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The βA4 amyloid precursor protein binding to copper

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Abstract

Previously it has been shown that the extracellular domain of transmembrane βA4 amyloid precursor protein (APP) includes binding sites for zinc(II) and for molecules of the extracellular matrix such as collagen, laminin and the heparin sulfate chains of proteoglycans (HSPGs). Here we report that APP also binds copper ions. A copper type II binding site was located within residues 135–155 of the cysteine-rich domain of APP_{695} which is present in all eight APP splice isoforms known so far. The two essential histidines in the type II copper binding site of APP are conserved in the related protein APLP2. Copper(II) binding is shown to inhibit homophilic APP binding. The identification of a copper(II) binding site in APP suggests that APP and APLP2 may be involved in electron transfer and radical reactions.

Key words: Alzheimer's disease; Copper binding site; Cell-adhesion; βA4 amyloid

1. Introduction

The βA4 amyloid precursor protein (APP) and its closest relative amyloid precursor-like protein 2 (APLP2) are multifunctional glycoproteins which both undergo similar alternative splicing and are ubiquitously expressed (for a recent survey see [1,2]). Only APP is the source of the characteristic βA4 amyloid deposits found in Alzheimer's disease since the βA4 sequence is not conserved in APLP2 [3,4].

APP is able to bind to different molecules of the extracelluar matrix (ECM) such as collagen, laminin [5,6,7] and heparin sulfate side-chains of proteoglycans (HSPG's) [5,8,9]. APP has also been reported to contain sequences with growth promoting activity [10] neurite promoting activity [11] and excitoprotection of neurons [11,12].

These activities are conferred by the soluble, non-amyloidogenic secretory products of APP which are generated by proteolytic cleavage within the βA4 region, proximal to the transmembrane domain of APP [13,14]. This shows that the extracellular part of APP is able to bind to molecules of the ECM and suggests that APP may participate in cell–cell interaction and cellular growth.

The binding of APP to heparin sulfate is modulated by the APP-ligand zinc(II) [5]. Zinc(II) binding was identified to correspond to residues 181–200, at the border between the cysteine-rich and the acidic domain of APP [8,15]. This sequence is included in all eight different APP isoforms which are produced by alternative splicing. The zinc(II) binding site is also highly conserved among the three so-far known members of the APP gene family [8]. Zinc(II) is an important cofactor in tissue-remodelling and wound-healing and reaches concentrations of 200–300 μM in brain regions which are damaged in AD and reaches similar concentrations in platelets. Since APP is highly concentrated at both sites, APP is suggested to be involved in repair processes.

Because tissue damage and subsequent wound-healing may be associated with respiratory burst and release of free radicals [16,17], and radical attack has been suggested to be involved in initiation of βA4 amyloid aggregation [18], we have analysed APP binding to metalions such as iron(II) and copper(II) that are involved in redox reactions. Copper is the third most abundant trace element in humans, after iron and zinc [19]. Since APP did not bind to iron(II)-loaded but did bind to copper-loaded chelating Sepharose we concentrated on copper(II) binding. We isolated and sequenced a putative type II copper(II) binding site of APP. We then determined the affinity constant and the influence of copper(II) binding on the homophilic interaction of APP. Our findings of a specific type II copper binding site on APP suggests that APP-copper binding may participate in electron transfer reactions and thus contribute to the aggregation and toxicity of βA4 amyloid.

2. Experimental

2.1. Construction of expression vectors

The plasmids pFd-$APP_{N262IgA}$ and pFd-APP_{770IgA} are based on pFd-APP [14] and pFd-APP_{N262} [8]. To obtain pFd-APP_{770IgA} the plasmid pFd-APP_{770} was linearized with *Kpn*I, treated with alkaline phosphatase, and religated in the presence of oligonucleotides A and B,

A: 5′-CGAACCCACGTCCACCAACACCGCTGGAGGTAC-3′
B: 5′-CTCCAGCGGTGTTGGTGGACGTGGGTTCGGTAC-3′

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which together form cohesive ends complementary to KpnI restriction sites. After transformation of *E. coli* a colony was selected by polymerase chain reaction containing the plasmid pFd-APP_{770IgA} in the desired orientation. The resulting clone pFd-APP_{770IgA} encoded a fusion protein with a recognition sequence for the IgA protease from *Neisseria gonorrhoeae* [20,21].

The plasmid pFd-$APP_{N262IgA}$ was derived from pFd-APP_{770IgA} and pFd-APP_{N262} by exchanging the corresponding *Acc*I fragments of pFd-APP_{N262} (6.5 kbp).

