Identification of Basic Amino Acid Residues Important for Citrate Binding by the Periplasmic Receptor Domain of the Sensor Kinase CitA

Tanja Gerharz,[‡] Stefan Reinelt,^{§,||} Sibylle Kaspar,[‡] Leonardo Scapozza,[§] and Michael Bott*,[‡]

Institut für Biotechnologie 1, Forschungszentrum Jülich, D-52425 Jülich, Germany, and Departement Pharmazie, Eidgenössische Technische Hochschule, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

Received January 13, 2003

ABSTRACT: The sensor kinase CitA and the response regulator CitB of Klebsiella pneumoniae form the paradigm of a subfamily of bacterial two-component regulatory systems that are capable of sensing trior dicarboxylates in the environment and then induce transporters for the uptake of these compounds. We recently showed that the separated periplasmic domain of CitA, termed CitAP (encompasses residues 45-176 supplemented with an N-terminal methionine residue and a C-terminal hexahistidine tag), is a highly specific citrate receptor with a K_d of 5.5 μ M at pH 7. To identify positively charged residues involved in binding the citrate anion, each of the arginine, lysine, and histidine residues in CitAP was exchanged for alanine, and the resulting 17 muteins were analyzed by isothermal titration calorimetry (ITC). In 12 cases, the K_d for citrate was identical to that of wild-type CitAP or slightly changed (3.9– 17.2 μ M). In one case (R98A), the K_d was 6-fold decreased (0.8 μ M), whereas in four cases (R66A, H69A, R107A, and K109A) the K_d was 38- to >300-fold increased (0.2 to >1 mM). The secondary structure of the latter five proteins in their apo-form as deduced from far-UV circular dichroism (CD) spectra did not differ from the apo-form of wild-type CitAP; however, all of them showed an increased thermostability. Citrate increased the melting point (T_m) of wild-type CitAP and mutein R98A by 6.2 and 9.5 °C, respectively, but had no effect on the $T_{\rm m}$ of the four proteins with disturbed binding. Three of the residues important for citrate binding (R66, H69, and R107) are highly conserved in the CitA subfamily of sensor kinases, indicating that they might be involved in ligand binding by many of these sensor kinases.

In bacteria, adaptive responses to changing environmental conditions are often accomplished by two-component regulatory systems, which in their simplest form consist of two modular proteins, a sensor kinase, and a response regulator. The sensor kinase responds to a certain stimulus by autophosphorylation of a conserved histidine residue, and the phosphoryl group is subsequently transferred to a conserved aspartate residue of the response regulator, which triggers changes in gene expression or cell behavior depending on its phosphorylation status (1-4). In the majority of cases, sensor kinases are transmembrane proteins with an extracellular N-terminal sensor domain (typically 100-200 amino acids in length) flanked by two transmembrane helices and a cytoplasmic C-terminal autokinase core (~220 amino acids) that is connected to the second transmembrane helix either by a short linker region or by an additional domain. This architecture enables the proteins to sense external stimuli and transduce information to the cytoplasm.

A typical representative of these proteins is the sensor kinase CitA from the enterobacterium *Klebsiella pneumoniae* (Figure 1). Together with its cognate response regulator CitB, it induces the expression of genes encoding enzymes that



FIGURE 1: Schematic representation of the domain organization of the *K. pneumoniae* sensor kinase and of the arginine, lysine, and histidine residues within CitAP. Abbreviations: TMH, transmembrane helix; Q, Q linker (*30*); H, H-box containing the conserved autophosphorylated histidine residue; N, G1, F, G2, conserved boxes involved in ATP binding; PAS, acronym formed from the names of the proteins in which the features characteristic for this signaling module were first recognized (*31*).

are involved in citrate fermentation (5, 6) (i.e., the sodiumdependent citrate carrier CitS (7), citrate lyase (8-10), and the sodium-ion pump oxaloacetate decarboxylase (11-13)). Expression requires the presence of citrate, sodium ions, and anoxic conditions (5) and is subject to catabolite repression (14). It was recently shown that the periplasmic domain of the sensor kinase CitA serves as a highly specific citrate receptor (15). The periplasmic domain (residues 45–176)

^{*} Corresponding author. Tel.: +49-(0)2461-615515. Fax: +49-(0)-2461-612710. E-mail: m.bott@fz-juelich.de.

[‡] Forschungszentrum Jülich.

[§] Eidgenössische Technische Hochschule.

^{II} Present address: Max-Planck-Institut für medizinische Forschung, Jahnstrasse 29, D-69120 Heidelberg, Germany.

name	sequence $(5' \rightarrow 3')$	new r.s.
R7f	ATTACCGAGGAAGCTTTGCATTATCAGGTC	HindIII
H9f	ACCGAGGAGCGGCTAGCTTATCAGGTCGGG	NheI
R15f	CAGGTCGGGCAAGCGGCATTAATTCAGGCGATGCAGATTTCG	AsnI
K35f	GAGGCAGTGCAGGCGCGCGATCTCGCC	BssHII
R36f	GCAGTGCAGAAAGCAGATCTCGCCAGA	BglII
R40f	AAACGAGATCTCGCCGCAATCAAAGCCCTT	BglII
K42f	CGCGATCTCGCGCGCATCGCAGCCCTTATCGAC	BssHII
R49f	CTTATCGACCCCATGGCTTCGTTCTCCGAC	NcoI
R66f	GATGCCAGCGGCCAGGCCCTGTATCACGTCAAT	MvaI
H69f	CAGCGCCTCTATGCCGTTAACCCTGATGAA	HpaI
K77f	GATGAAATCGGCGCCTCGATGGAAGGC	NarI
K92f	GAGGCGTTGATTAATGCTGCAAGCTACGTGTCA	AsnI
R98f	AGCTACGTGTCAGTCGCGAAAGGCTCGCTG	NruI
K99f	GTGTCAGTGCGCGCAGGATCCCTGGGATCG	BamHI
R107f	GGATCGTCGCTGGCCGGCAAATCGCCG	NaeI
K109f	TCGCTGCGCGGTGCTAGCCCGATTCAGGAC	NheI
K118f	GACGCAACCGGCGCCGTGATCGGCATT	NarI

^{*a*} Only the sequence of the forward primers is shown, that of the reverse primers (R7r, H9r, etc.) is exactly complementary to the corresponding forward primers. The last column designated New r.s. lists the new restriction sites introduced into plasmid pET-CitAP for the easy identification of positive clones.

was overproduced as a soluble, cytoplasmic protein with a carboxyterminal hexahistidine tag, termed CitAP (Figure 1), and purified by Ni²⁺-NTA¹ affinity chromatography. Binding studies with CitAP using either ¹⁴C-labeled citrate or ITC revealed that CitAP bound citrate with a K_d of 5.5 μ M in 50 mM sodium phosphate buffer pH 7. Binding was driven by the enthalpy change ($\Delta H = -76.3$ kJ/mol), whereas the entropy change was unfavorable ($T\Delta S = -46.3$ kJ/mol). Under the conditions tested, CitAP behaved as a monomeric protein, independent of the presence or absence of citrate. Binding was strongly dependent on the pH, with a maximal affinity at pH 5.7, at which the concentration of the divalent citrate species H-citrate²⁻ is maximal. This was used as an argument that H-citrate²⁻ is the ligand bound by CitAP (*15*).

Binding of a 2-fold negatively charged citrate ion to CitAP might involve positively charged amino acid residues. Therefore, each of the arginine, lysine, and histidine residues in CitAP was exchanged to the neutral amino acid alanine, and after purification the resulting 17 mutated CitAP derivatives were analyzed for citrate binding by ITC. In addition, the structure and thermal stability of selected CitAP mutants were studied by CD spectroscopy. In this way, five residues were identified that are highly important for citrate binding.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. For the propagation and isolation of mutated derivatives of plasmid pET-CitAP, supercompetent cells of *Escherichia coli* XL1-Blue (Stratagene) were used. For the overproduction of the mutated CitAP derivatives, *E. coli* BL21(DE3) (*16*) was used. The strains were routinely cultivated aerobically at 37 °C in Luria–Bertani medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) supplemented with 50 μ g/mL kanamycin.

Construction of CitAP Derivatives Containing Single Arg-Ala, Lys-Ala, or His-Ala Exchanges. CitAP is a derivative of the sensor kinase CitA from K. pneumoniae encompassing residues 45–176 supplemented with an N-terminal methionine residue and a C-terminal hexahistidine tag (Figure 1). The protein is encoded by plasmid pET-CitAP, and its synthesis is controlled by a phage T7 promoter and the translation initiation signals of the gene 10 protein of phage T7 (15). To exchange each of the nine arginine, six lysine, and two histidine residues within CitAP by alanine, pET-CitAP was subjected to site-directed mutagenesis using the QuikChange site-directed mutagenesis kit according to the instructions of the supplier (Stratagene, Heidelberg, Germany). The oligonucleotides used for mutagenesis (Table 1) contained not only the mutations required for the desired amino acid exchange but also silent mutations that introduced new restriction sites into pET-CitAP. In this way, the plasmids obtained after mutagenesis could be first screened by restriction mapping for those containing the mutation. The CitAP coding region of these plasmids was then sequenced on both strands (17) to confirm that no spurious mutations had been introduced.

Overproduction and Purification of CitAP Derivatives Containing Single Arg-Ala, Lys-Ala, or His-Ala Exchanges. For the purification of the mutated CitAP derivatives, the corresponding plasmids were transferred to E. coli BL21 (DE3). Cultivation of the transformed cells and purification by Ni²⁺-NTA affinity chromatography of the mutated CitAP derivatives were performed as described for wild-type CitAP (15). The purity of the proteins was checked by SDSpolyacrylamide gel electrophoresis and staining of the gels with colloidal Coomassie (Pierce, Rockford, IL). Protein concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient of 6.4 mM⁻¹ cm⁻¹ (18). In some cases, the protein solutions were concentrated using Centricon-3 or Centricon-10 ultracentrifugation devices (Millipore, Eschborn, Germany) before being used for ITC measurements.

Mass Spectrometric Analysis of CitAP Muteins. To confirm the amino acid exchanges in the mutated CitAP proteins, several of them were analyzed by peptide mass fingerprinting. To this end, the desired protein spots were excised from Coomassie-stained SDS—polyacrylamide gels and subjected to digestion in the gel with trypsin as described (19). The trypsinogenic peptides were extracted and analyzed using a

¹ Abbreviations: CD, circular dichroism; HPK, histidine protein kinase; ITC, isothermal titration calorimetry; NTA, nitrilotriacetic acid.

