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The Nature of the Stimulus and of the Fumarate Binding Site of the Fumarate Sensor DcuS of *Escherichia coli**

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Holger Kneuper‡, Ingo G. Janausch‡, Vinesh Vijayan§, Markus Zweckstetter§¶, Verena Bock‡, Christian Griesinger§, and Gottfried Unden‡‖

From the ‡Institut für Mikrobiologie und Weinforschung, Johannes Gutenberg-Universität Mainz, 55099 Mainz, Germany and §Max Planck Institut für Biophysikalische Chemie, 37077 Göttingen, Germany

DcuS is a membrane-associated sensory histidine kinase of *Escherichia coli* specific for C_4-dicarboxylates. The nature of the stimulus and its structural prerequisites were determined by measuring the induction of DcuS-dependent *dcuB′-′lacZ* gene expression. C_4-dicarboxylates without or with substitutions at C2/C3 by hydrophilic (hydroxy, amino, or thiolate) groups stimulated gene expression in a similar way. When one carboxylate was replaced by sulfonate, methoxy, or nitro groups, only the latter (3-nitropropionate) was active. Thus, the ligand of DcuS has to carry two carboxylate or carboxylate/nitro groups 3.1–3.8 Å apart from each other. The effector concentrations for half-maximal induction of *dcuB′-′lacZ* expression were 2–3 mM for the C_4-dicarboxylates and 0.5 mM for 3-nitropropionate or D-tartrate. The periplasmic domain of DcuS contains a conserved cluster of positively charged or polar amino acid residues (Arg^{107}-X_2-His^{110}-X_9-Phe^{120}-X_{26}-Arg^{147}-X-Phe^{149}) that were essential for fumarate-dependent transcriptional regulation. The presence of fumarate or D-tartrate caused sharpening of peaks or chemical shift changes in HSQC NMR spectra of the isolated C_4-dicarboxylate binding domain. The amino acid residues responding to fumarate or D-tartrate were in the region comprising residues 89–150 and including the supposed binding site. DcuS(R147A) mutant with an inactivated binding site was isolated and reconstituted in liposomes. The protein showed the same (activation-independent) kinase activity as DcuS, but autophosphorylation of DcuS was no longer stimulated by C_4-dicarboxylates. Therefore, the R147A mutation affected signal perception and transfer to the kinase but not the kinase activity *per se*.

Bacterial cells contain large numbers of sensors for monitoring changes in chemical and physical parameters. The most common type of sensory system is represented by two-component sensors consisting of sensory histidine kinases and response regulators (1, 2). The response regulators mostly control the expression of target genes by transcriptional activation or repression. Many sensor kinases have a transmembrane arrangement with the sensory domain in the periplasm and the kinase domain in the cytoplasm. The activity of the kinase domain typically is regulated by the sensory domain by binding of an effector molecule, resulting in autophosphorylation of the kinase at a conserved histidine residue and subsequent transfer of the phosphoryl group to the response regulator. For most of the 28 identified suggested histidine kinases of *Escherichia coli* (3), neither the exact nature of the stimulus nor the stimulus binding sites are known. One of the few His kinases characterized in this respect is the citrate sensor CitA of enteric bacteria. Stimulus and stimulus binding site of CitA are well characterized (4–6).

The DcuS[1]-DcuR two-component system (C_4-dicarboxylate uptake) of *E. coli*, consisting of the membrane-associated His kinase DcuS and of the response regulator DcuR, has been identified as a sensory system for fumarate and other C_4-dicarboxylates (7–9). DcuS-DcuR stimulates the expression of genes encoding carriers and enzymes for the catabolism of externally supplied C_4-dicarboxylates. One of the main targets of the DcuS-DcuR system is the *dcuB* gene, which encodes the C_4-dicarboxylate carrier DcuB catalyzing fumarate/succinate antiport during fumarate respiration (10, 11). *dcuB* is transcriptionally activated by the phosphorylation of DcuR in the presence of C_4-dicarboxylates and by the oxygen sensor FNR during anaerobiosis (7, 8, 12–14).

As a member of the CitA family of sensor kinases, DcuS shares sequence similarity and domain organization with the citrate sensor CitA (7, 9, 15). DcuS is predicted to be a transmembraneous protein with the sensory domain exposed to the periplasm. The periplasmic domain of 140 amino acids is arranged between the two transmembraneous helices of DcuS. The structure of the periplasmic domain of DcuS in solution has been determined using NMR spectroscopy (16, 17). The sensory domain consists of a novel $\alpha\beta$-fold with α-helices close to the N- and C-terminal ends of the periplasmic domain. The binding domain shows similarity to the PAS (Per-Arnt-Sim) domain of the photoactive yellow protein PYP of *Halorhodospira halophila* (18). The cytoplasmic part of DcuS consists of a further PAS domain of unknown function and a transmitter domain with the predicted phosphorylation site. DcuS has been solubilized in detergent and functionally reconstituted in liposomes (12). In this system, kinase activity of DcuS responds to the presence of fumarate and other C_4-dicarboxylates (12).

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S The on-line version of this article (available at http://www.jbc.org) contains Supplemental Figs. 1 and 2.

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‖ To whom correspondence should be addressed: Institut für Mikrobiologie und Weinforschung, Johannes Gutenberg-Universität Mainz, Becherweg 15, 55099 Mainz, Germany. Tel.: 49-6131-3923550; Fax: 49-6131-3922695; E-Mail: unden@uni-mainz.de.

[1] The abbreviations used are: DcuS, dicarboxylate uptake sensor (protein kinase); DcuS-PD, periplasmic domain of DcuS; DcuR, dicarboxylate uptake regulator response regulator); CitA, citrate sensor kinase; FNR, fumarate and nitrate reductase regulator; PAS domain, Per-Arnt-Sim domain; PYP, photoactive yellow protein.

TABLE I

Oligonucleotides used for site-directed mutagenesis of the periplasmic domain of DcuS and DcuS-PD in plasmids pMW181, pMW180, and pMW145 and of DcuR in plasmid pMW151, respectively

The sequence of the second oligonucleotide used for mutagenesis corresponds to the complementary sequence of the oligonucleotide shown. The changed nucleotide sequences of *dcuS* resulting in the amino acid exchange are underlined.

