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Effect of cysteine replacements on the properties of the turgor sensor KdpD of *Escherichia coli*

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Abstract

Escherichia coli responds rapidly to K^+-limitation or high osmolarity by induction of the *kdpFABC* operon coding for the high affinity K^+-translocating Kdp-ATPase. This process is controlled by the membrane-bound histidine kinase KdpD and the response regulator KdpE. Here, it is demonstrated that replacements of the native Cys residues at positions 409, 852, and 874 influence distinct activities of KdpD, whereas replacements of Cys residues at positions 32, 256, and 402 have no effect. Replacements of Cys^{409} in KdpD reveal that transmembrane domain I is important for perception and/or propagation of the stimulus. When Cys^{409} is replaced with Ala, *kdpFABC* expression becomes constitutive regardless of the external stimuli. In contrast, when Cys^{409} is replaced with Val or Tyr, induction of *kdpFABC* expression in response to different stimuli is drastically reduced. KdpD with Ser at position 409 supports levels of *kdpFABC* expression comparable to those seen in wild-type. Since neither the kinase nor phosphatase activity of these proteins is affected, it is proposed that different amino acid side-chains at position 409 alter the switch between the inactive and active forms of the kinase. When Cys^{852} or Cys^{874} is replaced with Ala or Ser, kinase activity is reduced to 10% of the wild-type level. However, kinetic studies reveal that the apparent ATP binding affinity is not affected. Surprisingly, introduction of Cys^{852} and Cys^{874} into a KdpD protein devoid of Cys residues leads to full recovery of the kinase activity. Labeling studies support the idea that a disulfide bridge forms between these two residues.

Keywords: Signal transduction; Sensor kinase; Turgor; Cysteine replacement

1. Introduction

KdpD and KdpE regulate the expression of the *kdpFABC* operon in *Escherichia coli* [1] and are members of the large family of sensor kinase/response regulator systems (see [2,3] for review). The corresponding genes are organized in the *kdpDE* operon, which is adjacent to the *kdpFABC* operon that encodes the structural genes of the high affinity K^+-transport complex, KdpFABC [4,5]. K^+ is an important osmotic solute for the maintenance of turgor in bacterial cells [6], and the Kdp system can be characterized as an optional system to scavenge K^+ from

Abbreviations: DTT, dithiothreitol; biotin maleimide, 3-(*N*-maleimidyl propionyl)biocytin; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Amino acid replacements are designated as follows: the one-letter amino acid code is used followed by a number indicating the position of the residue in the wild-type KdpD. The second letter denotes the amino acid replacement at this position

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the environment. The stimulus that KdpD senses is believed to be turgor pressure or some effect thereof [7,8].

Expression of the Kdp-ATPase is induced under conditions of K^+-limited growth (under 2 mM K^+). In mutants lacking all other K^+-translocating transport systems, the Kdp-ATPase is expressed in media containing 50 mM K^+ or less. Therefore, neither the external nor the internal concentration of K^+ per se seems to be sensed, but what might be called the 'need' for K^+ to maintain turgor. Control by turgor is supported by the finding that a sudden increase in medium osmolarity, which reduces turgor, transiently turns on expression of the *kdpFABC* operon [6,7]. This model has been challenged recently by the finding that there is a difference in expression of *kdpFABC* when the osmolarity of the medium is increased by a sugar or a salt [9,10]. Analysis of mutant forms of KdpD that express constitutively *kdpFABC* regardless of the K^+-concentration of the medium, but retain the ability to respond to changes in medium osmolarity led Sugiura et al. [11] to suggest that KdpD senses two stimuli, decrease in turgor and K^+ concentration.

The *kdpD* gene has been cloned, sequenced and overexpressed [12]. The gene product KdpD is an integral protein of the cytoplasmic membrane [13]. Recently, KdpD was purified and reconstituted in an active form in proteoliposomes [14]. This purified protein has autokinase activity, and the phosphoryl group can be transferred to the response regulator KdpE. Moreover, KdpD is the only protein that mediates the dephosphorylation of purified KdpE∼P [14].

Recently, we demonstrated that the phosphatase activity of KdpD can be increased significantly in the presence of ATP or non-hydrolyzable ATP analogs, whereas other nucleotides had no effect [15]. According to hydropathy analysis of its primary amino acid sequence, KdpD consists of an extended cytoplasmic N-terminal region, four putative transmembrane domains, and an extended cytoplasmic C-terminal region (Fig. 1). This model is strongly supported by the analysis of a series of KdpD-alkaline phosphatase (*kdpD-phoA*) and KdpD-β-galactosidase (*kdpD-lacZ*) fusions, as well as by protease susceptibility experiments [16]. Furthermore, characterization of KdpD proteins carrying different truncations in the N-terminal region revealed that the membrane-spanning domain is mandatory for the in vitro and in vivo activity of the turgor sensor [17].

Site-specific modification of engineered proteins containing single Cys residues has become a powerful approach to study structural and dynamic aspects of proteins. This approach has been applied successfully for, e.g., bacteriorhodopsin [18], lactose permease [19] or aspartate receptor [20]. KdpD contains six cysteine residues. Two are located in the N-terminal region, two in transmembrane domain I, and two in the C-terminal region (Fig. 1). The aim of this work was to study the effect of replacements of these Cys residues on KdpD function and to obtain a functional Cys-free protein. We found that different replacements of the Cys residues at positions 409, 852 and 874 influence distinct functions of KdpD.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP, [α-^{35}S]ATP, and streptavidin-conjugated horseradish peroxidase were purchased from Amersham Buchler. Goat anti-rabbit IgG-alkaline phosphatase conjugate was purchased from Biomol. The 'Altered Sites II' in vitro mutagenesis system was from Promega. ATP was removed using NAP 10 columns from Pharmacia. Ni-NTA spin columns were obtained from Qiagen. All other materials were reagent grade and obtained from commercial sources.

