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Journal of Chromatography A, 800 (1998) 29–37

JOURNAL OF CHROMATOGRAPHY A

Molecular characterization of metal-binding polypeptide domains by electrospray ionization mass spectrometry and metal chelate affinity chromatography[1]

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Abstract

Metal ion-binding of synthetic peptides containing HxH and CxxC motifs was investigated by electrospray ionization mass spectrometry (ESI-MS) and metal chelate affinity chromatography. A high affinity of Ni^{2+} and Cu^{2+} to HxH containing sequences was found. Based on their natural metal ion-binding potential it was possible to include metal affinity chromatography in the purification process of two proteins without using an additional His-tag sequence: ATPase-439, a P type ATPase from *Helicobacter pylori* and the amyloid precursor protein (APP). © 1998 Elsevier Science B.V.

Keywords: Metal chelate affinity chromatography; Peptides; Proteins; Metal chelates

1. Introduction

A number of peptides and proteins containing certain motifs of histidine and cysteine residues are known to specifically bind divalent transition metal ions [1,2]. Typical binding sites for Cu^{2+}, Zn^{2+} and Ni^{2+} ions comprise CxxC-motifs [3] which, for example, are present in zincfinger proteins [4]. Furthermore, histidine residues with the N-terminal motif NH_2-xxH in albumins have been reported to bind Cu^{2+} and Ni^{2+} ions [1,5]. In recent publications, electrospray ionization mass spectrometry (ESI-MS) has been shown to be a powerful method for the characterization of non-covalent complexes of biomacromolecules [6]. The successful application of ESI-MS has also been demonstrated in studies of transition metal ion–peptide complexes of zincfinger [7] and angiotensin peptides [8]. These studies have prompted our interest in a systematic study on the application of ESI-MS to identify specific metal ion–peptide interactions in polypeptides and proteins.

Two P type ATPases (ATPase-439 [9] and ATPase-948 [10,11]) from *Helicobacter pylori*, a human gastric pathogen associated with chronic

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[1]Presented at the 45th ASMS Conference on Mass Spectrometry and Allied Topics, Palm Springs, CA, 1–5 June 1997.

0021-9673/98/$19.00
PII S0021-9673(97)00877-7

gastritis [12], have found particular interest for their specific metal ion-binding properties. ATPase-948 shows considerable homology to enterococcal CopA-ATPase and has therefore been proposed to be a copper-transporting protein. The function of ATPase-439 is yet unknown, however it has been suggested to play a role in nickel ion transport. Of particular interest have been peptides at the N-termini of both ATPases which contain typical ion-binding motifs suggesting regulatory functions for ion transport. The sequences of both N-terminal ATPases contain CxxC-motifs that are characteristic for P type ATPases. However, the ATPase-439 contains an additional HxH motif which is not present in ATPase-948. Because of a possible role of ATPase-439 in nickel ion transport, we proposed that Ni^{2+} ions bind to the N-terminal sequence.

The metal ion-binding capability of histidine has been already employed in the purification of proteins containing adjacent histidine residues [13–15]. His-tagged recombinant proteins are usually purified by nickel nitrilotriacetate (NTA) metal chelate affinity chromatography [16]. The ion selectivity of corresponding natural binding sites in protein sequences should make it possible to develop a specific purification procedure for metal ion-binding proteins such as P type ATPases based on this technique, without using an additional His-tag. We have evaluated this method for the purification of ATPase-439.

Furthermore, we report here results with the amyloid precursor protein (APP), another protein containing potentially specific metal ion-binding motifs. Alzheimer's disease is a chronic neurodegenerative process characterized by deposits of β-amyloid polypeptides in the brain parenchyma and in cerebral blood vessels [17]. β-Amyloid is derived by proteolytic degradation from APP, a membrane-spanning precursor protein of which multiple isoforms are produced by alternative splicing from a single gene on human chromosome 21. Putative functional domains have been found which contain Cu-/Zn- as well as heparin-binding domains [18–20]. For a detailed structural characterization, a Cu-binding peptide comprising a HxHxH-motif, APP (135–157) was synthesized and investigated by metal chelate chromatography [21].