Table 2: Thermodynamic Parameters for Citrate Binding to Wild-Type CitAP and 17 CitAP Muteins as Determined by Isothermal Titration Calorimetry^a

-						
protein	n	$K_{\rm d}$ (μ M)	$K_{\rm d}$ (mutein)/ $K_{\rm d}$ (wild type)	ΔH (kJ/mol)	$\frac{T\Delta S}{(\text{kJ/mol})}$	ΔG (kJ/mol)
Wt	0.95 ± 0.06	5.3 ± 1.1	1.0	-80.3 ± 8.7	-48.7 ± 9.0	-30.7 ± 0.6
R7A	0.51 ± 0.03	6.0 ± 0.1	1.1	-45.8 ± 0.9	-16.0 ± 0.9	-29.8 ± 0.1
H9A	0.87 ± 0.03	9.8 ± 1.4	1.9	-45.2 ± 2.8	-16.6 ± 3.1	-28.6 ± 0.3
R15A	0.94 ± 0.02	9.3 ± 2.8	1.8	-48.6 ± 1.2	-19.8 ± 0.6	-28.8 ± 0.7
K35A	0.74 ± 0.01	4.8 ± 0.7	0.9	-67.9 ± 1.4	-36.7 ± 3.0	-31.2 ± 1.6
R36A	0.73 ± 0.01	4.3 ± 0.3	0.8	-73.9 ± 8.4	-43.3 ± 8.6	-30.6 ± 0.2
R40A	0.69 ± 0.01	4.6 ± 0.4	0.9	-71.3 ± 2.0	-40.8 ± 2.2	-30.5 ± 0.2
K42A	0.74 ± 0.02	7.0 ± 0.6	1.3	-59.1 ± 3.1	-29.7 ± 3.2	-29.4 ± 0.2
R49A	0.63 ± 0.04	16.7 ± 5.0	3.2	-80.3 ± 5.8	-52.9 ± 6.2	-27.4 ± 0.7
R66A	(1.00, fixed)	(7067 ± 42)	(1333)	(-1.9 ± 1.0)	(10.4 ± 1.0)	(-12.3 ± 0.1)
H69A	(0.68 ± 0.18)	(2087 ± 767)	(394)	(-32.9 ± 0.1)	(-17.6 ± 0.1)	(-15.4 ± 0.1)
K77A	0.56 ± 0.15	17.2 ± 3.9	3.3	-84.4 ± 8.0	-57.1 ± 8.3	-27.3 ± 0.6
K92A	1.23 ± 0.25	12.2 ± 1.0	2.3	-66.9 ± 3.5	-38.9 ± 3.8	-28.0 ± 0.2
R98A	0.30 ± 0.01	0.8 ± 0.1	0.2	-56.6 ± 5.8	-21.9 ± 5.5	-34.7 ± 0.3
K99A	0.77 ± 0.03	4.7 ± 1.3	0.9	-61.6 ± 0.4	-31.2 ± 1.1	-30.5 ± 0.7
R107A	1.36 ± 0.18	205 ± 35	38.7	-61.5 ± 2.1	-40.5 ± 1.7	-21.1 ± 0.4
K109A	(0.53 ± 0.03)	(2054 ± 1949)	(388)	(64.5 ± 0.6)	(46.3 ± 3.4)	(18.2 ± 2.8)
K118A	0.79 ± 0.02	3.9 ± 0.6	0.7	-71.0 ± 4.2	-40.1 ± 4.5	-30.9 ± 0.4

^{*a*} All experiments were performed at 25 °C in 50 mM sodium phosphate buffer pH 7.0. The values represent the mean of at least two experiments. The binding stoichiometry is indicated as *n*. The values given in parantheses represent estimates rather than accurate data since in the corresponding experiments the *c* value, defined as the total protein concentration multiplied with the binding constant (20), was ≤ 1 and therefore too low to obtain reliable data (32).

MALDI-TOF mass spectrometer (Voyager STR, Applied Biosystems, Weiterstadt, Germany) as reported previously (19).

Isothermal Titration Calorimetry. All ITC measurements were performed using a VP-ITC microcalorimeter from MicroCal (Northampton, MA) with a cell volume of 1.40 mL at a temperature of 25 °C. Automated injections were made with a 250 μ L microsyringe while stirring at 310 rpm. The calorimeter and the equations used to fit calorimetric data have been described in detail previously (20). The reference cell was filled with water. Prior to loading into the microcalorimeter, the solutions were degassed for 10 min with gentle swirling under vacuum. All ITC measurements were performed in 50 mM sodium phosphate buffer pH 7.0, and the purified CitAP derivatives were extensively dialyzed against this buffer before being used for ITC. Solutions of the protein (62–2340 μ M) were filled into the sample cell and titrated with citrate using identical injections of 3-10 μ L. Sodium citrate was dissolved in the final dialysis buffer to a concentration 11-57 times higher than the protein concentration. Calorimetric data were analyzed using the ORIGIN software Version 5.0 (MicroCal, Northampton, MA). All binding parameters presented in Table 2 are the means of at least two experiments.

CD Spectroscopy. Prior to analysis by CD spectroscopy, the solution of purified CitAP was dialyzed against 50 mM sodium phosphate buffer pH 7.0. Citrate was added before the measurements to a final concentration of 10 mM. All CD spectra were recorded with a JASCO J720 spectropolarimeter at 20 °C. For far-UV spectra (185–260 nm), a 0.1 cm cuvette was used, for near-UV-spectra (260–380 nm) a 0.5 cm cuvette. The protein concentration used for all measurements was 1 mg/mL, corresponding to a CitAP concentration of 65 μ M. For protein and buffer solutions, the spectra of three scans were averaged. Protein spectra were converted to molar ellipticity after subtraction of the corresponding buffer spectrum. Estimates of secondary structure were obtained by using software provided by JASCO.

Thermal Denaturation of CitAP. Thermal denaturation was monitored by CD spectroscopy at a wavelength of 218 nm. Samples were prepared as described for far-UV CD spectroscopy. The temperature was increased from 25 to 90 °C at a rate of 30 °C/h. The midpoint of the transition curve (T_m) was determined by a fit to a two-state unfolding model following a standard protocol (21). When the temperature was decreased from 90 to 25 °C, about 90% of the initial CD signal at 25 °C was recovered; therefore, the process can be considered reversible. A subsequent increase to 90 °C led to the same T_m value as in the first denaturation.

RESULTS

Construction, Purification, and Mass Spectrometry of Mutated CitAP Proteins. CitAP is a derivative of the sensor kinase CitA from K. pneumoniae comprising the periplasmic domain of this protein (amino acids 45-176) supplemented with an N-terminal methionine residue and a C-terminal hexahistidine tag (15). To exchange all arginine, lysine, and histidine residues (except that of the His tag) for alanine, plasmid pET-CitAP was subjected to site-directed mutagenesis as described in the Experimental Procedures. The 17 CitAP muteins were overproduced in E. coli BL21(DE3) and purified by Ni²⁺-NTA affinity chromatography as described for wild-type CitAP (15). None of the mutations led to a significant decrease in solubility, and all proteins showed the expected size of 15 kDa and were highly pure (>98%) when analyzed by SDS-polyacrylamide gel electrophoresis and staining with Coomassie (see Figure 5 in ref 15). On average, 25 mg of protein was obtained from 1 g of cells (wet weight), and the concentration after affinity chromatography ranged from 5 to 14 mg/mL (0.3-0.9 mM). Using peptide mass fingerprinting by MALDI-TOF mass spectrometry, several of the amino acid exchanges (R7A, H9A, K42A, H69A, K77A, K92A, and K109A) in the muteins were confirmed at the protein level (data not shown). The numbers used to designate specific mutations refers to the amino acid position in CitAP. To obtain the corresponding



FIGURE 2: Citrate binding to wild-type CitAP (Wt) and muteins R98A, R66A, H69A, R107A, and K109A measured by ITC at 25 °C. The upper panels show the heat changes observed upon injection of $3-10 \,\mu$ L of a trisodium citrate solution in 50 mM sodium phosphate buffer pH 7.0 into 1.40 mL of a protein solution in the same buffer. The lower panels show the integrated heat changes of each injection (normalized per mol citrate) plotted against the molar ratio of citrate to protein. The solid line represents a nonlinear least-squares fit according to a single-site binding model. The conditions for the individual experiments were as follows: Wt, 324 μ M protein, 6.48 mM trisodium citrate, injection volume 3 μ L except for the first injection (5 μ L); R98A, 222 μ M protein, 4.40 mM trisodium citrate, injection volume 5 μ L; R66A, 1200 μ M protein, 60.0 mM trisodium citrate, injection volume 5 μ L; H69A, 580 μ M protein, 33.0 mM trisodium citrate, injection volume 10 μ L; R107A, 78 μ M protein, 2.0 mM trisodium citrate, injection volume 5 μ L; K109A, 125 μ M protein, 2.5 mM trisodium citrate, injection volume 5 μ L.

position in the entire CitA protein, 42 has to be added to the numbers given here.

Isothermal Titration Calorimetry of the CitAP Muteins with Citrate as Ligand. The 17 CitAP muteins were analyzed for citrate binding by isothermal titration calorimetry, and the calorimetric data are summarized in Table 2. In Figure 2, the binding curves for wild-type CitAP and particularly interesting muteins are shown. According to their K_d values, the muteins can be divided into three groups. The first one includes 12 proteins, whose K_d ranged between 0.7- to 3.3-fold of the value of wild-type CitAP (i.e., R7A, H9A, R15A, K35A, R36A, R40A, K42A, R49A, K77A, K92A, K99A, and K118A). The weak effect of these mutations on citrate binding argues against a direct participation of the corresponding residues in the binding process. As in wildtype CitAP, citrate binding of all muteins listed above was driven by the enthalpy change; however, in the case of muteins R7A, H9A, and R15A, the binding enthalpy was significantly decreased as compared to wild-type CitAP. This decrease was compensated by a gain of entropy. Enthalpyentropy compensation is known to be a general feature of many chemical reactions and processes in biological systems (22). More recently, it has been shown that redistribution of side chain entropy is an important factor influencing the thermodynamics of ligand binding events (23). On the basis of these observations and the ITC data, a different dynamics of the muteins R7A, H9A, and R15A can be hypothesized.

The second group identified by ITC includes only a single mutein, CitAP-R98A, which showed a 6-fold decreased K_d value of 0.8 μ M (Table 2, Figure 2). This increased affinity appears to be caused by the fact that the entropy change is less unfavorable than in the case of the wild-type protein. It seems likely that the R98 residue is located close to the citrate

binding site and that the positively charged guanidinium group interferes with binding by influencing the protein movement, as indicated by the near-UV CD spectra described below. A remarkable feature of the R98A mutein was the low binding stoichiometry of ~ 0.3 , which cannot be explained yet. As in the case of wild-type CitAP (15), titration of isocitrate or tricarballylate to the R98 mutein did not cause enthalpy changes attributable to binding, showing that the ligand specificity was unchanged.

The third group includes the four muteins R66A, H69A, R107A, und K109A, whose apparent K_d values are drastically increased (i.e., at least more than 38-fold (Table 2, Figure 2)). For the R107A mutein a K_d of 205 μ M was determined, whereas the K_d of the other three muteins presumably was above 1 mM. For the latter proteins, a reliable quantitative evaluation of the ITC data was not possible. An entropically driven binding of citrate by the group III muteins cannot be completely excluded but seems very unlikely. Moreover, the CD spectra presented below argue against the possibility that the increased K_d values are due to misfolding. Therefore, the ITC data indicate that residues R66, H69, R107, and K109 are directly involved in citrate binding.

