The NMR structure of the sensory domain of the membranous two-component fumarate sensor (histidine protein kinase) DcuS of *Escherichia coli*

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Running title: structure of the periplasmic domain of DcuS in solution
1 Summary

The structure of the water soluble, periplasmic domain of the fumarate sensor DcuS (DcuS-pd) has been determined by NMR spectroscopy in solution. DcuS is a prototype for a sensory histidine kinase with transmembrane signal transfer. DcuS belongs to the CitA-family of sensors that are specific for sensing di- and tri-carboxylates. The periplasmic domain is folded autonomously and shows helices at the N- and the C-terminus suggesting direct linking or connection to helices in the two transmembrane regions. The structure constitutes a novel fold. The nearest structural neighbor is the PAS domain of the photoactive yellow protein that binds small molecules covalently. Residues R107, H110, and R147 are essential for fumarate sensing and are found clustered together. The structure constitutes the first periplasmic domain of a two component sensory system and is distinctly different from the aspartate sensory domain of the Tar chemotaxis sensor.

2 Introduction

The fumarate sensor DcuS is a prototype for a two component sensory histidine kinase with signal perception in the periplasm, transmembrane signal transfer\textsuperscript{1, 2} and autophosphorylation of a His residue in the kinase domain in the cytoplasm\textsuperscript{3}. DcuS belongs to the CitA-family of sensors that are specific for sensing di- and tri-carboxylates\textsuperscript{1, 2, 4, 5}. The periplasmic domain of the histidine autokinase CitA works as a highly specific citrate receptor whereas DcuS uses any type of C\textsubscript{4}-dicarboxylate like fumarate, succinate and malate as a stimulus\textsuperscript{1, 4, 5, 6}. DcuS is predicted to consist of two transmembrane helices and of a periplasmic sensory domain enclosed by the transmembrane helices. The second transmembrane helix is followed by a cytoplasmic PAS domain of unknown function, and the kinase with the consensus histidine residue for autophosphorylation.
The periplasmic citrate binding domain of CitA is conserved in DcuS and presumably responsible for binding of fumarate and other C$_4$-dicarboxylates. Preliminary results suggest that fumarate sensing occurs by this domain in the periplasm$^2,4,5$. After phosphorylation by DcuS the response regulator DcuR of the DcuSR system activates the expression of the target genes like $dcuB$ and $frdABCD$ encoding an anaerobic fumarate carrier DcuB and fumarate reductase$^4,5$. Despite their prevalence no structural information is available for transmembraneous sensory kinases, in particular not for signal perception and transmission across the membrane. Only the structures of cytoplasmic sensory kinases, or of domains not involved in transmembrane signalling have been determined.

Purified DcuS is active after reconstitution in proteoliposomes and capable of transmembraneous stimulation of the kinase by fumarate$^2$. For a more detailed understanding of signal perception representing the first step of signal transduction in transmembraneous histidine kinases of two-component systems, the structure of the periplasmic C$_4$-dicarboxylate binding domain of DcuS (DcuS-pd) was determined after stable over-production of the domain.

### 3 Experimental Procedures

*Over-production of DcuS$_{45-180}$ (‘DcuS-pd’)*

The sequence of $dcuS$ coding for the periplasmic domain of DcuS (DcuS$_{45-180}$ or DcuS-pd) enclosed by the two transmembrane helices was cloned into the $Nde$I and $Hind$III sites of plasmid pET28a (Novagen) resulting in plasmid pMW145. The DNA fragment was amplified with oligonucleotides pdcus-Nde2 (ATT TAC TTC TCG CAT ATG AGT GAT ATG) and pdcuS-Hind (GAC CAG ATA
AAG CTT CAG CGA CTG) by PCR of genomic *E. coli* K-12 AN387 DNA. The cloned fragment codes for DcuS-pd starting with Ser45 and ending with Arg180. The N-terminal extension contains in addition a His<sub>6</sub>-tag followed by a thrombin cleavage site in front of DcuS-pd. Over-production of His<sub>6</sub>-DcuS-pd was performed in *E. coli* BL21DE3(pMW145) grown aerobically in LB broth or supplemented M9 medium containing <sup>13</sup>C-glucose (6 mM) and/or <sup>15</sup>N-NH<sub>4</sub>Cl (7 mM) as suitable, after induction with 1 mM isopropyl-α-D-thiogalactopyranoside. The washed cells (1.2 g) were broken by two passages through a French Pressure Cell. The soluble protein fraction was used for isolation of His<sub>6</sub>-DcuS-pd on a column of Ni<sup>2+</sup>-NTA-agarose (3 ml bed volume). His<sub>6</sub>-DcuS-pd was eluted in 6 to 9 ml of buffer containing 500 mM imidazole in 50 mM Na/K phosphate buffer and 200 mM NaCl at pH 7.0.

