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Interaction of Human Angiogenin with Copper Modulates Angiogenin Binding to Endothelial Cells

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Abbreviations: rAng, human recombinant angiogenin; CPAE, calf pulmonary artery endothelial; FBS, fetal bovine serum; IDA, iminodiacetate; IDA-M^{2+}, chelate of transition metal ion (M^{2+}); IMAC, immobilized metal ion affinity chromatography.

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ABSTRACT

Angiogenin is a potent inducer of blood-vessel formation with ribonucleolytic activity. Angiogenin binds to high affinity endothelial cell receptors and with lower affinity to extracellular matrix components. Here we report the effect of copper and zinc on these interactions. There was a 4.3-fold increase in angiogenin binding to calf pulmonary artery endothelial cells in the presence of Cu^{2+}, *in vitro*. A 3.8-fold increase was observed with Zn^{2+} whereas Ni^{2+}, Co^{2+}, or Li^{+} had no effect. Specific angiogenin binding to the lower affinity matrix sites was increased by 2.7 and 1.9-fold in the presence of Cu^{2+} and Zn^{2+} respectively. Metal ion affinity chromatography and atomic absorption spectrometry were used to show the direct interaction of angiogenin with copper and zinc ions. Angiogenin bound 2.4 mol of copper per mol protein. We suggest that copper, a modulator of angiogenesis *in vivo*, may be involved in the regulation of the biological activity of angiogenin.

INTRODUCTION

Angiogenin, a 14 kDa polypeptide, has been first purified as an inducer of angiogenesis from HT-29 human adenocarcinoma cell-conditioned medium [1]. It has also been purified from human plasma [2]. Angiogenin is secreted by anchorage-dependent cells, including vascular endothelial and aortic smooth muscle cells, fibroblasts and tumor cells [3]. Its RNA transcript has been detected in human epithelial and endothelial cells, fibroblasts, peripheral blood cells [3,4] as well as in various rat and human tissues [5,6]. The regulation of angiogenin synthesis at the cell or tissue level is still unknown.

Angiogenin has specific ribonuclease activity [7,8] which enables it to block protein synthesis in cell-free systems [9,10] or when injected in Xenopus oocytes [11]. The integrity of its ribonuclease catalytic site is necessary but not sufficient for its angiogenic activity which also requires a cell binding domain [12-15]. Angiogenin binds to high affinity receptors on endothelial cells [16-18] and triggers several intracellular events [19,20].

The relationship between the interaction of angiogenin with cells *in vitro*, and its ribonucleolytic and angiogenic activities is unknown at present. Since binding of angiogenin to its receptors might be the initial step in intracellular signaling and/or internalization, studies were undertaken to evaluate potential agonists or antagonists of these interactions. Among the effectors that modulate angiogenesis [21], divalent copper has been shown to induce motility of endothelial cells [22]. In this study, we report that angiogenin binds to copper, and describe the effect of different metal ions on angiogenin binding to calf pulmonary artery endothelial cells.

MATERIALS AND METHODS

Materials. Recombinant human angiogenin (rAng) was produced in *Escherichia coli*, renatured and purified to homogeneity as described previously [23]. It differs from natural human angiogenin in possessing an N-terminal methionine residue (Met-[-1] Ang) followed by glutamine instead of the natural N-terminal pyroglutamic residue (<Glu-1 Ang), [24]. rAng shows identical biological properties to natural angiogenin in the chick embryo chorioallantoic membrane and rabbit cornea assays [23,25,26] and exhibits characteristic ribonucleolytic activity [23,25]. Human recombinant <Glu-1 Ang was a gift from Dr. R. Shapiro (Harvard Medical School, Boston, MA, USA [25]). Bovine serum albumin (fraction V), lysozyme (EC 3.2.1.17; Grade I), pancreatic RNase A (EC 3.1.27.5; type I) and IgG were from Sigma Chemical Co. (St Louis, MO, USA). Culture chemicals were from Gibco BRL (Gaithersburg, MD, USA). Fast flow chelating iminodiacetate-Sepharose 6B (IDA-Sepharose) was from Pharmacia (Uppsala, Sweden). All metal salts were analytical grade reagents. Deionised, pyrodistilled water containing no detectable metal ions or pyrogens was used throughout. Assays were performed in polypropylene tubes (Nunc InterMed, Naperville, IL, USA) to minimize adsorptive losses of protein.

Protein determination. Protein concentrations were determined spectrophotometrically, assuming an ε_{280} of 12500 M^{-1} cm^{-1} for angiogenin at neutral pH [27]), and by the procedure of Bradford [28] using the Bio-Rad protein microassay with BSA as a standard

(Bio-Rad Laboratories, Melville, NY, USA). For atomic absorption spectrometry, angiogenin concentration was determined by amino acid analysis [24].

Cell culture. Calf pulmonary artery endothelial cells (CPAE cells) were obtained from the American Type Culture Collection (CCL209; Rockville, MD, USA) and subcultured for less than 20 passages in Eagle's minimum essential medium/26 mM $NaHCO_3$/20% heat-inactivated fetal bovine serum (FBS) without antibiotics. Cells were maintained at 37 °C in humidified 5% CO_2 /95% air and were passaged at confluence by harvesting with a solution containing 0.05% (w/v) trypsin and 0.02% (w/v) EDTA, and seeding at a 1:5 ratio. Cell numbers were determined using a Coulter counter (Coultronics, France) or a haemocytometer.

