Identification of the Site of [α-^{32}P]ATP Adduct Formation in the ArsA Protein†

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ABSTRACT: The ArsA protein, the catalytic subunit of the oxyanion-translocating ATPase encoded by the bacterial plasmid-encoded *ars* operon, and truncated derivatives were labeled with α-[^{32}P]ATP. The labeled polypeptides were cleaved with cyanogen bromide. From gas-phase microsequencing the site of labeling was deduced to lie within residues 283–323 of the ArsA protein, a region postulated to be in a flexible linker connecting the two homologous halves of the ArsA protein.

The ArsA protein encoded by *Escherichia coli* plasmid R773 is the catalytic subunit of an arsenical extrusion pump and is an oxyanion-stimulated ATPase (Kaur & Rosen, 1992a). A motif commonly found in many nucleotide binding proteins (the Walker A motif) contains the glycine-rich cluster (GXXGXGKTT/S, where X is any residue) that forms a flexible loop, most likely connecting a β sheet to an α helix (Walker et al., 1982). From mutational analysis (Senior & Al-Shawi, 1992; Yohda et al., 1988) and X-ray structure determination (Pai et al., 1977; Story & Steitz, 1992), this region appears to interact with the β and τ phosphates of ATP and is referred to as the phosphate binding loop (P-loop). From analysis of the predicted amino acid sequence the ArsA protein contains two halves, A1 and A2, that are homologous to each other and probably the result of gene duplication and fusion of a gene ancestral to the *arsA* gene (Chen et al., 1986). Both the N-terminal A1 and C-terminal A2 halves of the ArsA protein contain Walker A motifs (G_{15}KGGVGKTS$_{23}$ and G_{334}KGGVGKTT$_{342}$), respectively. Purified ArsA protein has been shown to bind two molecules of ATP per molecule of the ArsA protein (Karkaria & Rosen, 1991), indicating that both sequences are indeed components of two independent nucleotide binding sites. Light-activated adduct formation of α-[^{32}P]ATP with the ArsA protein has been used to distinguish between the nucleotide binding sites; only the A1 nucleotide binding site formed an adduct with ATP (Karkaria et al., 1990; Kaur & Rosen, 1992b, 1993).

In this study, identification of the region of the A1 domain in contact with the adenine ring of ATP was determined by direct photoaffinity labeling with α-[^{32}P]ATP. Identification of the site of photoadduct formation was facilitated by biochemical reconstitution of a catalytically active species from peptide fragments (Kaur & Rosen, 1994). The reconstituted complex exhibited both ATPase activity (Kaur & Rosen, 1994) and light-activated adduct formation with α-[^{32}P]-ATP. Although the chemistry of the photolabeling reaction is not known, adduct formation is thought to occur through the ring of the nucleotide base (Kierdaszuk & Eriksson, 1988). Thus, the Walker A sequence defines the location of the phosphoryl groups of ATP, while the site of adduct formation defines the location of the adenine ring. By a combination of chemical and enzymatic cleavage of the native ArsA protein and the truncated polypeptides, the use of polypeptide specific antisera, and by amino acid sequencing of labeled peptides, the region of the ArsA protein labeled with [α-^{32}P]ATP was identified. This region is approximately 280 amino acyl residues away from the A1 phosphate binding loop, defining a portion of a nucleotide binding site composed of residues distant from each other in the primary sequence.

MATERIALS AND METHODS

Purification of Proteins. Native ArsA protein was purified from the cytosolic fraction of *E. coli* cells expressing the *arsA* gene from plasmid pUM3 (Hsu & Rosen, 1989). Truncated ArsA-derived polypeptides were produced by expression of subclones of the *arsA* gene and purified from inclusion bodies as described previously (Kaur & Rosen, 1994). Inclusion bodies were washed twice with a buffer consisting of 10 mM Tris–acetate, pH 7.5, and solubilized in 50 mM Tris–acetate buffer containing 6 M guanidine hydrochloride. Polypeptides N18, N28, and N35 contained portions of the A1 half of the ArsA protein, while polypeptides C35 and C46 were chimeras of the C-terminal portion of the ArsA protein fused with the N-terminal 23 amino acids of the ArsD protein. N and C refer to the N-terminal A1 and C-terminal A2 halves of the ArsA protein respectively. The numbers indicate the molecular mass of the ArsA portion of the polypeptides; the total mass varied depending on the nature of the genetic construction (Kaur & Rosen, 1994).

