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The Structure and Function of the Histidine-Containing Phosphotransfer (HPt) Signaling Domain of the *Escherichia coli* ArcB Sensor[1]

Akinori Matsushika and Takeshi Mizuno[2]

Laboratory of Molecular Microbiology, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464-8601

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The *Escherichia coli* ArcB sensor is involved in anaerobic signal transduction. ArcB is an unorthodox His-kinase, in that it contains three types of phosphotransfer signaling domains in its primary amino acid sequence, namely, transmitter (or His-kinase), receiver, and histidine-containing phosphotransfer (HPt) domains. In this study, we first conducted an *in vivo* experiment to determine whether or not the phosphorylation of the HPt domain is crucial for ArcB/ArcA-mediated anaerobic signal transduction. The results are best interpreted as meaning that the HPt domain of ArcB is important for the anaerobic signal transduction, as far as the expression of the succinate dehydrogenase (*sdh*) operon is concerned. We then isolated a set of ArcB mutant each with a single amino acid substitution in the HPt domain, which has lost the ability to function as a phospho-transmitter. The results of such mutational analyses, together with the three-dimensional crystal structure of the HPt domain, provided an insight into the structure and function of the HPt domain of ArcB.

Key words: ArcB sensor, *Escherichia coli*, HPt domain, phosphotransfer signal transduction.

Bacteria have devised phosphotransfer signaling mechanisms for eliciting a variety of adaptive responses to their environment. They are collectively referred to as "two-component signaling systems" (*1-4*). A typical two-component system consists of two types of signal transducers, a "sensor kinase" and a "response regulator" (*5, 6*). These signal transduction proteins contain one or both of the following common phosphotransfer signaling domains, a "transmitter (or His-kinase)" and a "receiver." The transmitters contain a crucial histidine site, which is autophosphorylated, whereas the receivers contain an invariant aspartate site, which can acquire a phosphoryl group from the phosphohistidine of the cognate transmitter. Besides these two signaling domains, some signal transducers were recently found to have another common signaling domain, termed the "histidine-containing phosphotransfer (HPt) domain." In the most sophisticated case, a phosphoryl group moves from a transmitter to a receiver, then to a HPt domain, and finally to another receiver, during a signal transduction process. Thus, such the conceptual view as to the bacterial signal transduction mechanism can be referred to as "multistep His to Asp phosphotransfer systems" (*7, 8*).

A typical HPt domain was first discovered in the *Escherichia coli* ArcB sensor (*9, 10*), which is responsible for anaerobic responses (*11-13*). ArcB is an unorthodox sensor, in that it contains both a transmitter and a receiver in its primary amino acid sequence (see Fig. 1). In this hybrid sensor, these two authentic phosphotransfer signaling domains are followed by a HPt domain at the C-terminal end. As pointed out previously, inspection of the entire nucleotide sequence of *E. coli* revealed that this bacterium has five presumed hybrid signal transducers that each contain a HPt domain (*i.e.*, ArcB, BarA, EvgS, TorS, and YojN) (*14*). The *Bordetella pertussis* BvgS sensor also contains a typical HPt domain, of which the functional importance has been demonstrated (*15*). Another striking example of a HPt domain was found recently in the yeast, *Saccharomyces cerevisiae*, osmoregulatory signaling system (*16-18*). The Sln1p-Ypd1p-Ssk1p pathway, which is currently the best-characterized eukaryotic two-component system, represents yet another multistep phosphotransfer signaling strategy, in which Ypd1p, comprising only a HPt domain, plays a crucial role (*17*). Taking all these examples together, it has been proposed that the HPt domains in a number of signal transducers function as a common device involved in the phosphotransfer signal transduction (*8*). However, examination of the structure and function of this newly-emerging phosphotransfer signaling domain is still at a very early stage.

