Rotational On-off Switching of a Hybrid Membrane Sensor Kinase Tar-ArcB in *Escherichia coli**

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Signal transduction in biological systems typically involves receptor proteins that possess an extracytosolic sensory domain connected to a cytosolic catalytic domain. Relatively little is known about the mechanism by which the signal is transmitted from the sensory site to the catalytic site. At least in the case of Tar (methyl-accepting chemotaxis protein for sensing aspartate) of *Escherichia coli*, vertical piston-like displacements of one transmembrane segment relative to the other within the monomer induced by ligand binding has been shown to modulate the catalytic activity of the cytosolic domain. The ArcB sensor kinase of *E. coli* is a transmembrane protein without a significant periplasmic domain. Here, we explore how the signal is conveyed to the catalytic site by analyzing the property of various Tar-ArcB hybrids. Our results suggest that, in contrast to the piston-like displacement that operates in Tar, the catalytic activity of ArcB is set by altering the orientation of the cytosolic domain of one monomer relative to the other in the homodimer. Thus, ArcB represents a distinct family of membrane receptor proteins whose catalytic activity is determined by rotational movements of the cytosolic domain.

The ArcB/A (anoxic redox control) two-component system of *Escherichia coli* regulates the expression of more than 30 operons depending on the redox conditions of growth (1–3). This system consists of ArcB as the transmembrane sensor kinase and ArcA as the cognate response regulator (Fig. 1). ArcB is unorthodox in possessing an elaborate cytosolic structure that comprises three catalytic domains: a primary transmitter with a conserved His residue at position 292, a receiver with a conserved Asp at position 576, and a secondary transmitter with a conserved His at position 717 (4, 5). ArcA is a typical response regulator possessing an NH_2 terminus receiver domain with a conserved Asp residue at position 54 and a COOH terminus helix-turn-helix DNA binding domain (1).

Under reducing conditions, ArcB undergoes autophosphorylation at His^{292}, a reaction that is enhanced by several fermentation metabolites such as D-lactate, pyruvate, and acetate (6). The phosphoryl group is then sequentially transferred to ArcA via a $His^{292} \rightarrow Asp^{576} \rightarrow His^{717} \rightarrow Asp^{54}$ phospho-relay (7–9). Phosphorylated ArcA (ArcA-P), in turn, represses the expres-

sion of many operons involved in respiratory metabolism (1) and activates other operons encoding proteins involved in fermentative metabolism (10, 11). Under oxidizing conditions, ArcB autophosphorylation is curtailed and the protein catalyzes the dephosphorylation of ArcA-P via an $Asp^{54} \rightarrow His^{717} \rightarrow Asp^{576}$ reverse phospho-relay (12).

Another unorthodox structural feature of ArcB is its short periplasmic sequence of only about 16 amino acid residues (13), in contrast to typical sensor kinases that have a substantial periplasmic domain for environmental sensing. Unexpectedly, replacing various segments within the ArcB membrane region by a counterpart of MalF (a subunit of maltose permease without any sequence homology with ArcB) was without significant impact on the signal transduction process (13). From these observations it was concluded that the stretch of ArcB delimited by the two transmembrane segments (amino acid residues 22–77) does not directly participate in signal recognition but rather serves as an anchor to keep the protein close to the source of the signal (13). Recently, the oxidized forms of ubiquinone and menaquinone electron carriers, accumulating during aerobiosis, were shown to act as direct negative signals that inhibit autophosphorylation of ArcB (14). This would explain the importance of tethering the sensor kinase to the membrane. Otherwise, the lipophilic quinones located exclusively in the membrane bilayer would only be able to silence a small fraction of the ArcB molecules at any given time, since most of them would be distributed throughout the cytosol. Indeed, liberation of ArcB from the membrane by genetic excision of its NH_2-terminal transmembrane region was shown to result in a constitutively active kinase *in vivo* (13). Although the signals, as well as several effectors, for ArcB have been identified and considerable knowledge about the steps of signal transduction and decay has been gained, the mechanism by which the signal is conducted from the quinone sensory site to the autophosphorylation site of ArcB remains elusive.

