Function of DcuS from *Escherichia coli* as a Fumarate-stimulated Histidine Protein Kinase *in Vitro*

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The two-component regulatory system DcuSR of *Escherichia coli* controls the expression of genes of C4-dicarboxylate metabolism in response to extracellular C4 dicarboxylates such as fumarate or succinate. DcuS is a membrane-integral sensor kinase, and the sensory and kinase domains are located on opposite sides of the cytoplasmic membrane. The intact DcuS protein (His6-DcuS) was overproduced and isolated in detergent containing buffer. His6-DcuS was reconstituted into liposomes made from *E. coli* phospholipids. Reconstituted His6-DcuS catalyzed, in contrast to the detergent-solubilized sensor, autophosphorylation by [γ-32P]ATP with an approximate *Kₐ* of 0.16 mM for ATP. Up to 7% of the reconstituted DcuS was phosphorylated. Phosphorylation was stimulated up to 5.9-fold by C4-dicarboxylates such as fumarate, but not by other carboxylates. The phosphoryl group of DcuS was rapidly transferred to the response regulator DcuR. Upon phosphorylation, DcuR bound specifically to *dcuB* promoter DNA. The reconstituted DcuSR system therefore represents a defined *in vitro* system, which is capable of the complete transmembrane signal transduction by the DcuSR two-component system from the stimulus (fumarate) to the DNA, including signal transfer across the phospholipid membrane.

Bacteria have to adapt to frequent changes in environmental conditions. For sensing of environmental parameters most frequently two-component sensor-regulator systems are used by the bacteria. The two-component systems are composed of sensory histidine (His) protein kinases and of response regulator proteins (1, 2). The protein kinases typically are located in the cytoplasmic membrane and comprise of a N-terminal sensory and a conserved C-terminal transmitter domain. The transmitter domain contains a kinase and a His residue as the site for phosphorylation. After autophosphorylation of the His residue, the phosphoryl group is transmitted to the response regulator. Typically, the N-terminal sensory domain is located at the periplasmic aspect of the membrane, whereas the kinase domain is located on the opposite cytoplasmic side of the membrane. Information on the partial reactions like stimulus perception, kinase activity, and phosphoryl transfer to the response regulator and the function of individual domains were gained mainly by studies with soluble His protein kinases like CheA or NtrB or, in the case of membranous kinases, with solubilized domains of the protein obtained by genetic truncation. In this way autophosphorylation, transphosphorylation between the subunits of the homooligomeric kinase domains, and phosphoryl transfer to the response regulators has been studied (Refs. 3–9, and for reviews, see Refs. 10 and 11). Similarly, binding of citrate to the periplasmic domain of the citrate sensor CitA, which binds citrate with high affinity, has been carefully characterized after overproduction of the solubilized domain (12, 13).

Due to lack of suitable experimental systems for studies in membranes, His protein kinases with transmembrane arrangement of the sensory and kinase domains have not been studied in detail *in vitro*. The most intriguing and characteristic property of this class of His protein kinases is signal transduction across the cytoplasmic membrane and the control of kinase activity by stimulus binding in the periplasm. Therefore a system suitable for biochemical studies on sensors of this type in the membrane capable of all functions, in particular control of the kinase by stimulus binding would be required for studies on signal transfer.

In *Escherichia coli* the DcuSR two component system (C4-dicarboxylate uptake) is responsible for the detection of C4-dicarboxylates in the medium (14–17). DcuSR stimulates the expression of genes involved in C4-dicarboxylate metabolism, including *dcuB* encoding the C4-dicarboxylate carrier DcuB of fumarate respiration (18–21). The sensor DcuS is a member of the CitA family of His protein kinases (15, 16, 22). For regulation by DcuS, no uptake of the C4-dicarboxylates into the cells is required (14, 15). Binding of the C4-dicarboxylates presumably occurs by a periplasmic domain close to the N-terminal domain, which is framed by two transmembrane helices. This periplasmic domain is homologous to the citrate binding domain of CitA, which binds citrate with high affinity (12). The kinase domain is located in the cytoplasm at the C-terminal end of the protein and separated by a putative further sensory (PAS or “linker”) domain of unknown function from the second transmembrane helix (14, 15).

