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# Arg-220 of the PstA Protein Is Required for Phosphate Transport through the Phosphate-Specific Transport System in *Escherichia coli* but Not for Alkaline Phosphatase Repression

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**The *pstA* gene encodes an integral membrane protein of the phosphate-specific transport system of *Escherichia coli*. The nucleotide change in the previously described *pstA2* allele was found to be a G→A substitution at position 276 of the nucleotide sequence, resulting in the premature termination of translation. Three mutations in the *pstA* gene were produced by site-directed mutagenesis. The amino acid substitutions resulting from the three site-directed mutations were Arg-170→Gln, Glu-173→Gln, and Arg-220→Gln. These amino acid residues were selected because a previous PstA protein structure prediction placed them within the membrane. The Arg-220→Gln mutation resulted in the loss of phosphate transport through the phosphate-specific transport system, but the alkaline phosphatase activity remained repressed. Neither the Arg-170→Gln nor the Glu-173→Gln mutation affected phosphate transport. The results are discussed in relation to a proposed structure of the PstA protein.**

The phosphate-specific transport (Pst) system of *Escherichia coli* comprises four distinct subunits encoded by the *pstS* (*phoS*), *pstA*, *pstB*, and *pstC* genes (1, 19). These genes, together with the *phoU* gene, form the *pst* operon, which maps at about min 83.5 on the *E. coli* chromosome (2). The nucleotide sequences of all five genes have been determined, and the amino acid sequences of the corresponding proteins have been deduced (1, 19). The PhoU protein is not required for phosphate transport through the Pst system but is involved in regulation of the phosphate regulon (19). The PstS protein, the most studied of the four subunits, is the phosphate-binding protein located in the periplasmic space (7). The PstA and PstC proteins are hydrophobic and are likely to form the transmembrane portion of the Pst system. The PstB protein is likely to interact on the cytoplasmic side with the PstA and PstC proteins, since the *pstB* gene does not encode an N-terminal leader sequence. A conserved sequence associated with a nucleotide-binding site has been located on the PstB protein (17). Other transport systems known to be associated with periplasmic binding proteins also possess analogous structural features (8).

In the present paper, we characterize the nucleotide change in the previously described *pstA2* allele and describe the properties of strains in which Arg-220, Arg-170, and Glu-173 of the *pstA* protein have been changed by site-directed mutagenesis.

## MATERIALS AND METHODS

**Enzymes and chemicals.** All chemicals and enzymes used were of the highest quality available. Restriction endonucleases were obtained from Bethesda Research Laboratories, Gaithersburg, Md. T4 polynucleotide kinase, T4 DNA ligase, [α-$^{32}$P]dATP, and [γ-$^{32}$P]dATP were obtained from Amersham Pty. Ltd., Australia. The nucleotide sequencing kit was obtained from BRESA, Adelaide, South Australia. The site-directed mutagenesis kit was obtained from Amersham Pty. Ltd. Oligonucleotides were synthesized by K. Newell, C.S.I.R.O. Division of Plant Industry, Canberra, Australia.

**Bacterial strains and plasmids.** All of the bacterial strains used were derived from *E. coli* K-12 and are described, together with the plasmids used, in Table 1.

**Media and cell culture.** Cells were grown overnight with shaking at 37°C in medium 56 with supplements, as previously described (12), and were harvested in stationary phase. A phosphate-free, buffered medium, used in the assay of $P_i$ uptake and for starvation of cells of phosphate (uptake medium) contained 40 mM KCl, 10 mM $(NH_4)_2SO_4$, and 1 mM $MgSO_4$ and was buffered with 25 mM MOPS [morpholinepropanesulfonic acid] at pH 6.5 (measured at 37°C). The uptake medium was supplemented with a carbon source and other relevant supplements to the same extent as the growth medium. This uptake medium, supplemented with 0.08% (wt/vol) Neopeptone (Difco Laboratories, Detroit, Mich.) and 0.2% (wt/vol) yeast extract, constituted the low-phosphate medium used for growing cells under the condition of *pho* derepression.

**Preparation of plasmids.** Plasmid DNA was prepared as described by Selker et al. (15). Chromosomal DNA was prepared by the method described by Saito and Miura (13).