2.2. Purification of APP

APP_{695} was isolated from rat brain (rn-APP) as described previously for human brain [22] with the exception that the purification step on phenyl-Sepharose was replaced by affinity chromatography on lentil lectin-Sepharose. Preparations of purified 130- and 110 kDa APP (hs-APP, present as a 1:1 mixture), were derived from human brain membrane extracts and contained soluble holoprotein [22].

Full-length (Fd-APP_{770} and Fd-APP_{751}) recombinant forms and a truncated recombinant form of human APP corresponding to the N-terminal 262 residues of APP_{695} (Fd-APP_{N262}) [8] were prepared and purified in the form of a prokaryotic expressed Fd fusion protein by methods essentially as described by Weidemann et al. [14]. After preparative SDS-PAGE and electroelution the soluble protein was separated from salts and SDS by Excellulose GF-5 columns (Pierce) and renatured in 10 mM Tris-HCl, pH 7.5.

Fd-$APP_{N262IgA}$ and Fd-APP_{770IgA} fusion proteins were treated with IgA-protease from *Neissseria gonorrhoeae* at 37°C for 16 h according to the manufacturer's protocol (Boehringer, Mannheim). The cleavage products APP_{N262} and APP_{770} were purified by SDS-PAGE as given for Fd-APP_{N262}, Fd-APP_{751} and Fd-APP_{770}.

Protein concentrations were determined by amino acid analysis.

2.3. APP binding to Cu(II)-charged chelating Sepharose

Human APP was iodinated by the chloramine-T method (Iodo-Beads, Pierce) and separated from free iodine by heparin-Sepharose chromatography (specific activity: 700,000 cpm/pmol). Increasing concentrations of [^{125}I]APP (100,000 cpm/pmol) were loaded in starting-buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5) and incubated for 2 h at room temperature with 20 μl of a 1:1 suspension of Cu(II)-charged chelating Sepharose (Pharmacia) and end-over-end rotation. Before incubating with APP, Sepharose has been equilibrated with starting-buffer containing 1% ovalbumin, followed by loading buffer (50 mM $CuCl_2$, 10 mM Tris-HCl, pH 6.8) and starting-buffer again. The amount of APP bound was quantified by liquid scintillation counting of Sepharose after centrifugation. Values were corrected for non-specific binding to Sepharose CL-6B.

Purified APP fusion proteins were loaded onto a Cu(II)-charged chelating Sepharose column (200 μl of a 1:1 suspension in disposable filtration columns (Bakerbond spe), pre-equilibrated as described before) in starting-buffer. The column has been washed three times with 400 μl of washing-buffer A (400 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.05% NP-40), and buffer B (800 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.05% NP-40) and eluted with 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 50 mM EDTA.

Recombinant fusion proteins Fd-APP_{770} and Fd-APP_{N262} were treated with 3 μg of endoproteinase Lys-C (Boehringer, Mannheim) in starting-buffer at 37°C overnight. The resulting peptides were incubated with 300 μl of copper-loaded chelating Sepharose. After washing, elution was performed in the presence of EDTA and the eluate was dried down. The peptide fraction was redissolved in 5% acetic acid and injected onto a RP-HPLC column (Aquapore RP-300, Applied Biosystems). The peptides were eluted with a linear gradient of 70% acetonitrile in 0.1% TFA.

2.4. Western blotting of APP

Western blotting was done as described using the monoclonal antibodies 22C11 and Alz90 (Boehringer, Mannheim) and the problot alkaline phosphatase system (Promega) [14].

2.5. Homophilic APP binding

The 'homophilic' synthetic peptide (corresponding to residues 448–478 of APP_{695}) (manuscript in preparation) was adsorbed to microtiter wells (5μg/well) and incubated with 1% BSA in PBS for 1 h, ^{125}I-labelled hs-APP was added (1×10^3 to 2×10^6 cpm/well) together with the divalent ions to be tested in PBS. After 3 h incubation at room temperature the wells were emptied, dried, cut and subjected to liquid scintillation counting.

The mean of duplicate measurements is given which was corrected for non-specific binding (BSA alone).

2.6. Surface plasmon spectroscopy [23]

Surface plasmon resonance analysis (BIAcore, Pharmacia Biosensor) of copper(II) binding was done in 1 × HBS (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% Surfactant P20 (Pharmacia)) at a flow rate of 5 μl/min according to the instructions of the manufacturer. For all experiments 200–400 ng of proteins (C1q diluted to 50 μg/ml, rn-APP to 10 μg/ml and Fd-APP_{751} and Fd-APP_{N262} to 80 μg/ml in 10 mM NaOAc, pH 3.4) were used for coupling to the CM5 sensor chip (BIAcore amine coupling kit) and gave 9,000–19,000 response units. Copper(II) was injected for 1 min to reach final concentrations of 100 μM. Ligands bound to proteins immobilized onto the sensor chip surface were removed by injecting 30 μl of regeneration solution I (1 mM EDTA, 5 mM DTT, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5) and 20 μl of solution II (100 mM HCl). Injections were repeated two times independently and the increase of response units taken from the dissociation phase were averaged.

