Acylphosphate formation by the Menkes copper ATPase

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Abstract The Menkes ATPase is the product of the *MNK* gene, defective in some inherited human disorders of copper metabolism. We here show the formation of an acylphosphate intermediate by the murine *MNK* homologue in membranes from normal and copper resistant Chinese hamster ovary cells. In the latter, fivefold higher levels of acylphosphate were formed. Challenging these cells with copper, which induces relocation of the *MNK* ATPase from the *trans*-Golgi network to the plasma membrane, did not influence acylphosphate formation. The kinetics of phosphorylation, metal dependence, and sensitivity to inhibitors were investigated. The results show that the *MNK* ATPase is an active P-type ATPase and provide a direct functional test for this enzyme.

Key words: Menkes disease; P-type ATPase; Copper; Phosphorylation; Copper resistance; (Hamster)

1. Introduction

Copper is an essential trace element that functions as a redox center in over 30 enzymes, but if not carefully controlled, can form highly reactive radicals that are detrimental to cells [1]. However, how copper can access mammalian cells and their subcellular compartments is still largely unknown. Two putative human copper pumping ATPases that may be involved in the process, *MNK* and *WND*, have recently been cloned. They are associated with the inherited disorders of copper metabolism, Menkes disease and Wilson disease [2–6]. Similar putative copper pumps were also cloned from yeast [7,8] and bacteria and over 20 putative copper ATPases are known today [9–13].

All these heavy metal ATPases cloned so far belong to the class of P-type ATPases, exemplified by Ca^{2+}- and Na^+K^+-ATPases. A key feature of these enzymes is the formation of an aspartylphosphate intermediate from the γ-phosphate of ATP as part of the reaction cycle. The aspartic acid that is phosphorylated is part of the sequence Asp-Lys-Thr-Gly-Thr that is uniquely conserved in all P-type ATPases [14]. However, apart from this and some other strongly conserved domains, heavy metal ATPases are quite distinct from non-heavy metal ATPases. For one, their predicted membrane topology is C-m-m-m-m-C-m-m-C-m-m-C (C, cytoplasmic domain; m, transmembranous helix), compared to C-m-m-C-m-m-C-m-m-m-m-C for non-heavy metal ATPases. Also, heavy metal and non-heavy metal ATPases clearly occupy separate evolutionary branches [13,15].

While strong indirect evidence for a function in copper transport exists for several of the putative copper ATPases, direct measurements of ATPase activity, acylphosphate formation, or copper transport have so far only been reported for the CopB ATPase of *Enterococcus hirae* [16,17]. Particularly for the human Menkes and Wilson ATPases, direct functional test have met with great difficulty and the evidence for the role of these enzymes remains indirect. In Menkes disease, copper is accumulated by mucosal cells, fibroblasts, kidney epithelial cells and other cell types, but not by hepatocytes, and fails to reach peripheral organs. The resultant deficiency of copper dependent enzymes causes severe neurological disorders, defective keratinization and arterial and bone abnormalities, and leads to death in early infancy. It had been shown that mutations in the *MNK* ATPase are responsible for Menkes disease [18]. These and the following observations suggested that the *MNK* ATPase is required for copper export from cells: (i) cultured fibroblasts of Menkes patients are deficient in copper secretion [19], and (ii) copper resistant Chinese hamster ovary (CHO) cells overexpress *MNK* ATPase and exhibit enhanced copper efflux [20]. In these overexpressing CHO cells, the *MNK* ATPase was localized to a *trans*-Golgi compartment under normal growth conditions, but was found to relocate to the plasma membrane if the cells were challenged with copper [21]. We here show acylphosphate formation by the Menkes ATPase of CHO cells, its response to heavy metal ions and inhibitors, and the kinetics of phosphorylation and dephosphorylation. We also show that *MNK* ATPase activity is elevated in copper resistant CHO cells, supporting its role in cellular copper secretion.

2. Materials and methods

2.1. Cell lines and tissue culture

Copper resistant CHO cells overexpressing Menkes ATPase (CUR3), the corresponding control cell line (K1), and the growth conditions have been described before [20]. Cells were cultured in 225 cm^2 flasks without the addition of copper (CUR3−). Basal media copper content was 0.8 μM. At 80% confluence, flasks were washed at room temperature with twice 10 ml 0.2 M Tris-SO_4 and suspended in 10 ml of the same buffer by scraping. Following centrifugation for 5 min at 500×*g*, cell pellets were snap-frozen in liquid nitrogen. To generate 'induced' cells (CUR3+), 80% confluent cultures were exposed for 4 h to fresh media containing 500 μM $CuSO_4$ and all the buffers were supplemented with 150 μM $CuSO_4$.