Polyacrylamide Gel Electrophoresis and Immunoblotting. SDS PAGE[1] was performed as described by Laemmli (1970). Immunological blotting was carried out as described previously (Tisa and Rosen, 1990). The N18, N35, or C46 polypeptides prepared from inclusion bodies were used to raise polyclonal antibodies in New Zealand white rabbits. To remove potential antibodies against contaminants, the sera were preabsorbed with extracts from cells bearing a control plasmid without the *arsA* gene. Antisera were shown by immunoblot analysis to be specific for the predicted regions of the ArsA protein. The peptides generated by cyanogen bromide cleavage of native and N35 proteins were probed with antisera specific to the three different regions of the protein. The peptides were transferred to PVDF membrans as described earlier. The membranes were sequentially probed with antiserum to N18, N35, and C46 polypeptides. After each probing, antibodies were stripped by incubating the membrane for 1 h at 65 °C in a solution consisting of 60 mM Tris, pH 8.0, containing 1%

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; DTT, dithiothreitol.

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SDS and 2% mercaptoethanol. The membrane was washed with several changes of the blotting buffer before probing with the next antiserum.

Reconstitution of Complexes. The guanidine HCl-solubilized proteins were renatured by dialysis, as described previously (Kaur & Rosen, 1994). The N- and C-terminal peptides were mixed either before renaturation to form complexes or after separate renaturation, as indicated. The combinations of polypeptides N18 and C46 or N28 and C35 peptides form full length ArsA proteins without overlap.

Light-Activated Reaction with [α-^{32}P]ATP. Ultraviolet light-mediated photoreaction of the ArsA protein and ArsA-derived polypeptides with [α-^{32}P]ATP was performed as described previously (Kaur & Rosen, 1994). Samples were mixed with 5 μM ATP, 5 mM $MgCl_2$, and 10 μCi [α-^{32}P]-ATP in 0.1 mL total volume in a 96-well microtitration plate. The samples were irradiated with 254-nm light using a lamp placed directly on top of the microtitration plate for 30 min at 4 °C. The samples were precipitated and washed three times with 10% trichloroacetic acid, followed by SDS PAGE and autoradiography.

Cleavage with Cyanogen Bromide. CNBr cleavage was performed as described by Gross and Witkop (1967). Cleavage was carried out either in solution or in the gel slices. In solution 0.5 mg of native ArsA protein was labeled with [α-^{32}P]-ATP by a light-activated reaction and precipitated with 10% trichloroacetic acid. After centrifugation, the precipitate was washed three times with ether and allowed to air dry. The dried pellet was dissolved in 70% formic acid (v/v). Approximately 3 mg of crystalline cyanogen bromide was added, and the sample was mixed thoroughly. The mixture was incubated in the dark at room temperature for 24 h, following which it was diluted with 20 volumes of water and lyophilized to remove excess cyanogen bromide. The last step was repeated twice. The lyophilized sample was dissolved in a solution of 0.1% SDS and centrifuged in an Eppendorf microcentrifuge for 5 min at room temperature to remove insoluble material. The supernatant solution was subjected to gel filtration chromatography on a 100-mL Superose 12 column equilibrated with a buffer consisting of 50 mM ammonium bicarbonate, pH 7.8. Radioactive fractions were pooled, lyophilized, dissolved in 0.1 mL of the same buffer, and analyzed by SDS PAGE on 15% polyacrylamide gels. The peptide bands were transferred electrophoretically onto a PVDF membrane (Matsudaira, 1987) and the labeled peptides detected by autoradiography.

Cyanogen bromide cleavage was carried out in gel slices when the truncated peptides were photolabeled in reconstituted complexes. The N- and C-terminal peptides were separated by SDS PAGE on 10% polyacrylamide gels. The gels were dried and subjected to autoradiography to locate the labeled proteins. The radioactive bands corresponding to the C35, C46, or N35 proteins were excised and the gel slices equilibrated with 70% formic acid (v/v) at room temperature for 30 min. The gel slices were soaked in a solution of 5% (w/v) CNBr in 70% formic acid. After incubation for 90 min at room temperature, the gel slices were equilibrated with several changes of a buffer consisting of 0.1 M Tris–HCl, pH 8.0, and then with 2× concentrated SDS sample buffer for 15 min. To separate the peptides the gel slices were applied directly onto a 15% polyacrylamide gel and electrophoresed in a Tricine SDS buffer. Electrophoresis was carried out until the bromophenol blue marker dye was within 1–2 cm of the bottom of the gel. The gel was dried and subjected to autoradiography.