For understanding of DcuS function as a transmembranous sensor and signal transducing protein, DcuS was solubilized in detergent, isolated, and reconstituted functionally in liposomes. By this an *in vitro* system for studies on DcuS function was established, which allows studies on stimulus perception and transmembrane signal transfer to the kinase domain and the complete signal transduction pathway to the DNA. Such a system seems to be a prerequisite for studies extending beyond the function of single domains, in particular of control of kinase activity by extracellular stimuli and transmembrane signaling.

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This work is dedicated to the late Prof. A. Kroeger (Frankfurt).

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**Experimental Procedures**

**Genetic Methods**—Standard molecular genetic methods were performed according to Sambrook et al. (23) or as recommended by suppliers. Plasmid DNA was isolated according to Chen and Kwo (24), plasmids by boiling lysis (25) or using kits (Qiagen, Hilden). PCR products were purified with the Qiagene PCR Purification Kit (Qiagen). DNA from agarose gels was extracted with the Qiagene Gel Extraction Kit (Qiagen). E. coli strains were transformed after pretreatment with RbCl. dcuS-encoding plasmid DNA was isolated by the hot start method with oligonucleotide primers DcuS-N (5'-CACA-CAGGAAACCATAGAGACATT-3') and DcuS-C (5'-ATTAAAGCTTT-GATCATCCTTGGC-3') from E. coli AN387 (29) genomic DNA. The PCR product was cloned via the NdeI and HindIII sites of the oligonucleotide into PET28a behind the inducible T7 promoter (Novagen). The resulting construct (pMW151) codes for complete DcuS, including Met (1) and carries an N-terminal His$_6$-tag and a thrombin cleavage site. For overproduction of DcuR, the deur gene was cloned into the NdeI and HindIII sites of PET28a after amplification of the gene from E. coli AN387 DNA PCR with primers pdcuR-Nde-22 (5'-GAGGTC-GAAGGAAGCATATGACATTC) and pdcuR-Hind-22 (5'-TAACCGCG-GAAGCTTATGTGCG). The resulting plasmid pMW180 codes for the complete dcuR gene, the thrombin site, and the His$_6$-tag.

**Overexpression and Isolation of His$_6$-DcuS and of DcuR**—For isolation of DcuS, E. coli BL21DE3pMW151 was grown in 0.8 liter of LB medium (30) at 30 °C under aerobic conditions to OD$_{600}$ = 0.5 and then induced with 1 mM isopropyl-$eta$-D-thiogalactopyranoside (IPTG) for 3 h (OD$_{600}$ = 1.5). Where appropriate, antibiotics were included. Before and after induction, 1 ml of the bacterial culture was dissolved in SDS loading buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (31). The cells were harvested, washed, and resuspended in buffer 1 (50 mM Tris/HCl, pH 7.7, and 10 mM MgCl$_2$). The bacteria were broken by three passages through the French press, and after removal of debris (9000 × g), the membrane fraction was pelleted by centrifugation (200,000 × g) for 65 min. All further steps were performed at 4 °C. The membranes were washed twice (1 mM Tris/HCl, 3 mM EDTA at pH 7.7) and homogenized (1.5 mg/ml) in buffer 2 (50 mM Tris/HCl, 10% glycerol, 2 mM dithiothreitol). The membrane fraction was mixed with Triton X-100 (0.58% w/v) or Triton X-100 (0.5% w/v). Isolated His$_6$-DcuS in elution buffer was added at a phospholipid:protein ratio of 2000:1. The suspension was incubated overnight at 4 °C. The supernatant was run by gravity through a Ni$_2$-NTA column (3 ml) equilibrated with buffer 4 (50 mM Tris/HCl, pH 7.7, 10% glycerol, 0.5 M NaCl, 10 mM imidazole, 0.04% dodecyl maltoside). The column was rinsed with 40 ml of buffer 2, and bound protein was eluted with 3 ml of buffer 3 (50 mM Tris/HCl, pH 7.7, 10% glycerol, 0.5 M NaCl, 10 mM imidazole, 100 mM Na$_2$-phosphate at pH 7, 500 mM NaCl, 10 mM imidazole). After washing with 30 ml of buffer 2 (buffer 4 with 20 mM imidazole), the His$_6$-DcuS protein was eluted with 30 ml of buffer 3 with 300 mM NaCl, 500 mM imidazole. The eluted protein was adjusted to 40% glycerol and stored at −80 °C

