

# Multimerization of Phosphorylated and Non-phosphorylated ArcA Is Necessary for the Response Regulator Function of the Arc Two-component Signal Transduction System\*

Received for publication, May 28, 2001, and in revised form, August 24, 2001  
Published, JBC Papers in Press, August 29, 2001, DOI 10.1074/jbc.M104855200

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To adapt to anaerobic conditions, *Escherichia coli* operates the Arc two-component signal transduction system, consisting of a sensor kinase, ArcB, and a response regulator, ArcA. ArcA is converted to the active form, phosphorylated ArcA (ArcA-P), by ArcB-mediated phosphorylation. The active ArcA-P binds to the promoter regions of target genes, thereby regulating their transcriptional activities. The phosphoryl group of ArcA-P is unstable with a half-life of 30 min. However, we were able to inhibit the dephosphorylation for more than 12 h by the addition of EDTA; this allowed us to characterize ArcA-P. Gel-filtration and glycerol sedimentation experiments demonstrated that ArcA exists as a homo-dimer. ArcA phosphorylated by either ArcB or carbamyl phosphate multimerizes to form a tetramer of dimers; this multimer binds to the ArcA DNA binding site. Isoelectric focusing gel electrophoresis and nitrocellulose-filter binding analyses indicated that the ArcA multimer is composed of both ArcA-P and ArcA in a ratio, 1:1. The ArcA(D54E) mutant protein was unable to be phosphorylated by ArcB. This defect resulted in the inability of ArcA(D54E) to form a multimer or to bind to the ArcA DNA binding site. These results indicate that phosphorylation of ArcA induces multimerization prior to DNA binding, and the multimerization is a prerequisite for binding. Our results suggest a novel model that phosphorylation of ArcA by ArcB regulates multimerization of ArcA, which in turn functions as a response regulator.

Bacteria are able to adapt to a wide variety of environmental conditions through regulation of gene expression (1, 2). This gene regulation occurs by the activation of two-component signal transduction systems, a number of which have been identified. One such system found in *Escherichia coli*, the Arc two-component signal transduction system, is activated in response to anaerobic growth conditions (3). The Arc system is comprised of a transmembrane sensor kinase, ArcB, and a response regulator, ArcA (4, 5). Upon a shift from aerobic to anaerobic conditions, ArcB undergoes autophosphorylation,

presumably due to a change of redox signals in the membrane (6, 7). It is known that the phosphoryl group on phosphorylated ArcB (ArcB-P)<sup>1</sup> undergoes intramolecular phospho-relay from His-292 to Asp-576 to His-717 and then is finally transferred to ArcA (8, 9). Although the exact phosphorylation site of ArcA is unknown, comparison of the *arcA* gene sequence with other response regulators such as *cheY* suggests a putative phosphorylation site at Asp-54 (6, 8).

Phosphorylated ArcA (ArcA-P) positively and negatively regulates the transcription of a number of operons involved either directly or indirectly in cellular metabolism under both aerobic and anaerobic conditions (5). In some of these genes, for example *pfl* (10), *cyd* (11), and *sodA* (12), it has been demonstrated that ArcA-P functions as a transcriptional regulator by binding their promoter region. Recently, it was reported that ArcA-P binds to *oriC*, the origin of chromosomal replication, thereby specifically inhibiting *in vitro* *E. coli* chromosomal replication (13).

It has been reported that, in DNase I protection assays, ArcA-P protects a wide region of DNA, more than 60 bp (up to 150 bp in the *oriC* region), from the DNase I cleavage (10–12). In this report, we examined the interaction of ArcA protein with the wide regions of DNA as defined by the DNase I protection assays, and we show that phosphorylation of an ArcA dimer induces formation of an ArcA multimer, consisting of a tetramer of dimers that contains both ArcA and ArcA-P in a 1:1 ratio. In addition, we demonstrate that this multimerization occurs prior to DNA binding.

## MATERIALS AND METHODS

**Reagents and Proteins**—Sources were as follows: [ $\gamma$ -<sup>32</sup>P]ATP (5000 Ci/mmol), Amersham Pharmacia Biotech; Long Ranger polyacrylamide, FMC BioProducts; DNase I, Life Technologies, Inc.; *Pfu* DNA polymerase and *DpnI*, Stratagene; T4 polynucleotide kinase, New England BioLabs; calf intestinal alkaline phosphatase and restriction enzymes, Promega Corp. Other reagents, unless otherwise indicated, were purchased from Sigma Chemical Co.

ArcA and ArcB proteins were purified as previously described (13).

**Purification of ArcA(D54E)**—To generate mutant ArcA(D54E) protein, polymerase chain reaction (PCR)-based site-directed mutagenesis was conducted as recommended by Stratagene. Mutagenic primers (5'-GGTGGATCATGGAATCAATCTGCC-3' and 5'-GGCAGATTGATTCCATGATCACC-3') were designed to incorporate a Glu substitution at Asp-54; PCR was performed using pBADarcA (13) as template DNA. Parental DNA templates were removed from the PCR products using *DpnI* restriction enzyme digestion, then the mixture containing nicked plasmids harboring the desired mutation was transformed into DH5 $\alpha$  (14). Plasmids were isolated, and the proper amino acid substitution

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\* This work was supported in part by grants from the Korea Research Foundation (KRF-2000-015-DP0320, through the Research Institute of Basic Sciences, Seoul National University) and from the Basic Research Program of Korea Science and Engineering Foundation (1999-1-209-004-5). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a research fellowship from the Korean Ministry of Education (BK21).