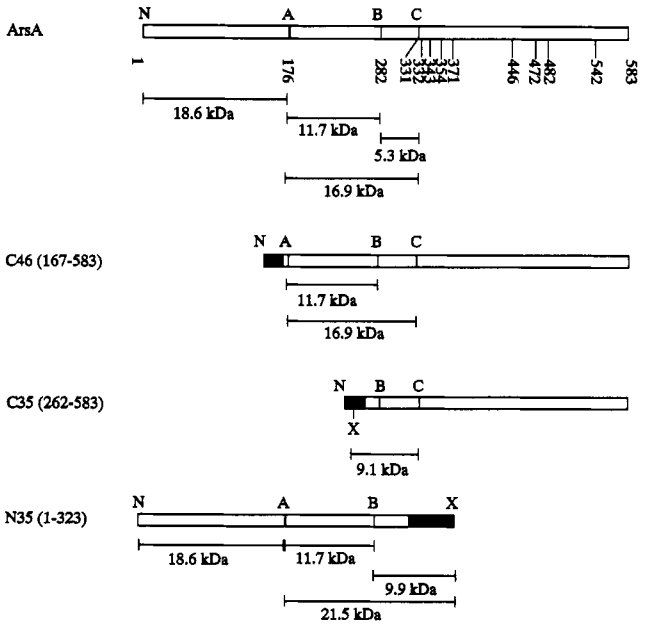

FIGURE 1: Diagrammatic representation of cyanogen bromide cleavage fragments of native ArsA protein and derivatives. The numbers under the linear representation of the ArsA protein indicate the position of the 12 methionyl residues and cyanogen bromide cleavage sites. The three relevant sites in the N-terminus are designated as A, B, and C. The calculated molecular masses of the relevant peptides are shown in kDa. X represents a methionine residue in the ArsD-derived sequence in polypeptide C35 and the end of the vector derived sequence in polypeptide N35.

Digestion with V-8 Staphylococcal Protease. For complete digestion of the labeled proteins the samples were precipitated with 10% trichloroacetic acid for 30 min on ice. The precipitated protein was washed with ether three times, air dried, dissolved in 0.1 mL of 4 M urea, pH 8.0, and incubated with V-8 protease for 12–16 h at 37 °C. V-8 protease was used at a 10:1 ratio of protein to protease. The samples were analyzed by SDS PAGE, and the gel was autoradiographed.

Amino Acid Sequencing. Proteins were transferred from 15% polyacrylamide gels to the PVDF membrane at a constant voltage of 25 V at 4 °C for 12–16 h. After autoradiography the labeled band was excised from the membrane and subjected to amino acid sequencing using a gas-phase sequencer. For sequencing from polyacrylamide gels, the gels were dried and autoradiographed briefly. The autoradiograph was used to locate the labeled band, which was excised, and the peptide eluted into a buffer of 50 mM ammonium bicarbonate, pH 8.0. The elution efficiency was monitored by scintillation counting of the eluate and the gel slice. The eluted sample was lyophilized and subjected to amino acid sequencing. Microsequencing was performed in the Macromolecular Core Facility of Wayne State University.

RESULTS

Light-Activated Reaction with α-[^{32}P]ATP. Native and truncated ArsA polypeptides were purified as described previously (Kaur & Rosen, 1994). The portion of the ArsA protein contained in the truncated polypeptides is shown in Figure 1. The polypeptides were photolabeled individually or in biochemically reconstituted complexes. None of the truncated ArsA polypeptides (N18, N28, C35, or C46) formed a Mg^{2+}-dependent adduct with ATP (Figure 2, lanes 3–6 and 19–22). However, the N35 polypeptide (corresponding to the entire A1 half) retained the ability to form a Mg^{2+}-dependent adduct after renaturation from inclusion bodies