K_d value for copper induced structural changes of rn-APP has been calculated from equilibrium measurements where the net binding rate is zero and association and dissociation are balanced. Using R_{eq} for the equilibrium response, substituting K_a for k_{ass}/k_{diss} and rearranging and by plotting R_{eq}/C against R_{eq}, K_a and R_{max} could be calculated from the slope and intercept respectively according to:

$$R_{eq}/C = K_a \times R_{max}(\text{intercept}) - K_a \times R_{eq}(\text{slope})$$

(see also BIAcore Methods Manual, chapter 8).

Complement component C1q (human) and hemocyanin (Megathura crenulata) were from Sigma, Munich.

2.7. Peptide synthesis

Peptides were synthesized according to Barany and Merrifield [24], purified on C-18 columns with a linear gradient of acetonitrile in 0.1% TFA and sequences confirmed using pulse liquid gas phase technology and on-line PTH analysis (Applied Biosystems) [25].

3. Results

3.1. APP binding to copper(II) and identification of a type II copper binding site

APP_{695} purified from rat brain, recombinant proteins APP_{770} and APP_{N262} (APP residues 1–262) bind to copper(II)-charged chelating Sepharose. The elution of the retained APPs is shown in Figs. 1B and B. The binding of APP_{N262} places a copper(II) binding site within the N-terminal 262 residues. Binding of [^{125}I]APP to copper(II)-charged chelating Sepharose is saturable (Fig. 2) at a dissociation constant of 10 nM at pH 7.5 (inset of Fig. 2).

Putative Cu^{2+} binding sites of APP were identified by affinity chromatography of peptides obtained by Endo Lys-C digestion of the fusion protein Fd-APP_{770}. HPLC separation of peptides displaced from the copper-loaded column was followed by amino-terminal sequencing. The four peptides, C1, C2, C3, and C4 present in the major peaks originated from different positions within the APP sequence (Fig. 3). Peptide C1 is encoded within exon 9, C4 is encoded within APP exons 9 and 10, C2 within exon 12 and C3 within exon 4 [26,27]. Only pep-

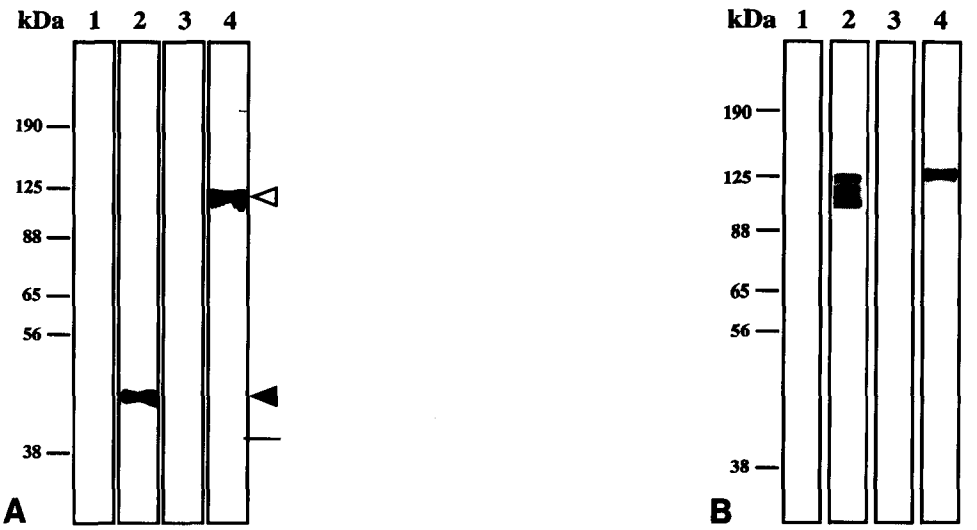

Fig. 1. Copper-charged chelating Sepharose chromatography of APP. (a) Immunostaining of recombinant APP proteins APP_{N262} (750 ng loaded, lanes 1 and 2) and APP_{770} (750 ng loaded, lanes 3 and 4) with monoclonal antibody 22C11; eluate (lanes 2 and 4); flow-through (lanes 1 and 3). (b) Immunostaining of rn-APP (1 μg loaded, lane 2) and APP_{770} (750 ng loaded, lane 4) with monoclonal antibody ALZ90; eluate (lanes 2 and 4); flow-through (lanes 1 and 3). 'loaded' = amount of proteins applied to the affinity column.

tide C3 was isolated from APP_{N262} (data not shown). The zinc(II) binding peptide located in exon 5 was not identified in both digests (Fig. 3). This shows that the copper(II) and zinc(II) binding is exhibited by different sites.