2.2. Bacterial strains and plasmids

E. coli JM 109 [*recA1 endA1 gyrA96 thi hsdR17 supE44* λ^- *relA1* Δ(*lac-proAB*)/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q}*lacZ*ΔM15] [21] was used as carrier for the plasmids described. *E. coli* TKR2000 [Δ*kdpFABCDE trkA405 trkD1 atp706*] [22] harboring plasmid pPV5-1 or its derivatives carrying different *kdpD* mutations was used for expression of *kdpD* from the *tac* promoter. *E. coli* HAK006 [Δ*kdpABCD* Δ(*lac-pro*) *ara thi*] [23] and *E. coli* TKV2208 [Δ*kdpD trkA405 trkD1*] [17] transformed with plasmid pPV5-1 or pBD and its derivatives were used for studying signal transduction in vivo. *E. coli* ES1301 *mutS* [*lacZ53*

mutS201::Tn5 *thyA36 rha-5 metB1 deoC* IN(*rrnD-rrnE*)] transformed with plasmid pALTER-1 was used for the mutagenesis with the 'Altered Sites II' system.

In plasmid pPV5 [12], *kdpD* was cloned into vector pKK223-3; expression of *kdpD* is under the control of the *tac* promoter. In plasmid pBD, *kdpD* was cloned into pBAD18 [24], in which expression of *kdpD* is under the control of the arabinose promoter.

2.3. Oligonucleotide-directed site-specific mutagenesis

In order to facilitate mutant construction, five unique restriction sites [*Xho*I (395), *Sac*I (843), *Spe*I (1291), *Not*I (1395), and *Hin*dIII (2869), numbers in parenthesis correspond to the nucleotide position in the *kdpD* sequence] were introduced by silent mutation into plasmid pPV5 to create plasmid pPV5-1. For this purpose the *kdpD* gene was cloned into plasmid pALTER-1. Mutagenesis was carried out following the manufacturer's protocol of the 'Altered sites II' in vitro mutagenesis system. Restriction analysis and DNA sequencing confirmed the existence of the new restriction sites. Subsequently, individual substitution of the six native Cys residues was achieved by PCR mutagenesis. All site-specific mutations were directed by synthetic, mutagenic oligonucleotide primers. Oligonucleotide primers were designed to change codons of the six native Cys residues to Ala or Ser and, in addition, to change Cys^{409} to Tyr or Val. The PCR overlap extension method [25] was employed. PCR products were purified in agarose gels and digested with appropriate restriction enzymes. The DNA-fragments were isolated from agarose gels and ligated to similarly treated pPV5-1. *KdpD* devoid of codons for Cys residues was constructed by introducing C852A into pPV5-1-C874A by PCR using the same primers as described above. DNA fragments encoding replacements of Cys codons 32, 256, 402 with Ala codons and Cys codon 409 with a Ser codon were subcloned into this plasmid using different restriction sites, as described above. All of these *kdpD* constructs were cloned into pBAD18 [24] using *Xma*I and *Hin*dIII restriction sites, resulting in plasmid pBD and its derivatives.

2.4. DNA sequencing

Mutations were verified by sequencing the length of the PCR-generated segment through the ligation junctions in double-stranded plasmid DNA, using the dideoxynucleotide termination method [26] and synthetic sequencing primers after alkaline denaturation of the DNA [27].

2.5. Preparation of everted membrane vesicles

E. coli strain TKR2000 transformed with plasmid pPV5-1 carrying the different *kdpD* mutations was grown aerobically at 37°C in KML complex medium (1% tryptone, 0.5% yeast extract and 1% KCl) supplemented with ampicillin (100 μg/ml). Cells were harvested at an absorbance at 600 nm of ~1.0. Everted membrane vesicles were prepared as described previously [14]. Vesicles were resuspended in 50 mM Tris-HCl, pH 7.5, 10% glycerol, frozen in liquid nitrogen and stored until use at −80°C.

2.6. Phosphorylation and dephosphorylation assays

Everted membrane vesicles containing KdpD (2 mg protein/ml) were incubated at room temperature in phosphorylation buffer containing 50 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 M NaCl, 10 mM $MgCl_2$, and 2 mM DTT. Phosphorylation was initiated by addition of 20 μM [γ-^{32}P]ATP (2.38 Ci/mmol). At different times, aliquots were removed and mixed with an equal volume of two-times concentrated SDS-sample buffer [28]. After incubation for 4.5 min, an equimolar amount of KdpE was added to the KdpD-containing samples and the incubation was continued. Further aliquots were removed at different times and mixed with SDS-sample buffer, as described above.

To test dephosphorylation, purified KdpE (prepared as described [17]) was first phosphorylated in the following manner. Wild-type KdpD (4 mg/ml) was incubated in phosphorylation buffer, except that $MgCl_2$ was replaced with 5 mM $CaCl_2$ and phosphorylation was initiated with 20 μM [γ-^{32}P]-ATP (2.38 Ci/mmol). After 5 min, purified KdpE (0.1 mg/ml) was added, and the incubation was continued for 1 min. KdpD-containing mem-

brane vesicles were removed by centrifugation. ATP was removed by gel filtration through Sephadex G-25 (preincubated in 50 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM DTT, and 0.5 M NaCl). Purified KdpE∼P was used immediately. Dephosphorylation was initiated by addition of 20 mM $MgCl_2$, 20 μM ATP, and KdpD in everted membrane vesicles (1 mg/ml). Aliquots were removed at different times, and the reaction was stopped by addition of SDS-sample buffer.

All samples were immediately subjected to SDS-polyacrylamide gel electrophoresis (PAGE) [28]. Shortly before stopping SDS-PAGE, an ATP standard was loaded on the gels. Gels were dried and phosphorylation of the proteins was detected by exposure of the gels to a Storage Phosphor Screen. Phosphorylated proteins were quantified by image analysis using the Phosphorimager SI of Molecular Dynamics.

2.7. Probing signal transduction in vivo

In vivo signal transduction was probed with *E. coli* strain HAK006 transformed with the plasmids described. Cells were grown in TY medium (1% tryptone, 0.5% yeast extract) [11] or minimal medium [30] supplemented with NaCl and/or KCl, as indicated. Cells were grown to mid-logarithmic growth phase and harvested by centrifugation. β-Galactosidase activity was determined as described [29] and is given in Miller units.

Complementation of the *kdpD* deletion in strain TKV2208 was tested after transformation with plasmids pBD carrying the different *kdpD* mutations. The ability to grow under K^+-limiting conditions was the measure of the degree of complementation. Cells were grown in minimal medium containing 0.1 mM K^+ [30] and 0.4% glucose as carbon source.