Several mechanisms have been proposed for signaling across biological membranes by receptors that possess an external ligand-binding sensory domain connected to a cytosolic catalytic domain. The mechanisms include conformational changes of the functional dimer brought about by ligand-induced vertical displacement of one transmembrane segment relative to the other within the monomer (piston mechanism) or by rotational movement of the cytosolic domain (15, 16). Although the rotational model is yet to be demonstrated experimentally, good evidence for the piston model emerged from studies on Tar, the methyl-accepting chemotaxis protein for sensing aspartate in *E. coli*. Tar has two transmembrane segments, TM 1 (amino acids residues 7–30) and TM 2 (amino acids residues 189–212), and forms a stable dimer in the cytoplasmic membrane (17). The periplasmic portion of Tar functions as the signal input domain by binding various ligands such as aspartate. Results from disulfide cross-

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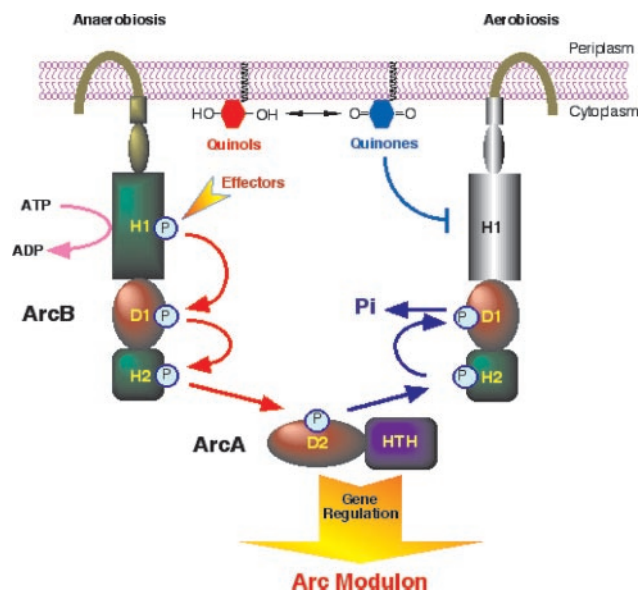
FIG. 1. **A schematic representation of signal transduction by the Arc two-component system.** Oxidized forms of quinone electron carriers behave as inhibitory signals that curtail ArcB autophosphorylation. Fermentative metabolites such as D-lactate, pyruvate, and acetate behave as effectors that promote ArcB autophosphorylation. For details see text.

linking experiments suggested that the aspartate binding does not affect the dimerization status but induce global changes in the conformational structure of Tar (18). Subsequent experiments based on spin-labeling electron paramagnetic resonance spectroscopy indicated that binding of the periplasmic domain to aspartate results in a piston-like displacement of TM 2 relative to TM 1 of Tar (19). Such a displacement would decrease an allosteric interaction between the receptor and the membrane by pushing a cytoplasmic domain away from the membrane, thereby altering the methylation and kinase cascades (16, 19, 20).

The piston mechanism has been proposed to operate also in membrane bound sensor kinases of two-component signal transduction systems (21) and has been demonstrated for the bacterial EnvZ protein, an orthodox sensor kinase with only a single cytoplasmic transmitter domain (22). The study of EnvZ involved replacing its transmembrane domain with the corresponding domain of Tar. It was found that the kinase activity of the Tar-EnvZ hybrid sensor protein came under the control of periplasmic aspartate (22). In contrast to EnvZ, ArcB has no periplasmic signal reception domain. The question arises as to whether signal propagation could still involve a piston mechanism. Here we address the problem by analyzing the signaling property of a Tar-ArcB chimeric kinase in which the transmembrane domain of the Tar sensor protein is fused to the cytosolic portion of the ArcB sensor kinase.

EXPERIMENTAL PROCEDURES

Growth Conditions—Cells were grown in Luria-Bertani (LB) broth or on LB agar, unless otherwise specified. Ampicillin, tetracycline, kanamycin, and chloramphenicol were provided at a final concentration of 50, 12, 40, and 20 μg/ml, respectively.