2. Experimental

2.1. Peptides

All polypeptides were synthesized on a semi-automated peptide synthesizer, Abimed EPS221 (Abimed, Langenfeld, Germany), using fluorenylmethoxycarbonyl (Fmoc) chemistry [22,23]. Fmoc-protected amino acids (Novabiochem, Germany) were coupled by a benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP)–*N*-methylmorpholine coupling procedure in dimethylformamide [24,25]. After completion of the synthesis, the peptides were cleaved from the resin with a solution of 90% trifluoroacetic acid (TFA), 5% water, 5% triethylsilane for 2 h at room temperature. The raw products were then precipitated with a 7-fold volume of diethyl ether at −20°C. All peptides were purified by RP-HPLC (semi-preparative μBondapak-C_{18}, Waters, UK) using a linear gradient of 0.1% TFA in water and 0.07% TFA in acetonitrile, and were then characterized by matrix-assisted laser desorption mass spectrometry (MALDI-MS).

2.2. Proteins

The recombinant ATPase-439 was overexpressed using the *E. coli*-strain MM294 carrying the plasmid PY25 (*E. coli*-PY25) containing the ATPase-439 gene in the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible vector pTI2-1 [26]. The cells were grown in Luria-Bestani medium at 37°C. Protein overexpression was induced with 0.3 m*M* IPTG for 1 h and cells were harvested by centrifugation. For the preparation of the membrane fraction, the cells were washed at 4°C with TBS buffer (0.004 *M* Tris–HCl, pH 7.5, 0.1 *M* NaCl) and suspended in 50 m*M* Tris–HCl, pH 7.5, at 5 ml per g wet cells. Lysozyme 100 μg/l and 1 m*M* EDTA were then added and the suspension was gently stirred on ice. After 1 h, 5 m*M* β-mercaptoethanol (β-ME), 1 m*M* 4-(2-amino-ethyl)-benzole sulfonylfluoride hydrochloride (Pefabloc), 50 μg/ml L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride (TLCK) and 100 μg/ml L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone (TPCK) were added and the suspension

was passed twice through a Gaulin-homogenizer (Micron Lab40, APV, Gaulin, Lübeck, Germany) at 700 bar. Intact cells were sedimented at 10 000 *g* for 15 min at 4°C. Ultracentrifugation of the supernatant (140 000 *g*, 60 min, 4°C) yielded a crude membrane fraction as pellet. The pellet was washed twice by homogenization in 3 m*M* Tris, 3 m*M* EDTA, 0.5 m*M* Pefabloc, 5 m*M* β-ME and ultracentrifugation. The washed membrane fraction was suspended in 50 m*M* NaH_2PO_4–NaOH, pH 7.5, 0.1 *M* NaCl, 10% glycerol, 5 m*M* β-ME at a protein concentration of 1 mg/ml. *n*-Dodecyl-β-D-maltoside was added to a final concentration of 1% (w/v) and the mixture was stirred on ice for 60 min. The solution was clarified by ultracentrifugation and used for Ni-NTA chromatography.

APP from human brain was pre-purified by Q-sepharose and heparin-sepharose chromatography as described [27].

2.3. Mass spectrometry

MALDI mass spectra were recorded on a Bruker Biflex time-of-flight spectrometer (Bruker-Franzen, Bremen, Germany) equipped with a UV-nitrogen laser (337 nm) and a dual microchannel plate detector. The acceleration voltage was set to 20 kV and mass spectra were calibrated with insulin. The matrix was α-cyano-4-hydroxycinnamic acid (HCCA). The standard amount of peptide for each analysis was 0.1 μg. On-target tryptic digestion [28] was carried out in the presence of 6-aza-2-thiothymine as matrix with a freshly prepared trypsin solution (0.5 μl; 0.2 mg/ml trypsin (TPCK-treated) in 50 m*M* NH_4HCO_3, pH 7.8). The reaction mixture was allowed to dry at room temperature (approx. 30 min).

ESI mass spectra were recorded on a Vestec A 201 single quadrupole mass spectrometer (Vestec, Houston, TX, USA). The peptide concentration was 0.1 μg/μl for each analysis. The solvent system, 5 m*M* NH_4OAc in water–methanol (9:1) was generaly used, and the specific pH for each analysis adjusted with acetic acid (pH 4 for the peptides ATPase-439 (1–51) and ATPase-948 (1–52); pH 5 for the peptides M1, M2, M3, M4; pH 3 for the peptide APP (135–157)). The spray temperature was 40°C and the repeller voltage (desolvation potential) was 40 V. Peptide transition metal ion complexes were measured in the presence of a 10-fold molar excess of $NiCl_2$ over peptide.