2.2. Preparation of membranes from CHO cells

Frozen pellets of 2×10^6 cells were suspended in 2 ml of ice cold 0.2 M Tris-SO_4, 0.5 mM dithiothreitol, pH 7.4, and the cells disrupted by sonication for 10 s with an Mk2 sonifier (MSE Scientific Instruments) at the lowest energy setting. Following centrifugation for 5 min at 6000×*g*, the membrane fraction was collected by ultracentrifugation for 10 min at 76000×*g*. The resultant pellets were suspended in 200

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Abbreviations: CHO, Chinese hamster ovary; K1, control CHO cell line; CUR3, copper-resistant CHO cell line; CUR3−, CUR3 cells grown without added copper; CUR3+, CUR3 cells grown in 500 μM $CuSO_4$; SDS, sodium dodecyl sulfate

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μl of 20 mM Tris-SO_4, 250 mM sucrose, 5 mM dithiothreitol, pH 7.4 and membrane protein concentrations determined by the method of Bradford [22], using bovine serum albumin as a standard. Membranes could be stored frozen at −20° without detectable loss of activity for several weeks. The addition of a protease inhibitor cocktail (0.5 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 0.5 mg/ml pefabloc) to the membrane preparation did not appear to influence the phosphorylation patterns or the stability of the membranes.

2.3. Formation of acylphosphate intermediates

Membrane samples containing 20 μg of protein were phosphorylated at 37°C in a total volume of 50 μl of 20 mM Tris-SO_4, pH 7, 250 mM sucrose, 5 mM $MgSO_4$, 5 mM dithiothreitol, 1 μM $CuSO_4$, and other additions as specified under Section 3. The reactions were started by the addition of 5 μCi of [γ-^{32}P]ATP (100 μCi/nmol, Bresatec, Adelaide, Australia) and terminated with 5 μl of 20% SDS. After 3 min at room temperature, the reactions were diluted 20-fold with 0.1 M NaP_i, pH 6, 1% Triton X-100, 2 mM EDTA, containing 1 : 1000 diluted Menkes antiserum [21] and 1 : 50 diluted, fixed *Staphylococcus aureus* cells (Pansorbin, Calbiochem). After shaking for 2 h at room temperature, the *S. aureus* cells were collected by centrifugation for 1 min at 6000×*g* and washed once with 0.5 ml of the same buffer without antibody and Pansorbin. The final pellets were suspended in 20 μl of acidic sample buffer and allowed to dissociate for 5 min at room temperature before loading onto acidic SDS gradient gels. In pulse-chase experiments, 2 mM cold ATP was added as indicated. Sensitivity to hydroxylamine was tested by treating samples for 5 min with 250 mM NH_2OH in 100 mM Na-acetate, pH 5.6, prior to immunoprecipitation and SDS gel electrophoresis.

2.4. Acidic SDS gels

SDS polyacrylamide 4 to 13% gradient gels were prepared by a modification of the method of Dame and Scarborough [23]. Polymerization of acrylamide was catalyzed by adding 1/1000 volume of each, 10% ascorbic acid, 0.3% $FeSO_4 \cdot 7H_2O$, and 3% H_2O_2 before pouring gels. Gradient gels were prepared by drawing 3 ml of 4% acrylamide (1/50 bis-acrylamide) in 0.25 M NaP_i, pH 2.4 (0.25 M *ortho*-phosphoric acid adjusted to pH 2.4 with NaOH), 1% SDS, into a 10 ml pipette, followed by 1.6 ml of 13% acrylamide, 17% sucrose, in the same buffer. The two phases were partially mixed by drawing eight air bubbles trough the solutions and the mixture dispelled into the gel plates of a Bio-Rad Mini Protean gel apparatus (Bio-Rad, Hercules, CA). After the gel had set, a 3% stacking gel in 50 mM NaP_i, pH 2.4, 1% SDS, was added. One gel per apparatus was run at room temperature at 70 V for 2 h in 50 mM NaP_i, pH 2.4, 1% SDS. Prestained molecular weight markers (Kaleidoscope, Bio-Rad) were mixed with one of the samples. After the run, free [γ-^{32}P]ATP was removed by washing the gels in 20% ethanol, 10% acetic acid for 15 min. Gels were dried and ^{32}P visualized with a PhosphoImager (Storm 830, Molecular Dynamics) and bands quantified by the program ImageQuant from the same manufacturer.