**DNA sequencing.** Nucleotide sequences were determined by the dideoxy-chain termination method of Sanger et al. (14) using either [α-$^{32}$P]dATP or α-$^{35}$S-labeled dATP with *pstA*-specific oligonucleotide primers (Table 2).

**Site-directed mutagenesis.** The method used for site-directed mutagenesis was that outlined in the Amersham handbook titled "Oligonucleotide-directed in vitro mutagenesis system".

**Measurement of phosphate uptake.** The assay for phosphate uptake (12) and the apparatus used (10) were described elsewhere. Specific uptake through the Pst system was measured at 0.5 μM $^{32}P_i$ (4).

**Assay of alkaline phosphatase.** Alkaline phosphatase activity in colonies was detected by a rapid spray method (3) or measured quantitatively by a method described previously (4).

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TABLE 1. Strains of *E. coli* and plasmids

| Bacterial strain or plasmid | Relevant genotype | Reference or other information |
|---|---|---|
| Bacteria | | |
| AN1685 | *pstA2 argH pyrE entA srl*::Tn*10 recA* | 18 |
| AN818 | *uncD409 argH pyrE entA recA* | |
| AN2658 | pAN411 *uncD409 argH pyrE entA recA* | AN818 transformed with pAN411 |
| AN724 | *ilvC argH pyrE entA recA* | 9 |
| AN2793 | pAN468 *pstA2 argH pyrE entA srl*::Tn*10 recA* | AN1685 transformed with pAN468 |
| AN2794 | pAN469 *pstA2 argH pyrE entA srl*::Tn*10 recA* | AN1685 transformed with pAN469 |
| AN2795 | pAN470 *pstA2 argH pyrE entA srl*::Tn*10 recA* | AN1685 transformed with pAN470 |
| AN2812 | pAN481 *pstA2 argH pyrE entA srl*::Tn*10 recA* | AN1685 transformed with pAN481 |
| AN2796 | pAN471 *pstA2 argH pyrE entA srl*::Tn*10 recA* | AN1685 transformed with pAN471 |
| Plasmids | | |
| pHC79 | $Ap^r$ $Tc^r$ | pBR322 derivative, obtained from B. Hohn |
| pAN411 | $Ap^r$ *pstA2* | Cosmid carrying *pstA2* allele |
| pAN45 | $Cm^r$ $Tc^s$ *uncB*$^+$ *E*$^+$ *F*$^+$ *H*$^+$ *A*$^+$ *G*$^+$ *D*$^+$ *C*$^+$ | 5 |
| pBR328 | $Cm^r$ $Tc^r$ $Ap^r$ | |
| pAN468 | $Ap^r$ $Cm^s$ $Tc^s$ *pstA402* | *Alu*I fragments from site-directed *pstA* mutants cloned into an *Eco*RI-*Bam*HI derivative of pBR328[a] |
| pAN469 | $Ap^r$ $Cm^s$ $Tc^s$ *pstA403* | |
| pAN470 | $Ap^r$ $Cm^s$ $Tc^s$ *pstA404* | |
| pAN471 | $Ap^r$ $Cm^s$ $Tc^s$ *pstA*$^+$ | |
| pAN481 | $Ap^r$ $Cm^s$ $Tc^s$ *pstA406* | |

[a] This describes plasmids pAN468, pAN469, pAN470, pAN471, and pAN481.