**Reconstitution of His$_6$-DcuS in Liposomes**—50 mg of E. coli phospholipids (polar lipid extract, 20 mg/ml in chloroform, Avanti Polar Lipids, Alabaster, AL) were evaporated and dissolved in 5 ml K$^+$-phosphate buffer (20 mM) containing 80 mg N-octyl-$eta$-D-glucopyranoside similar to a procedure by Jung et al. (32). The solution was dialyzed overnight against 3 × 1 liter buffer (20 mM potassium phosphate, pH 7.5). The resulting membrane suspension was frozen for three cycles in liquid N$_2$ and thawed slowly at 20 °C. The liposomes were destabilized by addition of dodecylmaltoside (0.58% w/v) or Triton X-100 (0.5% w/v). Isolated His$_6$-DcuS in elution buffer was added at a phospholipid:protein ratio of 20:1 (mg/mg) and stirred gently for 10 min at 20 °C. For every mg of Triton X-100 and dodecyl maltoside, 10 and 15 mg degassed Bio-Beads pretreated and described by Holloway (33) were added to remove detergent. The suspension was incubated overnight at 4 °C. The supernatant was then incubated with fresh Bio-Beads for 1 h at 20 °C. The suspension was removed with a pipette, frozen in liquid N$_2$, and stored at −80 °C.

**Phosphorylation of His$_6$-DcuS and Phosphoryl Transfer**—80 μl of the proteoliposome suspension were adjusted to 10 mM MgCl$_2$, 1 mM dithiothreitol, and 20 mM fumarate and frozen rapidly in liquid N$_2$, and slowly thawed at 20 °C for three cycles (34). After the final thawing, the proteoliposomes were kept for 1 h at 20 °C. Then 2.5 μl of γ-$^32$P]-ATP (110 TBq/mmol) were added at final concentrations of 0.1–10,000 μM ATP. Where indicated, isolated DcuR (4 μg/μg of DcuS) was mixed with the phosphorylation assay. At the time indicated, 10 μl were withdrawn, mixed with 10 μl SDS loading buffer, and 2 μl of DcuS per lane was subjected to SDS gels electrophoresis.

After electrophoresis the gels were exposed to an imaging plate (Fuji BAS-MP2040) to determine the radioactive bands in a phosphorimager (Fuji BAS 1500). For quantitative measurement of the radioactivity, the gels were stained for protein with Coomassie Blue. The protein bands were excised and incubated for 12 h at 46 °C in capped scintillation vials with small volumes (200 μl) of 30% H$_2$O$_2$. The solution with the dissolved bands was mixed with 4.5 ml of scintillation mixture (Rotisint Ecoplus) and counted for radioactivity (Beckman LS6000 SC). From the protein, the radioactivity, and the specific radioactivity, the amount of phosphate per protein (mol of P/mol of DcuS) was calculated.

**RESULTS**

**Overexpression and Isolation of DcuS and DcuR**—DcuS and DcuR are minor proteins in E. coli, therefore the dcuS and dcuR genes were cloned separately in expression vectors for overproduction of DcuS and DcuR as recombinant proteins with N-terminal His$_6$-extensions (Fig. 1). The dcuS expression strain overproduced a protein of $M_r$ 61,000, which is close to the predicted mass of 62,637 Da of His$_6$-DcuS. Upon cell fractionation, the $M_r$ 61,000 protein was found in the non-soluble cell fraction. From the membrane fraction the proteins were extracted with buffers containing the detergents LDAO or Empigen BB. Upon fractionation by centrifugation, most of the $M_r$ 61,000 protein was found in the soluble fraction. The solubilized proteins were applied to a Ni$_2^+$-NTA column, and LDAO or Empigen BB were replaced by rinsing with buffer containing dodecyl maltoside as the detergent. The $M_r$ 61,000 protein was eluted specifically with imidazole. The recombinant response regulator His$_6$-DcuR was present mostly in the supernatant of the cell homogenate after overexpression and fractionation (Fig. 1). His$_6$-DcuR was isolated in pure form using a Ni$_2^+$-NTA column. A considerable part of the protein was lost in the particulate cell fraction, presumably in the form of inclusion bodies. From 100 mg of cell protein about 2 mg of His$_6$-DcuS or 15 mg of His$_6$-DcuR were obtained.