2.4. Metal chelate affinity chromatography

For metal chelate affinity chromatography, nitrilotriacetic acid (NTA) agarose (Quiagen, Hilden, Germany) was used. The NTA was pre-loaded with $CuCl_2$, $NiCl_2$ or $ZnCl_2$. The preloaded material was packed into a 1-cm column and connected to a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden). Peptides and pre-purified APP were applied to the NTA columns in 20 m*M* Tris–HCl buffer (pH 7). For the elution of the peptides, 250 m*M* imidazole in Tris–HCl buffer (pH 7) was used. The chromatograms were UV-monitored and each fraction analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

For the purification of ATPase-439, the Ni-NTA-agarose was equilibrated with 50 m*M* NaH_2PO_4–NaOH (pH 7.5), 0.1 *M* NaCl, 10% glycerol, 5 m*M* β-ME, 0.1% dodecylmaltoside. A 1-ml aliquot of the prepared NTA material was added to 10 ml of the membrane fraction of the *E. coli* cells and incubated for 30 min at 4°C with gentle shaking. The resulting material was packed in a column and washed with 30 column volumes of 50 m*M* NaH_2PO_4–NaOH (pH 7.5), 0.1 *M* NaCl, 10% glycerol, 5 m*M* β-ME, 0.1% dodecylmaltoside. Elution was accomplished with 15 column volumes each of 5, 10, and 20 m*M* imidazole in 50 m*M* NaH_2PO_4–NaOH (pH 7.5), 0.1 *M* NaCl, 10% glycerol, 5 m*M* β-ME, 0.1% dodecylmaltoside. The fractions were collected and characterized by SDS–PAGE and Western blot analysis.

2.5. Polyacrylamide gel electrophoresis

Proteins were characterized by SDS–PAGE. SDS–PAGE was performed using slab gels (0.75 mm or 1 mm thickness, 9 cm width, 6 cm resolving gel, 1.5 cm stacking gel) in a BioRad gel electrophoresis apparatus (BioRad, Munich, Germany). The gels were prepared according to Schaegger and Von

Jagow [29]. The gels were silver stained or used for Western blot analysis directly following SDS–PAGE.

2.6. Western blots

Proteins from one-dimensional SDS–PAGE gels were transferred to nitrocellulose membranes (Protran BA85, Schleicher & Schuell, Dassel, Germany) using a constant voltage of 15 V for 60 min in a semi-dry-blotting apparatus (Transblot SD, BioRad) and the buffer system according to Towbin et al. [30]. Immunodetection of ATPase-439 was performed with an antiserum raised in rabbits against a synthetic peptide of the phosphorylation domain of ATPase-439, (382–394) which was coupled to serum albumin and the enhanced chemoluminescence (ECL)-detection system (Amersham-Buchler, Braunschweig, Germany). Immunodetection of APP was carried out with the monoclonal antibody, anti-APP A4 (Boehringer Mannheim, Pensberg, Germany).

3. Results

For a systematic investigation of the role of histidine residues in nickel ion binding, a number of model peptides were synthesized by Fmoc solid-phase peptide synthesis (SPPS) (M1, Ac-WEYHIHNLDG-NH_2; M2, Ac-WEYHIAHNLD-NH_2; M3, Ac-WEYHIRNLDG-NH_2; M4, Ac-WEYRIRNLDG-NH_2). These peptides contain variations of the His residues of the putative N-terminal metal ion-binding motif of ATPase-439, HxH, but no cysteine-containing binding motif. The peptides were investigated by ESI-MS in the presence of $NiCl_2$ at increasing molar peptide:metal ion concentration ratios. The resulting ESI spectra showed increasing metal ion adducts with increasing concentration of $NiCl_2$, with a maximum of complex ion abundances reached at an approximately 10-fold molar excess. Therefore, the results discussed in the following were all obtained in the presence of a 10-fold excess of $NiCl_2$. The ESI mass spectra of the peptides showed most abundant singly charged molecular ions due to the addition of Ni^{2+} ions, as illustrated in Fig. 1 for the peptides M1 and M3. The peptide M1 (Fig. 1) containing two His residues showed the $[M+H]^+$ ion at m/z 1325 and several adduct ions with Ni^{2+}. The base peak corresponds to the complex ion by addition of one Ni^{2+} and one deprotonation, $[M+Ni-H]^+$ (m/z 1383). A further molecular ion is observed at m/z 1404 which corresponds to the addition of one Ni^{2+} and one sodium ion, $[M+Ni^{2+}+Na^+-2H^+]^+$. Molecular ion adducts with two and three Ni^{2+} ions are also observed, the latter with low abundance. In addition to singly charged molecular ions, doubly charged ions were also observed but with considerably lower abundances (not shown in Fig. 1). The ESI spectrum of M2 (not shown) showed a similar behaviour, with nickel adduct peaks at a higher relative abundance than the peak for the peptide without nickel ions. M3 (Fig. 1b) and M4 (not shown) yielded different spectra, the most abundant peak being the protonated peptide ion $[M+H]^+$ at m/z 1344 (calc., 1343.4). There was also a nickel adduct ion at m/z 1401 (calc. 1401.1 for $[M+Ni-H]^+$), and a small peak due to a second nickel ion adduct.

