

Nucleotide binding by membrane components of bacterial periplasmic binding protein-dependent transport systems

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Bacterial periplasmic binding protein-dependent transport systems require the function of a specific substrate-binding protein, located in the periplasm, and several membrane-bound components. We present evidence for a nucleotide-binding site on one of the membrane components from each of three independent transport systems, the *hisP*, *malK* and *oppD* proteins of the histidine, maltose and oligopeptide permeases, respectively. The amino acid sequence of the *oppD* protein has been determined and this protein is shown to share extensive homology with the *hisP* and *malK* proteins. Three lines of evidence lead us to propose the existence of a nucleotide-binding site on each of these proteins. (i) A consensus nucleotide-binding sequence can be identified in the same relative position in each of the three proteins. (ii) The *oppD* protein binds to a Cibacron Blue affinity column and can be eluted by ATP but not by CTP or NADH. (iii) The *oppD* protein is labelled specifically by the nucleotide affinity analogue 5'-*p*-fluorosulphonylbenzoyl-adenosine. The identification of a nucleotide-binding site provides strong evidence that transport by periplasmic binding protein-dependent systems is energized directly by the hydrolysis of ATP or a closely related nucleotide. The *hisP*, *malK* and *oppD* proteins are thus responsible for energy-coupling to their respective transport systems.

Key words: gene fusions/histidine transport/maltose transport/nucleotide-binding site/oligopeptide permease

Introduction

Bacterial active transport systems can broadly be divided into two classes, those which require the function of specific periplasmic substrate-binding proteins and those which consist solely of membrane-bound components. These two classes of transport system were originally distinguished by the sensitivity of binding protein-dependent systems to cold osmotic shock (which causes loss of binding proteins from the periplasm) and by the nature of energy coupling to transport (Berger, 1973; Berger and Heppel, 1974). While osmotic shock-resistant transport systems are energized by coupling to electrochemical ion gradients, two lines of evidence have led to the proposal that osmotic shock-sensitive systems are energized by direct hydrolysis of ATP or a related nucleotide. (i) Arsenate specifically inhibits shock-sensitive transport systems yet such systems are relatively insensitive to uncouplers of oxidative phosphorylation. (ii) ATPase mutants cannot utilize substrates such as D-lactate to drive shock-sensitive transport while glucose is able to drive transport, presumably as a result of substrate-level phosphorylation. Such evidence is, however, somewhat indirect. Since Berger and Heppel's original definitions a number of observations have

led to suggestions that the energization of shock-sensitive transport systems may be more complex than originally supposed. Thus, under certain conditions there is a lack of correlation between transport and intracellular ATP levels (Plate *et al.*, 1974; Lieberman and Hong, 1976; Ferenci *et al.*, 1977). A role for the electrochemical proton gradient has also been proposed (Plate *et al.*, 1974; Hunt and Hong, 1983; Singh and Bragg, 1979), and there have also been suggestions that the driving force may be acetyl phosphate (Hong *et al.*, 1979) or a metabolite closely related to succinate (Hunt and Hong, 1983), rather than ATP. A more direct means of resolving the role, if any, of ATP hydrolysis in energizing binding protein-dependent transport systems is to determine whether or not any of the components of these transport systems can bind and/or hydrolyse ATP. This question is addressed here.

Periplasmic, binding protein-dependent transport systems each require the function of several cytoplasmic membrane proteins. Those systems which have been most completely characterized, and possibly all such systems, require three membrane-bound components (Ames and Higgins, 1983). The mechanisms by which the membrane-bound components function are poorly understood. The high-affinity histidine transport system of *Salmonella typhimurium* consists of a periplasmic histidine-binding protein (the *hisJ* protein) and three membrane-bound components, the *hisQ*, *hisM* and *hisP* gene products; the amino acid sequences of each of these proteins have been deduced from the nucleotide sequences of the corresponding genes (Higgins *et al.*, 1982). The maltose/maltodextrin transport system of *Escherichia coli* also has three cytoplasmic membrane components, the *malF*, *malG* and *malK* proteins. The amino acid sequences of two of these, the *malK* and *malF* proteins, have been determined (Gilson *et al.*, 1982b; Froshauer and Beckwith, 1984). Interestingly, the *hisP* and *malK* proteins share extensive homology with one another (Gilson *et al.*, 1982a).

A third binding protein-dependent transport system, the oligopeptide permease of *S. typhimurium*, is encoded by four genes, *oppA*, *oppB*, *oppC* and *oppD* (Higgins *et al.*, 1983; Hogarth and Higgins, 1983). *oppA* encodes a periplasmic protein (Higgins and Hardie, 1983) while the products of the other three genes are membrane associated (Jamieson and Higgins, unpublished data). Here we present the amino acid sequence of the *oppD* protein, deduced from the nucleotide sequence of its gene, and show that this protein shares extensive homology with the *hisP* and *malK* proteins. A consensus nucleotide-binding sequence (Walker *et al.*, 1982) is identified in each of these three proteins, within those regions of the proteins which show greatest sequence similarity. We also present experimental evidence that the predicted ATP-binding site on the *oppD* protein is functional. Evidence that the *hisP* protein binds ATP analogues has also been obtained recently (Hobson *et al.*, 1984). It therefore seems likely that the *hisP/malK/oppD* proteins function as energy-coupling subunits for their respective transport systems. Each of these systems presumably functions by a similar mechanism, coupling transport directly to ATP hydrolysis in an analogous manner to proton or ion-translocating ATPases.