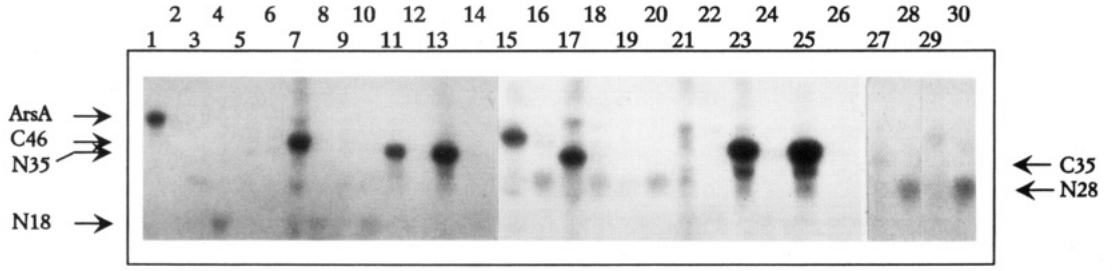

FIGURE 2: Photoadduct formation of wild-type ArsA, truncated polypeptides, and reconstituted polypeptides with [α-^{32}P]ATP. Light-activated adduct formation between wild-type ArsA protein or subclones (each protein at 5 μM, final concentration) and 5 μM [α-^{32}P]ATP was performed as described in the Materials and Methods. Samples were analyzed by SDS PAGE on 12% polyacrylamide gels followed by autoradiography. Each lane contained 15 μg of protein: lanes 1, 2 wild-type ArsA protein; lanes 3, 4, N18; lanes 5, 6, C46; lanes 7, 8, N18 + C46 corenatured; lanes 9, 10, N18 + C46 mixed after separate renaturation; lanes 11, 12, N35; lanes 13, 14, N35 + C46 corenatured; lanes 15, 16, N28 + C46 corenatured; lanes 17, 18, N28 + C35 corenatured; lanes 19, 20, N28; lanes 21, 22, C35; lanes 23, 24, N35 + C35 corenatured; lanes 25, 26, N35; lanes 27, 28, N28 + C35 mixed after separate renaturation; lanes 29, 30, N28 + C46 mixed after separate renaturation. The reactions were performed with (odd-numbered lanes) or without (even-numbered lanes) 5 mM $MgCl_2$.

(Figure 2, lanes 11 and 12), demonstrating that the N35 polypeptide refolded to a conformation containing a functional nucleotide binding site. When the N18 and C46 polypeptides were renatured separately and then mixed, specific photolabeling was not observed (Figure 2, lanes 9 and 10). Similar results were observed with the N28 and C35 polypeptides (Figure 2, lanes 27 and 28). However, when either the N18 and C46 polypeptides or the N28 and C35 peptides were coreconstituted by mixing prior to renaturation, specific Mg^{2+}-dependent adduct formation with [α-^{32}P]ATP was observed (Figure 2, lanes 7, 8, 17, and 18). Interestingly, the label was found exclusively on the C46 or C35 polypeptide, respectively. Similarly, renaturation of the N28 peptide in combination with the C46 polypeptide enabled specific photoadduct formation on the C46 polypeptide (Figure 2, lanes 15 and 16). In contrast, the label always appeared on the N35 polypeptide, even if it was renatured in the presence of the C46 or C35 polypeptides (Figure 2, lanes 13, 14, 23, and 24).

Cleavage of Native ArsA Protein with Cyanogen Bromide. Native ArsA protein and derivatives were labeled with [α-^{32}P]-ATP and cleaved with cyanogen bromide, with the predicted peptides shown in Figure 1. The ArsA protein has 12 methionyl residues in addition to the initiating methionine. There are two adjacent methionyl residues (M_{483} and M_{484}), so that on complete cleavage of the native ArsA protein 12 cyanogen bromide fragments would be expected. The A1 P-loop sequence is contained in the N-terminal 18.6-kDa fragment. The labeled CNBr fragment was fractionated by gel filtration chromatography on Superose 12, followed by SDS PAGE. Four major peaks of 280-nm absorbing material were observed, with most of the radioactivity in the second peak (data not shown). The fractions from the second peak were pooled and lyophilized. The sample was dissolved and electrophoresed on a 15% polyacrylamide gel, followed by autoradiography. The label was found predominantly in a single band of approximately 17 kDa (data not shown). The band was electrophoretically transferred to a PVDF membrane and subjected to six cycles of gas-phase microsequencing. A mixed signal comprised of two consecutive cyanogen bromide fragments sequences was observed. From the known sequences of the cyanogen bromide fragments, the two sequences were deduced to be MQFLQN and MAGLEK. These correspond to the N-terminal sequences of peptides NA (18 593 Da) and AB (11 651 Da), respectively (Figure 1). If complete cleavage had occurred, the sequence of peptide AB would not have been observed at that position on the gel. However, partial cleavage at methionine 282 would result in peptide AC (16 899 Da), which would be difficult to separate from peptide NA. Attempts to purify the CNBr peptides by HPLC using reversed-phase columns C8 or C18 were unsuccessful because the [α-^{32}P]ATP-containing peptides could not be removed from the matrix. Since CNBr fragments are very hydrophobic and tend to aggregate, they are generally difficult to purify on reversed-phase supports. Hence, whether the label existed on peptide NA or AC could not be resolved from these experiments.