Mutation	Plasmid	Sequence (5′ → 3′)
DcuS-K76A	pMW235	GCT TGC AGA AAG CGC CGC AGG AGA GTG G
DcuS-M103A	pMW296	CGT TAC CGA TGC CCA AAG TCT TCG C
DcuS-M103R	pMW297	CGT TAC CGA TAG GCA AAG TCT TCG C
DcuS-R107A	pMW234	GAT ATG CAA AGT CTT GCA TAC TCG CAT C
DcuS-H110A	pMW236	CTT CGC TAC TCG GCA CCT GAA GCC CAG C
DcuS-F120A	pMW292	CGT ATT GGT CAG CCA GCC AAA GGT GAT GAC
DcuS-F120M	pMW293	CGT ATT GGT CAG CCA ATG AAA GGT GAT GAC
DcuS-N134A	pMW294	GGC GAA GAA GCC GTC GCT ATC AAT CGC
DcuS-N134Y	pMW295	GGC GAA GAA TAT GTC GCT ATC AAT CGC
DcuS-R139I	pMW182	GTC GCT ATC AAT ATC GGT TTT CTG GCG C
DcuS-R147A	pMW237	GGC GCA GGC TTT AGC AGT ATT TAC CCC C
DcuS-R147I	pMW183	GGC GCA GGC TTT AAT CGT ATT TAC CCC C
DcuS-K158A	pMW238	GAT GAA AAT CAT GCG CAA ATT GGC GTG G
DcuS-Q159A	pMW284	GAA AAT CAT AAA GCA ATT GGC GTG GTG GCG
DcuS-Q159V	pMW290	GAA AAT CAT AAA GTA ATT GGC GTG GTG GCG
DcuS-A164S	pMW298	GGC GTG GTG TCG ATC GGC CTT GAG TTA AGC
DcuS-L167A	pMW302	GGC GTG GTG GCG ATC GGC GCC GAG TTA AGC
DcuS-L167Y	pMW301	GGC GTG GTG GCG ATC GGC TAT GAG TTA AGC
DcuS-F149A	pMW277	GCA GGC TTT ACG CGT AGC TAC CCC CAT CTA CG
DcuS-Q225R	pMW291	CTG TTT GAG CAA CGC CGC GCC ATG TTG CAG
DcuS-F149K	pMW278	GGC GCA GGC TTT ACG CGT AAA AAC CCC CAT CTA CG
DcuR-D46N	pMW267	GAC CTG ATA TTG CTC AAT ATC TAT ATG C

Previously, it has been suggested that DcuS uses C_4-dicarboxylates as the stimuli (7, 8, 12). This is in contrast to the high specificity of the homologous His kinase CitA for citrate, isocitrate, and tricarballylate (4, 19). So far the structure of DcuS-PD could be solved only without bound C_4-dicarboxylates (17), which precludes direct identification of the effector binding site. Therefore, we aimed at an identification of the stimulus binding site by genetic, biochemical, and NMR spectroscopic methods. The studies showed that DcuS has a remarkably broad specificity for stimulus molecules and suggested a defined binding pocket for the C_4-dicarboxylates and the presence of distinct amino acid residues for C_4-dicarboxylate binding. By these properties, DcuS can be clearly differentiated from the citrate sensor CitA. In addition, the effect of mutations in the effector binding site on the activity of the kinase domain and of its sensitivity to stimulation was tested for a better understanding of the protein in signal perception and transfer.

EXPERIMENTAL PROCEDURES

Genetic Methods—Standard molecular genetic methods were used (20). Genomic DNA was isolated according to Chen and Kuo (21). Plasmids were isolated using kits. PCR products were purified with the QIAquik PCR purification kit (Qiagen). DNA was extracted from agarose gels with the QIAquik gel extraction kit (Qiagen).

E. coli strains were transformed via electroporation or after treatment with RbCl (22, 23). The *dcuS* gene including the promoter region from *E. coli* AN387 (24) genomic DNA was amplified using the hot start method with oligonucleotide primers reHindDcuS (5′-TCG GCG ATT AAG CTT CGC GAC C-3′) and liXbaDcuS (5′TTT CAG GCT TTC ATC TAG ATC GGT ATG-3′). The product was cloned via the HindIII and XbaI sites into pET28a (Novagen). The resulting plasmid, pMW181, carried the *dcuS* gene and its complete promoter region and was used as the template for mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene). The mutations in DcuS, verified by DNA sequencing, were created by replacing the codon for an amino acid residue with the codon for alanine or another amino acid (Table I). The plasmids carrying the mutations were used for expression of the corresponding mutant proteins from the *dcuS* promoter.

For overexpression of *dcuS*, the *dcuS* coding region was amplified by PCR and cloned into pET28a (Novagen) behind the inducible T7 promoter (12). The resulting construct, pMW151, codes for the complete DcuS protein including the initial Met residue and carries an N-terminal His_6 tag and a thrombin cleavage site. The recombinant pET28a derivatives pMW180 and pMW145 were used for overproduction of DcuR and DcuS-PD (12, 17). For overproduction of the DcuS, DcuS-PD, and DcuR mutant proteins, the mutations were introduced into pMW151, pMW145, and pMW180, respectively, by site-directed mutagenesis as described above. Primers used for mutagenesis and resulting plasmids with the mutations are listed in Table 1.

Isolation of DcuS and DcuR—DcuS was isolated after solubilization with detergent (12). The DcuS(R147A) mutant protein was isolated in similar yields using the same procedure. DcuR and DcuR(D56N) were isolated following the procedure of Janausch *et al.* (12).

Reconstitution of DcuS in Liposomes—Purified DcuS and DcuS(R147A) were each reconstituted in *E. coli* phospholipids (Avanti Polar Lipids Inc.) as described for DcuS (12). The liposomes were destabilized by the addition of Triton X-100 (0.8% w/v). Isolated DcuS or DcuS(R147A) in elution buffer was added at a phospholipid:protein ratio >20:1 (mg/mg) and stirred gently for 10 min at 20 °C. Degassed Bio-Beads SM-2 (Bio-Rad) pretreated as described by Holloway (25) were added to remove the detergent (10 mg beads/mg Triton X-100). The suspension was incubated overnight at 4 °C. The supernatant was then incubated with fresh Bio-Beads (10 mg beads/mg Triton X-100) for 1 h at 20 °C. The supernatant was removed with a pipette, frozen in liquid N_2, and stored at −80 °C.

Phosphorylation of DcuS and Phosphoryl Transfer to DcuR—An 80-μl aliquot of the proteoliposome suspension was adjusted to 10 mM $MgCl_2$, 1 mM dithiothreitol, and 20 mM fumarate (or other carboxylic acids as indicated) and subjected to three cycles of rapid freezing in liquid N_2 and slow thawing at 20 °C (26). After the final thawing, the proteoliposomes were kept for 1 h at 20 °C. 2.5 μl of [γ-^{33}P]ATP (110 TBq/mmol) then was added to attain final concentrations of 0.1–100 μM ATP. Isolated DcuR or DcuR(D56N) was added to the suspension in 4-fold excess over DcuS. At the times indicated, 10-μl samples were mixed with 10 μl of SDS loading buffer and a volume corresponding to 2 μg of DcuS/lane was applied to an SDS-polyacrylamide gel. After electrophoresis, the gel was exposed to an imaging plate (Fuji BAS-MP2040) for measuring the radioactivity in the bands in a PhosphorImager (12).