OppD	Met Ser Leu Ser Glu Thr Ala Thr Gln Ala Pro Gln Pro Ala Asn Val Leu Leu Glu Val Asn Asp Leu Arg Val Thr Phe Ala Thr Pro	30
HisP		15
MalK		12
OppD	Asp Gly Asp Val Thr Ala Val Asn Asp Leu Asn Phe Thr Leu Arg Ala Gly Glu Thr Leu Gly Ile Val Gly Glu Ser Gly Ser Gly Lys	60
HisP	Tyr Gly Gly His Glu Val Leu Lys Gly Val Ser Leu Gln Ala Arg Ala Gly Asp Val Ile Ser Ile Ile Gly Ser Ser Gly Ser Gly Lys	45
MalK	Trp Gly Glu Val Val Val Ser Lys Asp Ile Asn Leu Asp Ile His Glu Gly Glu Phe Val Val Phe Val Gly Pro Ser Gly Cys Gly Lys	42
OppD	Ser Gln Ser Arg Leu Arg - Leu Met Gly Leu Leu Ala Thr Asn - Gly Arg Ile Gly Gly Ser Ala Thr Phe - Asn Gly - Arg	86
HisP	Ser - Thr Phe Leu Arg Cys Ile Asn Phe Leu Glu Lys Pro Ser Glu Gly Ala Ile Ile Val Asn Gly Gln Asn Ile Asn Leu Val Arg	74
MalK	Ser - Thr Leu Leu Arg Met Ile Ala Gly Leu Glu Thr Ile Thr Ser Gly Asp Leu Phe Ile - Gly Glu Lys - - - Arg	66
	Thr	
OppD	Glu Ile Leu Asn Leu Pro Glu Arg Glu Leu Asn Thr - Arg Arg Ala - - Glu Gln Ile Ser Met Ile Phe Gln Asp Pro Met Thr	113
HisP	Asp Lys Asp Gly Gln Leu Lys Val Ala Asp Lys Asn Gln Leu Arg Leu Leu Arg Thr Arg Leu Thr Met Val Phe Gln His Phe Asn -	103
MalK	Met Asn Asp Thr Pro - - Pro Ala Glu - - - Arg - - - Gly - Val Gly Met Val Phe Gln Ser Tyr Ala -	84
OppD	Ser Leu Asn Pro Tyr Met Arg Val Gly Glu Gln Leu Met Glu Val Leu Met Leu His Lys Gly Met Ser Lys Ala Glu Ala Phe Glu Glu	143
HisP	- Leu Trp Ser His Met Thr Val Leu Glu Asn Val Met Glu Ala Pro Ile Gln Val Leu Gly Leu Ser Lys His Asp Ala Arg Glu Arg	132
MalK	- Leu Tyr Pro His Leu Ser Val Ala Glu Asn - Met Ser Phe Gly Leu Lys Pro Ala Gly Ala Lys Lys Glu Val Ile Asn Gln Arg	113
OppD	Ser Val Arg Met Leu Asp Ala Val Lys Met Pro Glu Ala Arg Lys Arg Met Lys Met Tyr Pro His Glu Phe Ser Gly Gly Met Arg Gln	173
HisP	Ala Leu Lys Tyr Leu - Ala - Lys Val Gly Ile Asp Glu Arg Ala Gln Gly Lys Tyr Pro Val His Leu Ser Gly Gly Gln Gln Gln	160
MalK	Val Asn Gln - Val - Ala - Glu Val Leu Gln Leu Ala His Leu Leu Asp Arg Lys Pro Lys Ala Leu Ser Gly Gly Gln Arg Gln	140
OppD	Arg Val Met Ile Ala Met Ala Leu Leu Cys Arg Pro Lys Leu Leu Ile Ala Asp Glu Pro Thr Thr Ala Leu Asp Val Thr - - Val	201
HisP	Arg Val Ser Ile Ala Arg Ala Leu Ala Met Glu Pro Asp Val Leu Leu Phe Asp Glu Pro Thr Ser Ala Leu Asp Pro Glu Leu Val Gly	190
MalK	Arg Val Ala Ile Gly Arg Thr Leu Val Ala Glu Pro Ser Val Phe Leu Leu Asp Glu Pro Leu Ser Asn Leu Asp Ala Ala Leu Arg Val	170
	Arg - - - Gly - - - H H H H Asp	
OppD	Gln - Ala Gln Ile - Met Thr Leu Leu Asn Glu Leu Lys Arg Glu Phe Asn Thr Ala Ile Ile Met Ile Thr His Asp Leu Gly Val	229
HisP	Glu Val Leu Arg Ile - Met Gln Gln Leu Ala Glu Glu - Gly Lys - - Thr Met Val Val - Val Thr His Glu Met Gly Phe	215
MalK	Gln - Met Arg Ile Glu Ile Ser Arg Leu His Lys Arg Leu Gly Arg - - Thr Met Ile Tyr - Val Thr His Asp Gln Val Glu	196
OppD	Val Ala Gly Ile Cys Asp Lys Val Leu Val Met Tyr Ala Gly Arg Thr Met Glu Tyr Gly Lys Ala Arg Asp Val - Phe - Tyr Gln	257
HisP	Ala Arg His Val Ser Ser His Val Ile Phe Leu His Gln Gly Lys Ile Glu Glu Glu Gly Asp Pro Glu Gln Val - Phe - Gly Asn	243
MalK	Ala Met Thr Leu Ala Asp Lys Ile Val Val Leu Asp Ala Gly Arg Val Ala Gln Val Gly Lys Pro Leu Ala Val Pro Leu Ser Gly Arg	226
OppD	Pro Val His Pro Tyr Ser Ile Gly Leu Leu Asn Ala Val Pro Arg Leu Asp Ser Glu Gly Ala Glu Met Leu Thr Ile Pro Gly Asn Pro	287
HisP	Pro Gln Ser Pro Arg Leu Gln Gln Phe Leu Lys Gly Ser Leu Lys END	258
MalK	Pro Phe Cys Arg Arg Ile Tyr Arg Phe Ala Lys Asp Glu Leu Leu Pro Val Lys Val Thr Ala Thr Ala Ile Asp Gln Val Gln Val Glu	256
OppD	Pro Asn Leu Leu Arg Leu Pro Lys Gly Cys Pro Phe Gln Pro Arg Cys Pro His Ala Met Glu Ile Cys Asn Asn Ala Pro Pro Leu Glu	317
MalK	Leu Pro Met Pro Asn Arg Gln Gln Val Trp Leu Pro Val Glu Ser Arg Asp Val Gln Val Gly Ala Asn Met Ser Leu Gly Ile Arg Pro	286
OppD	Ala Phe Ser Pro Gly Arg Leu Arg Ala Cys Phe Lys Pro Val Glu Glu Leu Leu END	335
MalK	Glu His Leu Leu Pro Ser Asp Ile Ala Asp Val Ile Leu Glu Gly Glu Val Gln Val Val Glu Gln Leu Gly Asn Glu Thr Gln Ile His	315
MalK	Ile Gln Ile Pro Ser Ile Arg Gln Asn Leu Val Tyr Arg Gln Asn Asp Val Val Leu Val Glu Glu Gly Ala Thr Phe Ala Ile Gly Leu	346
MalK	Pro Pro Glu Arg Cys His Leu Phe Arg Glu Asp Gly Thr Ala Cys Arg Arg Leu His Lys Glu Pro Gly Val END	370