Inspection of the four major copper(II) binding peptides reveals that C1, C2 and C3 contain 2 or 4 His residues whereas C4 includes a single His residue. Since the Endo Lys-C fragments C1 and C3 contained the characteristic His-Xxx-His sequence of type II copper binding proteins [28] peptides C1 and C3 were synthesized and found to bind to copper(II)-charged chelating Sepharose (data not shown). This His-Xxx-His motif of peptide C3 but not of C1 is conserved in APLP2. We therefore concentrated on peptide C3. The characteristics of this peptide C3 which is contained within APP_{N262} are described in this study.

The amino acid residues of C3 that are responsible for binding of copper(II) were identified with synthetic peptides carrying the amino acid substitutions indicated in Table 1. All of these peptides except mmAPLP1 and huAPPNNNNM6 were found to bind to Cu^{2+}. This indicates that at least two of the histidines found, residues 13, 15 or 17, have to be conserved, and that one of the two adjacent His-Xxx-His motifs is sufficient for the copper-binding properties of C3. Binding properties of peptides huAPPS10, huAPPT7, mmAPLP1 and

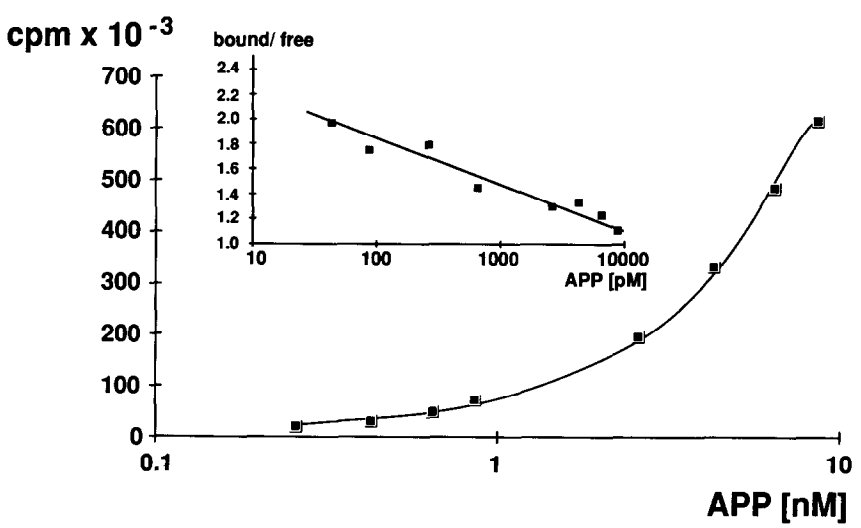

Fig. 2. Binding of [^{125}I]hs-APP to copper-charged chelating Sepharose. Chelating Sepharose was incubated with [^{125}I]hs-APP at various concentrations. The inset indicates a Scatchard plot for the binding curve.

