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Two periplasmic transport proteins which interact with a common membrane receptor show extensive homology: Complete nucleotide sequences

(histidine and arginine transport/gene duplication/*Salmonella*/chimeric binding protein/signal peptides)

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ABSTRACT **The *hisJ* and *argT* genes of *Salmonella typhimurium* encode two periplasmic binding proteins, J and LAO, which are involved in histidine and arginine transport, respectively, and which interact with a common membrane-bound component, the P protein. The complete nucleotide sequences of these two genes have been determined. The two genes show extensive homology (70%) and presumably arose by tandem duplication of a single ancestral gene. The two encoded proteins now perform distinct functions but still retain sufficient homology to permit interaction with the same site on the membrane-bound P protein. Three lines of evidence have allowed both the amino acid-binding site and the site involved in the interaction with the P protein to be assigned to specific regions of each binding protein: (*i*) the distribution of amino acid differences between the two proteins; (*ii*) the properties of a functional chimeric protein, produced by a deletion mutant in which the first half of the *argT* gene is fused to the second half of the *hisJ* gene; (*iii*) the sequence change in a mutant J protein unable to interact with P.**

Periplasmic binding proteins are essential components of many membrane transport and chemotactic systems in bacteria. The binding proteins function as the initial receptor in these processes, recognizing and binding a specific substrate—a sugar, an amino acid, or an inorganic ion. Recently it has become clear that membrane-bound components are also required for binding protein-dependent transport and chemotaxis, although it is still unknown how the periplasmic and membrane-bound components function together.

The high-affinity histidine transport system of *Salmonella typhimurium* is one of the best characterized of the binding-protein-dependent systems. Three genes, *hisJ*, *hisQ*, and *hisP*, are required for histidine uptake. Together with a regulatory locus, *dhuA*, these genes form an operon located at 48.5 min on the recalibrated *S. typhimurium* chromosomal map (1) (see Fig. 1). These genes encode, respectively, a periplasmic histidine-binding protein, J, a protein of as yet unknown location, Q, and an inner-membrane protein, P (2, 3). The periplasmic histidine-binding protein, J, is known to interact with the membrane-bound P protein during transport (4). A second periplasmic binding protein, LAO (the lysine-arginine-ornithine-binding protein), which participates in the uptake of arginine as a nitrogen source (5), is also known to require the Q and P proteins in order to function (2). By analogy with J, the LAO protein is also presumed to interact with the P protein, implying that the two binding proteins interact with a common membrane-bound receptor. Although such an interaction between periplasmic and membrane components has not been demonstrated for other transport systems, several other binding-protein-dependent systems have been shown to require membrane-bound components: it therefore seems likely that such interactions occur.

The LAO protein is known to be related to the J protein in several respects. The two proteins overlap somewhat in binding specificities (2), the LAO protein is known to crossreact with antibody raised against purified J protein (5), and the structural gene for LAO, *argT*, is located adjacent to the *hisJ* gene on the *S. typhimurium* chromosome (5) (see Fig. 1). These facts suggested that the *hisJ* and *argT* genes may have evolved as a result of gene duplication followed by divergent evolution.

In this paper we show that the nucleotide sequences of these two genes, and their translated protein sequences, strongly support this hypothesis. The fact that these two proteins interact with a common receptor and must therefore have retained some homology, yet have diverged with respect to substrate specificity, makes analysis of this case of gene duplication a particularly interesting one. We also describe the properties of a functional chimeric protein, resulting from a fusion of the *argT* and *hisJ* genes, which allows specific functions to be assigned to different regions of the binding proteins.

MATERIALS AND METHODS

Restriction endonucleases were purchased from New England BioLabs and digestions were carried out as recommended by the manufacturer. Polynucleotide kinase was purchased from P-L Biochemicals. [γ-^{32}P]ATP was synthesized and kindly provided by R. Myers. Plasmids pFA2, pFA7, and pFA9 are derivatives of pBR322 and carry fragments *sa-5*, *sa-4*, and *sa-21* (Fig. 1), respectively, which were derived from a clone of the entire histidine transport operon (6). These plasmids were constructed by standard procedures and used as a source of DNA for sequence determination. The sequence of a small portion of the *hisJ* gene, from the *Bgl* II site to *Kpn* I site a, was determined by using DNA derived from LA 1, a clone of the entire histidine transport operon in the vector λgt4 (6). End-labeling of DNA prior to sequence determination was carried out by the exchange reaction using [γ-^{32}P]ATP and polynucleotide kinase (7). Single end-labeled fragments were obtained by cleavage with appropriate restriction endonucleases. All sequence determinations were carried out by the procedure of Maxam and Gilbert (7); both strands were subjected to sequence determination in their entirety.