*Sample preparation for NMR*

Isolated His<sub>6</sub>-DcuS-pd (10 to 20 mg) was dialysed (MWCO 10 000, ZelluTrans, Roth) for 3 h against 100 vol of buffer containing 5 mM imidazole and 200 mM NaCl in 50 mM Na/K phosphate at pH 6.5. The sample (6 ml) was then concentrated by centrifugation at max. 5000 x g in a Vivaspin Concentrator tube (exclusion limit 10 000 Da, Vivaspin, Sartorius) to a final concentration of 10 to 25 mg protein/ml. The sample (300 to 900 µl, in fractions of 300 µl each) was frozen in liquid N<sub>2</sub> and stored at -80 °C. For the NMR measurement the His<sub>6</sub>-tag was removed by incubation of 300 µl samples with thrombin (20 U / mg DcuS) (Amersham Pharmacia Biotech) in 700 µl buffer for 1 h at 20 °C. The digested sample was passed through a Ni<sup>2+</sup>-NTA column to remove the His<sub>6</sub>-tag. The eluate (6 to 9 ml) was concentrated in a Vivaspin concentrator tube as described above. Overall 3 to 7.5 mg periplasmic domain of DcuS-pd with a Gly-Ser-His-Met extension at the N-terminal end in front of Ser45 of DcuS was obtained in 300 µl buffer. When required, for complete exchange of
H$_2$O against D$_2$O (99.9 %, Deutero GmbH, Kastellaun), the protein solution was frozen in liquid N$_2$, freeze-dried for 45 min, and resuspended in 1 ml D$_2$O followed by lyophilization as described for 3 cycles. The last suspension was kept for 1 h at 4°C and freeze-dried. The dry sample was dissolved at a final concentration of 30 mg protein / ml D$_2$O, and stored at -80°C till use.

**Assignment**

The assignment of His tagged DcuS-pd has been published\textsuperscript{7}, it is put into BRMB under the accession 4821. The assignment of DcuS-pd without the His tag differs only slightly from the one with the His-tag.

**Restraints**

Distance restraints were obtained from the intensities of NOE cross-peaks extracted from $^{15}$N-edited-3D-NOESY-HSQC, 2D NOESY and $^{13}$C-edited-3D-NOESY-HSQC spectra. Analysis, assignment and integration of NOESY spectra were accomplished with XEASY\textsuperscript{8}. NOEs were classified as *strong*, *medium*, *weak* and *very weak* with an upper distance restraint of 3.0, 3.8, 4.6 and 5.4 Å, respectively. No lower distance limit was applied during initial runs. The lower limit was restrained to 2 Å at a later stage of the calculations. Hydrogen bond restraints were applied in a standard way to slowly exchanging amide protons involved in α-helix and antiparallel β-sheet structures.

Restraints for $(\phi, \psi) = (-57 \pm 20, -47 \pm 20)$ and $(\phi, \psi) = (-139 \pm 20, 135 \pm 20)$ were applied to the amino acid residues involved in α-helix and antiparallel β-sheet secondary structures, respectively, as determined by NOE patterns and TALOS prediction using $^{13}$C chemical shifts\textsuperscript{9} for non regular secondary structure elements.
A set of $^1D_{\text{NH}}$, $^1D_{\text{NC}}$, $^1D_{\text{C'C}}$, and $^1D_{\text{C'\alpha}}$ residual dipolar couplings (rdc) of DcuS were calculated from the difference in the corresponding $J$ splitting measured in protein sample containing 10mg/ml Pf1 filamentous phage$^{10,11}$ and in protein sample in the absence of phage$^{12}$. $^1D_{\text{NH}}$ and $^1D_{\text{NC'}}$ rdc were measured simultaneously using a modified interleaved 3D-TROSY-HNCO experiment$^{13}$ and $^1D_{\text{C'\alpha}}$ and $^1D_{\text{C'\alpha}}$ rdc using a modified interleaved 3D-CBCACONH experiment$^{14}$. The data sets were processed and analysed using the NMRPipe/NMRDraw$^{15}$ software package. The magnitude of the alignment tensor (Da) obtained from the histogram of measured dipolar couplings is 10.7 Hz for $^1D_{\text{NH}}$ and the corresponding Rhombicity (R) is 0.63. Dipolar couplings were applied in the structure calculation with different weight factors 1.0 ($^1D_{\text{NH}}$), 0.4 ($^1D_{\text{C'\alpha}}$), 44 ($^1D_{\text{NC'}}$) and 2.5 ($^1D_{\text{C'\alpha}}$).