2.8. Detection of disulfide bridges

Everted membrane vesicles (5 mg/ml) containing different derivatives of KdpD were incubated with 10 mM *N*-ethylmaleimide (NEM) at room temperature. After incubation for 5 min, DTT (50 mM) was added to stop the reaction with NEM and to reduce existing disulfide bridges. In parallel, everted membrane vesicles were incubated for the same time; however, instead of NEM/DTT an equal volume of 50 mM Tris-HCl buffer, pH 7.5 containing 10% glycerol was added. All samples were washed with Tris-HCl/glycerol buffer three times by ultracentrifugation. Subsequently, all samples were labeled with 3-(*N*-maleimidyl propionyl)biocytin (biotin maleimide) (0.2 mM) for 15 min. The reaction was stopped by addition of an excess of β-mercaptoethanol (50 mM). Proteins were solubilized with lauryldimethylamine oxide and purified by affinity chromatography on Ni-NTA spin columns according to a recently published protocol [14]. Labeled and purified proteins were subjected to SDS-PAGE and Western blotting. Biotin maleimide-labeled KdpD was detected with streptavidin-horseradish peroxidase. In a control experiment, everted membrane vesicles containing wild-type KdpD were reduced with 50 mM DTT, extensively washed, and labeled with 10 mM NEM. Subsequently, vesicles were carefully washed, labeled with biotin maleimide, and treated as described above.

2.9. Analytical procedures

Protein was assayed by a modified Lowry method [31], using bovine serum albumin as standard. Proteins were separated by SDS-PAGE [28] using 9 or 11% acrylamide gels. Immunodetection of KdpD proteins or Kdp-ATPase with polyclonal antibodies against KdpD or KdpABC, respectively, was performed as described [13].

3. Results

3.1. Influence of Cys replacements in KdpD on the phosphorylation and phosphotransfer activities in vitro

All KdpD proteins with single Cys replacements were tested for kinase activity. The time courses of phosphorylation of KdpD proteins are shown in Fig. 2. Autophosphorylation was found to be linear within 0.5 min. All KdpD proteins with Cys replacements could be phosphorylated. However, the rates of phosphorylation of KdpD-C852A and KdpD-C874A were reduced to 10% of the rate of wild-type KdpD. Based on these results, Cys^{852} and

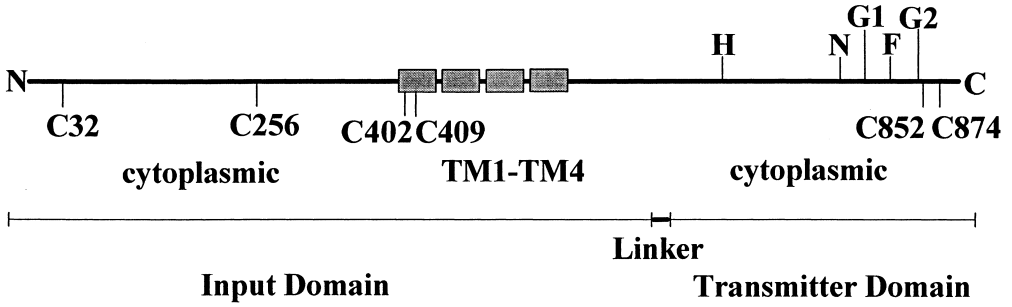

Fig. 1. Schematic presentation of the sensor kinase KdpD. The model is based on hydropathy and LacZ/PhoA fusion analyses [16]. Boxes represent the four transmembrane domains (TM1–TM4). Sequence motifs characteristic of the transmitter domains of sensor kinases (H, N, G1, F and G2) [38] are indicated above. Below, the positions of Cys residues are marked.

Cys^{874} were also replaced with Ser. Again, the rates of autophosphorylation of KdpD-C852S or KdpD-C874S were drastically reduced in comparison to wild-type KdpD (Fig. 2). Western blot analysis demonstrated that the differences seen in the kinase activity were not due to different amounts of the various KdpD proteins in the membrane (data not shown). Furthermore, the transfer of the phosphoryl group to KdpE was very fast, being complete within 15 s. Phosphotransfer was detectable for all KdpD variants with single Cys replacements (data not shown).

3.2. Kinetic studies of kinase activity of wild-type KdpD and KdpD variants with reduced kinase activity

Cys^{852} and Cys^{874} are located close to the proposed ATP binding site of KdpD (G1, F, G2 motif) (Fig. 1). Therefore, it was analyzed whether the reduced kinase activity was due to an altered apparent ATP affinity of the KdpD variants. The data obtained are summarized in Table 1. For wild-type KdpD, the apparent K_m value for ATP was determined to be 164 μM. Replacements of Cys^{852} or Cys^{874} with Ala or Ser did not change the apparent K_m values significantly. The V_{max} values of the kinase reaction of these proteins were about 7-fold lower than that of the wild-type protein.

3.3. Dephosphorylation of KdpE∼P by KdpD in vitro

It was recently demonstrated that KdpD is the only protein that mediates dephosphorylation of KdpE∼P [14]. KdpE∼P itself is very stable; within 2 h, no significant loss of the phosphoryl group was detected. Dephosphorylation of KdpE∼P was initiated by the addition of KdpD in everted membrane vesicles in the presence of 20 mM $MgCl_2$ and 20 μM ATP. Within 5 min, about half of the phosphorylated KdpE was dephosphorylated (Fig. 3). In the absence of ATP, very slow dephosphorylation was observed (data not shown). The time courses of the phosphatase activities of KdpD proteins revealed that replacements of single Cys residues do not influence this activity significantly (Fig. 3).

3.4. Influence of Cys replacements in KdpD on signal transduction in vivo

It has previously been found that in wild-type strains *kdpFABC* is expressed when K^+ in the me-

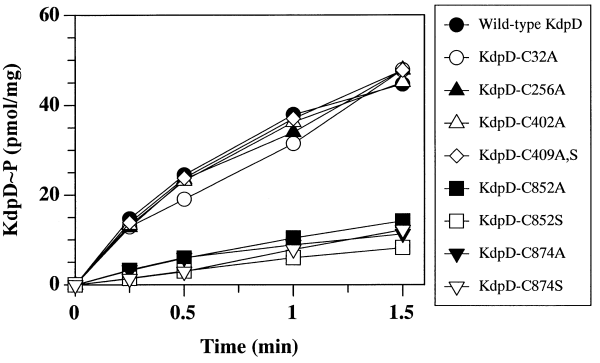

Fig. 2. Autophosphorylation of wild-type KdpD and KdpD variants with single Cys replacements with [γ-^{32}P]ATP. Time-dependent phosphorylation of everted membrane vesicles was carried out as described in Section 2. Proteins were separated by SDS-PAGE. The amount of KdpD∼P was quantified with a phosphorimager, using [γ-^{32}P]ATP as standard. KdpD-C409A and KdpD-C409S behave identically and are marked with KdpD-C409A,S.