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<sup>1</sup> The abbreviations used are: ArcB-P, phosphorylated ArcB; ArcA-P, phosphorylated ArcA; CP, phosphorylation reaction with carbamyl phosphate; IEF, isoelectric focusing; PCR, polymerase chain reaction; TP, transphosphorylation reaction; bp, base pair(s).

was confirmed by DNA sequencing. The resultant mutated DNA, pBADarcAD54E, was transformed into MC4100ΔarcA (gift from Edmund C. C. Lin) for purification to avoid contamination of wild type protein during purification procedures. ArcA(D54E) protein was purified in the same way as wild type ArcA purification as previously described (13), except that the FastS column step was omitted. Approximately 6.5 mg of ArcA(D54E) protein was purified from 18 liters of MC4100ΔarcA(pBADarcAD54E) culture.

**Transphosphorylation Reaction**—Transphosphorylation reactions (TP) containing ArcB and ArcA or ArcA(D54E) were performed as previously described (13) with minor modifications. 10 μl of TP mixture contained 6 μg of ArcA, 0.6 μg of ArcB, 0.1 mM ATP, 70 mM KCl, 10 mM MgCl<sub>2</sub>, 33 mM HEPES-KOH (pH 7.4), 0.1 mM EDTA, 2 mM dithiothreitol, and 10% glycerol. After incubation at 25 °C for 10 min, 0.5 M EDTA was immediately added to the reaction mixture for a final concentration of 50 mM (except for DNase I protection assays).

To visualize the phosphorylated proteins, [ $\gamma$ -<sup>32</sup>P]ATP was included in the TP mixture. The reaction was terminated by the addition of an equal volume of gel-loading buffer (20% glycerol, 8% SDS, 10% β-mercaptoethanol, 0.1 M EDTA, and 0.003% bromophenol blue). After incubation at 55 °C for 3 min, the mixture was subjected to 13% SDS-polyacrylamide gel electrophoresis. The gel was dried, and bands were visualized by autoradiography.

The phosphorylation reaction of ArcA with carbamyl phosphate (CP) was performed as previously described (11). The reaction was terminated by the addition of 0.5 M EDTA to 50 mM.

**DNA Binding Assays**—To construct pBS<sub>pfl</sub> used for DNA binding assays, the *pfl* promoter regulatory region of -458 to +23 (10) was amplified by PCR using the primers, 5'-CGGGGATCCACGCGTTT-GCTGCACATCAG-3' and 5'-CGGGAATTCAGCTTTTCACACTAATCTCTC-3' (*Bam*HI and *Eco*RI sites are in boldface), using *E. coli* genomic DNA as a template. The PCR product was digested with both *Bam*HI and *Eco*RI and cloned into the same sites of a pBluescript II SK(+) (Stratagene).

Gel-shift assays using the 324-bp *Bam*HI/*Dra*I fragment isolated from pBS<sub>pfl</sub> were performed essentially as previously described (11). 21.5 fmol of 5'-end-labeled DNA was mixed with 20 μl of TP mixtures containing 50 mM potassium chloride, 2.5 μg of bovine serum albumin, 40 mM HEPES-KOH (pH 7.6), and 10% glycerol. After incubation at 32 °C for 10 min, each sample was loaded onto a 4.5% polyacrylamide gel containing 10% glycerol.

For the DNase I protection assay, the 532-bp *Xba*I/*Xho*I fragment from pBS<sub>pfl</sub> was 5'-end-labeled at the *Xba*I restriction site, and the assay was conducted as previously described (13), unless 10 ng of DNase I (in 1.5 μl of H<sub>2</sub>O) was used.

**IEF Gel Electrophoresis Assays**—The IEF gel contained 5% polyacrylamide, 9 M urea, 2% Triton X-100, 2% ampholine (pH 4.0–6.0), and 0.4% ampholine (pH 3.0–10.0). 10 mM phosphoric acid and 20 mM sodium hydroxide were used as anolyte and catholyte, respectively. Each of the protein samples was mixed with an equal volume of IEF gel loading buffer (9 M urea, 2% Triton X-100, 2% ampholine (pH 4.0–6.0), 0.4% ampholine (pH 3.0–10.0), 1% β-mercaptoethanol, and 0.02% bromophenol blue). After removing insoluble materials through centrifugation for 10 min in a microfuge, samples were loaded to the anode. The gel was run at 150 V for 30 min, followed by 200 V for 2.5 h.

**Quantification of ArcA-P through Nitrocellulose Filter Binding**—TP reaction mixtures containing [ $\gamma$ -<sup>32</sup>P]ATP were filtered through nitrocellulose filters (Millipore, HA), equilibrated with buffer A (25 mM HEPES-KOH (pH 7.8), 1 mM EDTA, 2.8 mM β-mercaptoethanol, 50 mM KCl, and 10% glycerol), followed by an additional washing with 5 ml of this buffer. The filters were then dried, and radioactivities were determined by liquid scintillation counting.

**Gel Filtration and Glycerol Gradient Sedimentation**—Gel filtration analysis was carried out using the SMART system (Amersham Pharmacia Biotech, LKB). A Superose 12 column (PC 3.2/30) or Superose 6 column (PC 3.2/30) was equilibrated with buffer A. 30 μl of each indicated reaction mixture was applied to the column and eluted at a flow rate of 40 μl/min. Concentration of proteins in the eluent was monitored by measuring optical density at 280 nm, and 30 μl of each fraction was collected after an initial 0.93 ml was eluted. Each of these fractions was analyzed using IEF gel electrophoresis and gel-shift assays.

For glycerol gradient sedimentation, 100 μl of TP or CP mixture was loaded onto 4.5 ml of a 25–45% glycerol gradient in buffer A, followed by a centrifugation for 25 h at 48,000 rpm in an SW 50.1 rotor (Beckman) at 4 °C. 110 μl each of fraction was collected from the bottom.