Radiolabeling of proteins. rAng, <Glu-1 Ang and RNase A, were iodinated using the chloramine-T method [29]. For affinity chromatography, large amounts of the proteins were labeled as follows: In a final volume of 150 µl, 20 μg of protein (9.2 μM) was added to a 0.4 M sodium phosphate buffer, 3.3 mM polyethylene glycol 1000, pH 7.4. After the addition of 9.3 μM NaI containing 14% of $Na^{125}I$ (Oris, Gif-sur-Yvette, France) and 80 μM chloramine-T, the mixture was shaken for 2 min at room temperature. The reaction was stopped with 100 μM sodium bisulfite and 1.25 mM NaI and shaken for 1 min. The ^{125}I-protein was then separated from the reagents by chromatography on a PD10 Sephadex G25-M column equilibrated with 20 mM sodium phosphate buffer, 1 M NaCl, pH 6.8. Under these conditions, near 1 atom of iodine was bound per angiogenin or RNase A molecule. Ribonucleolytic activity of iodinated rAng was assessed as described [23]. For binding assays on cells, labeling conditions were as follows: In a final volume of 150 μl, 2 μg of rAng or <Glu-1 Ang (0.92 μM) was added to a 0.45 M sodium phosphate buffer, pH 7.4, containing 3.3 mM polyethylene glycol 1000. One mCi $Na^{125}I$ (3.3 μM; 1 Ci = 37 GBq) and 80 μM chloramine-T were added and the mixture shaken for 2 min at room temperature. The reaction was stopped as before and the volume adjusted to 0.5 ml either with PBS (6.5 mM Na_2HPO_4, 1.46 mM KH_2PO_4, 3 mM KCl, 140 mM NaCl, pH 7.4) or Hepes-buffered saline (20 mM Hepes, 140 mM NaCl, pH 7.2), both containing 1 mg/ml BSA. The ^{125}I-angiogenin was then purified by chromatography on a PD10 Sephadex

column equilibrated with the same buffer. Specific activity varied from 37 500 to 159 000 cpm/ng (0.3 to 1.3 Ci/μmole) corresponding to 0.2-0.8 atoms of iodine bound per molecule of angiogenin. Radioactivity was measured using a LKB Minigamma counter with 78% efficiency (LKB Wallac, Turku, Finland).

Angiogenin radioligand binding studies. Cells were seeded at 5 x 10^3/cm^2 in 24-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ, USA) and cultured for 2 days. Plates were cooled on ice for 30 min and washed three times with 0.5 ml per 2 cm^2-well of binding buffer (20 mM Hepes, 130 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM KCl, 1 mg/ml BSA, pH 7.2, with additional metal ions as indicated). Cells were incubated with ^{125}I-angiogenin in binding buffer (0.25 ml per 2 cm^2-well), for 4 h on a shaking-table, at 4 °C. They were rinsed three times with the same buffer and washed for 2 min at 4 °C with washing buffer (binding buffer containing 0.6 M NaCl). This buffer was shown to release ^{125}I-rAng bound with low affinity to pericellular components [16]. Cells were solubilized overnight at 4 °C with 20 mM Hepes, 10% glycerol, 1% Triton X-100, 1 mg/ml BSA, pH 7.2, with gentle shaking. Residual extra cellular components were recovered from the plastic wells by incubation with a 4 M guanidine hydrochloride solution containing 2% Triton X-100, 50 mM sodium acetate, pH 5.8, for 24 h at 4 °C. Radioactivity was determined as above. Each value was the mean of triplicate determinations. Non-specific binding was defined as the amount of radioactivity bound in the presence of the indicated excess of unlabeled rAng. Saturation experiments were carried out by increasing the concentration of ^{125}I-angiogenin and saturation reached by adding increased amounts of unlabeled angiogenin. After incubation, cells were washed three times with binding buffer and then solubilized with the 4 M guanidine hydrochloride buffer. Total cell lysates were counted for radioactivity. Binding data were analyzed using the LIGAND fitting program (version 2.3.11, [30]).

Immobilized metal ion affinity chromatography. Metal chelating IDA-Sepharose (5 ml-bed volume) was thoroughly washed with water, degassed, equilibrated with 0.1 M sodium acetate, 1 M NaCl, pH 4.0, and packed into a 1.1 cm-diameter column. The column was loaded at a flow rate of 0.25 ml/min with 50 ml of either $CuSO_4$ or $ZnSO_4$ (5 mg/ml)

dissolved in the same buffer. Unbound metal ions were washed out with 100 ml of metal-free buffer. The IDA-M^{2+} columns were washed with 100 ml of 20 mM sodium phosphate buffer, 1 M NaCl, pH 6.8 (buffer A) containing 1 mM imidazole, then with 100 ml of 10 mM imidazole in the same buffer, and finally equilibrated with 100 ml of buffer A with or without 1 mM imidazole [31]. All buffers contained 1 M NaCl to minimize ionic interactions, and were filtered (0.22 μm) and degassed before use. The columns were run at room temperature at a flow rate of 0.25 ml/min (1 ml/fraction) and monitored with a FPLC system (Pharmacia). Each protein run was preceded by a blank gradient. Proteins (0.2-0.5 mg, 2-5 mg/ml) were applied in the equilibrating column buffer. In some experiments, trace amounts of ^{125}I-labeled proteins were added (see results). Columns were developed with a linear imidazole gradient (0- or 1-10 mM, 50 ml) in buffer A, then washed with 50 ml of buffer A containing 10 mM imidazole. An acid elution was then performed stepwise (0.1 M sodium acetate, 1 M NaCl, pH 4.0). Protein content of the fractions was determined by absorbance at 280 nm. The presence of copper was detected at 750 nm with a Perkin-Elmer Lambda 3 spectrophotometer (Perkin-Elmer Corporation, Norwalk, CT, USA). Wavelength scanning was performed using an Uvikon 930 spectrophotometer (Kontron Instruments, Everett, MA, USA).

Metal analysis. To determine the stoichiometry of copper binding to angiogenin, separation of bound and free copper was performed at 4 °C by ultrafiltration using Centricon-10 micro concentrators (Amicon Corporation, Lexington, MA, USA). Filters were washed with 1 ml of 10 mM HCl by centrifugation at 4300 x *g* for 30 min, rinsed with 0.5 ml of diphenylthiocarbazone-extracted 5 mM Hepes buffer, pH 7.5 [32] and then washed with 1 ml of Hepes buffer by centrifugation. Samples (0.8 ml) in Hepes buffer containing 25 μM rAng and increasing amounts of $CuSO_4$ were loaded and pre-centrifuged at 4300 x *g* for 5 min. The filtrates were discarded and the runs continued for 20 min. Copper contents of starting solutions, retentates and filtrates were determined by graphite furnace atomic absorption spectrometry using a Perkin-Elmer model 5000 or 4100 ZL with Zeeman correction atomic absorption spectrophotometer.