Fig. 1. Aligned amino acid sequences of the *oppD*, *hisP* and *malK* proteins. The amino acid sequences of the *oppD*, *hisP* and *malK* proteins have been aligned for maximum homology. Amino acids identical in two or more of the proteins are boxed. Sequence data for the *hisP* and *malK* proteins are from Higgins et al. (1982) and Gilson et al. (1982b), respectively. Due to an ambiguity in the nucleotide sequence of *oppD*, there is some doubt as to the accuracy of the designation of amino acids 98–101.

Results

Extensive sequence homology between the *oppD*, *hisP* and *malK* proteins

The four genes encoding the oligopeptide permease of *S. typhimurium* have been cloned (Powell and Higgins, in preparation) and the nucleotide sequence of the *oppD* gene determined (Hiles and Higgins, unpublished data). The amino acid sequence of the *oppD* protein, as deduced from the nucleotide sequence,

is presented in Figure 1. The protein is 335 amino acids long with a mol. wt. of 36 986. In Figure 1 the sequence of the *oppD* protein is aligned with that of the *hisP* and *malK* proteins to show the extensive sequence similarity between these three proteins. Over its entire length, 29% of the amino acids of the *hisP* protein are identical to those in the same position in the aligned *oppD* protein; a further 12% are conservative substitutions. A similar percentage identity is found whichever two of the three proteins are compared. The percentage identity is much greater for specific

Protein	Residue	Sequence
A. Bovine ATPase β	149-169	Lys Gly Gly Lys Ile Gly Leu Phe - Gly Gly Ala Gly Val Gly Lys Thr - Val Phe Ile Met
<i>E. coli</i> ATPase β	142-161	Lys Gly Gly Lys Val Gly Leu Phe - Gly Gly Ala Gly Val Gly Lys Thr - Val Asn Met Met
<i>E. coli</i> ATPase α	161-180	Arg Gly Gln Arg Glu Leu Ile Ile - Gly Asp Arg Gly Thr Gly Lys Thr - Ala Leu Ala Ile
Adenylate kinase	6-26	Lys Lys Ser Lys Ile Ile Phe Val Val Gly Gly Phe Gly Ser Gly Lys Gly - Thr Gln Cys Glu
RecA-protein	57-80	Phe Met Gly Arg Ile Val Glu Ile Tyr Gly Phe Glu Ser Ser Gly Lys Thr - Thr Leu Thr Leu
<i>oppD</i> -protein	46-66	Ala Gly Glu Thr Leu Gly Ile Val - Gly Glu Ser Gly Ser Gly Lys Ser Gln Ser Arg Leu Arg
<i>hisP</i> -protein	31-50	Ala Gly Asp Val Ile Ser Ile Ile - Gly Ser Ser Gly Ser Gly Lys Ser - Thr Phe Leu Arg
<i>malK</i> -protein	28-47	Glu Gly Gly Phe Val Val Phe Val - Gly Pro Ser Gly Cys Gly Lys Ser - Thr Leu Leu Arg
Consensus		- Gly - - - - - - - Gly - - - Gly Ser Gly Lys Ser - Thr - - -
B. Bovine ATPase β	241-263	Val Ala Glu Tyr Phe Arg Asp Gln Glu Gly Gln Asp Val Leu Leu Phe Ile Asp Asn Ile Phe Arg
<i>E. coli</i> ATPase β	227-248	Met Ala Glu Lys Phe Arg Asp - Glu Gly Arg Asp Val Leu Leu Phe Val Asp Asn Ile Tyr Arg
<i>E. coli</i> ATPase α	265-286	Met Gly Glu Tyr Phe Arg Asp - Arg Gly Glu Asp Ala Leu Ile Ile Tyr Asp Asp Leu Ser Lys
ATP/ADP translocase	275-296	Ser Asn Val Leu - Arg Gly Met Gly Gly Ala Phe Val Leu Val Leu Tyr Asp Glu Ile Lys Lys
<i>oppD</i> -protein	174-195	Arg Val Met Ile Ala Met Ala Leu Leu Cys Arg Pro Lys Leu Leu Ile Ala Asp Glu Pro Thr Thr
<i>hisP</i> -protein	161-182	Arg Val Ser Ile Ala Arg Ala Leu Ala Met Glu Pro Asp Val Leu Leu Phe Asp Glu Pro Thr Ser
<i>malK</i> -protein	141-162	Arg Val Ala Ile Gly Arg Thr Leu Val Ala Glu Pro Ser Val Phe Leu Leu Asp Glu Pro Leu Ser
Consensus		- - - - - Arg - - - Gly - - - H H H H Asp Asp - - -