RESULTS

Comparison of Amino Acid and Nucleotide Sequences. The entire histidine transport operon and the *argT* gene have been cloned (6) and the positions of the genes have been located accurately with respect to a number of restriction sites (8). Specific fragments, known to cover the *hisJ* and *argT* genes, were subcloned into plasmid vectors and their sequences were deter-

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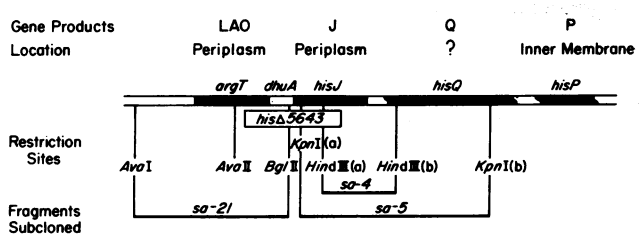

FIG. 1. Physical map of the histidine transport region of the *S. typhimurium* chromosome. Each structural gene on the chromosome is represented by a solid black bar. Where the precise end points of the genes are known from DNA sequence studies, the end of the gene is squared off; a wavy end indicates that the exact end point is not known. Restriction sites are marked by appropriately labeled thin vertical lines below the chromosome; the horizontal lines below the chromosome indicate specific restriction fragments subcloned. The location of deletion *hisJΔ5643* is indicated by the box just below the chromosome. The map is drawn to scale; *argT* and *hisJ* are each 780 base pairs long.

mined. The aligned nucleotide sequences of the *hisJ* and *argT* genes so obtained are presented in Fig. 2*A*. Translation of these sequences into amino acids yielded the protein sequences shown in Fig. 2*B*.

The nucleotide sequences of the *hisJ* and *argT* genes have been identified as those coding for the J and LAO proteins, respectively. Translation of the nucleotide sequences known to cover the entire *hisJ* gene revealed only one reading frame that could encode a protein with a molecular weight (25,000) similar to that of the J protein. The translated sequence in this reading frame matched the known amino acid sequence of the J protein (9), confirming this to be the *hisJ* structural gene. (In contrast with our data, the published amino acid sequence of the J protein indicates asparagine, as opposed to threonine, at residue 173.) The amino acid sequence obtained by translation of the nucleotide sequence (Fig. 2*B*) was found to have an additional 22 amino acids at its NH_2 terminus that were absent from the mature protein. These extra amino acids are typical of, and therefore assumed to be, a signal peptide, characteristic of periplasmic proteins (10).

The *argT* structural gene was also identified as being the only suitable reading frame in sequences known to include that gene. Translation of the nucleotide sequence revealed a protein similar to J. A preliminary sequence of the 19 NH_2-terminal amino acid residues of the LAO protein (R. W. Hogg, personal communication) matched this sequence, confirming it to be the *argT* structural gene. Again, a presumed signal peptide of 22 amino acids was found at the NH_2 terminus of the protein. The orientation of *hisJ* and *argT* on the chromosome indicates that both are transcribed in the same direction (from left to right in Fig. 1). The two genes are separated by the *dhuA* region which occupies 240 base pairs (unpublished data).

We consider first the aligned amino acid sequences of the mature J and LAO proteins (i.e., excluding the signal peptides). These sequences align perfectly and differ only by amino acid substitutions: no sequence rearrangements have occurred. Both J and LAO have 238 amino acids, their calculated molecular weights being 26,127 and 25,968, respectively. It should be noted that the apparent molecular weight of LAO, as derived from $NaDodSO_4$/polyacrylamide gel electrophoresis, is 1000–2000 less than that of J (5). However, this discrepancy between the experimental and the actual molecular weights is not surprising because it is known that even a single amino acid substitution in the J protein can alter its mobility considerably on $NaDodSO_4$/polyacrylamide gels (11). The similarity between the LAO and J proteins is striking: the two proteins are 70% homologous. Among the 72 amino acids that differ between J and LAO, 31 do not lead to a charge change and simply constitute the replacement of one amino acid by another of similar chemical characteristics. Nineteen of the amino acid differences between J and LAO result in a change in charge at that particular residue. However, the total number of glutamate and aspartate residues is identical in the two proteins, as is the total number of arginine and lysine residues. Thus, the overall charge on the two molecules remains the same.