RESULTS

Effect of metal ions on angiogenin binding to CPAE cells. The amount of ^{125}I-rAng specifically bound to CPAE cell cultures increased in the presence of $CuSO_4$, $CuCl_2$ and $ZnSO_4$ (Fig. 1) but not with other transition metal ions such as Ni^{2+} and Co^{2+} nor with Li^{+} (not shown). Iodinated-rAng specific binding to high capacity/low affinity matrix sites (released by 0.6 M NaCl wash [16]) was increased in the presence of 10 μM Cu^{2+} and Zn^{2+} (Fig. 1A), whereas specific binding to the high affinity cell surface receptor (detergent-extractable) sites increased to a maximum at 100 μM Cu^{2+} and Zn^{2+} (Fig. 1B).

Radioligand binding experiments, performed at steady state, at 4 °C, confirmed the metal-induced increase in angiogenin binding to CPAE cell cultures (Fig. 2). Conglomerate analyses using the LIGAND fitting program were done with independent experiments involving the same number of cells (cultures at 1/3 confluence) since the number of angiogenin receptors decreases when cell density increases [16]. In the control experiments, CPAE cells bound 187 000 rAng molecules at high affinity sites and 20 million at low affinity sites. In the presence of metal ions, analyses converged to a higher number of rAng molecules bound to CPAE cell cultures (Table 1). The number of rAng molecules bound to high-affinity sites increased by 4.3 fold in the presence of Cu^{2+} whereas lower affinity interactions increased by a factor 2.7 (conglomerate analysis from 4 independent experiments involving 3 different preparations of rAng). A similar effect was observed in the presence of Zn^{2+} with an increase of 3.8-fold and 1.9-fold in the number of rAng molecules bound to high and low affinity sites respectively (Table 1). Neither Cu^{2+} nor Zn^{2+} significantly affected the apparent dissociation constant of either high-affinity binding sites (1 to 2.2 nM) or low-affinity/high-capacity interactions (0.14 to 0.17 μM). Although replicates differed by less than 9%, LIGAND analysis resulted in large percentage coefficients of variation when fitting these data to a two independent binding site model (Table 1) suggesting that angiogenin interaction with CPAE cells may be more complex than this model.

Zinc and copper affinity chromatography. When rAng was applied to an IDA-Zn^{2+} column at pH 6.8, it was retarded and eluted as a broad peak at the beginning of the imidazole gradient, whereas in a parallel experiment RNase A was found in the breakthrough fractions (Fig. 3). IgG used as a positive control was retained [33] and eluted by 6 mM imidazole. The overall recovery, estimated by co-chromatography of 26 x 10^6 cpm of ^{125}I-rAng was 58% compared with 92% yield for ^{125}I-RNase A. No significant further radioactivity was released by acid wash at pH 4.0 (not shown).

Chromatography of rAng on a Cu^{2+} column yielded surprising results. BSA and RNase A used as control proteins were both retained by the column and eluted by the imidazole gradient as expected at 5.5 mM and 8 mM respectively (Fig. 4A). However, rAng appeared in the breakthrough peak which showed a much higher absorbance at 280 nm than could be accounted for by the amount of rAng applied to the column (Fig. 4B). Wavelength scanning of this peak showed an absorbance maximum at 725 nm characteristic of the presence of Cu^{2+} (Fig. 4B) suggesting that copper was co-eluted with rAng in the breakthrough volume. Furthermore, bleaching of the gel was observed when large scale purification of rAng was attempted using IDA-Cu^{2+} chromatography. These observations strongly suggest that rAng forms a tight complex with the chelated copper resulting in its release from the IDA column.

It should be noted that ^{125}I-rAng behavior on both IDA-M^{2+} columns slightly differed from that of the unlabeled molecule. Elution of ^{125}I-rAng from IDA-Zn^{2+} column slightly preceded that of the unlabeled molecule whereas ^{125}I-rAng was found to be retained on IDA-Cu^{2+} column (see discussion).

Stoichiometry of the rAng/copper complex. The direct interaction between angiogenin and copper in solution was quantitated by atomic absorption spectrometry. Copper determinations showed that saturation of copper binding to rAng was reached in the presence of a 4 to 5-fold molar excess of the metal ions and involved 2.4 atoms of copper per molecule (Fig. 5).

DISCUSSION

Our earlier observations that angiogenin binding to endothelial cell surface is enhanced in the presence of copper [16] led us to investigate the direct interaction between angiogenin and copper using immobilized metal ion affinity chromatography (IMAC) and atomic absorption spectrometry. The results obtained with IMAC suggest that rAng binds copper very tightly since, unlike most metal-binding proteins, rAng is able to displace copper from the IDA-groups linked to the column while RNase A, which shares 35% sequence identity with angiogenin [34], cannot. Atomic absorption spectrometry showed that, in solution, rAng binds about 2.4 mol/mol of Cu^{2+} at pH 7.5.

The precise identity of the amino-acids involved in copper binding remains to be determined. However, some possibilities may be proposed by comparing angiogenin and RNase A structures. RNase A binds copper [35] at several sites including His-12, His-105 and His-119 at pH 5 [36] and like angiogenin [37], RNase A enzymatic activity is inhibited by copper and zinc [38,39]. His-105 is not conserved but replaced by Asn-102 in angiogenin while His-12 and His-119 are conserved as His-13 and His-114, respectively, and are accessible to the solvent [40]. In addition, both His-13 and His-114 are part of the catalytic site of angiogenin. These observations taken together suggest that either or both of these histidine residues could bind copper. Angiogenin has three additional histidines which are not present in RNase A: His-8, His-65 and His-84 [24,40,41]. All three of these could also bind copper since they are well exposed. The similarity between the copper-binding plasma tripeptide Gly-His-Lys and the copper binding sites of albumin and α-fetoprotein where copper binds to a histidyl residue adjacent to a basic residue (Arg or Lys) suggests that His-65, adjacent to Arg-66 on angiogenin might be involved in copper binding [42-44].