Recombinant DNA Techniques and PCR—Chromosomal and plasmid DNA were isolated by using respectively the Wizard genomic DNA purification kit (Promega) and Quiaprep spin miniprep kit (Quiagen). DNA fragments were recovered from agarose gel by using Quiaquick gel extraction kit (Quiagen). Oligonucleotides used in this study were synthesized by Integrated DNA technologies Inc. PCR were carried out by using the TaqPlus precision PCR system (Stratagene). PCR products were purified by using QIAquick PCR purification kit (Quiagen). Sequence verification of PCR-amplified DNA was performed at Micro Core Facility of the Department of Microbiology and Molecular Genetics, Harvard Medical School.

Construction of Δtar Strains—To construct a Δ*tar* strain, a 1.4-kb flanking sequence of *tar* was prepared by two-step PCR (13) from the chromosomal DNA of strain MC4100 with primers DTR-5N (5′-CGCG-CGAGATCTATGACCGGTATGACG-3′), DTR-M (5′-CGCAGTCGTGG-CTGAGCCGGATCCAACAGCGTGACTACG-3′), and DTR-C (5′-CCCG-GGAGATCTTGCGCGTCCACCACAGC-3′). The purified PCR product was digested with *Bgl*II and cloned into the *Bam*HI site of pKO3 (23), resulting in pDTR. Plasmid pDTR was transformed into strains ECL5002 (λΦ[*lldP′-lacZ*]) and ECL5012 (Δ*arcB*::tet[r] λΦ[*lldP′-lacZ*]) to delete *tar* by homologous recombination as described previously (23), respectively, yielding strains ECL5041 (Δ*tar* λΦ[*lldP′-lacZ*]) and 5042 (Δ*tar* Δ*arcB*::tet[r] λΦ[*lldP′-lacZ*]). Deletion of *tar* was confirmed by PCR.

Construction of tar-arcB Fusions—To construct Φ(*tar-arcB*) fusions, PCR were performed using the chromosomal DNA of strain MC4100 (13) as a template and TAB-N (5′-CCAGGATCCCATATGATTAACCG-TATCCGCG-3′) and TAB-1 (5′-CGTGACTCCTCCAGTTGCTCAATGC-CGTACCACGCCACC-3′), TAB-2 (5′-CGTGACTCCTCCAGTTGCTCG-CGAATGCCGTACCACGCC-3′), TAB-3 (5′-CGTGACTCCTCCAGTTG-CTCTCGACGAATGCCGTACCACG-3′), TAB-4 (5′-CGTGACTCCTCC-AGTTGCTCCATTCGACGAATGCCGTACC-3′), or TAB-5 (5′-CGTTGT-CGTGACTCCTCCATTCGACGAATGCCGTACC-3′) as primers. Each purified PCR product and B3NRU (5′-GTAATGTCGCGACCAAAGCC-CATCAAACCG-3′) were used as primers for the second PCR by using pABS (13) as a template. The PCR products were digested with *Nde*I and *Pst*I. The 0.7-kb fragments were gel-purified and cloned between the corresponding sites of the pIBW (13). This process yielded, respectively, pTAB1 through pTAB5. These plasmids were used to integrate the Φ(*tar-arcB*) sequences into the chromosome of the strain ECL5042 by the gene replacement techniques as described previously (13). This process yielded, respectively, ECL5043 (Φ[*tar*$^{1-212}$-*arcB*$^{78-778}$]::kan[r] Δ*tar* Δ*arcB* λΦ[*lldP′-lacZ*]), ECL5044 (Φ[*tar*$^{1-213}$-*arcB*$^{78-778}$]::kan[r] Δ*tar* Δ*arcB* λΦ[*lldP′-lacZ*]), ECL5045 (Φ[*tar*$^{1-214}$-*arcB*$^{78-778}$]::kan[r] Δ*tar* Δ*arcB* λΦ[*lldP′-lacZ*]), ECL5046 (Φ[*tar*$^{1-215}$-*arcB*$^{78-778}$]::kan[r] Δ*tar* Δ*arcB* λΦ[*lldP′-lacZ*]), and ECL5047 (Φ[*tar*$^{1-215}$-*arcB*$^{81-778}$]::kan[r] Δ*tar* Δ*arcB* λΦ[*lldP′-lacZ*]).