Two specific regions of exceptional homology between the two proteins stand out: amino acids 75–116 and 168–198. These regions, which comprise 30% of each protein, are 92% homologous. In addition, the few amino acid differences observed in these regions are probably of only minor importance, involving the replacement of one amino acid by another of similar chemical characteristics.

The remarkable similarity between the *hisJ* and *argT* sequences is also observed at the nucleotide level: the two sequences are 71% homologous. Among the 205 nucleotides that differ, 40% do not effect an amino acid change. Only 10 codons show differences in all three bases.

The amino acid sequences of the presumed signal peptides of both J and LAO are similar to other known bacterial signal peptides: they have two basic amino acids close to the NH_2 terminus and an alanine residue at the cleavage point and are otherwise hydrophobic (10). The two peptides show only 41% and 45% homology at the amino acid and nucleotide levels, respectively. Excluding what are generally considered to be essential residues of any signal sequence—the initial methionine, the two basic residues at the NH_2 terminus, and the alanine at the cleavage site—the homology between the J and the LAO signal peptides is much poorer than that between the mature proteins: only 23% of the amino acids and 27% of the nucleotides are identical. However, the general hydrophobicity is maintained in both signal peptides, indicating that hydrophobicity, rather than the specific amino acid sequence, is the most important feature of these peptides. It is also of interest to note that no significant homology is observed in the noncoding regions immediately flanking *hisJ* and *argT* (unpublished data).

Mutant Proteins. Mutation *hisJ5625*, which prevents the normal interaction of J with the P protein (4), is known to cause an arginine-to-cysteine change in the J protein (11). This change was identified as being at residue 176 by determining the total amino acid composition of the tryptic peptide containing the altered residue. Comparison of this composition with the theoretical compositions of each tryptic peptide identified from the amino acid sequence of J allowed the altered peptide to be located, identifying the single arginine residue in that peptide, arginine-176, as the amino acid altered by the mutation.

Deletion *hisΔ5643* results in the fusion of a portion of *argT* to a portion of *hisJ* (Fig. 1), causing the production of a chimeric protein. The position of the end points of this spontaneously derived deletion have been accurately mapped with respect to various restriction sites (8). The nucleotide sequences of the *argT* and *hisJ* genes now allow us to determine the exact location of the genes with respect to the same restriction sites and, therefore, to locate the deletion end points within the genes. We know (*i*) that the left-hand end point of *hisΔ5643* does not extend as far as the *Ava* II site at base pairs 422–426 in *argT* (8), and (*ii*) that the right-hand end point of the deletion must be to the left of base pair 494 in *hisJ*.* Thus, the fusion point must be

* This limitation is imposed by the right-hand end point of *hisΔ6776*, which cannot be more than 170 base pairs to the right of *Hin*dIII site a (8). Because the right-hand end point of *hisΔ5634* is contained within *hisΔ6776*, it too cannot be more than 170 base pairs to the right of that site. Thus, because the *Hin*dIII site a is located at base pair 324 in the *hisJ* gene, this places a limit for the right-hand end of *hisΔ5643* at base pair 494.