Secondary Structure Estimation. The secondary structure of wild-type CitAP and muteins R98A, R66A, H69A, R107A, and K109A was evaluated by far-UV CD spectroscopy. Typical spectra obtained for wild-type CitAP in free and complexed form are shown in Figure 3. The characteristic minima at 209 and 222 nm reflect the high content of α -helical structure of approximately 40%. Addition of excess citrate resulted in only minor differences of the spectra, which indicates the absence of significant folding processes induced by ligand binding. The spectra obtained for the mutant proteins in the absence and presence of citrate are similar to the spectra of wild-type CitAP, which excludes a significant



FIGURE 3: Far-UV CD spectrum of wild-type CitAP in the absence and presence of citrate. The measurement was performed with a 0.1 cm cuvette containing a 1 mg/mL protein solution in 50 mM sodium phosphate buffer pH 7.0.



FIGURE 4: Near-UV CD spectrum of wild-type CitAP in the absence and presence of citrate. The measurement was performed with a 0.5 cm cuvette containing a 1 mg/mL protein solution in 50 mM sodium phosphate buffer pH 7.0.

influence of the amino acid exchanges on the secondary structure of the protein.

Near-UV CD Spectroscopy. To investigate changes of tertiary protein structure upon ligand binding, CD spectra were recorded in the near-UV range in the absence and presence of citrate. In the case of wild-type CitAP, a significant change of the spectrum was observed upon addition of citrate (Figure 4). The formation of the two minima at 281 and 287 nm indicates a structural rearrangement induced by ligand binding. The role of residues R98, R66, H69, R107, and K109 on this process was evaluated by analyzing the corresponding muteins. The alteration of the R107A spectrum upon addition of citrate was comparable to that of wild-type CitAP; therefore, a significant role of R107 for the rearrangement can be excluded. The spectra obtained from muteins R98A, H69A, R66A, and K109A in free and complexed form showed high similarity to the spectrum of the complexed wild-type protein. In the case of the high-affinity mutein R98A, the apparent lack of rearrangement is in agreement with the fact that the entropy loss upon citrate binding is smaller than in the case of wild-type CitAP and supports the idea that the guanidinium group of R98 may necessitate the structural rearrangement in wildtype CitAP upon ligand binding. The similarity of the near-UV CD spectra of H69A, R66A, and K109A to that of complexed wild-type CitAP indicates that the corresponding residues are involved in the changes of the tertiary structure upon ligand binding and that the replacement by alanine possibly has fixed the unliganded muteins in a more rigid conformation that resembles the liganded state of wild-type CitAP. This fixation could explain the drastically decreased citrate affinity of these three muteins.



FIGURE 5: Thermal denaturation of wild-type CitAP and mutein R98A measured by CD spectroscopy at 218 nm. The measurement was performed with a 0.1 cm cuvette containing a 1 mg/mL protein solution in 50 mM sodium phosphate buffer pH 7.0. The temperature was increased from 25 to 90 $^{\circ}$ C at a rate of 30 $^{\circ}$ C/h.

Table 3:	Thermal	Stability	of	Wild-Type	CitAP	and	Selected
Muteins ^a							

protein	$T_{\rm m}$ (°C) without citrate	$T_{\rm m}$ (°C) with citrate	$T_{\rm m}$ (mutein) – $T_{\rm m}$ (WT)	$T_{\rm m}(+{\rm citrate}) - T_{\rm m}(-{\rm citrate})$
WT R98A R66A H69A R107A K109A	$62.7 \pm 0.7 65.8 \pm 0.3 69.5 \pm 0.1 67.1 \pm 0.1 68.5 \pm 0.6 66.1 \pm 0.3 $	$68.9 \pm 2.0 75.3 \pm 0.0 69.1 \pm 0.2 66.4 \pm 0.9 68.7 \pm 1.0 67.7 \pm 0.3$	n.a. 3.1 6.8 4.4 5.8 3.4	$ \begin{array}{r} 6.2 \\ 9.5 \\ -0.4 \\ -0.7 \\ 0.2 \\ 1.6 \\ \end{array} $

^{*a*} Thermal denaturation was monitored by CD spectroscopy at 218 nm using a 0.1 cm cuvette filled with dialyzed CitAP (1 mg/mL) in 50 mM sodium phosphate buffer pH 7.0. The temperature was increased from 25 to 90 °C at a rate of 30 °C/h. The midpoint of the transition curve ($T_{\rm m}$) was determined by a fit to a two-state unfolding model following a standard protocol (21). N.A., not applicable.

Thermal Stability of Wild-Type CitAP and Relevant Muteins. The stability of wild-type CitAP and muteins R98A, R66A, H69A, R107A, and K109A in the absence and presence of citrate was evaluated by thermal denaturation experiments using CD spectroscopy at 218 nm. As shown in Figure 5, the presence of citrate (10 mM) shifted the $T_{\rm m}$ value of wild-type CitAP from 62.7 to 68.9 °C, showing significant stabilization of the protein structure by the bound ligand. The increased stability was found to be independent from the increased ionic strength of the buffer containing citrate (data not shown). As summarized in Table 3, the melting point of all five muteins analyzed in the absence of citrate was increased between 3.1 and 6.8 °C as compared to wild-type CitAP, showing that the mutations have a stabilizing effect on the unliganded state of the protein. Citrate (10 mM) did not significantly change the $T_{\rm m}$ values of the muteins with disturbed binding (R66A, H69A, R107A, K109A). Under the experimental conditions used, the fraction of the muteins having citrate bound was calculated to be between 59 (R66A) and 98% (R107A) according to the K_d values given in Table 2. This indicates that a further stabilization by citrate is not possible in these mutants. By contrast, citrate increased the stability of the high-affinity receptor CitAP-R98A by 9.5 °C. Thus, high citrate binding affinity correlates with strong stabilization by citrate, and low binding affinity correlates with a lack of stabilization.

DISCUSSION

In this paper, we tested the influence of all arginine, lysine, and histidine residues in the periplasmic domain of the K.

species	SK	RR	regulated genes	presumed or verified ligand
Klebsiella pneumoniae	CitA	CitB	<i>citS</i> , oadGAB, citAB, citCDEFG (5)	citrate (15)
E. coli	CitA	CitB	citT, citCDEFXG (33), and others (34)	citrate, isocitrate (29)
E. coli	DcuS	DcuR	<i>dcuB</i> , <i>frdABCD</i> (35, 36)	C_4 dicarboxylic acids (35–37)
B. subtilis	CitS	CitT	<i>citM</i> , <i>yflN</i> (38)	Mg-citrate (38)
B. subtilis	DctS	DctR	dctP (28)	C ₄ -dicarboxylate binding protein (DctB) and/or C ₄ dicarboxylates (28)
^{<i>a</i>} The genes encoding c	arriers for citrate or	C ₄ -dicarboxylic acid	s are shown in boldface. SK, sensor ki	nase; RR, response regulator.

