Osmosensing Properties of the Histidine Protein Kinase MtrB from Corynebacterium glutamicum

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The MtrB-MtrA two component system of Corynebacterium glutamicum was recently shown to be involved in the osmotic stress response as well as cell wall metabolism. To address the question of whether the histidine protein kinase MtrB is an osmosensor, the kinase was purified and reconstituted into liposomes in a functionally active form. The activity regulation was investigated by varying systematically physicochemical parameters, which are putative stimuli that could be used by the bacterial cell to detect osmotic conditions. Membrane shrinkage was ruled out as a stimulus for activation of MtrB. Instead, MtrB was shown to be activated upon the addition of various chemical compounds, like sugars, amino acids, and polyethylene glycols. Because of the different chemical nature of the solutes, it seems unlikely that they bind to a specific binding site. Instead, they are proposed to act via a change of the hydration state of the protein shifting MtrB into the active state. For MtrB activation it was essential that these solutes were added at the same side as the cytoplasmic domains of the kinase were located, indicating that hypertonicity is sensed by MtrB via cytoplasmically located protein domains. This was confirmed by the analysis of two MtrB mutants in which either the large periplasmic loop or the HAMP domain was deleted. These mutants were regulated similar to wild type MtrB. Thus, we postulate that MtrB belongs to a class of histidine protein kinases that sense environmental changes at cytoplasmatic protein domains independently of the periplasmic loop and the cytoplasmic HAMP domain.

Bacteria harbor large numbers of sensors for monitoring changes in chemical and physical parameters in their environment. The most common type of a bacterial sensory and signal transduction system is the two-component system. This system typically consists of a homodimeric membrane-bound histidine protein kinase and a cytoplasmic response regulator acting in most cases as a transcription factor. The histidine protein kinase, which is activated by an environmental stimulus, autophosphorylates in an ATP-dependent manner on a conserved histidine residue. The resulting high energy phosphoryl group is subsequently transferred to an aspartate residue of the response regulator. In turn, this affects the DNA binding properties of the response regulator effector domain (1, 2).

Two-component systems are frequently used by bacteria to detect environmental changes. The functions of these different signal transduction systems are diverse, ranging from the detection of nutrients (e.g. citrate, fumarate, or nitrogen) or toxic agents (e.g. copper) to other sensing processes like the perception of envelope, osmotic, or chill stress (3–9). Recently it was proposed that MtrB-MtrA from Corynebacterium glutamicum regulates expression of the genes important for both cell wall biosynthesis and osmostress response because altered mRNA levels of different genes were determined in the ΔmtrAB mutant (9). On the one hand, the expression of mepA, ppmA, and lpgB was elevated. These genes encode, respectively, a putative secreted metalloprotease, a putative membrane-bound protease modulator, and a lipoprotein of unknown function and are suggested to be important for peptidoglycan biosynthesis (9). On the other hand, RNA hybridization experiments showed that three of four genes coding for the osmoregulated carriers for the uptake of compatible solutes are under the control of MtrB-MtrA. After a hyperosmotic upshift, the expression of proP, betP, or lcpB was either dramatically decreased (proP) or completely abolished (betP and lcpB) in the mutant strain ΔmtrAB (9). These observations led to the suggestion that the response regulator MtrA could act either as a repressor or as an activator (9). Recently binding of MtrA to the promoter regions of mepA, betP, and proP has been confirmed (10), proving that the MtrB-MtrA system indeed directly regulates different cellular processes. Presumably, to control both cell wall metabolism and osmoregulation MtrB should possess different sensing capabilities. Because the genes coding for the uptake systems for compatible solutes are induced gradually depending on the extent of the osmotic stress applied, we suggested that MtrB is an osmosensor, capable of sensing the stress intensity.

The question arose of which stimulus is recognized by the histidine kinase MtrB as a measure of osmotic changes in order to induce the autophosphorylation reaction and, thus, to transduce the osmotic stress signal into the cell. An increase in the environmental osmolality concomitantly leads to changes in many other external and internal parameters, including concentrations of specific solutes, ionic strength, and internal osmolality as well as the physical state of the membrane (11). Therefore, it is not possible to analyze the effect of individual physicochemical parameters in whole cells. The complexity of the cell can be reduced by using...
proteoliposomes in which the sensing properties of a membrane protein can be examined by varying one parameter at a time (12, 13). Recently, we succeeded in reconstituting MtrB in functionally active form in liposomes. The sensor kinase showed all the characteristic enzymatic activities in this in vitro system (14). Interestingly, MtrB was efficiently activated in the presence of monovalent cations, like K\(^+\), Rb\(^+\), NH\(_4\)^+, and to a lesser extent by Na\(^+\). In principle, increasing ionic strength could signal hyperosmotic conditions in vivo, because the induced efflux of water leads to an increased concentration of cytoplasmic K\(^+\). However, the tested cations were shown to have a general stabilizing effect on reconstituted histidine protein kinases rather than to act as an osmo-specific signal for MtrB. This was concluded from the observation that the reconstituted fumarate sensor DcuS from *Escherichia coli*, which is not involved in the osmo-stress response in this organism, was activated in a similar way by these cations (14). In this investigation we addressed the question of which stimulus is recognized by MtrB as a measure of the osmotic changes. Using DcuS as a control representing a protein without osmosensing capability, we were able to identify stimuli specific for the activation of MtrB under osmotic stress.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—The *E. coli* strains used in this study are DH5αmcr (15), BL21(DE3) (Novagen, Darmstadt, Germany) and DK8 (16). *E. coli* cells were routinely cultivated in LB (Luria Bertani) medium in shaking flasks under aerobic conditions between 30 and 37 °C. The type strain ATCC 13032 was cultivated aerobically in fresh brain heart infusion medium at an optical density (OD) of 0.9. After reaching the mid-exponential growth phase (OD of 0.5), these cells were harvested for purification of RNA immediately before and up to 180 min after the osmotic upshock. For this purpose 2 ml of cell culture were transferred to a nylon membrane using a FastPrep® 120 instrument (Q-Biogene, Heidelberg, Germany). RNA was purified from the disrupted cells with the Nucleospin® RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. Digoxygenin-labeled antisense probes were synthesized as described recently (9). Changes in gene transcription were monitored by RNA hybridization experiments using these antisense RNA probes. For this purpose 5 μg of total RNA was transferred to a nylon membrane using a Minifold Dot Blotter (Schleicher & Schuell). RNA was bound to the membrane by careful vacuum suction (15 mbar) and subsequently cross-linked by means of ultraviolet irradiation at 125 J/cm². Hybridization and detection steps were carried out according to the DIG application manual (Roche Applied Science). Chemiluminescence was detected using a Fuji luminescent image analyzer LAS1000 (Raytest, Straubenhardt, Germany).