Cyanogen Bromide and V-8 Protease Cleavage of Polypeptide N35. As described above, polypeptide N35 was capable of forming an adduct with [α-^{32}P]ATP. The band corresponding to labeled polypeptide N35 was excised from an SDS gel and the gel slice treated with cyanogen bromide. The mixture was analyzed by SDS PAGE followed by autoradiography (Figure 3A). The major labeled species from the native ArsA protein corresponded to a polypeptide of approximately 17 kDa (Figure 3A, lane 1). The major radioactive species from polypeptide N35 corresponded to a peptide of approximately 21 kDa (Figure 3A, lane 2). In addition, radioactive species of approximately 40 kDa (uncleaved polypeptide N35) and 10 kDa were also present in lesser amounts. The ArsA-derived portion of polypeptide N35 ends at residue 323 of the ArsA protein and contains two methionyl residues at positions 176 and 282. On complete CNBr cleavage it would generate fragments NA (18.6 kDa) and AB (11.7 kDa) in common with those from native ArsA protein (Figure 1). A third peptide (BX) would contain additional vector-derived sequence and was predicted to be 9.9 kDa. The size of the major radioactive peptide from polypeptide N35 did not correspond to a product of complete cleavage and most likely was peptide AX (21.5 kDa). This fragment would be bigger than the corresponding fragment AC (17 kDa) from native ArsA due to the additional C-terminal vector sequence. Hence, it appears that methionyl residue 282 was somewhat resistant to cleavage by cyanogen bromide.

Peptide NA (18.6 kDa) would be generated from cleavage of both the native ArsA protein and the N35 polypeptide. No radioactive band in the range of 17–18 kDa was produced by cyanogen bromide cleavage of polypeptide N35, suggesting that peptide NA was not labeled. A radioactive band corresponding to the putative mixture of peptides NA and AC was observed in the cyanogen bromide fragments from the native ArsA protein. Cleavage at methionyl 282 would generate peptides AB (11.7 kDa) in both the intact ArsA and N35 proteins and either peptide BC (5.3 kDa) or BX (9.9 kDa) in intact ArsA or N35 proteins, respectively. No

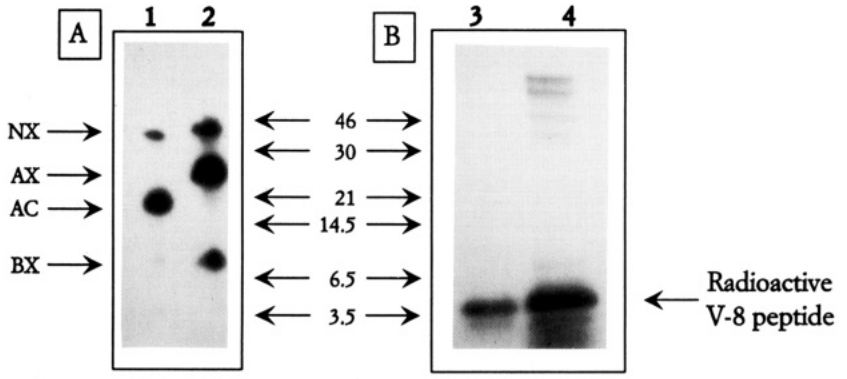

FIGURE 3: CNBr and V-8 protease cleavage patterns of native ArsA and truncated polypeptide N35 labeled with [α-^{32}P]ATP. Light-activated adduct formation between native ArsA protein or N35 polypeptide and [α-^{32}P]ATP was performed as described in the Materials and Methods. (A) The labeled polypeptides were excised from an SDS gel and subjected to cyanogen bromide cleavage. The samples were analyzed by SDS PAGE on 15% polyacrylamide gels followed by autoradiography. Lane 1: native ArsA protein. Lane 2: polypeptide N35. The presumed location of the peptides predicted from the cleavage map in Figure 1 are indicated. (B) The native ArsA and N35 proteins were subjected to complete V-8 cleavage as described in the Materials and Methods. The peptides were resolved by SDS PAGE on a 15% gel followed by autoradiography. Lane 1: N35. Lane 2: native ArsA protein. The migrations of protein standards of known molecular mass in kDa are indicated.

radioactive band corresponding to peptide AB was identified in the cyanogen bromide mixtures from either the intact ArsA or N35 proteins. However, a labeled band corresponding to peptide BX derived from the N35 polypeptide was observed, and a faint signal corresponding to BC was identified in the intact ArsA protein, indicating partial cleavage at methionyl 282 (Figure 3A). Hence, peptide BX is a subfragment of peptide AX originating from partial cleavage at M_{282}. Thus, the site of labeling lies between residues 283 and 323. As described below, the origin and nature of the cyanogen bromide fragments was verified by the use of peptide specific antisera.