β-Galactosidase Activity Assay—Strains were grown in minimal medium (24) containing 20 mM D-xylose with 20 mM L-lactate as a specific inducer (25). For aerobic growth, cells were cultured in 5 ml of medium in 250-ml baffled flasks at 37 °C with shaking (300 rpm). For anaerobic growth, cells were cultured in a screw-capped tube filled with medium up to the rim at 37 °C and stirred by a magnet. β-Galactosidase activity was assayed and expressed in Miller units.

Western Blot Analysis—Aerobically grown cultures were harvested by centrifugation during mid-exponential growth. The cell pellet was resuspended in 5× SDS sample buffer and separated by SDS-PAGE (12% polyacrylamide gel), and the proteins were transferred to a Hybond-ECL filter (Amersham Biosciences). The filter was equilibrated in TTBS buffer (25 mM Tris, 150 mM NaCl, and 0.05% Tween 20) for 10 min and incubated in blocking buffer (0.5% bovine serum albumin in TTBS) for 1 h at 37 °C. ArcB polyclonal antibodies, raised against His$_6$-ArcB$^{78-520}$ (9), was added at a dilution of 1:10,000 to the filter and incubated for 1 h at room temperature. The bound antibody was detected by using anti-rabbit IgG antibody conjugated to horseradish peroxidase and the ECL detection system (Amersham Biosciences).

RESULTS AND DISCUSSION

Cellular Expression of a Tar-ArcB Hybrid—An in-frame fused *tar-arcB* sequence encoding Tab-1 (Fig. 2, *A* and *B*), composed of the transmembrane domain of Tar (residues 1–212) and the cytosolic domains of ArcB (residues 78–778), was prepared. The DNA was incorporated into the chromosome by homologous recombination at the *arcB* locus of a host strain bearing an ArcA-P repressible λΦ(*lldP′-lacZ*) operon fusion (9, 13). In all strains studied the chromosomal *tar* sequence was deleted to prevent heterodimer formation between the wild-type Tar and Tar-ArcB hybrids. Western blot analysis of the cell extract with polyclonal antiserum raised against purified His$_6$-ArcB$^{78-520}$ (9) showed the presence of the hybrid protein but not the wild-type ArcB (Fig. 2*C*). Moreover, Tab-1 was produced at about the same level as that found in the isogenic *arcB*$^+$ strain and migrated electrophoretically at a rate corresponding to a molecular weight of 103 kDa instead of the 88 kDa expected of the wild-type ArcB. This increment in molecular weight is consistent with the extra 135 amino acid residues present in the Tar-ArcB hybrid.
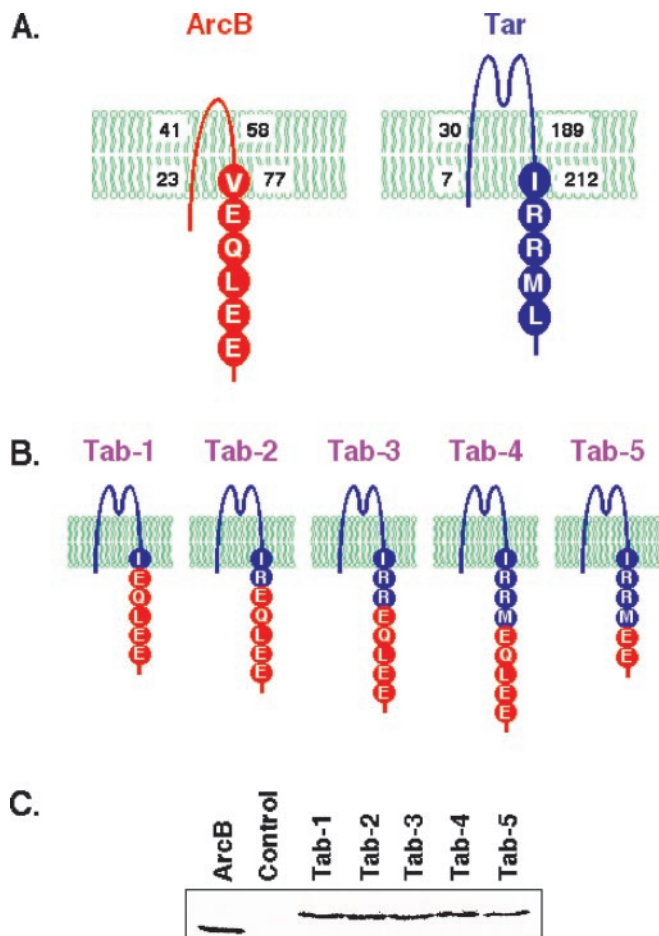
FIG. 2. **Chimeras of Tar and ArcB.** *A*, schematic depiction of the membrane-associated region of ArcB and Tar. *B*, junctions of Tar-ArcB chimeric kinases. *Tab-1*, Tar$^{1-212}$-ArcB$^{78-778}$; *Tab-2*, Tar$^{1-213}$-ArcB$^{78-778}$; *Tab-3*, Tar$^{1-214}$-ArcB$^{78-778}$; *Tab-4*, Tar$^{1-215}$-ArcB$^{78-778}$; and *Tab-5*, Tar$^{1-215}$-ArcB$^{81-778}$. *C*, analysis of extracts from cells expressing the various chimeric proteins by Western blot using ArcB antibodies (9).