**Construction of pASK-IBA3-mtrB-strep and Mutant Forms of MtrB**—The construction of the plasmid used for the synthesis of MtrB carrying the Strep Tag II at the C-terminal end was described recently (14). For the construction of the mtrB variant, mtrBΔ190, missing the sequence encoding the first 190 amino acids, the forward primer (5′-CCC GGG GAC CGC GGT ACCCAA CAG GTC ACC GC), the reverse primer (CAC TTC ACA GGT CAA GC), resulting in a 3′-end fusion to the DNA sequence encoding the Strep Tag II. The correct sequence and orientation of the mtrBΔ190 gene was verified by sequencing. To construct a gene where the sequence encoding the 134 amino acids of the periplasmic loop, which has a total of 144 amino acids, was deleted, the following PCR strategy was used. The primers (forward primer, 5′-AGC GAC GAA TCC TCT CTT G, and the reverse primer, 5′-AAT ATC GAT TTT CTG ATC CAC) annealed at the sequence of mtrB flanking the deletion in the template pIBA3-mtrB-strep. In this case the complete sequence of the plasmid except the desired deletion region was amplified, and the resulting fragment was re-ligated. The sequence encoding mtrBΔ134 was isolated using SacII and HindIII and ligated into the SacII- and HindIII-restricted pASK-IBA3 vector (IBA GmbH, Göttingen), resulting in a 3′-end fusion to the DNA sequence encoding the Strep Tag II. The correct sequence and orientation of the mtrBΔ134 gene was verified by sequencing. To construct a gene where the sequence encoding the 134 amino acids of the periplasmic loop, which has a total of 144 amino acids, was deleted, the following PCR strategy was used. The primers (forward primer, 5′-AGC GAC GAA TCC TCT CTT G, and the reverse primer, 5′-AAT ATC GAT TTT CTG ATC CAC) annealed at the sequence of mtrB flanking the deletion in the template pIBA3-mtrB-strep. In this case the complete sequence of the plasmid except the desired deletion region was amplified, and the resulting fragment was re-ligated. The sequence encoding mtrBΔ134 was isolated using SacII and HindIII and ligated into the SacII- and HindIII-restricted pASK-IBA3 vector, resulting in a 3′-end fusion to the DNA sequence encoding the Strep Tag II. The correct sequence of the mtrBΔ134 gene was verified by sequencing. A similar PCR strategy was used for the construction of mtrBΔHAMP. In this case the forward primer, 5′-AAA TTG GAG GAA TAC GGC AA, and the reverse primer, 5′-GGT GGC TAGCCA TGCA, were used. As a result a mutant form of mtrB was constructed in which the DNA sequence encoding the amino acids 192–244 of MtrB was deleted. This sequence represents the HAMP domain starting directly downstream of the second transmembrane segment.

**Purification of MtrA-His⋅NH₄**—For the isolation and purification of MtrA-His⋅NH₄, *E. coli* BL21(DE3) cells transformed with pET224b-mtrA in which the sequence of *C. glutamicum* mtrA was fused in-frame at the 3′-end with the sequence encoding a

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3 The abbreviations used are: PEG, polyethylene glycol; DDM, N-dodecyl α-α-maltoside.
His tag with 10 histidine residues\(^4\) were used. The purification was performed as recently described (14).

**Purification of MtrB-Strep, MtrBΔ190-Strep, MtrBΔL134-Strep, and MtrBΔHAMP-Strep by Strep Tag II/StrepTactin Affinity Chromatography**—For the isolation and purification of MtrB-Strep, E. coli BL21(DE3) cells transformed with the different pASK-IBA7-mtrB strep derivatives were used. The heterologous expression of mtrB was induced by the addition of 40 \(\mu\)l/g liter anhydrotetracycline. Cells were harvested 3 h after induction, washed (100 mM Tris/HCl, pH 7.5), diluted in lysis buffer (100 mM Tris/HCl, pH 7.5, Complete (Roche Diagnostics), 5 \(\mu\)g/ml DNase1), and disrupted by three passages through a French pressure cell press. Cell debris was removed by low speed centrifugation at 4 °C. Membranes were isolated by centrifugation (223,000 \(\times\) g, 60 min, 4 °C), washed once in lysis buffer, and suspended in buffer A (50 mM Tris/HCl, pH 8.0, 10% glycerol, 10 mM \(\beta\)-mercaptoethanol, and 1 mM EDTA). Membrane proteins were extracted at 4 °C with N-dodecyl-\(\alpha\)-C-maltoside (DDM), which was added stepwise until a final concentration of 2% was reached. After stirring at 4 °C for 30 min, the solubilized proteins were centrifuged for 20 min at 67,000 \(\times\) g. After pre-equilibration of the StrepTactin column (IBA, Göttingen, Germany) with buffer B (50 mM Tris/HCl, pH 8.0, 200 mM NaCl, 10% glycerol, 10 mM \(\beta\)-mercaptoethanol and 0.1% DDM), the supernatant containing MtrB-Strep was diluted with 4 volumes of buffer A (lacking DDM) and then applied to the column. The column was washed with 20 volumes of buffer C (50 mM Tris/HCl, pH 8.0, 500 mM NaCl, 10% glycerol, 10 mM \(\beta\)-mercaptoethanol, and 0.1% DDM) and 10 volumes of buffer B before MtrB-Strep was eluted with 10 ml of buffer B containing 5 mM desthiobiotin. MtrB-Strep was frozen in liquid nitrogen and stored at −80 °C. For the purification of MtrBΔ190-Strep, the cytoplasmic fraction was used instead of the membranes.

**Purification of His\(_6\)C-DcuS by Nickel-nitrilotriacetic Acid Affinity Chromatography**—For the isolation of His\(_6\)C-DcuS, a pET28a plasmid containing the dcuS gene was used that was fused at its 5’-end to the sequence of a His tag (18). For synthesis of DcuS the E. coli strain BL21 was used. The purification was performed essentially as described recently (18).