On the bases of the previous intensive *in vitro* studies in our and other laboratories (*10, 19, 20*), one can propose a plausible schema for the phosphotransfer circuitry as to ArcB, as shown in Fig. 1. First of all, His-292 in the ArcB transmitter domain acquires a γ-phosphoryl group from ATP through its own catalytic function (*i.e.*, autophosphorylation). This reaction is essential for the subsequent

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[2] To whom correspondence should be addressed. Phone: +82-52-789-4089, Fax: +81-52-789-4091, E-mail: i45455a@nucc.cc.nagoya-u.ac.jp

phosphotransfer, and in fact the phosphoryl group at His-292 moves to the intrinsic phospho-accepting aspartate (Asp-576) in the ArcB receiver domain. The C-terminal His-717 can also be modified by phosphorylation through the function of ArcB itself (possibly through Adp-576), in which His-292 plays a crucial role. The final destination of the phosphoryl group at His-717 appears to be Asp-54 in the ArcA receiver domain. However, it should be emphasized that this current scenario is solely based on *in vitro* biochemical results (*10, 20*). Furthermore, the importance of the HPt domain containing His-717 was only recognized recently. Therefore, the physiological (or *in vivo*) relevance of this multistep phosphotransfer signaling remains unknown (*13, 20*). In particular, it has not yet been determined whether or not the phosphorylation of His-717 is crucial for the *in vivo* ArcB-ArcA signaling. In this study, we first address this issue with regard to the functional importance of the HPt-domain. We then characterize the structure and function of the HPt-domain at the molecular level, taking advantage of the recently determined three-dimensional crystal structure of this domain (*19*).

MATERIALS AND METHODS

Bacterial Strains and Media—*E. coli* K-12 strain MC4100 (F⁻ *ΔlacU169, araD139 rpsL relA thiA flbB*) was used (*21*). OG903 is a derivative of MC4100, which carries an *sdh-lacZ* transcriptional fusion gene on the chromosome. An *arcB*::Cm^r allele was further introduced into OG903, by means of P1 transduction, to yield DAC903. This *arcB*::Cm^r allele was constructed previously (*9*). Strains YAC1 and YAC2 are derivatives of CSH26 (*Δ[lac-pro] ara thi*) (*22*), and were constructed previously (*23, 24*), These derivatives carry an *ompC-lacZ* transcriptional fusion gene and an *ompF-lacZ* transcriptional fusion gene, respectively. These cells were grown in Luria-broth, unless otherwise noted.

Plasmids—Plasmid pLIA001 and pLIA002 were constructed previously (*10*), both of which carry the *arcB* gene, but the latter has a base-substitution resulting in a His-717 to Leu substitution (see Fig. 1). Plasmid pAM5 carries a truncated form of the *arcB* gene (see Fig. 1), which is designated as "*arcB*^{c}." This artificial gene was constructed previously (*9*), so as to encode only the C-terminal region of ArcB (named ArcB^c), that corresponds to the HPt domain. The original plasmid carrying this particular gene was named pSN002 (*9*), and in this study the gene was recloned into a derivative of pBR322, to yield pAM5.

Recombinant DNA Techniques—DNA-manipulating enzymes, such as restriction endonucleases, the Klenow fragment of *E. coli* DNA polymerase I and T4 DNA ligase, were used under the conditions recommended by the suppliers (Takara Shuzo or Toyobo). Other recombinant DNA techniques were all carried out according to a standard laboratory manual (*25*). DNA was sequenced by the dideoxy chain termination method, using a sequencing kit and an automated sequencer (Perkin Elmer, ABI PRISM™ 310 Genetic Analyzer).

Enzyme Assays—β-Galactosidase activity was determined by Miller's method with slight modifications (*22*). Cells were grown to the mid-logarithmic phase in either Luria-broth or M9-glucose minimal medium, under both standard aerobic and anaerobic conditions. The cells were collected and then suspended in one volume of 250 mM sodium phosphate (pH 7.1) for accurate determination of the cell density. A portion of the cell suspension was subjected to β-galactosidase assaying, after permeabilization with toluene. Each figure shows the values for triplicate cultures (note that, for clarity, error bars are omitted).

Hydroxylamine Mutagenesis—Plasmid pAM5-DNA was treated in a buffer comprising 0.5 M potassium phosphate (pH 6.0), 0.4 M hydroxylamine, and 5 mM EDTA for 48 h at 37°C, and then for 10 min at 70°C. The sample was dialyzed against distilled water at 4°C overnight. The mutagenized plasmids were transferred into *E. coli* strain K58 harboring an *ung*⁻ allele, and then the plasmids were re-isolated. A short *Eco*RI-*Hin*dIII fragment was prepared, and then ligated back into the same sites of pAM5.