Fig. 2. Consensus nucleotide-binding sequences. The *oppD*, *hisP* and *malK* proteins are aligned with several other nucleotide-binding proteins to illustrate the two sequence blocks (A and B) which comprise the consensus nucleotide-binding fold (boxed residues). All amino acid sequences (except those of the *oppD*, *hisP* and *malK* proteins), their alignment and the identification of a consensus nucleotide-binding sequence are from Walker *et al.* (1982). H in the consensus sequence indicates a conserved hydrophobic residue.

regions of the three proteins. Both the *oppD* and the *malK* proteins are rather larger than the *hisP* gene product, with additional amino acids at the C terminus. Interestingly the C-terminal regions of the *oppD* and *malK* proteins show no significant homology. Thus, each of the three proteins has an N-terminal segment which is extensively conserved and a C-terminal segment (absent from the *hisP* protein) which shows no sequence conservation.

Identification of a consensus nucleotide-binding site

A comparison of a large number of nucleotide-binding proteins has led to the identification of a consensus amino acid sequence required to form a nucleotide-binding fold (Walker *et al.*, 1982). To determine whether a consensus nucleotide-binding fold is present on any of the membrane components of the histidine transport system, the amino acid sequences of the *hisQ*, *hisM* and *hisP* proteins were compared with that of the β -subunit of bovine mitochondrial ATPase using the DIAGON program (Staden, 1982; Walker *et al.*, 1982). A single region of significant homology was identified, between that portion of the β subunit of ATPase which corresponds to its nucleotide-binding site and a region of the *hisP* protein, between amino acids 32 and 46. This potential nucleotide-binding sequence falls in a region of the *hisP* protein which is highly conserved in both the *oppD* and *malK* proteins. The consensus sequence is thus present in the same relative positions on all three proteins. Figure 2A shows the *oppD*, *hisP* and *malK* proteins aligned with various nucleotide-binding proteins to show this consensus sequence. Many nucleotide-binding proteins also possess a second conserved sequence which forms part of the substrate-binding fold (Walker *et al.*, 1982). A sequence closely related to this consensus is also found on the *hisP*, *malK* and *oppD* proteins (Figure 2B).

While the *hisP*, *malK* and *oppD* proteins show sequence similarities throughout their entire length, certain regions of these proteins are more highly conserved than others. Thus, amino acids 27-85 and 150-185 of the *hisP* protein are highly conserved in both the *oppD* and *malK* proteins yet are separated by sequences which are very much less well conserved. Significantly, the two sequence blocks which comprise the putative nucleotide-binding fold fall within these two highly conserved regions.

The presence of a consensus nucleotide-binding sequence on the *oppD*/*hisP*/*malK* proteins does not, of course, prove that these

proteins possess a functional nucleotide-binding site. To demonstrate the functionality, or otherwise, of this site, two types of biochemical experiments were undertaken using the *oppD* protein as a model; binding to an affinity column and labelling with an affinity analogue.

OppD binds to a Cibacron Blue affinity column

The dye Cibacron Blue has proved useful in the purification of many proteins containing a nucleotide-binding fold (Thompson *et al.*, 1975). If the predicted nucleotide-binding sites on the *hisP*, *malK* and *oppD* proteins are functional, these proteins might be expected to bind to a Cibacron Blue affinity column and to be eluted by ATP. Because it is not yet possible to assay these three membrane-bound transport components directly, we took advantage of hybrids between the *oppD* protein and β -galactosidase. These hybrid proteins consist of the *oppD* protein with its carboxyl terminus replaced by β -galactosidase. The β -galactosidase activity is retained and the hybrids are therefore easily detected and assayed. The hybrid proteins were produced from *oppD-lacZ* gene fusions constructed *in vivo* using the MudIII301 (Ap, *lac*) phage (Casadaban and Chou, 1984) as described elsewhere (Jamieson and Higgins, in preparation). Strains CH489 and CH491 carry independently isolated *oppD-lacZ* gene fusions in which the hybrid protein is membrane associated and contains almost the entire *oppD* sequence, including that region of *oppD* containing the putative nucleotide-binding site. Strain CH267 carries an *oppD-lacZ* operon fusion (Jamieson and Higgins, 1984) which produces native β -galactosidase and serves as a suitable control. Although the lack of an assay necessitated the use of hybrid proteins rather than the native *oppD* protein, we believe that the experimental demonstration of a nucleotide-binding site on these hybrid proteins provides a valid indication that such a site is also present on the native *oppD* protein. Firstly, the hybrids contain almost the entire *oppD* sequence, sufficient that they become membrane-associated despite being relatively hydrophilic proteins. Secondly, it seems likely that the *oppD* and *lacZ* portions of the hybrid protein form two entirely independent domains; β -galactosidase activity is retained by the hybrids and, in addition, initial proteolytic events during degradation cleave the hybrid at or close to the *oppD-lacZ* junction (unpublished results) indicating that this is an exposed region between two domains. Thirdly, it seems highly improbable that a nucleotide-

binding site should be formed by the hybrid if it were not present in either of the two separate constituent proteins. To preclude the possibility that the β -galactosidase portion of the hybrids is responsible for any observed reactions, native β -galactosidase (from strain CH267) was used in all cases as a control.