**Liposome Preparation**—For the preparation of liposomes, E. coli phospholipids (polar lipid extract, 20 mg/ml in chloroform, Avanti polar lipids, Alabaster, AL) were first dried under a nitrogen atmosphere during preparation. Before use, the lipids were lyophilized with 100 mM KPi, pH 7.5, and 2 mM \(\beta\)-mercaptoethanol to a final concentration of 20 mg of phospholipids/ml, frozen in liquid nitrogen, and stored at −80 °C. To avoid oxidation, the lipids were kept under a nitrogen atmosphere during preparation. Before use, liposomes were formed by extrusion (Liposofast; Avestin, Ottawa, Canada) 14–20 times through polycarbonate filters (pore diameter, 400 nm). The liposomes were partly solubilized by the stepwise addition of 2-μl aliquots of 20% Triton X-100 solution until saturation was reached. Insertion of the detergent into the liposomes was followed by measuring the turbidity at 540 nm. Upon saturation with detergent, liposomes were mixed with purified MtrB-Strep, MtrBΔ134-Strep, MtrBΔHAMP or His\(_6\)C-DcuS at a lipid-to-protein ratio of 20:1 (w/w). The mixture was incubated for 35 min at 20 °C under gentle stirring. To remove detergent, Bio-Beads prewashed with deionized water were added at a Bio-Bead to Triton X-100 ratio of 5 (w/w) and a Bio-Bead to DDM ratio of 10 (w/w). Bio-Beads were added four times, and the mixture was kept under gentle stirring at room temperature for 1 h after each addition. The third treatment differed from these conditions; in this case the double amount of Bio-Beads was added, and the incubation time was prolonged to 16 h at 4 °C. After removal of Bio-Beads, the proteoliposomes in the supernatant were sedimented at 337,000 \(\times\) g, extruded 14–20 times through polycarbonate filters (pore diameter, 400 nm) in 50 mM Tris/HCl, pH 8.0, and washed once. The proteoliposomes were adjusted to 0.8–1 μg of protein/μl, frozen in liquid nitrogen, and stored at −80 °C.

**Autophosphorylation of MtrB-Strep in Proteoliposomes**—To test autophosphorylation activity of membrane-bound MtrB-Strep, 1.6–2 μg of reconstituted sensor kinase were slowly thawed at room temperature and diluted into phosphorylation buffer (50 mM Tris/HCl, pH 8.0) to a final volume of 950 μl. The proteoliposomes were extruded 14–20 times through polycarbonate filters (pore diameter, 400 nm) and collected by ultracentrifugation (337,000 \(\times\) g). Subsequently they were adjusted to the desired concentration with the phosphorylation buffer supplemented with 20 mM KCl, 1 mM dithiothreitol, and 5 mM MgCl\(_2\) to a final volume of 10 μl. If indicated, the buffer contained up to 600 mM concentrations of various solutes. The autophosphorylation reaction was initiated by the addition of 0.22 μM \([\gamma\text{-}^{33}\text{P}]\)ATP (110 TBq mmol\(^{-1}\)). Samples were incubated at 30 °C. Under standard conditions the reaction was stopped after 20 min by the addition of 9 μl of 2.4 \(\times\) SDS loading buffer, and samples were directly subjected to SDS-PAGE. The gels were dried and exposed to phosphorimaging plates (LAS-IP MP 2025, Fujifilm, Düsseldorf, Germany). Phosphorylation of proteins with \([\gamma\text{-}^{33}\text{P}]\) was detected using Fuji BAS-1800 (Fujifilm). For quantification of the phosphorylation signals, PcBAS version 2.09c was used. In control experiments, as shown in supplemental Fig. S1, it was verified that the autophosphorylation reaction still linearly increased after 20 min and, therefore, was not completed or saturated.

**Phosphotransfer from Membrane-bound MtrB-Strep to MtrA-His\(_{10}\)**—To analyze the MtrB-MtrA phosphotransfer activity in the presence of different solutes, reconstituted MtrB-Strep was phosphorylated by the addition of 0.22 μM \([\gamma\text{-}^{33}\text{P}]\)ATP as described above in the presence of up to 600 mM concentrations of several solutes. After incubation at 30 °C for 10 min, purified MtrA-His\(_{10}\) was added at a 4:1 MtrA/MtrB ratio (132 and 33 pmol, respectively) and further incubated at 30 °C. At the indicated time points, the reaction was stopped by

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\(^4\) M. Brocker and M. Bott, unpublished data.
the addition of 9 μl of 2.4× SDS loading buffer, and the samples were directly subjected to SDS-PAGE.

**Determination of High Osmolarity Induced Shrinkage of E. coli Polar Lipid Extract Liposomes**—The measurements of high osmolarity induced shrinkage of *E. coli* lipid liposomes was performed as described by Racher et al. (20). For this purpose *E. coli* liposomes were slowly thawed at room temperature and collected by ultracentrifugation. Calcein at 10 mM concentration (Invitrogen) was diluted in 2 mM KP, containing 50 mM NaCl, and the pH was titrated to 7.2 by the addition of KOH. To entrap 10 mM calcein in the liposomal lumen, liposomes were diluted in the calcein solution to a concentration of 20 mg/ml and extruded 14–20 times through polycarbonate filters (pore diameter, 400 nm). External calcein was removed by three gel filtration steps using G75-Sepharose (Amersham Biosciences). Calcein-loaded liposomes were eluted in 1-ml fractions of 20 mM NaPi buffer, pH 7.4, containing 100 mM KCl. Osmotic upshifts were imposed by diluting 5–15 μl of calcein-loaded liposomes (5 mg/ml) into 500 μl of reaction buffer with or without added osmolyte in a 1-ml cuvette. Samples were kept at room temperature in the dark for exactly 1 min. Fluorescence measurements were performed with an Amino Bowman Series 2 spectrometer (SLM Aminco, Büttelborn, Germany) at an excitation wavelength of 495 nm and an emission wavelength of 520 nm (slit width of 8 nm).

**Determination of the Orientation of Reconstituted MtrB-Strep by Proteolysis**—Accessibility of the C-terminal domain of MtrB-Strep was determined using either intact or solubilized liposomes. After thawing, MtrB-proteoliposomes were diluted in 50 mM sodium citrate buffer, pH 6.0, to meet the conditions required for peptidase activity and extruded 14–20 times in citrate buffer. Subsequently, 2.25 μg of reconstituted MtrB-Strep were diluted in the identical buffer to a final volume of 20 μl. The reaction was performed in the presence of 0.2 μg/μl carboxypeptidase Y. In a control reaction, MtrB liposomes were disintegrated by the addition of 0.1% Triton X-100 before the protease was added. Proteolysis was carried out at 25 °C. At the given time intervals (up to 24 h) the reaction was stopped by the addition of SDS-loading buffer. The proteins were subjected to SDS-PAGE, and the gels were either stained with Coomassie Brilliant Blue, or MtrB was transferred to a polyvinylidene difluoride membrane. Integrity of the C-terminal Strep Tag II of MtrB was analyzed by Western blot analysis using the Strep-tag antibody.

