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Characterisation by immobilised metal ion affinity chromatographic procedures of the binding behaviour of several synthetic peptides designed to have high affinity for Cu(II) ions

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Abstract

In this investigation, several peptides containing an increasing number of histidine residues have been designed and synthesised. The peptides involved repeat units of either the pentameric EAEHA or the tetrameric HLLH sequence motifs. Adsorption isotherms for these synthetic peptides and hexahistidine (hexa-His) as a control substance were measured under batch equilibrium binding conditions with an immobilised Cu(II)–iminodiacetic acid (IDA) sorbent. The experimental data were analysed in terms of Langmuirean binding behaviour. In common with previous studies with synthetic peptides, these investigations have demonstrate that the sequential organisation of the histidine side chains in these peptides can affect the selectivity of the coordination interactions with borderline metal ions in immobilised metal ion affinity chromatographic systems. The results also confirm that peptides selected on the basis of their potential to form amphipathic secondary structures with their histidine residues presented on one face of the molecule can exhibit equivalent or higher affinity constants towards copper ions than hexa-His, although they contain fewer histidine residues. These findings are thus relevant to the selection of peptides produced inter alia by combinatorial synthetic procedures to have enhanced binding properties for Cu(II) or Ni(II) ions, or intended for use as peptide tags in the fusion handle approach for the affinity chromatographic purification of recombinant proteins.

Keywords: Immobilised metal affinity chromatography; Adsorption isotherms; Peptides; Histidine

1. Introduction

Immobilised metal ion affinity chromatography (IMAC) is a widely used technique for the purification of wild type or recombinant proteins. Commonly, Cu(II), Ni(II) or Zn(II) ions are immobilised onto support materials modified with a tridentate chelating compound, e.g., iminodiacetic acid (IDA), or a tetradentating compound, e.g., nitrilotriacetic acid (NTA), permitting selective interactions between the immobilised M^{n+}–chelate complex with surface accessible histidine residues [1,2] of the protein. In this mode of adsorption chromatography, selectivity is achieved by exploiting the Lewis base donor properties of the nitrogen free electron pair or N-π-electrons of the imidazolyl side chains of histidine, coupled with the favourable protonation dependency of the side chains themselves. This behaviour also permits relatively straightforward elution protocols to be employed. When operated in this selectivity mode,

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IMAC takes advantage of the fact that histidine residues occur rather infrequently in proteins in terms of natural abundance [3]. As a consequence, IMAC procedures exploiting histidine-mediated interactions with immobilised M^{n+}–IDA sorbents (where the immobilised M^{n+} ion corresponds to a weak or borderline Lewis acid acceptor as defined by Pearson [4]) have gained considerable popularity since this variant of immobilised chelating complex chromatography (IMCC) was introduced by Porath et al. in 1975 [5].

In an increasing number of biotechnology applications [6,7], proteins produced by recombinant techniques have been isolated through the expediency of an introduced peptide fusion sequence, whereby the natural protein sequence is extended by a short segment of six to eight residues, often the same amino acid, at the N- or C-terminal position [8–13]. Currently, the hexahistidine (hexa-His) tag represents the most popular example of the use of this concept as an ''affinity handle'' for the purification at small scale of recombinant proteins [14–16], exploiting the ability of this residue to bind to immobilised borderline metal ions such as Cu^{2+} or Ni^{2+}. In some cases, however, the hexa-His moiety, when included as part of a recombinant protein, does not exhibit the expected affinity for immobilised metal ions [17,18] in IMAC systems, possibly due to the tag becoming buried within the internal structure of the folded recombinant protein.