Table 1
Kinetic analysis of KdpD kinase activity

Construct	K_m (μM)	V_{max} (pmol/min × mg protein)
Wild-type	164	242
KdpD-C852A	190	36
KdpD-C852S	143	35
KdpD-C874A	135	36
KdpD-C874S	161	35
KdpD-Cys-less	150	25

Initial rates of autophosphorylation were measured at substrate concentrations from 2 μM to 2 mM. The data were plotted according to Eadie-Hofstee. The values presented are the average of two independent experiments.

dium is below 2 mM. Furthermore, an increase in the osmolarity of the medium at a constant external K^+-concentration, a maneuver that reduces turgor, induces expression of *kdpFABC* [7]. Influence of Cys replacements on signal transduction was tested in two different ways.

(1) A *kdpD* null strain (*E. coli* TKV2208) was transformed with wild-type and mutant pBD plasmids, in which *kdpD* is under control of the arabinose promoter [24]. Even when cells were grown in the absence of inducer (arabinose) and in the presence of the repressor glucose the amount of KdpD produced was sufficient to complement the *kdpD* null strain so that cells were able to grow in minimal

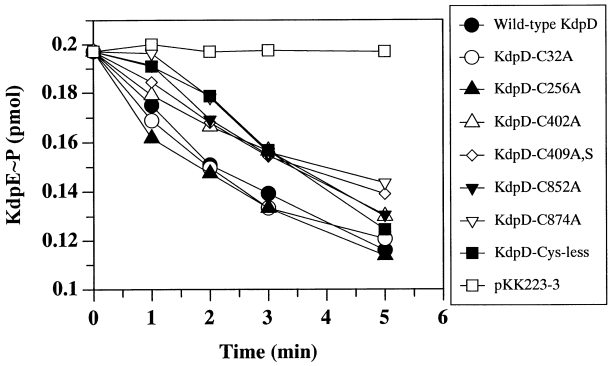

Fig. 3. Dephosphorylation of KdpE~P. KdpE was phosphorylated as described in Section 2. Dephosphorylation was initiated by addition of membrane-bound KdpD (everted membrane vesicles), 20 mM $MgCl_2$ and 20 μM ATP. As a control, everted membrane vesicles without KdpD were added. Proteins were separated by SDS-PAGE. The amount of phosphorylated KdpE was determined with a phosphorimager, using [γ-^{32}P]ATP as standard. KdpD-C409A and KdpD-C409S behave identically and are marked with KdpD-C409A,S.

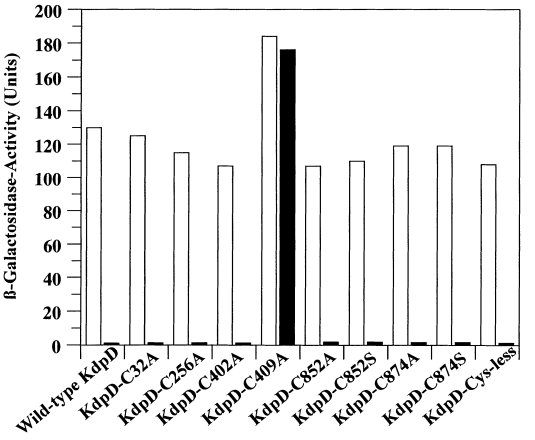

Fig. 4. Signal transduction mediated in vivo by KdpD proteins with Cys replacements as determined by β-galactosidase activity. *E. coli* strain HAK006 was transformed with plasmid pPV5-1 or its derivatives carrying the indicated mutations in *kdpD*. Cells were grown to mid-logarithmic growth phase in TY medium in the presence of 0.6 M NaCl (white bars) or in the presence of 0.6 M NaCl and 40 mM KCl (black bars). β-Galactosidase activity was determined as described in Section 2 and is given in Miller units [29]. The basal concentration of K^+ in the medium was determined to be 6 mM by flame photometry. The data presented represent average values obtained in at least three independent experiments.

medium at a K^+-concentration of 0.1 mM (data not shown). As a control, *E. coli* TKV2208 was transformed with pBAD18; no growth was observed under these conditions. These results indicate that replacements of single Cys residues in KdpD do not strongly interfere with signal transduction in vivo. In contrast, no growth of TKV2208 was observed under these conditions when *kdpD* expression was under the control of the strong *tac* promoter.

(2) Transcriptional control of *kdpFABC* was characterized using a *lacZ* fusion. For this purpose, *E. coli* strain HAK006 [11], which lacks a functional *kdpFABC* operon as well as the *kdpD* gene in the chromosome but contains the intact *kdpE* gene under the control of its own promoter, was used. This strain harbors a *kdpFABC* promoter-*lacZ* fusion gene in the chromosome. *E. coli* HAK006 transformed with plasmid pPV5-1 or its derivatives was grown in a medium of high osmolarity (0.6 M NaCl) to which different concentrations of KCl were added, and steady-state levels of β-galactosidase activity were determined (Fig. 4). High osmolarity of a complex medium containing a basal concentration of

KCl (6 mM) resulted in induction of *kdpFABC* expression, and hence high β-galactosidase activities. Addition of KCl at 40 mM was sufficient to repress *kdpFABC* expression (Fig. 4). Expression patterns of cells producing KdpD proteins with single Cys residues were comparable to that of wild-type KdpD, with the exception of KdpD-C409A (Fig. 4). This mutant exhibited high β-galactosidase activities, reflecting constitutive *kdpFABC* expression, regardless of the external stimuli (Figs. 4 and 5).

3.5. Influence of different amino acids at position 409 in KdpD on signal transduction in vivo

Since Cys^{409} appeared to be critical for KdpD activity, this amino acid was also replaced with Ser, Val, or Tyr to study signal transduction in more detail. KdpD-C409A induced expression of *kdpFABC* regardless of the ionic strength (Fig. 5A) or K^+-concentration (Fig. 5B) of the medium. β-Galactosidase activity in cells synthesizing KdpD-C409A remained at a constant, high level when the osmolarity of the medium was raised by addition of increasing concentrations of sucrose (data not shown). Ser substituted for Cys at position 409 in KdpD resulted in *kdpFABC* expression patterns comparable to that of wild-type KdpD (Fig. 5). In contrast, KdpD proteins carrying Val or Tyr at position 409 were unable to respond to an increase in osmolarity (Fig. 5A). However, modestly increased levels of β-galactosidase were detectable when these two mutants were grown under K^+-limiting conditions (Fig. 5B).