**RESULTS**

**Expression Regulation of betP and proP upon Hyperosmotic Shock in C. glutamicum**—Recently, activation of the MtrB-dependent gene expression of *betP* and *proP* was analyzed by increasing the external osmolality of the medium with NaCl (9). To test whether the external ionic strength or osmolality induces gene expression, an osmotic shift with trehalose was applied. Trehalose is suitable for this purpose since it cannot be taken up by *C. glutamicum* (21), thus creating an osmotic gradient across the membrane. After reaching the mid-exponential growth phase, *C. glutamicum* wild type or ΔmtrAB cells were shifted from the external osmolality of 0.3–1.7 osmol/kg by the addition of 1.5 M trehalose. Cells were harvested at time intervals up to 180 min after the osmotic shift, and total RNA was isolated. RNA hybridization experiments revealed that in *C. glutamicum* wild type cells expression of the carrier genes was significantly increased after the hyperosmotic shift. The *betP* gene showed a relatively high basal expression already at low osmolalities, whereas the *proP* expression was not detectable before the shift and showed a higher extent of induction. In contrast, in the ΔmtrAB mutant, the expression was abolished (betP) or markedly reduced (proP) (Fig. 1). Thus, induction of the two genes after an osmotic upshift by trehalose was almost identical to the response to the NaCl addition (Ref. 9 and Fig. 1). These results can be explained in two ways. If MtrB senses osmotic stress via the external protein domains, then osmolality rather than ionic strength is detected by MtrB. On the other hand, it is also possible that an internal parameter is sensed by MtrB. Applying a hyperosmotic shock with both trehalose or NaCl will lead to the efflux of water out of the cytoplasm and, thus, to a change of the internal conditions as well as the membrane strain.

**Orientation of MtrB in Liposomes**—The orientation of a membrane protein after reconstitution is unpredictable. The reconstituted protein can be integrated in liposomes either randomly or in a unidirectional “right-side-out” or “inside-out” orientation. To analyze possible physicochemical stimuli related to hyperosmotic stress and to distinguish whether cytoplasmic or periplasmic protein domains of MtrB were involved in osmosensing, it was essential to determine the orientation of MtrB in proteoliposomes. We used a proteolytic approach to investigate the accessibility of the large catalytic C-terminal domain of MtrB, which is located in the cytoplasm *in vivo*. For this purpose either intact freshly extruded or solubilized MtrB-proteoliposomes were incubated with carboxypeptidase Y for up to 24 h (Fig. 2). At the indicated time points aliquots were withdrawn. The reaction was stopped by the addition of SDS, and subsequently proteins were separated by SDS-PAGE. The
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![Absence of triton-X-100](Image)

![Presence of triton-X-100 [0.1 %]](Image)

**FIGURE 2.** Proteolytic digestion of MtrB-Strep in proteoliposomes. MtrB-Strep in intact or disintegrated proteoliposomes (after treatment with Triton X-100) were mixed with 0.2 μg/μl carboxypeptidase Y. At the indicated time points samples were taken and quenched with SDS. MtrB was subjected to gel electrophoresis and stained with Coomassie Brilliant Blue (upper panel) or detected after immunoblot analysis (lower panel). Left, MtrB-proteoliposomes. Right, disintegrated MtrB-proteoliposomes. The dataset is an example of 4–5 independent experiments using different proteoliposome preparations with almost identical results.

rate of degradation of MtrB was slightly faster in intact proteoliposomes than in the solubilized material. Starting at 30 min after the addition of the peptidase, a small but significant mass decrease of the intact MtrB was observed, but no smaller bands were detected during the course of the experiment. The limited degradation of MtrB at the C-terminal end is likely to be caused by the presence of several glycine residues in this region that are known to retard carboxypeptidase Y activity. To prove that degradation occurred in fact at the C-terminal end, Western blot analysis was carried out using an antibody raised against the Strep Tag II. In other words the truncated forms were N-terminally degraded by proteases of E. coli during synthesis. The smallest active form of MtrB was estimated to contain at least the second transmembrane segment of MtrB and the complete cytoplasmically located catalytic domains (Fig. 3A). From this observation it can be concluded that in these variants the HAMP, the phosphorylation, and the dimerization domains of MtrB were intact. Nevertheless, this is not proof that these truncated variants still contain the unchanged sensing domain, which would be essential for the characterization of the regulatory properties of the kinase. Thus, for quantification of MtrB phosphorylation, only the full-length form was taken into consideration, which was recently determined by Edman sequencing to be the largest protein active in our assay (14).

The MtrB was stimulated 4–9-fold by glutamate, proline, alanine, lysine, and betaine. The L-glutamate stimulated MtrB to the highest extent (Fig. 3, B and C). In this assay we used sodium glutamate; therefore, 300 mM Na⁺ was present, which in principle could be the stimulating agent. However, 300 mM NaCl led only to a minor activation of MtrB (Fig. 3D). Consequently the stimulation must be mainly caused by the L-glutamate. In an independent assay carried out at 50 or 600 mosmol/kg of the tested substances, it was proven that the activation of MtrB depended on the concentration of the solutes because low concentrations of the amino acids led to a decreased activation level (data not shown). Taken together, these results suggest that MtrB may be significantly activated not only by amino acids or amino acid derivatives, which are known to be accumulated by *C. glutamicum* under conditions of hyperosmotic stress, but also by amino acids not related to the osmo-stress adaptation.

We extended this investigation of the specificity of MtrB stimulation by testing the influence of different sugars on MtrB activity in the standard phosphorylation assay as described above. We found that monosaccharides (glucose, fructose) and disaccharides (sucrose, trehalose, or maltose) as well as higher oligomers (maltotriose, maltotetraose) were able to stimulate MtrB activity up to 4-fold (Fig. 4, A and B, not shown for maltotetraose). Also in this case a concentration-dependent activation was observed (data not shown).