IMAC of proteins is based on the co-ordination between the electron donor groups on the protein surface and chelated transition metal ions [45]. In addition to histidine, cysteine and tryptophan can also participate in IMAC of proteins. In angiogenin, cysteines are most probably not involved in copper binding since all 6 cysteinyl residues form disulfide bonds

(26-81, 39-92 and 57-107). The single tryptophan (Trp-89) is extremely well exposed, has high solvent accessibility and could bind copper.

Iodination of rAng modifies its chromatographic behavior on IDA-Cu^{2+} column since ^{125}I-rAng binds to but does not elute copper from the column suggesting that ^{125}I-rAng has a lower affinity for copper than rAng. At neutral pH, iodination occurs essentially on tyrosine residues. Since direct interaction of tyrosine with copper is not documented, it is likely that tyrosine iodination affects copper binding indirectly. Monoiodotyrosine has a lower pK that could modify its micro-environment and affect the interaction of neighboring histidines with copper as has been shown for other biologically active peptides [46]. Indeed, Tyr-6 and Tyr-14, which are likely candidates for iodination owing accessibility of their phenolic side chains [40], are also in close proximity to His-8 and His-13. Iodination of either or both of these tyrosines could therefore affect copper binding by His-8 and His-13. Two more tyrosines, Tyr-25 and Tyr-94 are present in angiogenin but both have their phenolic ring buried in the tertiary structure of the molecule [40]. The corresponding residues in RNase A, Tyr-25 and Tyr-97 are not easily iodinated [47].

Divalent copper and zinc ions increased the apparent number of angiogenin molecules bound to CPAE cells but the apparent dissociation constant remains unchanged (Table 1). This effect cannot be ascribed to the presence of the additional Met-[-1] in rAng since it is also observed when using ^{125}I-<Glu-1 Ang (not shown). The increased binding could result from interactions of copper with the cell surface or from aggregation of angiogenin molecules at the binding sites. Experiments designed to demonstrate angiogenin polymers, in vitro, using ^{125}I-rAng and copper, revealed the presence of mainly dimers (not shown). A functional change in the interactions of rAng with its receptors in the presence of copper remains the most probable explanation.

We obtained broadly similar results with Cu^{2+} and Zn^{2+} except for the metal chelate affinity chromatography experiments where a stronger affinity of rAng for Cu^{2+} was observed. Zinc is abundant in plasma (1 mg/ml) [48] and an essential component of many enzymes and DNA-binding proteins [49] but the involvement of zinc in angiogenesis has never been reported. In contrast, there is abundant evidence for an active role for copper in

angiogenesis. Accumulation of copper has been detected in rabbit cornea prior to capillary invasion during prostaglandin E1-induced angiogenesis [50], whereas copper-deficient rabbits did not respond to angiogenic stimuli in the cornea [50] and in brain tumors [51]. Several angiogenic growth factors have been reported to bind immobilized copper such as fibroblast growth factor [52], transforming growth factor β (reported in [53]) and vascular endothelial growth factor [54], and angiotropin, an angiogenic ribonucleoprotein, contains one atom of copper per molecule [55]. More recently SPARC (Secreted Protein Acidic and Rich in Cysteine), an extracellular matrix-binding protein expressed by endothelial cells during vascular remodeling, has been shown to release copper-binding peptides that could regulate angiogenesis [56]. The differences in copper interactions between the different angiogenic growth factors [57] added to the capacity of molecules such as heparin, ceruloplasmin and its fragments, and liver growth factor to induce angiogenesis in the presence of copper [58] suggest that copper might modulate the angiogenic activity of a given factor in a specific spatio-temporal micro-environment. Since angiogenin concentration in plasma, 100-400 ng/ml [3,59] is 20 to 800-fold higher than that needed to induce intracellular events in cultured endothelial cells [19,20] or angiogenesis in experimental models [1], tight regulation of angiogenin functions and/or strict compartimentalization would be necessary to reconcile its angiogenic potency with its high intravascular concentration. Copper exchange at sites where angiogenesis takes place might be part of the mechanism by which angiogenin functions are regulated in vivo. The data presented here support such an hypothesis.

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TABLE 1

Parameters of angiogenin binding to CPAE Cells

	K_{D1} (nM)	%CV	N_1 molecules/cell	%CV	K_{D2} (μM)	%CV	N_2 molecules/cell	%CV
Control n = 3	1.0	68	1.8×10^5	79	0.14	70	20×10^6	61
+ $CuSO_4$ (100 μM) n = 4	2.2	72	8.0×10^5	86	0.14	41	54×10^6	30
+ $ZnSO_4$ (100 μM) n = 2	1.2	33	7.1×10^5	35	0.17	52	38×10^6	44

Cells ($3\text{-}4 \times 10^4/cm^2$) were treated as indicated in Fig. 2. The values were deduced by the " LIGAND " program [30] to fit the data to a two-site independent model (conglomerate analysis from n independent experiments). High affinity sites with an apparent dissociation constant K_{D1}, and a number of interacting molecules N_1, have been shown to represent cell-specific interactions [16]. Low-affinity/high-capacity sites with an apparent dissociation constant K_{D2}, and a number of interacting molecules N_2, were essentially associated with pericellular components [16]. The relatively large coefficient of variations (%CV) indicates that angiogenin binding follows a more complex model.