The Redox Signaling Activity of Tar$^{1-212}$-ArcB$^{78-778}$ Hybrid Sensor Kinase—The *in vivo* signaling ability of the hybrid sensor was determined by analyzing the expression of an ArcA-P repressible reporter, Φ(*lldP'-lacZ*). Surprisingly, Φ(*lldP'-lacZ*) was strongly repressed in both aerobically and anaerobically grown cells (Fig. 3). This would indicate that Tar$^{1-212}$-ArcB$^{78-778}$ is highly active as an autokinase even under aerobic conditions, despite its being anchored to the plasma membrane. It thus appears that a proper mode of anchorage of the cytosolic domains of ArcB might be critical for redox signal transduction. To test whether aspartate could influence the signaling activity of Tab-1, the cells were grown aerobically or anaerobically in the presence or absence of the ligand. No aspartate effect on the expression of Φ(*lldP'-lacZ*) was found (data not shown), suggesting that a piston-like mechanism is not involved in the regulation of the ArcB kinase activity.

Functional Comparison of a Series of Tar-ArcB Hybrids with Altered Relative Orientation of the Catalytic ArcB Domain— The unfavorable evidence for a piston mechanism in ArcB signal transduction led us to examine the alternative model that involves rotational movements of the cytosolic domain around its membrane anchor. It should be noted that ArcB, like many other sensor kinases of two-components systems, functions as a homodimer that catalyzes intermolecular phosphoryl group transfers.[1] Consequently, the rotational orientation of
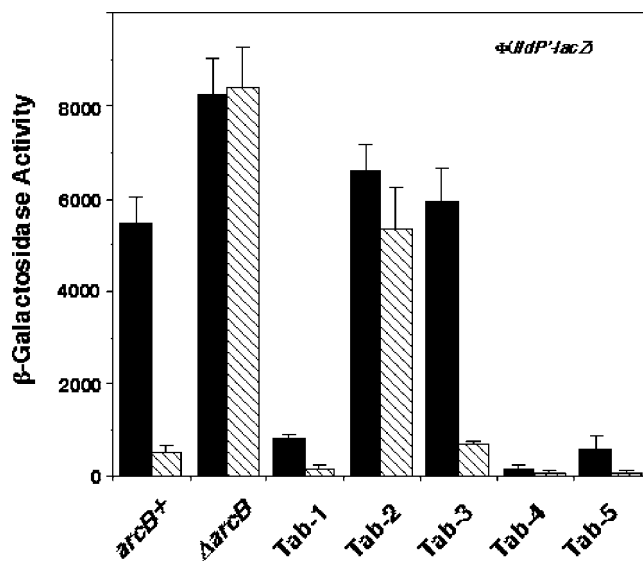
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[1] D. Georgellis, O. Kwon, and E. C. C. Lin, unpublished data.