A representative selection of these solutes was chosen to investigate their effect on the MtrB-MtrA signal transduction.

which accumulated in *C. glutamicum* after a hyperosmotic shift (26–29), were able to activate MtrB. For this purpose MtrB proteoliposomes were subjected to a hyperosmotic shift. To create a similar increase of external osmolality, either 600 mM concentrations of uncharged amino acids or 300 mM sodium glutamate or L-lysine·Cl⁻ were added. The proteoliposomes were incubated with 0.22 μM [³²P]ATP at 30 °C for up to 20 min. Membrane-integrated MtrB was subjected to SDS-PAGE, and the amount of autophosphorylation of MtrB was determined. We found that MtrB was stimulated by all tested amino acids (Fig. 3). However, not only the protein band corresponding to the expected molecular mass of 56 kDa but also proteins of lower masses were phosphorylated. As recently proven by immunodetection (14), these bands are truncated MtrB variants that were co-purified because they contain an intact Strep Tag II, which is located at the C terminus of MtrB. In other words the truncated forms were N-terminally degraded by proteases of *E. coli* during synthesis. The smallest active form of MtrB was estimated to contain at least the second transmembrane segment of MtrB and the complete cytoplasmically located catalytic domains (Fig. 3A). From this observation it can be concluded that in these variants the HAMP, the phosphorylation, and the dimerization domains of MtrB were intact. Nevertheless, this is not proof that these truncated variants still contain the unchanged sensing domain, which would be essential for the characterization of the regulatory properties of the kinase. Thus, for quantification of MtrB phosphorylation, only the full-length form was taken into consideration, which was recently determined by Edman sequencing to be the largest protein active in our assay (14).

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cascade. For this purpose MtrB was phosphorylated for 15 min in the presence of 0.22 μM [33P]ATP together with various solutes, which increased the external osmolality to about 600 mosmol/kg. Subsequently, MtrA was added at an MtrB:MtrA ratio of 1:4 (mol:mol), and the phosphorylation reaction was continued for further 20 min. Under all tested conditions, a small basal amount of phosphorylated MtrB was detected (data not shown), but the major fraction of phosphorylation was found in MtrA (Fig. 5). Quantification of the signals clearly showed that the stimulation pattern of MtrA was similar to that of MtrB (Figs. 3 and 4). Thus, a more efficient stimulation of MtrB, e.g. by glutamate, was reflected by a higher amount of phosphorylation detected in MtrA. Obviously, the stimulation of MtrB is transduced to MtrA. Furthermore, MtrA does not influence the effect of different solutes on MtrB activity.

To investigate the specificity of MtrB stimulation, we carried out the standard phosphorylation assay with the reconstituted fumarate sensor DcuS from E. coli as a control. DcuS is a well characterized histidine kinase that senses C₂-carboxylates under anaerobic conditions by ligand binding of the carboxylate to the periplasmatic loop (41). Recently, we showed that DcuS was not stimulated in vivo by increasing osmolalities. In other words it does not sense osmotic stress, although it was, like MtrB, efficiently activated by monovalent cations in the reconstituted state (14). From these experiments we concluded (i) that DcuS is obviously not able to sense osmotic stress in vivo and that (ii) monovalent cations may have a general activating influence on histidine kinases at least in the reconstituted state. Because of the described properties, DcuS seems to be an appropriate tool to distinguish between osmo-specific stimuli...
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Influence of sugars on the autophosphorylation activity of MtrB-Strep.

Proteoliposomes enriched with MtrB were incubated in buffer containing 600 mM concentrations of different sugars, respectively. The reaction was performed as described in Fig. 3. A, autoradiogram. The arrow indicates the intact form of MtrB used for the quantification of the autophosphorylation. B, quantification of the phosphorylation signal corresponding to intact MtrB (arrow), glucose; fructose; trehalose; maltose; melibiose; maltotriose. The experiments were carried out at least twice with almost identical results.

Influence of different solutes on the phosphotransfer reaction between MtrB and MtrA.

Proteoliposomes enriched with MtrB-Strep were incubated in buffer containing either 300 or 600 mM concentrations of different solutes (compare Figs. 3 and 4). After 10 min of autophosphorylation of MtrB, MtrA was added in a 4-fold molar excess, and the mixture was incubated for additional 20 min. The further treatment was performed as described in Fig. 3. A, autoradiogram. B, quantification of the MtrA phosphorylation signals. The experiments were carried out at least two times with almost identical results. glu, sodium glutamate; gluc, glucose; tre, trehalose; lys, lysine-Cl; ala, alanine; pro, proline.

Which Domain of MtrB Is Involved in Osmosensing?

The PEGs offer a possibility of using chemically similar compounds with different molecular size. Thus, they provide a tool to examine whether membrane permeability of a compound is a critical factor in the activation process of MtrB. The PEGs with low molecular size ranging from monoethylene glycol to hexaethylene glycol are thought to be membrane permeant, whereas higher molecular weight PEGs are membrane-impermeant (20). Intact MtrB-liposomes containing the cytoplasmic domain in an inside-out orientation were mixed with different PEGs (varying from PEG 2 to PEG 28) at PEG concentration of 150 mM. In these experiments lower concentrations were used because the solutions of higher molecular weight PEGs became viscous in higher concentrations, interfering with our assay. In the presence of diethylene glycol (PEG 2) no stimulation of MtrB activity was observed. Starting from PEG 4, the autophosphorylation signal increased with the PEG size in the following pattern: PEG 4 < PEG 6 < PEG 8 < PEG 12 < PEG 28 (Fig. 6). The 2.5-fold activation was lower compared with the other tested solutes. However, it has to be taken into account that a PEG concentration of only 150 mM was used in the assay. A higher stimulation was observed if 300 mM PEG 4, PEG 6, and PEG 8 were applied; thus, PEGs also stimulate MtrB in a concentration-dependent manner (Fig. 6A, right panel).

Because some small PEGs were able to stimulate MtrB, although they are described as membrane impermeant (PEG 4 and PEG 6), we additionally tested the effect of the membrane-permeant compounds urea and glycerol. These solutes failed completely to stimulate MtrB when present in 150 mM (Fig. 6, A and B; data not shown for urea). In a control experiment (supplementary Fig. S3), we showed that under...
identical assay conditions PEGs and glycerol had no stimulating effect on DcuS activity, indicating that the addition of PEGs represents a specific osmostress-related signal for MtrB.