Comparative studies by NTA-affinity chromatography revealed high Ni^{2+} and Cu^{2+} affinities for the model peptide sequences (Table 1). Peptides containing only one His residue were found to bind Cu^{2+} but no Ni^{2+} and Zn^{2+} ions. Peptides containing two His residues close to each other were found to bind both Cu^{2+} and Ni^{2+} ions. The APP partial peptide APP(135–157) containing three histidine residues, HxHxH, was found to bind Cu^{2+}, Ni^{2+} and Zn^{2+} ions.

Two N-terminal polypeptides of the *Helicobacter pylori* P type ATPases, ATPase-439 (1–51) containing a HxHxxxCxxC motif and ATPase-948 (1–52) containing GMxCxxC, were synthesized by SPPS, purified by HPLC, and characterized by MALDI-MS and on-target tryptic digestion [28]. Both peptides contain a CxxC motif characteristic for P type ATPases. However, the ATPase-439 (1–51) peptide contains an additional HxH-motif which is not present in ATPase-948 (1–52). Because of its putative role in nickel ion transport, we considered that nickel ions may bind to the N-terminus of ATPase-439.

Metal chelate affinity chromatography revealed a high affinity of the ATPase-439 peptide to both Ni^{2+} and Cu^{2+} ions. Fig. 2 shows the ATPase-439 (1–51) peptide which was bound to the Ni-NTA column in

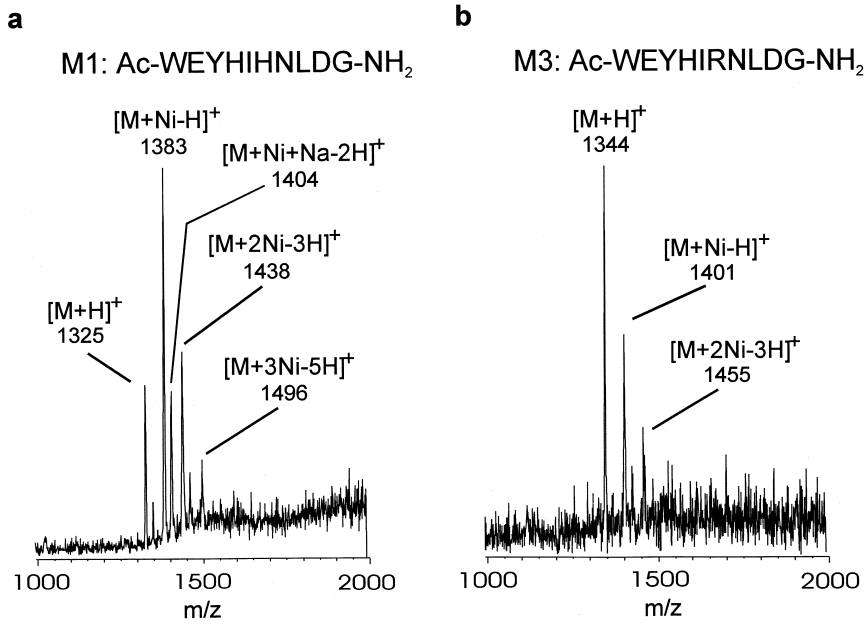

Fig. 1. ESI-spectra of model peptides (a) Ac-WEYHIHNLDG-NH_2 (M1), and (b) Ac-WEYHIRNLDG-NH_2 (M3). Spectra were determined in 5 m*M* ammonium acetate–methanol (9:1), pH 5 in the presence of a 10-fold molar excess $NiCl_2$.