									-	•
Kpn-CitA	45	DITEERLHY	OVGORALIOAM	QISAMPELVEA	VQKRDL	AR 🛙 K.	ALIDPMRS	FSDATYITV	GDASGORL	YHVNPD
STM0053	40	DITKERLHY	VGORALIOAM	OISAMPELVEA	VEAHDL	SRIK	ALIDPMRS	FSDATYITV	GNEKGORL	YHVNPD
STY0062	40	DITKERLHY	VGORALIOAM	OISAMPELVEA	VEAHDL	SRIK	ALIDPMRS	FSDATYITV	GNEKGORL	YHVNPD
VC0791	44	OTLSETLOD	TSTKALTOAR	ETATOPNI TVI.	100-NR	LAEVO	AKTDRUOR	TSDANFTVT	GDANGTRT	AHPDEO
Eco-CitA	43	ASEEDVITL		TTASNDSVTSA	WKT-RD	VKR A	TTANKUOR	DTDEDVVT	GDRHSTRL	VHPNPP
STM0625	42			TTASNDSTTAA	WKN-RD	VKP - A	TTANKUOR	GTOFOVVVI	CORHSTRL	VHDNDE
STM0025	42	CACEEDVIACI		TTACNDOTTA			TTANK	GIDFDIVVI GTDFDVVVI	CORHSTRI	VHDNDIS
VC1605	33	MVVKUGOFOI	TYCOKAT CVAA	FUNCTEDAUTNM	TRUCDA		OCVERUTO	LICANETVI	CDNOCIPI	VUDTDIS
VC1005	25		NGERATEVAA		FCAPDD		DIARCUDY	RECARDING	CUTTLE TRY	AHDIDE
002251	30	CVER OF O		AVGETPERKER	FUORD		PIAESKV	D CNARF IVV	GNIDLIRI	
083251	50	SIERDQIRQI		AVSEIPEVORV	DOMOD		PFIER RK	QSNAEF IVI	GORNSIRI	
SC05829	57			TTAQQPQVVRD		TANGP Q	REAERVRE	AIRAEYVVV	MDRQGVRW	SHITDED
Eco-Deus	38	-LIYFSQISDMTRDO	JIANKALAVAR	THADSPEIRQG	LQKKPQ.	ESG-IQA	LAE-AVRK	RNDLLFIVV	TOMOSIERY	SHPEAQ
STM4304	38	-LIYFSQISSMTRD	ALADKALAVAR	SHADSPAVREG	KKPPA.	ESG-IQT	LAE-AVSQ	HNGFLFIVV	TNMQGIRY	SHPETQ
STY4502	38	-LIYFSQISSMTRD	ALADKALAVAR	SHADSPAIREG	KKPPA	ESG-IQP	LSE-AVSQ	HNGFLFIVV	TNMQGIRY	SHPETQ
Sfl-DcuS	38	-LIYFSQISDMTRDO	JI ANKALAVA R	THADSPEIRQG	QKKPQ.	ESG-IQA	IAE-AVRK	RNDLLFIVV	TIDMQSHRY	SHPEAQ
Bsu-YufL	32	AQTTKRIRD(JEKATAL QTAE	MVAEAPMTAAA	ESGKK	QKE-LQS	YTK-RVQK	ITGTEFVVV	MDMNGTRK	THEOPS
BH0397	28	DLLVSVATSERLQSI	NIEEKAIAISR	TVAKAQWVIDG	LENEEE	EWR-VQT	YTM-EIQS	ATDVLFIVV	MDMEGIRK	SHPNPE
OB3220	28	DLLINYATGERIKE	NIEEKAVIISR	TMAKSEWVING	LQNKDE	EKY-IQE	YTN-EISR	YIDLIFIVV	MDMDGIRK	SHPNPE
CPE0531	30	EDKLSY	DVRNTLKETA F	SISEIPFIQED	SNGEI	NSR-IQE	YTKHFIEA	INDVDIIVV	ADMRGVKY	SHILDEK
Bsu-DctS	34	NIQHTEER	ELKKRLMNTAR	TVSEMTEVKEA	LARKKQ	TEAVR	HAVEEIRM	INEADYIVV	MOMNHURY	THPVST
BH2752	34	GYVTSIKED	ELSNRTMITAQ	LVAQNHTVQQW	VDAKPE:	EASRTIQ	PIVERIRV	INDHDYIVL	LNMDRIRI	THPIPE
SC05435	38	DRGQAEE	AAGRQARAVSL	AIADSPSVAEA	IRTPDP	FAL∥Q	PYAVRVMR	DIDVDFVII	MNPEGIRW	THPEPT
SPY1107	32	HDTHQSIKN(DETHLLTSTGK	MLASHQAIIKEL	LINNQPI	NAK-TTA	YTN-SIAS	IYNLDYVVV	MNMKGIRL	THENEK
SAG1921	28	-YVTIHQSYRMVRV	DEEKILKNTGY	ALSRNPQVIQT	LKDNHY	DQS-LQK	QML-FUSK	KSNLDYIVL	INLKGIRF	THEDST
Bsu-CitS	35	VQHTQGERR	DAEQL <u>AVQTAR</u>	TISYMPPVKEL	ERKDG:	HAA-QTQ	EVIEQ₫KE	QuGAFAUYV	LNEKCD	ISASG
					-	_	-			
V Cith	117									
Kpn-CitA	117		- LINAKSYVSVR	KGSLGSSLRGK	SPIQDA'	IGKV	IGIVSVGY	TIEQLENWL	s	-
Kpn-CitA STM0053	117 112	EIGKSMEGGDSDEAI	- INAKSYVSVR INAKSYVSVR	KGSLG <mark>S</mark> SLRGK KGSLG <mark>S</mark> SLRGK	SPIQDA SPIQDS	IGKV IGKV	IGIVSVGY IGIVSVGY	TIEQLENWI. TLEQLESWI.	s	-
Kpn-CitA STM0053 STY0062	117 112 112	EIGKSMEGGDSDEAI EIGKYMEGGDSDDAI EIGKYMEGGDSDDAI	□ INAKSYVSVR YNAKSYVSVR YNAKSYVSVR	KGSLGSSLRGK KGSLGSSLRGK KGSLGSSLRGK	SPIQDA' SPIQDS' SPIQDS'		IGIVSVGY IGIVSVGY IGIVSVGY	TIEQLENWL TLEQLESWL TLEQLESWL	s N N	-
Kpn-CitA STM0053 STY0062 VC0791	117 112 112 116	ETGKSMEGGDSDEAT ETGKYMEGGDSDDAT ETGKYMEGGDSDDAT KTGLPMQGGDSRRAT	UINAKSYVSVR JYNAKSYVSVR JYNAKSYVSVR JKEGEYYTSTQ	KGSLGSSLRGK KGSLGSSLRGK KGSLGSSLRGK KGSLGWAIRGK	SPIQDA' SPIQDS' SPIQDS' AAIVAP:		IGIVSVGY IGIVSVGY IGIVSVGY LGVVSVGY	TIEQLENWL TIEQLESWL TIEQLESWL LIDNISSWL	S N N RVYSY	-
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA	117 112 112 116 115	ETGKSMEGGDSDEAI ETGKYMEGGDSDDAI ETGKYMEGGDSDDAI KTGLPMQGGDSRRAI KTGLPMQFTKQG-AI	UNAKSYVSVR VNAKSYVSVR VNAKSYVSVR KEGEYYTSTQ EKGESYFITG	KGSLGSSLRGK KGSLGSSLRGK KGSLGSSLRGK KGSLGWAIRGK KGSNGMAMRAK	SPIQDA' SPIQDS' SPIQDS' AAIVAP: TPIFDD!		IGIVSVGY IGIVSVGY IGIVSVGY LGVVSVGY IGVVSIGY	TIEQIENWI TIEQIESWI TIEQIESWI LIDNISSWI LVSKIDSWR	S N N RVYSY AEFLLP	-
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625	117 112 112 116 115 115	EIGKSMEGGDSDEAI EIGKYMEGGDSDDAI EIGKYMEGGDSDDAI KIGLPMQGGDSRRAI KIGYPMQFTKQG-AI KIGYPMQFTKPG-AI	INAKSYVSVR YNAKSYVSVR YNAKSYVSVR KECEYYTSTO EKCESYFITG	KGSLGSSLRGK KGSLGSSLRGK KGSLGSSLRGK KGSLGWAIRGK KGSMGMAMRAK KGSIGMAMRAK	SPIQDA' SPIQDS' SPIQDS' AAIVAP TPIFDDJ TPIFDDJ		IGIVSVGY IGIVSVGY IGIVSVGY LGVVSVGY IGVVSIGY IGVVSIGY	TIEQIENWI TIEQIESWI TIEQIESWI LIDNISSWI LVSKIDSWR LVSKIDSWR	S N RVYSY AEFLLP LDFLLP	-
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674	117 112 112 116 115 115	EIGKSMEGGDSDEA EIGKYMEGGDSDDA EIGKYMEGGDSDDA KIGLPMQGGDSRA KIGYPMQFTKQG-A KIGYPMQFTKPG-A KIGYPMQFTKPG-A	INAKSYVSVR YNAKSYVSVR YNAKSYVSVR IKECEYYTSTQ IEKCESYFITG IERCESYFITG	KGSLGSSLRGK KGSLGSSLRGK KGSLGMAIRGK KGSLGMAIRGK KGSNGMAMRAK KGSIGMAMRAK KGSIGMAMRAK	SPIQDA' SPIQDS' SPIQDS' AAIVAP TPIFDDI TPIFDNI TPIFDNI		IGIVSVGY IGIVSVGY IGIVSVGY LGVVSVGY IGVVSIGY IGVVSIGY IGVVSIGY	TIEQLENWI TIEQLESWI TIEQLESWI LIDNISSWI LVSKIDSWR LVSKIDSWR LVSKIDSWR	S N RVYSY AEFLLP LDFLLP LDFLLP	-
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605	117 112 112 116 115 115 115	EIGKSMEGGDSDEA EIGKYMEGGDSDDA EIGKYMEGGDSDDA KIGLPMQGGDSRAI KIGYPMQFTKQG-A KIGYPMQFTKPG-AI KIGYPMQFTKPG-AI RIGKPMVGGDNERA	LINAKSYVSVR VNAKSYVSVR VNAKSYVSVR LKEGEYYTSTQ JERGESYFITG JERGESYFITG LERGESYFITG LERGESYFITG	GSLCSSLRGK KGSLCSSLRGK KGSLGSSLRGK KGSLGWAIRGK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK SGSLGKSVRGK	SPIQDA' SPIQDS' SPIQDS' TPIFDDI TPIFDNI TPIFDNI AAVVDQI		IGIVSVGY IGIVSVGY IGIVSVGY LGVVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSVGY	TIEQIENWI TIEQIESWI LIDNISSWI LVSKIDSWR LVSKIDSWR LVSKIDSWR LVSKIDSWR LIERIQDRV	S N RVYSY AEFLLP LDFLLP LDFLLP E	-
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839	117 112 112 116 115 115 106 108	EIGKSMEGGDSDEA EIGKYMEGGDSDDA EIGKYMEGGDSDDA KIGLFMQGGDSRAA KIGYPMQFTKQG-A KIGYPMQFTKPG-A RIGYPMQFTKPG-A RIGKPMVGGDNERA RIGCRMVGGDNERA	LINAKSYVSVR VNAKSYVSVR VNAKSYVSVR LKEGEYYTSTQ LEKGESYFITG LEKGESYFITG LEKGESYFITG LVEGEAYVSFA VHGESYVSKA	GSLGSSLRGK KGSLGSSLRGK KGSLGSSLRGK KGSLGWAIRGK KGSMGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK VGSLGPSIRGK	SPIQDA' SPIQDS' SPIQDS' TPIFDDI TPIFDNI TPIFDNI AAVVDQI VPVFDDI		IGIVSVGY IGIVSVGY IGIVSVGY LGVVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSVGY IGIVSVGP	TIEQIESWI TIEQIESWI LUDNISSWI LVSKIDSWR LVSKIDSWR LVSKIDSWR LIERIQDRV LIERIQDRV LMEDIQQVI	S N RVYSY LDFLLP LDFLLP E GERLI	-
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839 OB3251	117 112 112 116 115 115 106 108 103	EIGKSMEGGDSDEAT EIGKYMEGGDSDDAT EIGKYMEGGDSDDAT KIGLPMQGGDSRAT KIGYPMQFTKQG-AT KIGYPMQFTKPG-AT KIGYPMQFTKPG-AT RIGKPMVGGDNERAT RIGQRMVGGDNERAT KVGMQMVGGDNEQAT	LINAKSYVSVR YNAKSYVSVR YNAKSYVSVR KEGEYYTSTQ LEKGESYFITG LERGESYFITG VEGEAYVSFA VHGESYVSFA VHGESYVSKA VHGESYVSKA	KGSLGSSLRGK KGSLGSSLRGK KGSLGWAIRGK KGSLGWAIRGK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK VGSLGKSVRGK VGSLGPSIRGK	SPIQDA' SPIQDS' SPIQDS' AAIVAPS TPIFDDI TPIFDNI TPIFDNI AAVVDQI VPVFDDI SPIFNS		IGIVSVGY IGIVSVGY IGIVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSVGY IGIVSVGY	TIEQIENWI TIEQIESWI LIDNISSWI LVSKIDSWR LVSKIDSWR LVSKIDSWR LVSKIDSWR LIERIQDRV LIERIQDRV LMEDIQQVI MISYVDSLF	S N RVYSY AEFLLP LDFLLP LDFLLP E GERLI KQ	-
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839 OB3251 SC05829	117 112 112 116 115 115 106 108 103 128	EIGKSMEGGDSDEAI EIGKYMEGGDSDDAI EIGKYMEGGDSDDAI KIGLPMQGGDSRAI KIGYPMQFTKQG-AI KIGYPMQFTKPG-AI RIGKPMVGGDNERAI RIGQRMVGGDNERAI KVGMQMVGGDNEQAI RIGEVVST-DPGQAI	INAKSYVSVR YNAKSYVSVR YNAKSYVSVR YKEGEYYTSTQ JEKGESYFITG JERGESYFITG VEGEAYVSFA VHGESYVSKA VHGESYVSKA VHGENYVSIA JA-GREVMEID	KGSLGSSLRGK KGSLGSSLRGK KGSLGSSLRGK KGSLGWAIRGK KGSLGWAIRGK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSLGASVRGK NGSLGASVRGK DGTLGRSARGK	SPIQDA SPIQDS' SPIQDS' AAIVAP TPIFDDI TPIFDNI TPIFDNI AAVVDQI VPVFDDI SPIFNSI VPLRDGI		IGIVSVGY IGIVSVGY IGIVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSVGF IGIVSVGF IGIVSVGF IGIVSVGF	TIEQIENWI THEQIESWI LUDNISSWI LVSKIDSWR LVSKIDSWR LIERIQDRU LIERIQDRU LMEDIQQVI MISYVDSLF AYDSVRARI	S N RVYSY AEFLLP LDFLLP LDFLLP GERLI GERLI KQ IHAIP	-
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839 OB3251 SC05829 Eco-Dcus	117 112 116 115 115 106 108 103 128 115	EIGKSMEGGDSDEAI EIGKYMEGGDSDDAI EIGKYMEGGDSDDAI KIGLPMQGGDSRAI KIGYPMQFTKQG-AI KIGYPMQFTKPG-AI KIGYPMQFTKPG-AI RIGKPMVGGDNERAI RIGORMVGGDNERAI RIGEVVST-DPGQAI RIGOPFKGDDILKAI	INAKSYVSVR YNAKSYVSVR YNAKSYVSVR YEEEYYTSTQ IEEESYTSTQ IEEESYFITG IEEESYFITG VEEEAYVSFA VHEESYVSFA VHCENYVSIA IA-GREVMEID IN-GEENVAIN	GSLGSSLRGK KGSLGSSLRGK KGSLGSSLRGK KGSLGWAIRGK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSIGASVRGK NGSLGASVRGK DGILGRSARGK	SPIQDA' SPIQDS' SPIQDS' AAIVAP: TPIFDDI TPIFDDI AAVVDQ VPVFDDI SPIFNSI VPLRDGI TPIYDE		IGIVSVGY IGIVSVGY IGIVSVGY IGVVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSVGF IGIVSVGF IGIVSVGY IGVVSUGI IGVVAIGL	TIEQIESWI TIEQIESWI LUDNISSWI LVSKIDSWR LVSKIDSWR LVSKIDSWR LIERIQDRU LMEDIQQVI MISYDSLF AYDSVRARI ESSVTQQI	S N REFLLP LDFLLP LDFLLP GERLI GERLI IHAIP NDSRW	-
