Role of the Linker Region of the Anion-stimulated ATPase ArsA

EFFECT OF DELETION AND POINT MUTATIONS IN THE LINKER REGION*

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The anion-stimulated ATPase ArsA in *Escherichia coli* consists of two homologous halves, A1 and A2, which are connected by a 40-amino acid long stretch of residues designated as the linker region. The linker region of ArsA lies in close proximity of the nucleotide-binding domain(s) of ArsA and is involved in significant conformational changes on binding of the substrates. Hence, it has been suggested earlier that the linker may play an important role in the function of ArsA. The aim of the present study was to determine the role of the linker by deletion and by site-directed mutagenesis of specific residues. Effect of deletion of the linker was determined by using the *in vivo* complementation approach where two halves of ArsA were co-expressed either with or without the linker region. Two co-expressed halves of ArsA conferred arsenite resistance only if the linker region was present on one of the halves. Of the six different point mutations created in the linker region, three (G284S, R290S, and D303G) were seen to drastically affect the catalytic function of ArsA. In addition, these three mutant alleles conferred arsenite sensitivity in cells carrying the wild type *arsB* gene. Trypsin proteolysis studies carried out with the purified proteins showed that the A1 nucleotide-binding domain in D303G protein has a conformation different from the wild type ArsA, suggesting that the linker region interacts with the nucleotide-binding domain(s) of ArsA. Based on the studies presented here, we propose that, in addition to providing flexibility, the nature of the residues themselves in the linker region is important for the conformation of the nucleotide-binding domains and for the catalytic function of ArsA.

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ArsA is the peripheral membrane component of the anion-translocating ATPase ArsAB of *Escherichia coli*. ArsB forms the carrier for the export of the anion while ArsA functions as the catalytic subunit that transduces the energy of ATP hydrolysis to the export via ArsB. ArsA is made up of two homologous halves, A1 and A2, which are connected by a flexible linker consisting of roughly a 40-amino acid long stretch of residues (1). Each half of ArsA contains one nucleotide-binding domain. The A1 half carries out low-level (unisite) catalysis in the absence of antimonite (2). Multisite catalysis with about 30–40-fold stimulation of the activity occurs on addition of antimonite and it requires intact A1 and A2 domains (2). It has previously been proposed that binding of antimonite opens up the A2 site for ATP binding, followed by interaction between A1 and A2 which then results in multisite catalysis (2, 3). One possible mechanism that has been proposed for formation of the A1/A2 interface is via coordination of antimonite to two cysteine residues in the A1 half and to one cysteine in the A2 half of ArsA, which then pulls the A1 and A2 domains together (4). Whether the linker region that connects the A1 and A2 halves of ArsA plays an important role in the function of ArsA is an open question. A recent study from B. P. Rosen’s (5) group has proposed that the linker is a connector that holds the A1 and A2 domains together and its only function is to provide flexibility. They have suggested that only the length of the linker is important and, as such, it is not required for function of ArsA (5).

Previous studies have, however, suggested that the linker region lies in close proximity to the nucleotide-binding site(s) in ArsA. It has been observed that under catalytic conditions, ADP, the product of the catalytic reaction, is associated with the linker so that upon exposure to UV, ADP is cross-linked to the linker region (2). Furthermore, the linker is the site of major conformational changes upon binding of the substrate ATP and the ligand antimonite to ArsA. Residue Arg290 in the linker is the site for initial trypsin cleavage in ArsA. However, this residue becomes unavailable for cleavage upon simultaneous binding of ATP and antimonite (3), thus suggesting a major conformational change involving the two domains of ArsA and the linker. These findings suggest that the linker plays an important role in the function of ArsA. Hence, the present investigation was aimed at determining if linker, in addition to serving as a flexible tether that holds the two domains together, plays a crucial role in the catalytic function of the protein. In this work, we studied the role of the linker by creating a deletion of the linker region as well as by introducing point mutations in residues in the linker. The data suggest that the deletion of the linker disrupts arsenite resistance. We also find that some point mutations in the linker region, such as G284S, R290S, and D303G, drastically affect function of ArsA while some others have no significant effect. Furthermore, some linker mutations also affect folding of the nucleotide-binding domains, indicating that residues in the linker interact with residues in the nucleotide-binding domains. Thus, based on the previous observations (2, 3) and the data presented in this paper, we propose that residues in the linker play an essential role in the function of ArsA and that the nature of the residues themselves is important.