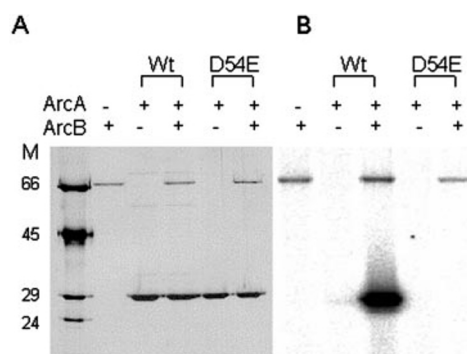


FIG. 1. ArcA(D54E) is inert to the phosphorylation. TP reactions with the indicated proteins and [ $\gamma$ -<sup>32</sup>P]ATP were carried out as described under "Materials and Methods." 2 μl of each reaction mixture was subjected to 13% SDS-polyacrylamide gel electrophoresis, followed by visualization using silver staining (A) or autoradiography (B). *Wt* and *D54E* indicate wild type ArcA and mutant ArcA(D54E) protein, respectively. Molecular mass markers (*M*) were as follows: bovine serum albumin, 66 kDa; egg albumin, 45 kDa; carbonic anhydrase, 29 kDa; and trypsinogen, 24 kDa.

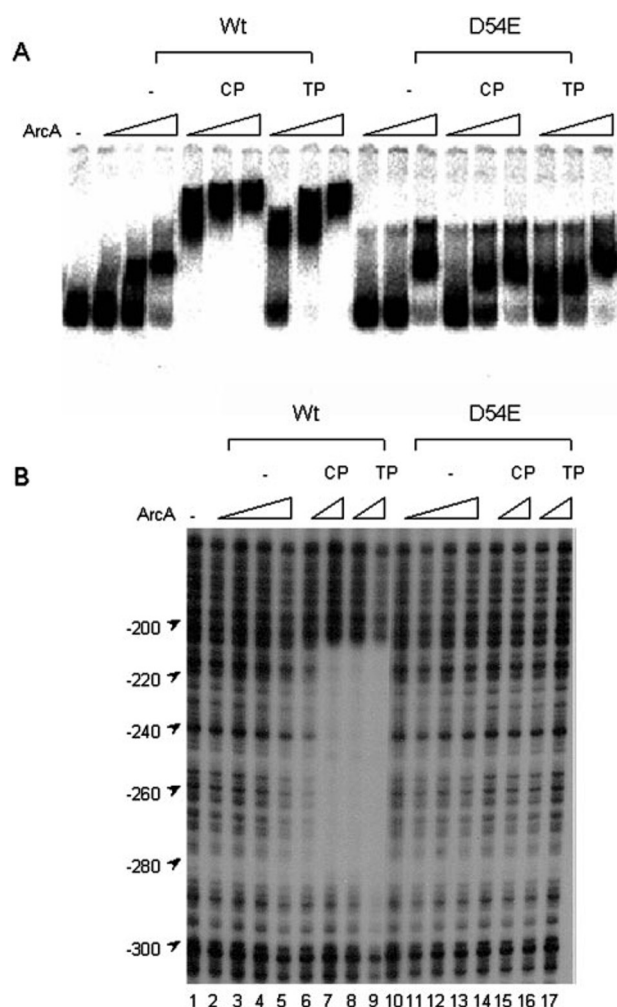
## RESULTS

**Asp-54 Is Essential for the Phosphorylation of ArcA**—ArcA and ArcB proteins were purified as previously described (13); both purified proteins were active in transphosphorylation reactions (Fig. 1). In addition, ArcB-P, which was formed by the autophosphorylation of ArcB in the presence of ATP, was able to phosphorylate ArcA as previously shown (6, 13). Because the ArcB protein can function as both a kinase and a phosphatase, it was necessary to optimize the ArcA to ArcB ratio to maximize ArcA-P formation. Using transphosphorylation reactions, we determined that a 10:1 ratio of ArcA to ArcB produced the largest concentration of ArcA-P.

Although the ability of ArcB-P protein to phosphorylate ArcA has clearly been established, it is unknown which ArcA amino acid(s) is phosphorylated by ArcB-P. Previously, the ArcA amino acid sequence was compared with other two-component response regulators, revealing a putative phosphorylation site at amino acid 54, an aspartic acid residue (15). Thus, we wanted to determine the importance of Asp-54 in ArcA phosphorylation by ArcB-P. To do this, we constructed the ArcA mutant protein ArcA(D54E), in which Asp-54 of ArcA was substituted with glutamate using site-directed mutagenesis. The ArcA(D54E) mutant protein was purified to near homogeneity using column chromatography. ArcB-P which was able to phosphorylate wild type ArcA was unable to phosphorylate the purified ArcA(D54E) (Fig. 1).

Next, we wanted to determine the ability of wild type ArcA and mutant ArcA(D54E) protein to bind the *pfl* promoter. Under anaerobic conditions, binding of ArcA-P to the *pfl* promoter induces the expression of pyruvate-formate lyase. Because small phosphoryl donors such as carbamyl phosphate and acetyl phosphate can phosphorylate two-component response regulators in the absence of cognate sensor kinases and ATP (1), ArcA was incubated with either carbamyl phosphate or ArcB. ArcA-P, phosphorylated by either carbamyl phosphate or ArcB, exhibited efficient binding to the *pfl* promoter as determined by gel-shift assays (Fig. 2A). The ability of ArcA (unphosphorylated) to bind the *pfl* promoter was also examined; although ArcA was also able to bind the *pfl* promoter, its binding pattern was different from that of ArcA-P. In addition, ArcA(D54E), incubated with either carbamyl phosphate or ArcB, showed a similar binding pattern to the *pfl* promoter as ArcA (unphosphorylated).