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839 OB3251 SC05829 Eco-DcuS STM4304	117 112 116 115 115 106 108 103 128 115 115	EIGKSMEGGDSDEA EIGKYMEGGDSDDA EIGKYMEGGDSDDA KIGLPMQGGDSRA KIGYPMQFTKQG-A KIGYPMQFTKPG-A KIGYPMQFTKPG-A RIGYPMVGGDNERA RIGQRWVGGDNERA RIGEVVST-DPGQA RIGEPFKGDDILLA	INAKSYVSVR YNAKSYVSVR YNAKSYVSVR KECEYYTSTQ EKCESYFITG LERCESYFITG VECEAYVSFA VHCESYVSFA VDCENYVSIA JA-CREVMEID N-CEENVAIN Q-CKENVAIN	GSLGSSLRGK KGSLGSSLRGK KGSLGSSLRGK KGSLGWAIRGK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK GGSLGSVRGK GGSLGASVRGK GGFLAQALRVF RGFLAQALRVF	SPIQDA' SPIQDS' AAIVAP' TPIFDDI TPIFDNI TPIFDNI TPIFDNI AAVDQI VPVFDDI SPIFNSI VPIRDGI TPIYDEI	ПС КV ПС КV ГС КV ГС КV СС КV СС КV СС КV КС К СС СП ПС СП ПП КQ НН КQ	IGIVSVGY IGIVSVGY IGIVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSVGI IGIVSVGY VGAVSVGI IGVVAIGL IGVVAIGL	TIEQIESWI TUEQIESWI LUDNISSWI LVSKIDSWR LVSKIDSWR LVSKIDSWR LIERIQDVI MEDIQQVI MISYVDSLF AYDSVRARI EISRVTQQI	S N RVYSY AEFLLP LDFLLP E GERLI KQ IHAIP NDSRW NNSR	-
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839 OB3251 SC05829 Eco-DcuS STM4304 STY4502	117 112 112 116 115 115 106 108 103 128 115 115	EIGKSMEGGDSDEA EIGKYMEGGDSDDA EIGKYMEGGDSDDA KIGLPMQGGDSRAA KIGLPMQFTKQG-A KIGYPMQFTKPG-A KIGYPMQFTKPG-A RIGYPMVGGDNERA RIGQRMVGGDNEQA KVGMQMVGGDNEQA RIGQPFKGDDILLA RIGQPFKGDDILLA	INAKSYVSVR YNAKSYVSVR YNAKSYVSVR KECESYFITG ERCESYFITG UECESYFITG VECEAYVSFA VDCENYVSFA VDCENYVSFA A-CREVMEID A-CREVMEID A-CREVMIN Q-CKENVAIN	GSLCSSIRCK KGSLCSSIRCK KGSLCSSIRCK KGSLCWAIRCK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK QGSLCKSVRCK VGSLCPSIRCK NGSLCASVRCK DGTLCRSARCK RGFLACALRVF RGFLAKALRVF	SPIQDS SPIQDS AAIVAP TPIFDD TPIFDD TPIFDN TPIFDN SPIFNS VPVFDD TPIYDG TPIYDG TPIYDE		IGIVSVGY IGIVSVGY IGVVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSVGY IGIVSVGF IGIVSVGI IGVVAIGL IGVVAIGL	TIEQIESWI TIEQIESWI LIDNISSWI LVSKIDSWR LVSKIDSWR LVSKIDSWR LIERIQDVI MEDIQVI MESYVDSLF AYDSVRARI EISSVTQQI EISHVTQQI	S N AEFLLP LDFLLP E GERLI KQ NDSRW NNSR	-
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839 OB3251 SC05829 Eco-DcuS STM4304 STY4502 Sfl-DcuS	117 112 112 116 115 115 106 108 103 128 115 115 115	EIGKSMEGGDSDEA EIGKYMEGGDSDDA EIGKYMEGGDSDDA KIGLPMQGGDSRAI KIGYPMQFTKQG-A KIGYPMQFTKPG-A KIGYPMQFTKPG-A RIGKPMVGGDNERAI RIGQRWVGGDNERAI RIGQPFKGDDILKAI RIGQPFKGDDILLAI RIGQPFKGDDILLAI	INAKSYVSVR YNAKSYVSVR YNAKSYVSVR KEGEYTSTQ IERGESYFITG UEGESYFITG UEGEAYVSFA VHGESYVSKA VHGESYVSKA INAGENYVSIA IA-GREVMEID IA-GEENVAIN IQ-GKENVAIN IN-GEENVAIN	KGSLGSSLRGK KGSLGSSLRGK KGSLGSSLRGK KGSLGWAIRGK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSLGSVRGK NGSLGASVRGK NGSLGASVRGK RGFLAQALRVF RGFLAQALRVF RGFLAQALRVF	SPIODA SPIODS SPIODS AAIVAPJ TPIFDN TPIFDN TPIFDN SPIFNS VPVFDD SPIFNS TPIYDE TPVYDE TPVYDE TPIYDE		IGIVSVGY IGIVSVGY IGIVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSVGY IGIVSVGF IGIVSVGY VGAVSVGI IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL	TIEQIENWI TIEQIESWI LUDNISSWI LVSKIDSWR LVSKIDSWR LVSKIDSWR LVSKIDSWR LVSKIDSWR LVSKIDSWR LVSKIDSWR LIERIQDRU MISYVDSLF AYDSVRARI EUSRVTQQI EUSRVTQQI	S N RVYSY LDFLLP LDFLLP GERLI GERLI HAIP IHAIP NDSRW NNSR NNSR	-
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839 OB3251 SC05829 Eco-DcuS STM4304 STY4502 Sfl-DcuS Bsu-YufL	117 112 112 116 115 115 106 108 103 128 115 115 115 115	EIGKSMEGGDSDEAI EIGKYMEGGDSDDAI EIGKYMEGGDSDDAI KIGLPMQGGDSRAI KIGYPMQFTKQG-AI KIGYPMQFTKPG-AI RIGKFMVGGDNERAI KIGQFMVGGDNERAI RIGQPFKGDDILKAI RIGQPFKGDDILLAI RIGQPFKGDDILLAI RIGQPFKGDDILLAI RIGQPFKGDDILLAI	INAKSYVSVR YNAKSYVSVR YNAKSYVSVR KEGEYYTSTQ JEKGESYFITG JERGESYFITG JERGESYFITG VHCESYVSKA VHCESYVSKA VHCESYVSKA UNGENYVSIA A-GREVMEID IN-GEENVAIN Q-GKENVAIN Q-GKENVAIN N-GEENVAIN K-GHVHISTA	KGSLGSSLRGK KGSLGSSLRGK KGSLGWAIRGK KGSIGWAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSIGASVRGK VGSLGPSIRGK NGSLGASVRGK DGTLGRSARGK RGFLACALRVF RGFLACALRVF SGTLGKSQRAF	SPIQDA SPIQDS SPIQDS AAIVAP TPIFDDI TPIFDN AAVVDQ VPVFDDS SPIFNS VPVFDG TPVYDE TPVYDE TPVYDE TPVYDE TPVYDE TPVYAE	ПС К V ГС	IGIVSVGY IGIVSVGY IGVVSVGY IGVVSVGY IGVVSIGY IGVVSIGY IGVVSVGF IGIVSVGF IGIVSVGF IGIVSVGF IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL	TIEQIENWI TIEQIESWI TIEQIESWI LUDNISSWI LVSKIDSWR LVSKIDSWR LIERIQDRU MISYUDSLF AYDSVRARI EISKVTQQT EISKVTQQT EISKVTQQT EISKVTQQT TVNEIDEVT	S N RVYSY AEFLLP LDFLLP E KQ IHAIP NDSRW NNSR NNSR SHSLR	
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839 OB3251 SC05829 Eco-DcuS STM4304 STY4502 Sfl-DcuS Bsu-YufL BH0397	117 112 112 115 115 106 108 103 128 115 115 115 115 105 106	G FIGKSMEGGDSDDAI EIGKYMEGGDSDDAI EIGKYMEGGDSDDAI KIGLPMQGDSRAI KIGYPMQFTKQG-AI KIGYPMQFTKPG-AI KIGYPMQFTKPG-AI RIGKPMVGGDNERAI KVGMQMVGGDNERAI RIGOPFKGDDILLAI RIGOPFKGDDILLAI RIGOPFKGDDILLAI RIGOPFKGDDILLAI RIGOPFKGDDILLAI RIGOPFKGDDILLAI RIGOPFKGDDILLAI RIGVFKGDDILLAI	INAKSYVSVR YNAKSYVSVR YNAKSYVSVR YEEEYYTSTQ IEEESYTSTQ IEEESYFITG IEEESYFITG VEEEAYVSKA VHEESYVSKA VHEESYVSKA INGENIVSIA A-GREVMEID IN-GEENVAIN Q-GKENVAIN IN-GEENVAIN IN-GEENVAIN IN-GEENVAIN IN-GEENVAIN IN-GEENVAIN	KGSLGSSLRGK KGSLGSSLRGK KGSLGWAIRGK KGSLGWAIRGK KGSLGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSLGSSRGK NGSLGASVRGK DGTLGRSARGK RGFLACALRVF RGFLAKALRVF RGFLAKALRVF SGTLGKSQRAF TGTLGSSLRAF	SPIQDA SPIQDS SPIQDS AAIVAP TPIFDDI TPIFDDI TPIFDNI SPIFNSI VPVFDDI SPIFNSI VPVFDGI TPIVDE TPVVDE TPVVDE TPIVDE TPIVDE TPIYDE	□G K V IG K V SG E I DG K V EG K V EG N V EG N V HG E I NH KQ HH KQ NG	IGIVSVGY IGIVSVGY IGVVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSVGF IGIVSVGF IGVVSVGI IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL UGVAVGI	TIEQIENWI TIEQIESWI TIEQIESWI LUSKIDSWR LVSKIDSWR LVSKIDSWR LIERIQDRU MISYUDSLF AYDSVRARI EISRUTQQI EISRUTQQI EISRUTQQI EISRUTQQI EISRUTQQI EISRUTQQI	S N RVYSY AEFLLP LDFLLP GERLI KQ IHAIP NNSR NNSR NNSR NNSR ADNHTSIL	- - - - - - - - - - - - - - - - - - -
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839 OB3251 SC05829 Eco-DcuS STM4304 STY4502 Sfl-DcuS Bsu-YufL BH0397 OB3220	117 112 112 115 115 106 108 103 128 115 115 115 115 105 106 106	EIGKSMEGGDSDEA EIGKYMEGGDSDDA EIGKYMEGGDSDDA KIGLPMQGGDSRA KIGYPMQFTKQG-A KIGYPMQFTKPG-A KIGYPMQFTKPG-A RIGKPMVGGDNERA KVGMQMVGGDNERA RIGORWGGDNERA RIGOFFKGDDILLA RIGOFFKGDDILLA RIGOFFKGDDILLA RIGOFFKGDDILLA RIGOFFKGDDILLA RIGOFFKGDDILLA RIGOFFKGDDILLA RIGOFFKGDDILLA RIGOFFKGDDILLA RIGOFFKGDDILLA RIGOFFKGDDILLA RIGOFFKGDDILLA	INAKSYVSVR YNAKSYVSVR YNAKSYVSVR YEEEYYTSTQ IEEESYFITG IEREESYFITG	KGSLGSSIRGK KGSLGSSLRGK KGSLGSSLRGK KGSLGWAIRGK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSIGSIRGK NGSLGASVRGK DGTLGRSARGK RGFLACALRVF RGFLACALRVF RGFLACALRVF RGFLACALRVF RGFLACALRVF RGFLACALRVF RGFLACALRVF RGFLACALRVF	SPIODA SPIODS AAIVAP TPIFDDI TPIFDDI TPIFDNI TPIFDNI VPVFDDI SPIFNS VPLRDGI TPIVDEI TPVYDEI TPIYDEI TPIYDEI TPIFNDI SPIFNE		IGIVSVGY IGIVSVGY IGVVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSVGY VGAVSVGI IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL IGVVAVGI IGAVSVGI	TIEQIENWI TIEQIESWI LIDNISSWI LVSKIDSWR LVSKIDSWR LVSKIDSWR LIERIQRVI MEDIQQVI MISY(DSLF AYDSVRARI EUSRVTQQI EUSHVTQQI EUSHVTQQI EUSRVTQQI EUSRVTQQI EUSRVTQQI EUSRVTQQI EUSRVTQQI SUQEIDDVI	S N AEFLLP LDFLLP GERLI GERLI IHAIP NNSR NNSR NNSR SHSLR SHSLR ADNHTSIL	- - - - - - - - - - - - - - - - - - -
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839 OB3251 SC05829 Eco-DcuS STM4304 STY4502 Sf1-DcuS Bsu-YufL BH0397 OB3220 CPE0531	117 112 116 115 115 106 108 103 128 115 115 115 106 106 106	EIGKSMEGGDSDEA EIGKYMEGGDSDDA EIGKYMEGGDSDDA KIGYPMQGTSQGDSRA KIGYPMQFTKQG-A KIGYPMQFTKPG-A KIGYPMQFTKPG-A RIGYPMVGGDNERA RIGQRWVGGDNERA RIGQPFKGDDILA RIGQPFKGDDILA RIGQPFKGDDILA RIGQPFKGDDILA RIGQPFKGDDILA RIGQFFKGDDILA RIGQFFKGDDILA RIGQFFKGDDILA RIGQFFKGDDILA RIGQFFKGDDILA RIGQFFKGDDILA RIGQFFKGDDILA	INAKSYVSVR YNAKSYVSVR YNAKSYVSVR KECEYYTSTQ EKCESYFITG LERCESYFITG LERCESYFITG LERCESYFITG AVECENYVSFA VHCENYVSFA LACEVMEID N-CEENVAIN Q-CKENVAIN Q-CKENVAIN Q-CKENVAIN K-CHVHISTA LECCEHTSIS TQCSSYYSLM	GSLGSSIRGK KGSLGSSIRGK KGSLGSSIRGK KGSLGWAIRGK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK GGSLGKSVRGK GGSLGSVRGK GGFLAQAIRVF RGFLAQAIRVF RGFLAQAIRVF RGFLAQAIRVF SGTLGKSQRAF GGTLAQAIRVF EGTLGDSIRAF EGTLGDSIRAF	SPIODS SPIODS SPIODS AAIVAP TPIFDDI TPIFDNI TPIFDNI VPVFDDI SPIFNS VPVFDDI TPVYDE TPVYDE TPVYDE TPIYDE SPIFNE SPIFNE QPVMYN		IGIVSVGY IGIVSVGY IGVVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSVGY IGIVSVGY VGAVSVGI IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL IGAVSVGI VGAVSVGI VGFIMVGK	TIEQIENWI TUEQIESWI LUDNISSWI LUSKIDSWR LVSKIDSWR LVSKIDSWR LIERUQRV MISYUDSLF AYDSVRARI EUSRUTQQI EUSRUTQQI EUSRUTQQI EUSRUTQQI EUSRUTQQI SIQEINDVI SIQEIDVI YYNEIQLLT	S N AEFLLP LDFLLP E GERLI KQ NDSRW NNSR NNSR NNSR SHSLR SHSLR SHSLR SHSLR HKTLIK	- - - - - - - - - - - - - - - - - - -
