The ArcB Sensor Kinase of *Escherichia coli* Autophosphorylates by an Intramolecular Reaction

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Received 26 October 2009/Accepted 7 January 2010

The Arc two-component system, comprising the ArcB sensor kinase and the ArcA response regulator, modulates the expression of numerous genes in response to the respiratory conditions of growth. ArcB is a tripartite histidine kinase whose activity is regulated by the oxidation of two cytosol-located redox-active cysteine residues that participate in intermolecular disulfide bond formation. Here we show that ArcB autophosphorylates through an intramolecular reaction which diverges from the usually envisaged intermolecular autophosphorylation of homodimeric histidine kinases.

The Arc (Anoxic redox control) two-component system is an important element in the complex transcriptional regulatory network that allows facultative aerobic bacteria to sense and respond to various respiratory conditions (9, 18, 25). This system comprises ArcB as the membrane-bound sensor kinase and ArcA as the cognate response regulator (13, 14). ArcA is a typical response regulator that has an N-terminal receiver domain and a C-terminal helix-turn-helix DNA-binding domain, whereas ArcB is a tripartite protein that has, in addition to the primary transmitter domain, a receiver domain and a secondary transmitter or phosphotransfer domain (12, 15). Moreover, in the linker region, there is a putative leucine zipper (6) and a PAS domain (38).

Under anoxic growth conditions, ArcB autophosphorylates, a process that is enhanced by certain fermentative metabolites, such as D-lactate, pyruvate, and acetate (7, 30), and transphosphorylates ArcA through a His292 → Asp576 → His717 → Asp576 phosphorelay (10, 19). Phosphorylated ArcA, in turn, represses the expression of many operons involved in respiratory metabolism and activates others that encode proteins involved in the expression of many operons involved in respiratory metabolism (1). Here, the ArcB autophosphorylation reaction was analyzed by using wild-type and mutant proteins unable to bind ATP or blocked at the autophosphorylation site.

(ArcB292Q) and one blocked at the autophosphorylation site (ArcB78-778/H9253, hereafter referred to as ArcB78-778/H9253, as Histagged proteins. For the purposes of this study and to facilitate the purification of the protein variants, amino acid residues 1 to 77, which constitute the transmembrane segments, were omitted. Previous studies on sensor kinases, including ArcB, showed that removal of the transmembrane segments does not affect the processes of autophosphorylation and the subsequent transphosphorylation of the cognate regulator proteins (10, 11, 16, 17, 23, 29). To construct the mutant ArcB-expressing plasmids, we used primer 5'-CCCGGATCCCATATGGAGCAACTGGAGGAGTCACGCAC-3' together with mutagenic primer 5'-CGGCCAGAGCAATAGCCGCGGAGCCGC-3' (G470A and G472A or 5'-GGGTACGGCAATTCTTGACTGATGGTGG-3' (H9253) in PCRs with plasmid pQ30ArcB78-778 (10) as the template. The products of these reactions were purified and used as megaprimers in PCRs together with primer 5'-CCCGGATCCCATGCGGAGTCACGCAC-3' with pQ30ArcB78-778 as the template. The products of the second PCRs were digested with NdeI and NruI and used to replace the NdeI-NruI wild-type fragment of pQ30ArcB78-778, generating plasmids pMX025 (ArcBG*) and pMX028 (ArcBH*) in PCRs with plasmid pQ30ArcB78-778 as the template. The products of the second PCRs were digested with NdeI and NruI and used to replace the NdeI-NruI wild-type fragment of pQ30ArcB78-778, generating plasmids pMX025 (ArcBG*) and pMX028 (ArcBH*). Plasmids pQ30ArcB78-778, pMX025, and pMX028 were used to overexpress and purify ArcB78-778 (hereafter referred to as ArcB), ArcB292Q, and ArcB78-778 as described earlier (10). The...
purified proteins were incubated with [γ-32P]ATP, and their ability to autophosphorylate was tested. As expected, ArcBG* and ArcBH* failed to phosphorylate, whereas ArcB was rapidly phosphorylated (Fig. 1A to C). We then argued that in a mixture of both defective proteins, hybrid heterodimers should phosphorylated (Fig. 1A to C). We then argued that in a mixture of both defective proteins, hybrid heterodimers should