Because it was not possible to find an unequivocal correlation between membrane permeability and the ability to activate MtrB, we investigated the membrane permeability of several tested substances under our assay conditions. For this purpose shrinkage of liposomes made from E. coli polar lipid extract was analyzed in the presence of the solute of interest. Liposomes were loaded with 10 mM concentrations of the fluorescent dye calcein by means of the extrusion method, separated from excess external dye by repeated steps of gel filtration, and diluted into buffer in which the osmolality was increased to about 300 mosmol/kg by the addition of several solutes including amino acids, sugars, PEGs, and glycerol. In the case of PEG 28 only 150 mM concentrations of solute were added due to the above described problems with the high viscosity at elevated concentrations. Fluorescence was measured after sample incubation for 1 min in the dark. The liposome shrinkage leads to an increased luminal calcein concentration causing self-quenching of the fluorescein derivative. The analyzed osmolytes like salts, amino acids, and sugars produced almost identical results (Fig. 7), namely a reduction of the signal from 2.6 arbitrary units (AU) to 0.5–0.7 AU. In contrast, no shrinkage was detected in the presence of PEG 2, PEG 4, PEG 6, or glycerol. The PEG 8 caused partial shrinkage. The high molecular weight PEGs, PEG 12, and PEG 28 caused signal reductions similar to those found for other membrane impermeant solutes. Taken together, the fluorescence measurements revealed that with the exception of PEG 4 and 6, MtrB stimulation was found only if membrane-impermeant solutes were used.

To elucidate this situation in more detail, we tested whether liposome shrinkage is essential for MtrB activation. Glucose added to the buffer has been shown to activate MtrB in our standard assay in which shrinkage is induced (Fig. 4). To measure the influence of osmolytes under iso-osmotic conditions, i.e. when no shrinkage occurred, the assay was varied as follows. Thawed proteoliposomes were extruded in the presence of 600 mM glucose (i.e. elevating the osmolality by about 600 mosmol/kg) to equilibrate the osmolyte concentration across the membrane. Then, the liposomes were washed twice in an iso-osmotic glucose-free buffer before they were suspended again in a buffer, in which the osmolality was increased to 600 mosmol/kg by the addition of either glucose, proline, glutamate, or LiCl. The LiCl was recently shown to have neither a stimulatory nor inhibitory effect on MtrB activity (14). Thus, it represents an ideal osmolyte to balance the osmolality in our assay without influencing MtrB activity. We observed that glucose, when present only in the liposomal lumen, did not cause activation of MtrB. Only if glucose, glutamate, or proline was present in the external buffer under iso-osmotic conditions, was MtrB stimulated

**FIGURE 6.** Influence of polyethylene glycols with various molecular size on the autophosphorylation activity of MtrB-Strep. Proteoliposomes enriched with MtrB were incubated in buffer containing 150 mM PEG 2, PEG 4, PEG 6, PEG 8, PEG 12, PEG 28, or glycerol (gly) (left panel), or 300 mM PEG 4, PEG 6, and PEG 8 (right panel). The reaction was performed as described in Fig. 3 A, autoradiogram. The arrow indicates the intact form of MtrB used for the quantification of the autophosphorylation. B, quantification of the MtrB autokinase activity. The experiments were carried out at least two times with almost identical results.

**FIGURE 7.** Measurement of liposome shrinkage using calcein. Osmotic upshifts were imposed on calcein-loaded liposomes by dilution of liposomes in buffer containing either 150 mM salts or PEG 28 or 300 mM concentrations of all other solutes. Fluorescence measurements were performed as described under “Experimental Procedures.” glu, sodium glutamate; tre, trehalose; gluc, glucose; tre, trehalose; malt, maltose; pro, proline; ala, alanine; gluc, glucose. AU, arbitrary units.
Histidine Protein Kinase MtrB

The topology prediction of MtrB reveals that this protein belongs to the prototypical histidine protein kinases (subfamily HPK1a (30)). According to this prediction, MtrB has a very short N-terminal domain of only five amino acids and two transmembrane segments that are connected by a large periplasmic loop consisting of 144 amino acids. The 310 amino acids composing the C-terminally located cytoplasmic part contain the kinase and phosphatase domain as well as a putative HAMP domain, which is supposed to be involved in signal transduction processes in histidine protein kinases, adenylate cyclases, methyl-accepting chemotaxis proteins, and phosphatases (31). To test the hypothesis that the osmosensing domain of MtrB is located within the cytoplasmic part of the kinase and not in the periplasmic loop, as typically found in other histidine protein kinases, three different mutants were constructed. In one mutant, MtrBΔL134, only the soluble cytoplasmically located part of MtrB was present, i.e. the N-terminal 190 amino acids containing the two transmembrane segments were deleted. In the second mutant, MtrBΔL134, a major part of the large periplasmic loop comprising 134 of 144 amino acids, was deleted. In addition a mutant was constructed carrying a deletion of the complete HAMP domain. All mutants were constructed as fusion proteins carrying Strep Tag II at the C terminus. The induction of the mutant genes in the E. coli strain BL21 was performed as recently described for wild type derivatives mtrB-strep (14). Subsequently, the MtrB derivatives were purified according to the protocol developed for the wild type protein. The SDS analysis of the isolated MtrB derivatives revealed that similar to the wild type protein, in all mutants the truncated forms of the Strep-tag MtrB derivatives were co-purified with the full-length protein (supplemental Fig. S4).

In the phosphorylation assay either the soluble form of MtrBΔL134 or proteoliposomes containing MtrBΔHAMP were used. In contrast to many examples found in the literature, the soluble domain of MtrB was inactive (data not shown). For example, in E. coli, 27 of 30 predicted two-component sensor histidine kinases were purified in the truncated form containing the cytoplasmic part only. Of these 27 truncated derivatives, 25 were shown to autophosphorylate in vitro (32). Because dimerization is the prerequisite for autokinase activity, truncated and, thus, soluble histidine kinases should in principle be able to form dimers. Thus, it seems unlikely that in the case of MtrBΔL134 the loss of activity is a consequence of failure to dimerize. Rather, it should be taken as an indication that integration into a membrane environment is a prerequisite for MtrB activity. This is in accordance with the observation that solubilized MtrB was also inactive (14). The loop mutant MtrBΔL134, however, was fully active after reconstitution. In the standard phosphorylation assay this mutant was stimulated by the addition of a variety of solutes similar to the wild type protein (Fig. 9, A and B). The analysis of MtrBΔL134 indicated that the loss of the periplasmic loop of MtrB did not change its sensory properties. Thus, the sensing domain seems to be located within the cytoplasmic parts of MtrB and not in the periplasmic loop.

The deletion of the HAMP domain resulted in a MtrB mutant, which was active and stimulated by the same solutes like wild type MtrB, although the activation levels were found to be lower (Fig. 9, C and D). Therefore, we suggest that the HAMP domain is in principle dispensable for the sensing and signal transduction process of the osmostress-dependent activation. However, this part of MtrB seems to be at least important for an effective activation of MtrB under conditions of osmotic stress.