NMR Studies on Isolated Periplasmic Domain of DcuS (DcuS-PD)—Wild-type DcuS-PD and the mutant forms, DcuS(F120M)-PD, DcuS(R147A)-PD, and DcuS(H110A)-PD, were dissolved in a NMR buffer at pH 7.0 containing 45 mM sodium/potassium phosphate, 10% D_2O, 200 mM NaCl, 0.01% NaN_3 50 μM Pefabloc, 50 mM glycine, and 4.5 mM imidazole. Titrations with the effectors were conducted by the stepwise addition of properly buffered fumarate and tartrate solutions (pH 7.0 and up to 300 mM final concentration) to a sample of 1.2 mM periplasmic domain of DcuS. Two-dimensional ^{15}N-^{1}H heteronuclear single quantum coherence (HSQC) experiments were recorded for each step of the titration. Experiments were performed on Bruker DRX 800

TABLE II
Effect of C_4-dicarboxylates and related compounds on the stimulation of dcuB′-′lacZ expression in vivo

E. coli IMW237 [MC4100 λ(Φ*dcuB′-′lacZ*)] was grown anaerobically in M9 medium containing glycerol and dimethyl sulfoxide as the growth substrates plus the effector (30 mM) indicated. The activity and K_D values were determined in experiments similar to those shown in Fig. 1. Activities are given in Miller units (MU). ND, not determined.

Effector	β-Galactosidase activity	Apparent K_D
	MU	*mM*
None	48	
Fumarate	537	ND
Succinate	437	3.0
Maleate	463	2.0
Malonate	89	13.0
Glutarate	91	ND
Citrate	173	7.0
1,2-Ethane-disulfonate	18	ND
1,3-Propane-disulfonate	20	ND
3-Nitropropionate[a]	485	0.45
Butyrate	18	ND
Formate + acetate	5	ND
Formate + propionate	10	ND
Succinamic acid	50	ND
Succinamide[b]	127	ND
Succinyl-dimethyl ester	25	ND

[a] 5 mM.
[b] 3 mM.

and 600 MHz NMR spectrometers at 30 °C. The data were processed using NMRPIPE (27) and analyzed using SPARKY.[2]

Expression Studies with dcuB′-′lacZ—E. coli IMW237 (MC4100 λ(Φ*dcuB′-′lacZ*) grown anaerobically in M9 mineral medium with glycerol and dimethyl sulfoxide as the energy substrates (7) was used for expression studies. For determining the K_D and V_{max} values for the various carboxylic acids, effectors (0–50 mM) were added to the M9/glycerol/Me_2SO growth medium from stock solutions (0.5 or 1 M, taken to pH 7 by NaOH) before growth. Samples of the cultures at an $A_{578\ nm}$ of 0.5–0.7 were withdrawn for measurement of β-galactosidase activity (28).

RESULTS

C_4-Dicarboxylates as Effectors of DcuS-dependent Regulation—Expression of *dcuB* depends under anaerobic conditions only on active DcuR and reflects the degree of DcuS stimulation by external stimuli (7, 8). The expression of *dcuB* during anaerobic growth of *E. coli* IMW237 [MC4100 λ(Φ*dcuB′-′lacZ*)] in the presence of a high concentration of various C_4-dicarboxylates and related compounds was measured *in vivo* (Table II). Each of the C_4-dicarboxylates, such as fumarate, succinate, or maleate, caused a high level of expression of the *dcuB′-′lacZ* reporter gene fusion. With the C_3- and C_5-dicarboxylates, malonate and glutarate, and with citrate, the level of expression was much lower. Potential effectors in which one or two of the carboxylate groups were replaced by the anionic sulfonate group had no stimulatory effect on *dcuB′-′lacZ* expression, whereas 3-nitropropionate caused strong stimulation. The amides and the methyl esters of the carboxylates retained only a low or no stimulating effect. The monocarboxylates, butyrate, propionate, acetate, and formate, did not lead to any significant induction, even when supplied in combination, *e.g.* propionate plus formate or acetate plus formate.

Effect of Substitutions at C2 or C3 of the C_4-Dicarboxylates on Induction Efficiency—Various C_4-dicarboxylates with substitutions of hydroxy, thiol, amino, methyl, and related groups at positions C2 and C3 were tested for their effects on *dcuB′-′lacZ* expression. The presence of a hydroxy (malate), thiol (thiosuccinate), or amino (aspartate) group did not significantly change the induction compared with succinate (Table III). Even tartrate, containing two hydroxy groups, stimulated DcuS-dependent expression to a high extent. With methylfumarate, the stimulation was decreased to about half the stimulation by fumarate. Methylenesuccinate, 2,3-dimethylsuccinate, or phthalate, an aromatic dicarboxylate with an "internal" maleate-like C_4-dicarboxylate structure, had no longer a stimulatory effect on *dcuB* expression.

TABLE III
Effect of C_4-dicarboxylates with substitutions at C2 or C3 on the stimulation of dcuB′-′lacZ expression

E. coli IMW237 [MC4100 λ(Φ*dcuB′-′lacZ*)] was grown anaerobically in M9 medium containing glycerol plus dimethyl sulfoxide as the growth substrates plus the effector (30 mM) indicated. The activities and approximate K_D values were determined in experiments similar to those shown in Fig. 1. Activities are given in Miller units (MU). ND, not determined.

Effector	β-Galactosidase activity	Apparent K_D
	MU	*mM*
None	48	
Fumarate	537	ND
Succinate	437	3.0
Malate	435	ND
D-Tartrate	613	0.5
L-Tartrate	329	2.8
Mercaptosuccinate (thiomalate)	337	ND
Aspartate	434	2.0
2-Methylfumarate (mesaconate)	276	ND
2-Methylenesuccinate (itaconate)	22	ND
2,3-Dimethylsuccinate	10	ND
Phthalate (1,3-dicarboxybenzene)	18	ND

Apparent K_D Values for C_4-Dicarboxylates and Related Compounds—Some of the C_4-dicarboxylates are not significantly metabolized under the test conditions (anaerobic growth on glycerol + dimethyl sulfoxide), and for some of these effectors, the expression of *dcuB′-′lacZ* was measured as a function of the concentration (Fig. 1). With increasing effector concentration, the expression of *dcuB′-′lacZ* increased and reached saturation with Michaelis-Menten-like kinetics. The apparent K_D values for induction by succinate and maleate were 3.5 and 2.0 mM, respectively (Fig. 1 and Tables II and III). Citrate and malonate showed significantly higher apparent K_D values, which is in agreement with the role of DcuS as a sensor for C_4-dicarboxylates. The K_D values for 3-nitropropionate and D-tartrate, which both are not regarded as physiological stimuli of DcuS but are structurally closely related, were distinctly lower (0.45 and 0.5 mM). L-Tartrate, which is used as a growth substrate, showed an apparent K_D of 2.8 mM.