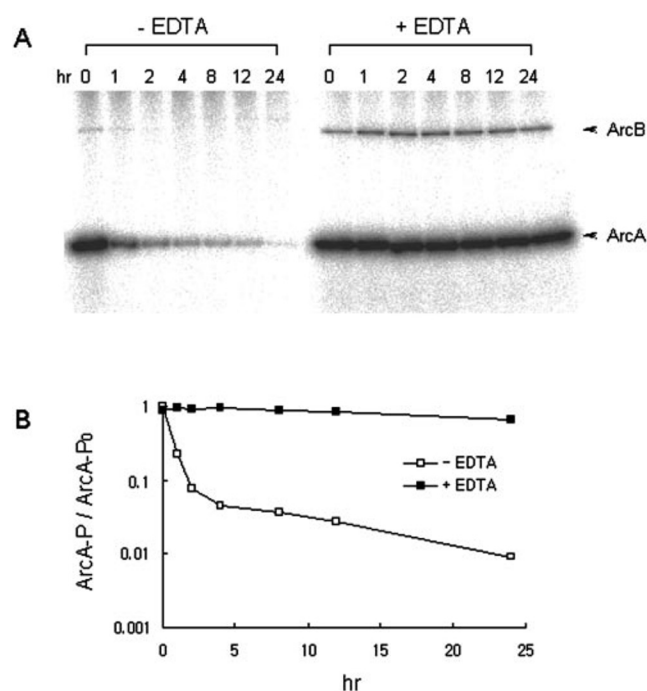
Given the unexpected binding of unphosphorylated ArcA and ArcA(D54E) to the *pfl* promoter in gel-shift assays, their bind-



**FIG. 2. Phosphorylation of ArcA is necessary for specific DNA binding.** Gel shift (A) and DNase I protection (B) assays containing the *pfl* promoter region as a probe were performed with ArcA (Wt) or ArcA(D54E) (D54E) protein as described under "Materials and Methods." A, 50, 100, and 200 ng of ArcA wild type or mutant proteins unphosphorylated or phosphorylated with carbamyl phosphate (CP) or ArcB and ATP (TP) were used for gel-shift assays. B, 75, 150, 300, and 600 ng of unphosphorylated ArcA were used for lanes 2–5 and lanes 10–13. 75 and 150 ng each of ArcA proteins phosphorylated with carbamyl phosphate (CP) or ArcB and ATP (TP) were used for lanes 6–9 and lanes 14–17, respectively. The numbers to the left correspond to the numbers from the mRNA start site of *pfl* promoter (10).

ings were examined using DNase I footprinting analysis (Fig. 2B). The ArcA-P formed by incubation with either carbamyl phosphate or ArcB bound to its binding locus, –285 to –208 relative to the start site of transcript P6, in the *pfl* promoter as shown previously (10). Although a 3-fold excess (600 ng) of unphosphorylated ArcA as compared with the unphosphorylated ArcA (200 ng) in gel-shift assay (Fig. 2A) was used in footprint analysis, the protein did not yield a significant protection from DNase I cleavage. In addition, ArcA(D54E), incubated with either carbamyl phosphate or ArcB, was unable to exhibit the protection. Therefore, the binding of unphosphorylated ArcA or ArcA(D54E) in the gel-shift assays can be attributed to nonspecific binding to DNA. This nonspecific binding persisted in the presence of poly(dI-dC). These results indicate that the phosphorylation of ArcA is required for specific DNA binding, and Asp-54 of ArcA is necessary for proper DNA binding.

**EDTA Stabilizes the Phosphoryl Group of ArcA-P**—It has been reported that the phosphoryl group of response regulators such as NtrC (16) and CheY (17) are unstable. We found that



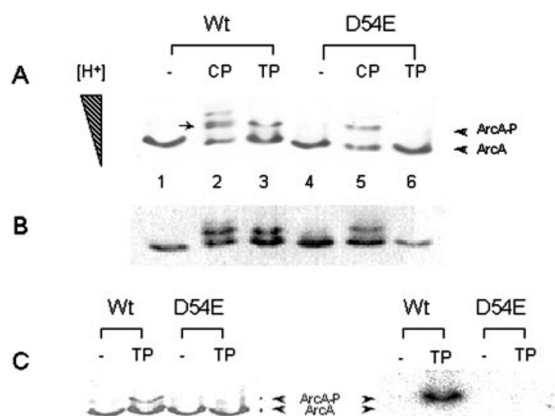
**FIG. 3. EDTA inhibits the dephosphorylation of ArcA-P.** After the TP reaction, 50 mM of EDTA (final concentration) was added (+EDTA) or not (–EDTA) and the resultant mixture kept at 4 °C. A, 2  $\mu$ l of each mixture was withdrawn at the indicated time and subjected to 13% SDS-polyacrylamide gel electrophoresis. The gel was dried and visualized by autoradiography. B, the radioactivities of ArcA- $^{32}$ P in each lane were measured using a FUJIFILM Bio-imaging Analyzer (BAS 1000) and indicated as ratios to the radioactivity of zero time.

ArcA-P rapidly lost its phosphoryl group with a half-life of about 30 min (Fig. 3). In addition, the phosphoryl group of ArcB-P also rapidly dissociated. Addition of EDTA immediately following the transphosphorylation reaction prevented the dephosphorylation of both ArcA-P and ArcB-P. In the presence of EDTA, the phosphoryl groups of both phosphorylated proteins did not significantly dissociate for more than 12 h. The effect of EDTA is presumably due to chelation of the  $Mg^{2+}$  ion, which might be a required cofactor in the dephosphorylation process. Therefore, EDTA was added to a concentration of 50 mM for further experiments to stabilize the phosphoryl group.