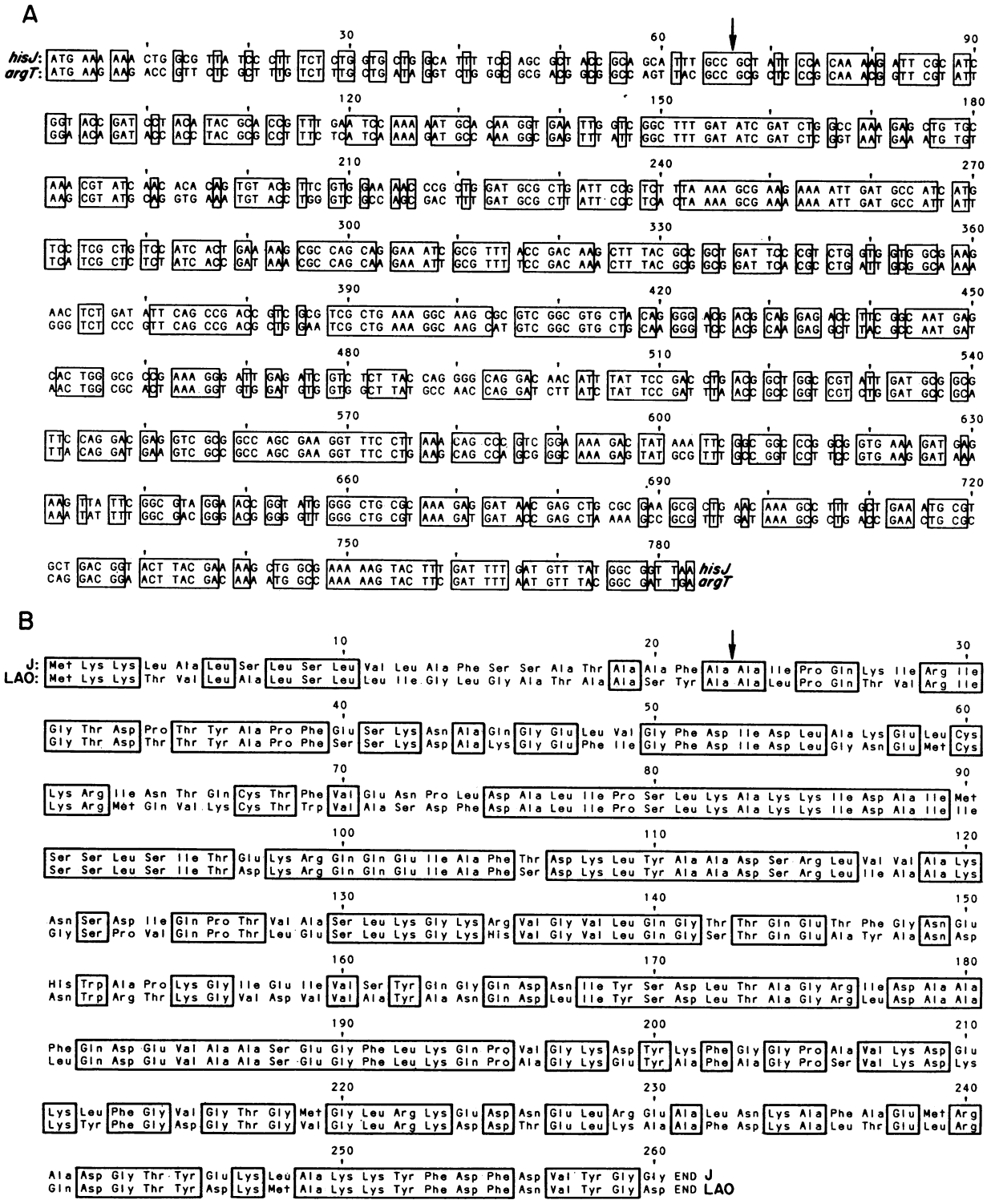

FIG. 2. Sequences of the *hisJ* and *argT* genes and of their translated products, the J and LAO proteins. (*A*) Aligned nucleotide sequences of the *hisJ* and *argT* genes. The sequences of both DNA strands of each gene were determined in their entirety. Bases that are identical in the two genes are boxed. The arrow after nucleotide 66 indicates the end of the sequence coding the signal peptides (see text). The antisense strand is shown. (*B*) Aligned amino acid sequences of the J and LAO proteins. Amino acid sequences were determined by translation of the nucleotide sequences in *A*. Amino acids identical in the two sequences are boxed. The point of cleavage for removal of the signal peptide from the mature protein is indicated by the vertical arrow after amino acid 22.

located between amino acids 143 and 164 of the two proteins.

More precise localization of the fusion point was obtained by determining the number of histidine residues in the chimeric protein. The wild-type J and LAO proteins each contain one histidine residue (LAO, histidine-135; J, histidine-151). If the fusion point were between residues 135 and 151, the chimeric protein would contain two histidine residues; if the fusion were downstream from residue 151, the chimeric protein would contain a single histidine residue. Cells producing either the chimeric or the wild-type LAO protein were double-labeled with [^{3}H]histidine and [^{14}C]leucine, and the periplasmic proteins were separated by two-dimensional acrylamide gel electropho-

resis (12). The chimeric protein and wild-type LAO (plus 12 additional proteins as controls) were eluted and the $^3H/^{14}C$ ratios were determined. The chimeric protein was found to contain the same ratio of histidine to leucine as the wild-type LAO protein, after correction for the different number of leucine residues in the chimeric protein compared with wild-type LAO. Thus, the fusion point must be downstream from histidine-151, between residues 152 and 164.

