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Amplification of Signaling Activity of the Arc Two-component System of *Escherichia coli* by Anaerobic Metabolites

AN *IN VITRO* STUDY WITH DIFFERENT PROTEIN MODULES*

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In *Escherichia coli*, changes in redox condition of growth are sensed and signaled by the Arc two-component system. This system consists of ArcB as the membrane-associated sensor kinase and ArcA as the cytoplasmic response regulator. ArcB is a tripartite kinase, possessing a primary transmitter, a receiver, and a secondary transmitter domain that catalyzes the phosphorylation of ArcA via a His → Asp → His → Asp phosphorelay, as well as the dephosphorylation of ArcA-P by a reverse phosphorelay. When ArcA and ArcB were incubated with ATP, the peak levels of phosphorylated proteins increased in the presence of the fermentation metabolites D-lactate, acetate, or pyruvate. In this study, we report that these effectors accelerate the autophosphorylation activity of ArcB and enhance the transphosphorylation of ArcA, but have no effect on the dephosphorylation of ArcA-P. Moreover, the presence of the receiver domain of ArcB is essential for the effectors to influence the autophosphorylation rate of the primary transmitter domain of ArcB.

In prokaryotes, environmental changes are often signaled by a family of two-component systems, resulting in adaptive gene expressions. Typically, such a system comprises a membrane-associated sensor kinase and its cognate response regulator (1, 2). Signal reception by the sensor kinase stimulates an ATP-dependent autophosphorylation at a conserved histidine residue in the cytosolic transmitter domain. Subsequently, the phosphoryl group is transferred to a conserved aspartate residue in the receiver domain of the cognate response regulator. The phosphorylated response regulator in general serves as a transcriptional regulator. Upon cessation of signaling, both the cognate response regulator and the sensor kinase undergo dephosphorylation that results in silencing of the system.

The Arc (anoxic redox control) two-component system of *Escherichia coli*, consists of the ArcB protein as a transmembrane sensor kinase and the ArcA protein as the response regulator (3, 4) (Fig. 1). Phosphorylated ArcA controls the expression of some 30 operons (the Arc modulon) in response to redox conditions of growth (5, 6). ArcB contains three catalytic domains: an N-terminal transmitter domain (H1) with a conserved His-292 residue, a central receiver domain (D1) with a conserved Asp-576 residue, and a C-terminal secondary transmitter domain (H2) with a conserved His-717 residue (7, 8) (Fig. 1). Upon stimulation, ArcB autophosphorylates at the expense of ATP. The phosphoryl group is then transferred to ArcA by a His → Asp → His → Asp phosphorelay, sequentially involving His-292 of H1, Asp-576 of D1, His-717 of H2, and Asp-54 of ArcA (9). Upon cessation or subsidence of stimulation, ArcA-P dephosphorylation occurs by reverse transfer of the phosphoryl group to His-717 of H2 and then to Asp-576 of D1, where release of P_i takes place. H1 plays no apparent role in the process of signal decay (10). Signal transmission by His → Asp → His → Asp phosphorelay has also been reported for the Kin/Spo system of *Bacillus subtilis* (11), the Sln1p/Ypd1p/Ssk1p of *Saccharomyces cerevisiae*, the BvgS/A of *Bordetella pertussis* (12), and the TorS/R of *E. coli* (13), although the number of proteins involved differs from system to system.