Other strategies, which can be contemplated for the generation of peptide affinity tags for the selective isolation of recombinant proteins [19,20] in the presence of soft or borderline metal ions chosen from the first row of the transition series of elements (i.e., Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+} or Cr^{3+}), involve presenting fewer histidine residues, but in well organised geometrical array such as α-helical or β-sheet secondary structures. Analogous considerations also apply to the use of affinity tagged proteins in binding assays arrayed in 96-well formats based on the directional immobilisation of recombinant proteins. Application of such approaches with IMAC systems can be found in the studies by Haymore and co-workers [21,22] with bovine growth hormone, or in the investigations by Hutchens and Yip [23] with synthetic peptides related to metal ion transport proteins, by Smith et al. [24] with luteinising hormone releasing factor analogues, and by Sulkowski et al. [25,26] with α-interferon-related peptides. Surprisingly, few investigations have systematically examined the influence of the position and number of histidine residues within the sequence of such peptide structures, particularly those that have the potential to adopt preferred secondary structures. Studies reported by Sulkowski et al. [25,27], Hutchens and Yip [28], Smith et al. [29] and Hansen et al. [30] have largely focused on synthetic peptides containing only a single histidine, where it has been shown that a histidine residue at the N-terminal position makes a smaller binding contribution in the ligand–ligate interaction than a histidine residue that is located more centrally or towards the C-terminal position. This finding is consistent with the anticipated change in the electronic properties of the lone pair electrons of the donor N-atom due to the effect of the peptide main chain dipole on the π-electron cloud of the imidazole ring. Moreover, recent work [21,22,31,32] with recombinant proteins has suggested that histidine residues in a $i \rightarrow i+4$ arrangement (i.e., His–XXX–His, where X is another amino acid) in α-helical presentation potentially can exhibit different binding affinities in IMAC systems than when presented in a β-sheet arrangement.

When Cu^{2+}, Ni^{2+} and the other borderline M^{2+} ions are chelated to the immobilised IDA ligate, potentially three coordination sites are available within the coordination sphere of the metal ion for interaction with donor groups on a protein, such as histidine residues. Two histidine residues, separated by a pitch of 5.4 Å in a regular α-helical structure or within a pitch range of 6.4 to 6.8 Å that characterises the parallel and antiparallel β-sheet-like structures of many globular proteins, can potentially interact simultaneously with the coordination sphere of the immobilised metal ion. As part of our current work [33–36] with new ligates and fusion tags for use in IMAC systems generated by, inter alia, combinatorial synthetic methods, we have investigated procedures for the selection of short peptide sequences with the appropriate orientational display of their amino acid side chains. The binary, or in some cases tertiary, binding sites that peptides and proteins can exhibit, due to the unique disposition of specific amino acid side chains, in their interaction with immobilised metal ion complexes has been exploited

as part of these earlier investigations [34,37–40]. These highly selective peptide sequences can then be used as binding ligands in conjunction with these new chelating ligates as a cassette approach for the biospecific affinity chromatographic isolation of recombinant proteins. Thus, various peptides containing multiple histidine residues have been examined for their potential to form amphipathic α-helical structures whereby the His residue(s) are conformationally constrained onto one side of the molecule. These studies have led us to design, synthesise and evaluate different peptides with enhanced binding and selectivity features for a specific metal ion, and which thus represent alternative peptidic structures for use as IMAC fusion tags with recombinant proteins. In the present paper, we describe the binding properties of two sets of peptides with histidine residues in different sequential arrangements selected on the basis of these considerations. The results further extend the information available on the importance of composition and sequential arrangements of peptides in their interaction with IMAC sorbents, allowing structures with equilibrium binding constants of equivalent or greater magnitude than hexa-His for immobilised Cu^{2+}–IDA sorbents to be identified.

2. Experimental

2.1. Abbreviations

The abbreviations for amino acids follow the recommendations of the IUPAC-IUB Commission of Biochemical Nomenclature. TFA=Trifluoroacetic acid; Fmoc=9-fluorenylmethyloxycarbonyl; DIC=diisopropylcarbodiimide; HBTU=*O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate; HOBt=1-hydroxybenzotriazole; DIEA=1,3-diisopropylethylamine; DMF=*N*,*N*-dimethylformamide; EDT=ethane-1,2-dithiol; HPLC=high-performance liquid chromatography; TNBSA=trinitrobenzene-sulphonic acid; TPCK=L-1-tosyl-amide-2-phenylethylchloromethyl ketone; DMAP=4-dimethylamino-pyridine; PBS=phosphate-buffered saline.