The chimeric protein has been identified on NaDodSO$_4$/polyacrylamide gels and has a mobility very similar to that of LAO. Table 1 shows the substrate-binding properties of this chimeric protein. Despite the fact that about half of the chimeric protein is derived from J, it has binding properties similar to those of LAO. It binds arginine to the same extent as does wild-type LAO; both lysine and ornithine compete for arginine binding. Arginine-binding activity is also increased by the nitrogen regulatory mutation, *gln-139*, to exactly the same extent as in LAO. Like LAO, but unlike J, the chimeric protein does not bind histidine and is unable to function in D-histidine transport. Thus, the fusion must be in phase and must be a fairly precise fusion of the two genes.

DISCUSSION

The availability of the complete nucleotide sequences of the *hisJ* and *argT* genes, which encode the histidine-binding and lysine-arginine-ornithine-binding proteins, respectively, is an important step in our attempt to understand the evolution and molecular mechanisms of transport systems that are dependent on binding proteins.

It seems clear that the *hisJ* and *argT* genes originated by tandem duplication of an ancestral gene, followed by divergent evolution. The two genes show 70% homology and are located adjacent to each other on the chromosome, separated by only 240 base pairs (unpublished data). Because *E. coli* is also known to have both a histidine-binding and a lysine-arginine-ornithine-binding protein similar to those of *S. typhimurium* (6, 13, 14), the duplication event giving rise to these two genes must therefore have occurred before the divergence of *Escherichia* and *Salmonella* (or transferred to both by a common vector). Since the original duplication, the two proteins have diverged, developing different affinities and specificities for their substrates: the J protein acquired high affinity for histidine and the LAO protein, a high affinity for lysine, arginine, and ornithine. The high incidence of silent base differences between the two genes indicates that they diverged sufficiently long ago to allow almost complete randomization of those bases not under selective pressure (15).

Table 1. Arginine binding by shock fluids

Relevant genotype	Binding protein	Total arginine binding: No addition	Total arginine binding: With 1 μM lysine	Total arginine binding: With 1 μM ornithine	Lysine-inhibited arginine binding
argT$^+$	LAO	8.5	7.9	5.0	0.6
argT$^+$ *gln-139*	LAO	23.0	6.7	6.5	16.3
argT526 gln-139	LAO$^-$	6.3	7.3	5.2	0
hisΔ5643	Fusion	8.0	6.0	6.3	2
hisΔ5643 gln-139	Fusion	25.9	8.2	8.1	17.7
hisΔ5643 argT526 gln-139	Fusion	3.6	5.7	8.5	0

Binding activity of shock fluids was assayed by equilibrium dialysis (2) and is expressed as pmol of [^{3}H]arginine bound per A_{650} at an arginine concentration of 10 nM. Lysine-inhibited arginine binding values are a measure of that portion of the total arginine binding activity due to LAO or the fusion protein and are obtained by subtracting column 2 from column 1. All shock fluids were assayed for histidine-binding activity; all contained <1% of wild-type J activity. All strains lack the J protein. Mutation *gln-139* is a regulatory mutation that increases the LAO protein (5). Mutation *argT526* completely eliminates production of LAO or the fusion protein.

It has often been postulated that gene duplication is an important evolutionary mechanism (reviewed in ref. 16). An excellent example of this is illustrated by the multiple globin genes of eukaryotic cells. However, in bacteria, possible examples of stabilized gene duplications are less well-characterized. Duplicate genes for several tRNAs, ornithine transcarbamylase, and the translational elongation factor EF-Tu, as well as seven nontandem copies of the rRNA genes, are present in the *E. coli* genome (reviewed in ref. 17). However, in each of these cases, the two gene products appear to serve identical functions within the cell and do not seem to have undergone significant evolutionary divergence. Duplications have also been postulated as being responsible for groups of functionally or structurally related proteins [e.g., the serine proteases (18)].

The present studies on the histidine-binding and lysine-arginine-ornithine-binding proteins provide an excellent example of a naturally occurring gene duplication event in which the two genes have subsequently diverged to perform different, although in this case closely related, functions. This provides strong support for the view that new genetic functions can evolve as a result of gene duplication and divergence within the bacterial genome. It is interesting to note that gene duplication and divergence may also have occurred for other periplasmic binding proteins. The genes encoding the leucine-specific-binding and the leucine-isoleucine-valine-binding proteins map close to each other on the *E. coli* chromosome (19). Comparison of the complete amino acid sequence of the leucine-isoleucine-valine-binding protein (20) with partial nucleotide and amino acid sequences of the leucine-specific-binding protein indicates that they share extensive homology (19). Some minor homology (23%) has also been observed between the *E. coli* ribose-binding and galactose-binding proteins (21); these two proteins have been postulated to interact with a common membrane component in chemotaxis (22), and the 23% homology, though minor, may reflect this interaction (23).