20 m*M* Tris–HCl (pH 7) and eluted rapidly when the solvent was changed to 250 m*M* imidazole in 20 m*M* Tris–HCl (pH 7) or 0.1% trifluoroacetic acid in water (pH 2, not shown). The ATPase-948 (1–52) peptide was not adsorbed on a Ni-NTA column (Fig. 2) but showed high affinity to a Cu-NTA column (data not shown).

The ESI mass spectrum of the N-terminal peptide (1–51) of ATPase-439 in the presence of a 10-fold excess of $NiCl_2$ (Fig. 3) revealed high abundances of 4+ and 5+ charged molecular ions representing Ni^{2+} adducts. The Ni^{2+} ion complex formation was demonstrated to be specific for the ATPase-439 (1–51) peptide while no Ni^{2+} ion adducts were observed for the ATPase-948 (1–52) domain, which was consistent with the results of the NTA-chelate chromatography described above. By contrast, both peptides yielded copper ion adducts in the presence of $CuCl_2$, under identical conditions. This difference in binding specificity has been previously described [31]. Generally, an increase of charge states for molecular ions was observed upon binding of metal ions, such as for the ATPase peptide (Fig. 3) from 3+ to 5+ charged ions up to 7+ charge states upon metal ion complexation [31]. The peptide–metal ion adducts with higher-charge states showed lower

Table 1
Synthetic model peptides and their affinity to NTA-agarose

Peptide	Sequence	Affinity[a]		
		Ni-NTA	Cu-NTA	Zn-NTA
M1	Ac-WEY**H**I**H**NLDG-NH_2	+	+	−
M2	Ac-WEY**H**IA**H**NLD-NH_2	+	+	−
M3	Ac-WEY**H**IRNLDG-NH_2	−	+	−
M4	Ac-WEYRIRNLDG-NH_2	−	−	−
APP (135–157)	FLHQERMDVCET**H**L**H**W**H**TVAKE-NH_2	+	+	+

Binding studies were performed according to Fig. 2, as described in Section 2.
[a] +, quantitative binding to NTA-agarose column; −, no affinity to NTA-agarose column.

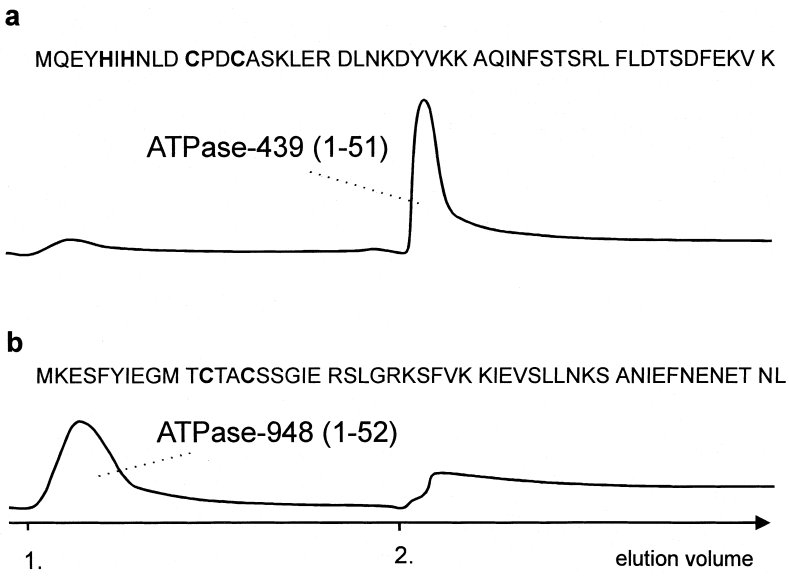

Fig. 2. Ni-NTA chromatography of ATPase-439 (1–51) (a) and ATPase-948 (1–52) (b). The numbers denote: 1, peptides were loaded on the column in 20 m*M* Tris–HCl buffer (pH 7); 2, peptides were desorbed from the column with 250 m*M* imidazole in 20 m*M* Tris–HCl buffer (pH 7).

relative abundances than the protonated molecular ions. However, in contrast to the small model peptides, low-charge state ions could not be observed for the metal ion complexes of the ATPase peptides because of their m/z values (>2000) exceeding the mass range of the quadrupole instrument.