MATERIALS AND METHODS

Subcloning of the ArsA Fragments, N30, N35, C31, and C35—N30 (residues 1–279) and N35 (1–323) fragments of ArsA were subcloned into *Nde*I and *Hind*III sites of pET16b vector. The C31 (221–583) and C35 (278–583) fragments of ArsA were subcloned into the *Nde*I and *Hind*III sites of pDX101 (6), which is a derivative of pSU2718. The ArsA fragments in each case were amplified by polymerase chain reaction and the appropriate restriction sites were introduced into the primers.
Site-directed Mutagenesis of Residues in the Linker—Site-directed mutations in the linker region of ArsA were made by using the Quick-change™ in vitro mutagenesis kit (Stratagene). pUC18arsAB(7) was used as the template. The sequence of the primers used to introduce changes in arsA is given below. The changed residues are underlined.

| Change | Sequence |
|--------|----------|
| G284S  | GTCGCGTACGCTTCATGGTTGCTGGCCGTC | |
| R290S  | GCATCAGCGCTTCATGGTTGCTGGCCGTC | |
| D303G  | GCATCAGCGCTTCATGGTTGCTGGCCGTC | |

The A1 and A2 nucleotide-binding domains and the sequence of the linker region of ArsA are shown. The residues in the linker which were subjected to mutagenesis are underlined. The location of various subclones of ArsA, discussed in this article, is also shown.

**Deletion**

Effect of the deletion of linker on arsenite resistance was studied by testing for in vivo complementation between two half-clones of arsA (corresponding to the A1 and the A2 domain) which lack the sequence for the linker region. It has been shown previously that two half-clones of ArsA (for example, N35 and C46, Fig. 1) can complement each other in vivo in E. coli to confer arsenite resistance (7). In the present study, an A1 clone, N30 (residues 1–279), was tested in combination with two A2 clones, one, C35-(278–583), containing the linker sequence, and the other, C31-(321–583), without the linker sequence. The rationale for the experiment described here is as follows: if the linker region of ArsA is merely a flexible connector that pulls together two halves of ArsA, thus resulting in an A1/A2 interface, then two half-clones of ArsA lacking the linker sequence would exhibit the same level of arsenite resistance as seen with half-clones containing the linker. Since in this experiment, the A1 and the A2 halves of ArsA are on separate polypeptides, the flexibility of the domains would not be a critical element. On the other hand, if the linker is required for function of the catalytic domains, then such a combination would lose arsenite resistance. The data in Fig. 2 suggest that the co-expression of the A1 clone N30 and the A2 clone C31 does not confer arsenite resistance in cells containing the wild type arsB gene, whereas co-expression of N30 and C35 results in complementation so that an almost wild type level of arsenite resistance is seen. Since no arsenite resistance is seen in the absence of the linker, this experiment suggests that the linker may be important for the function of ArsA. To rule out the possibility that the absence of arsenite resistance in the N30 and C31 combination may be due to the inability of C31 to fold properly, complementation assay was also performed between N35 and C31. The data in Fig. 2 suggest that the co-expression of N35 and C31 results in arsenite resistance to the same level as seen with N30 and C35, thus indicating that C31 is folded properly. (It should be pointed out that the intermediate level of arsenite resistance seen in cells containing the C-clones (C31 and C35) results from the presence of the wild type arsB gene on these plasmids; this is due to the ability of ArsB to carry out pmf-driven export in the absence of ArsA, thus conferring a low-level arsenite resistance (8).
Point Mutations

To understand the role of the linker and to determine if the nature of the residues in the linker is important, five residues of the 40-amino acid-long linker region were initially chosen for site-directed mutagenesis. The linker sequence contains one glycine (Gly284), two basic (Arg290 and Arg309) and three acidic (Asp303, Asp311, and Asp320) residues. In this study, we targeted five (Gly284, Arg290, Asp303, Asp311, and Asp320) of these six residues. Arg290 was mutated to two different residues, one a highly conserved lysine, and the other a semiconserved serine. Gly284 was changed to a serine and the three “Asp” residues were each changed to a glycine. The resulting six mutant alleles were subcloned into pET16b vector to create fusions of the mutant proteins with the His6 tag at their N termini.