Membrane fractions from each of the three strains were isolated, solubilized in 1.0% Triton X-100, and loaded onto a 6 ml Cibacron Blue affinity column. The resin was washed with two column volumes of loading buffer and any bound proteins eluted with loading buffer containing the appropriate nucleotide

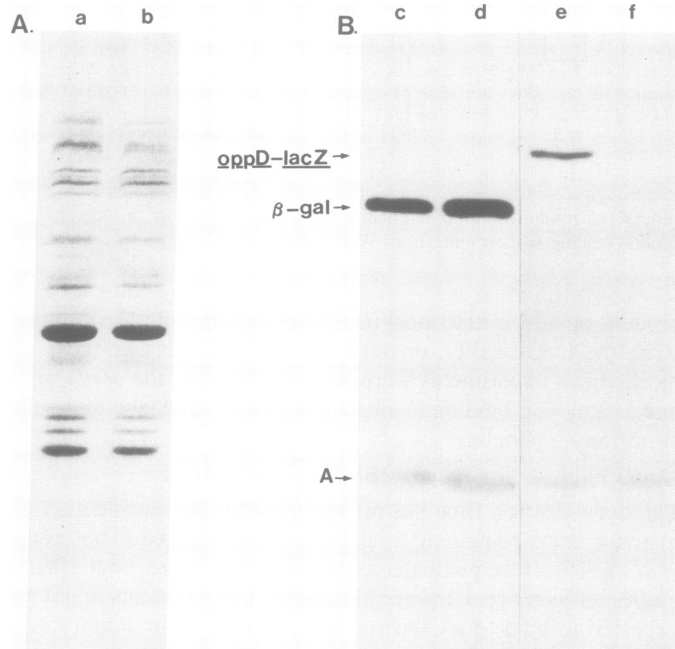


Fig. 3. Binding of *oppD-lacZ* hybrid proteins to a Cibacron Blue affinity column. (A) SDS-polyacrylamide gel of Triton-solubilized membrane proteins from strain CH267 loaded onto a Cibacron Blue column (lane a) and of those which pass straight through the column in the loading eluate (i.e., fail to bind; lane b). The proteins passing through the column in the loading eluate were concentrated by freeze-drying and resuspended in a volume of buffer equivalent to that of the original sample, prior to loading onto the column. (B) Binding of *oppD-lacZ* hybrid proteins to a Cibacron Blue affinity column. Samples of membrane proteins loaded onto the column (lanes c and e) and which wash through in loading buffer (i.e., fail to bind; lanes d and f) were separated by 12% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and reacted sequentially with anti- β -galactosidase antibodies and ^{125}I -labelled *S. aureus* Protein A. The total protein which washed through in loading buffer was pooled, freeze-dried and resuspended in an identical volume to that originally loaded onto the column, prior to gel electrophoresis. Lanes c and d are the 'loading' and 'wash-through' proteins, respectively, from CH267 (*oppD-lacZ* operon fusion) and lanes e and f the 'loading' and 'wash-through' proteins from CH489 (*oppD-lacZ* gene fusion).

or NaCl. Figure 3A shows a polyacrylamide gel of the protein sample (from strain CH267) loaded onto the column (lane a) and of those proteins which fail to bind and wash straight through in the loading eluate (lane b). Clearly, the majority of membrane proteins fail to bind to the column under these conditions. Because the *oppD-lacZ* hybrid proteins are poorly expressed they cannot be identified in these gels by Coomassie staining. Thus, the hybrid proteins were detected using anti- β -galactosidase antibodies. Proteins were transferred from the polyacrylamide gel to nitrocellulose and detected by reaction with anti- β -galactosidase antibodies and ^{125}I -labelled Protein A (Towbin *et al.*, 1979). Figure 3B shows an autoradiogram of such an immunoblot. While native β -galactosidase (from strain CH267) passes straight through the column in loading buffer (lanes c and d show the proteins loaded, and which fail to bind to the column, respectively), the *oppD-lacZ* hybrid protein from strain CH489 is almost fully retained (lanes e and f). The hybrid from CH491 was similarly retained (data not shown). The band labelled A is an unidentified protein also precipitated by our preparation of anti- β -galactosidase antibodies and serves as an internal control.

Although the data in Figure 3 show that the *oppD-lacZ* hybrid proteins are bound by the Cibacron Blue affinity column, it is important to show that this is not non-specific binding, but that the proteins can be specifically eluted with ATP. Because of the susceptibility of the hybrid proteins to proteolytic attack, it was not possible to obtain quantitative data from polyacrylamide gels showing the hybrid proteins eluted by various nucleotides. We therefore monitored the elution of β -galactosidase activity as a measure of *oppD-lacZ* hybrid elution (Table I). While native β -galactosidase passes through the column in loading buffer, *oppD-lacZ* hybrids are retained but can be eluted with 10 mM ATP. It is not clear why only ~50% of the hybrid protein can be eluted by ATP. 10 mM CTP or 10 mM NADH are far less efficient eluants than 10 mM ATP. In addition, the bound hybrid fails to elute with 100 mM NaCl, demonstrating that it is not ionic strength alone that is required to elute the hybrid. In the absence of ATP the hybrid can be eluted by 2 M NaCl. The eluted proteins were shown to be the intact hybrid, and not simply a hydrolytic fragment retaining β -galactosidase activity, by immunoprecipitation and gel electrophoresis.