**Reconstitution of DcuS and Autophosphorylation**—The solubilized DcuS protein was incorporated into liposomes prepared from E. coli phospholipids by dialysis. The liposomes were
incubated with low amounts of Triton X-100 or dodecyl maltoside to achieve "onset solubilization" (34) and mixed with isolated His<sub>6</sub>-DcuS in dodecyl maltoside. The detergent was removed from the mixture by treatment with Bio-Beads, resulting in the insertion of DcuS in the liposomes and the formation of proteoliposomes. The proteoliposomes were freeze-thawed to increase their size and to enable access of the stimulus (fumarate or succinate) to the intraliposomal space.

The histidine kinase activity of DcuS was measured as the autophosphorylation of DcuS in the presence of [γ-<sup>32</sup>P]ATP. After incubation, the proteoliposomes were dissolved in SDS and subjected to SDS gel electrophoresis. The phosphorylation of DcuS was detected by autoradiography (Fig. 2). The labeling of the reconstituted DcuS increased with time of incubation. Isolated DcuS protein in detergent, however, was not labeled by [γ-<sup>32</sup>P]ATP under the same conditions. Even with 10 times the amount of solubilized DcuS, no labeling was detected (Fig. 2).

**Kinetics of DcuS Autophosphorylation**—In a quantitative kinetic analysis, the phosphorylation of reconstituted DcuS increased with time (Fig. 3) and approached a maximum after 45 min with a t<sub>0.5</sub> value of about 13 min. Quantitative evaluation of phosphorylation by scintillation counting of the protein bands after excision and digestion showed that up to 7% of DcuS became phosphorylated. Presumably, the other portion of DcuS was not functional.

When the DcuS-proteoliposomes were sonicated after addition of the ATP, the same rate and final extent of phosphorylation of DcuS was obtained (not shown). Therefore the site of DcuS phosphorylation seems to be accessible to ATP under the experimental conditions. Since ATP was added after formation of the proteoliposomes, the kinase domain seems to be accessible from the outside suggesting an inside-out orientation of DcuS. The osmosensor KdpD, which was reconstituted by a similar procedure, was found in an unidirectional inside-out orientation in the liposomes (32). Alternatively, the result could indicate that the proteoliposomes are not closed and permit the ATP to access the internal space.

The kinetics of DcuS phosphorylation was followed for different ATP concentrations ranging from 10 to 10,000 μM in the phosphorylation buffer (Fig. 4). The experiments were performed similar to that from Fig. 3, and the rates were calculated after 15 min of reaction. Plotting the inverse rate of phosphorylation (1/v) versus the inverse concentration of ATP (1/[ATP]) gave a nearly linear relation (not shown). From the Lineweaver-Burk plot a K<sub>m</sub> for ATP of 0.16 mM can be estimated.

**Stimulation of Kinase Activity by C<sub>4</sub>-dicarboxylates—In vivo,** C<sub>4</sub>-dicarboxylates serve as stimuli of DcuS/DcuR-dependent gene expression (14–16). The stimulation of DcuS autophosphorylation by C<sub>4</sub>-dicarboxylates was tested in the proteoliposomes (Fig. 5 and Table I). The proteoliposomes were prepared without or with addition of succinate or fumarate during the freeze-thaw step to enable the effectors to gain access to the internal space of the liposomes. Then the phosphorylation of DcuS was measured after incubation with [γ-<sup>32</sup>P]ATP, separation of the proteins by SDS-PAGE, and phosphorimagery analysis (Fig. 5). The phosphorylation of DcuS was significantly increased in the presence of fumarate or succinate.

The stimulation of DcuS phosphorylation by mono- and di-
carboxylates was quantified by the phosphorimager and is compared in Table I to the effect of the carboxylates on 
dcuB expression, using a dcuB-::lacZ reporter gene fusion (14). Fumarate showed the highest stimulation of DcuS phosphorylation by factors up to 5.9, followed by succinate. Monocarboxylates like acetate had no positive, or even an inhibitory, effect on phosphorylation. Fumarate and succinate caused the highest stimulation of dcuB expression (Table I), whereas monocarboxylates did not stimulate (14, 15). Thus DcuS phosphorylation and DcuS-dependent regulation in principle had similar specificities for the carboxylates. Stimulation of dcuB expression, however, was generally higher than the increase in DcuS phosphorylation as would be expected due to an amplification of the stimulus by the sensor/regulator. As a consequence, the response of the target (dcuB expression) to the stimulus would be stronger than that of the sensor/regulator (DcuS phosphorylation).