FIGURE LEGENDS

Fig. 1. Metal-ion-concentration-dependence of ^{125}I-rAng binding to CPAE cells. Cells (1.4 x 10^5/2 cm^2) were incubated at 4 °C with ^{125}I-rAng (0.9 nM; 2.4 x 10^5 cpm) in binding buffer containing increasing concentrations of $CuSO_4$ (··), $ZnSO_4$ (▪▪) and $CuCl_2$ (▪▪). Non-specific binding was determined using a 1000-fold excess of unlabeled rAng. Radioactivity bound specifically with low affinity was released by 0.6 M salt wash [16] (A) and that specifically bound to high affinity sites was obtained by solubilizing the cells (B). Ni^{2+}, Co^{2+} and Li^{+} in the same concentration range had no effect on rAng binding (not shown). Values are the mean ± s.e. of triplicate determinations.

Fig. 2. Scatchard plot of total rAng binding to CPAE cells at 4 °C in the presence or absence of Cu^{2+} and Zn^{2+}. CPAE cell cultures (6.7 x 10^4 cells/2 cm^2) were incubated with increasing concentrations of ^{125}I-rAng and unlabeled rAng when needed, for 4 h at 4 °C in binding buffer containing 100 μM $CuSO_4$ (··), $ZnSO_4$ (··) or in the absence of metal ions ( ). After incubation, cells were washed three times with binding buffer and then solubilized with the 4 M guanidine hydrochloride buffer as described in the text. The data from representative experiments are plotted ± s.e. of triplicate determinations. The curves were deduced by the " LIGAND " program [30] as indicated in Table 1.

Fig. 3. Interaction of rAng and RNase A with an iminodiacetate-Sepharose column precharged with Zn^{2+}. ( ) rAng (400 μg, 0.2 ml), ( ) RNase A (400 μg, 0.2 ml) or ( ) IgG (500 μg, 0.1 ml), was applied to IDA-Zn^{2+} columns which were developed with a linear imidazole concentration gradient (0-10 mM). Elution was monitored by absorbance at 280 nm.

Fig. 4. Chromatography of rAng and RNase A on iminodiacetate-Sepharose columns precharged with Cu^{2+}. Protein (400 μg, 0.2 ml): (··) rAng, ( ) RNase A or ( ) BSA, was applied on a 5 ml IDA-Cu^{2+} column in equilibrating buffer. The columns were developed with a linear imidazole concentration gradient (1-10 mM). (A) Elution profiles at 280 nm. (B) Absorption spectra of the breakthrough fraction: (1) Blank column; (2) rAng column.

Fig. 5. Stoichiometry of rAng-copper interaction. rAng (25 μM, 0.8 ml) in Hepes buffer pH 7.5, was incubated for 5 min at room temperature in the presence of increasing amounts of $CuSO_4$. Bound and unbound copper was filtered through Centricon 10 by centrifugation at 4 °C and analyzed by graphite furnace atomic absorption spectrometry.

Figure 1

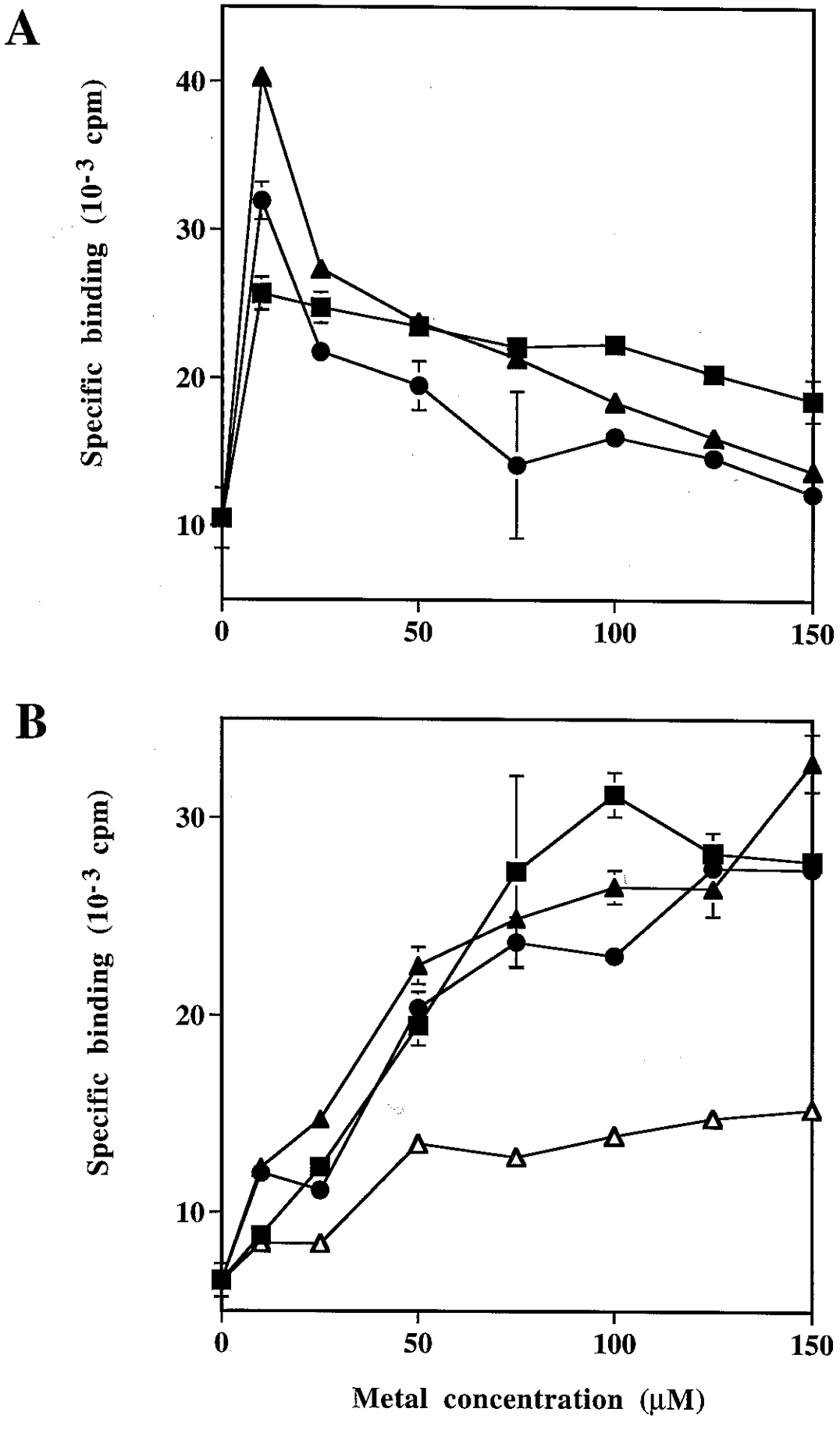

A
Specific binding (10-3 cpm)
40
30
20
10
0
50
100
150
B
Specific binding (10-3 cpm)
30
20
10
0
50
100
150
Metal concentration (μM)

