse treatments.

Inhibition of the phosphate-binding protein-mediated restoration of phosphate uptake in cold-shocked E. coli by rabbit antiserum to phosphate-binding protein

Rabbit anti-phosphate-binding protein serum effectively inhibited phosphate binding by phosphate-binding protein, whereas the control serum had no effect (Fig. 9). The effect of the antiserum on the reconstitution of uptake is shown in Fig. 10. The stimulation of the phosphate uptake by phosphate-binding protein is completely abolished in the presence of the antiserum but not of the control serum.

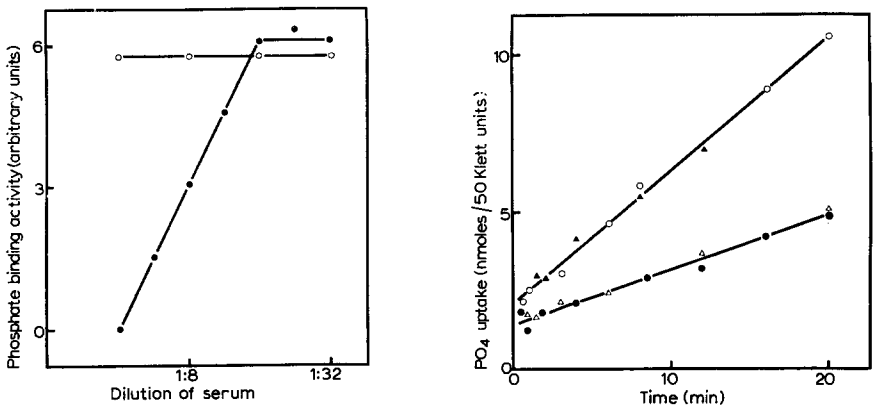


Fig. 9. The inhibition of phosphate-binding protein by rabbit anti-phosphate-binding protein serum. Phosphate-binding protein (0.2 ml, 100 mg/ml) was incubated with 0.2 ml dialysed rabbit serum of the appropriate dilution at 4° for 8 h. Phosphate-binding activity was measured by the method of PEARLMAN *et al.*[20]. ○—○, control serum; ●—●, antiserum.

Fig. 10. The inhibition of phosphate-binding protein-stimulated P_i uptake in cold-shocked *E. coli* by rabbit antiserum to phosphate-binding protein. The phosphate-binding protein was incubated with 0.2 ml dialysed rabbit serum for 2 h at 0°. The conditions for uptake are described in the legend for Fig. 5. ●—●, cold-shocked cells alone; ○—○, cold-shocked cells with phosphate-binding protein; ▲—▲, cold-shocked cells with phosphate-binding protein and control serum; △—△, cold-shocked cells with phosphate-binding protein and antiserum.

Comparison between the phosphate-binding protein and the R2 protein

In our previous communication[6], we commented on the relationship between the phosphate-binding protein and the R2 protein described by GAREN AND OTSUJI[21]. On the basis of certain physiological parameters (especially response to phosphorus starvation) we claimed that they were different. This claim is now further supported since we were able to test several of the mutants, kindly provided by Dr. A. GAREN. We found that functional phosphate-binding protein was present in all the strains in approximately equal amounts even though one of the strains completely lacked the

R2 protein (Table IV). The phosphate-binding protein is therefore distinct from the R2 protein of *E. coli*.

TABLE IV

A COMPARISON OF THE PHOSPHATE-BINDING ACTIVITIES IN SHOCK FLUIDS OF *E. coli* STRAINS K-10, C-93, C-85a AND AB 3311

The phosphate-binding activity was determined as described in Table I.

Strain	*Presence of the R2 protein (see ref. 21)*	*Phosphate binding (nmoles/mg shock protein)*
AB3311	Not determined	3.1
K-10	+	3.6
C-93	−	3.0
C-85a	+	3.7

DISCUSSION

The phosphate-binding protein from *E. coli* resembles the other known binding proteins in a number of its properties. It is removed by osmotic shock to an extent of over 80%, it binds one molecule of phosphate per molecule of protein and its binding constant for P_i ($K_D = 0.8\ \mu M$) is of the same order of magnitude as those of the leucine- and galactose-binding proteins but is considerably lower than that of the sulphate-binding protein. The molecular weight of 42000 is somewhat larger than those of the other bacterial binding proteins described (30000–36000)[1].

Although the rapid uptake of phosphate appears only in cells that have been phosphate-starved, phosphate starvation does not appear to be essential for the production of phosphate-binding protein. Cells grown in 10 mM P_i, which show virtually no phosphate uptake (unless starved) contain functional phosphate-binding protein. Cells grown in TMY, not starved for phosphate, contain about $2 \cdot 10^4$ molecules of phosphate-binding protein per cell. This is in the range quoted for the other bacterial binding proteins, *e.g.* derepressed *Salmonella typhimurium* contain about $1 \cdot 10^4$ molecules of the sulphate-binding protein per cell[1].

It is believed that the known binding proteins are concerned with transport. Direct histological evidence[22,23] and the fact that the binding proteins are released by osmotic shock show that they are associated with the membrane. Mutants which lack a particular binding protein, also lack the corresponding specific transport function[1,4,5]. The loss of the transport of a number of compounds concomitant with the release of their corresponding binding proteins by osmotic shock is also indicative of the involvement of these proteins in transport. The value of the dissociation constant of the binding proteins from their 'substrates' is approximately the same as the K_m for the transport of those molecules into intact cells[1].

Proof of the participation of binding proteins in transport must involve the restoration of a transport function by a specific binding protein. Several such claims have been made, but these have been treated with reservation[1,24]. The most probable artifact in restoration experiments is the nonspecific retention (mediated by the binding protein) of labelled substrate on the filter membrane—either by direct

adsorption to the membrane or by adsorption to the cells themselves. In all such cases the substrate is not actually transported into the cell. In the experiments described above, entry of phosphate into the cells was clearly demonstrated by the time-dependent labelling of cellular material. The electrophoretic method used[10] clearly separated P_i from other compounds and it is unlikely that adsorbed phosphate could be esterified without entering the cell.

Another control was provided by the use of the two mutants. The restoration of transport in the mutant lacking the phosphate-binding protein, by the addition of this protein is encouraging, as is the fact that the cells had to be cold-shocked for the phosphate-binding protein to be effective. However, the most significant observation is the failure of phosphate-binding protein to stimulate any uptake in a mutant defective in phosphate transport through the lack of a function other than phosphate-binding protein. Nonspecific phosphate-binding protein-mediated adsorption of phosphate to cells would produce an observed 'uptake' in this mutant. Finally, the restoration of transport is not merely due to a nonspecific 'recovery' caused by the shock protein; very small amounts of pure phosphate-binding protein were effective in our experiments, and unlike other systems[25], the pure phosphate-binding protein was much more effective than the crude shock fluid. The abolition of restoration by the anti-phosphate-binding protein serum is further evidence in this direction. We therefore believe that restoration of phosphate transport by the phosphate-binding protein in *E. coli* has been demonstrated in these experiments. This and the demonstration that the phosphate-binding protein is distinct from the R2 protein[21] implicate the binding protein in phosphate transport.

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