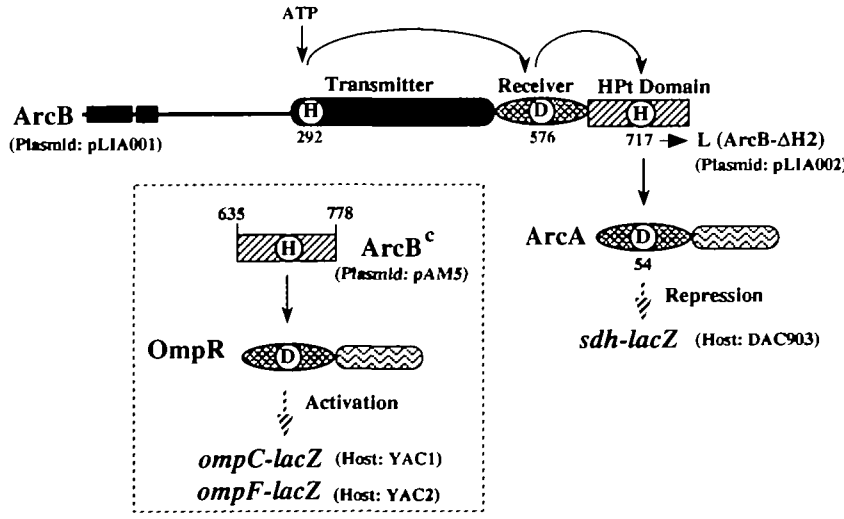

Fig. 1. **A schema for the multistep phosphotransfer circuitry of ArcB.** The ArcB sensor contains a transmitter, a receiver, and a C-terminal HPt domain, which are all involved in His to Asp phosphotransfer reactions, as schematically shown. The ArcA protein is the cognate response regulator containing a receiver domain, which acquires a phosphoryl group from ArcB. Arrows indicate the flows of phosphoryl groups, which were demonstrated *in vitro* previously (*10, 20*). The resulting phospho-ArcA functions as a transcriptional repressor for the expression of a certain set of genes involved in anaerobiosis, including the *sdh* operon. The OmpR protein is the non-cognate response regulator containing a receiver domain. Nonetheless, OmpR can acquire a phosphoryl group through the phospho-HPt domain of ArcB (named ArcB^c), if this polypeptide is overexpressed in *E. coli*. As a consequence, *in vivo* and *in vitro*, ArcB^c comprising only a HPt domain can function as an artificial phospho-donor in such a manner that OmpR is phosphorylated even in the absence of EnvZ, which is the cognate sensor kinase for OmpR. As demonstrated previously (*9, 10*), this event can be monitored *in vivo* by measuring the enhanced expression of *ompC* and *ompF* in an *envZ*⁻ and *arcB*⁺ background (host strains YAC1 and YAC2). Note that the set of plasmids and strains used in this study are indicated in this schema (see the text).

RESULTS AND DISCUSSION

The HPt-Domain Is Crucial for the Anaerobic Signaling through the ArcB-ArcA System—Previous studies established that the expression of the *sdhCDAB* operon is markedly elevated by aerobiosis and suppressed severely during growth under anaerobic conditions, mainly through the ArcB-ArcA signal transduction system (*11-13, 26*). Succinate dehydrogenase (SDH) is an enzyme of the tricarboxylic acid cycle, participating in the aerobic electron-transport pathway, and is encoded by the *sdhCDAB* operon. To examine *in vivo* the ArcB-ArcA anaerobic signal transduction with special reference to the HPt domain, an *E. coli* strain carrying an *sdh-lacZ* transcriptional fusion gene on the chromosome was constructed (strain OG903), in which the promoter region of the *sdhCDAB* operon was fused to the *lacZ* gene encoding β-galactosidase. This newly constructed *sdh-lacZ* fusion gene should provide a convenient reporter for monitoring the *in vivo* function of the ArcB-ArcA signaling system. An *arcB*-deletion (*arcB*::Cm^r) mutant was also constructed from OG903, to yield DAC903 carrying both the *sdh-lacZ* and *arcB*::Cm^r alleles.