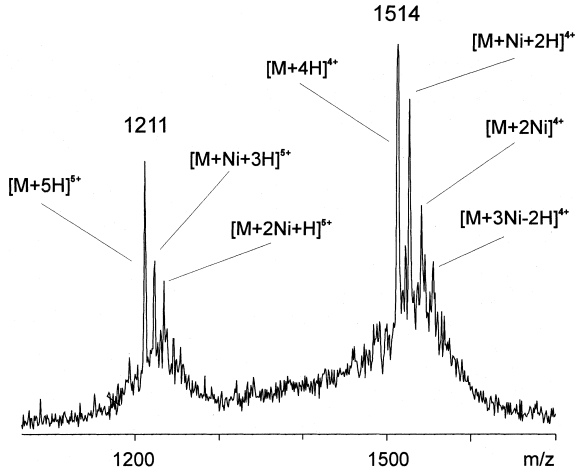

Fig. 3. ESI-spectrum of ATPase-439 (1–51) in the presence of a 10-fold molar excess of $NiCl_2$. The spectrum was obtained in 5 m*M* ammonium acetate–methanol (9:1), pH 4.

In order to purify the intact ATPase, the membrane fraction of recombinant ATPase-439 from *E. coli* was adsorbed on a Ni-NTA-agarose column. Competitive elution with imidazole using a step gradient with increasing concentration of imidazole (5, 10, 20 m*M* imidazole) yielded several fractions (Fig. 4). ATPase-439 was detected in each fraction as shown by SDS–PAGE and Western blot analysis. Such multiple affinities to the column by a single protein may be explained by different metal ion-binding sites within the same sequence [9]. The region between the phosphorylation site and the ATP-binding site contains a considerable number of His and Cys residues that may constitute further metal ion-binding sites. SDS–PAGE analysis (Fig. 4b) indicated a rather high purity (>79% as estimated from the UV-absorption) for the ATPase-439 in the eluted fractions.

A high affinity to Cu^{2+} ion binding has been recently found for an N-terminal peptide domain of APP [20]. A corresponding peptide, APP(135–157)

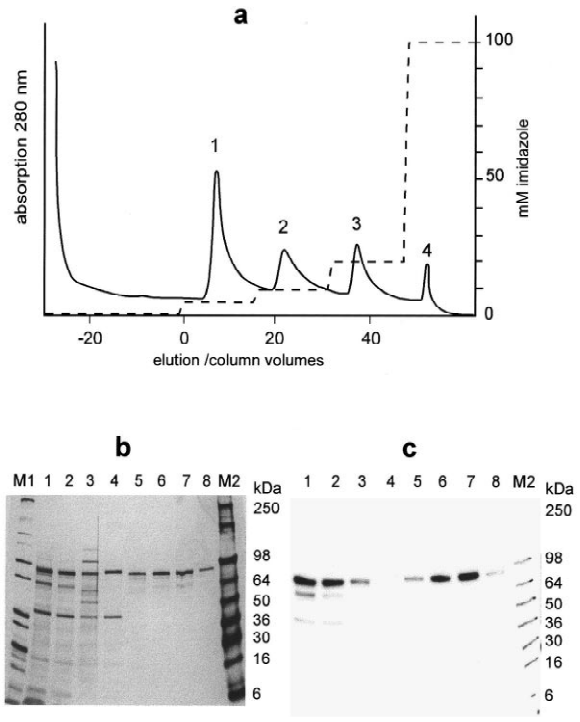

Fig. 4. Purification of ATPase-439 by Ni-NTA chromatography. (a) Elution profile of ATPase-439 by a step gradient with 5, 10 and 20 m*M* imidazole; (b,c) SDS–PAGE (silver stain) and Western blot analysis of collected fractions. Lane 1, membrane fraction from *E. coli*; 2, solubilized membrane proteins; 3, non-soluable residue; 4, flow through; 5–8, fractions 1–4. M1, M2, molecular mass/standards (kDa, kilodaltons).