The properties of the two mutant proteins that have the same size and both of which possess a His6 tag do not permit the experimental identification of heterodimers in the above-described reaction mixtures. Therefore, we created a plasmid carrying an ArcBG* version fused to the maltose-binding protein (MBP-ArcBG*) by cloning the BamHI-HindIII fragment from plasmid pMX025 into pMAL-c2. The constructed plasmid was used to overexpress and purify MBP-ArcBG*. The properties of the two mutant proteins that have the same size and both of which possess a His6 tag do not permit the experimental identification of heterodimers in the above-described reaction mixtures. Therefore, we created a plasmid carrying an ArcBG* version fused to the maltose-binding protein (MBP-ArcBG*) by cloning the BamHI-HindIII fragment from plasmid pMX025 into pMAL-c2. The constructed plasmid was used to overexpress and purify MBP-ArcBG*.
resin and processed the same way. Eluents of the above-described experiments were analyzed by Western blotting using MBP-specific antibodies. As expected, in the absence of ArcB<sup>B14</sup>, MBP-ArcB<sup>B14</sup> failed to interact with the resin and eluted in the washing fractions (Fig. 1G). In contrast, in the presence of ArcB<sup>B14</sup>, a considerable fraction of the MBP-ArcB<sup>B14</sup> protein was retained in the column and coeluted with ArcB<sup>B14</sup> (Fig. 1H), indicating the formation of heterodimers.

Subsequently, we tested whether ArcB<sup>G16</sup> and ArcB<sup>H16</sup> are able to receive the phosphoryl group at the conserved Asp<sup>576</sup> of their receiver domain and/or His<sup>717</sup> of their phosphotransfer domain from an ArcB protein that is able to autophosphorylate. Purified ArcB<sup>G16</sup> and ArcB<sup>H16</sup> were incubated with [γ-<sup>32</sup>P]ATP, and after 90 s of incubation, ArcB<sup>B78-661</sup> (10) was added to the reaction mixture (Fig. 1I and J). Although ArcB<sup>G16</sup> and ArcB<sup>H16</sup> were not able to autophosphorylate, both mutant ArcB proteins were able to rapidly receive the phosphoryl group from ArcB<sup>B78-661</sup>-P at Asp<sup>576</sup> and/or His<sup>717</sup> through an intermolecular reaction. It therefore appears that ArcB, like other sensor kinases (4, 33, 35, 36), functions as a dimer and that the G<sup>747</sup>A-G<sup>772</sup>A and H<sup>792</sup>Q substitutions do not affect the overall structure of the protein.

The structural integrity of the purified ArcB proteins was also assessed by analysis of their secondary structure by far-UV circular dichroism (CD) spectra. Purified ArcB, ArcB<sup>G16</sup>, and ArcB<sup>H16</sup> proteins (1.5 mM) were dialyzed against 9.32 mM Na<sub>2</sub>HPO<sub>4</sub>, 6.8 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM β-mercaptoethanol, and scans from 190 to 260 nm were performed. The CD spectra showed that all three polypeptides maintain their structural integrity during purification and contain the characteristics of globular proteins (Fig. 1K).

To verify the in vitro results by in vivo experiments, a plasmid-borne arc<sup>B1-778</sup> mutant allele (pMX041) was coexpressed with a chromosomal arc<sup>B1-778</sup> mutant allele (pMX041) and their ability to complement each other was analyzed by monitoring the in vivo levels of phosphorylated ArcA, as indicated by the expression of the λ(lldP<sup>-lacZ</sup>) and λ(acydA<sup>-lacZ</sup>) target operons. The lldP<sup>-lacZ</sup> and cydA<sup>-lacZ</sup> target operons were chosen as an ArcA-P-repressible reporter and as an ArcA-P-activatable reporter, respectively. To construct plasmid pMX041, we first created plasmid pMX712 by cloning the BamHI-HindIII fragment from plasmid pIBW (20), which carries the arcB promoter, the arcB ribosomal binding site, an introduced NdeI site that includes the initiation codon of arcB, and the arcB open reading frame (ORF) and stop codon into pBlueScript II KS<sup>+</sup>. Subsequently, the MluI-HindIII fragment of pMX025 was used to replace the MluI-HindIII wild-type ArcB fragment of pMX712, generating plasmid pMX040. Finally, the BamHI-HindIII fragment of pMX712 and pMX040 was cloned into low-copy-number plasmid pET22 (5) to generate plasmids pMX039 (carrying the wild-type arcB allele) and pMX041 (carrying the arc<sup>B1-778</sup> mutant allele), respectively.