In a separate experiment with *E. coli* strain TKV2208, expression of the *kdpFABC* operon was examined directly by immunodetection of the KdpATPase. Data obtained in Western blots developed with anti-KdpABC antibodies confirmed that KdpD-C409A causes constitutive expression of *kdpFABC* (data not shown).

3.6. In vitro activities of KdpD proteins with different amino acids at position 409

Replacements of Cys^{409} with the amino acids described above change neither the kinase nor phosphatase activities of the corresponding proteins significantly (data for KdpD-C409A and KdpD-C409S are shown in Figs. 2 and 3; data for the other proteins are not shown). Furthermore, the transfer of the phosphoryl group to KdpE was detectable for each protein tested (data not shown).

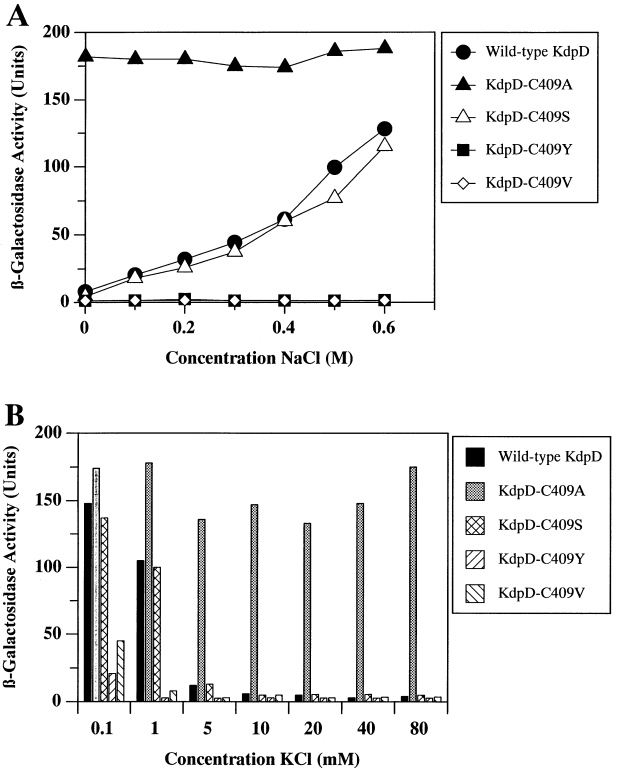

Fig. 5. Signal transduction mediated in vivo by KdpD proteins with different amino acids at position 409 as determined by β-galactosidase activity. *E. coli* strain HAK006 was transformed with plasmid pPV5-1 or its derivatives carrying the indicated mutations in *kdpD*. Cells were grown to mid-logarithmic growth phase in TY medium in the presence of different NaCl-concentrations (the basal concentration of K^+ in the medium was determined to be 6 mM by flame photometry) (A) or in minimal medium containing different amounts of K^+ (B). β-Galactosidase activity was determined as described in Section 2 and is given in Miller units [29]. The data presented represent average values obtained in at least three independent experiments.

3.7. KdpD-Cys-less and KdpD with single Cys residues

Since none of the single Cys replacements abolished the in vitro or in vivo activity of KdpD, all of the native Cys residues were replaced to create KdpD-Cys-less. Based on the results obtained for the single replacements, five Cys residues were changed to Ala and Cys^{409} was replaced with Ser. Cells producing KdpD-Cys-less responded normally

to the ionic strength and K^+ concentration of the medium as shown in the in vivo signal transduction test (Fig. 4). In vitro, the phosphatase activity of KdpD-Cys-less was indistinguishable from that of wild-type KdpD (Fig. 3), although its kinase activity is drastically reduced (Fig. 6). Determination of the kinetics of the kinase reaction revealed that KdpD-Cys-less has an apparent K_m value for ATP comparable to that of wild-type KdpD but shows a 10-fold decrease in V_{max} value (Table 1). These values are comparable to those determined for KdpD proteins with single Cys replacements at positions 852 or 874.

To determine if reintroduction of any specific Cys residue can restore activity, native single Cys residues were then put back in KdpD-Cys-less, and kinase activities were determined for these proteins. All KdpD proteins with a single Cys residue were characterized by kinase activities that were comparable to that of KdpD-Cys-less (Fig. 6). Since replacement of either Cys^{852} or Cys^{874} with Ala led to a decrease in kinase activity, both Cys residues were reintroduced in KdpD-Cys-less. The resulting protein (KdpD-Cys-less-C852,C874) showed wild-type kinase activity (Fig. 6).

3.8. Detection of disulfide bridges

To determine whether a disulfide bridge forms between Cys^{852} and Cys^{874}, we used a sulfhydryl labeling technique. Wild-type KdpD and KdpD-Cys-less-C852,C874 were labeled with the thiol reagent biotin maleimide with and without pretreatment with NEM and a strong reductant. The results of a blot using streptavidin-horseradish peroxidase for detection are shown in Fig. 7A. As judged from a Coomassie blue-stained gel all lanes contained comparable amounts of KdpD (Fig. 7B). In the case of wild-type KdpD, free thiol groups could be labeled with biotin maleimide. Additional thiol groups became accessible after reduction, indicating the existence of disulfide bridges. Although some of the thiol groups of KdpD-Cys-less-C852,C874 were accessible to biotin maleimide without reduction, further thiol groups became accessible after reduction. As expected, KdpD-Cys-less could not be labeled. As a further control, KdpD proteins with single Cys residues at positions 852 or 874 were treated with or without NEM/DTT and labeled with biotin maleimide. In both cases only a faint band was detectable. To make sure that all free thiol groups could be blocked with NEM, wild-type KdpD was first reduced, then labeled with NEM and finally labeled with biotin maleimide. In this case, no labeling was observed (Fig. 7A). These results provide clear evidence for

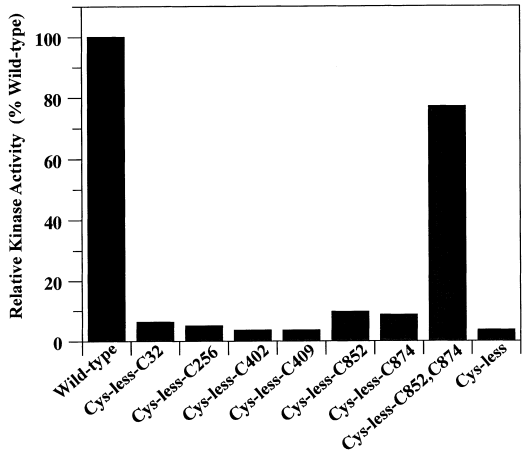

Fig. 6. Relative kinase activities of KdpD-Cys-less and KdpD with single Cys residues. Data are presented as percentages of the initial rate of phosphorylation of KdpD by [γ-^{32}P]ATP relative to wild-type KdpD. For wild-type KdpD, the phosphorylation activity (100% value) was 50 pmol/min×mg protein.