Figure 2

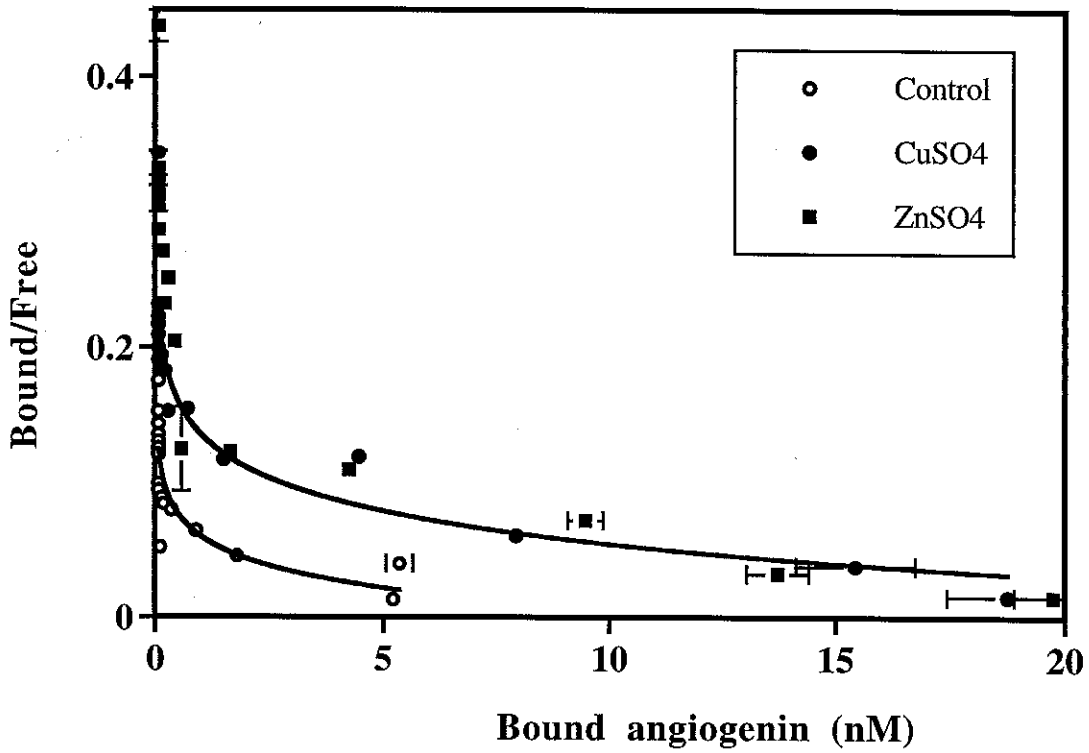

Control
CuSO4
ZnSO4
Bound/Free
0.4
0.2
0
0
5
10
15
20
Bound angiogenin (nM)