The Amino Acid Residues of the Periplasmic Domain Involved in Fumarate Binding—The binding site of the C_4-dicarboxylates in DcuS is proposed to be located in the periplasmic domain (7–9, 12). An alignment of the amino acid sequence of the periplasmic domain of DcuS from *E. coli* with those from the citrate sensor CitA from *Klebsiella* (4, 5) and other DcuS and CitA proteins revealed conserved residues in the members of the CitA/DcuS family (Fig. 2, *unshaded boxes*). Most of the conserved amino acid residues are hydrophobic (Ala, Gly, Ile, Leu, Val) and might be structurally relevant. Charged residues are conserved in both types of proteins (Asp^{102}, Arg^{107}, His^{110}, and Arg^{147}, according to the numbering of *E. coli* DcuS).

In addition (Fig. 2, *shaded boxes*), amino acid residues Met^{103}, Phe^{120}, Phe^{149}, and Gln^{159} are conserved in the DcuS-like proteins, whereas Gly^{143}, Lys^{149}, Val/Ile^{159}, and Ser^{164} (according to the numbering of *E. coli* CitA) are found in CitA-like proteins. In particular, the change of the pair Phe^{149}-Gln^{159} in DcuS representing two polar but uncharged amino acid residues to Phe^{149}-Val/Ile^{159} in the CitA proteins representing basic and hydrophobic residues seems to be coupled. This change from a polar/polar pair in DcuS to a basic/hydro-

[2] T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco.

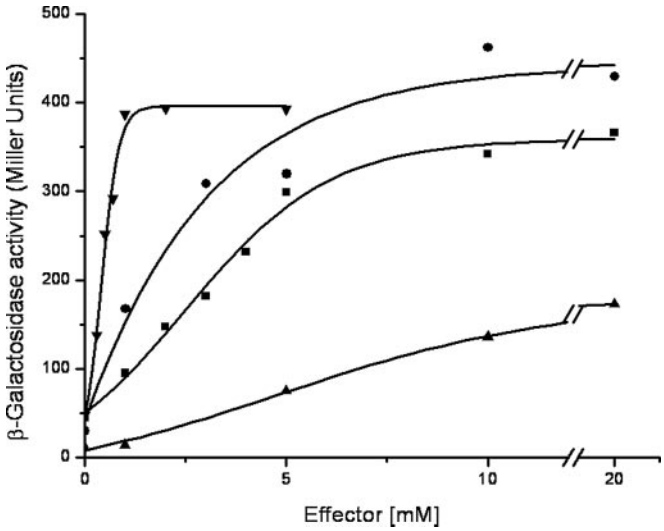

FIG. 1. **Expression of *dcuB′-′lacZ* as a function of succinate (●), maleate (■), 3-nitropropionate (▼), or citrate (▲) in the medium.** Bacteria were grown anaerobically in M9 medium supplemented with glycerol and dimethyl sulfoxide as growth substrates, plus the effectors succinate (●), maleate (■), 3-nitropropionate (▼), or citrate (▲) to an $A_{578\ nm}$ of 0.5. β-galactosidase activity were then measured.

phobic pair in CitA is indicative for the distinction of DcuS and CitA proteins (Fig. 2).

Positively charged amino acid residues would be expected to be important for the binding of the anionic C_4-dicarboxylates. Therefore, conserved and other significant Arg, Lys, and His residues of the DcuS periplasmic domain were replaced using site-directed mutagenesis by Ala or Ile residues. The mutant DcuS protein was tested for function in *E. coli* with a deletion of the wild-type chromosomal *dcuS* gene and a *dcuB′-′lacZ* reporter gene fusion (Fig. 3). In the *dcuS* deletion strain, the expression of the *dcuB′-′lacZ* reporter gene fusion was very low as predicted (<6% of the strain carrying wild-type chromosomal *dcuS*), whereas in the presence of the plasmid-encoded wild-type *dcuS* gene, the *dcuB′-′lacZ* reporter gene fusion was expressed at high levels. Of the mutations with substituted basic amino acid residues, R107A, H110A, R147A, and R147I caused a nearly complete loss of stimulation of *dcuB′-′lacZ* expression similar to the level of the Δ*dcuS* mutant. In contrast, mutations in which the other positively charged residues were replaced (K76A, R139I, and K158A) showed stimulation of *dcuB′-′lacZ* expression similar to the wild type.

Another set of mutations was created in amino acid residues that are specifically conserved in DcuS-type proteins (Met^{103}, Phe^{120}, Phe^{149}, and Gln^{159}) or in CitA-type proteins (Asn^{134}, Ala^{164}, and Leu^{167} of DcuS corresponding toTyr^{134}, Ser^{164}, and Tyr^{167} of *E. coli* CitA) (Fig. 2). By mutating these residues to Ala, only F120A, F149A, and L167A were strongly impaired in *dcuB′-′lacZ* expression, whereas the mutation of Met^{103}, Asn^{134}, Gln^{159}, and Ala^{164} only marginally impaired or even slightly stimulated the expression of *dcuB′-′lacZ*. The mutations resulting in inactivation of DcuS (F120A, F149A, and L167A) were further analyzed by replacing the wild-type residues by the corresponding residues of CitA. Conversion of Phe^{120} to a Met residue created a protein that was still inactive in stimulating *dcuB′-′lacZ* expression. Mutation of Phe^{149} to Lys resulted in DcuS, which had retained some activity (~24% wild type) (Fig. 3). The L167Y mutation caused even a slight stimulation of expression compared with the wild type, which contrasts the nearly complete loss of stimulation in the L167A mutation (Fig. 3). Therefore, only Phe^{120} and Phe^{149} appear to be essential, whereas Leu^{167} is not specifically required for DcuS function, but the presence of a bulky residue at this position might be important.

The results obtained for double mutants were consistent with those of the single mutations (Fig. 3). A combination of a mutation that resulted in a decrease in stimulation (*e.g.* R147I or F149K) with a second mutation, which was silent on its own, led to the same decrease in expression as the single former mutation. The combination of two silent mutations (*e.g.* A164S/L167Y) also did not affect *dcuB′-′lacZ* expression (Fig. 3).