3. Results and discussion

Acylphosphate formation by a P-type ATPase is usually an easy and sensitive tool to detect and functionally test such an enzyme. Standard conditions are the reaction of membranes or purified enzyme with [γ-^{32}P]ATP, followed by precipitation of the protein with trichloroacetic acid and resolution on acidic SDS gels. However, phosphorylation of the Menkes ATPase could not be detected by this procedure. It was found that precipitation of membranes with trichloroacetic acid or other agents led to irreversible aggregation of the Menkes ATPase. It was therefore necessary to developed the modified method described under Section 2. Key steps in this procedure were the termination of the labeling reaction and dissociation of the membranes with 5% SDS, followed by immunoprecipitation of the *MNK* ATPase. Without immunoprecipitation, no labeled Menkes product could be detected above the background of other labeled proteins. Acylphosphate formation was dependent on the presence of dithiothreitol in the reactions. No phosphorylation could be observed at 0°C, as had previously been reported for the CopB copper ATPase of *E. hirae* [17].

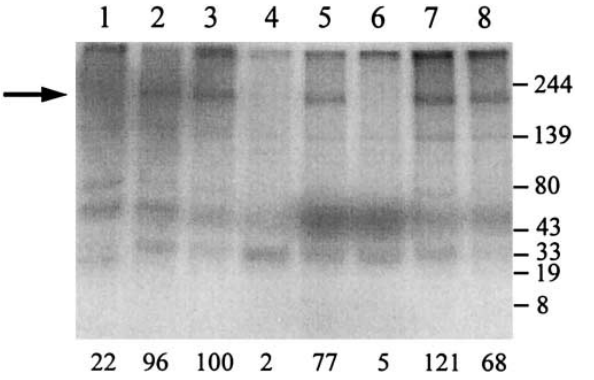

Fig. 1. Phosphoenzyme formation by Menkes ATPase. Lanes 1–3, 20 μg of membranes from CHO control cells (K1), CHO mutant cells overexpressing *MNK* ATPase and grown without added copper (CUR3−), and from overexpressing CHO cells grown in the presence of 0.5 mM copper (CUR3+), respectively, were labeled for 90 s with [γ-^{32}P]ATP, SDS dissociated, immunoprecipitated with anti-*MNK* sera, resolved on a 4 to 13% SDS-polyacrylamide gel and radioactivity visualized with a phosphoimager. Lanes 4–8, labeling as in lane 3, with the following modifications: lane 4, hydroxylamine treatment prior to immunoprecipitation; lane 5, immunoprecipitation with affinity purified *MNK* antibodies; lane 6, immunoprecipitation with pre-immune sera; lane 7, labeling in the presence of 100 μM vanadate; lane 8, labeling in the presence of 10 μM Cu^+. Other details are described under Section 2. The arrow indicates the 200 kDa band corresponding to *MNK* ATPase, the numbers below the tracks the relative intensity of the 200 kDa band, and the vertical scale the migration of marker proteins of the molecular masses indicated in kDa.

Fig. 1 shows the phosphorylation products of crude membrane fractions from different Chinese hamster ovary cell lines. A weak phosphorylated band[1] of apparent molecular mass 200 kDa could be detected in control cells (K1), whereas the same band was elevated 5-fold in copper resistant CHO cells overexpressing the *MNK* ATPase (CUR3−, CUR3+). Immunoprecipitation was complete in these experiments, as assessed by a second round of immunoprecipitation with the same samples. Also, gels similar to the one shown in Fig. 1 were subjected to Western blotting, establishing that the radiolabeled band co-migrated with the immunolabeled *MNK* ATPase band (not shown). This suggested that the labeled 200 kDa band represents phosphorylated *MNK* ATPase. Further support for this conclusion was gained from the following control experiments in Fig. 1: (i) the 200 kDa band was not observed with pre-immune sera, (ii) this band was of similar intensity when precipitated with whole or affinity purified antisera, (iii) the label was sensitive to hydroxylamine, a test reagent for acylphosphates [24], and (iv) the label of the band could be chased with cold ATP, indicating continuous turnover. The labeled 200 kDa band was also sensitive to alkaline pH, another hallmark of acylphosphate intermediates, since it could not be detected on SDS gels run at pH 8.8.