The oppD protein reacts with 5'-p-fluorosulphonylbenzoyl-adenosine

5'-p-Fluorosulphonylbenzoyl-adenosine (5'-FSBA) is a relatively specific affinity label for ATP-binding sites (Colman, 1983). Membrane fractions from cells containing either native β -galactosidase (CH267) or the *oppD-lacZ* hybrid proteins (CH489, CH491) were reacted with ^{14}C -labelled 5'-FSBA and immunoprecipitated with anti- β -galactosidase antibodies as described in Materials and methods. Figure 4 shows an autoradiogram of the

Table I. Binding of *oppD-lacZ* hybrid proteins by Cibacron Blue

Strain	Fusion Protein	Units β -galactosidase Loaded onto column	Eluant					
			Loading eluate	10 mM ATP	10 mM CTP	10 mM NADH	100 mM NaCl	2 M NaCl
CH267	native β -galactosidase	9150	8760(96%)	87 (1%)	99 (1%)	138 (2%)	240 (3%)	230 (3%)
CH489	<i>oppD-lacZ</i> hybrid	2600	198 (8%)	1284(49%)	312(12%)	330(13%)	112 (4%)	1980(76%)
CH491	<i>oppD-lacZ</i> hybrid	1430	176(12%)	798(56%)	220(15%)	142(10%)	185(13%)	1183(83%)

Membrane fractions from each of the three strains were loaded onto the Cibacron Blue affinity column. The total number of units of β -galactosidase in the samples loaded, the loading eluate, and eluted by various nucleotides or NaCl is shown. The figures in parentheses indicate the percentage of the total loaded β -galactosidase which is eluted under each set of conditions.

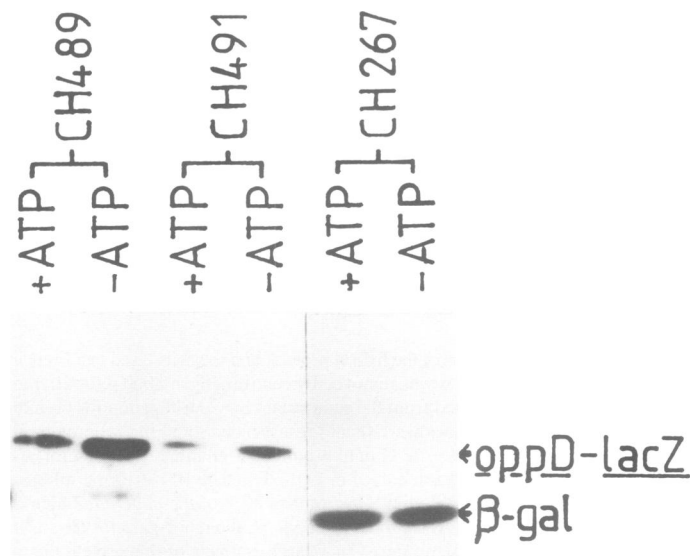


Fig. 4. Reaction of *oppD-lacZ* hybrid proteins with 5'-FSBA. Membrane fractions from appropriate strains were reacted with ^{14}C -labelled 5'-FSBA in the presence or absence of 10 mM ATP as indicated. The labelled proteins were immunoprecipitated with anti- β -galactosidase antibodies, separated by polyacrylamide gel electrophoresis, fluorographed and exposed to X-ray film. Membrane fractions from CH267 contained about six times as many units of β -galactosidase per mg protein as those from CH489 and CH491. In addition, the labelled proteins from CH267 were exposed to X-ray film for five times as long as the proteins from CH489 and CH491. The hybrid proteins have not been purified, and absolute values for their specific activities relative to that of β -galactosidase can be obtained by comparison of β -galactosidase activity with the amount of protein as estimated from the intensity of bands on X-ray film after immunoblotting (using anti- β -galactosidase antibodies and ^{125}I -labelled protein A). Such comparisons show that in no case is the specific activity of either of the two hybrid proteins greater than that of the pure β -galactosidase (Jamieson and Higgins, in preparation). Thus, the efficiency with which native β -galactosidase is labelled is at least 30-fold less than that with which the *oppD-lacZ* hybrid is labelled under the conditions used.

precipitated proteins separated by polyacrylamide gel electrophoresis. Clearly, 5'-FSBA reacts with both the *oppD-lacZ* hybrid proteins. To determine whether this reaction is specific, the reactions were also carried out in the presence of 10 mM ATP. As shown in Figure 4, ATP inhibits the reaction between 5'-FSBA and the *oppD-lacZ* hybrid protein. However, like other such analogues, 5'-FSBA is not totally specific and will react non-specifically with all proteins to some extent. Indeed, under the conditions used, 5'-FSBA reacts with native β -galactosidase (Figure 4). This reaction is, however, very much less efficient than the reaction with *oppD-lacZ* hybrids (see legend to Figure 4). In addition, the labelling of native β -galactosidase is not inhibited by ATP (Figure 4). Thus, while 5'-FSBA does react with native β -galactosidase, this reaction is both inefficient and non-specific when compared with the labelling of *oppD-lacZ* hybrid proteins under identical conditions.