Figure 3

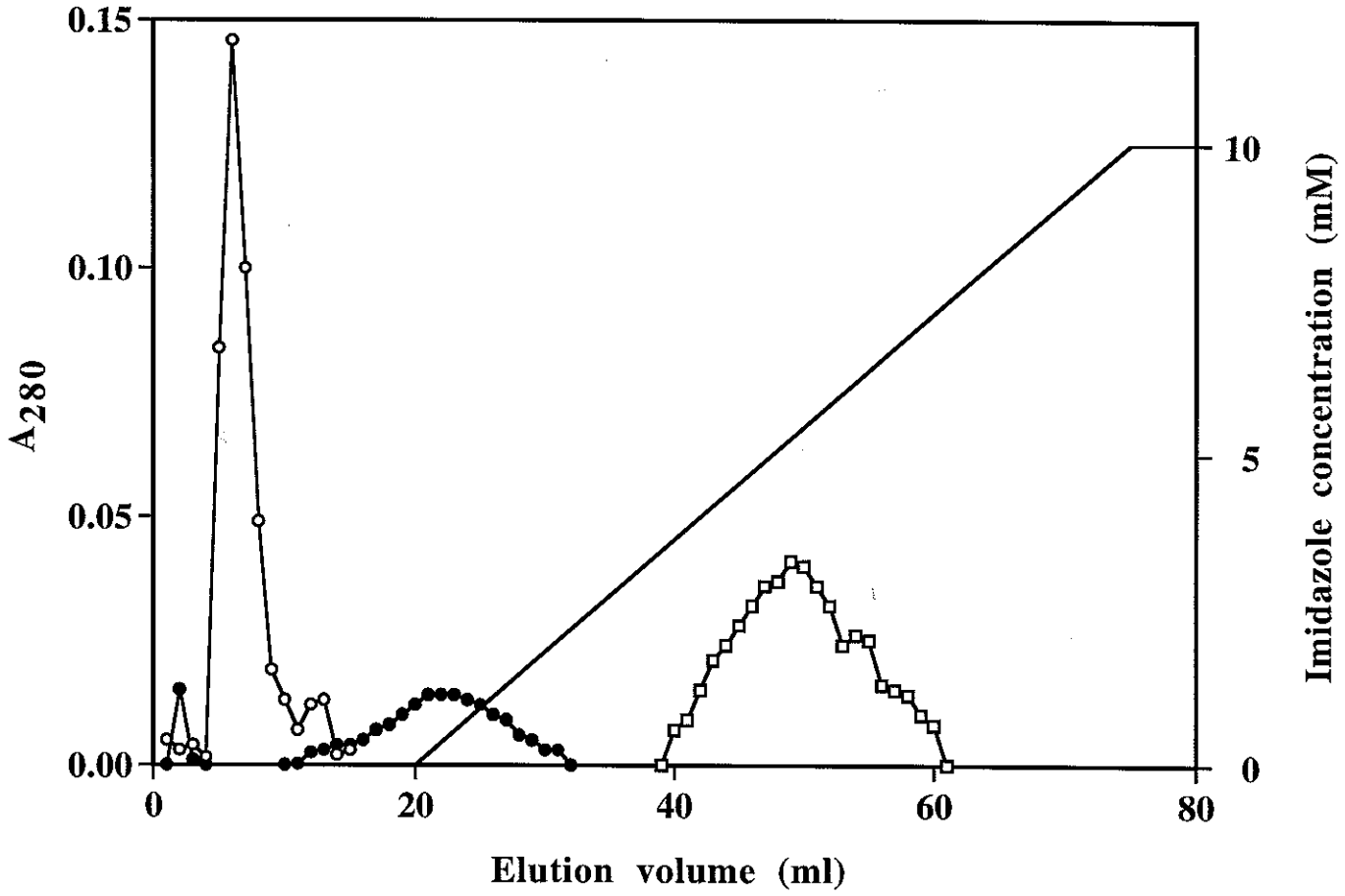
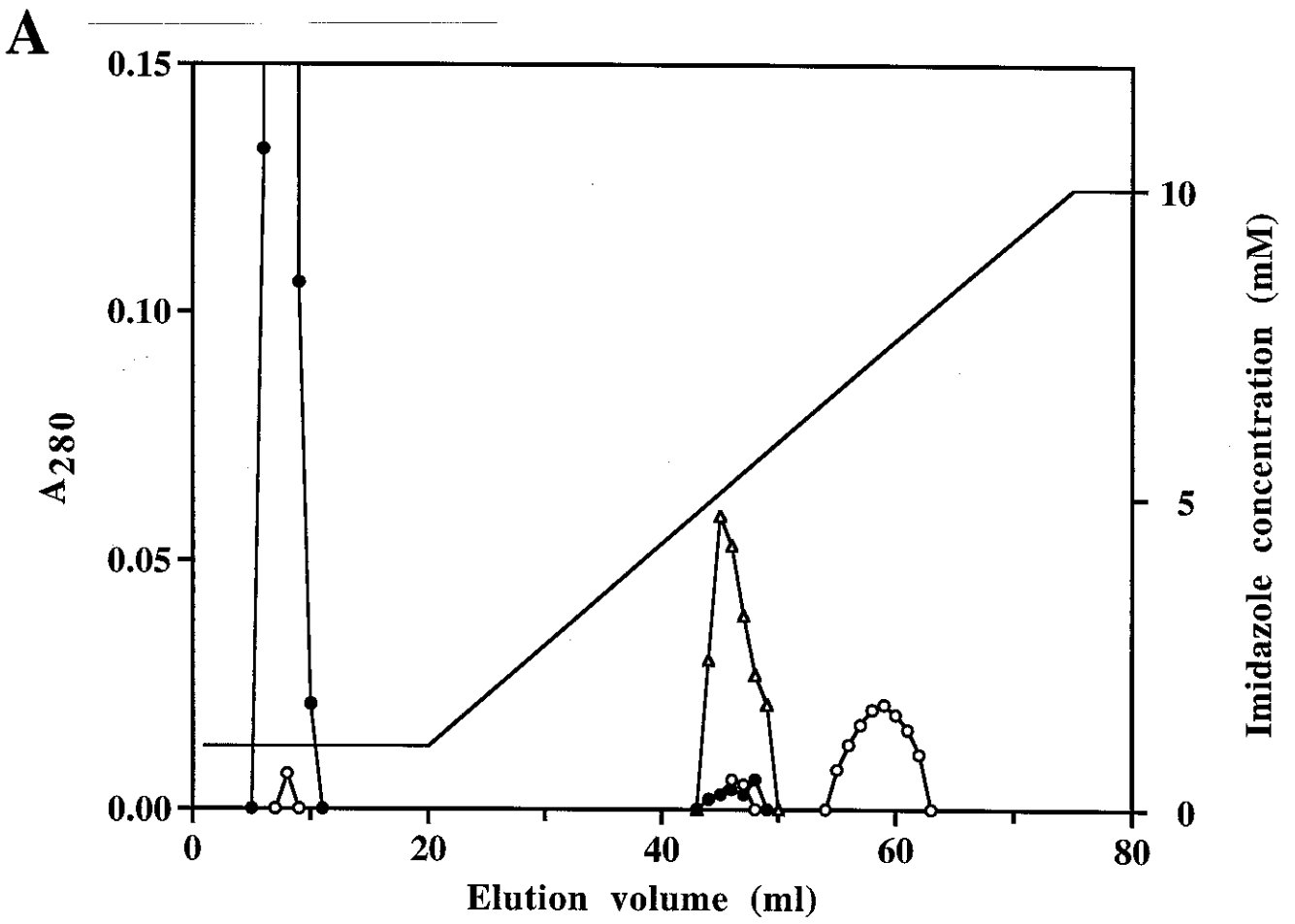

0.15
0.10
0.05
0.00
A_{280}
0
20
40
60
80
Elution volume (ml)
10
5
0
Imidazole concentration (mM)

Figure 4


A
0.15
0.10
0.05
0.00
A280
10
5
0
Imidazole concentration (mM)
0
20
40
60
80
Elution volume (ml)