Labeled ArsA protein and N35 polypeptide were digested with V–8 protease, as described in the Materials and Methods, and the peptides analyzed by SDS PAGE and autoradiography (Figure 3B). Only one major radioactive species of approximately 5 kDa was observed from digestion of both, indicating that the site of adduct formation was the same in both native ArsA protein and the N35 polypeptide. A 5-kDa V-8-labeled fragment would be predicted from cleavage between residues E_{258} and E_{304}. Thus, both sets of data argue that the site of adduct formation is located between residues 282 and 323.

Identification of Labeled Peptides using Polypeptide Specific Antisera. Polyclonal antisera to polypeptides N18, N35, and C46 were examined for cross-reactivity with other regions of the ArsA protein. The N18 antiserum did not show cross-reactivity with the polypeptide C46 antigen (data not shown). Similarly, C46 antiserum did not cross-react with the polypeptide N18 antigen. N35 antiserum cross-reacted with all three antigens (polypeptides N18, N35 and C46), as well as with intact ArsA protein; this would be expected, since polypeptide N35 has regions of overlap with both polypeptides N18 and C46. The three antisera were used to identify peptides generated by cyanogen bromide cleavage of the intact ArsA and N35 proteins. Partial cyanogen bromide cleavage of the intact ArsA and N35 proteins would be expected to yield at least three fragments in common (NB, NA, and AB), of which only NB and NA should have reacted with N18 antiserum (Figure 4A). Peptides from the intact ArsA protein that cross-reacted with N18 antiserum were approximately 36, 30, and 18 kDa, corresponding to peptides NC, NB, and NA, respectively (Figure 4A, lane 1). Peptides from polypeptide N35 that reacted with the N18 antiserum were approximately 40, 30, and 18 kDa, corresponding to peptides NX, NB, and NA, respectively (Figure 4A, lane 2). When the blot was reprobed with N35 antiserum, additional bands could be identified. With polypeptide N35, peptides AB (12 kDa), AX (21 kDa), and BX (10 kDa) were observed (Figure 4B, lane 2). With the intact ArsA protein, two additional bands were observed, corresponding to peptides AB (12 kDa) and BC (6 kDa) (Figure 4B, lane 1). Peptide AC was not identified because it comigrated with peptide NA. Peptide AX generated from polypeptide N35 was larger than peptide AC and could be identified as a separate species (Figure 4B, lane 2). The radioactive peptide BX derived from polypeptide N35 was identified when immunoblotted with N35 antiserum (Figure 4B) but not N18 antiserum (Figure 4A). Hence, this peptide originated from peptide AX by cleavage at methionine 282, generating peptide BX. These data support the conclusion that the site of adduct formation is located between residues 282 and 323 of the ArsA protein. Cleavage at residue 282 was probably incomplete in both native ArsA and N35 proteins; however, it was somewhat more amenable to cleavage in polypeptide N35. This smallest labeled fragment was clearly observed in cleavage patterns from polypeptide N35, whereas only a faint signal was observed with the native ArsA protein.

The immunoblot was stripped of N35 antibodies and reprobed with C46 antiserum (Figure 4C). The band corresponding to peptide NA was not immunoreactive, while five other immunoreactive bands could be identified in the polypeptide N35 cleavage products (Figure 4C, lane 2). Similarly, in the corresponding position in the native ArsA, a weakly immunoreactive band corresponding to peptide AC was observed (Figure 4C, lane 1). Additional peptides arising from the C-terminal portion of native ArsA were observed when reacted with C46 antiserum.

Cyanogen Bromide Cleavage of Labeled Peptides in Reconstituted Complexes. When polypeptides N18 or N28 were reconstituted with C-terminal polypeptides C35 or C46, the label was found on the C-terminal fragments (Figure 2). The labeled peptides were isolated from the reconstituted complexes as described in the Materials and Methods and subjected to CNBr cleavage (Figure 5). The radioactivity was found exclusively in a 17-kDa band derived from the C46 polypeptide (Figure 5, lane 1), whereas it appeared in a band of approximately 9 kDa after cyanogen bromide cleavage of the C35 polypeptide (Figure 5, lane 2). In the C46 and C35 polypeptides 166 and 261 residues, respectively, were deleted from the N-terminus of the ArsA protein. Following cleavage