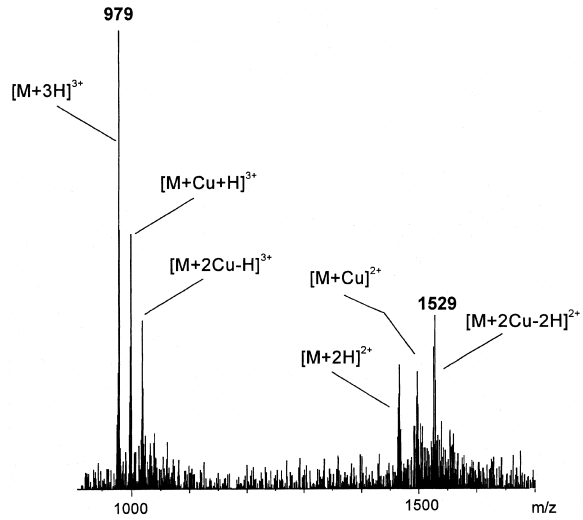

Fig. 5. ESI-spectrum of synthetic APP (135–157) in the presence of a 10-fold molar excess of $CuCl_2$. The spectrum was obtained in 5 m*M* ammonium acetate–methanol (9:1), pH 3.

was synthesized which contains a HxH motif suggesting Ni^{2+} ion-binding specificity. This peptide showed binding to Ni-NTA, Zn-NTA as well as Cu-NTA in metal chelate chromatography (Table 1). ESI-mass spectra of APP(135–157) were obtained in the presence of 10-fold molar excess of $NiCl_2$ and $CuCl_2$ at pH 3 which provided the identification of peptide complex ions with both metal ions, as illustrated in Fig. 5 by the Cu complex.

Because of the Ni^{2+} ion specificity to the HxH-motif and the observed binding of the synthetic peptide APP(135–157) to Ni-NTA and Cu-NTA columns [21] (Table 1), the purification of intact APP pre-purified from human brain by Q-sepharose and heparin-sepharose chromatography [27] was attempted with both Cu- and Ni-NTA-agarose columns. The isolated fractions were characterized by SDS–PAGE and Western blot analysis (Fig. 6). However the interaction of APP with the Ni-NTA column was found too weak for providing effective adsorption. By contrast, using the Cu-NTA column, APP could be separated from non-copper-binding proteins. In this case the NTA-chromatography was not selective enough to directly yield homogeneous protein, and the isolated APP still contained several contamination products.

4. Discussion

One goal of the present study was the structural characterization of the metal ion-binding sites in *Helicobacter pylori* P type ATPases. Since direct mass spectrometric studies on metal ion binding with large proteins have not yet been successfully performed, we initially investigated synthetic peptides containing the putative metal ion-binding motifs in order to elucidate their ion selectivity. The comparison of several peptides with variations of His positions revealed that peptides containing two histidine residues showed a high affinity to Cu^{2+} and Ni^{2+} ions. The complex with one Ni^{2+} ion was

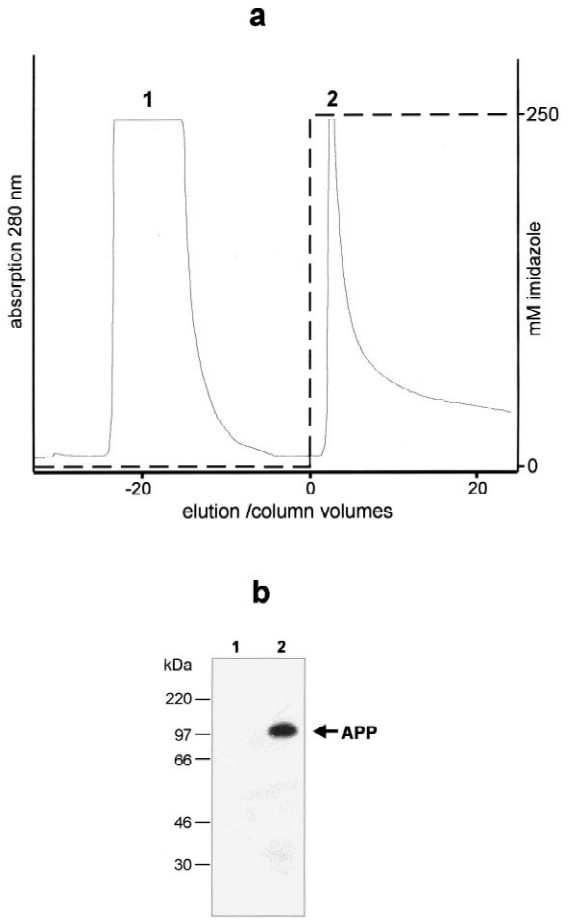

Fig. 6. Purification of APP by Cu-NTA chromatography. (a) Elution profile of APP isolated from human brain; 1, prepurified APP was loaded on the column in 20 m*M* Tris–HCl buffer (pH 7); 2, APP was desorbed from the column with 250 m*M* imidazole in 20 m*M* Tris–HCl buffer (pH 7). (b) Western blot analysis of fractions 1 and 2.