FIG. 3. **Redox signaling ability of Tar-ArcB chimeric kinases as a function of the orientation of the catalytic domain.** Cells were grown aerobically or anaerobically in minimal medium (24) containing D-xylose and L-lactate (25). β-Galactosidase activity specified by the Φ(*lldP'-lacZ*) operon fusion (13) was expressed in Miller units. The data are an average of four experiments, and the S.D. values are indicated. *Solid bars*, aerobic growth; *hatched bars*, anaerobic growth. *Tab-1*, Tar$^{1-212}$-ArcB$^{78-778}$; *Tab-2*, Tar$^{1-213}$-ArcB$^{78-778}$; *Tab-3*, Tar$^{1-214}$-ArcB$^{78-778}$; *Tab-4*, Tar$^{1-215}$-ArcB$^{78-778}$; and *Tab-5*, Tar$^{1-215}$-ArcB$^{81-778}$.

one subunit relative to the other subunit may be critical for the phosphorylation cascade.

To test the importance of the relative orientations of the two ArcB segments imposed by the dimeric membrane anchor, we constructed a series of hybrids with different spatial relationships between the Tar anchor and the cytosolic ArcB parts. This was accomplished by fusing a set of Tar transmembrane domains with progressively extended COOH-terminal region to a constant cytosolic portion of ArcB: Tar$^{1-213}$-ArcB$^{78-778}$ (Tab-2), Tar$^{1-214}$-ArcB$^{78-778}$ (Tab-3), and Tar$^{1-215}$-ArcB$^{78-778}$ (Tab-4) (Fig. 2*B*). The procedure used was similar to that described for the construction of Tab-1. Since the rigid helical structure of the second transmembrane segment of Tar protrudes into the cytoplasm, each extra residue of Tar added at the Tar-ArcB junction should rotate the attached ArcB kinase by ~100°. Western blot analysis showed that all Tab hybrids were produced about the same level as Tab-1 and the wild-type ArcB. Moreover, all the Tab hybrids migrated electrophoretically at approximately the same rate (Fig. 2*C*).

The *in vivo* signaling properties of these fusion proteins were then analyzed by comparing the aerobic and anaerobic Φ(*lldP'-lacZ*) expression (Fig. 3). Tab-2, possessing one more amino acid residue of Tar at the junction than Tab-1, was inactive as a kinase, as indicated by the derepression of Φ(*lldP'-lacZ*) under both aerobic and anaerobic growth conditions (Fig. 3). The catalytic inactivity of this hybrid is likely due to failure of intermolecular phosphoryl group transfer at one or more steps of the phospho-relay, irrespective of whether the ArcB moiety is in the "on" or "off" conformation. Tab-3, possessing two additional amino acid residues at the junction, behaved like wild-type ArcB in response to altered redox conditions of growth. Tab-4, possessing three additional amino acid residues should have a similar spatial relationship between the two ArcB moieties as in Tab-1. This dimer once again became constitutively active as a kinase.

Because insertion of amino acids in the junction region of the chimeric protein not only alters the topological relationship of the anchor to its attached catalytic domain but also the local
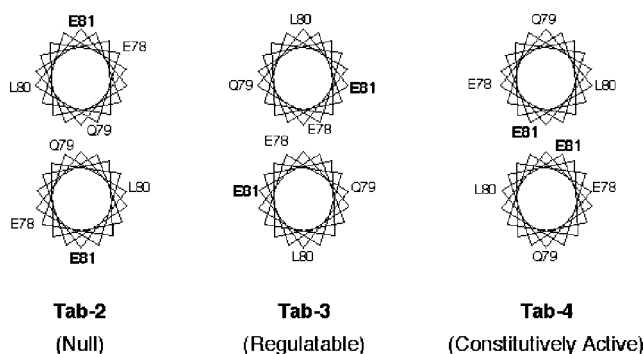
FIG. 4. **Relative orientation of the cytosolic ArcB domains of Tab-2, Tab-3, and Tab-4 homodimers.** Glu[78] (the first NH$_2$ residue of the cytoplasmic portion of ArcB), Gln[79], Leu[80], and Glu[81] of ArcB are depicted in each Tab subunit of the homodimer, as viewed from the periplasmic space. This configuration is based on the known topological structure of the α-helix of the second transmembrane segment of Tar (26–28).