**Structure calculation and analysis**

Structures were calculated using the Xplor-NIH program package$^{16}$. A standard four-stage molecular dynamics protocol was used with an initial high temperature annealing stage (1000 step/15 ps torsion angle MD at $T=50,000$), followed by a first (1000 step/15 ps cooling to 0) and a second (3000 step/15 ps Cartesian MD cooling from $T=2,000$ to 0) slow-cool annealing stage and a final minimization stage. The final structures were analysed by the programs MOLMOL$^{17}$ and ProCheck$^{18}$. 
Effect of mutations in the putative fumarate binding site of DcuS on the fumarate plus DcuR dependent expression of dcuB'-lacZ

The activity of the DcuS/DcuR two-component system was measured by the dcuB'-lacZ reporter gene fusion. Expression of dcuB depends strongly on the presence of active DcuS/DcuR two-component system. Expression was measured after growth of the bacteria under anaerobic conditions (A578nm = 0.5) with 50 mM fumarate as the substrates to achieve optimal induction of dcuB. All strains contain a chromosomal dcuB'-lacZ reporter gene fusion. E. coli IMW260 is a derivative of E. coli K-12 with a chromosomal dcuS mutation (MC4100, but dcuS::camR dcuB'-lacZ). The other strains were the same as IMW260, but contained various mutant forms of dcuS cloned in plasmid pET28a.

4 Results

Secondary structure of DcuS-pd

The secondary structure of DcuS-pd (Fig. 1) consists of a long N-terminal α-helix (α1) ranging from amino acid 46 to 64 with a continuation from 68 to 72 (α2). After a short loop there is another α-helix (α3a: 77 to 79) and (α3b: 83 to 92) that is connected to the first β-strand (β1: 97 to 102) of the four stranded antiparallel β-sheet. β1 is connected via an α-helix (α4: 126 to 128) and a long loop to the second β-strand (β2: 134 to 138), which is connected by a short loop to the third strand (β3: 145 to 153). Yet, another turn connects to the fourth β strand (β4: 159 to 167). From this strand the C-terminal helix follows after a short helix (α5: 174 to 179). The secondary structural elements have been established by secondary chemical shifts as well as characteristic sequential
NOEs and the connectivity of the four stranded antiparallel β-sheet by HN, Hα, and Hα, Hα cross strand NOEs (Fig. 1). The secondary structure of DcuS including the transmembrane helices most probably is as follows: Transmembrane helix 1 (TM1 21-42) to form a contiguous helix from 21 to 64. By the same token, it is expected that the C-terminal helix (α5) extends into the membrane uninterruptedly via TM2 forming a helix from 174 to 202.

Structure determination

The total number of non-ambiguous NOEs is 2245 (16.5 per residue) with 1074 (8 per residue) being inter-residual. We measured 382 dipolar couplings including 107 NH, 95 HαCα, 114 NC’, and 66 CαC’. 46 hydrogen bonds have been included as described above. 187 φ and ψ angles were derived from the carbon chemical shifts using Talos. 1J(Cα,Hα) couplings were used to define the φ angle: negative for 1J(Cα,Hα) > 137 Hz and in the α-helix19 range for 1J(Cα,Hα) > 145 Hz. Figure 2 shows the distribution of NOEs and the resulting backbone RMSD as a function of sequence position. The backbone RMSD clearly anticorrelates with the number of NOE restraints per amino acid identifying several loops with reduced restraint density. Out of the 200 structures we took 10 structures that had the lowest energies and displayed them using the program MOLMOL17. They had converged with an RMSD to the average structure of 0.68Å in the backbone of the structured regions. The structure is well ordered in the region 46 to 168 while the first residues at the N-terminus and the C-terminal residues (169 to 178) are not well ordered. Especially the C-terminal helix does not show long range NOEs.

Figure 3 shows a stereo view of the mean structure derived from the 10 structures with the lowest energy, fitted for minimal RMSD (backbone rmsd 0.582, heavy rmsd 1.035) of the region with secondary structure elements (residues 55-88,94-105,123-136,144-154,158-167). These structures were
submitted to the Protein Data Bank, PDB ID 1ojg. The $^1\text{N},^{15}\text{N}$ NOEs (Fig. 2) do not vary strongly over the structure of DcuS, except for the C-terminal helix.