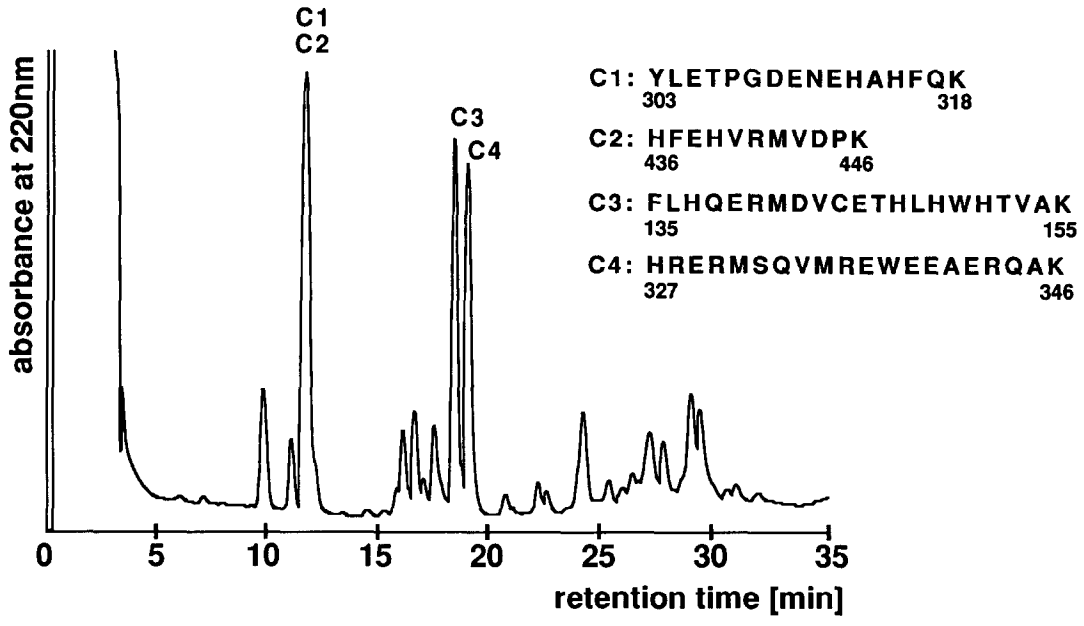

Fig. 3. Reverse-phase chromatography of copper binding peptides obtained by digestion of Fd-APP_{770} with endoproteinase Lys-C. Peptides were those eluted from chelating Sepharose loaded with copper. N-Terminal sequences of copper binding peptides are given with the numbering of APP_{695}.

huAPPNNNNM6 show that Cys10 and Met7 are not essential for copper(II) binding (Table 1).

3.2. *Regulation of homophilic binding of APP by copper(II)*

We used surface plasmon resonance [23] to detect changes in the APP conformation in dependence of increasing concentrations of copper(II). With this method changes in the refractive index of proteins which are immobilized to the sensor chip surface can be detected if the surface area covered by the protein is altered upon binding of a ligand. These changes are expressed in response units (RU) [29]. The surface plasmon resonance spectra (sensorgrams) of the binding of copper(II) to purified rat brain APP and to the bacterial fusion proteins Fd-APP_{751} and Fd-APP_{N262} are shown in Fig. 4. These sensorgrams reveal that APP undergoes a conformational change in the presence of μM copper(II).

In a typical experiment injection of copper(II) to reach the final concentration of 100 μM resulted in an increase from 17400 RU to 17580 RU (Fig. 4, rn-APP), 19000 RU to 19300 RU (Fig. 4, Fd-APP_{N262}), and 23600 RU to 24100 RU (Fig. 4, Fd-APP_{751}). Upon removal of copper(II) by EDTA (regeneration solution I) the initial RU (baseline) was not reached indicating that an irreversible conformational change of APP occurred. A return to baseline values was found when DTT was added to the regeneration solution I (data not shown). This suggests that cystine formation had occured. A formation of S–S bridges was also found to occur with the APP peptides

Table 1
Binding to copper-charged chelating Sepharose of the synthetic peptide C3 (representing amino acids 135–156 of APP_{695}), C3 variants and the related peptide of APLP2

		Copper binding	M_r	Monomer	Dimer
C3	FLHQE RMDVC ETHLH WHTVA KE	y	2747	y	y
C3-G5; G11	FLHQG RMDVC GTHLH WHTVA KE	y	2603	y	y
C3-N3	FLNQE RMDVC ETHLH WHTVA KE	y	2727	y	y
C3-T7	FLHQE RTDVC ETHLH WHTVA KE	y	2717	y	y
C3-S10	FLHQE RMDVS ETHLH WHTVA KE	y	2731	y	n
C3-N13	FLHQE RMDVC ETNLH WHTVA KE	y	2724	y	y
C3-N17	FLHQE RMDVC ETHLH WNTVA KE	y	2724	y	y
C3-NNNNM6	FLNQE RTDVS ETNLN WNTVA KE	n	2609	y	n
C3-N3; N17	FLNQE RMDVC ETHLH WNTVA KE	y	2701	y	y
C3-N3; T7	FLNQE RTDVC ETHLH WHTVA KE	y	2694	y	y
mm-APLP1	FLHQE RMDQC ESSTR RHQEA QE	n	2746	y	y
APLP2	FFHKE RMEVC ENHQH WHTVV KE	y	2851	y	y