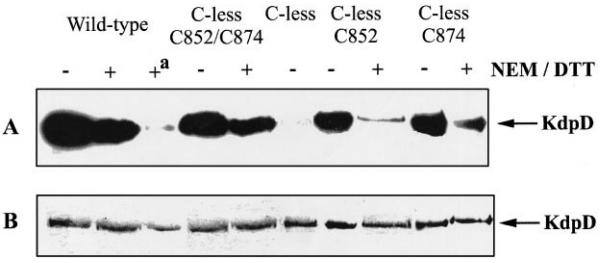

Fig. 7. Accessibility of thiol groups before and after exposure to DTT. (A) Free thiol groups of wild-type KdpD, KdpD-Cys-less-C852,C874, KdpD-Cys-less-C852, KdpD-Cys-less-C874, or KdpD-Cys-less were labeled with biotin maleimide. The proteins were purified, and labeled KdpD was detected using streptavidin-horseradish peroxidase as described in Section 2. The corresponding lanes are marked with '−NEM/DTT'. In a parallel experiment (marked with '+NEM/DTT') everted membrane vesicles were first incubated with NEM to block free thiol groups. Subsequently, 50 mM DTT was added to reduce existing disulfide bridges. After extensive washing, free thiol groups were detected using biotin maleimide as described above. [a]Control experiment in which wild-type KdpD was first treated with 50 mM DTT followed by incubation with NEM and biotin maleimide as described in Section 2. (B) Detection of labeled proteins after staining with Coomassie blue.

the existence of a disulfide bridge between Cys^{852} and Cys^{874} in KdpD.

4. Discussion

In this communication we show that replacement of Cys^{409} (transmembrane domain I) or Cys^{852} and Cys^{874} (C-terminal cytoplasmic domain) influences distinct functions of KdpD, whereas replacement of Cys^{32} and Cys^{256} (N-terminal cytoplasmic domain) or Cys^{402} (transmembrane domain I) does not affect the in vivo or in vitro activities of KdpD.

KdpD-C409A is the first KdpD variant to cause constitutive expression of *kdpFABC*, regardless of the K^+-concentration or osmolarity of the medium. A Tyr or Val substituted at this position resulted in KdpD proteins that were not able to respond to an increase in osmolarity but were to some extent sensitive for K^+. When Ser was introduced at position 409, *kdpFABC* expression remained at wild-type levels. The observation that Ala and Tyr or Val caused deregulation of *kdpFABC* expression, although in the opposite direction, supports the notion that the size of the amino acid side-chain at residue 409 is important for the function of KdpD. Furthermore, it appears that hydrogen bonding is involved in signal transduction since Cys^{409} and Ser^{409} are equivalent. These results clearly indicate that the osmosensing and K^+-sensing capabilities of KdpD are distinct processes. In addition, these results provide evidence that transmembrane domain I is involved in stimulus perception and/or stimulus propagation.

Earlier, Mizuno's group [11] isolated different mutants in transmembrane domains III and IV that were characterized by their altered sensing properties. These mutants were unable to sense the K^+ signal, but they became highly sensitive to changes in osmolarity. The importance of the transmembrane domains was further supported by KdpD proteins that lack the four transmembrane domains and are inactive in vivo and in vitro [17]. In other sensor proteins, the transmembrane domains play also an important role. In the case of the aspartate chemoreceptor, the transmembrane helices transduce the signal between the periplasmic and the cytoplasmic domain (reviewed in [32]). Based on recent studies with the osmolarity-sensing histidine kinase EnvZ, it was suggested that the primary stimulus is perceived by the membrane-spanning domains of this protein [33].

The nature of the stimulus sensed by KdpD and the mechanism of KdpD sensing are still unknown. Our current model proposes that a stimulus (changes in turgor and/or membrane stretch) is perceived by the transmembrane domains [2]. Two possibilities can be envisaged of how the stimulus is propagated: (1) there is a switch from an inactive to an active form of the kinase; and (2) since KdpD is a bifunctional enzyme, there could be a shift in the ratio of kinase to phosphatase activity.

Different side-chains at position 409 in KdpD influenced neither its kinase nor phosphatase activity. In this regard, it is worth mentioning that we recently isolated another KdpD variant (KdpD-R511Q) that causes constitutive expression of *kdpFABC*. In this case, replacement of a positively charged amino acid by a neutral amino acid leads to the loss of phosphatase activity. Furthermore, replacement of Arg at position 506 with Gln causes an expression pattern that is comparable with that of KdpD-C409V or KdpD-C409Y. In this case, the ratio of phosphatase to kinase activity is shifted towards the phosphatase (K.J. and K.A., unpublished information).

Taking these results together, we propose that replacement of Cys^{409} perturbs switching between an inactive and an active kinase. KdpD-C409A is held in a permanently active form, whereas KdpD-C409V or KdpD-C409Y are locked in an inactive form. However, kinase activity is not completely locked, since under extreme K^+ limitation, *kdpFABC* expression was observed and kinase activity was detectable in vitro.

Introduction of different side-chains at position 409 might disrupt helix–helix dimerization. This situation has been seen with the glycophorin A transmembrane helix, for which Ala scanning mutagenesis has been used to map helix–helix interactions [34]. Preliminary results indicate that KdpD is a dimer (K.J., R.H., unpublished information). Dimerization has also been shown for other sensor kinases, e.g. EnvZ, NR_{II} and CheA [35–37]. Therefore, an influence of substitutions at residue 409 on the formation of KdpD dimers can be envisaged.