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839 OB3251 SC05829 Eco-DcuS STM4304 STY4502 Sf1-DcuS Bsu-YufL BH0397 OB3220 CPE0531 Bsu-DctS	1177 1122 1121 115 1155 1066 1088 103 1155 1155 1055 1055 1066 1064 1064	EIGKSMEGGDSDEA EIGKYMEGGDSDDA EIGKYMEGGDSDDA KIGLPMQGGDSRAA KIGLPMQFTKQG-A KIGYPMQFTKPG-A KIGYPMQFTKPG-A KIGYPMQGDNERAA RIGQRMVGGDNERAA KVGMQMVGGDNERAA RIGEVVST-DPGQA RIGEVVST-DPGQA RIGEPFKGDDILLAA RIGOFFKGDDILLAA RIGOFFKGDDILLAA RIGOFFKGDDILLAA RIGOFFKGDDILLAA RIGOFFKGDDILLAA LIGKFFVGGDESAA LIGKFFVGGDESAA LIGKFFVGGDEDTV QIGQVFVNEDKKEV	INKSYVSVR YNAKSYVSVR YNAKSYVSVR KECEYYTSTQ EKCESYFITG ERCESYFITG UECEAYVSFA VHCEYVSFA VDCENYVSFA A-CREVMEID N-CEENVAIN Q-CKENVAIN Q-CKENVAIN K-CHVHISTA E-CREHVSIS EQCIEHISIS TQCSSYYSLM FA-EHIYFSEA	CSLCSSIRCK KGSLCSSIRCK KGSLCSSIRCK KGSLCMARAK KGSICMAMRAK KGSICMAMRAK KGSICMAMRAK CSICCSVRCK VGSLCSVRCK VGSLGSVRCK JGTLCSSRCK RGFLACALRVF RGFLACALRVF RGFLACALRVF RGFLACALRVF FGTLGSIRAF EGTLCDSLRAF KGSMCETLRWF	SPIODA SPIODS SPIODS TPIFDD TPIFDD TPIFDN TPIFDN SPIFNS TPIYDE TPYYDE TPYYDE TPYYDE TPIYDE SPIFNE SPIFNE QPVMYN YPVKDQ		IGIVSVGY IGIVSVGY IGVVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSVGI IGVVSVGI IGVVSVGI IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL IGAVSVGI VGFIMVGK IGVVLVGK	TIEQIENWI TIEQIESWI LIDNISSUL LIDNISSUL LISKIDSWR LISKIDSWR LIEKIQDVU MISYUDSLF AYDSVRARI EISRUTQQI EISHUTQQI EISHUTQQI EISHUTQQI SUQEUNDVI SUQEUDVI YYNEIQLLT TIPGIADII	S N AEFLLP LDFLLP E GERLI KQ NDSRW NNSR NNSR NNSR SHSLR ADNHTSIL ADNHTSIL NNNHI HKTLIK LHLKRD	- - - - - - - - - - - - - - - - - - -
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839 OB3251 SC05829 Eco-DcuS STM4304 STY4502 Sf1-DcuS Bsu-YufL BH0397 OB3220 CPE0531 Bsu-DctS BH2752	1117 112 112 115 115 115 108 103 128 103 128 115 115 115 105 106 106 106 106 109	EIGKSMEGGDSDEAI EIGKSMEGGDSDDAI EIGKYMEGGDSDDAI KIGLPMQGGDSRAAI KIGYPMQFTKQG-AI KIGYPMQFTKPG-AI RIGKPMVGGDNERAI KVGMQMVGGDNERAI KVGMQMVGGDNERAI RIGOPFKGDDILKAI RIGOPFKGDDILLAI RIGOPFKGDDLLAI RIGOPFKGDDILLAI	INAKSYVSVR INAKSYVSVR INAKSYVSVR IKEGEYYTSTQ JEKGESYFITG IERGESYFITG IERGESYFITG IERGESYVSKA IVEGESYVSKA IVEGESYVSKA IVEGESYVSKA IVEGESYVSKA IVEGESYVSKA IVEGESYVSIA IN-GEENVAIN IQ-GKENVAIN IQ-GKENVAIN IQ-GKENVAIN IA-GHVHISTA IE-GREHVSIS ITQGSSYSIM FA-EHIYFSEA FA-EHIYTSKA	KGSLGSSLRGK KGSLGSSLRGK KGSLGWAIRGK KGSIGWAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK VGSLGPSIRGK VGSLGPSIRGK VGSLGPSIRGK VGSLGPSIRGK VGSLGASVRGK VGSLGASVRGK VGSLGASVRGK VGSLGASVRGK VGSLGASVRGK VGSLGASVRGK VGSLGASVRGK KGSLGASLRAF GGTLGGSLRAF GGTLGGSLRAF KGSMGTLRWF KGGTLGTAVRAF	SPIQDA SPIQDS SPIQDS TPIFDDI TPIFDDI TPIFDNI AAVVDQI VPVFDDI SPIFNS VPIRDG TPIYDEI TPVYDEI TPYYDEI TPYYDEI TPIYDEI SPIFNDI SPIFNDI SPIFNDI SPIFNEI QPVMYLL YPVKQQ MPILNQ	ПС К V ГС К V ГС К V SC Е I DC К V EC Г V V CC Г I DC Е I DC Е I DC Е I DC К Q HH К Q HH К Q HH К Q HH К Q HC Е Q C E	IGIVSVGY IGIVSVGY IGVVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSVGY IGVVSVGY IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL IGAVSVGI IGAVSVGI IGAVSVGI IGVVLVGK VGVAVQGS	TIEQIENWI TIEQIESWI LUDNISSWI LVSKIDSWR LVSKIDSWR LVSKIDSWR LVSKIDSWR LVSKIDSWR LVSKIDSWR MISYVDSLF AYDSVRARI EISRVTQQI EISRVTQQI EISRVTQQI TVNEIDEVI SIQEVDVI SIQEVDVI SIQEIDDVI TVNEIQLIT TVNEIQLIT	S N RVYSY AEFLLP LDFLLP GERLI GERLI NDSRW NNSR NNSR SHSLR SHSLR SHSLR ADNHTSIL NNNHI LHLKRD QEFWQ	- - - - - - - - - - - - - - - - - - -
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839 OB3251 SC05829 Eco-DcuS STM4304 STY4502 Sfl-DcuS Bsu-YufL BH0397 OB3220 CPE0531 Bsu-DctS BH2752 SC05435	1177 1122 116 1155 1155 1056 108 103 128 1155 1055 1056 1066 1066 1066 1099 1099	EIGKSMEGGDSDEAI EIGKSMEGGDSDDAI EIGKYMEGGDSDDAI KIGLPMQGGDSRAI KIGYPMQFTKQG-AI KIGYPMQFTKPG-AI RIGKPMVGGDNERAI KUGMQWVGGDNERAI KUGMQWVGGDNERAI RIGQPFKGDDILKAI RIGQPFKGDDILLAI RIGQPFKGDDILLAI RIGQPFKGDDILLAI KIGKFRGGDESEV EIGKRFVGGDESEV EIGKRFVGGDEDTV QIGQVFVNGGDEDTV QIGQVFVGGDEDAI SIGKKSEGADEEAAI	INAKSYVSVR YNAKSYVSVR YNAKSYVSVR YNEGEYYTSTQ JEKGESYFITG JERGESYFITG JERGESYFITG JERGESYFITG JERGESYFITG JERGESYFITG JERGESYFITG JERGESYFITG JERGENVAIN N-GEENVAIN N-GEENVAIN N-GEENVAIN N-GEENVAIN N-GEENVAIN N-GEENVAIN N-GEENVAIN S-GUENTSIS JEGGIEHTSIS JEGGIEHTSIS JEGGIEHTSIS JEGGIEHTSIS JEGGIEHTSIS JEGGIEHTSIS JEGGIEHTSIS JEGGIEHTSIS JEGGIEHTSIS JEGGIEHTSIS	KGSLGSSLRGK KGSLGSSLRGK KGSLGSSLRGK KGSLGWAIRGK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSLGASVRGK DGTLGRSARGK DGTLGRSARGK DGTLACALRVF RGFLACALRVF RGFLACALRVF SGTLGKSQRAF TGTLGSSLRAF EGTLGDSLRAF KGSMGETLRWF KGSMGETLRWF	SPIQDA SPIQDS SPIQDS AAIVAP TPIFDDI TPIFDNI AAVVDQ VPVFDDI SPIFNSI VPVFDGI TPVYDE TPVYDE TPVYDE TPVYDE TPIVDE		IGIVSVGY IGIVSVGY IGVVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSVGF IGIVSVGF IGIVSVGF IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGU VGAVAVGI IGVVVVG VGVAVVGS VGLVSAGI	TIEQIENWI TIEQIESWI TIEQIESWI LUNKIDSWR LVSKIDSWR LIERIQDRY MISYVDSLF AYDSVRARI EISKVTQQT EISKVTQQT EISKVTQQT TVNEIDEVT SIQEVNDVI SIQEVNDVI SIQE DDVT TIPGIADII VIPSYADMI KVEEISKRA	S N RVYSY AEFLLP LDFLLP E IHAIP IHAIP NNSR NNSR NNSR SHSLR SHSLR SHSLR ADNHTSIL NNNHI HKTLIK QEFWQ QEQ	- - - - - - - - - - - - - - - - - - -
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839 OB3251 SC05829 Eco-DcuS STM4304 STY4502 Sfl-DcuS Bsu-YufL BH0397 OB3220 CPE0531 Bsu-DctS BH2752 SC05435 SPY1107	1177 1122 116 1155 1155 1056 108 103 128 1155 1055 1055 106 106 104 106 109 109 109	EIGKSMEGGDSDEA EIGKSMEGGDSDDA EIGKYMEGGDSDDA KIGLPMQGGDSRA KIGYPMQFTKQG-A KIGYPMQFTKPG-A KIGYPMQFTKPG-A RIGKPMVGGDNERA KIGYPMQGDNERA KVGMQMVGGDNEQA RIGOPFKGDDILLA RIGOPFKGDDILLA RIGOPFKGDDILLA RIGOPFKGDDILLA RIGOPFKGDDILLA RIGOPFKGDDILLA RIGOPFKGDDILLA RIGOPFKGDDILLA RIGOPFKGDDILLA RIGOPFKGDDILLA RIGOPFKGDDILLA RIGOPFKGDDILLA RIGOPFKGDDILLA RIGVFVGGDESEV EIGKRFVGGDESEV SIGKKSEGADEAA RIQTFVGGDEDA EIGHLFQG-HIERA	INAKSYVSVR YNAKSYVSVR YNAKSYVSVR YEEEYYTSTQ IEEESYTSTQ IEEESYFITG IEEESYFITG IEEESYFITG IEEESYFITG IEEENYSIA A- CREVMEID IN- CEENVAIN IA- CREVNAIN IA- CREVNAIN IA- CREVNAIN IA- CREVNAIN IA- CREVNAIN IA- CREVNAIN IA- CREVSIS IEQCIEHISIS ICCSSYSIM FA- EHIYFSAA FA- EHIYISKA IR- COTFTETY IA- CKKVISTA	KGSLGSSLRGK KGSLGSSLRGK KGSLGSLRGK KGSLGWAIRGK KGSLGMAMRAK KGSLGMAMRAK KGSLGMAMRAK KGSLGASVRGK OGSLGPSIRGK NGSLGASVRGK DGTLGRSARGK RGFLACALRVF RGFLAKALRVF RGFLAKALRVF RGFLAKALRVF RGFLACALRVF SGTLGSSLRAF EGTLGDSLRAF EGTLGDSLRAF EGTLGDSLRAF TGTLGSSLRAF TGTLGSSLRAF KGELGTAVRAF	SPIQDS SPIQDS SPIQDS TPIFDDI TPIFDDI TPIFDNI TPIFDNI SPIFNSI VPVFDDI SPIFNSI VPVFDG SPIFNSI VPVYDE TPIVDE TPIVDE TPIVDE TPIVDE TPIVDE TPIVDE SPIFNE VPVYAE VPVFDG SPIFNE VPVFDG		IGIVSVGY IGIVSVGY IGVVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSUGY IGVVSVGF IGVVSVGF IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGI IGVVAVGI VGAVAVGI VGAVAVGI IGVVLVGK VGVAVVGS VGLVSAGI IGALAVGI	TIEQIENWI TIEQIESWI TIEQIESWI LUSKIDSWR LVSKIDSWR LVSKIDSWR LIERIQDRU MISYUDSLF AYDSVRARI EISRUTQQI EISRUTQQI EISRUTQQI EISRUTQQI TVNEIDEVI SIQEVDVI SIQEIDDVI YYNEIQLIT TVPGIADII KVEEISKRA KUTTINDVA	S N REFLLP LDFLLP LDFLLP E KQ IHAIP NNSR NNSR NNSR NNSR ADNHTSIL NNNHI HKTLIK LHLKRD QEFWQ QEQ LTSKRN	- - - - - - - - - - - - - - - - - - -
Kpn-CitA STM0053 STY0062 VC0791 Eco-CitA STM0625 STY0674 VC1605 BH3839 OB3251 SC05829 Eco-DcuS STM4304 STY4502 Sfl-DcuS Bsu-YufL BH0397 OB3220 CPE0531 Bsu-DctS BH2752 SC05435 SPY1107 SAG1921	1177 1122 116 1155 1155 106 108 1155 1155 1055 106 106 104 106 109 109 105 105	EIGKSMEGGDSDEA EIGKYMEGGDSDDA EIGKYMEGGDSDDA KIGLPMQGGDSRA KIGYPMQFTKQG-A KIGYPMQFTKPG-A KIGYPMQFTKPG-A KIGYPMQFTKPG-A RIGVPMQGDNERA KVGMQMVGGDNERA KVGMQMVGGDNERA RIGOPFKGDDILLA RIGOPFKGDDILLA RIGOPFKGDDILLA RIGOFFKGDA RIGOFFKGDA RIGOFFKGDA RIGOFFKGDA RIGOFFKGDA RIGOFFKGDA RIGOFFKGA RI	INAKSYVSVR YNAKSYVSVR YNAKSYVSVR YEEEYYTSTQ IEECESYFITG IEECESYFI IEECESYFITG IEECESYFITG IEECESYFITG IEECESYFITG IEECESYFITG IEECESYFITG IEECESYFI IEECESYFITG IE	KGSLGSSIRGK KGSLGSSLRGK KGSLGSSLRGK KGSLGWAIRGK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSIGMAMRAK KGSIGSVRGK DGTLGPSVRGK DGTLGSSRGK KGFLAKALRVF RGFLAKALRVF RGFLAKALRVF RGFLAKALRVF RGFLAKALRVF KGFLGALRVF KGSGGETLRWF KGSMGETLRWF KGSMGETLRWF KGSMGETLRWF KGSMGETLRWF KGSMGETLRWF KGSMGETLRWF KGSMGETLRWF KGSMGETLRWF KGSMGETLRWF KGSMGETLRWF KGSMGETLRWF KGSMGETLRWF KGSMGETLRWF	SPIQDA SPIQDS SPIQDS AAIVAP TPIFDDI TPIFDDI TPIFDDI TPIFDDI TPIFDDI SPIFNS SPIF	IG KV IG KV IG KV IG KV IG KV IG KV IG KI IG KI IG KQ IHH RQ IHH RQ IH	IGIVSVGY IGIVSVGY IGVVSVGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSIGY IGVVSVGY VGAVSVGI IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL IGVVAIGL VGAVAVGI VGFIMVGK VGVAVVGS VGVSAGI IGATAVGI	TIEQIENWI TIEQIESWI TIEQIESWI LUDNISSWI LVSKIDSWR LVSKIDSWR LVSKIDSWR LVSKIDSWR LIERIQRVI MISYUDSLF AYDSVRARI EUSRUTQQI EUSRUTQQI EUSRUTQQI EUSRUTQQI SUQEVDVI SUQEVDVI SUQEVDVI SUQEVDVI SUQEVDVI SUQEVDVI SUQEVDVI SUQEXIDI VIPSYADMI KVEEISKRA KUTTINDVA	S N AEFLLP LDFLLP GERLI GERLI IHAIP IHAIP NNSR NNSR NNSR NNSR ADNHTSIL NNNHI HKTLIK LHLKRD QEFWQ LTSKRN QSSIKEFS	- - - - - - - - - - - - - - - - - - -