## RESULTS

**Characterization of the *pstA2* allele.** Sprague et al. (16) isolated a mutant strain defective in $P_i$ transport, and the mutant allele responsible for the defect was designated *pst-2* by Zukier and Torriani (20) and, later, *pstA2* by Cox et al. (4). Chromosomal DNA was prepared from strain AN1685 (*pstA2*) partially digested with the restriction endonuclease *Hin*dIII and ligated to *Hin*dIII-digested cosmid vector pHC79. The concatamers of appropriate size were then packaged in vitro into bacteriophage lambda particles. Strain AN818 (*uncD409*) was transformed with the lambda-packaged DNA, and transformants were selected on ampicillin-succinate minimal medium. The *uncD* gene and the *pst* genes are on the same *Hin*dIII restriction fragment. One colony was selected and purified (AN2658), and plasmid DNA (*pAN411*) was prepared. The plasmid DNA was digested with the restriction endonuclease *Alu*I, and the fragments were separated by electrophoresis in low-melting-point agarose. The fragment (1,603 base pairs) carrying the *pstA* gene was identified by hybridization with a $^{32}P_i$-labeled, *pstA*-specific oligonucleotide. The *Alu*I fragment carrying the *pstA* allele was cloned into the *Sma*I site of the vector M13mp18, and the nucleotide sequence was determined by using *pstA*-specific oligonucleotide primers. The nucleotide sequence of the *pstA2* allele differed from that of the *pstA* gene only in that a G→A substitution had occurred at position 276 of the nucleotide sequence (Fig. 1). This substitution converts the triplet TGG coding for tryptophan at position 92 into the chain-terminating triplet TGA. The resultant short polypeptide is unlikely to be assembled in the membrane, and strain AN1685 (*pstA2*) was therefore used as a recipient strain for plasmids carrying *pstA* alleles (see below).

TABLE 2. Synthetic oligonucleotides used in the preparation of mutants and as sequencing primers for sequencing the *pstA* gene

| Synthetic oligonucleotide | Relevant amino acid substitution |
|---|---|
| 5′-CCAGCAATTTGGGCAATCG[a] | Arg-220→Gln |
| 5′-TCGGTGGTTTGGATAACAA[a] | Arg-170→Gln |
| 5′-TCAGCATGTTCTGGGTGGT[a] | Glu-173→Gln |
| 5′-GAGAGGGTTAGGGTGAGG[b] | |
| 5′-TCGCAGAGATCATCTTCC[b] | |
| 5′-ACCGAATACCGTGGCCCA[b] | |

[a] Underlined nucleotides denote differences from the normal sequence.
[b] *pstA*-specific sequencing primers.

**Substitution of Arg-220 by glutamine, Arg-170 by glutamine, and Glu-173 by glutamine in the PstA protein.** A secondary and tertiary structure of the integral-membrane PstA protein has been proposed (17). A number of the

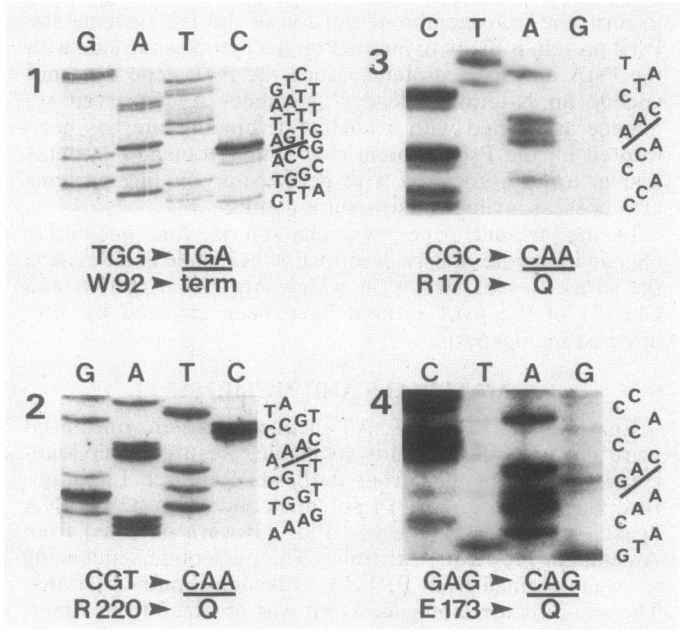


FIG. 1. Portions of DNA sequencing gels of various *pstA* alleles. (1) *pstA2*; (2) *pstA402*; (3) *pstA403*; (4) *pstA404*. The mutant triplets are underlined, and the resulting amino acid changes are indicated.

transmembrane helices in the proposed structure included charged amino acids, for example, arginine-170 and glutamate-173 in helix 4 and arginine-220 in helix 5. It has been suggested that such residues only exist in transmembrane helical segments if they are functionally important (9a). The residues arginine-170, glutamate-173, and arginine-220 were therefore each replaced with glutamine by using site-directed mutagenesis.