(i) In Vivo Effects—To determine the effect of the linker mutations on the phenotype, arsenite resistance of cells expressing the mutant arsA alleles was determined. E. coli cells containing plasmids with mutant arsA alleles were grown in LB containing different concentrations of sodium arsenite and the growth was measured at 600 nm after 12 h of growth at 37 °C with aeration. *, pET16b (vector); ■, pET16b arsA303G; □, pET16b arsA309G; ●, pET16b arsA311G; ■, pET16b arsA320G; ○, pET16b arsA290S; †, pET16b arsA290K; ×, pET16b arsA290E; ●, pET16b arsA290B; ○, pSU2718 arsA290E; †, pSU2718 arsA290B; ○, pSU2718 arsA290S; ●, pSU2718 arsA290K.

(ii) In Vitro Effects—The mutant proteins were purified using Ni-NTA affinity chromatography. The purified mutant proteins were tested for ATPase activity by measuring release of \( ^{32} \text{P} \) on hydrolysis of \([\gamma^{32} \text{P}]\)ATP. Wild type ArsA normally shows about 30–40-fold stimulation of ATPase activity on addition of antimonite which is also seen in the data shown in Table 1. In addition, these data show that some point mutations in the linker significantly affect the multisite catalytic activity (obtained in the presence of antimonite) of ArsA. In G284S mutation, no stimulation of activity is seen in the presence of antimonite, hence no multisite catalysis occurs which is consistent with complete loss of arsenite resistance in this mutant seen in Fig. 3. R290S is also significantly affected so that only a 4-fold stimulation of activity is seen. Of the three Asp to “Gly” mutations, D303G is the most affected and it shows only about 5-fold stimulation of activity in presence of antimonite which is also seen in the data shown in Fig. 2. R290K, D311G, and D320G are 5-fold stimulation of activity. R290K, D311G, and D320G are not significantly affected.

To determine if the mutant proteins have a conformation different from the wild type protein, sensitivity of proteins to trypsin treatment in the presence of antimonite and ATP was determined. Previous studies have shown that an initial cleavage with trypsin at Arg290 in the linker cleaves ArsA into a 32-kDa N-terminal and a 27-kDa C-terminal fragment (3). The 27-kDa fragment is further cleaved into smaller fragments while the 32-kDa N-terminal fragment, being more compact, is not cleaved further. In the presence of both antimonite and ATP, however, Arg290 is protected from trypsin proteolysis so that most of the protein is found in the original 63-kDa species, thus suggesting that binding of ATP and antimonite produces a conformational change which makes Arg290 inaccessible (3). This trypsin resistant conformation, in which most protein is present in the 63-kDa species, has previously been designated “AS” while the conformation in the presence of ATP, in which most protein is in the 32-kDa species and some in the 54-kDa species, is designated “A” (3). Proteins containing mutations in the linker region of ArsA, with the exception of D303G and D324S, behave very much like the wild type ArsA when subjected to trypsinolysis as shown in Fig. 4. D303G was found to be much more sensitive to trypsin both in the absence and presence of ATP and antimonite. Data in Fig. 4A show that the 32-kDa N-terminal fragment, which in the wild type protein is very compact and hence trypsin resistant, is easily digested in D303G protein. Intensity of the 32-kDa band in D303G in lane 5 is much less as compared with wild type in lane 2 in Fig.
A breakdown product smaller than 25 kDa in size (shown with the arrow) is seen in D303G (lanes 5–7), but this fragment is not seen in wild type ArsA (Fig. 4, A or B, lanes 2–4) or in the other linker mutants shown in Fig. 4A (lanes 8–13) and Fig. 4B (lanes 5–13). Furthermore, in D303G protein the AS conformation is not achieved so that there is no difference between lanes 6 and lane 7 in D303G (Fig. 4A) while in the wild type (lanes 3 and 4) and in other linker mutants the A and AS are distinct conformations (Fig. 4, A and B). R290S lacks the trypsin site, hence its pattern does not conform to the pattern of other mutants (Fig. 4B). G284S gives an intermediate phenotype hence its pattern does not conform to the pattern of other linker mutants shown in Fig. 4, A and B.

To determine if the smaller fragment in D303G (mentioned above) results from breakdown of the 32-kDa N-terminal fragment, Western blot analysis using anti-N18 or the anti-C31 serum was carried out. Data in Fig. 5 show the Western blots. Samples in Fig. 5A correspond to the Coomassie-stained gel shown in Fig. 4A. Similarly, samples in Fig. 5B correspond to the samples in Fig. 4B. The upper panel in each case (Fig. 5, A or B) is probed with anti-N18 and the lower panel is probed with anti-C31 serum. Data in Fig. 5A (upper panel) show that the 25-kDa breakdown fragment seen in D303G indeed belongs to the N terminus, hence it is the result of cleavage of the 32-kDa N-fragment. This fragment is not seen with the anti-C31 antibody (lower panel, Fig. 5A) nor is it seen with anti-N18 antibody in other point mutants shown in Fig. 5, A or B.