To assess the function of the *arcB* gene, a low-copy number plasmid (named pLIA001) carrying the wild-type *arcB* gene was introduced into DAC903, and then the expression of *sdh-lacZ* was monitored in cells grown in M9-glucose minimal medium under both aerobic and anaerobic conditions (Fig. 2; see Wild). As expected, the expression of *sdh-lacZ* was severely repressed under the anaerobic growth conditions. To examine the function of the HPt-domain of ArcB, in terms of the anaerobic regulation of *sdh-lacZ*, the same strain was transformed with another plasmid, pLIA002, which carries a gene encoding an ArcB mutant with a His to Leu substitution at the position of His-717. It was found that the anaerobic repression of *sdh-lacZ* was completely abolished in the cells carrying the ArcB mutant lacking the His-717 phosphorylation site (Fig. 2; see ΔH2). However, the level of β-galactosidase in the ΔH2 cells grown under aerobic conditions was lower than that in the wild-type cells. The reason for this is not clear at present. In any event, our results are best interpreted as meaning that the phosphorylated His-717 site in the HPt-domain is crucial for the anaerobic regulation of *sdh-lacZ* through the ArcB-ArcA signaling system. This suggests that the presumed phosphotransfer between His-717 of ArcB and Asp-54 of ArcA is physiologically crucial (see Fig. 1). More comprehensive studies to address the relevant issues will be described elsewhere.

Experimental Design for Mutational Analyses of the HPt Domain—Since the above finding clearly implies the functional importance of the HPt-domain of ArcB, we then characterized its structure and function in more detail. To this end, we characterized in this study a truncated form of the *arcB* gene, which was constructed previously (the gene was named "*arcB*^{c}"). Its gene-product, named "ArcB^c," consists of only a HPt domain, extending from Val (Met)-653 to Lys-778 (C-terminus), as schematically shown in Fig. 1 (the plasmid carrying this artificial *arcB*^{c} gene was named pAM5). It was previously demonstrated that ArcB^c is capable of functioning *in vivo* as a phospho-transmitter (*9*). In other words, when ArcB^c is expressed in the *arcB*$^+$ background, it is phosphorylated at His-717 through the function of the His-kinase domain of the wild-type ArcB protein produced by the gene on the chromosome. More specifically, provided that ArcB^c is expressed from pAM5 in an *envZ* deletion mutant (*e.g.*, strains YAC1 and YAC2), it is capable of phosphorylating the OmpR response regulator (*9, 10*). The resulting phospho-OmpR can activate the *ompC-lacZ* fusion gene in YAC1 (or the *ompF-lacZ* gene in YAC2) (*27-29*), as schematically shown in Fig. 1. This is because of the phosphotransfer from His-717 of ArcB^c to Asp-55 of OmpR, as demonstrated previously (*9, 10*). Consequently, YAC1 and YAC2 each harboring pAM5 exhibit the Lac^+ phenotype, and give red colonies on lactose-MacConkey agar plates in a manner dependent on the ArcB^c-OmpR phosphotransfer signaling. This particular event can be used as a clear hallmark of the *in vivo* function of the HPt domain itself. Based on this experimental rationale, we designed a screening strategy that allowed us to isolate a set of ArcB^c mutants, which should provide with an insight into the structure and function of the HPt domain, as will be described below.

Isolation of a Set of ArcB^c Mutants—We attempted to isolate a set of ArcB^c mutants that fail to activate the *ompC-lacZ* fusion gene in YAC1. To do so, plasmid pAM5-DNA encompassing the *arcB*^{c} gene was subjected to *in vitro* localized-mutagenesis with hydroxylamine. The *Eco*RI-*Hin*dIII restriction fragment containing the *arcB*^{c}-coding region was isolated from the mutagenized DNA and then ligated back into the original plasmid, in order to localize the mutagenized region specifically within the *arcB*^{c}-coding sequence. With this mixture of plasmids, YAC1 carrying the *ompC-lacZ* fusion gene was transformed and then spread on lactose-MacConkey plates. A number of white colonies appeared on the plates, which were each suspected to contain a mutant plasmid producing an altered ArcB^c.

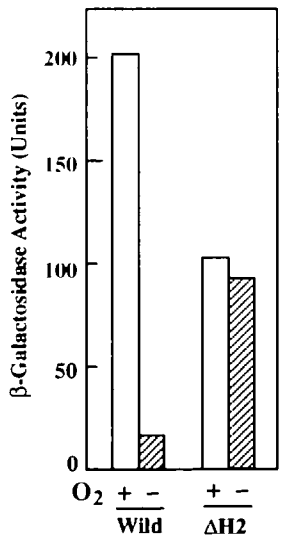

Fig. 2. **β-Galactosidase activity expressed by the *sdh-lacZ* transcriptional fusion gene.** Strain DAC903 carrying the *arcB*::Cm^r and *sdh-lacZ* alleles was transformed with either pLIA001 carrying the wild-type *arcB* gene (denoted as "Wild") or pILA002 carrying the mutant *arcB* gene specifying a ArcB mutant with a His-to-Asp substitution at His-717 (denoted as "ΔH2") (see Fig. 1). These transformants were grown in M9-glucose medium under both aerobic (O_2+) and anaerobic (O_2−) conditions. The harvested cells were assayed for β-galactosidase.