NMR Studies on the Binding of Ligands to the Periplasmic Domain of DcuS—The isolated DcuS-PD was used for studies of the binding of the effectors to the domain by NMR spectroscopy (17). After the addition of fumarate, sharpening of peaks was observed for some of the amino acid residues of the periplasmic domain in ^{15}N-^{1}H HSQC spectra (Fig. 4*A*). The affected residues cluster in a defined region in the structure of the periplasmic domain of DcuS involving amino acid residues 107–168 (Fig. 5*A*). This region corresponds to the binding pocket of citrate in the periplasmic domain of CitA (6). There were no chemical shift changes observed during the titration.

When DcuS-PD was titrated with D-tartrate chemical shift changes was observed for a number of residues (Figs. 4*B* and 5*B* and Supplemental Fig. 1). Most of these residues belong to the same region that was affected by fumarate binding. Such chemical shift changes were only observed for D-tartrate but not for L-tartrate. There was a set of common residues that were affected both by the fumarate and tartrate titration (Ala^{113}, Lys^{121}, Asn^{134}, Ala^{145}, and Thr^{150}). Other residues were specifically affected by fumarate (Arg^{107}, Ile^{116}, Ala^{128}, Ala^{136}, Ala^{143}, Gln^{144}, Arg^{147}, and Phe^{149}) or D-tartrate addition (Val^{89}, Lys^{91}, Leu^{96}, Phe^{97}, Val^{100}, His^{110}, Gln^{114}, Gln^{118}, Asp^{124}, and Gly^{140}). A comparison of the affected residues indicates that fumarate and tartrate bind to the same positively charged binding pocket in the DcuS-PD domain (Fig. 5*C*). In addition, some residues located outside this defined binding pocket are specifically affected by D-tartrate and more nonpolar residues are affected by fumarate than D-tartrate, the latter containing two additional hydroxyl groups.

^{15}N-^{1}H HSQC spectra of mutant DcuS(F120M)-PD and DcuS(H110A)-PD showed a very small chemical shift dispersion characteristic for unfolded proteins (Supplemental Fig. 2*A*). On the other hand, for DcuS(R147A)-PD, a broadening of the NH resonances was observed (Supplemental Fig. 2*B*). This indicates a higher oligomerization state, in agreement with an increased rate of precipitation of DcuS(R147A)-PD.

Effect of the R147A Mutation on Autophosphorylation and Histidine Kinase Activity of DcuS—The mutant protein DcuS(R147A), which lost *in vivo* activity for stimulation of gene expression nearly completely, was tested *in vitro* for autokinase activity and its response to fumarate. DcuS(R147A) with an N-terminal His_6 tag was overproduced and purified as a homogenous preparation. After reconstitution (12), the proteoliposomes were incubated with [γ-^{33}P]ATP in the presence of fumarate and the kinetics of DcuS(R147A) and DcuS phosphorylation were determined (Fig. 6). Phosphorylation of DcuS after separation of the proteins by SDS-polyacrylamide gel electrophoresis was quantified using a PhosphorImager. Similar to wild-type DcuS, DcuS(R147A) was capable of autophosphorylation. Phosphorylation of both proteins showed saturation after 45 min, but the rates and degrees of phosphorylation of the two proteins differed. Phosphorylation of the mutant protein amounted to ~50% phosphorylation of the wild-type DcuS protein. When the purified response regulator DcuR was added to the phosphorylated samples, >80% radioactivity from DcuS and from DcuS(R147A) was transferred to DcuR within 1 min, which indicated that DcuS(R147A) was not impaired in phosphoryl transfer to DcuR.

Lack of Stimulation of Histidine Kinase Activity of DcuS(R147A)—The stimulation of DcuS and DcuS(R147A)