In the absence of ArcB, phosphorylated ArcA (ArcA-P) is quite stable, exhibiting a half-life of more than 1 h (10). By contrast, phosphorylated ArcB (ArcB-P), which can exist in numerous states of phosphorylation (singly phosphorylated at one of the three sites, doubly phosphorylated, or completely phosphorylated) is relatively labile (9, 14). Consequently, addressing the complex question of ArcB-P half-life remains a technical challenge. Iuchi reported (14) that in a reaction mixture containing ArcB and ATP, the net autophosphorylation of ArcB reaches a peak in about 2 min and then decays. The presence of certain fermentation intermediates, namely D-lactate (but not L-lactate which is not a fermentation product), acetate, and pyruvate, heightened the level of net ArcB phosphorylation. These results, together with the observation that the rate of release of P_i from the protein was retarded in the presence of D-lactate, led to the suggestion that the effectors inhibited autophosphatase activity. At that time, however, it was not known that ArcB mediates a His → Asp → His → Asp phosphorelay and that ArcB serves also as an ArcA-P phosphatase.

In a subsequent study of the phospho-decay pathway of ArcA-P, we failed to detect any influence of the effectors on this process (10). The successful characterization of both the phosphorylating and the dephosphorylating pathways of the Arc system by the use of various combinations of the ArcB modules afforded us a chance to undertake a more systematic investigation of the mode of effector action. Here we present the results of experiments designed to analyze the influence of the effectors on the phosphorylation and dephosphorylation pathways.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Oligonucleotides—E. coli strain M15 and plasmids pREP4 and pQE30 were obtained from Qiagen Ltd. Plasmids pQE30ArcB$^{78-778}$, pQE30ArcB$^{78-520}$, pQE30ArcA, and

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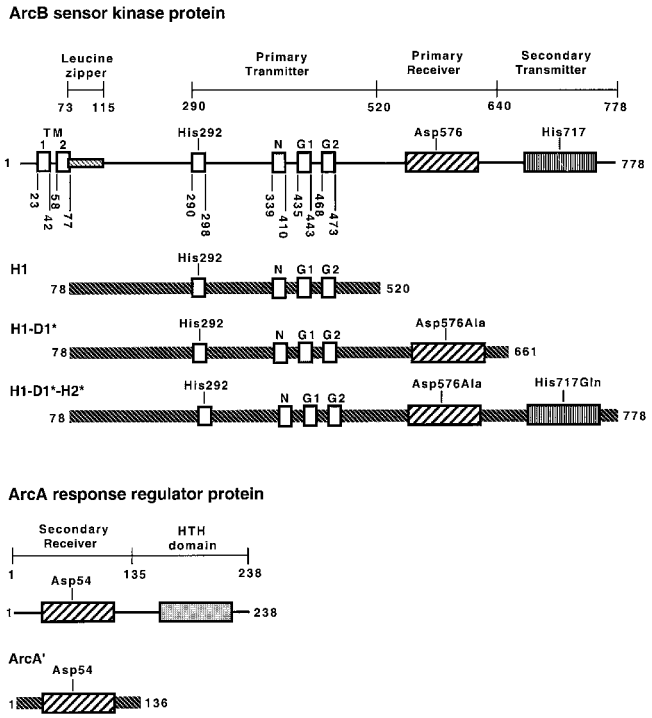

FIG. 1. **The schematic representation of ArcB and ArcA.** *Top panel*, the two transmembrane segments (*TM*) in the N-terminal domain were predicted on the basis of hydrophobicity plot (4) and ArcB-PhoA fusion analysis.[1] Residues 73–115 contain a putative leucine zipper motif (17). The primary transmitter domain (H1) is shown with the conserved His-292 and the catalytic determinants N, G1, and G2. The sequences of G1 and G2 resemble nucleotide-binding motifs. The receiver domain (D1) is shown with the conserved Asp-576, and the secondary transmitter domain (H2) is shown with the conserved His-717. The *hatched bars* indicate the lengths of H1, H1-D1*, and H1-D1*-H2*. The *stars* of D1 and H2, respectively, represent the Asp-576 → Ala substitution and the His-717 → Gln substitution in the conserved sequences shown as *hatched boxes*. *Bottom panel*, ArcA is shown with its N-terminal receiver domain containing the conserved Asp-54 and its C-terminal helix-turn-helix domain (*HTH domain*). The *hatched bar* indicates the length of ArcA′.