Cys^{852} and Cys^{874} are located in the C-terminal

region of KdpD, close to the G1, F, and G2 motifs that are parts of the proposed ATP binding site in sensor kinases [38] (Fig. 1). Replacement of these residues led to drastically reduced kinase activities in vitro but did not influence signal transduction in vivo. Obviously, a very low amount of phosphorylated KdpD is sufficient to mediate signal transduction. Furthermore, the apparent K_m for ATP of wild-type KdpD is 164 μM and KdpD-Cys-less has a comparable apparent K_m value, suggesting that ATP binding is not influenced by the replacement of Cys residues at positions 852 and 874. Furthermore, replacement of Cys^{852} or Cys^{874} with Ala or Ser had the same effect. Therefore, it seems unlikely that Cys^{852} or Cys^{874} are involved in hydrogen bonding, which should be restored by placing Ser at these positions.

Reintroduction of Cys^{852} and Cys^{874} into KdpD-Cys-less restored kinase activity to wild-type levels. In addition, thiol labeling studies with wild-type KdpD and KdpD-Cys-less-C852,C874 provide evidence for the existence of a disulfide bridge between Cys^{852} and Cys^{874}. Since KdpD exists as a dimer (K.J. and R.H., unpublished information), an intermolecular as well as an intramolecular disulfide bridge could be envisaged. However, both residues are located in a domain of the protein that is proposed to be cytoplasmic. Due to the more reducing environment of the cytoplasm [39], and the presence of thioredoxin reductase encoded by *trxB* gene [40] disulfide bridges are usually absent in cytoplasmic proteins. Indeed, the thiol labeling experiment demonstrates that KdpD-Cys-less-C852,C874 represents a mixture of free and oxidized thiol groups. Therefore, conformational changes in KdpD might favor a structural arrangement of the C-terminal domain that allows sequential oxidation of these residues even in the reducing environment of the cytoplasm. This oxidation could even be part of the activation or deactivation of KdpD. However, taking into account that *kdpFABC* expression is not altered when all Cys residues in KdpD are removed, this putative disulfide bridge seems not to be essential for the activity of KdpD. In agreement with this finding is the observation that the kinase activity of KdpD is not dependent on the oxidized state of the protein (K.J. and R.H., unpublished information). Therefore, a physiological role of the postulated disulfide bridge is unclear. $CheA_s$, the N-terminally truncated form of the histidine kinase CheA, which is involved in chemotactic signaling, appears to form intermolecular disulfide bonds. This disulfide crosslink eliminates the ability to bind to CheZ, the phosphatase that accelerates dephosphorylation of the response regulator CheY [41]. However, the disulfide bridge of $CheA_s$ seems not to be functionally important under in vivo conditions. Furthermore, for the outer membrane protein LamB of *E. coli* a disulfide bond between subunits is important for the thermal stability of this protein. However, the disulfide bond in LamB is not required for its transport function [42].

In summary, we have analyzed the effect of Cys replacements on the sensing and signal transduction mechanism of the turgor sensor KdpD. The KdpD-C409A, KdpD-C409V or KdpD-C409Y forms of KdpD seem to be locked in a conformation that deregulates *kdpFABC* expression. Therefore, these KdpD proteins are especially interesting for further structural analysis. In addition, we propose that Cys^{852} and Cys^{874} form a disulfide bridge. Finally, a functional KdpD protein devoid of Cys residues is now available which forms the basis for engineering KdpD proteins containing only one Cys residue. These single reactive sulfhydryl groups will be useful as labeling sites to study the dynamics of the sensor in relation to different stimuli.

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References

[1] J.W. Polarek, G. Williams, W. Epstein, The products of the *kdpDE* operon are required for expression of the Kdp-ATPase of *Escherichia coli*, J. Bacteriol. 174 (1992) 2145–2151.