Among them, we were not interested in those producing no or only a reduced amount of ArcB^c (designated as type-1 mutants). To identify such undesirable colonies, the cellular content of ArcB^c in each candidate was examined, by means of immunoblotting analysis with an anti-ArcB^c antiserum. Among the candidates thus analyzed (146 white colonies), the majority were found to be undesirable (*i.e.*, type-1 mutants), as anticipated (data not shown). Only 11 colonies were found to produce ArcB^c in an amount comparable to that produced by the wild-type (designated as type-2 mutants). These mutant plasmids were expected to specify a functionally-altered ArcB^c, which most likely lacks the ability to signal to OmpR. These plasmids were thus isolated and then directly subjected to DNA-sequencing, in order to identify substituted amino acids in these presumed ArcB^c mutants. As a reference, we also determined the nucleotide sequences of 10 representative type-1 mutants, which were found to produce markedly reduced amounts of ArcB^c. The results of these analyses are shown in Figs. 3 and 4.

Identification of Amino Acids Crucial for the Function of ArcB^c—As shown in Fig. 3A, five distinct ArcB^c mutants were identified as type-2 ones, each of which has a single amino acid substitution at a certain position. For clarity hereafter, these ArcB^c mutants will be designated as to the inferred amino acid changes. For example, ArcB^c-G685R refers to a mutant that has a glycine to arginine substitution at amino acid position 685. Likewise, four others, ArcB^c-E714K, -H717Y, -G721D, and -G724E, were identified (Figs. 3A and 4). Among these ArcB^c mutants thus identified, it should be emphasized that some were independently selected multiple-times, as indicated in Fig. 3A. It should also be emphasized that the one with the amino acid substitution at the crucial His-717 site was included (ArcB^c-H717Y), as expected.

It was confirmed that these type-2 ArcB^c mutants exhibited a markedly reduced ability to signal OmpR, as judged from the results of β-galactosidase assays with YAC2 carrying the *ompF-lacZ* fusion genes (Fig. 3A), as well as with YAC1 carrying the *ompC-lacZ* fusion gene (data not shown). In these experiments, the transformants were grown under anaerobic conditions. Note also that even when the cells were grown under aerobic conditions, essentially the same results were obtained, but of course, the levels of β-galactosidase were significantly lower than those under the former conditions. We thus succeeded in identifying certain amino acid sites in ArcB^c that are most likely crucial for its function, as far as the phosphotransfer signaling to OmpR is concerned.

In addition, in type-1 mutants, we identified three amino acid substitutions, each of which appeared to make ArcB^c very unstable in the cells. They were ArcB^c-K718E, -L734P, and -W746R (note that others were found to have a termination codon within the coding-sequence). In any case, these altered ArcB^c had lost the *in vivo* ability to signal OmpR, as expected (Fig. 3B).

Structure of the HPt Domain—The crystal structure of ArcB^c consisting of 125 amino acid residues was deter-