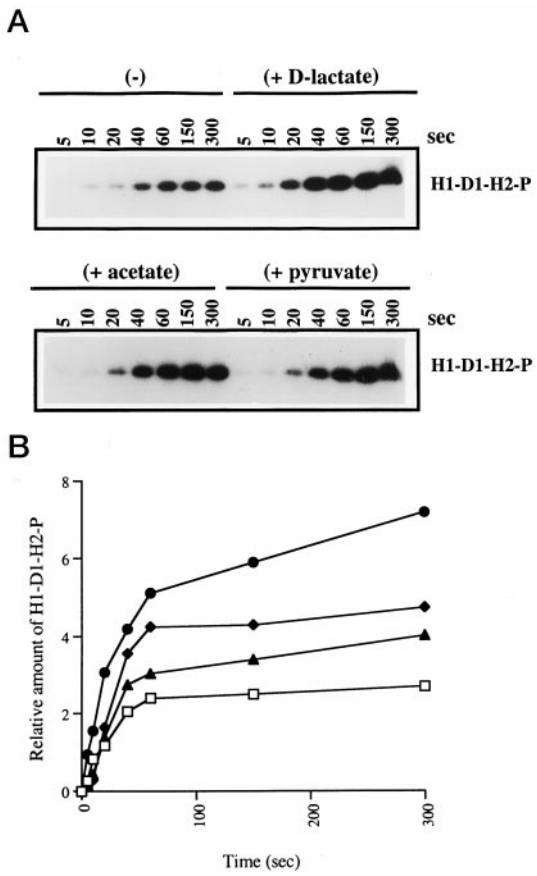

FIG. 2. **Effect of D-lactate, acetate, and pyruvate on the net phosphorylation of ArcB.** H1-D1-H2 was incubated with [γ-^{32}P]ATP at 15 °C in the presence or absence of a metabolite. At various time points, samples were taken for assay of radiolabeled ArcB by gel electrophoresis. D-lactate, acetate, and pyruvate were used at 1 mM. *A*, autoradiogram of the dried gel; *B*, the kinetics of H1-D1-H2 net phosphorylation. *Open squares*, without effector; *solid circles*, in the presence of D-lactate; *solid diamonds*, in the presence of acetate; *solid triangles*, in the presence of pyruvate.

pQE30ArcA′ used for expression of His-tagged derivatives of ArcB and ArcA, have been described previously (9, 10, 15). To create pQE30ArcB$^{78-661,Asp-576\rightarrow Ala}$, primers 5′-CCCGGATCCCATATGGAGCAACTGGAGGAGTCACGAC-3′ and 5′-CCCGGATCCATGCATCATTATGATTTACTGTTCTCTCTTCTGTCGTC-3′ were used in the polymerase chain reaction with pBB35 (7) as template. The polymerase chain reaction product was digested with *Bam*HI and *Nsi*I and cloned between *Bam*HI and *Pst*I of pQE30.

Plasmid pQE30ArcB$^{78-778,Asp-576\rightarrow Ala,\ His-717\rightarrow Gln}$ was constructed by inserting the *Bam*HI-*Eco*RV fragment from plasmid pQE30-ArcB$^{78-661,\ Asp-576\rightarrow Ala}$ into *Bam*HI-*Eco*RV-restricted pQE30ArcB$^{521-778,His-717\ \rightarrow\ Gln}$ (10).

The DNA fragments cloned from polymerase chain reaction amplified material were verified by sequencing at Micro Core Facility of the Dept. of Microbiology and Molecular Genetics, Harvard Medical School.