FIGURE 6: Alignment of the periplasmic domain of members of the CitA subfamily of sensor histidine kinases. The residues identified in this work with the *K. pneumoniae* sensor kinase CitA as important for citrate binding are marked by filled squares, those without a drastic effect on binding are indicated by open squares. Abbreviations: BH, *Bacillus halodurans*; Bsu, *B. subtilis*; CPE, *Clostridium perfringens*; Eco, *E. coli*; Kpn, *K. pneumoniae*; OB, *Oceanobacillus iheyensis*; SAG, *Streptococcus agalactiae*; SCO, *Streptomyces coelicolor*; Sfl, *Shigella flexneri*; SPY, *Streptococcus pyogenes*; STM, *S. tyhimurium*; STY, *S. typhi*; and VC, *Vibrio cholerae*. Either the designation of the corresponding genes is given or the number according to the protein table of the entire genome (http://www.ncbi.nlm.nih.gov/ PMGifs/ Genomes/micr.html).

pneumoniae sensor kinase CitA on citrate binding by replacing the corresponding residues with alanine. ITC analysis of the resulting 17 muteins led to the identification of five residues that are highly important for binding (Table 2). In four cases, the apparent K_d values were increased 38-fold (R107A) or >300-fold (R66A, H69A, K109A). By contrast, the K_d value of the R98A mutein was 6-fold decreased. Thermal denaturation experiments (Table 3) revealed that citrate significantly stabilized wild-type CitAP and mutein R98A but had only weak effects on the four proteins with disturbed binding. Since the far-UV CD spectra did not reveal major changes of secondary structure in these mutants, a direct involvement of R98, R66, H69, R107, and K109 in citrate binding and related conformational changes of the protein appears likely.