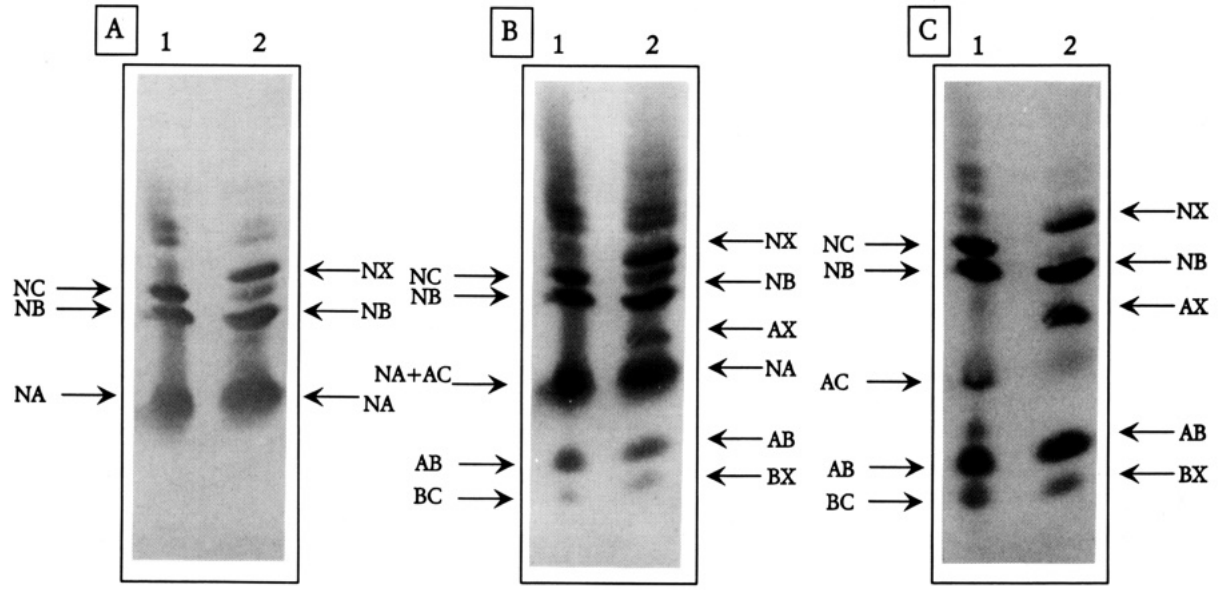

FIGURE 4: Immunoblot analysis of the CNBr-treated ArsA and N35 proteins using region specific antisera. The native ArsA and N35 polypeptides were labeled with [α-^{32}P]ATP and subjected to cyanogen bromide cleavage. The peptides were resolved by SDS PAGE on 15% polyacrylamide gels and transferred to a PVDF membrane. The membrane was probed sequentially with antisera to N18 (A), N35 (B), and C46 (C) polypeptides. Lane 1: native ArsA protein. Lane 2: N35 polypeptide. The presumed locations of the peptides predicted from the cleavage map in Figure 1 are indicated.

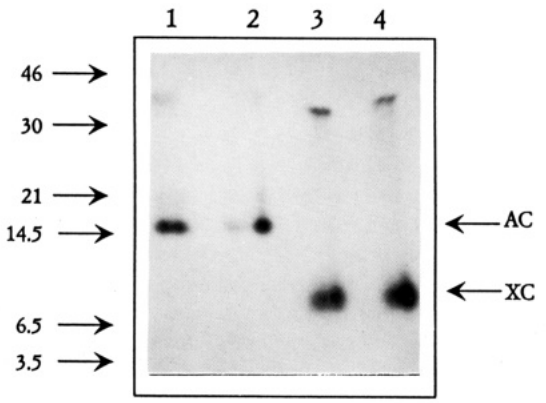

FIGURE 5: CNBr cleavage pattern of truncated polypeptides C46 and C35 labeled with [α-^{32}P]ATP. Light-activated adduct formation between the C46 or C35 polypeptides and [α-^{32}P]ATP was performed in reconstituted complexes as described in the Materials and Methods. The labeled bands were excised from the gel and subjected to cyanogen bromide cleavage. The peptides were analyzed by SDS PAGE on a 15% polyacrylamide gel followed by autoradiography. Lane 1; C46. Lane 2: C35. The presumed location of the peptides predicted from the cleavage map in Figure 1 are indicated. The migrations of protein standards of known molecular mass in kDa are indicated.

of the C46 polypeptide the radioactivity was found in a fragment of the same size as in the native ArsA protein, suggesting again that the site of adduct formation was not within the first 176 residues of the ArsA protein. The labeled fragment would result from cleavage at the methionyl residues M_{176} and M_{331} in the ArsA protein (corresponding to peptide AC from intact ArsA protein), again indicating that only partial cleavage occurred at residues M_{282}. Similarly, lack of cleavage of polypeptide C35 at residue M_{282} would generate a fragment of about 9 kDa between methionyl residues M_{11} in ArsD derived sequence and M_{331} in the ArsA protein. If the site of adduct formation was between residues 282 and 323, then this peptide would be radioactive, as the data demonstrate (Figure 5, lane 2).