**Separation of ArcA-P from ArcA**—Isoelectric focusing (IEF) gel electrophoresis separates proteins based on differences in isoelectric points. Because phosphorylation increases the acidity of a protein, a phosphorylated form of a protein can be separated from its non-phosphorylated form using IEF gel electrophoresis. ArcA, possessing a pI value of 5.4, and ArcA(D54E) migrated as a single protein band (Fig. 4A, lanes 1 and 4). ArcA-P, phosphorylated by ArcB, yielded an additional band, the migration of which was shifted. Western blot analysis using anti-ArcA serum indicated that the protein bands shown in the IEF gel corresponded to ArcA or its modified forms (Fig. 4B). Interestingly, the shifted band was not exhibited in the lane of ArcA(D54E) treated with ArcB. To determine if this was due to an ability of ArcB to phosphorylate ArcA, ArcA and ArcA(D54E) were incubated with ArcB and [ $\gamma$ - $^{32}$ P]ATP, then subjected to IEF gel electrophoresis followed by silver staining or autoradiography (Fig. 4C). The  $^{32}$ P label was incorporated in the shifted ArcA band and was not incorporated into ArcA(D54E). These results indicate that the shift of ArcA incubated with ArcB and [ $\gamma$ - $^{32}$ P]ATP was due to the phosphorylation of ArcA and the shifted band corresponded to ArcA-P.

To determine the effect of carbamyl phosphate phosphorylation on ArcA and ArcA(D54E), both proteins were incubated





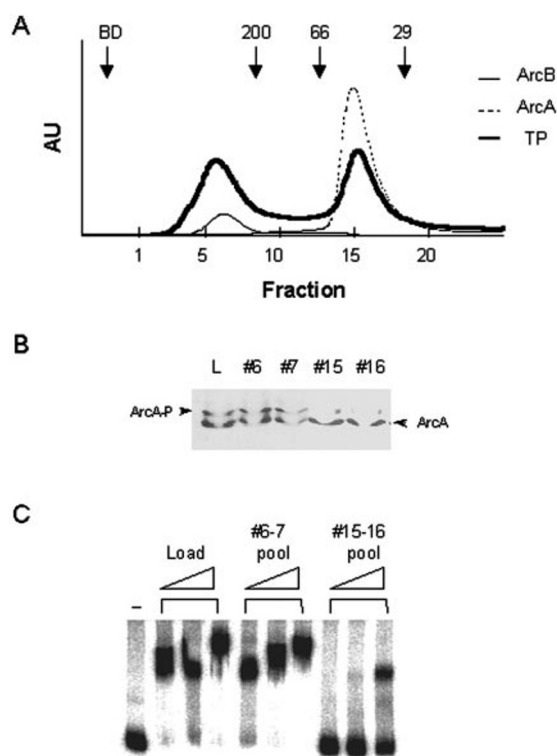
**FIG. 4. Separation of ArcA-P from ArcA through IEF gel electrophoresis.** The indicated proteins and their modified forms were separated using IEF gel electrophoresis as described under "Materials and Methods." The proteins were visualized using silver staining (A) or Western blot analysis using anti-ArcA serum (B). TP reactions containing [ $\gamma$ - $^{32}$ P]ATP were subjected to an IEF gel electrophoresis followed by visualization using silver staining (C, left panel) or autoradiography (C, right panel).

with carbamyl phosphate (Fig. 4A). IEF analysis generated other shifted protein bands, which were not seen following the ArcB treatment. The arrow in Fig. 4A indicates two close bands; the migration of the lower band was identical to that of the ArcA-P generated by ArcB but not seen in the ArcA(D54E) lane treated with carbamyl phosphate. However, the upper band was also observable in the ArcA(D54E) treated with carbamyl phosphate. These results indicate that carbamyl phosphate modifies amino acids of ArcA other than Asp-54, probably through nonspecific phosphorylation or carbamylation. Because of the lack of resolution in the previous IEF gel electrophoresis, the other bands modified by carbamyl phosphate could not be separated from the ArcA-P band (18).

**Multimerization of ArcA Dimer Is Induced by Phosphorylation**—Although the *arcA* gene encodes a 27-kDa polypeptide (6), ArcA-P footprints have shown that ArcA-P protects wide regions (at least 60 bp) of DNA from DNase I cleavage (10, 11, 13). Therefore, we investigated how those wide regions could be bound by the 27-kDa polypeptide of ArcA.

ArcA-P (transphosphorylation mixture) and ArcA were subjected to Superose-12 gel filtration chromatography (Fig. 5). ArcA eluted as a single peak of ~50-kDa protein, whereas the transphosphorylation mixture yielded an additional, earlier-eluted peak that contained larger molecular size forms of the protein (Fig. 5A). IEF gel electrophoresis of the peaks revealed that the earlier peak contained both ArcA-P and ArcA, and that the later-eluted peak contained only ArcA (Fig. 5B). In gel-shift assays, most (>95%) of the *pfl* promoter binding activity was recovered in the earlier peak (Fig. 5C). Because the earlier peak contained higher molecular weight forms of the protein, these results indicate that the phosphorylation of ArcA induces multimerization of ArcA prior to DNA binding. Both ArcB (Fig. 5A) and ArcB-P co-eluted at the earlier peak (data not shown).