Purification of His$_6$-tagged Proteins, Isolation of ArcA$^{1-136}$-P (ArcA′-P), and Dephosphorylation Assays—Expression and purification of the His$_6$-tagged proteins were described previously (9). Phosphorylation of ArcA′ (about 0.4 nmol) was carried out at room temperature in 20 μl of buffer A (33 mM HEPES at pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol) containing ArcB$^{78-778}$ (H1-D1-H2) (~50 pmol) and 40 μM [γ-^{32}P]ATP (specific activity 2 Ci/mmol, NEN Life Science Products). After 10 min, the reaction was terminated by addition of 100 μl of buffer B (50 mM Tris-HCl at pH 7.0, 150 mM KCl, 5 mM EDTA, and 3% Triton X-100). H1-D1-H2 was separated from the reaction mixture by ultrafiltration, using a Nanosep 30K device (Pallfiltron). The filtrate, containing ArcA′-P, was passed through a Nanosep 10K device, and the retained material was washed 4 times with 500 μl of buffer B and once with 500 μl of buffer A. Finally, the retained material containing ArcA′-P (essentially free of H1-D1-H2, nonhydrolized ATP and P_i) was diluted to a final volume of 200 μl with buffer A. Dephosphorylation assays were carried out at room temperature in mixtures of 35 μl containing ~50 pmol ArcA′-P and 5 pmol of H1-D1-H2. At various time points, a 5-μl sample was withdrawn, mixed with 5 μl of SDS sample buffer, and kept on ice until the last portion was taken. The radioactivity of proteins, resolved on SDS-gels, was determined qualitatively by autoradiography with X-Omat AR (Eastman Kodak) and quantitatively by a PhosphorImager (Molecular Dynamics).

Autophosphorylation and Transphosphorylation Assays—Unless otherwise specified, autophosphorylation and transphosphorylation assays were carried out at 15 °C in the presence of 40 μM [γ-^{32}P]ATP (specific activity 2 Ci/mmol, NEN Life Science Products), 33 mM HEPES (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol. D-Lactate, acetate, and pyruvate were used at 1 mM. The reactions were initiated by addition of [γ-^{32}P]ATP, terminated by addition of an equal volume of 2× SDS sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis on 12.5% gels. Radioactivity of proteins, resolved in the gels, was determined qualitatively by autoradiography of the dried gels with X-Omat AR (Kodak) and quantitatively by a PhosphorImager (Molecular Dynamics).

RESULTS AND DISCUSSION

D-Lactate, Acetate, and Pyruvate as Effectors on the Net Phosphorylation of ArcB and ArcA—To probe the influence of the effectors on the phospho-transfer reactions, we used ArcB$^{78-778}$ (hereafter referred to as H1-D1-H2), deprived of amino acid residues 1–77 that constitute the transmembrane segments. Absence of residues 1–77 of ArcB, facilitates the purification of

[1] O. Kwon and D. Georgellis, unpublished data.