**Phosphoryl Transfer from DcuS to DcuR—**The phosphoryl transfer from DcuS to DcuR was studied in the in vitro system. DcuS-proteoliposomes were incubated in the presence of fumarate with [γ-32P]ATP after 15 min the proteoliposomes were dissolved in SDS sample buffer and subjected to SDS-PAGE. The radioactivity and phosphate content were determined using the phosphorimager and scintillation counting. The radioactivity in the fumarate-stimulated samples was taken as 100% (70 mmol of phosphate/mol of DcuS). The expression of dcuB-::lacZ was measured in strain IMW260pMW181 after growth in the presence of the correspond- 

| Stimulus (20 mM) | Phosphorylation of DcuS | Expression of dcuB-::lacZ |
|-----------------|------------------------|------------------------|
| None            | 17 – 30                | 48                     |
| Fumarate        | 100                    | 573                    |
| Succinate       | 51                     | 437                    |
| Butyrate        | ND                     | 18                     |
| Acetate         | 20                     | 65                     |

**TABLE I**

Effect of carboxylates on autophosphorylation of DcuS reconstituted in proteoliposomes and on the expression of dcuB-::lacZ in vivo

His6-DcuS proteoliposomes in the presence of the carboxylates (20 mM) were incubated with 100 nM [γ-32P]ATP. After 15 min the proteoliposomes were dissolved in SDS sample buffer and subjected to SDS-PAGE. The radioactivity and phosphate content were determined using the phosphorimager and scintillation counting. The radioactivity in the fumarate-stimulated samples was taken as 100% (70 mmol of phosphate/mol of DcuS). The expression of dcuB-::lacZ was measured in strain IMW260pMW181 after growth in the presence of the corresponding stimuli (14). ND, not determined.

**FIG. 5.** Autophosphorylation in proteoliposomes by [γ-32P]ATP in the presence of 20 mM succinate (A), 20 mM fumarate (C), or without addition (B). Autoradiography with the phosphorimager was performed after SDS-PAGE of the proteins. The samples were incubated with 0.1 mM [γ-32P]ATP for 5–30 min, and proteoliposomes containing 2 μg of DcuS were subjected to SDS-PAGE. Autoradiography was performed with the phosphorimager, and the radioactivity incorporated into DcuS (corresponding to autophosphorylation activity) is given below the panels (100% corresponding to the phosphorylation of DcuS in the presence of fumarate after 30 min).

**FIG. 6.** Phosphoryl transfer from DcuS to DcuR in proteoliposomes. DcuS-proteoliposomes were phosphorylated for 30 min with [γ-32P]ATP (0.1 μM), and then a 4-fold excess of His6-DcuR was added. At the time points indicated, samples corresponding to 2 μg DcuS were withdrawn, quenched with SDS sample buffer, and subjected to gel electrophoresis. Other conditions as for Fig. 3. The gel was stained for protein (A), and autoradiography was performed in the phosphorimager (B). Quantitative evaluation (C) of DcuS (■) and DcuR (●) phosphorylation was obtained by phosphorimager analysis, which was calibrated for 32P by liquid scintillation counting with excised and dissolved gel bands.

Based on a quantitative evaluation using the phosphorimager and calibration by scintillation counting, the rates of DcuS phosphorylation and of dephosphorylation can be estimated from the gradient of the reaction graph in Fig. 6C. The phosphoryl transfer occurred with rates exceeding those of DcuS phosphorylation by a factor of 40 or more.

**DNA Binding of DcuR Phosphorylated by DcuS—**The DNA binding of phosphorylated DcuR was tested in a gel retardation assay (Fig. 7). DcuR was phosphorylated by incubation with ATP and DcuS-proteoliposomes (Fig. 7A) or for control by incubation with acetyl phosphate (Fig. 7B). Phosphorylated DcuR was then incubated with radioactively labeled DNA containing the dcuB promoter, and the mixture was subjected to native DNA gel electrophoresis. When DcuR was phosphorylated with acetylphosphate (Fig. 7B), low amounts (0.7 μM) of DcuR-phosphate (DcuR-P) caused disappearance of the band of free DNA. In parallel a band of retarded DNA turned up with decreased mobility, which presumably represents the DNA-DcuR-P complex. The retardation was not observed for non-phosphorylated DcuR (Fig. 7C) or for DNA derived from promoters not regulated by DcuSR (not shown).