Discussion

To understand better the mechanisms of energy coupling to bacterial periplasmic binding protein-dependent transport systems we have investigated the possibility that one or more of the membrane components of such transport systems is able to bind ATP or a closely related nucleotide. Three lines of evidence strongly

suggest the existence of such a nucleotide-binding site. Firstly, we have identified, by amino acid sequence homology, a consensus nucleotide binding site on one of the membrane components from each of three independent transport systems, the *hisP*, *malK*, and *oppD* gene products of the histidine, maltose and oligopeptide transport systems, respectively. Secondly, we have shown that the *oppD* protein binds to a Cibacron Blue affinity column, which is known to retain many ATP-binding proteins, and can be specifically eluted with ATP and to a lesser extent by CTP and NADH. Thirdly, the ATP affinity analogue 5'-FSBA has been shown to react with the *oppD* gene product; this reaction is inhibited by ATP. In addition, the *hisP* protein of the histidine transport system has been recently shown to react with the photoaffinity analogue 8-azido ATP (Hobson *et al.*, 1984).

There has been some controversy in recent years concerning the mechanisms by which energy is coupled to binding protein-dependent transport systems. Despite Berger and Heppel's elegant experiments which led to their suggestion that direct hydrolysis of ATP was the driving force, these conclusions were based on somewhat indirect evidence and have subsequently been questioned. Several groups have shown a requirement for an electrochemical proton gradient (Plate *et al.*, 1974; Ferenci *et al.*, 1977; Plate, 1979). However, it is clear that protons are not co-transported with substrate (Henderson *et al.*, 1977; Darawalla *et al.*, 1981) and that the chemical potential gradient of substrate which can be achieved by these transport systems is far in excess of that which the proton motive force (pmf) could account for (Hengge and Boos, 1983). Thus, the role of the pmf cannot be that of primary driving force but must play an indirect or subsidiary role such as maintenance of membrane integrity. From studies, mainly on membrane vesicles, alternative energy sources to ATP have also been proposed such as acetyl phosphate (Hong *et al.*, 1979) and succinate or a closely related metabolite (Hunt and Hong, 1983). However, the present demonstration of a nucleotide-binding site on one of the membrane components of each of three independent transport systems strongly supports Berger and Heppel's original proposal that energization is by direct hydrolysis of ATP or a closely related nucleotide. Unfortunately, the high background levels of ATPase activity in isolated membranes preclude experiments designed to demonstrate ATPase activity of a single specific transport component such as the *oppD* protein (G.F.-L. Ames, personal communication); any direct demonstration of ATPase activity must await the purification of one or more of these proteins. Thus, until such experiments have been performed, the possibility that nucleotide binding simply plays a regulatory role cannot be excluded. The fact that the *oppD* protein is eluted from a Cibacron Blue column by ATP, but much less well by CTP or NADH, implies that ATP is the nucleotide for which this site has greatest affinity. Thus, it seems probable that the periplasmic binding protein-dependent transport systems function by a common mechanism involving conformational changes induced by direct hydrolysis of ATP, perhaps in a manner analogous to the ATP-driven ion pumps (Cantley, 1981; Kyte, 1981; Amzel and Pedersen, 1983). It is not clear whether one or more of the proteins forms a phosphorylated intermediate although, despite an intensive search (Ames and Nikaido, 1981), none has yet been identified.

There is increasing evidence for structural and functional similarities between binding protein-dependent transport systems (Ames and Higgins, 1983). For those systems which have been most completely characterized, and possibly for all such systems, three cytoplasmic membrane proteins are required in addition

to the periplasmic substrate-binding protein. Little is known about the functions of these membrane components and the amino acid sequences of only five such proteins have previously been published (the *hisQ*, *hisM*, *hisP*, *malF* and *malK* gene products). The *hisP* and *malK* proteins have previously been shown to share extensive amino acid sequence homology (Gilson *et al.*, 1982a) and we show here that the *oppD* protein is also homologous with these two proteins. Such extensive homology between components of transport systems which handle very different substrates (an amino acid, a sugar and oligopeptides, respectively) is intriguing and might either reflect a common evolutionary origin or a functional constraint on the amino acid sequence. From the available data the latter alternative seems to be the most likely. While the similar number of components and organization of each of these three transport systems would suggest a common evolutionary origin, amino acid sequence comparison suggests this common origin must be relatively distant. The binding proteins are very different in size (*hisJ* = 26 000; *malE* = 40 000 and *oppA* = 52 000) and no significant sequence homology is found between them. The *malF* protein is also very different in mol. wt. from either the *hisM* or *hisQ* gene products (57 000 compared with 24 500 and 26 500) and no sequence similarity can be identified (Froshauer and Beckwith, 1984; Higgins *et al.*, 1982). Thus, at the amino acid sequence level, the only similarity between these three systems is the nucleotide-binding domain of the *hisP/malK/oppD* proteins. Indeed, these three proteins themselves differ considerably at their carboxy terminus, the additional amino acids at the C terminus of *malK* and *oppD*, but absent from *hisP*, show no sequence conservation and presumably serve a function specific to the individual systems (e.g., regulation). Thus, it seems probable that the homology between the *hisP/malK/oppD* proteins reflects constraints imposed by the requirement for nucleotide binding rather than a close evolutionary origin.

There has previously been no direct evidence as to which components of the periplasmic transport systems are involved in energy coupling. However, certain indirect evidence indicates that the *hisP*, *malK* and *oppD* proteins are the most likely candidates. The *hisQ* and *hisM* components are relatively hydrophobic proteins and there is evidence that *hisM*, *hisQ*, *malF* and *malG* (Shuman, 1982a; Ames and Higgins, 1983) are involved in substrate binding within the membrane. The *hisP*, *malK* and *oppD* proteins, however, differ considerably in that they are relatively hydrophilic (Gilson *et al.*, 1982a). The *malK* protein has been shown to be a peripheral inner membrane protein, located on the inner surface of the membrane (Shuman and Silhavy, 1981) and on this basis it was suggested that the *malK* protein might be an energy-coupling subunit (Shuman, 1982b). The *malK* protein is also believed to play a regulatory role in interaction with factor III^{Glc} during inducer exclusion (M.H.Saier, personal communication). The present results now provide strong evidence that the *malK*, *hisP* and *oppD* gene products do indeed serve as energy-coupling subunits for their respective transport systems.