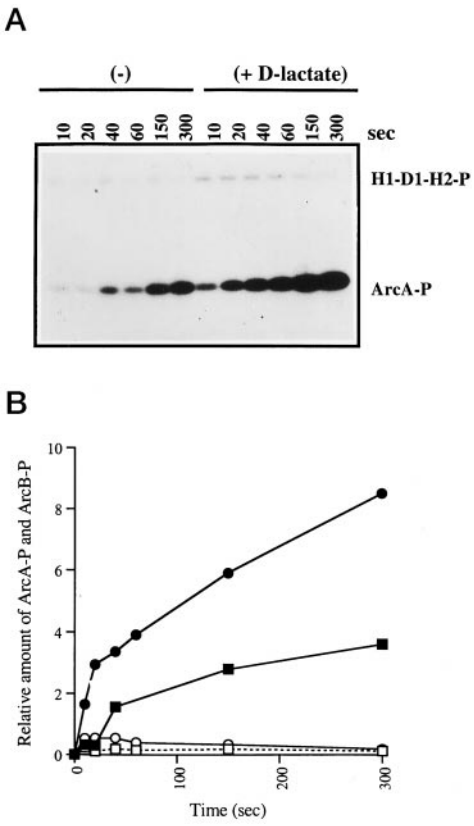

FIG. 3. **Effect of D-lactate on the net transphosphorylation of ArcA.** ArcA was incubated with H1-D1-H2 and [γ-^{32}P]ATP at 15 °C in the presence or absence of 1 mM D-lactate. At various time points, samples were taken for assay of the radiolabeled proteins by gel electrophoresis. D-lactate was used at 1 mM. *A*, autoradiogram of the dried gel; *B*, the kinetics of net protein phosphorylation. *Squares*, in the absence of D-lactate; *circles*, in the presence of D-lactate. *Solid symbols* represent phosphorylated ArcA; *open symbols* represent phosphorylated H1-D1-H2.

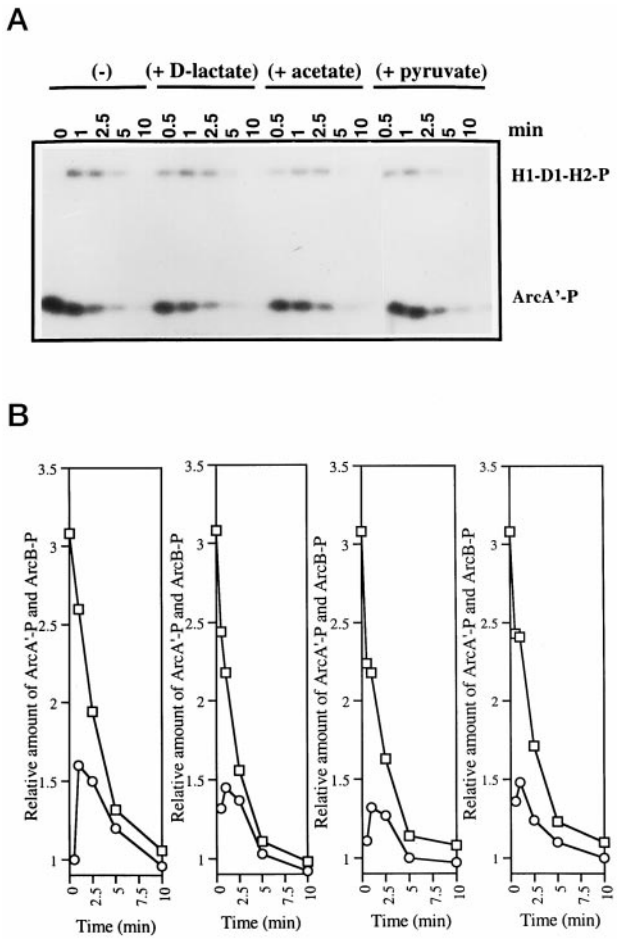

FIG. 4. **Effect of the metabolites on the phosphatase activity of ArcB.** Purified ArcA′-P was incubated with H1-D1-H2 in the presence or absence of an effector. At various time points, samples were taken for assay of the radiolabeled proteins by gel electrophoresis. *A*, autoradiogram of the dried gel; *B*, the kinetics of ArcA′-P dephosphorylation. *Squares*, ArcA′-P; *circles*, ArcB-P. *Panels* from left to right: no effector, in the presence of D-lactate, in the presence of acetate, and in the presence of pyruvate.

H1-D1-H2, which is able to catalyze both the phosphorylation of ArcA (9) and the dephosphorylation of ArcA-P (10). H1-D1-H2 was incubated with [γ-^{32}P]ATP at 15 °C in the presence or absence of each of the metabolites (at 1 mM, which is within the physiological range), and the time course of protein net phosphorylation was followed. The low temperature enabled the observation of the initial reaction rate. We noted, however, that at temperatures substantially below 15 °C the specific activity of H1-D1-H2 was drastically lost, possibly because of conformational changes. As shown in Fig. 2, both the initial rate of H1-D1-H2 phosphorylation and the overall level of the phosphorylated protein were increased about 3-fold by the presence of 1 mM D-lactate. Acetate and pyruvate exerted similar effects, but to lesser degrees (Fig. 2). In agreement with a previous study (14), L-lactate, which is not a fermentation product, did not affect the phosphorylation rate of H1-D1-H2 (data not shown). It is worthy of note that in the presence of D-lactate, the steady state level of protein phosphorylation in the reaction mixture was not reached within 300 s. Prolonged incubation eventually resulted in loss of radiolabel by H1-D1-H2-P in all incubation mixtures (data not shown), consistent with the results from previous studies, which were carried out at room temperature over a period of 20 min (9, 14).