The participation of positively charged residues in citrate binding has also been shown for other proteins. In citrate synthase from different species, three arginine and three histidine residues were identified as ligands by structural analysis (24, 25). Mutational analysis of the citrate chemore-ceptor Tcp from *Salmonella typhimurium* suggested that three arginine residues and one lysine residue are involved in citrate recognition (26). However, as in the case of CitAP, other possibilities for the observed effects of the mutations cannot be strictly excluded without a structural analysis.

CitA of K. pneumoniae represents the first characterized member of a subfamily of sensor kinases that share significant sequence identity not only in the conserved kinase domain but also in the periplasmic domain (15). In the classification scheme by Grebe and Stock, this subfamily was designated HPK₅ (27). Meanwhile, the function of several of these sensor kinases and their cognate response regulators have been characterized to some extent, and in all cases, a transporter for either citrate or C₄-dicarboxylic acids was found to be induced by the corresponding twocomponent systems (Table 4). In addition, many homologues of CitA (and its cognate response regulator CitB) were identified in the course of bacterial genome sequencing projects but not yet characterized. From an alignment of the periplasmic domains of the CitA homologues (Figure 6), it is evident that three of the residues essential for citrate binding in K. pneumoniae CitA (i.e., R66, H69, and R107) are highly conserved within the homologues. Thus, it appears possible that these residues are critical for ligand binding also in other members of the CitA family. In the case of the Bacillus subtilis C4-dicarboxylate sensor/regulator pair DctS/ DctR, the periplasmic binding protein DctB was shown to be essential for induction of the dicarboxylate carrier DctP (28), indicating an involvement of DctB in the sensing of dicarboxylates. It appears possible that an interaction between ligand-bound DctB and DctS regulates the autophosphorylation activity of DctS instead of or in addition to binding of C₄-dicarboxylates by DctS itself.

In contrast to R66, H69, and R107, residue K109 was conserved in only about half of the homologues, whereas the other half contained phenylalanine, leucine, valine, or serine at this position (Figure 6). According to the currently characterized members of the CitA family, it appears possible that lysine is preferred at this position in those members of the CitA family that bind tricarboxylic acids, whereas it is mostly absent in members that eventually bind C₄-dicarboxylic acids.

In the case of residue R98, only two homologues from S. typhimurium and Salmonella typhi also have an arginine residue at this position, whereas no other homologues contain a positively charged residue at this position. In the case of E. coli CitA, a glycine residue (G138) is present instead of an arginine residue. The periplasmic domain of E. coli CitA (comprising residues 38-177 with a C-terminal hexahistidine tag, named CitAP_{Ec}) was also shown to function as a citrate receptor with a K_d of about 0.5 μ M in 50 mM sodium phosphate buffer pH 7.0 as measured by ITC (29). Since this K_d value is very similar to that of CitAP-R98A from K. pneumoniae, we wondered whether an exchange of Gly102 (corresponds to G138 in the entire protein) in CitAP_{Ec} by arginine would result in a \sim 6-fold increased K_d value. The corresponding mutein CitAP_{Ec}-G102R precipitated when dialyzed against 50 mM sodium phosphate buffer, pH 7, indicating that the exchange has significantly altered the physicochemical properties of the protein. ITC binding studies were performed in a buffer in which the mutein did not precipitate (i.e., TNI400 buffer consisting of 20 mM Tris, 500 mM NaCl, and 400 mM imidazole adjusted to pH 7.9). No binding of citrate to CitAP_{Ec}-G102R was observed, whereas wild-type CitAP_{Ec} had a K_d value of 2.3 μ M for citrate in this buffer (29). Although it cannot be excluded that the G102R exchange caused structural changes, its dramatic effect on citrate binding supports the conclusion drawn from the R98A mutation in K. pneumoniae CitAP that the corresponding position is critical for citrate binding.

In this paper, the effects of replacing arginine, lysine, and histidine residues by alanine on citrate binding by the isolated periplasmic domain of the sensor kinase CitA was tested. Our future studies will focus on the structural analysis of CitAP and on the consequences of the amino acid exchanges on the signaling properties of the entire sensor kinase CitA. For this purpose, an in vivo system has to established that allows us to measure the expression of target genes of the CitA–CitB two-component system (e.g., *citS* or *citC*).

ACKNOWLEDGMENT

The authors thank Dean Madden for valuable comments on the manuscript and Hermann Sahm for continuous support.

REFERENCES

- Stock, J. B., Ninfa, A. J., and Stock, A. M. (1989) *Microbiol. Rev.* 53, 450–490.
- Bourret, R. B., Borkovich, K. A., and Simon, M. I. (1991) Annu. Rev. Biochem. 60, 401–441.
- 3. Parkinson, J. S., and Kofoid, E. C. (1992) Annu. Rev. Genet. 26, 71–112.
- Robinson, V. L., Buckler, D. R., and Stock, A. M. (2000) Nature Struct. Biol. 7, 626–633.
- 5. Bott, M., Meyer, M., and Dimroth, P. (1995) *Mol. Microbiol. 18*, 533–546.
- 6. Meyer, M., Dimroth, P., and Bott, M. (1997) J. Mol. Biol. 269, 719-731.
- Van der Rest, M. E., Siewe, R. M., Abee, T., Schwarz, E., Oesterhelt, D., and Konings, W. N. (1992) *J. Biol. Chem.* 267, 8971–8976.
- 8. Bott, M., and Dimroth, P. (1994) Mol. Microbiol. 14, 347-356.
- Schneider, K., Dimroth, P., and Bott, M. (2000) FEBS Lett. 483, 165–168
- Schneider, K., Dimroth, P., and Bott, M. (2000) *Biochemistry 39*, 9438–9450.
- Schwarz, E., Oesterhelt, D., Reinke, H., Beyreuther, K., and Dimroth, P. (1988) J. Biol. Chem. 263, 9640–9645.

- Laussermair, E., Schwarz, E., Oesterhelt, D., Reinke, H., Beyreuther, K., and Dimroth, P. (1989) *J. Biol. Chem.* 264, 14710– 14715.
- Woehlke, G., Laussermair, E., Schwarz, E., Oesterhelt, D., Reinke, H., Beyreuther, K., and Dimroth, P. (1992) *J. Biol. Chem.* 267, 22804–22805.
- 14. Meyer, M., Dimroth, P., and Bott, M. (2001) J. Bacteriol. 183, 5248-5256.
- Kaspar, S., Perozzo, R., Reinelt, S., Meyer, M., Pfister, K., Scapozza, L., and Bott, M. (1999) *Mol. Microbiol.* 33, 858–872.
- Studier, F. W., and Moffatt, B. A. (1986) J. Mol. Biol. 189, 113– 130.
- 17. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463–5467.
- 18. Gill, S. C., and von Hippel, P. H. (1989) Anal. Biochem. 182, 319-326.
- Schaffer, S., Weil, B., Nguyen, V. D., Dongmann, G., Günther, K., Nickolaus, M., Hermann, T., and Bott, M. (2001) *Electro*phoresis 22, 4404–4422.
- Wiseman, T., Williston, S., Brandts, J. F., and Lin, L. N. (1989) Anal. Biochem. 179, 131–137.
- Pace, N. C., and Scholtz, J. M. (1997) in *Protein structure: a practical approach* (Creighton, T. E., Ed.), 2nd ed., pp 299–321, University Press, Oxford.
- 22. Dunitz, J. D. (1995) Chem. Biol. 2, 709-712.
- Lee, A. L., Kinnear, S. A., and Wand, A. J. (2000) Nature Struct. Biol. 7, 72–77.
- 24. Remington, S., Wiegand, G., and Huber, R. (1982) J. Mol. Biol. 158, 111–152.

- Russell, R. J., Ferguson, J. M., Hough, D. W., Danson, M. J., and Taylor, G. L. (1997) *Biochemistry* 36, 9983–9994.
- Iwama, T., Nakao, K. I., Nakazato, H., Yamagata, S., Homma, M., and Kawagishi, I. (2000) *J. Bacteriol.* 182, 1437–1441.
- 27. Grebe, T. W., and Stock, J. B. (1999) Adv. Microb. Physiol. 41, 139-227.
- Asai, K., Baik, S. H., Kasahara, Y., Moriya, S., and Ogasawara, N. (2000) *Microbiology* 146, 263–271.
- 29. Kaspar, S., and Bott, M. (2002) Arch. Microbiol. 177, 313-321.
- Wootton, J. C., and Drummond, M. H. (1989) Prot. Eng. 2, 535– 543.
- Taylor, B. L., and Zhulin, I. B. (1999) *Microbiol. Mol. Biol. Rev.* 63, 479–506.
- Indyk, L., and Fisher, H. F. (1998) *Methods Enzymol.* 295, 350– 364.
- Ingmer, H., Miller, C. A., and Cohen, S. N. (1998) Mol. Microbiol. 29, 49–59.
- 34. Oshima, T., Aiba, H., Masuda, Y., Kanaya, S., Sugiura, M., Wanner, B. L., Mori, H., and Mizuno, T. (2002) *Mol. Microbiol.* 46, 281–291.
- Zientz, E., Bongaerts, J., and Unden, G. (1998) J. Bacteriol. 180, 5421–5425.
- 36. Golby, P., Davies, S., Kelly, D. J., Guest, J. R., and Andrews, S. C. (1999) J. Bacteriol. 181, 1238–1248.
- Janausch, I. G., Garcia-Moreno, I., and Unden, G. (2002) J. Biol. Chem. 277, 39809–39814.
- Yamamoto, H., Murata, M., and Sekiguchi, J. (2000) Mol. Microbiol. 37, 898–912.

BI0340595