2.2. Materials and methods

TFA, DMF, piperidine, HOBt, HBTU, *p*-alkoxybenzyl alcohol resin and the L-α-Fmoc-protected amino acids were obtained from Auspep (Melbourne, Australia). Unless otherwise stated, all the solvents were of analytical grade. Phenol was obtained from Merck Australia (Kilsyth, Australia). Thioanisole, ethanedithiol, acetic anhydride, diisopropylethylamine and trifluoromethanesulphonic acid were obtained from Aldrich (Milwaukee, WI, USA). Diisopropylcarbodiimide was obtained from Sigma (St. Louis, MO, USA). Fmoc-Glu-(Rink amide mBHA)-OtBu resin was obtained from Novabiochem (Sydney, Australia). Solid-phase peptide synthesis was performed either manually or using a PS3 Protein Technologies Automated Peptide Synthesiser, Rainin (Woburn, MA, USA).

2.3. Peptide synthesis

The peptides EAEHA, EAEHAAHEAEHA, LHLLH, LHLLHHL, LHLLHHLLH, LHLLHHLLHHLLH and HHHHHH were prepared using standard solid-phase peptide synthesis (SPPS) procedures, using either the Fmoc-L-α-His(Trt)-NovaSyn TGT resin (0.17 mmol/g, 1.176 g), the Fmoc-L-α-Leu-Wang resin (0.56 mmol/g, 0.405 mg) or Fmoc-Ala-Wang resin (0.65 mmol/g, 0.308 g), respectively, with standard Fmoc synthesis protocols and the HBTU/HOBt activation method [41]. The crude peptide was cleaved in one step with phenol (0.75 g), ethanedithiol (0.25 ml), thioanisole (0.50 ml), deionised water (0.50 ml) and TFA (10 ml). The cleavage mixture was stirred for 2 h before filtering, concentrating and precipitated with cold ether. The ether solution was kept in the freezer overnight before being filtered and taken up in 50% acetonitrile and lyophilised.

2.4. Peptide purification and analysis by reversed-phase (RP) HPLC

The crude synthetic peptides were purified by semi-preparative RP-HPLC with a TSK-ODS-120T C_{18} column (300×21.5 mm I.D., particle size 10 μm), obtained from Tosoh (Yamaguchi, Japan) which was protected by a guard column, using a

two-buffer system, with a gradient of 0–100% buffer B over 1 h and a flow-rate of 6 ml/min with detection at 254 nm. The following elution system was used: eluent A, 0.1% TFA in water; and eluent B, 0.1% TFA in water–acetonitrile (40:60, v/v). Fractions were collected using a Pharmacia (Frac-100) fraction collector. Analytical experiments were carried out with TSK C_{18} columns (150×4.6 mm, particle size 5 μm). Samples were eluted using the following protocol: eluent A, 0.1% TFA in water; and eluent B, 0.1% TFA in water–acetonitrile (40:60, v/v) with 0–85% eluent B over 25 min, using a linear gradient and a flow-rate of 1 ml/min with detection at 214 nm.

2.5. Mass spectra

Electrospray mass spectroscopy was carried out on a Perkin-Elmer Sciex mass spectrometer Model ''PE Sciex API///''. The scan range was set between 200 and 2400 u and 5 to 50 μl of the peptide sample was injected via a ''PE ISS 200'' autoinjector. The solvent used was 60% aqueous acetonitrile, 0.1% acetic acid (HPLC grade).

2.6. Amino acid analysis

Peptide solutions were dried and hydrolysed in constant boiling point 6 *M* HCl (0.4 ml/mg) at 110°C for 24 h under reduced pressure. Amino acid analyses were performed using the phenylisothiocyanate (PITC) derivatisation procedure [42].