The calculated molecular mass of the hamster *MNK* gene product is 160 kDa; the larger relative molecular mass derived from the SDS gels used here has previously also been observed by Petris et al. [21]. The difference between calculated

[1] This weak signal could readily be detected by the phosphoimaging system, but proved difficult for photographic reproduction.

and observed values could be due to glycosylation and/or abnormal migration on SDS gels. Labeling of the plasma membrane Ca^{2+}-ATPase could readily be detected by SDS gel electrophoresis of samples not immunoprecipitated. It served as an internal control that no significant amounts of acylphosphate were lost in the procedure and for the effectiveness of the hydroxylamine treatment (not shown).

By Southern and Northern blotting, Camakaris et al. had estimated that CUR3 cells overexpress the *MNK* ATPase up to 70-fold compared to K1 cells [20]. We here observed a 5-fold increase in acylphosphate formation in CUR3 cells compared to controls. Whether this discrepancy in overexpression levels is due to the presence of non-functional enzyme in CUR3 cells or to an experimental shortcoming is not clear at present. Copper induces trafficking of the *MNK* protein from a *trans*-Golgi location in cells grown without added copper (CUR3−) to a plasma membrane location in cells grown in the presence of 500 μM copper (CUR3+). This trafficking did not significantly affect the overall formation of phosphoenzyme (Fig. 1), suggesting that the activity of the enzyme was maintained in the process.

Micromolar concentrations of vanadate (VO_4^{3-}) inhibit phosphoenzyme formation by non-heavy metal P-type ATPases by acting as a phosphate analogue [25–27]. Vanadate is therefore considered to be a specific inhibitor of P-type ATPases. Phosphorylation of the *MNK* ATPase was not inhibited, but slightly stimulated, by 100 μM VO_4^{3-} and could not be inhibited by up to 1 mM vanadate (cf. also Table 1). Insensitivity to vanadate has also been reported for the Cd^{2+}-ATPase of *Staphylococcus aureus* [28] while the CopB copper ATPase of *E. hirae* required half millimolar vanadate for 90% inhibition [17]. These observations could suggest that heavy metal P-type ATPases are relatively refractory to vanadate inhibition. However, given the complexity of enzyme–vanadate interactions and the chemistry of vanadate, these effects observed on crude membranes and under strongly reducing conditions should be interpreted cautiously [29,30].

Turnover of the phosphoenzyme intermediate was investigated in pulse-chase experiments (Fig. 2). Steady-state labeling was reached after approximately 90 s at 37°C and with 1 μM ATP. Dephosphorylation was slower, exhibiting a half-time of 200 s. Compared to non-heavy metal P-type ATPases, such as eukaryotic Ca^{2+}- or Na^+K^+-ATPases, the turnover of the Menkes protein was slow [31,32]. Slow turnover may be a general property of heavy metal ATPases and may, at least in part, be responsible for the difficulties encountered in assaying the function of these enzymes. The only heavy metal ATPase for which purification has been reported to date, the CopB copper ATPase of *E. hirae*, exhibited a specific activity

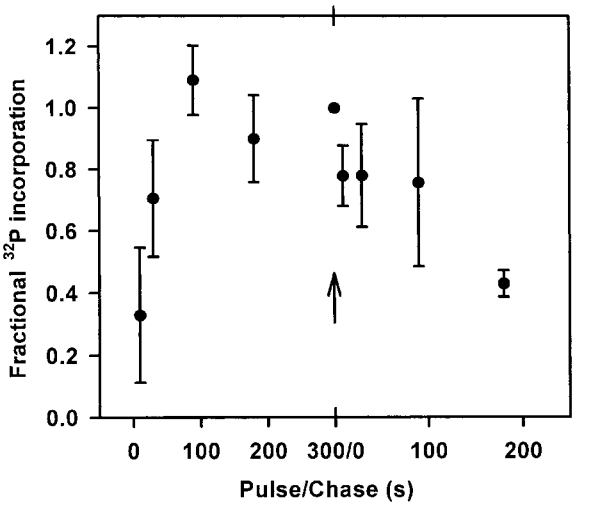

Fig. 2. Kinetics of phosphorylation and dephosphorylation. Phosphoenzyme formation by Menkes ATPase was determined as described in Fig. 1. The chase was initiated at 300 s by the addition of 2 mM cold ATP (arrow). Values for the ^{32}P incorporation were from three independent membrane preparations, normalized relative to the labeling at 300 s and expressed as fraction thereof (±standard deviation).

of 20 nmol/min/mg in the purified form, corresponding to a turnover number of 0.03 s^{-1} [17]. Of course it cannot be ruled out at present that a cofactor necessary for full activity is missing or inactive in these experiments.