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2 I. Garcia-Moreno and G. Unden, unpublished data.
E. coli with the turgor and osmolality sensors KdpD and EnvZ of rhizobia. The work was performed in a functional in vitro system. Detailed studies have been performed with the turgor and osmolality sensors KdpD and EnvZ of E. coli (32, 37, 38). KdpD controls the intracellular $K^+$ concentration by transriptional regulation of the kdpFABC operon, which encodes the high affinity $K^+$-translocating Kdp ATPase. The stimulus of KdpD is supposed to be the cytoplasmic $K^+$ concentration or a parameter related to it, like changes in the cytoplasmic ATP levels, which are supposed to reflect turgor changes (39, 40). KdpD requires membrane insertion for function like DcuS. KdpD contains (in addition to the C-terminal cytoplasmic kinase) a large cytoplasmic N-terminal domain, which is essential for sensing. Signal transfer from the sensory to the kinase domain of KdpD presumably requires direct interaction of the cytoplasmic sensor and kinase domains and does not depend on transmembrane signaling in contrast to DcuS. EnvZ contains a distinct periplasmic domain, but it is not clear whether this domain is involved in stimulus perception.

Another type of membranous histidine protein kinases is exemplified by the O$_2$-sensor FixL of Sinorhizobium (former Rhizobium) meliloti. In FixL, the membrane portion is not essential for sensing or signal transfer and might be required mainly for anchoring the protein to the membrane. The short N-terminal anchor domain of FixL is followed by sensory and kinase domains, which are both located in the cytoplasm. FixL obviously senses O$_2$ after its diffusion into the cell, and signal transfer to the kinase domain does not involve transmembrane processes. After genetic deletion of the membrane anchor, the sensor and kinase domains form one coherent soluble protein, which has been used for studies on O$_2$ binding and control of kinase function by O$_2$ (4, 35).

Use of DcuS for Functional Analysis of Transmembrane Sensor Kinases in Vitro—Isolated DcuS is active only after reconstitution into liposomes. Since autophosphorylation requires transphosphorylation between the monomers of dimeric or oligomeric His protein kinases (36), it is feasible that this oligomeric state is lost in detergent and regained upon reconstitution. 7% of the reconstituted DcuS were phosphorylated in the monomeric state is lost in detergent and regained upon reconstitution. Thus stimulus binding might control DcuS function at the kinase level; however, other activities like phosphoryl transfer or oligomerization. For other reconstituted membranous sensors the portion of functionally reconstituted sensor kinase has not been quantified and might be even lower. Reconstituted DcuS responded to the presence of C$_4$-dicarboxylates with an up to 5.9-fold stimulation of autophosphorylation. Thus stimulus binding might control DcuS function at the kinase level, however, other activities like phosphoryl transfer or phosphatase might be affected in addition.

For reconstituted DcuS the $K_m$ for ATP was determined (0.16 mM). In aerobically and anaerobically grown E. coli the cellular ATP levels (> 3 μmol ATP/g of dry cells, corresponding to 3 mM intracellular ATP) (29) significantly exceed this value. Therefore ATP would be present at saturating concentrations for DcuS phosphorylation during growth by fumarate respiration where DcuSR has its main function, and most other conditions, suggesting that intracellular ATP concentrations do not supply a regulatory signal.

**FIG. 7.** Gel retardation of dcbB promoter DNA by DcuR (DcuR-P) phosphorylated by DcuS and ATP (A) or with acetyl phosphate (B) or by unphosphorylated DcuR (C). The radioactively labeled dcbB promoter fragment (646 bp, 76 nM DNA) was incubated with DcuR phosphorylated for 20 min at 20 °C by DcuS proteoliposomes plus ATP (10 mM) (A) or with 5 mM acetyl phosphate for 60 min at 37 °C (B). DNA used in A was labeled with higher specific radioactivity than the DNA for B and C. The samples were subjected to native DNA-agarose electrophoresis, and the DNA bands were identified by autoradiography.