The ability of the linker mutants to form an adduct with ADP in the presence of ATP and magnesium on exposure to UV light was also tested. Earlier studies have shown that the UV-activated adduct between ArsA and ADP is localized in the linker region, thus suggesting that the linker is in close proximity to the nucleotide binding/catalytic site(s) (2). Data in Fig. 6 suggest that the three Asp mutants and the R290K mutant behave like the wild type protein, hence are normal in ADP adduct formation, however, the G284S and the R290S do not form the ADP adduct.

**TABLE I**

| ArsA protein | -Sb | +Sb | Stimulation | Remaining activity (fold) % |
|--------------|-----|-----|-------------|---------------------------|
| Wild type    | 33  | 863 | 26.2        | 100                       |
| G284S        | 15  | 21  | 1.4         | 2.4                       |
| R290S        | 21  | 86  | 4.1         | 10                        |
| R290K        | 33  | 560 | 17          | 64.9                      |
| D303G        | 42  | 191 | 4.6         | 22.1                      |
| D311G        | 31  | 454 | 14.6        | 52.6                      |
| D320G        | 31  | 529 | 17.1        | 61.3                      |

**FIG. 4. Trypsin cleavage patterns of wild type and mutant ArsA proteins.** Purified wild type and mutant ArsA proteins were subjected to trypsin proteolysis as described under “Materials and Methods.” The samples were drawn at different time points (in the presence of antimonite at 20, 40, 60, 80, and 100 s, while in the absence of antimonite at 1, 2, 3, 4, and 5 min) and analyzed by TLC as described. The data presented are means of two separate experiments.

**DISCUSSION**

The A1 and the A2 domains of ArsA are connected by a 40-amino acid long stretch of residues (283–320) which have been designated the linker region (1). Previous studies suggest that the linker region is in close proximity to the nucleotide binding/catalytic site(s) of ArsA (1) and it is involved in major conformational changes (3); hence, it has been proposed that residues in the linker region may have an important role in the catalytic function of ArsA. Alternatively, it is also possible that the linker is merely a flexible hinge that holds/brings the two...
domains of the protein together; if so, then the length of the linker would be more important than the nature of the residues themselves. Such a role for the linker has been proposed in a recent study carried out by Li and Rosen (5). In that study, progressively longer deletions of the linker were created. 5-Codon deletions, including 295–299, 300–304, and 305–309 showed significantly reduced activities ranging from 24 to 40% of the wild type ArsA. A 23-codon deletion (A291–313) contained only about 20% activity and this protein could not be allosterically activated by antimonite. The authors proposed that, because of the shortening of the linker, the three cysteines, Cys^{113} and Cys^{172} in the A1 half and Cys^{422} in A2, cannot be pulled together by antimonite to form the A1/A2 interface, thus suggesting that only the length of the linker is of significance. However, an alternate explanation of these data is that the linker affects the structure and function of the A1 and A2 domains, thus the effect seen may not be due to shortening of the linker but because of the role of the linker in the function of ArsA.

In the present work, we studied the role of the linker by determining the effect of the deletion of the linker as well as point mutations in certain residues in the linker region. Instead of creating a simple deletion of the linker, we studied the effect of the absence of the linker on in vitro complementation between the A1 and A2 halves of ArsA. We used an N-terminal A1 clone, N30, that lacks the linker sequence, with two different A2 clones, one without the linker (C31) and the other containing the linker sequence (C35). In the approach described here, each domain of ArsA is on a separate polypeptide, hence deletion of the linker will not constraint movement of the domains. Thus, absence of the linker is expected to have very little effect except if it is required for function of ArsA. The data suggest that N30 and C35 can complement each other but N30 and C31 cannot (Fig. 2), indicating that the linker is essential for function and not merely for flexibility. Since co-expression of N35 and C31 also results in complementation, it further suggests that the linker can function either in cis or trans. It is possible that the linker region lies at the interface between the A1 and A2 halves, hence whether it lies on the A1 or the A2 half does not affect the outcome.