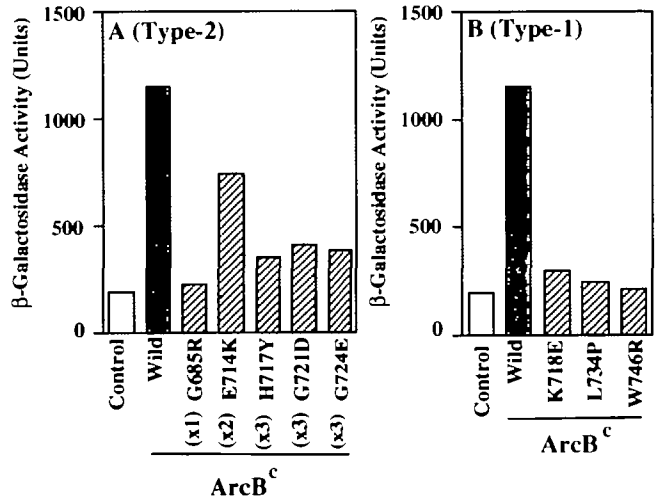

Fig. 3. **β-Galactosidase activity expressed by the *ompF-lacZ* transcriptional fusion gene.** Strain YAC2 carrying the *envZ*$^-$ and *ompF-lacZ* alleles was transformed with pAM5 carrying the *arcB*^{c} gene encoding the ArcB^c polypeptide containing the HPt domain (see Fig. 1). This strain was also transformed with a set of mutant plasmids, isolated in this study, each of which carries a mutant *arcB*^{c} gene encoding a ArcB^c mutant (panel A for the stable ArcB^c mutants, and panel B for the unstable ArcB^c mutants). The amino acid substitutions determined for each ArcB^c mutant are indicated (*e.g.*, G685R, see the text). Mutants which were selected twice or more are indicated by (×2) or (×3). These transformants were grown in Luria-broth under anaerobic conditions. The harvested cells were assayed for β-galactosidase.

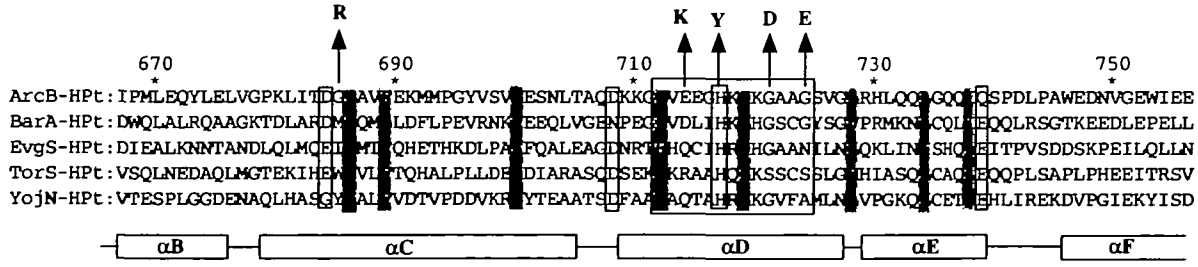

Fig. 4. **Alignment of the amino acid sequences corresponding to the HPt domains of the *E. coli* hybrid sensors.** The amino acid sequence of the HPt domain of ArcB was aligned with those of the HPt domains of other *E. coli* hybrid sensors (*14*). Among them, highly conserved amino acids including the crucial histidine residue (His-717) are boxed, and the highly conserved hydrophobic amino acids are shaded. Note that the numbers at the top are those for the amino acid sequence of ArcB. Note also that both the N-terminal and C-terminal portions of the HPt sequences are omitted, for clarity, in this figure. The amino acid substitutions, characterized in this study, are indicated by arrows. At the bottom, the secondary structure of the HPt domain of ArcB is schematically shown, in which the most N-terminal α helix (αA) is missing. Other helices are indicated by rectangles, which are denoted as αB to αF.

mined recently (*19*). X-ray analysis revealed an all-α structure of ArcB^c, consisting of six helices (αA to αF) (Fig. 4). The four helices (αC to αF) form a bundle-structure, in which the crucial His-717 site is located on the surface of helix-D, that lies on the internal curvature of the kidney-shaped molecule. As mentioned above, *E. coli* has four other signal transducers that contain a presumed HPt domain (BarA, EvgS, TorS, and YojN). The structural view as to ArcB^c allowed us to unambiguously align the sequences of these *E. coli* HPt domains, as shown in Fig. 4. Although they are highly variable in their primary amino acid sequences, helix-D containing the crucial histidine residue (His-717) comprises the most highly conserved region. It is also clear that among these HPt domains, a number of hydrophobic residues are conserved at certain positions. These hydrophobic residues mostly appear to play a role in the formation of the hydrophobic core that allows association of the four-helix bundle. In any event, it should be emphasized that four of the five critical amino acid substitutions, identified in this study, occur in helix-D. This finding supports the view that highly conserved helix-D of the HPt domain is functionally important. The other one (G685R) is located in helix-C. This issue will be addressed further below.