Because D-lactate was most effective in raising the rate of ArcB net phosphorylation, the effect of this metabolite on the transphosphorylation of ArcA by ArcB was also examined. For this purpose, ArcA and H1-D1-H2 were incubated with [γ-^{32}P]ATP in the presence or absence of D-lactate, and the time course of the reactions was followed. The rate of ArcA transphosphorylation by H1-D1-H2 was stimulated about 3-fold by the presence of the effector (Fig. 3), which is consistent with an earlier qualitative observation (14). In view of the observation that the net rates of H1-D1-H2 autophosphorylation and ArcA transphosphorylation are both stimulated by D-lactate by about 3-fold, it seems unlikely that the effector acts by stimulating the rate of phosphoryl group transfer to ArcA. Because the net phosphorylation of both H1-D1-H2 and ArcA reflects the balance of the rates of phosphorylation and dephosphorylation, it is not clear in which reaction pathway D-lactate exerts its effect. To clarify this issue, the rate of dephosphorylation of ArcA-P by H1-D1-H2, in the presence or absence of the effector, was next studied.

Influence of the Effectors on the Phosphatase Activity of ArcB—It has been shown that the dephosphorylation of ArcA-P depends on a reverse phosphorelay pathway involving the sequential action of H2 and D1 of ArcB (10). Moreover, a previous report suggested that the effectors act by inhibiting the autophosphatase activity of ArcB (14). We therefore asked whether any of the effectors act by inhibiting a step in the dephosphorylation pathway. To test this, ArcA$^{1-136}$ (hereafter referred to as ArcA′), containing the receiver domain but lacking the helix-turn-helix domain (the presumptive DNA binding domain) was used. Removal of this domain facilitates the preparation of

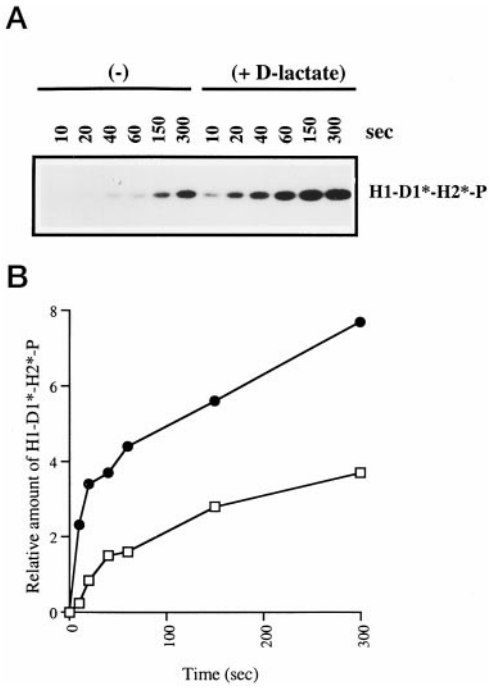

FIG. 5. **Effect of D-lactate on the autophosphorylation rate of ArcB.** H1-D1*-H2* ($ArcB^{78-778,\ Asp\text{-}576\rightarrow Ala,\ His\text{-}717\rightarrow Gln}$) was incubated with [$\gamma$-^{32}P]ATP at 15 °C in the presence or absence of 1 mM D-lactate. At various time points, samples were taken for assay of the radiolabeled protein by gel electrophoresis. *A*, autoradiogram of the dried gel; *B*, the kinetics of H1-D1*-H2* autophosphorylation. *Open squares*, in the absence of D-lactate; *closed circles*, in the presence of D-lactate.