Analysis of the distribution of amino acid differences between the J and LAO proteins might be expected to indicate which portions of the molecules are important for specific functions. The J protein is known to possess two distinct functional sites, a histidine-binding site and a site that interacts with the membrane-bound P protein; LAO is also presumed to have two analogous sites. In view of the similarities in structure between the J and LAO proteins and their presumed common origin, these two binding proteins are likely to interact with the same site on P as illustrated in Fig. 3. If the two binding proteins do indeed interact with the same site on the P protein, the regions of the two binding proteins involved in this interaction would be expected to have retained a good deal of homology. Comparison of the amino acid sequences of J and LAO shows two obvious regions of extensive homology (92%): between residues 75 and 116 and between residues 168 and 198. It therefore seems likely that one or both of these regions are involved in interactions with the P protein. This hypothesis is supported by the finding that mutation *hisJ5625*, which is known to affect specifically the interaction of J with the P protein but not to affect the histidine-binding site of J (24), is located within the second of these homologous regions (at arginine-176).

It is possible that the regions of high homology between J and LAO are maintained by "concerted evolution" [a mechanism for the retention of homology by recombination between closely related genes (25)]. This possibility was ruled out by calculating the corrected percentage sequence divergence for the conserved and nonconserved regions of the two proteins (15). Com-

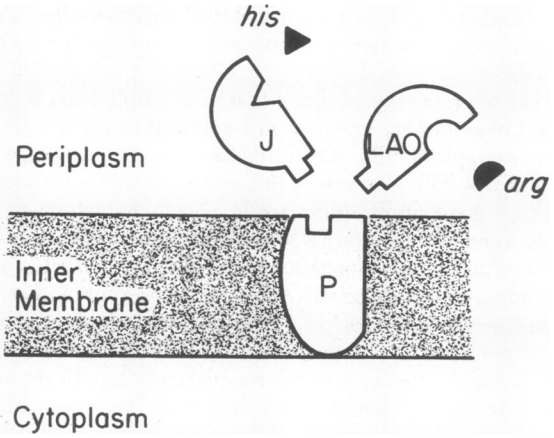

FIG. 3. Diagram illustrating the interaction of the two binding proteins, J and LAO, with the same site on the membrane-bound P protein. No implication as to the molecular mechanism by which the substrates are transported across the membrane is intended.

parison of these values showed no significant differences between the conserved and nonconserved regions, indicating that the maintenance of homology between these two proteins is simply due to selective pressure.

The major difference between the J and LAO proteins lies in their substrate specificities. The residue(s) important in determining the difference in substrate specificity between the binding sites can be localized by a study of *hisΔ5643*, a deletion that results in fusion of portions of the *argT* and *hisJ* genes. This deletion is presumed to be a precise fusion of the *argT* and *hisJ* genes and probably arose as a result of homologous recombination between the two adjacent genes. The chimeric protein produced as a result of this fusion consists of about the first 150 residues of LAO and the final 90 residues of J. Because the chimeric protein has substrate specificity indistinguishable from that of LAO, the amino acid residue(s) important in determining substrate specificity must be located within the first 150 residues of the proteins. This view is supported by the observation that several regions of nonhomology between J and LAO—for example, residues 23–74—lie within these first 150 residues and could account for the difference in substrate specificity. The difference in substrate specificity between J and LAO may require several amino acid differences; alternatively, a single amino acid change might be responsible, as in the case of trypsin and chymotrypsin (26). It is interesting to note that a similar gene/protein fusion, resulting in a functional product and arising as a result of recombination between two similar and adjacent genes, has been observed in the case of a hemoglobin hybrid in humans (27).

The complete amino acid sequences of the isoleucine-leucine-valine-binding, arabinose-binding, galactose-binding, and sulfate-binding proteins have been compared with each other and also with the histidine-binding protein J; no significant homology was observed (R. W. Hogg, personal communication). However, there is now increasing evidence that the tertiary structure of several binding proteins may be similar: a bilobate molecule with two clearly distinct domains separated by a cleft (28). This may reflect a common mechanism for all binding-protein-dependent processes. The work described here, together with the many mutations already characterized in the structural genes for the J and LAO proteins and with NMR (29) and x-ray crystallographic (F. A. Quiocho, personal communication) studies make this a good model system for structural and functional analysis of binding proteins.

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