Discussion of the structure

The structure is a novel $\alpha,\beta$-fold completely dissimilar of the four helix bundle structure of the aspartate sensor$^{20,21}$. Also, the structure is a monomer in solution dissimilar to the aspartate sensor. From relaxation data the dimer content can be estimated to be below 10%, which is in agreement with gel shift data. In a DALI$^{22}$ search the closest match (score=5) is photoactive yellow protein from *Halorhodospira halophila* (PYP)$^{23}$ which also shows an $\alpha,\beta$-fold, however, with 5 instead of 4 $\beta$-strands (Fig. 1). The topology of strands $\beta_3$, $\beta_4$ and $\beta_5$ of PYP is similar to the strands $\beta_2$, $\beta_3$ and $\beta_4$ of DcuS. However, the rest of the secondary structure is quite dissimilar. While in PYP the PAS-core domain connects the strands $\beta_2$ and $\beta_3$ by crossing the whole $\beta$-sheet in a diagonal manner there is no PAS core domain in DcuS and the connection between sheets $\beta_1$ and $\beta_2$ is achieved on one side of the $\beta$-sheet. Similar to PYP, there are two hydrophobic cores on both sides of the $\beta$-sheet formed. Helices $\alpha_1$ and $\alpha_{3b}$ bind to the bottom side of the $\beta$-sheet while helix $\alpha_4$ and the connector attach to the upper half of the $\beta$-sheet. In PYP the chromophore-binding site is formed by the PAS-core domain. Dissimilar to PYP, in DcuS residues located in the $\beta$-sheet (R147) as well as in the connector (R107 and H110) across the $\beta$-sheet contribute to the putative binding site of fumarate.
Relevance of the structure

The periplasmic domain used in this study is folded and the residues R107, H110 and R147 found from mutation to be essential for the signalling are in close proximity in the structure suggesting that the binding motif is retained in the periplasmic domain. Conservation of His and Arg residues in DcuS-pd which have been assigned in citrate binding in CitA by mutagenesis, suggest that the same residues are important for fumarate binding in DcuS-pd. After mutagenesis of R107, H110 or R147 functional data were obtained by measuring the activity of the DcuS/DcuR two-component system via a $dcuB^{-}·lacZ$ reporter gene fusion (Table 1), the expression of which depends on the presence of active DcuS/DcuR two-component system\(^2\). Replacement of the Arg residues 107 and 147 and of the His residue 110 by Ala by site directed mutagenesis abolished the stimulation by fumarate to the same extent as complete deletion of the $dcuS$ gene (Table 1), although the mutated protein was formed at normal levels.
Table 1

| Strain (genotype) | $\beta$-galactosidase activity (Miller units) | Wild-type/mutant activity |
|-------------------|---------------------------------------------|---------------------------|
| IMW260 ($dcuS^-$) | 7                                           | 0.033                     |
| IMW260 p($dcuS^+$) | 209                                         | 1                         |
| IMW260 p($dcuS – R107A$) | 6                                           | 0.029                     |
| IMW260 p($dcuS – H110A$) | 5                                           | 0.024                     |
| IMW260 p($dcuS – R147A$) | 11                                          | 0.053                     |
Figure Legends

Figure 1: Left: Secondary structure of DcuS together with the intrastrand NOEs from which the topology of the β-strands is derived. Right: Secondary structure of PYP

Figure 2: Statistics of number of NOE restraints, rms-deviation of the atomic positions and secondary structure elements for DcuS. The rmsd of each amino acid correlates very well with the number of restraints. This is expected since the heteronuclear NOE does not show appreciable variations across the structure of DcuS.

Figure 3: Stereoview of the structure of DcuS indicating the amino acids R107, H110 and R147 which cluster together to form the binding site of fumarate. The structure is a α, β-fold where both sides of the large β-sheet form hydrophobic cores with α-helices and the long connector between helix α4 and strand β2. The C-terminal helix is not fixed by NOE’s and shows only small dipolar couplings, in agreement with a flexible helix
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a. Numbers of restraints per residue, Het NOE value

b. RMS devs from mean coords: main-chain (black) and side-chain (grey)

c. Secondary structure & average estimated accessibility
The NMR structure of the sensory domain of the membrane-bound two-component fumarate sensor (histidine protein kinase) DcuS of Escherichia coli
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