2.7. Preparation of IDA-Sepharose CL-4B

Sepharose CL-4B was activated with epichlorohydrin according to a method by Porath and Olin [43]. In brief, 10g of Sepharose CL-4B was mixed with 10 ml of 2 *M* sodium hydroxide solution and 37.5 mg sodium borohydride. The mixture was incubated at room temperature for 2 h, after which 12 ml of epichlorohydrin was added and the mixture was shaken for 15 h at room temperature. The activated Sepharose CL-4B was recovered by vacuum filtration and washed extensively with 20 volumes of water. Iminodiacetic acid (1 g) was added to the activated Sepharose CL-4B (10 g) suspended in 2 *M* Na_2CO_3 solution, and the mixture was agitated on a shaking water bath at 60°C for 15 h. The IDA-Sepharose CL-4B was again recovered by vacuum filtration and washed successively with water, 50 m*M* acetate buffer, pH 4.0 and again with water. This IMAC sorbent was finally suspended in 20% (v/v) ethanol in water and stored at 4°C until used.

2.8. Adsorption isotherms

The IDA-modified Sepharose CL-4B was complexed with copper (II) ions by suspension in a 1 mg/ml $CuSO_4$ solution. The immobilised Cu^{2+}–IDA sorbent was extensively washed with Milli-Q water and then with the PBS equilibration buffer (10 m*M* KH_2PO_4/K_2HPO_4 buffer–137 m*M* NaCl, pH 7.4). The sorbent was vacuum filtrated, 2-g aliquots of the wet gel suspended in 2 ml PBS equilibration buffer, and 100-μl aliquots of this suspension placed into the adsorption tubes. Under these conditions, a conversion factor of ca. 40 applies, when the adsorption data are expressed as μmol peptide bound per g gel. Peptide solutions ranging in concentration from 0.06 to 0.6 mg/ml were prepared in PBS equilibration buffer. Aliquots were taken to permit precise determination of the peptide concentration by quantitative amino acid analysis and by RP-HPLC analysis using a modification of previously described protocols [26–28] with the elution system employed in the analytical separations described above and a set of standard curves determined in preliminary studies. A total volume of 5 ml of peptide solution was added to the IMAC sorbent in each adsorption tube and the suspension was agitated for 2 h at room temperature (20°C) after which the tubes were centrifuged. A sample of the supernatant was then taken for determination of the residual free peptide concentration. All experiments were performed in duplicate with the experimental error typically $\leq \pm 5\%$ of mean values.

2.9. Data evaluation

The experimental data were analysed from the plots of the free adsorbate concentration (c^*) versus amount bound (q^*), $1/c^*$ versus $1/q^*$, c^* versus c^*/q^* and q^* versus q^*/c^* were employed [44–46] to assess whether the adsorption can be approxi-

mated to a Langmuir isotherm [47], i.e., a one-to-one stoichiometry occurs in the interaction of the ligate with the adsorbate molecule, all binding sites are of equivalent binding energy, and the bound adsorbate molecules do not affect the binding at neighbouring sites. Significant deviations from linear behaviour in these plots indicate that non-Langmiurean binding mechanism(s) prevail in the interaction between the ligate and the adsorbed peptide molecules.