DISCUSSION

The two-component system MtrB-MtrA is highly conserved in actinobacteria (9, 33), which indicates its importance for the physiology of these organisms. In mycobacteria the response regulator MtrA seems to be essential (34), whereas it was possible to generate ΔmtrAB as well as ΔmtrA mutants in C. glutamicum (9, 10). It is not clear whether the MtrB-MtrA system has an identical function in all actinobacteria, because in many species its physiological role is not well characterized. In mycobacteria there is evidence that the system is involved in cell envelope biosynthesis, virulence, and DNA replication (35, 36). Also in C. glutamicum the MtrB-MtrA system seems to play a role in cell wall biosynthesis and, furthermore, is involved in the osmostress response (9). The question now arises of what stimulus (or stimuli) is sensed by MtrB to regulate genes involved in rather different cellular processes. To get an insight into its sensing mechanism we studied the ability of the histidine protein kinase MtrB to detect osmotic stress in vitro.

Four major categories of parameters, which in principle could be sensed as correlates to hyperosmotic stress, are discussed in literature (11). (i) Stimuli originating from the environment may directly affect receptors, e.g. external osmolarity, ionic strength, or the concentration of particular solutes. (ii) The same parameters can be sensed at the cytoplasmic side too, since an increase of the external osmolarity induces efflux of water that consequently leads to an increase in the concentration of all internal solutes and to crowding of macromolecules.

FIGURE 8. Effect of LiCl, glucose, glutamate, or proline on MtrB-Strep activity under iso-osmotic conditions. Proteoliposomes were loaded with 600 mM glucose, washed under iso-osmotic conditions, and subsequently suspended in iso-osmotic buffer containing 300 mM LiCl or sodium glutamate or 600 mM glucose or proline. Gluc, glucose; glu, glutamate; pro, proline. Intact MtrB is indicated by the arrow. The experiment was carried out twice with almost identical results.

LiCl  gluc  glu  pro

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FIGURE 9. Influence of amino acids and amino acid derivatives on the autophosphorylation activity of MtrBΔL134 and MtrBΔHAMP. Proteoliposomes enriched with MtrBΔL134 or MtrBΔHAMP were incubated in a buffer containing either 300 mM sodium glutamate or lysine-Cl or 600 mM concentrations of the other tested solutes. The reaction was performed as described in Fig. 3. A, autoradiogram of the autophosphorylation of MtrBΔL134. B, quantification of the MtrBΔL134 autokinase activity in comparison to the full-length MtrB-Strep. The experiments were carried out at least twice with almost identical results. wt, wild type. C, autoradiogram of the autophosphorylation of MtrBΔHAMP. The arrow indicates the intact form of MtrBΔHAMP used for the quantification of the autophosphorylation. D, quantification of the MtrBΔHAMP autokinase activity. glu, sodium glutamate; pro, proline; gb, glycine betaine; lys, lysine; gluc, glucose.

within the cell. (iii) Membrane-related parameters like cell turbidity or membrane strain may be relevant stimuli for membrane proteins. (iv) Changes in the external or internal osmolality may also directly influence soluble and membrane-embedded proteins by altering their surface hydration and, thus, their conformation. Because changes in most of these parameters are consequences of the water efflux from the cell, which occurs in milliseconds, it is very likely that the sensing events are very fast processes, too. This is one of the reasons why it is virtually impossible to discriminate in vivo which of the different putative stimuli is actually detected by the investigated osmosensor. Consequently, for successfully dissecting the sequence (or chain) of events, it is necessary to reduce the complexity of the system. This can be achieved by reconstitution of MtrB in proteoliposomes where both the external and internal solvent as well as the membrane composition are freely accessible to experimental variation.

Recently we showed that monovalent cations are able to stimulate MtrB autokinase activity in proteoliposomes, but these ions seem to activate reconstituted histidine kinases in general (14). Thus, we concluded that cation concentration cannot be the physical parameter sensed by MtrB, although other osmosensors, two component systems as well as uptake systems for compatible solutes, use cations as a measure for hypertonicity (12, 13, 22–25). Because of the observation that all osmosensors investigated so far detect a cytoplasmic correlate of hyperosmotic stress, like ionic strength or the concentration of an internal solute (12, 13, 22–25), we initially tested whether known cytoplasmic solutes have an effect on MtrB. Taking advantage of the unidirectional inside-out orientation of MtrB in the liposomal membrane, we were able to test the influence of different solutes on the cytoplasmic protein domains by adding them directly to the assay buffer. The in vitro investigation of MtrB autokinase activity in the presence of various osmolytes of different chemical nature showed that the sensor kinase was in fact stimulated by the presence of the tested sugars, compatible solutes, amino acids, and some PEGs (PEG 4 and larger) and that these effects were concentration-dependent. Within the range of tested solutes, membrane-impermeable solutes in general were able to stimulate MtrB, whereas membrane-permeable substances like glycerol, urea, or PEG 2 were not. The only exceptions from this rule were PEG 4 and PEG 6, which led to a significant activation of MtrB, although they were shown to be membrane-permeant under our assay conditions. Obviously, the molecular size scales for membrane permeability and activation of MtrB differed to some extent of each other. The same substances did not influence the in vitro activity of DcuS from E. coli, which served as a control. Hence, the stimulation by various osmolytes turned out to be specific for the MtrB sensor kinase.

What is the principle causing activation of MtrB by such a large variety of substances? It seems rather unlikely that MtrB is able to bind these chemically very diverse compounds in a specific binding pocket. Instead it seems more reasonable that MtrB uses a chemical or physical signal that is triggered by these different solutes. Osmolality can very likely be excluded due to the observation that membrane-permeant substances like PEG 2, glycerol, and urea failed to activate MtrB. The fact that, besides PEG 4 and PEG 6, only membrane-impermeant substances were able to stimulate MtrB led to the hypothesis that membrane shrinkage (or a correlate of shrinkage), which is induced by these types of solutes, activates MtrB. It is important to note that we were able to rule out this particular hypothesis, since membrane-impermeant substances like glucose or glutamate activated MtrB also under iso-osmotic assay conditions, when shrinkage does not occur.