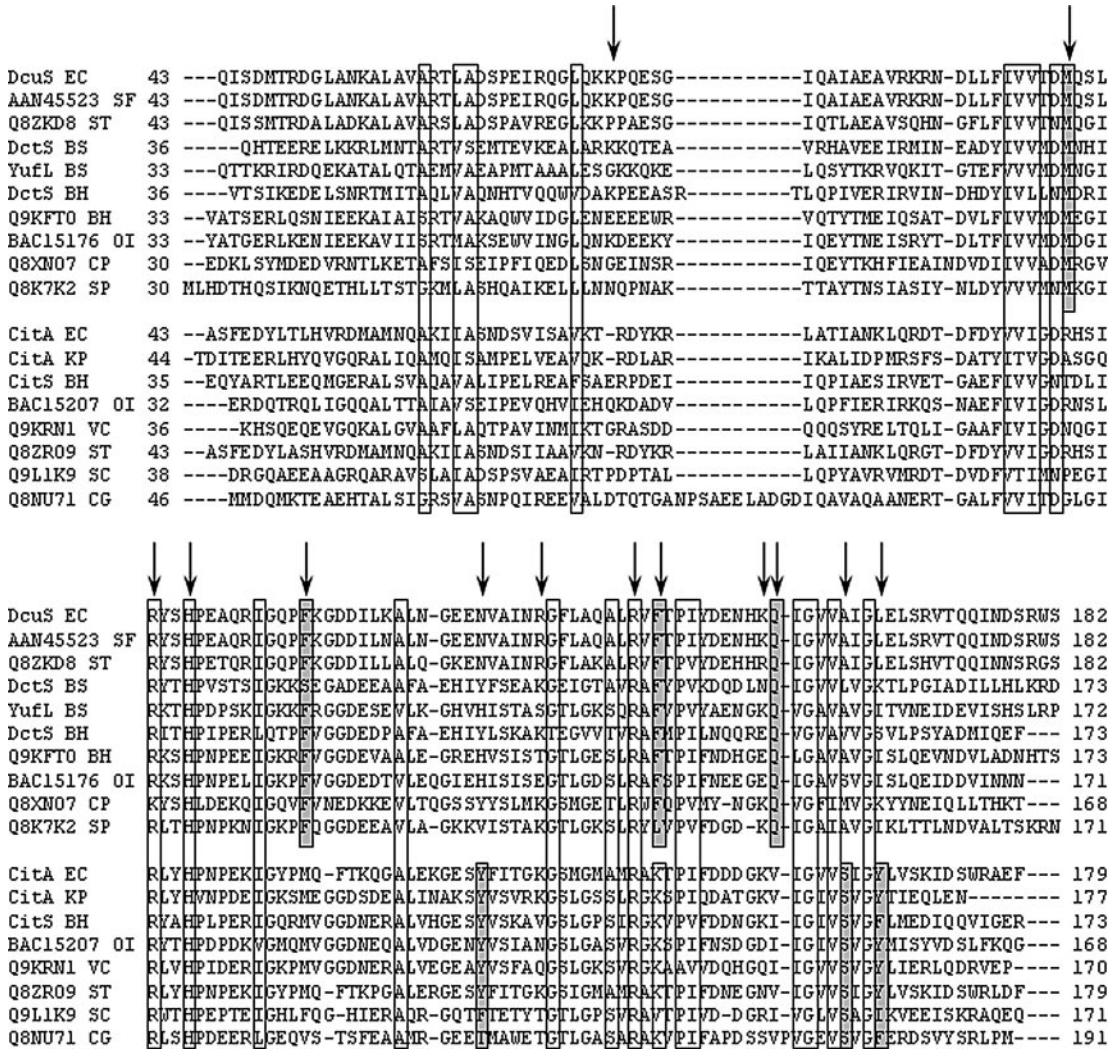

FIG. 2. **Comparison of the amino acid sequences of the periplasmic sensor domains of C_4-dicarboxylate or citrate sensory histidine kinases.** Amino acid residues conserved in both types of proteins are *boxed*; amino acids conserved in only one of the types of are *boxed* and *shaded*. Amino acid residues of *E. coli* DcuS that were changed by site-directed mutagenesis are indicated by *arrows*. *BS*, *Bacillus subtilis*; *BH*, *Bacillus halodurans*; *CG*, *Corynebacterium glutamicum*; *CP*, *Clostridium perfringens*; *EC*, *E. coli*; *KP*, *Klebsiella pneumoniae*; *OI*, *Oceanobacillus ihejensis*; *SC*, *Streptomyces coelicolor*; *SF*, *Shigella flexneri*; *SP*, *Streptococcus pyogenes*; *ST*, *Salmonella typhimurium*; *VC*, *Vibrio cholerae*.

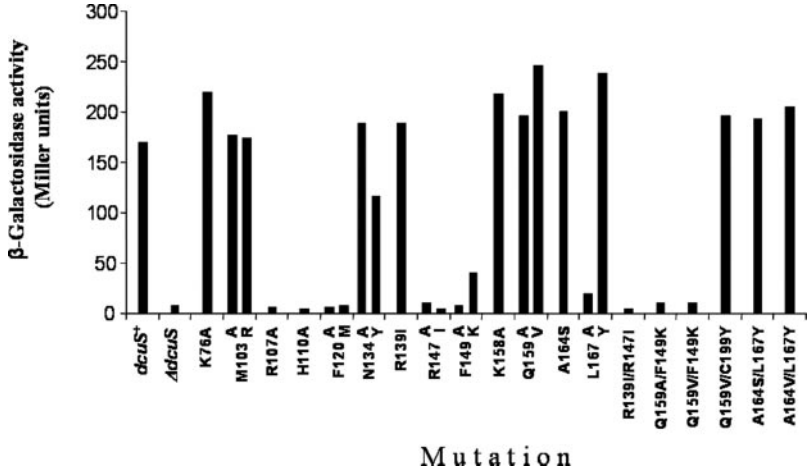

FIG. 3. **Effect of mutations in the DcuS-PD on DcuS-dependent *dcuB'-'lacZ* expression.** *E. coli* IMW260 (Δ*dcuS*) was transformed with low-copy number plasmids carrying the indicated mutated alleles of *dcuS*. β-Galactosidase activity of cultures at an $A_{578\ nm}$ of 0.5 was measured under standard conditions. The activity of *E. coli* IMW260 (Δ*dcuS*) carrying the plasmid-encoded wild-type *dcuS* allele [p(*dcuS*$^+$)] is shown for comparison.

autophosphorylation by C_4-dicarboxylates was tested. The relative amounts of the phosphorylated proteins were determined after 30 min of incubation, which provides a good measure for the differences in phosphorylation (Fig. 7). In the absence of a stimulus, wild-type and mutant DcuS showed the same degree of phosphorylation. Phosphorylation of wild-type DcuS increased ~2-fold in the presence of the C_4-dicarboxylates fumarate, succinate, and malate, whereas citrate and acetate had no effect. Phosphorylation of DcuS(R147A), however, was not stimulated by the C_4-dicarboxylates or by other effectors. Therefore, the basic (unstimulated) activity and function of the kinase domain in DcuS(R147A) appar-

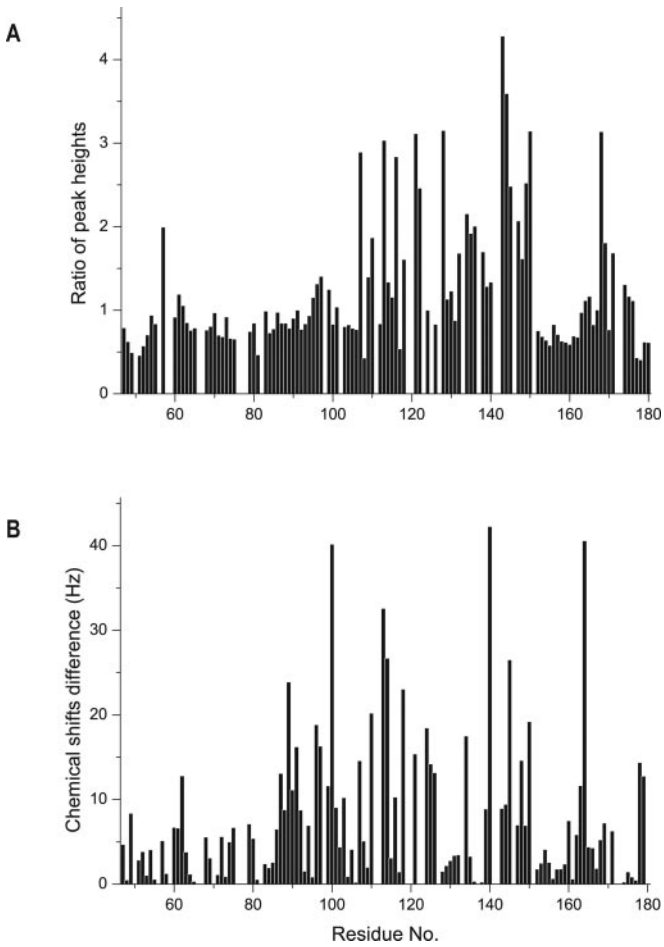

FIG. 4. **Interaction of fumarate and D-tartrate with the periplasmic domain of DcuS as characterized by NMR spectroscopy.** NMR signals of backbone amides constitute excellent probes of complex formation, providing maps of the interaction interfaces. Changes in peak heights between ^{1}H-^{15}N HSQC spectra of free DcuS-PD and DcuS-PD in the presence of fumarate (30-fold excess) (*A*) and changes in ^{15}N chemical shifts between the ^{1}H-^{15}N HSQC spectra of free DcuS-PD and DcuS-PD in the presence of D-tartrate (50-fold excess) (*B*) were observed. The concentration of DcuS-PD was 1.2 mM.

ently is not affected but the effector-induced stimulation of phosphorylation is lost.