The generated plasmids were transformed into the ΔarcB mutant strains ECL<sup>5004</sup> (carrying a λ(acydA<sup>-lacZ</sup>) operon fusion) and ECL<sup>5012</sup> (carrying a λ(lldP<sup>-lacZ</sup>) operon fusion) and the arc<sup>B1-778</sup>H<sup>292Q</sup> mutant strains ECL<sup>5022</sup> and ECL<sup>5030</sup> carrying λ(acydA<sup>-lacZ</sup>) and λ(lldP<sup>-lacZ</sup>) operon fusions, respectively (19). The transformants were grown aerobically or anaerobically in buffered Luria-Bertani broth containing 100 mM MOPS (morpholinepropanesulfonic acid), pH 7.4, and 20 mM D-xylose. In the case of the λ(lldP<sup>-lacZ</sup>)-bearing strains, the above-described medium was supplemented with 20 mM L-lactate as the inducer (3). At an optical density at 600 nm (OD<sub>600</sub>) of ~0.5, the cultures were harvested and β-galactosidase activity was assayed and expressed in Miller units (26). Empty bars represent aerobic growth, and solid bars represent anaerobic growth. The data are averages from four independent experiments (variations were less than 10% of the mean). (Bottom panel) Western blot analysis. A 1-ml sample of the above-described aerobic cultures was harvested at an OD<sub>600</sub> of 0.5. The pelleted cells were solubilized by incubation at 95°C for 10 min in 100 μl of 4X SDS sample buffer. Samples of 5 μl were subjected to electrophoresis in an SDS–8% polyacrylamide gel, and the resolved proteins were electrotransferred to a Hybond-ECL filter (Amersham). Immunoblot analyses were subsequently performed using ArcB polyclonal antibodies as previously described (20).
Increasing amounts of ArcBG* are not able to quench ArcB autophosphorylation. To provide independent evidence in support of the above conclusion, we argued that if increasing amounts of ArcBG* were added to phosphorylation reaction mixtures of ArcB78-520 (10), heterodimer formation should be favored, resulting in a decline of the ArcB78-520-P levels if an intermolecular autophosphorylation were involved. Also, the phosphoryl group of the ATP bound on ArcB78-520 should be transferred to His392 of ArcBG* and ArcBG*-P should be detected. On the other hand, no change in ArcB 78-520-P should be observed and no ArcBG*-P should be detected if an intramolecular autophosphorylation were involved.

To distinguish between the two possibilities, ArcB78-520 (10) and ArcBG* were purified and used in phosphorylation reaction mixtures with [γ-32P]ATP. ArcB78-520 (the primary transcript domain) was chosen because it contains the elements necessary for autophosphorylation but not the receiver domain, which is known to have an associated phosphatase activity and rapidly loses the phosphoryl group from Asp576 (6, 10). The purified ArcB78-520 protein (2 pmol) was incubated in nine phosphorylation reaction mixtures containing various amounts of ArcBG* (0 to 200 pmol) for 10 min at room temperature before [γ-32P]ATP was added. Each reaction was carried out for 1 min, terminated by the addition of an equal volume of 4× sodium dodecyl sulfate (SDS) sample buffer, and immediately subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 3A). As expected, ArcB78-520 was rapidly phosphorylated. However, the presence of ArcBG*, even at a 100-fold excess, failed to affect the autophosphorylation level of ArcB78-520 (Fig. 3A). Also, no ArcBG*-P was detected (Fig. 3A).

To verify the above result in vivo, plasmid pMX065, carrying an arabinose-induced arcb1-520,G470A,G472A mutant allele, was transformed into plasmid strains ECL5001 and ECL5002 harboring, respectively, the λΦ(cydA-lacZ) and λΦ(lldP-lacZ) reporter fusions. To construct this plasmid, we first generated plasmid pMX175 by replacing the Ndel-HindIII fragment of plasmid pMX020 (28) with the NdeI-HindIII fragment of plasmid pMX712, placing the arcB1-778 wild-type allele under the control of the arabinose promoter. Subsequently, the PstI-HincII ArcB fragment of pMX025, carrying the G770A and G472A mutations, was used to replace the PstI-HincII fragment of pQE30ArcB78-520 (10), generating plasmid pMX064. Finally the Ncol-HindIII fragment of pMX064 was used to replace the Ncol-HindIII fragment of pMX517, generating pMX065, in which the arcB1-720,G470A,G472A mutant allele is under the control of the arabinose promoter.

The transformants were grown aerobically or anaerobically in the presence of various concentrations of arabinose (0 to 100 μM), and at an OD600 of ~0.5, the cultures were harvested and the β-galactosidase activities were determined (Fig. 3). Western blot analysis of the cell extracts with polyclonal antibodies was done as described in the legend to Fig. 2. The transformants were grown aerobically or anaerobically in the presence of various concentrations of arabinose (0 to 100 μM), and at an OD600 of ~0.5, the cultures were harvested and the β-galactosidase activities were determined (Fig. 3). Western blot analysis of the cell extracts with polyclonal antibodies was done as described in the legend to Fig. 2.

Thus, both the in vivo and in vitro results of two independent experimental approaches indicate that ArcB, in contrast to most homodimeric histidine kinases, autophosphorylates through an intramolecular reaction, requiring the ATP-binding site and the site of autophosphorylation (His392) to be present in the same ArcB molecule.

We thank Claudia Rodriguez and Javier de la Mora for technical assistance and the Unidad de Biología Molecular of the Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, for oligonucleotide synthesis and sequencing.
This work was supported by grants 37342-N from the Consejo Nacional de Ciencia y Tecnología (CONACYT) and IN221106/17 from DGAPA-PAPIIT, UNAM.

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