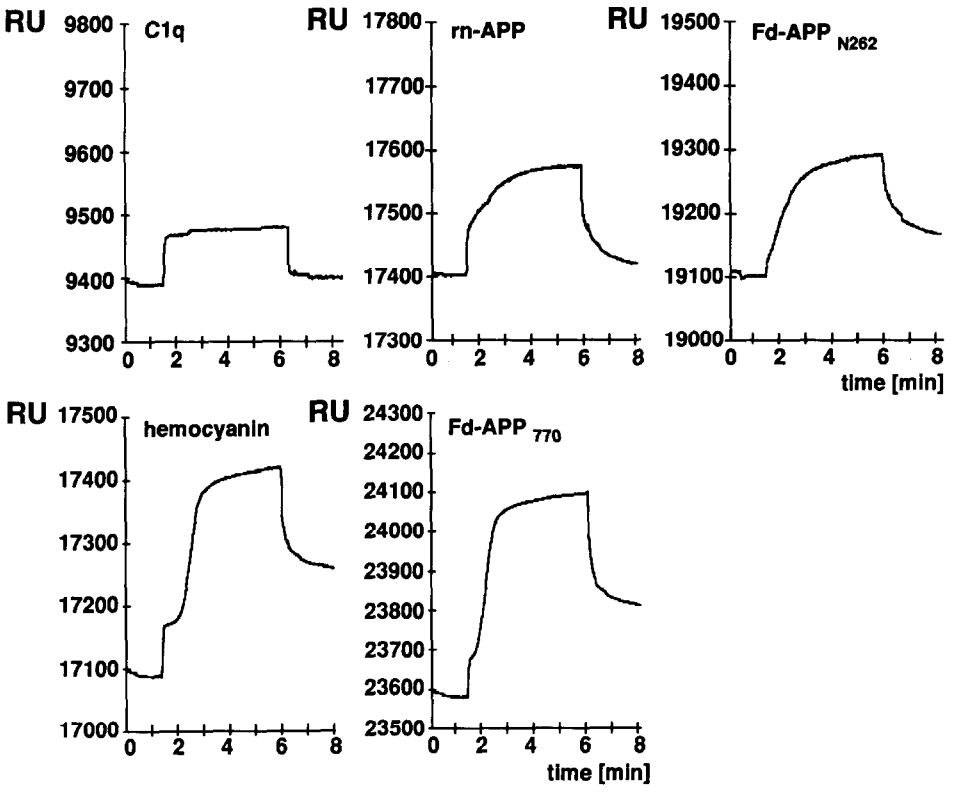

Fig. 4. Surface plasmon resonance (SPR) analysis of copper binding of rn-APP, Fd-APP_{N262}, Fd-APP_{751}, hemocyanin and C1q. Results are expressed as an increase of relative response units following addition of Cu^{2+} (100 μM) at time 1.5 min.

which bound and were eluted from the copper(II)-charged chelating Sepharose (dimerized peptides; Table 1).

The increase of APP response units was specific for copper(II) and was not observed for the metal ions Zn^{2+}, Co^{2+}, Mn^{2+} ad Ni^{2+} (data not shown). A similar increase in response units was measured for immobilized hemocyanin which is a multimeric copper containing protein (Fig. 4, hemocyanin). No effect is seen with C1q, a protein that does not bind copper(II) (Fig. 4, C1q).

The copper(II)-induced reversible structural change of rn-APP was used to determine the dissociation constant for copper(II) binding. Equilibrium measurement revealed a Kd value of 10 μM (Fig. 5). This value falls

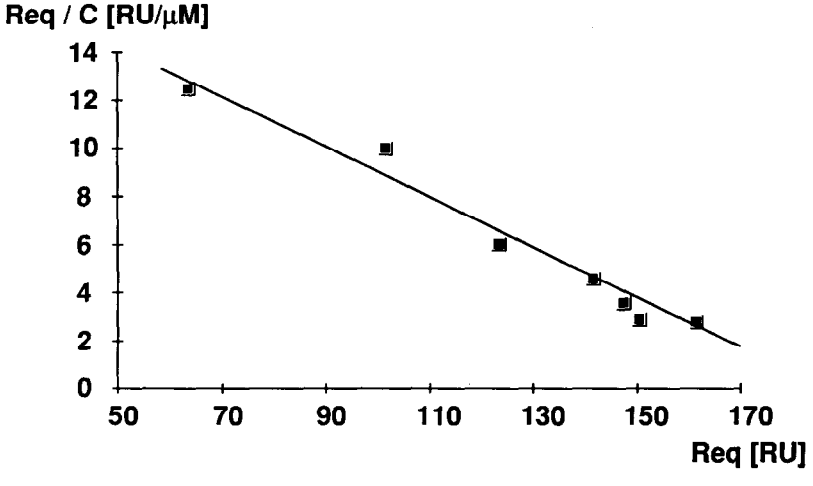

Fig. 5. Equilibrium measurement analysis of copper binding to APP. Rn-APP was coupled to the sensor chip surface to which Cu^{2+} was added at concentrations of 1–100 μM and analysed by surface plasmon resonance.