The ArcA treated with carbamyl phosphate also generated two peaks (Fig. 6A). In the IEF gel, the band corresponding to ArcA-P (Fig. 4) was seen in the earlier but not the later peak (Fig. 6B). In contrast, the other bands generated by carbamyl phosphate were present in both peaks. Most of the *pfl* promoter binding activity was recovered from the earlier peak (Fig. 6C). The observation that the earlier peak formed by carbamyl phosphate has an identical retention volume with the earlier peak formed by ArcB (Fig. 5A) excluded the possibility of a complex formation between ArcA-P and ArcB.



**FIG. 5. Phosphorylation of ArcA by ArcB induces multimerization of ArcA-P.** A, Superose 12 gel-filtrations with ArcA, ArcB, or TP mixture was performed in a SMART System as described under "Materials and Methods." B, 10  $\mu$ l each of the indicated fractions obtained from the gel-filtration with TP mixtures were subjected to IEF gel electrophoresis followed by visualization using silver staining. C, 50, 100, and 200 ng each of the indicated pooled fractions were assayed in gel-shift assays. L or Load indicates the TP mixture loaded onto Superose 12 column. AU indicates the relative absorbance at 280 nm. Molecular markers were blue dextran (BD, 2000 kDa),  $\beta$ -amylase (200 kDa), alcohol dehydrogenase (66 kDa), and carbonic anhydrase (29 kDa).

In addition, the elution profile of untreated ArcA(D54E) was obtained; a single peak with the same retention volume of the later peak of ArcA was generated, but its elution pattern was broader than that of ArcA (Fig. 7). This elution profile of ArcA(D54E) was not influenced by the treatment of ArcB or the carbamyl phosphate. The earlier peaks of ArcA, which were produced by incubation with either ArcB or carbamyl phosphate, contained ArcA-P (Figs. 5 and 6); those peaks were not observed in the elution of similarly treated ArcA(D54E) (Fig. 7, B and C), presumably because the protein could not be phosphorylated by ArcB (Figs. 1 and 4). The identical elution pattern of the earlier peak with that of untreated ArcB (Fig. 7A), and the absence of the earlier peak in the ArcA(D54E) incubated with carbamyl phosphate (Fig. 7C) indicated that the earlier peak shown in ArcA(D54E) incubated with ArcB was as due to elution of ArcB (Fig. 7B).

Multimerization of ArcA-P was confirmed using glycerol gradient sedimentation experiments (Fig. 8A). The glycerol gradient sedimentation of ArcA yielded a single peak, whereas ArcA-P, produced by ArcB phosphorylation, yielded two peaks; the slowly sedimenting peak coincided with the ArcA (untreated) peak. These results confirmed that ArcA-P forms multimers as observed in gel-filtration experiments (Figs. 5 and 6).

The sedimentation coefficient and Stokes radii of the slowly sedimenting and later, respectively, peak were estimated as 3.9 S (Fig. 8B) and 3.04 nm (Fig. 8C), respectively. These values predict a molecular mass of the ArcA protein (unphosphorylated) to be 50 kDa, indicating that ArcA is a homodimer of two 27-kDa polypeptides. The sedimentation coefficient as 11.0 S

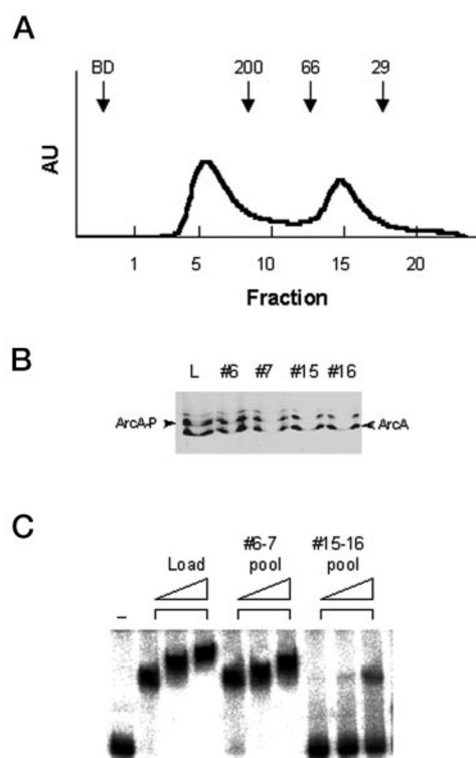


FIG. 6. **Multimerization of ArcA phosphorylated with carbamyl phosphate.** ArcA treated with carbamyl phosphate was subjected to Superose 12 gel-filtration chromatography (A) as described in Fig. 5. The column fractions were analyzed in IEF gel electrophoresis (B) or gel-shift assays (C) as described in Fig. 5.

and Stokes radii as 4.92 nm of the fast-sedimenting (Fig. 8B) and earlier, respectively, peak (Fig. 8D) containing ArcA-P predicts a molecular mass of a multimer of 230 kDa. These results suggest that the multimer is a tetramer of ArcA-P dimers or an octamer composed of 27-kDa ArcA-P polypeptides.