The *Alu*I restriction fragment carrying the *pstA* gene (see above) was prepared from the plasmid pAN45 by electrophoresis of the *Alu*I digest in low-melting-point agarose. The purified fragment was cloned into the *Sma*I restriction site of the vector M13mp18, and a single-stranded template DNA was prepared according to the Amersham handbook. The mutant oligonucleotides used for each site-directed mutagenesis are listed in Table 2. The site-directed mutagenesis method used was that described in the Amersham handbook. Four of the resultant plaques from each site-directed mutagenesis were screened by nucleotide sequencing. Two of four plaques were found to be mutant for both the Arg-170 and Glu-173 changes, whereas all four plaques of the Arg-220 change were mutant (Fig. 1). One of each mutant was selected, and the complete *pstA* gene was sequenced. The sequences confirmed that the only difference between the normal *pstA* gene and the site-directed mutant alleles was that expected, given the sequence of the particular mutant oligonucleotide. The replicative forms of vector M13mp18, carrying each of the mutant inserts, were prepared and treated with the restriction endonucleases *Eco*RI and *Bam*HI, utilizing restriction sites that were in the polylinker region of M13mp18. The vector pBR328 was also treated with the restriction endonucleases *Eco*RI and *Bam*HI, mixed with each of the three restricted M13mp18 preparations carrying the mutant inserts, and ligated with T4 DNA ligase, and the mixtures were used to transform strain AN724 to ampicillin resistance. The transformants from each of the three transformations were then screened for chloramphenicol sensitivity. Plasmids were prepared from four such transformants and screened by gel electrophoresis for the presence of the *Eco*RI-*Bam*HI fragment. One such plasmid from each transformation was retained, and these were used to transform strain AN1685 (*pstA2*) to ampicillin resistance. Four transformants from each of the three transformations were purified and screened for alkaline phosphatase activity by a rapid spray method (3). All four transformants from each of the three transformations lacked alkaline phosphatase activity, and one strain from each group was retained for further work. Strain AN2793 (Arg-220→Gln) carries plasmid pAN468 with the mutant allele *pstA402*, strain AN2794 (Arg-170→Gln) carries plasmid pAN469 with the mutant allele *pstA403*, and strain AN2795 (Glu-173→Gln) carries plasmid pAN470 with the mutant allele *pstA404*. The double *pstA* mutant (Arg-170→Gln and Glu-173→Gln) was prepared by site-directed mutagenesis from a single-stranded template DNA preparation from the plasmid carrying the Arg-170→Gln mutation. Strain AN2812 (Arg-170→Gln and Glu-173→Gln) carries plasmid pAN481 with the mutant allele *pstA406*.

**Phosphate transport and alkaline phosphatase activities.** Phosphate transport through the Pst system and alkaline phosphatase activities under conditions of both phosphate deprivation and phosphate sufficiency were measured in strains AN1685 (*pstA2*), AN2793 (Arg-220→Gln), AN2794 (Arg-170→Gln), AN2795 (Glu-173→Gln), AN2812 (Arg-170→Gln and Glu-173→Gln), and AN2796 (*pstA*$^+$). Strain AN2796 (*pstA*$^+$) was prepared from strain AN1685 (*pstA2*) by transformation using a pBR328 derivative plasmid carrying the wild-type *pstA* gene. This plasmid, pAN471, is identical to each of the mutant plasmids except for the mutations introduced by site-directed mutagenesis. The results obtained with these six strains are summarized in Table 3. Phosphate uptake through the Pst system was absent from both the parental mutant control strain AN1685 (*pstA2*) and strain AN2793 (Arg-220→Gln). Phosphate uptake also appeared slightly impaired in the double mutant strain AN2812 (Arg-170→Gln and Glu-173→Gln). The remaining strains had normal Pst activity. Surprisingly, alkaline phosphatase activity was not derepressed in strain AN2793 (Arg-220→Gln) in phosphate-sufficient medium even though phosphate transport through the Pst system was absent (Table 3).