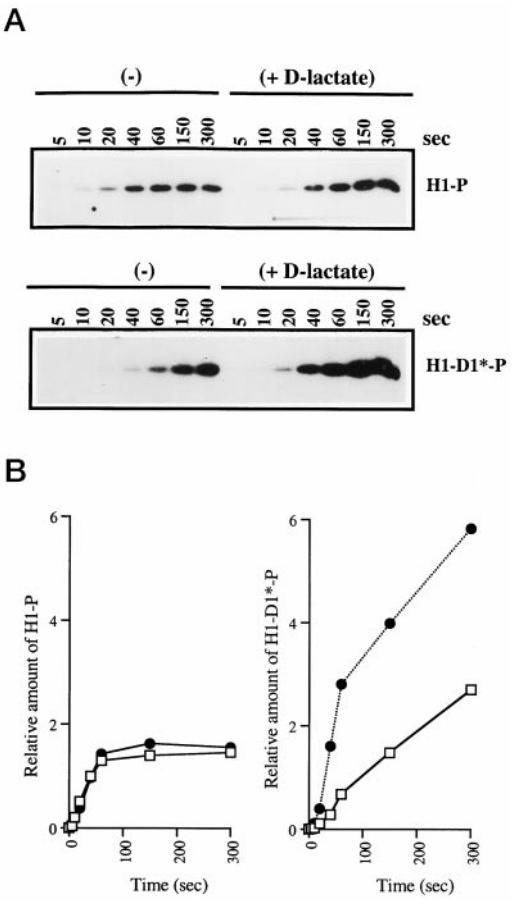

FIG. 6. **Probing the modular location of effector binding.** H1 ($ArcB^{78-520}$) and H1-D1* ($ArcB^{78-661,\ Asp\text{-}576\rightarrow Ala}$) were incubated with [$\gamma$-^{32}P]ATP at 15 °C in the presence or absence D-lactate. At various time points, samples were taken for assay of the radiolabeled proteins by gel electrophoresis. *A*, autoradiogram of the dried gels; *B*, the kinetics of H1 (*left panel*) and H1-D1* (*right panel*) autophosphorylation. *Open squares*, in the absence of D-lactate; *closed circles*, in the presence of D-lactate.

ArcA′-P, because, unlike the phosphorylated form of the intact protein, ArcA′-P does not aggregate during the purification process. It is also known that ArcA′ can be phosphorylated by H1-D1-H2 and that ArcA′-P can be dephosphorylated by H1-D1-H2 (10). To prepare ArcA′-P, the protein was incubated with H1-D1-H2 in the presence of [γ-^{32}P]ATP and subsequently isolated by ultrafiltration. ArcA′-P was then incubated with H1-D1-H2 in the presence or absence of the three metabolites, and the kinetics of the reactions were followed (Fig. 4). During the course of all four reactions, there was a rapid loss of radiolabel by ArcA′-P and a slight transient rise of labeled H1-D1-H2 followed by a gradual decay. None of the effectors showed any detectable effect on the rate of ArcA′-P dephosphorylation (Fig. 4). It thus appears that the phosphatase activity of ArcB is not modulated by the effectors, implying that they enhance the autophosphorylating activity of ArcB.