Implications—In this study, the physiological importance of the HPt domain of ArcB was first demonstrated. When the putative phosphorylation site (His-717) was substituted, the resulting ArcB mutant had completely lost the *in vivo* ability to transmit the anaerobic signal, thereby resulting in a constitutive expression of *sdh-lacZ*. This suggest that the HPt domain of ArcB plays an essential role in the anaerobic signal transduction. There have been previous observations that the HPt domain in the *B. pertussis* BvgS sensor and the *S. cerevisiae* Ypd1p protein play an essential role in the respective His-Asp phosphotransfer signal transduction systems (*15, 17*). The results of this study further support the view that the HPt domains function as a common device (or signal-transmitter) in widespread phosphotransfer signaling systems.

As mentioned above, we recently determined the crystal structure of the HPt domain of ArcB (*19*). This is the first presentation of the three-dimensional structure of the HPt domain. X-ray analysis revealed the all-α structure of the HPt domain, as schematically shown in Fig. 4. The amino acid residues which were demonstrated to be crucial for the proper functioning of the HPt domain in this study are highlighted in a space-filling model of the HPt domain (Fig. 5). They are Gly-685, Glu-714, Gly-721, Gly-724, as well as His-717. It was found that all these residues lie on the same concave surface of the kidney-shaped molecule. Note also that the phosphorylated residue, His-717, is also on the same surface. It is thus tempting to speculate that this concave surface is involved in the presumed interaction with a cognate receiver domain (either the receiver domain of ArcB containing Asp-576, the receiver domain of ArcA containing Asp-54, or the receiver domain of OmpR containing Asp-55, see Fig. 1). The three-dimensional structures of some receiver domains have been reported (*e.g.*, CheY, NarL, and Spo0F) (*30-32*), all of which fold into a similar $(\alpha/\beta)_5$ globular structure. Although the corresponding structure has not been determined for either for ArcB, ArcA, or OmpR, one can reasonably envisage that these receivers also have a similar globular structure. The

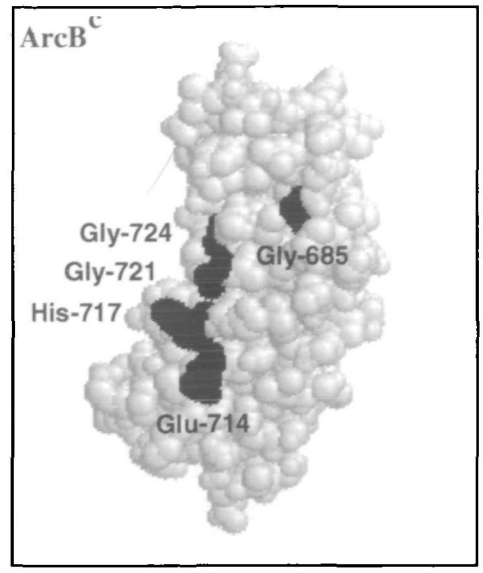

Fig. 5. **Three-dimensional structure of the HPt domain of ArcB.** The recently determined structure of the HPt domain is presented as a space-filling model (*19*). In this structure, the amino acid residues apparently crucial for the function of the HPt domain are highlighted. They are Gly-685, Glu-714, Gly-721, and Gly-724, as well as His-717.

concavity of the kidney-shaped molecule of the HPt domain may fit well the round surface of the globular receiver molecule. Thus, the concave surface of the HPt domain may be involved in the direct interaction with a cognate receiver. Such a presumed interaction may be crucial for the phosphotransfer reaction between them. In this context, the results of this study provided experimental evidence supporting this hypothetical view. For example, when certain bulky and/or charged amino acid residues (*i.e.*, Arg, Asp, and Glu) were used to replace the small glycine residues (Gly-685, Gly-721, and Gly-724), respectively, the presumed interaction between the HPt domain and the cognate receiver domains appears to be impaired. However, verification of this must await determination of the three-dimensional structure of a complex of HPt and receiver domains.

It is clear that the present set of ArcB^c mutants are defective in the ability to function as a phospho-transmitter, as discussed above. However, what remains unclear is whether these mutants are defective in the ability to acquire the phosphoryl group from the ArcB receiver or, alternatively, are defective in the ability to transfer the phosphoryl group to the OmpR receiver. To clarify this interesting point, a series of *in vitro* experiments involving the purified ArcB^c mutant proteins should be carried out. It will also be of interest to introduce these identified substitutions into the context of the intact ArcB protein, in order to see the consequence in ArcB-mediated anaerobic signal transduction *per se*. Such future analyses should shed light on the general function of the HPt domain.

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