**The ArcA Multimer Is Composed of Both ArcA and ArcA-P with a 1:1 Ratio**—The earlier peaks comprised multimers containing both ArcA and ArcA-P proteins (Figs. 5B and 6B). Because all the experiments were performed in the presence of EDTA, which inhibited dephosphorylation of ArcA-P (Fig. 3), the presence of ArcA due to dephosphorylation of ArcA-P in these multimers is negligible. Thus, we wanted to determine the ratio of ArcA and ArcA-P in these multimers. To do this, the amount of ArcA-<sup>32</sup>P in TP mixtures containing [ $\gamma$ -<sup>32</sup>P]ATP was quantified using nitrocellulose filter binding assays (Table I). The filter binding assay indicated that 19.2% of the input ArcA was converted to the ArcA-P. Although the filter binding assay detected both ArcA-<sup>32</sup>P and ArcB-<sup>32</sup>P in the TP mixture, the ArcB-<sup>32</sup>P radioactivities were negligible compared with the ArcA-<sup>32</sup>P radioactivities (Fig. 1B). Densitometric scanning of the ArcA-P band in the IEF gel (Fig. 4A) showed that 24% of the input ArcA was converted to ArcA-P. When the ArcB amount was taken out from the earlier peak (the earlier peak of TP mixture contained ArcB in addition to ArcA-P and ArcA) in Fig. 5A, the ratio of the protein amount in the earlier peak to that in the later peak was near 1. These results indicate that 38–48% of the multimer is composed of ArcA-P. This value appeared to be in agreement with the ArcA-P fraction shown in Fig. 5B. Therefore, we concluded that the multimer is composed of both ArcA and ArcA-P with a ratio of 1:1.

#### DISCUSSION

Among the response regulators of bacterial two-component signal transduction systems, the structure of CheY, which is

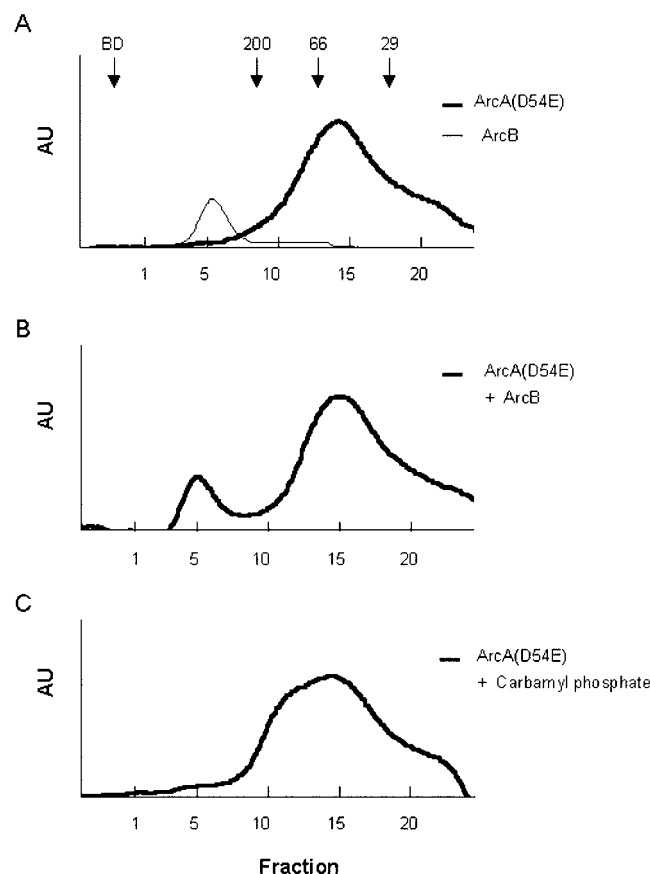


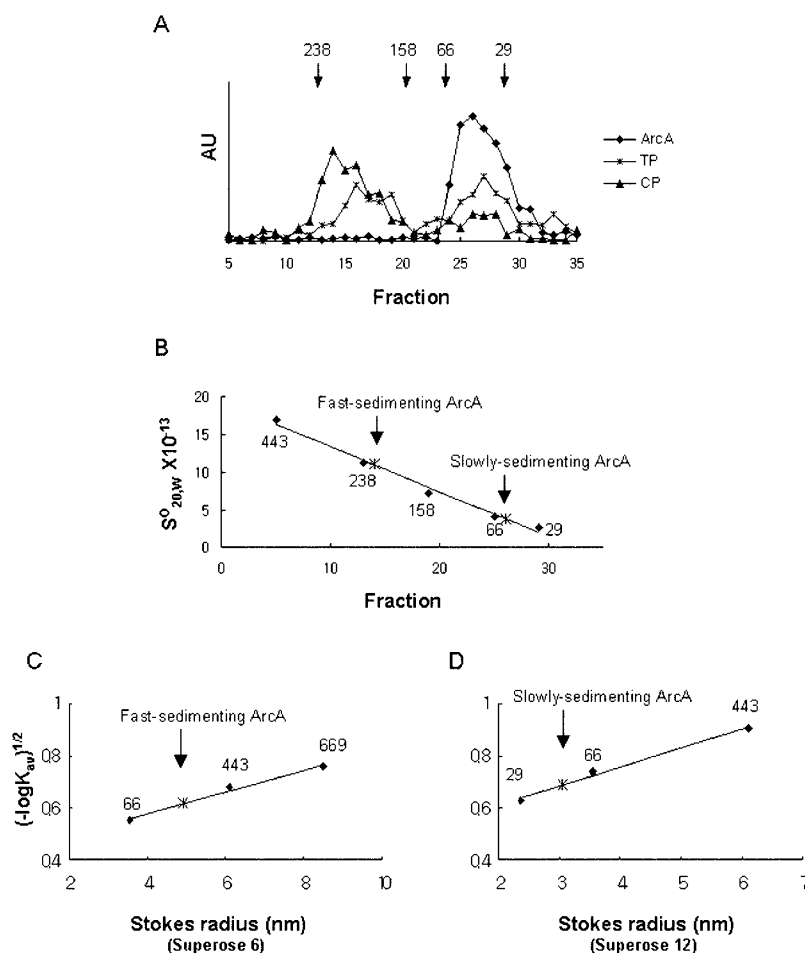
FIG. 7. **ArcA(D54E) mutant protein cannot be multimerized.** Untreated ArcA(D54E) or ArcB (A), ArcA(D54E) treated with ArcB and ATP (B), or ArcA treated with carbamyl phosphate (C) were subjected to Superose 12 gel-filtration chromatography.