D-Lactate Effect on the ArcB Autophosphorylating Activity—Because His-292 was shown to be the autophosphorylating site of H1-D1-H2 (7, 9) and Asp-576 is essential for the release of P_i by the phosphorylated protein (10), we generated $ArcB^{78-778,Asp\text{-}576\rightarrow Ala,\ His\text{-}717\rightarrow Gln}$ (hereafter referred to as H1-D1*-H2*) to test the effect of D-lactate on the initial rate of ArcB autophosphorylation. The mutant protein was incubated with [γ-^{32}P]ATP in the presence or absence of D-lactate, and the time course of the reaction was followed. As shown in Fig. 5, the rate of H1-D1*-H2* phosphorylation was at least 3-fold higher in the presence of D-lactate than in its absence. Moreover, prolonged incubation did not reveal any P_i release (data not shown). Because D-lactate enhances the net phosphorylation of H1-D1-H2 and H1-D1*-H2* at a similar rate, it seems most likely that the effect is exerted by accelerating the rate of autophosphorylation rather than inhibiting the rate of dephosphorylation. Although a direct answer regarding the dephosphorylation rate could be provided by determining the half-life of H1-D1-H2-P in the presence or absence of the effector, the lability of the phosphoprotein did not permit its isolation as an ATP-free preparation for such a study.

Probing the Modular Location of the Effector Binding Site—Efforts were then made to localize the domain of effector binding. Because phosphorylation of H1-D1*-H2* can occur only at the primary transmitter domain, we tested the effect of D-lactate on the phosphorylation of this individual domain. $ArcB^{78-520}$ (H1) was incubated with [γ-^{32}P]ATP in the presence or absence D-lactate. No effect of D-lactate on the rate of H1 phosphorylation was apparent (Fig. 6). By contrast, when D-lactate was added to a reaction mixture containing $ArcB^{78-661,\ Asp\text{-}576\rightarrow Ala}$ (H1-D1*) and [γ-^{32}P]ATP, a 3-fold enhancement of the level of protein phosphorylation occurred (Fig. 6), as in the case of H1-D1*-H2*- P (Fig. 5). It thus seems that either the effector binding site is located in D1 or the structural presence of D1 enables the effector to bind to H1.

Implications for Signal Generation and Decay—We have shown in this study that the effectors accelerated the autophosphorylation of the primary transmitter in the presence of the receiver domain, even when it is catalytically inactive (*e.g.* $D1^{Asp\text{-}576\rightarrow Ala}$). Although all of the proteins used lack the transmembrane segments of ArcB and some of the proteins sustained single or double amino acid substitutions, they exhibited the expected catalytic activities. Thus, it is most likely that the mechanisms of effector action observed in this study do not depart from the wild-type situation.

If H1 autophosphorylation is the rate-limiting step and the phosphorelay is the sole means of ArcA phosphorylation by ArcB, then stimulation of H1 autophosphorylation by the effectors would be sufficient to raise the level of ArcA-P. However, there may be an additional pathway for ArcA phosphorylation by ArcB that responds differently to the effectors. Both *in vivo* experiments using ArcB alleles borne by multicopy plasmids (8) and *in vitro* experiments with different ArcB modular units (9) showed a weak, but significant, ArcA phosphorylating activity by H1. In this case the acceleration of H1 autophosphorylation would augment ArcA-P level by the additional route of direct phosphotransfer. Indeed, the *in vivo* work led to the suggestion that signal transmission via the H1 pathway responds to the cellular metabolic state and that the H2 pathway responds to the redox state of the cell (16). The issue of single or dual pathway of signal transmission, however, would require further experimentation. *In vivo* experiments based on single copies of the various *arcB* alleles would be necessary. In addition, the *in vitro* effects of D-lactate, acetate, and pyruvate need to be confirmed by *in vivo* evidence based on the behavior of appropriate mutants strains.

ArcB is a paradigm for a tripartite sensor kinase participating in a His-Asp-His-Asp phosphorelay and provides the first example of such a kinase whose activity may be physiologically modulated by defined effectors, in this case metabolic intermediates of fermentation pathways. The discovery that different ArcB modular units can be prepared in catalytically active forms adds further promise that better understanding of the mechanisms of ArcB signal transmission will hasten the characterization of other tripartate kinases of two-component systems.

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