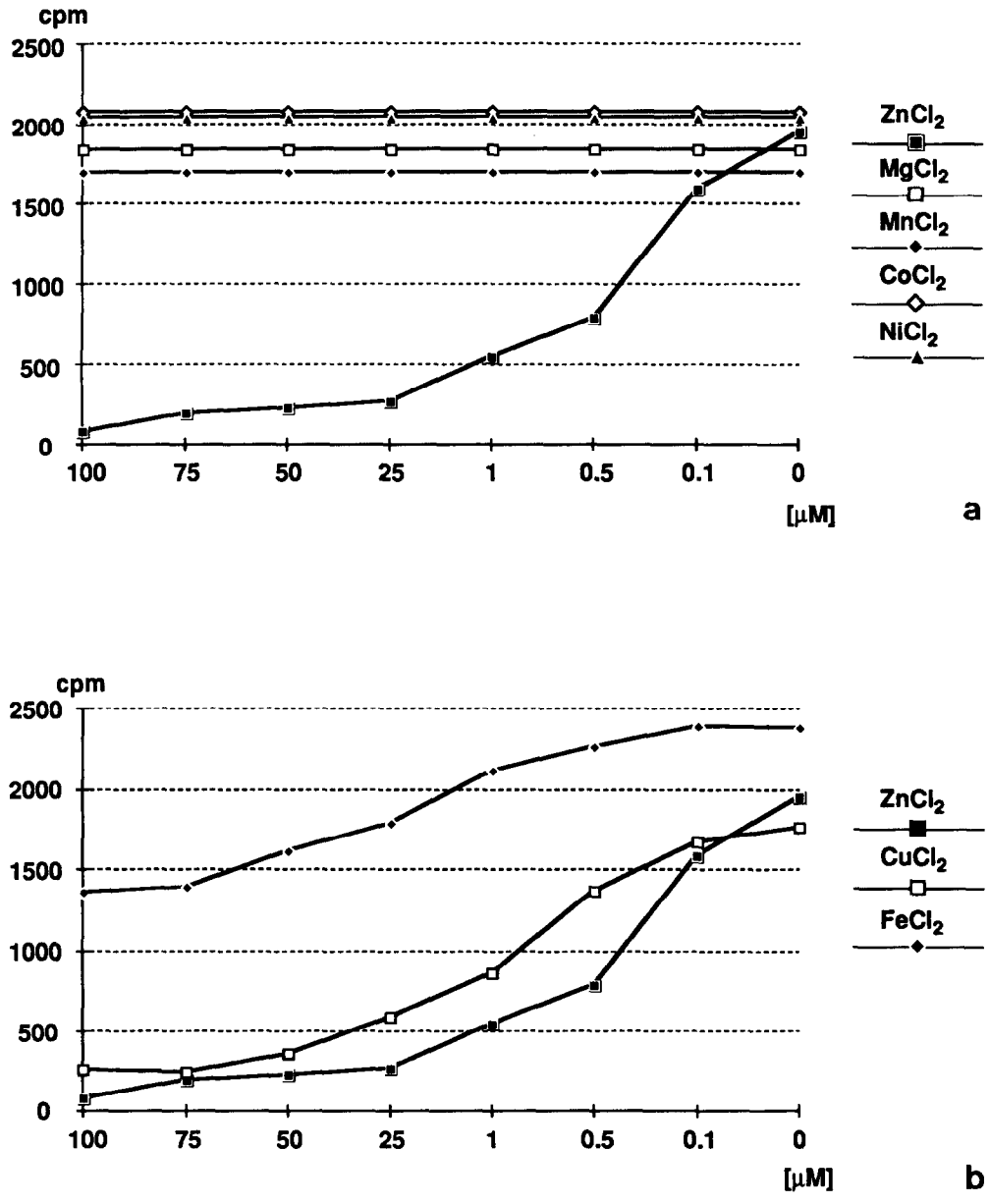

Fig. 6. Modulation of binding of the 'homophilic' APP peptide to [^{125}I]hs-APP. Divalent metal ions Zn^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} (a) and Zn^{2+}, Cu^{2+} and Fe^{2+} (b) were added at concentrations of 0–100 μM to microtiter wells coated with the 'homophilic' APP peptide and [^{125}I]hs-APP binding was analysed.

within the 20 μM range of physiological copper(II) concentrations [30,31].