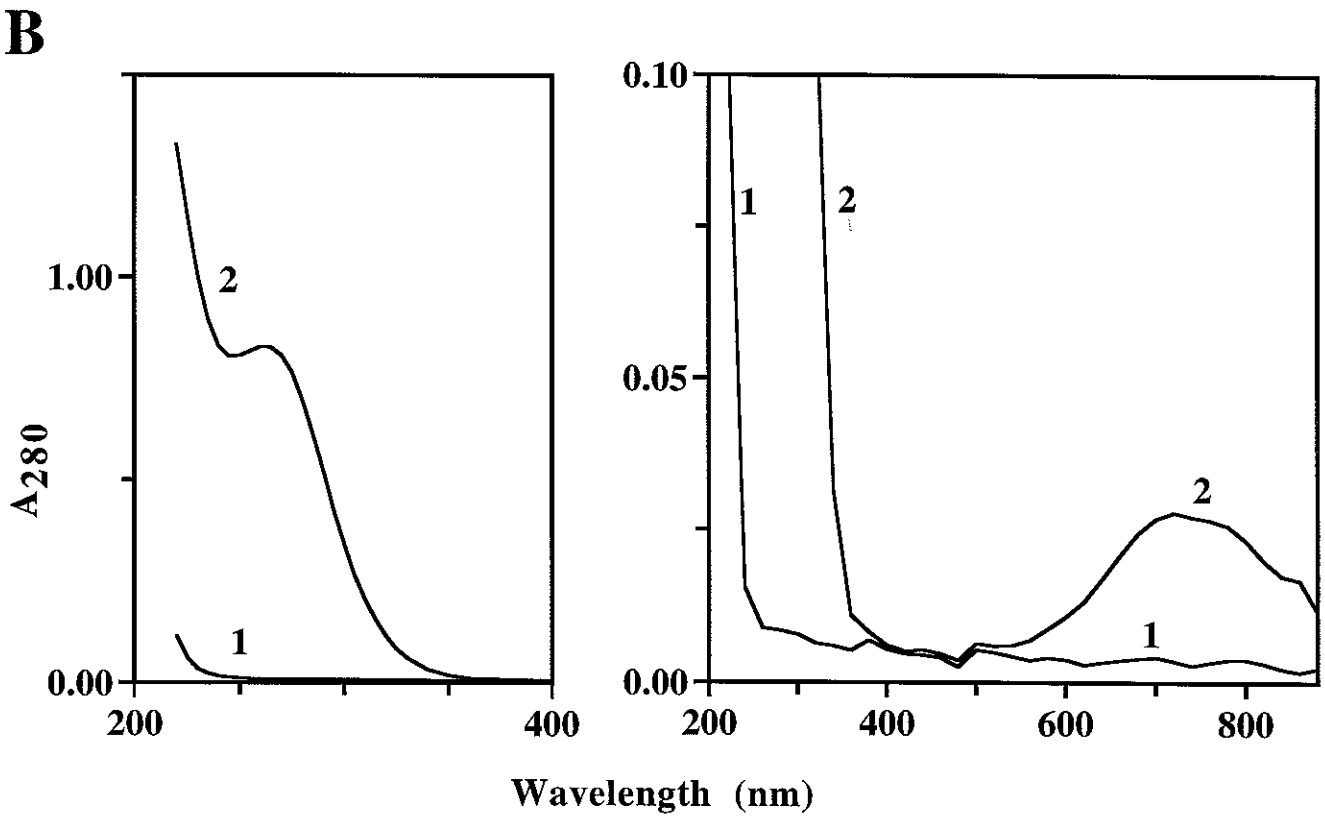
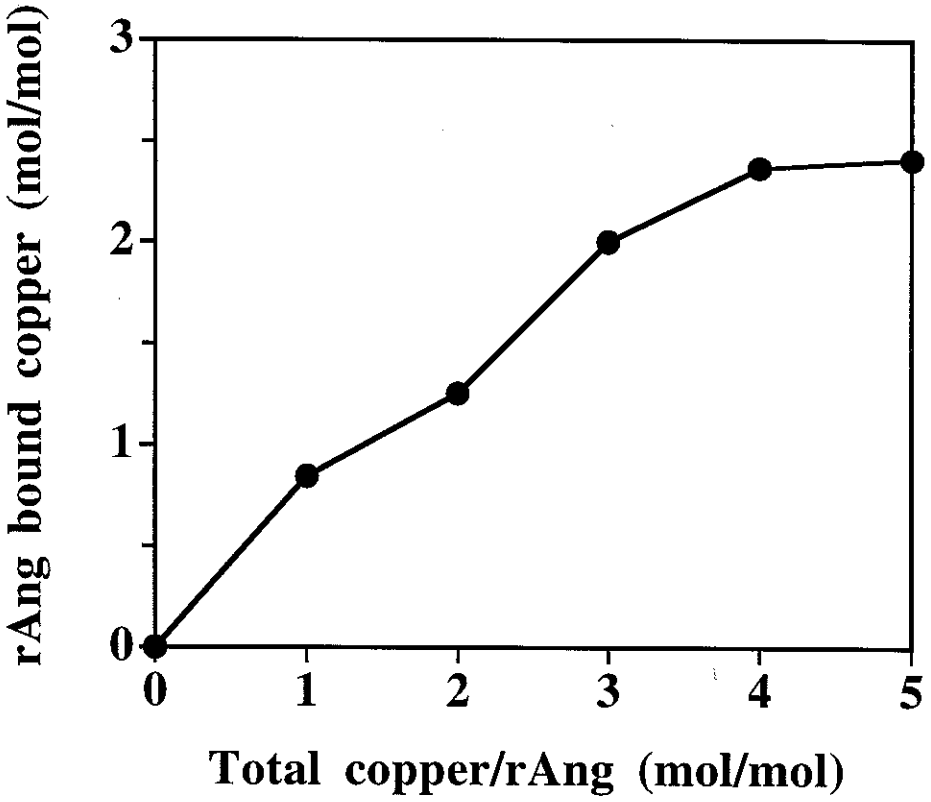

B
1.00
0.00
A280
2
1
200
400
0.10
0.05
0.00
1
2
2
1
200
400
600
800
Wavelength (nm)

Figure 5


rAng bound copper (mol/mol)
3
2
1
0
0
1
2
3
4
5
Total copper/rAng (mol/mol)