3. Results and discussion

In initial experiments, the ligate density of the IDA-modified Sepharose CL-4B was determined by frontal analysis with $CuSO_4$ solutions of various concentrations. Under the conditions employed, 1 ml of the IDA-modified Sepharose CL-4B bound 34.4 μmol of Cu(II) ions with an association constant of $1.05 \cdot 10^7$ mol/l. The adsorption properties of two sets of peptide structures, involving multiple copies of the pentapeptide EAEHA or the tetrapeptide HLLH motif, as well as hexa-His as a control substance, were then determined using batch equilibrium binding conditions. These peptides were selected on the basis of their calculated hydrophobic moments [48], their Edmundson wheel projections [49], their predicted propensity to form α-helical structures in the presence of immobilised metal ion ligates, and the disposition of their histidine and other flanking residues. In common with other small peptides of less than 15 amino acid residues, under aqueous buffer conditions, such as the 10 m*M* KH_2PO_4/K_2HPO_4 buffer–137 m*M* NaCl, pH 7.4, conditions employed in this study as the equilibration and/or adsorption buffers, such synthetic peptides are not expected to assume any significant extent of secondary structure as assessed from circular dichroism spectroscopic measurements. However, in the presence of a IMAC ligate, peptides with histidine residues in a $i \rightarrow i+2$ or $i \rightarrow i+4$ arrangement, as found in the well known HXH or HXXXH sequence arrangements, could in principle assume preferred secondary structures whereby the histidine residues become localised to the same face of the molecule.

In Fig. 1a and b are shown several projections of the peptides EAEHAAHEAEHA and LHLLHHLLHHLLH presented in their α-helical

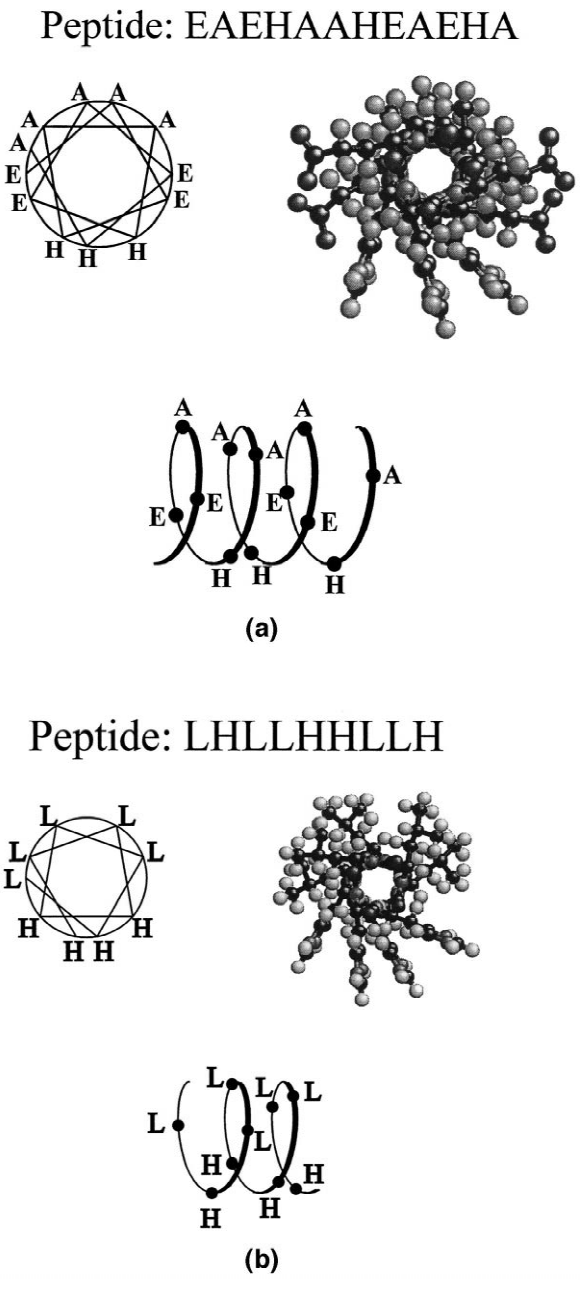

Fig. 1. Representation of the synthetic peptides EAEHAAHEAEHA (a) and LHLLHHLLHHLLH (b) in their α-helical conformations as stick-filled Edmundsen wheel, ball-filled Edmundsen wheel and cylinder-filled projections, with the histidine residues located on one face of the molecule highlighted.