[2] K. Altendorf, W. Epstein, The Kdp-ATPase of *Escherichia*

coli, in: A.G. Lee (Ed.), Biomembranes, Vol. 5, JAI Press London, 1996, pp. 403–420.
[3] K. Altendorf, P. Voelkner, W. Puppe, The sensor kinase KdpD and the response regulator KdpE control expression of the *kdpFABC* operon in *Escherichia coli*, Res. Microbiol. 145 (1994) 374–381.
[4] J.E. Hesse, L. Wieczorek, K. Altendorf, A.S. Reicin, E. Dorus, W. Epstein, Sequence homology between two membrane transport ATPases, the Kdp-ATPase of *Escherichia coli* and the Ca^{2+}-ATPase of sarcoplasmic reticulum, Proc. Natl. Acad. Sci. USA 81 (1984) 4746–4750.
[5] A. Siebers, K. Altendorf, K^+-translocating Kdp-type ATPases and other bacterial P-ATPases, in: E.P. Bakker (Ed.), Alkali cation transport systems in prokaryotes, CRC Press, Boca Raton, FL, 1992, pp. 225–252.
[6] W. Epstein, Osmoregulation by potassium transport in *Escherichia coli*, FEMS Microbiol. Rev. 39 (1986) 73–78.
[7] L.A. Laimins, D.B. Rhoads, W. Epstein, Osmotic control of *kdp* operon expression in *Escherichia coli*, Proc. Natl. Acad. Sci. USA 78 (1981) 464–468.
[8] W. Epstein, Kdp, a bacterial P-type ATPase whose expression and activity are regulated by turgor pressure, Acta Physiol. Scand. 146 (1992) 193–199.
[9] L. Sutherland, J. Cairney, M.J. Elmore, I.R. Booth, C.F. Higgins, Osmotic regulation of transcription: induction of the proU betaine transport gene is dependent on accumulation of intracellular potassium, J. Bacteriol. 168 (1986) 805–814.
[10] H. Asha, J. Gowrishankar, Regulation of the *kdp* operon expression in *Escherichia coli*: evidence against turgor as signal for transcriptional control, J. Bacteriol. 175 (1993) 4528–4537.
[11] A. Sugiura, K. Hirokawa, K. Nakashima, T. Mizuno, Signal-sensing mechanisms of the putative osmosensor KdpD in *Escherichia coli*, Mol. Microbiol. 14 (1994) 929–938.
[12] M.O. Walderhaug, J.W. Polarek, P. Voelkner, J.M. Daniel, J.E. Hesse, K. Altendorf, W. Epstein, KdpD and KdpE, proteins that control expression of the *kdpABC* operon, are members of the two-component sensor-effector class of regulators, J. Bacteriol. 174 (1992) 2152–2159.
[13] P. Voelkner, W. Puppe, K. Altendorf, Characterization of the KdpD protein, the sensor kinase of the K^+-translocating system of *Escherichia coli*, Eur. J. Biochem. 217 (1993) 1019–1026.
[14] K. Jung, B. Tjaden, K. Altendorf, Purification, reconstitution and characterization of KdpD, the turgor sensor of *Escherichia coli*, J. Biol. Chem. 272 (1997) 10847–10852.
[15] K. Jung, K. Altendorf, Truncation of amino acids 12 to 128 cause deregulation of the phosphatase activity of the sensor kinase KdpD of *Escherichia coli*, J. Biol. Chem., in press.
[16] P. Zimmann, W. Puppe, K. Altendorf, Membrane topology analysis of the sensor kinase KdpD of *Escherichia coli*, J. Biol. Chem. 270 (1995) 28282–28288.
[17] W. Puppe, P. Zimmann, K. Jung, M. Lucassen, K. Altendorf, Characterization of truncated forms of the KdpD protein, the sensor kinase of the K^+-translocating Kdp system of *Escherichia coli*, J. Biol. Chem. 271 (1996) 25027–25034.
[18] H.J. Steinhoff, R. Mollaaghababa, C. Altenbach, H.G. Khorana, W.L. Hubbell, Site directed spin labeling studies of structure and dynamics in bacteriorhodopsin, Biophys. Chem. 56 (1995) 89–94.
[19] K. Jung, H. Jung, J. Wu, G.G. Privé, H.R. Kaback, Use of site-directed fluorescence labeling to study proximity relationships in the lactose permease of *Escherichia coli*, Biochemistry 32 (1993) 12273–12278.
[20] S.A. Chervitz, J.J. Falke, Lock on/off disulfides identify the transmembrane signaling helix of the aspartate receptor, J. Biol. Chem. 270 (1995) 24043–24053.
[21] C. Yanisch-Perron, J. Vieira, J. Messing, Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors, Gene 33 (1985) 103–119.
[22] R. Kollmann, K. Altendorf, ATP-driven potassium transport in right-side-out membrane vesicles via the Kdp system of *Escherichia coli*, Biochim. Biophys. Acta 1143 (1993) 62–66.
[23] K. Nakashima, A. Sugiura, K. Kanamaru, T. Mizuno, Signal transduction between the two regulatory components involved in the regulation of the *kdpABC* operon in *Escherichia coli*: phosphorylation-dependent functioning of the positive regulator, KdpE, Mol. Microbiol. 7 (1993) 109–116.
[24] L.-M. Guzman, D. Belin, M.J. Carson, J. Beckwith, Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter, J. Bacteriol. 177 (1995) 4121–4130.
[25] S.N. Ho, H.D. Hunt, R.M. Horton, J.K. Pullen, L.R. Pease, Site-directed mutagenesis by overlap extension using the polymerase chain reaction, Gene 77 (1989) 51–59.
[26] F. Sanger, S. Nicklen, A.R. Coulsen, DNA sequencing with chain-terminating inhibitors, Proc. Natl. Acad. Sci. USA 74 (1977) 5463–5467.
[27] M. Hattori, Y. Sakaki, Dideoxy sequencing method using denatured plasmid templates, Anal. Biochem. 152 (1986) 232–238.
[28] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[29] J.H. Miller, in: A short course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1992, pp. 72–74.
[30] W. Epstein, M. Davies, Potassium-dependent mutants of *Escherichia coli*, J. Bacteriol. 101 (1970) 836–843.
[31] G.L. Peterson, A simplification of the protein assay method of Lowry et al., which is more generally applicable, Anal. Biochem. 83 (1977) 346–356.
[32] J.B. Stock, M.G. Surette, Chemotaxis, in: F.C. Neidhardt (Ed.), *Escherichia coli* and *Salmonella*, ASM Press, Washington, DC, 1996, pp. 1103–1129.
[33] M.R. Leonardo, S. Forst, Re-examination of the role of the periplasmic domain of EnvZ in sensing of osmolarity signals in *Escherichia coli*, Mol. Microbiol. 22 (1996) 405–413.

[34] I. Mingarro, P. Whitley, M.A. Lemmon, G. von Heijne, Ala-insertion scanning mutagenesis of the glycophorin A transmembrane helix: a rapid way to map helix–helix interactions in integral membrane proteins, Protein Sci. 5 (1996) 1339–1341.

[35] Y. Hidaka, H. Park, M. Inouye, Demonstration of dimer formation of the cytoplasmic domain of a transmembrane osmosensor protein, EnvZ, of *Escherichia coli* using Ni-histidine tag affinity chromatography, FEBS Lett. 400 (1997) 238–242.

[36] E.G. Ninfa, M.R. Atkinson, E.S. Kamberov, A.J. Ninfa, Mechanism of autophosphorylation of *Escherichia coli* nitrogen regulator II (NR_{II} or NtrB): *trans*-phosphorylation between subunits, J. Bacteriol. 175 (1993) 7024–7032.

[37] M.G. Surette, M. Levit, Y. Liu, G. Lukat, E.G. Ninfa, A. Ninfa, J.B. Stock, Dimerization is required for the activity of the protein histidine kinase CheA that mediates signal transduction in bacterial chemotaxis, J. Biol. Chem. 271 (1996) 939–945.

[38] J.S. Parkinson, E.C. Kofoid, Communication modules in bacterial signaling proteins, Annu. Rev. Genet. 26 (1992) 71–112.

[39] C. Hwang, A.J. Sinskeym, H.F. Lodish, Oxidized redox state of glutathione in the endoplasmic reticulum, Science 257 (1992) 1496–1502.

[40] A.I. Derman, W.A. Prinz, D. Belin, J. Beckwith, Mutations that allow disulfide bond formation in the cytoplasm of *Escherichia coli*, Science 262 (1993) 1744–1747.

[41] H. Wang, P. Matsumura, Characterization of the $CheA_S$/CheZ complex: a specific interaction resulting in enhanced dephosphorylating activity on CheY-phosphate, Mol. Microbiol. 19 (1996) 695–703.

[42] M. Luckey, R. Ling, A. Dose, B. Malloy, Role of a disulfide bond in the thermal stability of the LamB protein trimer in *Escherichia coli* outer membrane, J. Biol. Chem. 266 (1991) 1866–1871.