Materials and methods

Growth of bacteria

The genotypes of the bacterial strains used in this study are listed in Table II. Cells were grown at 30°C with aeration in LB liquid medium (Miller, 1972) containing 25 µg/ml⁻¹ ampicillin where appropriate.

Isolation of cell membranes

Cells (500 ml) were grown to O.D.₆₀₀ of 0.6, harvested by centrifugation, washed once with 250 ml Hepes buffer (10 mM Hepes, pH 7.4) and resuspended in 20 ml of Hepes buffer at 4°C. Benzamidine hydrochloride was added to 1

Table II. Genotypes of bacterial strains

Strain	Genotype
CH267	<i>galE503</i> Δ <i>bio-561 pro-662::Tn10 oppD316::Mud1</i> (Ap, <i>lac</i>)
CH489	<i>galE503</i> Δ <i>bio-561 pro-662::Tn10 oppD320::MudII301</i> (Ap, <i>lac</i>)
CH491	<i>galE503</i> Δ <i>bio-561 pro-662::Tn10 oppD322::MudII301</i> (Ap, <i>lac</i>)

Strain 267 (*oppD-lacZ* operon fusion) was constructed as described (Jamieson and Higgins, 1984). Strains 489 and 491 (*oppD-lacZ* gene fusions) were constructed in an identical manner using phage *MudII301* (Casadaban and Chou, 1984).

mg/ml, to reduce proteolysis of the hybrid protein, and the cells lysed in a French Pressure cell. Cellular debris was removed by centrifuging at 8000 g for 20 min and the membranes separated from the supernatant by centrifugation for 60 min at 120 000 g. The membranes were finally resuspended to 50 mg/ml protein in Hepes buffer and stored at -70°C until required. Membrane fractions for affinity chromatography were solubilized in 1.0% Triton X-100 at 10 mg/ml protein prior to loading on the column. Greater than 80% of the *oppD-lacZ* hybrid proteins is recovered in the membrane fractions. Although β -galactosidase is a cytoplasmic protein, CH267 produces such large amounts of this protein that a small proportion remains in the membrane fraction following these separation procedures (although this can be removed by more extensive washing). It is this residual β -galactosidase in the membrane preparations of CH267 which is assayed in the experiments described above.

Immunoprecipitation

Membranes (2.5 mg protein) were mixed with an equal volume of anti- β -galactosidase antibodies and incubated at 4°C overnight. 100 µl of *Staphylococcus aureus* (10% w/v in 0.5% Nonidet) was added and the mix incubated for a further 30 min at 4°C. The precipitate was sedimented by 10 min centrifugation in an Eppendorf centrifuge and the efficiency of sedimentation checked by assaying the supernatant for β -galactosidase activity. The pellet was washed five times with Hepes buffer before being resuspended in 2% SDS and boiled for 5 min to disaggregate the antigen-antibody complex. An equal volume of sample buffer (Laemmli, 1970) was added prior to loading onto SDS-polyacrylamide gels.

Gel electrophoresis and transfer to nitrocellulose

SDS-polyacrylamide gel electrophoresis was carried out on 12% gels (acrylamide to bisacrylamide ratio, 19:1) as described by Laemmli (1970) and modified by Ames (1974). Proteins were transferred from the gel to nitrocellulose (Schleicher and Schuell BA85) electrophoretically (Towbin *et al.*, 1979) and were detected immunologically using anti- β -galactosidase antibodies and ¹²⁵I-labelled *Staphylococcus* protein A (Amersham) as described by Towbin *et al.* (1979) except that all washes were carried out with 0.9% NaCl, 10 mM Tris-HCl pH 7.4, 0.05% Tween 20.

Reaction with 5'-FSBA

¹⁴C-labelled 5'-fluorosulphonylbenzoyl-adenosine (40 mCi/mmol) was obtained from New England Nuclear. Membrane fractions (2.5 mg protein) were reacted in Hepes buffer (pH 7.4) at 28°C for 30 min with 5'-FSBA (40 µM). The reaction was stopped by adding a 50-fold excess of cold, non-radioactive 5'-FSBA, and the membranes washed with Hepes buffer at 4°C prior to antibody precipitation.

β -galactosidase assays

β -galactosidase was assayed as described by Miller (1972). Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard.

Cibacron Blue affinity column

Cibacron Blue F3GA covalently attached to Sepharose CL-6B was obtained from Pharmacia. A column (bed volume 6 ml, 1 cm diameter) was equilibrated with loading buffer (10 mM Hepes, pH 7.4; 5 mM MgCl₂; 0.5 mM EDTA; 2 µM β -mercaptoethanol). Samples (50 µl) of Triton-solubilized membrane preparations (2.5 mg total protein) were loaded and the column washed with two column volumes of loading buffer prior to elution with loading buffer containing appropriate concentrations of ATP, CTP, NADH or NaCl. 1 ml fractions of eluate were collected and assayed for β -galactosidase activity or analysed by gel electrophoresis.

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Since submitting this paper the sequence of the *pstB* gene product of the phosphate transport system of *E. coli* has been determined (Surin, B.P. *et al.*, (1985) *J. Bacteriol.*, **161**, 189-198). We find the homologies between the *oppD/malK/hisP* proteins are also conserved in the *pstB* protein.