TABLE 3. Phosphate uptake rates and alkaline phosphatase activities of various *pstA* mutant strains

| Strain (amino acid change) | $P_i$ uptake[a] | APase activity[b] | |
|---|---|---|---|
| | | Unstarved cells | Starved cells |
| AN1685 (Trp-92→term) | 1.3 | 0.46 | 0.43 |
| AN2793 (Arg-220→Gln) | 1.5 | <0.05 | 0.36 |
| AN2794 (Arg-170→Gln) | 32 | <0.05 | 0.37 |
| AN2795 (Glu-173→Gln) | 33 | <0.05 | 0.34 |
| AN2812 (Arg-170→Gln, Glu-173→Gln) | 24 | <0.05 | 0.35 |
| AN2796 | 32 | <0.05 | 0.34 |

[a] $P_i$ uptakes are expressed as nanomoles of $P_i$ per minute per milligram of dry weight.
[b] Alkaline phosphatase (APase) activities are expressed as micromoles of *p*-nitrophenol formed per minute per milligram of protein.

## DISCUSSION

A structure for the PstA protein, comprising six transmembrane helices, has been previously proposed (17). In this structure, residues Arg-170, Glu-173, and Arg-220 were all buried within the membrane. Given the difficulty of submerging charged residues in membranes (6), it has been suggested that such residues only exist in transmembrane helical segments if they are functionally important (9a). The residue Arg-220 clearly fulfills this requirement, but it appears that neither Arg-170 nor Glu-173 is functionally important. The structure of the PstA protein has therefore now been reassessed, and the six transmembrane helices of the rationalized structure are depicted in Fig. 2. The major alteration from the previously proposed structure is in helix 4, where residues Arg-170 and Glu-173 are no longer within the membrane. Helix 5 of the proposed structure carries the functional residue Arg-220. The structure gains some support from a comparison of the PstA sequence with the sequence of the other integral membrane protein, PstC. Helices 4 and 5 appear to be conserved to some extent in both proteins, with about 35% amino acid homology, including the functionally important Arg-220 of the PstA protein (Fig. 2).

The lack of alkaline phosphatase synthesis in the strain carrying the Arg-220→Gln mutation is particularly interesting. It would appear that the Pho regulon is not controlled by the movement of phosphate through the Pst system. It is clear from mutant studies, however, that the Pst system is involved in the transmembrane signaling event that controls the Pho regulon (11). The interaction of the phosphate-charged PstS protein with the membrane portion of the Pst system is apparently sufficient to trigger the appropriate

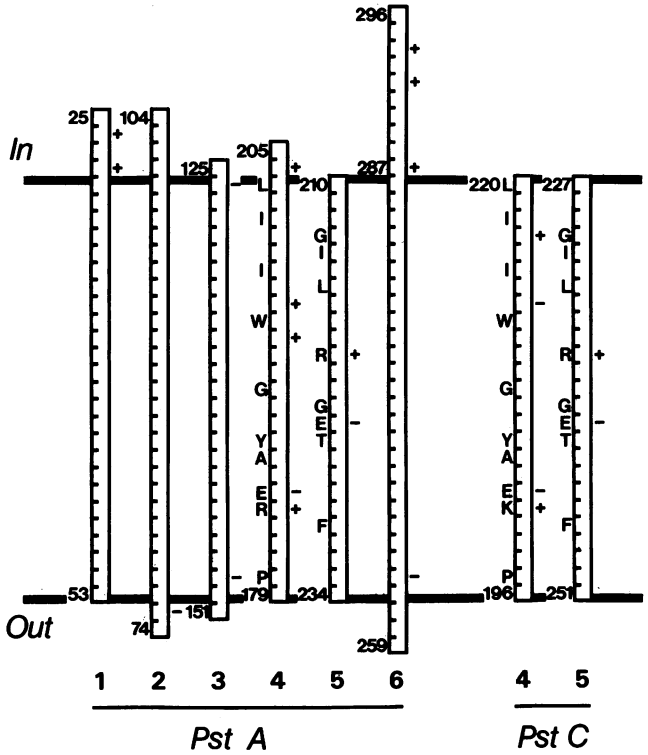


FIG. 2. Proposed transmembrane helices of the PstA protein and helices 4 and 5 of the PstC protein. The N-terminal and C-terminal residue numbers for each helix are indicated. All charged residues (+, −) are indicated, as are those residues in helices 4 and 5 of the PstA protein that are conserved in corresponding helices of the PstC protein.

transmembrane signal which results in repression of the Pho regulon. Previously described mutations, such as the *pstA2* allele, probably affect both phosphate uptake and the transmembrane signal because of lack of assembly of the Pst complex.

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