amino acid sequence of the linker region, a control hybrid kinase, Tar$^{1-215}$-ArcB$^{81-778}$ (Tab-5), was constructed as a control. In this construct, the Tar moiety of the hybrid was extended by three amino acid residues on the COOH-terminal side at the expense of three amino acid residues on the NH$_2$-terminal side of ArcB. Thus the ArcB portion of this hybrid should have the same orientation as that of the constitutively active Tab-1. Indeed, Tab-5 turned out to be also constitutively active as a kinase. Finally, the effect of aspartate on the signaling properties of all the chimeras was tested. None of the constructs responded to the ligand (data not shown), indicating that the piston model does not apply to ArcB. Therefore it seems reasonable to conclude that the quinone electron carriers, previously identified as the direct signal for ArcB, act by altering the orientation of the cytosolic domain of one monomer relative to that of the other monomer.

Relative Orientations of the Cytosolic ArcB Domains of Various Tar-ArcB Hybrid Homodimers—Since the helical structure of the second transmembrane segment of Tar (26–28) is known, it is possible to predict the relative orientation of the two ArcB moieties in each dimer. It is assumed that the α-helix of the second transmembrane segment remains in a constant position for each chimera. Fig. 4 presents the predicted orientation of the three representative chimeras: Tab-2, Tab-3, and Tab-4. If we use the Glu residue at position 81 of ArcB as a marker, it can be seen that the two Glu81 residues of Tab-2 are located on diametrically opposite sites of the homodimer. This configuration disables the kinase activity irrespective of the redox condition. The addition of an extra amino acid residue at the fusion junction brings the two Glu[81] residues closer together in Tab-3 because of a 100° clockwise rotation of each monomer relative to Tab-2. The relative orientation of the kinase moieties in Tab-3, which is redox-regulated, most likely represent that of the wild-type ArcB. The addition of two extra amino acid residues at the fusion junction brings the two Glu[81] residues even closer to each other in Tab-4 as the result of a 200° clockwise rotation of each monomer relative to Tab-2. The orientation of the cytoplasmic ArcB moieties in Tab-4 is very similar to that in Tab-1. Tab-4 exhibits constitutively kinase activity, like Tab-1.

A potentially informative approach to verify a signaling mechanism that depends on the relative orientation of the cytosolic portion of a membrane protein with respect to the membrane attached portion would be the use of cysteine-cysteine cross-linking analysis. Such a strategy has been successfully employed to study α-helix interactions in analogous systems (20, 29). In particular, inverted vesicles embedded with

various mutant hybrids containing cysteine substitutions in the helical cytosolic domain of the ArcB portion could be prepared and changes in the ability of different cysteine residues to form disulfide bridges depending on the type of hybrid could be examined by Western blotting.

On the basis of the catalytic properties of various Tab constructs, we postulate that the mechanism of ArcB signal transduction relies on rotational movements of its cytosolic portion. ArcB thus appears to represent a distinct family of membrane signal transduction proteins. It is of interest to note that the evolution of such a rotational switch mechanism would require coadaptation of a signal receptor protein to function as a dimer.

Possible Applications of the Tar Sensor Hybrids—The use of the transmembrane domain of Tar to study chimeric sensor proteins could be applied in two ways. First, this strategy should be useful in distinguishing the piston mechanism from the rotation mechanism in a signal transduction process by a membrane protein. Second, the approach should allow the generation of constitutively active sensor kinases. This can be achieved either by providing aspartate in the growth medium, as in the case of the Tar-EnvZ chimera (22), or by altering the orientation of the catalytic domain through addition or subtraction of amino acid residues in the helical region that links the transmembrane to the cytosolic domain, as in the case of the Tar-ArcB chimera. Because the actual signals of most membrane-bound sensor kinases remain unknown, constitutively active sensor kinases could serve as a tool for investigating the downstream processes of each system under *in vivo* conditions.

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