the response regulator upon chemotaxis, has been well studied by x-ray crystallography and other methods (17, 19, 20). The Asp-12, Asp-13, and Asp-57 of this protein form an acid pocket, and the Asp-57 is phosphorylated (15). Due to amino acid sequence homology with CheY and other response regulators of bacterial two-component systems, it was predicted that Glu-10, Asp-11, and Asp-54 of ArcA protein also form an acid pocket and the Asp-54 is the site for ArcB phosphorylation (8). However, the relevancies of these amino acids remain unstudied. Here we demonstrate that an ArcA mutation at Asp-11<sup>2</sup> as well as a mutation at Asp-54 (Fig. 1) resulted in the inability of these mutant proteins to be phosphorylated by ArcB. In addition, the broad elution pattern of ArcA(D54E) (Fig. 7A) as compared with that of wild type ArcA (Fig. 5A) in gel filtration studies suggests that ArcA(D54E) possesses a different conformation from ArcA. Although the phosphorylation site was not determined specifically, these results indicate that the Asp-11 and Asp-54 are crucial to constitute a proper conformation for functional ArcA.

Carbamyl phosphate and acetyl phosphate commonly phosphorylate the response regulators of two-component systems (1). However, specificity for the phosphorylation has not been reported. In addition to ArcA-P produced by ArcB phosphorylation, incubation of ArcA-P with carbamyl phosphate produced at least two additional ArcA proteins whose migrations were retarded in IEF gel electrophoresis (Figs. 4A and 6). The two modified ArcA proteins were also formed upon incubation of the ArcA(D54E) mutant protein with carbamyl phosphate. There-

<sup>2</sup> Y. Jeon, Y. S. Lee, J. S. Han, J. B. Kim, and D. S. Hwang, unpublished results.

**FIG. 8. The ArcA multimer is a tetramer of ArcA dimers.** A, ArcA (ArcA), ArcA transphosphorylated with ArcB and ATP (TP), or ArcA phosphorylated with carbamyl phosphate (CP) was subjected to 25–45% glycerol gradient centrifugation as described under “Materials and Methods.” Molecular mass makers were apoferritin (443 kDa), catalase (238 kDa), aldolase (158 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). B, sedimentation coefficients of the earlier and later peaks were determined using the values obtained from the glycerol gradient centrifugation as described previously (26). C, Stokes radii of ArcA multimers in the earlier peak were determined based on the values obtained from Superose 6 gel-filtration chromatography of the TP mixture as described previously (26). D, the Stokes radii of the ArcA dimer in the later peak were determined using the values obtained from Superose 12-gel filtration chromatography shown in Fig. 5A. Thyroglobulin (669 kDa), apoferritin (443 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were used as molecular mass markers for the gel-filtration chromatography.



**TABLE I**  
Determination of ArcA-P amount in TP mixture

Nitrocellulose filter binding experiments with TP mixtures containing [ $\gamma$ - $^{32}$ P]ATP were performed as described under “Materials and Methods.” Three independent assays were analyzed and averaged.

	Incorporated ATP = ArcA-P	Total ArcA	ArcA-P
	pmol	pmol	%
1st	4.44	22.2	20.0
2nd	4.28	22.2	19.3
3rd	4.08	22.2	18.4
Average			19.2 ( $\pm$ 0.82)

fore, these modified ArcA proteins could be produced by non-specific modifications irrelevant to ArcA-P function. The presence of these modified proteins in both the earlier- and later-peak in gel-filtration studies (Fig. 6) indicates that the nonspecific modification did not affect the multimerization of ArcA-P with ArcA.

Phosphorylation of proteins generally induces a conformational change, thus modifying protein activity. In bacterial two-component systems, a sensor kinase phosphorylates an aspartic acid residue of its cognate response regulator, resulting in activation of the regulator (21). Phosphorylation of the monomeric PhoB protein induces dimerization, and the resultant dimer binds to its DNA binding sites (22, 23). In addition, the phosphorylated OmpR forms dimers and trimers as evidenced by chemical cross-linking experiments (24). Also, phosphorylation of dimeric NtrC induces interaction between dimers that results in the formation of tetramers. The resulting tetrameric NtrC proteins cooperatively and sequentially bind to target DNA (25).

If a protein binds sequentially or cooperatively to DNA, the bound region seen in footprint experiments becomes broadened by the increasing amount of the protein. Interestingly, increasing the amount of ArcA-P did not broaden the bound region but enhanced the intensity of the bound region (Fig. 2B) and region of *oriC* (13). These observations support our conclusion that multimerization of ArcA occurs prior to DNA binding, and the ArcA multimer binds to DNA. Our results indicate that the phosphorylation of the ArcA dimer induces the formation of a tetramer of dimers (or an octamer) that is composed of both ArcA-P and ArcA. The ratio of ArcA-P to ArcA in the multimer appears to be  $\sim 1$ . These findings suggest two possibilities: 1) ArcA multimer is composed of four identical dimers, of which one subunit is phosphorylated, or 2) two dimers of ArcA-P, all of which subunits are phosphorylated, form a tetramer with two unphosphorylated ArcA dimers. The formation of an ArcA multimer with ArcA-P and ArcA would be advantageous in the aspect that all ArcA proteins do not need to undergo phosphorylation in response to an anaerobic situation. This will facilitate the activation process of ArcA, thereby contributing to a rapid adaptation of *E. coli* to anaerobic environments.

**Acknowledgment**—We thank Gillian Newman for careful editing of this manuscript.

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