The *MNK* ATPase is a member of the newly discovered sub-class of heavy metal P-type ATPases [13]. It was therefore of interest to compare some of its properties to those of non-heavy metal P-type ATPases. We tested a range of ATPase inhibitors that are diagnostic for various types of ATPases as well as some metal chelating agents (Table 1). As expected, inhibitors specific for V- or F-type ATPases and inhibitors for Ca^{2+}- or Na^+K^+-ATPases did not inhibit the labeling of the 200 kDa band to extents considered significant (discussion of vanadate see above). The copper(I) chelator bathocuproin disulfonate reduced phosphorylation by 71% at 0.5 mM. This would support a role of copper(I) in the Menkes ATPase. However, 1 μM Cu^+ that was routinely added to the phosphorylation reactions did not stimulate phosphorylation above the basal level and 10 μM Cu^+ caused a 32% inhibition (cf. Fig. 1, lane 8). The purified, reconstituted CopB copper ATPase of *E. hirae* displayed the same behavior towards copper and was 60% inhibited by 0.5 mM bathocuproin disulfonate [17]. Conceivably, there is always sufficient contaminating copper in these ATPase preparations to fully activate the

Table 1
Effects of ATPase inhibitors on acylphosphate formation by *MNK* ATPase

Inhibitor	Target ATPases	Concentration (mM)	% Acylphosphate[a]
None	–	–	100
$NaNO_3$	V-type	50	94±7
N-Ethylmaleimide	V-type	1	51±1
Bafilomycin-A_1	V-type	0.001	69±18
NaN_3	F-type	5	82±22
VO_4^{3-}	P-type	0.1	116±7
Ouabaine	P-type (Na^+/K^+)	1	81±3
EGTA	P-type (Ca^{2+})	1	94±27
Bathocuproin disulfonate	Cu^+-ATPases?	0.5	29±2

[a]$n=3$, ±standard deviation.

enzymes. The inhibition by higher copper concentrations remains unexplained at present. Other heavy metals like 100 μM Ag^{+}, Cd^{2+}, Fe^{2+}, Zn^{2+}, Ni^{2+}, or Pb^{2+} had no significant effects on either phosphorylation or dephosphorylation of the 200 kDa band (not shown).

Also of interest is the observation that the ATPase retained over 50% of the activity after treatment with 1 mM *N*-ethylmaleimide. There would be several potential target sites for this sulfhydryl reagent: human and hamster copper ATPases have, in the long polar N-terminal region, six repeats of a putative copper binding motive with consensus Cys-Xaa-Xaa-Cys and a conserved Cys-Pro-Cys motive in the predicted transmembranous helix six. These cysteines are either not very accessible to *N*-ethylmaleimide or not essential for function. While inaccessibility is a feasible explanation for the intramembranous Cys-Pro-Cys, it appears unlikely that the six Cys-Xaa-Xaa-Cys motives in the N-terminus are all buried, suggesting that at least some of them are not essential.

In conclusion, the present study shows acylphosphate formation by the Menkes ATPase of Chinese hamster ovary cells. In cells overexpressing the enzyme, increased amounts of acylphosphate were formed. This was independent of a *trans*-Golgi network or a plasma membrane location of the ATPase. The acylphosphate formation described here should provide an important functional tool for further investigation of the in vivo function and regulation of the Menkes ATPase.