DISCUSSION

The Signal Molecule for DcuS: Structural Properties—DcuS has a strong specificity for C_4-dicarboxylates, and the two carboxyl groups have to be within the same molecule. Under the conditions tested (pH 7), the C_4-dicarboxylates are present as dianions (pK_1 = 3.0 and pK_2 = 4.5 for fumarate), suggesting that the dicarboxylates are bound as dianions. Maleate (*cis*-butenedicarboxylate) has a distance of 3.1 Å between the C atoms of the carboxylic groups, whereas in the saturated C_4-dicarboxylates (succinate, malate, tartrate, and aspartate) and in fumarate, the distances amount to 3.8 Å, assuming a stretched conformation for the C_4-dicarboxylate. Thus, a remarkable variation in distance between the carboxyl groups is tolerated by DcuS. In the C_3- and C_5-dicarboxylates, malonate and glutarate, the carboxyl groups are 2.5 and 4.9 Å apart, respectively, which is outside this range. The carboxyl groups can be replaced by nitro and to some extent by amides of carbonates but not by sulfonate or the methyl ester groups. In 3-nitropropionate, the distance between the carboxyl carbon and the nitrogen of the nitro group is 3.8 Å, whereas the S atoms in 1,2-ethan disulfonate are 4.2 Å apart, which might contribute to the lack of stimulation by the latter.

DcuS tolerates a vast spectrum of substituents at the C2 or C3 position. Thus, small polar substitutions (hydroxy, thiolate, or amino groups) are tolerated, whereas large apolar substitutions like methyl groups prevent binding and stimulation. Thus, methylfumarate shows significantly decreased stimulation and dimethyl succinate has lost the capacity for stimulation. Citrate, on the other hand, which can be regarded as a C_4-dicarboxylate with an acetyl group as the substituent, is more efficient with respect to induction and apparent K_D than the C_3- and C_5-dicarboxylates, malonate and glutarate.

The C_4-Dicarboxylate Binding Site of DcuS—The sequence Arg-X_2-His-X_9-Phe-X_{26}-Arg-*X*-Phe presumably represents the signature sequence for binding of C_4-dicarboxylates by DcuS. The residues are conserved in DcuS-like proteins and have been shown by mutagenesis to be essential for DcuS function *in vivo*. Other residues (Met^{103} and Gln^{159}), which are also conserved specifically in DcuS-like proteins (but not in CitA-like proteins), were not required for function when tested *in vivo* after mutation to Ala or Arg and Ala or Val, respectively.

The periplasmic domain of DcuS shares many conserved residues with the citrate sensor CitA from *Klebsiella*, and the residues required for citrate binding have been identified by mutagenesis (4, 5). CitA binds citrate presumably as a divalent anion ($citrate^{2-}$) and requires residues Arg^{109}, His^{112}, Arg^{150}, and Lys^{152} for binding. The former three residues are equivalent to the (essential) residues Arg^{107}, His^{110}, and Arg^{147} of DcuS. Residue Lys^{152} of CitA is replaced by the (essential) Phe residue in DcuS (Phe^{149}), which cannot be replaced by a Lys residue. The essential Phe^{120} of DcuS obviously has no counterpart in CitA. Thus, Phe^{120} is specific for DcuS, whereas $Arg^{107/109}$, $His^{110/112}$, and $Arg^{147/150}$ are present in DcuS and CitA as well and Phe^{149}-Lys^{152} are homologous replacements. This set of residues obviously is responsible for the binding of the C_4-dicarboxylate and $citrate^{2-}$ in DcuS and CitA.

High resolution structures of the fumarate and succinate binding sites of fumarate reductases and succinate dehydrogenases have been determined. In *Wolinella succinogenes* and *E. coli* fumarate reductase, one carboxylate of the C_4-dicarboxylate is ligated by an Arg and a His residue and the second carboxylate is ligated by a His residue (Arg^{404} and His^{369} and His^{257} of *W. succinogenes* FrdA) (29, 30). Fumarate binding by DcuS might be similar, suggesting the binding of the first carboxylate by one Arg and one His residue and of the second carboxylate by the second essential Arg residue together with one of the conserved Phe residue. Arg^{301} from the active site of FrdA, which is required for fumarate reduction, has no homologue in DcuS.

Structural Properties of the C_4 Binding Site / Domain of DcuS and Comparison to the Citrate Binding Site of CitA—Structural information on the periplasmic domains of DcuS and of CitA, which has become available recently (6, 17), will help to understand C_4-dicarboxylate binding in future. The structure of the periplasmic domain of DcuS with bound fumarate, which is not available yet due to the low affinity binding of the C_4-dicarboxylates, will be required. However, from the comparison of the binding pockets of DcuS and CitA where the x-ray structure is available (6), it was tempting to propose a binding model of fumarate to DcuS. Indeed, the residues that are involved in the binding of one of the carboxylate groups of citrate ($Lys^{152} \rightarrow Phe^{149}$ and $Ser^{167} \rightarrow Ala^{165}$) have lost their positive charge in DcuS so that one could speculate that the residues of the other two carboxylate groups are used by DcuS to bind the two carboxylate groups of fumarate. However, due to the fact that the nonphysiological molybdate is bound very close to the citrate in the CitA, we refrain from a detailed discussion of binding modes.