Because copper(II) is compared to APP a small ligand the contribution of bound copper(II) for the increment of the specific refractive index of the copper(II)–APP complex is expected to be negligible. Therefore we concluded that the copper-induced changes in RU reflect primarily structural changes of APP. These structural changes could be due to dimerization since a conformational change of monomeric APP is unlikely to cause the observed RU changes. If dimerization occurs, APP binding to an 'homophilic' APP peptide (residues 448–478 of APP_{695}) (D. Beher and G. Multhaup, unpublished) should be inhibited by copper(II). This peptide binds to APP and is therefore 'homophilic'. The binding of the peptide was analysed in a solid phase assay. Radioiodinated rat brain APP was added to microtiter plates coated with the 'homophilic' peptide in the absence or presence of copper(II) and other divalent ions (Fig. 6). Binding of [^{125}I]APP was inhibited by copper(II) and zinc(II) in a concentration-dependent manner with an IC_{50} at 0.5–1.0 μM. This effect was specific for Cu^{2+} and Zn^{2+} and not observed for other divalent ions such as Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} (Fig. 6).

4. Discussion

We found saturable and specific binding of Cu^{2+} to APP. The binding constant was determined to be 10 nM which is 3 orders of magnitude below the physiological concentration of copper(II) [30,31]. Metal affinity chromatography of APP fragments obtained after digestion with Endo-Lys-C resulted four potential copper-binding peptides. Sequencing of the four peptides revealed two (C1 and C3 in Fig. 3) to include the consensus His-Xxx-His motif which is characteristic for type II copper proteins (Fig. 7). In this study we concentrated on peptide C3 since only the sequence of peptide C3 is highly conserved in APLP2 (Fig. 7) which is the closest relative of APP in the APP gene family [2]. The APLP2 peptide was synthesized and shown to bind copper(II). The corresponding peptide of APLP1 which lacks the typeII copper(II)-binding sequence did not bind (Table 1). Further characterization of peptide C3 was done by exchanging histidine, methionine and cysteine residues. Dimerization of the peptide upon binding to copper(II)-charged chelating Sepharose was not observed when the single cysteine residue was replaced by serine. Exchanges of the four histidines abolished copper(II) binding. Copper(II) binding was retained in all peptides with an intact His-Xxx-His sequence. A substitution of the single methionine residue by threonine had no effect on copper(II) binding. This confirms that copper binding requires the presence of histidine residues.

We then used surface plasmon resonance to analyze the effect of copper(II)-binding on APP structure. This method allows to monitor ligand binding if the bound ligand itself contributes to the changes in light refraction (refractive index increment) or if the protein undergoes a conformational change upon binding of the ligand. Because copper is a small ligand the change observed in response units for the APP–copper(II) complex could be due to homophilic aggregation or major molecular structural changes of APP. The latter appears less likely. We therefore analysed the effect of copper(II) on the binding

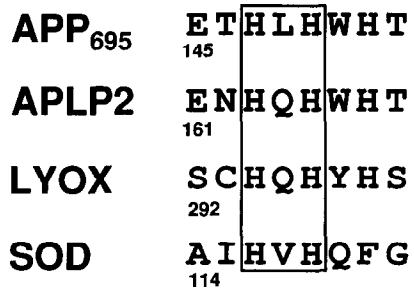

Fig. 7. Comparison of the copper binding region of APP peptide C3 with that of the type II copper binding proteins human Cu/Zn superoxide dismutase (SOD) and lysyl oxidase (LYOX) and of the corresponding sequence of APLP2. The proposed Cu^{2+} binding regions of APP, Cu/Zn superoxide dismutase and lysyl oxidase are boxed.

of radioiodinated APP to an APP fragment. This fragment shows homophilic binding to APP. Copper(II) and zinc(II) were found to inhibit this homophilic binding. We showed previously modulation of APP-binding to heparin by zinc(II) to be due to an allosteric effect [32]. This conformational change could also interfere with the homophilic interaction of APP. Because copper(II) binding causes a significant response in the surface plasmon resonance whereas zinc(II) binding does not, the inhibition of the homophilic interaction of APP by copper(II) may be only in part due to conformational changes of the monomer. The inhibition by copper(II) is more likely due to the formation of APP aggregates in the presence of copper(II). This aggregation appears to involve or mask the homophilic binding site of APP and inhibit the binding to the peptide on the solid support.

The presence of a type II copper-binding site in APP suggests that APP belongs to the same group of proteins to which the copper protein superoxide dismutase and lysyl oxidase belong. APP may therefore have a function in electron transfer and radical reactions.

It remains to be shown whether this putative APP function is altered in Alzheimer's disease and whether APP thus could contribute to the suggested radical-mediated initiation of βA4 amyloid aggregation [18].

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