When DcuR was phosphorylated by DcuS-proteoliposomes (Fig. 7A), the DNA band corresponding to free DNA disappeared at concentrations of DcuR-P very similar to those of the experiment of Fig. 7B, when DcuR was phosphorylated by acetyl phosphate. In a control experiment without ATP or DcuR, the band of free DNA did not disappear (not shown). The retarded DNA-protein complex, however, was not able to migrate into the gel and remained in the pockets of the gel (Fig. 7A). This suggests that the dcbB promoter DNA was bound specifically by phosphorylated DcuR and that a complex consisting of DcuS, DcuR-P, and DNA was formed, which was too large to migrate into the gel. Complexes consisting of sensors and response regulators have been described earlier (12, 35). Overall, DcuR phosphorylated via reconstituted DcuS, and ATP seems to be capable of specific binding to dcbB promoter DNA similar to DcuR phosphorylated by acetyl phosphate.

**FIG. 8.** Supposed topology of DcuS in proteoliposomes. The scheme shows transmembrane helices 1 and 2 (TM1 and TM2), the periplasmic domain (DcuS$_{per}$), the PAS, the kinase (transmitter) domain, and the conserved His residue (H, His$^{249}$) of DcuS. The orientation of DcuS in the proteoliposomes is inverse to that in the bacteria. In bacteria the topology was derived from the accessibility of the stimulus and phaA fusion studies and in liposomes from the accessibility of ATP to the kinase and of a protease to PAS. For details see text. Fum, fumarate.

**DISCUSSION**

**Function of DcuS as a Transmembranous Fumarate Sensor in Vitro—**DcuS has been isolated and reconstituted functionally in liposomes with inside-out orientation, i.e. the stimulus binding domain within and the kinase domain outside the lipid membrane (Fig. 8). This can be concluded from the accessibility of ATP to the kinase without disintegration of the liposomes and of a unique protease site. Reconstituted DcuS is capable of C$_4$-dicarboxylate-sensitive autophosphorylation and of phosphoryl transfer to DcuR, which is then able to bind to promoter DNA. Thus a functional in vitro system for complete signal transduction by DcuS/DcuR from the stimulus fumarate to DNA binding is available. Such a system will be important for understanding the overall function of two-component sensors with transmembrane arrangement of sensory and transmitter domains (5, 6, 12, 13). In particular, such a system can be used to study signal transduction and transmembrane signaling, which depends on the membrane intrinsic portions of the protein linking the periplasmic sensory and the cytoplasmic kinase domains.

**Comparison of DcuS Function to Other Membranous His Sensor Kinases—**There is only a small number of membrane intrinsic histidine kinases that have been studied in a functional in vitro system. Detailed studies have been performed with the turgor and osmolality sensors KdpD and EnvZ of E. coli (32, 37, 38). KdpD controls the intracellular $K^+$ concentration by transcriptional regulation of the kdpFABC operon, which encodes the high affinity $K^+$-translocating Kdp ATPase. The stimulus of KdpD is supposed to be the cytoplasmic $K^+$

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3 H. Kneuper and G. Unden, unpublished data.
Availability of an in vitro system for DcuSR should enable future studies on the complete signal transduction pathway and a more detailed understanding of the underlying processes. In particular it should be possible to study reactions that require the intact sensor kinase such as signal transduction across the membrane. Questions of this type cannot be addressed in a system using genetically truncated and solubilized domains due to the transmembranous arrangement of sensory and kinase domains and the need for the membrane intrinsic domains for signal transfer.