Amino Acid Sequencing. The N-terminal sequence of the 9.9-kDa radioactive peptide BX generated from CNBr cleavage of polypeptide N35 was determined by excising the radioactive band out of the gel and eluting out the peptide. A single N-terminal sequence was observed, and after 29 cycles of gas phase sequencing the sequence VXVXALXRLL-STQPVASPSSDEYLQQRPD was obtained (X indicates peaks obscured due to contaminating material from the polyacrylamide gel). This sequence corresponds to the ArsA protein from residue 282 to 311. The labeled residue was not identified from sequence analysis, perhaps due to its lability during the sequencing procedure or because PTH derivatives of amino acids photolabeled with nucleotides bind strongly to the positively charged filters.

DISCUSSION

The ArsA protein has two nucleotide binding sites with a characteristic Walker A sequence (Walker et al., 1982). Photolabeling of the ArsA protein with [α-^{32}P]ATP required an A1 but not A2 domain (Karkaria et al., 1990; Kaur & Rosen, 1992b). N-Terminal polypeptides N18 or N28 were found to be unable to form light-activated adducts with ATP, suggesting that these smaller polypeptides do not carry a complete A1 nucleotide binding domain. When reconstituted with C-terminal polypeptides C46 or C35, respectively, ability to form photoadducts with [α-^{32}P]ATP was restored, and the label was found on the C-terminal polypeptides. Since the adduct formation would occur through the adenine ring, the data suggest that the adenine binding site of the A1 nucleotide binding domain in ArsA lies distal to the end of N28 peptide but is contained within the N35 polypeptide. These results also demonstrate that the N-terminal adenylate binding site is composed of regions of the protein far apart in the primary sequence. The N-terminal sequence G_{15}KGGVGKTS$_{23}$ is similar to the P-loop of nucleotide binding sites and has been postulated to interact with the triphosphate moiety of the nucleotide. This would localize the position of the phosphoryl groups of the bound ATP at the N-terminus (residues 15–23) of the ArsA protein. Hence, in reconstituted complexes of polypeptides N18 and C46 or N28 and C35, the phosphate groups of the ATP molecule would interact with the P-loop region in the N-terminal peptide, and the adenine moiety would

interact with its binding site contained in the C-terminal fragment.

The N-terminal adenylate binding site in the ArsA protein lies in a region at the boundary of the A1 and A2 halves. As mentioned earlier, the A1 and A2 halves are homologous to each other. If amino acid sequence of the two halves are aligned (Chen et al., 1986), residues 281–319 appear to be located at the end of the A1 half of the ArsA protein. This region has no counterpart in the A2 half and might form an interdomain linker conferring conformational flexibility on the ArsA protein. The ArsA protein has been shown to undergo a specific conformational change upon ATP binding (Hsu & Rosen, 1989). Moreover, the labeled sequence is contained within a region rich in glutamine, proline, serine, and leucine, characteristic of the Q-linker motif frequently found in polypeptide connectors between domains of proteins (Wootton & Drummond, 1989). Q linkers typically occur in regulatory and sensory transduction proteins in bacteria and are located at the boundaries of functionally distinct domains. The function of the Q linker appears simply to tether two halves of proteins, and it probably has no functional role in transmitting signals. The catalytically active form of the ArsA protein is a dimer with four nucleotide binding sites, two in the A1 and two in the A2 half (Hsu et al., 1991). Their interaction produces cooperative effects and suggests a regulatory role for ATP binding in energy transduction to the Ars pump.

In conclusion, these studies suggest that the A1 adenylate binding site in ArsA protein is composed of regions of the protein distant from each other in the primary sequence. The glycine rich sequence of the A1 phosphate binding loop is contained within residues 15–23, at least 280 residues from the location of the residue that reacts with the adenine ring. Hence, the ArsA protein must be folded such that those two regions are spatially neighboring, defining the environment of the A1 ATP binding site. Further, since the adenine binding region of the A1 site is close in the linear sequence to the P-loop region of the A2 site, the A2 glycine-rich consensus site (and hence the A2 nucleotide binding site) must be in close proximity to the A1 site. This suggests an interface between the A1 and the A2 nucleotide binding domains.

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