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References

[1] Linder, M.C. and Hazegh Azam, M. (1996) Am. J. Clin. Nutr. 63, 797S–811S.
[2] Chelly, J., Tumer, Z., Tonnesen, T., Petterson, A., Ishikawa Brush, Y., Tommerup, N., Horn, N. and Monaco, A.P. (1993) Nature Genet. 3, 14–19.
[3] Mercer, J.F.B., Livingston, J., Hall, B., Paynter, J.A., Begy, C., Chandrasekharappa, S., Lockhart, P., Grimes, A., Bhave, M. and Siemieniak, D. et al. (1993) Nature Genet. 3, 20–25.
[4] Vulpe, C., Levinson, B., Whitney, S., Packman, S. and Gitschier, J. (1993) Nature Genet. 3, 7–13.
[5] Bull, P.C., Thomas, G.R., Rommens, J.M., Forbes, J.R. and Cox, D.W. (1993) Nature Genet. 5, 327–337.
[6] Tanzi, R.E., Petrukhin, K., Chernov, I., Pellequer, J.L., Wasco, W., Ross, B., Romano, D.M., Parano, E., Pavone, L. and Brzustowicz, L.M. (1993) Nature Genet. 5, 344–350.
[7] Fu, D., Beeler, T.J. and Dunn, T.M. (1995) Yeast 11, 283–292.
[8] Yuan, D.S., Stearman, R., Dancis, A., Dunn, T., Beeler, T. and Klausner, R.D. (1995) Proc. Natl. Acad. Sci. USA 92, 2632–2636.
[9] Kanamaru, K., Kashiwagi, S. and Mizuno, T. (1994) Mol. Microbiol. 13, 369–377.
[10] Phung, L.T., Ajlani, G. and Haselkorn, R. (1994) Proc. Natl. Acad. Sci. USA 91, 9651–9654.
[11] Ge, Z., Hiratsuka, K. and Taylor, D.E. (1995) Mol. Microbiol. 15, 97–106.
[12] Melchers, K., Weitzenegger, T., Buhmann, A., Steinhilber, W., Sachs, G. and Schäfer, K.P. (1996) J. Biol. Chem. 271, 446–457.
[13] Solioz, M. and Vulpe, C. (1996) Trends Biochem. Sci. 21, 237–241.
[14] Moller, J.V., Juul, B. and Le Maire, M. (1996) Biochim. Biophys. Acta Rev. Biomembr. 1286, 1–51.
[15] Lutsenko, S. and Kaplan, J.H. (1995) Biochemistry 34, 15607–15613.
[16] Solioz, M. and Odermatt, A. (1995) J. Biol. Chem. 270, 9217–9221.
[17] Wyler-Duda, P. and Solioz, M. (1996) FEBS Lett. 399, 143–146.
[18] Das, S., Whitney, S., Taylor, J., Chen, E., Levinson, B., Vulpe, C., Gitschier, J. and Packman, S. (1995) J. Inherit. Metab. Dis. 18, 364–365.
[19] Camakaris, J., Danks, D.M., Ackland, L., Cartwright, E., Borger, P. and Cotton, R.G. (1980) Biochem. Genet. 18, 117–131.
[20] Camakaris, J., Petris, M.J., Bailey, L., Shen, P., Lockhart, P., Glover, T.W., Barcroft, C.L., Patton, J. and Mercer, J.F.B. (1995) Hum. Mol. Genet. 4, 2117–2123.
[21] Petris, M.J., Mercer, J.F., Culvenor, J.G., Lockhart, P., Gleeson, P.A. and Camakaris, J. (1996) EMBO J. 15, 6084–6095.
[22] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[23] Dame, J.B. and Scarborough, G.E. (1980) Biochemistry 19, 2931–2937.
[24] Post, R.L. and Kume, S. (1973) J. Biol. Chem. 248, 6993–7000.
[25] Inesi, G. and Sagara, Y. (1994) J. Membr. Biol. 141, 1–6.
[26] Lingrel, J.B. and Kuntzweiler, T. (1994) J. Biol. Chem. 269, 19659–19662.
[27] Serrano, R. (1994) Arch. Biochem. Biophys. 306, 287–296.
[28] Tsai, K.J., Yoon, K.P. and Lynn, A.R. (1992) J. Bacteriol. 174, 116–121.
[29] Macara, I.G., Kustin, K. and Cantley Jr., L.C. (1980) Biochim. Biophys. Acta 629, 95–106.
[30] Chasteen, N.D. (1995) Met. Ions Biol. Syst. 31, 231–247.
[31] Pickart, C.M. and Jencks, W.P. (1984) J. Biol. Chem. 259, 1629–1643.
[32] Heyse, S., Wuddel, I., Apell, H.J. and Sturmer, W. (1994) J. Gen. Physiol. 104, 197–240.