REFERENCES
1. Parkinson, J. S., and Kofoid, E. C. (1992) Annu. Rev. Genet. 26, 71–112
2. Aizawa, S. I., Harwood, R. J., and Kadner, J. (2000) J. Bacteriol. 182, 1459–1471
3. Gilles-Gonzalez, M. A., Ditta, G. S., and Helinski, D. R. (1991) Nature 350, 170–172
4. Leis, A. F., Ditta, G. S., and Helinski, D. R. (1993) J. Bacteriol. 175, 1103–1109
5. Schroeder, I., Wolin, C. D., Cavichioli, R., and Gunsalus, R. P. (1994) J. Bacteriol. 176, 4985–4992
6. Bird, T. H., Du, S., and Bauer, C. E. (1999) J. Biol. Chem. 274, 16343–16348
7. Emmerich, R., Panglungthang, K., Strehler, P., Hennecke, H., and Fischer, H.-M. (1999) Eur. J. Biochem. 263, 455–463
8. Himpsel, S., Locht, C., and Supply, P. (2000) Microbiology 146, 3091–3098
9. Georgellis, D., Kwon, O., and Lin, E. C. C. (1999) J. Bacteriol. 274, 35950–35954
10. Ninfa, A. J., Atkinson, M. R., Kamberov, E. S., Feng, J., and Ninfa, E. G. (1995) in Two-Component Signal Transduction (Hoch, J. A., and Silhavy, T. J., eds) pp. 65–88, ASM Press, Washington, D. C.
11. Amsler, C. D., and Matsumura, P. (1995) in Two-Component Signal Transduction (Hoch, J. A., and Silhavy, T. J., eds) pp. 89–104, ASM Press, Washington, D. C.
12. Kaspar, S., Perozzo, R., Reinelt, S., Meyer, M., Pfister, L., Scapozza, L., and Bott, M. (1999) Mol. Microbiol. 33, 858–872
13. Kaspar, S., and Bott, M. (2002) Arch. Microbiol. 177, 313–321
14. Zientz, E., Bongarzis, J., and Unden, G. (1998) J. Bacteriol. 180, 5421–5425
15. Golby, P., Davies, S., Kelly, J. R., Guest, J. R., and Andrews, S. C. (1999) J. Bacteriol. 181, 1238–1248
16. Janasch, I. G., Zientz, E., Tran, Q. H., Krüger, A., and Unden, G. (2002) Biochim. Biophys. Acta 1553, 39–56
17. Parar, T. N., Coligaev, B., Zientz, E., Unden, G., Peti, W., and Griesinger, C. (2001) J. Bacteriol. 183, 17080–17085
18. Six, S., Andrews, S. C., Unden, G., and Guest, J. R. (1994) J. Bacteriol. 176, 6470–6478
19. Engel, P., Kramer, R., and Unden, G. (1994) Eur. J. Biochem. 222, 605–614
20. Golby, P., Kelly, D., Guest, J. R., and Andrews, S. C. (1998) J. Bacteriol. 180, 6588–6596
21. Zientz, E., Six, S., and Unden, G. (1996) J. Bacteriol. 178, 7241–7247
22. Bott, M., Meyer, M., and Dimroth, P. (1995) Mol. Microbiol. 18, 533–546
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Chen, W., and Kuo, T. (1993) Nucleic Acids Res. 21, 2260
25. Holmes, D. S., and Quigley, M. (1981) Anal. Biochem. 114, 193
26. Hanahan, D. (1983) J. Mol. Biol. 166, 557–565
27. Chung, C. T., Niemela, S. L., and Miller, R. H. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2172–2175
28. Inoue, H., Nojima, H., and Okayama, H. (1990) Gene (Amst.) 96, 23–28
29. Tran, Q. H., and Unden, G. (1998) Eur. J. Biochem. 251, 538–543
30. Miller, J. H. (1992) A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
31. Lasemli, U. K. (1970) Nature 227, 680–685
32. Jung, K., Tjaden, B., and Altendorf, K. (1997) J. Biol. Chem. 272, 10847–10852
33. Holloway, P. W. (1973) Anal. Biochem. 53, 304–308
34. Rigaud, J. L., Pitard, B., and Levy, D. (1995) Biochim. Biophys. Acta 1231, 223–246
35. Tuckerman, J. R., Gonzalez, G., and Gilles-Gonzalez, M. A. (2001) J. Mol. Biol. 308, 449–455
36. Stock, J. B., Surette, M. G., Levit, M., and Park, P. (1995) in Two-Component Signal Transduction (Hoch, J. A., and Silhavy, T. J., eds) pp. 25–51, ASM Press, Washington, D. C.
37. Jung, K., Hamann, K., and Revermann, A. (2001) J. Biol. Chem. 276, 40896–40902
38. Jung, K., Krábauch, M., and Altendorf, K. (2001) J. Bacteriol. 183, 3800–3803
39. Jung, K., and Altendorf, K. (1998) J. Biol. Chem. 273, 17406–17410
40. Heermann, R., Altendorf, K., and Jung, K. (2000) J. Biol. Chem. 275, 17080–17085