To determine if nature of the residues in the linker region is crucial for the function of ArsA, site-directed mutagenesis of certain residues in the linker region was carried out; the rationale being that if certain residues in the linker are crucial for function of A1 and A2 domains, then mutations in these residues would significantly affect the function of the protein. If only length of the linker is of import, then point mutations may not have any effect on the function per se.

Our studies suggest that of the five residues tested, Gly^{284}, Arg^{290}, and Asp^{303} are crucial for function of ArsA (Table I, Fig. 3). G284S demonstrates no multisite catalysis, while R290S and D303G show only about 4–6-fold stimulation of the ATPase activity on addition of antimonite. This trend in the ATPase activity of mutants is also generally reflected in the arsenite resistance phenotype shown in Fig. 3. Of the three Asp mutants D303G is the least arsenite resistant and behaves very much like the R290S and G284S mutants. D303G contains about 22% of the wild type activity; hence its very high arsenite sensitivity might suggest a role for Asp^{303} residue in interaction between ArsA and ArsB.

D303G mutation also affects conformation of ArsA. Trypsin proteolysis experiments suggest that this mutation affects folding of the A1 domain, thus making it more susceptible to trypsin, implying that an interaction occurs between residues in the linker region and residues in the A1 domain. These findings are consistent with the earlier observation which showed that linker is the site of UV-activated ADP adduct implying that linker lies in close proximity of the nucleotide-binding site(s) in ArsA (1, 2). Trypsin protection experiments show that D303G does not respond to the presence of antimonite so that no difference is seen between the A and AS conformations (Fig. 4). Since binding of antimonite results in multi-
site catalysis, it is reasonable to assume that antimonite brings about an interaction between the A1 and A2 domains. Hence, absence of the AS conformation in D303G suggests that this mutation affects conformation of the nucleotide-binding domains and interaction between them.

The three Asp mutants show no defect in the formation of the UV-activated ADP adduct, whereas G284S and R290S lose adduct formation. The inability of G284S to form an ADP adduct can be explained by its inability to hydrolyze ATP. R290S shows a low-level hydrolysis in the presence of antimonite, however, no ADP adduct is formed, whereas R290K is unaffected in its ability to form the UV adduct. These results suggest that, in this position, a positively charged residue is required for activity of ArsA (Table I), and that this residue is also involved in the formation of the UV-activated adduct with ADP (Fig. 6).

Linker sequences that connect multiple nucleotide-binding domains have also been identified in other proteins. In P-glycoprotein which is an ATP-dependent export pump, a 75-amino acid long stretch of residues connects the two homologous halves of the protein. This linker sequence contains sites for PKC and PKA phosphorylation but the role of phosphorylation at these sites in P-glycoprotein is still open to question (9). A recent study suggests that the linker in P-glycoprotein is a connector whose only function is to provide flexibility for interaction between the domains (10). However, in another protein, STE6 of yeast, the 100-amino acid long linker that connects two nucleotide-binding domains is essential for ubiquitination and fast turnover of the protein (11). The linker sequence in STE6 has been shown to contain certain motifs that are involved in ubiquitination. Hence, linker sequences in different proteins may carry out different functions. Response regulators, which play an important role in signal transduction, are another group of proteins that contain multiple domains connected by linkers. OmpR is one such bifunctional protein that contains an N-terminal phosphorylation domain and a C-terminal DNA-binding domain connected by a flexible linker which goes through conformational changes on phospho-rylation of the N-domain or on DNA binding to the C-domain. As in the case of ArsA, the linker in OmpR is protease sensitive, and the conformational changes can be easily resolved by proteolytic analysis (12). It has been suggested that the linker in OmpR may function as a signal transducer, actively communicating between the two domains in response to those signals (12).

The experiments described in this article provide strong support for the hypothesis that residues in the linker region of ArsA play an active role in the function of the protein and that there is interaction between the linker region and the nucleotide-binding domains. Mutations in five different residues tested in this study affect the activity of the protein to different extents. Of these five residues, Gly284 most likely provides flexibility which would be crucial for interaction between A1 and A2, hence no stimulation of activity is seen in the G284S mutant. Arg290 and Asp303 seem to be required for function of the protein, hence multisite catalysis in R290S and D303G is seen but to a much lesser extent than in the wild type protein. Further mutagenesis of residues in the linker, suppressor analysis as well as linker substitution experiments will be crucial for understanding the exact role of residues in the linker sequence.

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