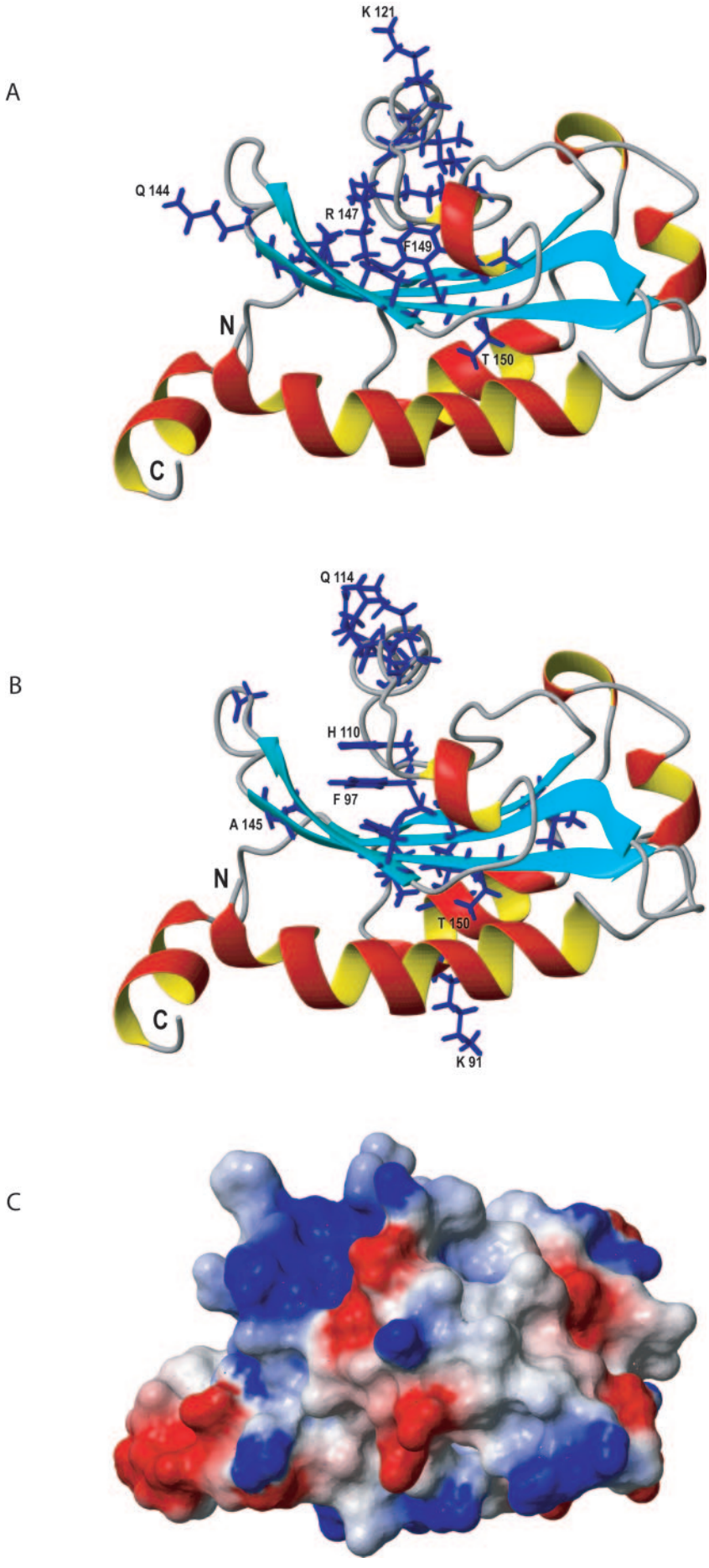

FIG. 5. Structure of the periplasmic domain of DcuS showing the residues most affected in peak height by fumarate (*A*) or in chemical shift by D-tartrate addition (*B*). Residues highlighted in *A* are Arg^{107}, Ala^{113}, Ile^{116}, Lys^{121}, Ala^{128}, Asn^{134}, Ala^{136}, Ala^{143}, Gln^{144}, Ala^{145}, Arg^{147}, Phe^{149}, and Thr^{150}, and residues in *B* are Val^{89}, Lys^{91}, Leu^{96}, Phe^{97}, Val^{100}, His^{110}, Ala^{113}, Gln^{114}, Gln^{118}, Lys^{121}, Asp^{124}, Asn^{134}, Gly^{140}, Ala^{145}, and Thr^{150}. *C*, electrostatic surface potential of the periplasmic domain of DcuS with positive and negative potential colored *blue* and *red*, respectively. Figures were generated using the program MOLMOL (31).

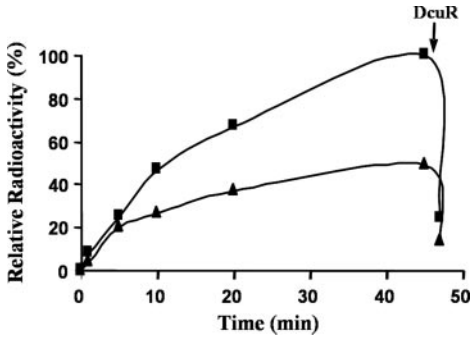

FIG. 6. **Autophosphorylation of reconstituted DcuS and DcuS(R147A) in the presence of fumarate.** Kinetics of DcuS (■) and DcuS(R147A) (▲) autophosphorylation by [γ-^{33}P]ATP in proteoliposomes relative to autophosphorylation of DcuS after 45 min (100%) are shown. DcuS-proteoliposomes and DcuS(R147A)-proteoliposomes containing 20 mM fumarate in the buffer and within the proteoliposomes were mixed with 100 μM [γ-^{33}P]ATP. After 46 min of autophosphorylation, a 4-fold excess of His_6-DcuR was added. At the time points indicated, aliquots (2 μg of DcuS protein) were withdrawn, quenched with SDS sample buffer, subjected to SDS-polyacrylamide gel electrophoresis, and evaluated using a PhosphorImager.

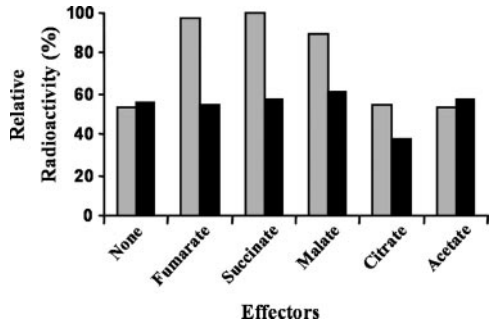

FIG. 7. **Stimulation of DcuS and DcuS(R147A) autophosphorylation by C_4-dicarboxylates, citrate, or acetate after reconstitution in liposomes.** Phosphorylation of DcuS (*gray bars*) and DcuS(R147A) (*black bars*) by [γ-^{33}P]ATP in proteoliposomes was measured in the presence of different carboxylic acids. After 30 min of incubation, the radioactivity bound by DcuS or DcuS(R147A) was determined after SDS-polyacrylamide electrophoresis and phosphorimaging analysis (see the legend to Fig. 6). The highest value obtained (DcuS with succinate) was set to 100%.

C_4-Dicarboxylate Binding Site and Signal Transfer in DcuS—Mutation in the fumarate binding site of DcuS affected only C_4-dicarboxylate-dependent expression *in vivo* and the fumarate-stimulated kinase activity *in vitro*. The basic kinase activity itself and the phosphoryl transfer to DcuR were not impaired, which demonstrated that kinase activity *per se* is independent of the sensory domain. In addition, the experiments directly show that binding of fumarate to the (periplasmic) binding domain controls the activity of the (cytoplasmic) kinase and transmitter domains. Future experiments will aim at an understanding of the reactions and structural changes induced by fumarate binding and their transmission from the binding site to the transmitter domain of DcuS.

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