Low molecular weight substances such as glucose were able to activate MtrB to a level comparable with that of maltotriose,
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maltoheptaose, and high molecular weight PEGs. This indicates that MtrB is regulated by the hydration state of the protein, influenced by the prevailing water activity in the cell rather than by macromolecular crowding, i.e. the concentration of macromolecules in the cytoplasm. An example of an enzyme activity directly regulated by water activity and, thus, by the hydration state of the system is hexokinase (37). The dissociation constant for glucose binding to hexokinase was found to decrease with increasing osmotic pressure of the assay medium varied by the addition of PEGs with molecular weight higher than 1000. It was concluded that high molecular weight PEGs were sterically excluded from clefts in the protein surface. Other studies confirmed that substances like glycerol and PEG 2 were not excluded from protein surfaces. In contrast, higher molecular weight PEGs (starting with PEG 4), osmoprotectants (e.g. glycine betaine), sugars (e.g. glucose), and amino acids (e.g. proline, alanine) showed preferential hydration of protein surfaces as a consequence of preferential or steric exclusion (38 – 40). Taken together, these data suggest that the activation of MtrB arises via preferential and/or steric exclusion of osmolytes from the surface of MtrB or from the interface between MtrB and the membrane, thus leading to a conformational change of the histidine kinase. This type of regulation was also suggested for the osmoregulated uptake system ProP of E. coli, which can be activated both by cations and by PEGs larger than PEG 4 (20, 25).

In a prototypical two-component system the periplasmic loop harbors the sensing function. Typical examples are DcuS, CitA, or PhoQ (7, 41, 42). In these cases the periplasmic location of the sensing domain seems reasonable, since they detect the presence of external nutrients like fumarate, citrate, or the external supply of divalent cations. In the case of MtrB, the question arises of which domain carries the sensor function. Based on our results, we suggest that at least the osmosensing function of MtrB is not associated with the periplasmic loop but rather with the cytoplasmic parts of MtrB, as indicated by the following data. (i) Under iso-osmotic conditions, MtrB was stimulated by different solutes only if they were provided at the same side as the cytoplasmically located catalytic domains of MtrB. Because shrinkage was proven not to be essential for activation, it is furthermore unlikely that membrane parts of MtrB together with the cytoplasmic domains are responsible for activation. (ii) The mutant MtrBΔL134 missing the major part of the periplasmic loop was still able to respond to the stimulus exerted by the different activating solutes. (iii) Within the cytoplasmic parts of MtrB, which are possible candidates for the detection of osmotic stress, the HAMP domain does not seem to be essential for the sensing process.

In contrast to MtrB, however, mutational analyses of EnvZ revealed that the amino acids that are critical for stress signalling in this sensor kinase are spread within the protein. The most critical domains were found to be located in the linker region (subsequently named HAMP domain) between the second transmembrane segment and the cytoplasmic transmitter domain (43) and around the site of autophosphorylation including the X-region (44). Interestingly, periplasmic loop deletions resulted in a constitutive OmpF<sup>−</sup> OmpC<sup>−</sup> phenotype (45). This indicates that the external loop, in contrast to MtrB, is functionally significant for osmosensing in EnvZ. With respect to the function of the periplasmic loop, MtrB seems to be more similar to the sensor kinase KdpD of E. coli. It was reported that a truncated derivative of KdpD possessing both the large N- and C-terminal domain but lacking all four transmembrane segments was still able to sense low K<sup>+</sup> concentrations. It was, thus, suggested that the sensing domain is located in the two large cytoplasmic parts of the KdpD protein (46).

More recently, the in vivo analysis of various truncated derivatives of KdpD revealed a more defined location of the sensing domain, which turned out to be situated within the soluble C-terminal part of this protein (47). Thus, at least KdpD and MtrB, which are involved in the detection of K<sup>+</sup> limiting conditions and/or osmosensing, differ from the prototypical histidine protein kinases described above, since their sensing function depends on the cytoplasmic protein domains.

Future analyses will reveal whether stimulus detection may function independently from the periplasmic loop in sensing processes where other types of environmental stress like heat, chill, oxidative, or envelope stress are involved. At least in the case of chill and envelope stress, evidence was provided that the parts of the sensor kinases other than the periplasmic loop are involved. Examples of systems involved in chill stress adaptation are DesK and Hik33 from Bacillus subtilis or Synechocystis (5, 48), respectively. For DesK, controlling the expression of a desaturase in fatty acid biosynthesis, it was shown that the membrane fluidity determines the activity status of the kinase in vivo (5). The periplasmic loop of the histidine protein kinase LiaS from B. subtilis, which is involved in the adaptation to envelope stress (49), contains only 12 amino acids. Thus, also in this case it seems rather unlikely that the sensor is located in this part of the protein. Consequently, sensing mechanism of the LiaS-type kinase was defined as “intramembrane sensing” (49).

Does the sensing mechanism of MtrB represent a general functional principle for other histidine protein kinases involved in the adaptation to osmotic stress? As a matter of fact, all the other osmoregulated two-component systems seem to use an internal stimulus for the detection of osmotic stress (Refs. 22 – 23 and 50 and this work). However, the sensing mechanisms differ in details. Whereas MtrB is stimulated by a broad variety of chemically different solutes, EnvZ, KdpD, and CpxA were shown to detect osmotic stress by sensing the increasing concentrations of monovalent cations (22 – 23, 50). Therefore, to date a general model for the osmosensing mechanism of histidine kinases cannot be proposed. The recent observation that under in vitro conditions reconstituted DcuS was also stimulated by monovalent cations (14) despite the fact that this system does not sense osmotic stress in vivo, it should be taken into consideration in future studies whether monovalent cations are indeed sensed by EnvZ, KdpD, and CpxA (22 – 23, 50) as a specific stimulus for the detection of osmostress.

In summary, we provide evidence that MtrB detects osmotic stress at cytoplasmic protein domains via the modulation of the hydration state of the protein, which is induced via preferential and/or steric exclusion of osmolytes from the protein surface. In the future more detailed analyses are necessary to reveal where the osmosensor is located in the cytoplasmic domains of
MtrB. In principle two scenarios are feasible. Either the hydration state of the protein is sensed by MtrB via the complete cytoplasmic domain or the osmosensitive region is restricted to a small area within the cytoplasmic domains of MtrB. Possible candidates for such smaller-sized units of stress detection are (i) the N-terminal extension, which is predicted to comprise the first five amino acids, or (ii) subdomains of the large catalytic domain, which is comprised of the HAMP (which does not seem to be essential in case of MtrB), the kinase, and the phosphatase domains (Fig. 3A). The latter parts of MtrB seem to be more suitable for this purpose than the short stretch of amino acids at the N terminus.

Because of the fact that the MtrB-MtrA system is responsible for the expression regulation of different target genes involved in osmoregulation and cell wall metabolism, future work has to address the question whether MtrB, which is multifunctional with respect to its targets, may possibly be also multifunctional with respect to the kind of stimulus input. In other words the interesting question arises of whether one single sensing input site is enough for the regulation of different cellular processes or whether different sensing domains related to different stimuli may be present.

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