found as the base peak by ESI-MS. This complex appears to be specific, and may result from deprotonation of two adjacent amide bonds and the presence of His-imidazole as anchor for the metal chelation (Fig. 7), as described previously for the N-terminus of albumins [1,5]. Since the ESI-mass spectra showed more than one adduct ion (Figs. 1 and 3), it appears likely that the complexes employ only one imidazole as an anchor group. The affinity of peptides containing two or more histidine residues to the Ni-NTA columns may result from the additive affinities of two or more imidazole groups. The evaluation of the detailed structures of the complexes will require additional methods such as NMR spectroscopy and X-ray crystallography. However, it must be considered that the structures of the complexes in solution, and complexes formed on nitrilotriacetate agarose are different, since in the latter case three coordination sites are already occupied by acetate groups of the NTA column.

In this study, a major point of interest has been the investigation of the N-terminal sequences of ATPase-439, for which high Ni^{2+} ion-binding affinity has been observed. By contrast, the N-terminus of ATPase-948 did not show Ni^{2+} ion affinity. The N-terminus of P type ATPases may play a role in the regulation of ion transport, while ion transport itself appears to occur via the long cytosolic loop of the ATPase [32]. Further studies of the cytosolic region between the phosphorylation site and the ATP-binding site, in which several cysteine and histidine motifs as potential metal ion-binding sites are located, will therefore be of particular interest.

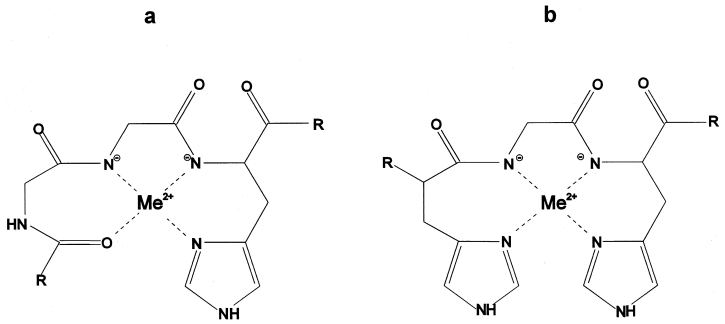

Fig. 7. Proposed structures of peptide complexes with transition metal ions. (a) Metal ion chelation by one His-imidazole residue and three adjacent amide bonds. (b) Metal ion chelation by two imidazole groups and two adjacent amide bonds.

5. Conclusions

N-terminal peptides of two P type ATPases from *Helicobacter pylori* have been synthesized. ESI-MS and metal chelate affinity chromatography revealed different metal ion selectivity of these peptide sequences. As general motifs for Ni^{2+} ion binding, sequences were identified which contain two or more histidine residues in close proximity, i.e. HxH and HxxH. Cysteine-containing motifs such as CxxC were capable to bind Cu^{2+} but not Ni^{2+} ions. Ni-NTA chromatography was used to effectively purify ATPase-439, and Cu-NTA chromatography to separate APP from non-metal-binding proteins. Since the isolation of the native APP-protein has been found difficult so far, the observed metal ion specificity may lead to an improved purification procedure. Furthermore, the present study demonstrates the possibility of protein purification by metal chelate chromatography without using an additional oligo-histidine (His-tag) sequence, indicating the efficient NTA chromatographic purification of proteins having suitable natural binding motifs.

Acknowledgements

We gratefully acknowledge helpful comments and critical discussions in preparing this manuscript by Dr. Kenneth Tomer, NIEHS, Research Triangle Park, during his guest stay at the University of Konstanz. This work has been supported by the Bundesministerium für Bildung und Forschung, Bonn, Germany, and by the Hungarian-Baden Württemberg science cooperation program.

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