conformations, where it can be seen that all histidine residues face onto one side of the molecule. In the case of the peptides involving the EAEHA sequence motifs, the contra-faces of these peptides are predicted to be highly charged due to the presence of Glu residues which intervene the clusters formed on the opposite faces by the His and Ala residues, whilst with the HLLH sequence motif peptides, typical amphipathic α-helical structural features are evident due to the presence of the Leu residues as a hydrophobic contra-face. In these arrangements, two or more imidazole groups within these peptides become, in spatial terms, sufficiently close to permit a bidentating-type interaction with the coordination sphere of a single immobilised copper ion as shown in Fig. 2, notwithstanding the small ionic radius of 0.72 Å for the Cu(II) species. This binary binding was expected to lead to favourable adsorbate–ligate interactions. Moreover, when employed as peptide fusion tags with recombinant proteins, the location of hydrophilic residues, such as glutamic acid residues, as contra-faced entities in such His residue constructs can prevent the insertion of these tag sequences into the predominantly hydrophobic protein interior, and therefore can maintain the surface accessibility of the imidazole side chains.

The adsorption isotherms for the peptide set involving the sequences EAEHA and EAEHAAHEAEHA are shown in Fig. 3a and b. Fig. 4a–c show the analogous isothermal results for the peptide set involving LHLLH, LHLLHHL and LHLLHHLLH, whilst Fig. 5 shows the adsorption results for HHHHHH. These adsorption measurements for these peptides were in each case carried out over a define concentration range to ensure adequate solubility and absence of self-association effects. For example, the corresponding adsorption isotherms for the LHLLHHLLHHLLH peptide could not be measured due to the intrinsic property of this peptide to self-association in aqueous buffers leading to precipitation effects. Shown as inserts in these figures are also the semi-reciprocal plots of c^* versus c^*/q^* for each of these peptide, with the degree of linearity in the binding data consistent with the adsorption being described in terms of a Langmuirean process. As has been documented previously [44,45], the advantage of employing the semi-reciprocal plot over other types of transformations (i.e., the Scatchard plot) with such IMAC binding data is that the emphasis is placed on the range involving higher peptide concentration values where the experimental error in the equilibrium adsorption measurement is smallest. Table 1 details the corresponding values for the dissociation constants (K_d values) and binding capacities (q_m values) of these peptides derived by fitting the experimental data to the double reciprocal, semi-reciprocal and Scatchard plots, assuming that Langmuirean adsorption behaviour was prevailing. Several conclusions can thus be drawn from these results.

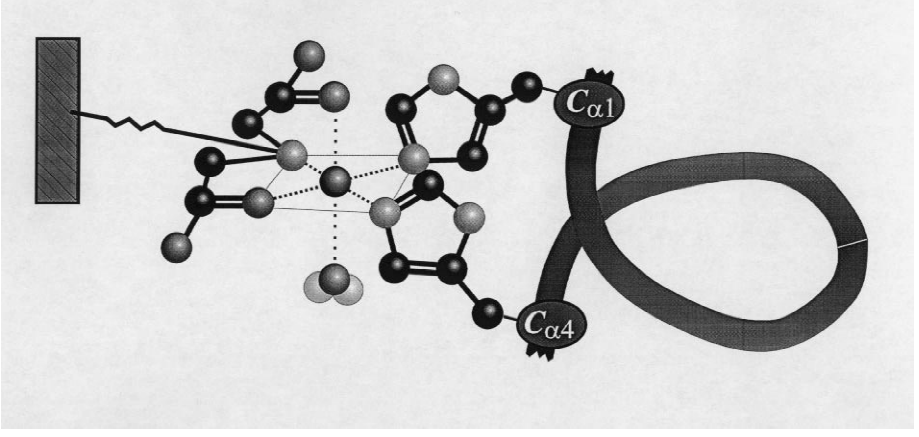

Fig. 2. Representation of the peptide EAEHAAHEAEHA bound to the immobilised Cu^{2+}–IDA complex via a bidentaing interaction which involves participation of two of the equatorial coordination sites being occupied by the imidazolyl groups of the histidine residues. Prior to the generation of this peptide-immobilised Cu^{2+}–IDA complex, these two coordination sites would have been occupied by water.

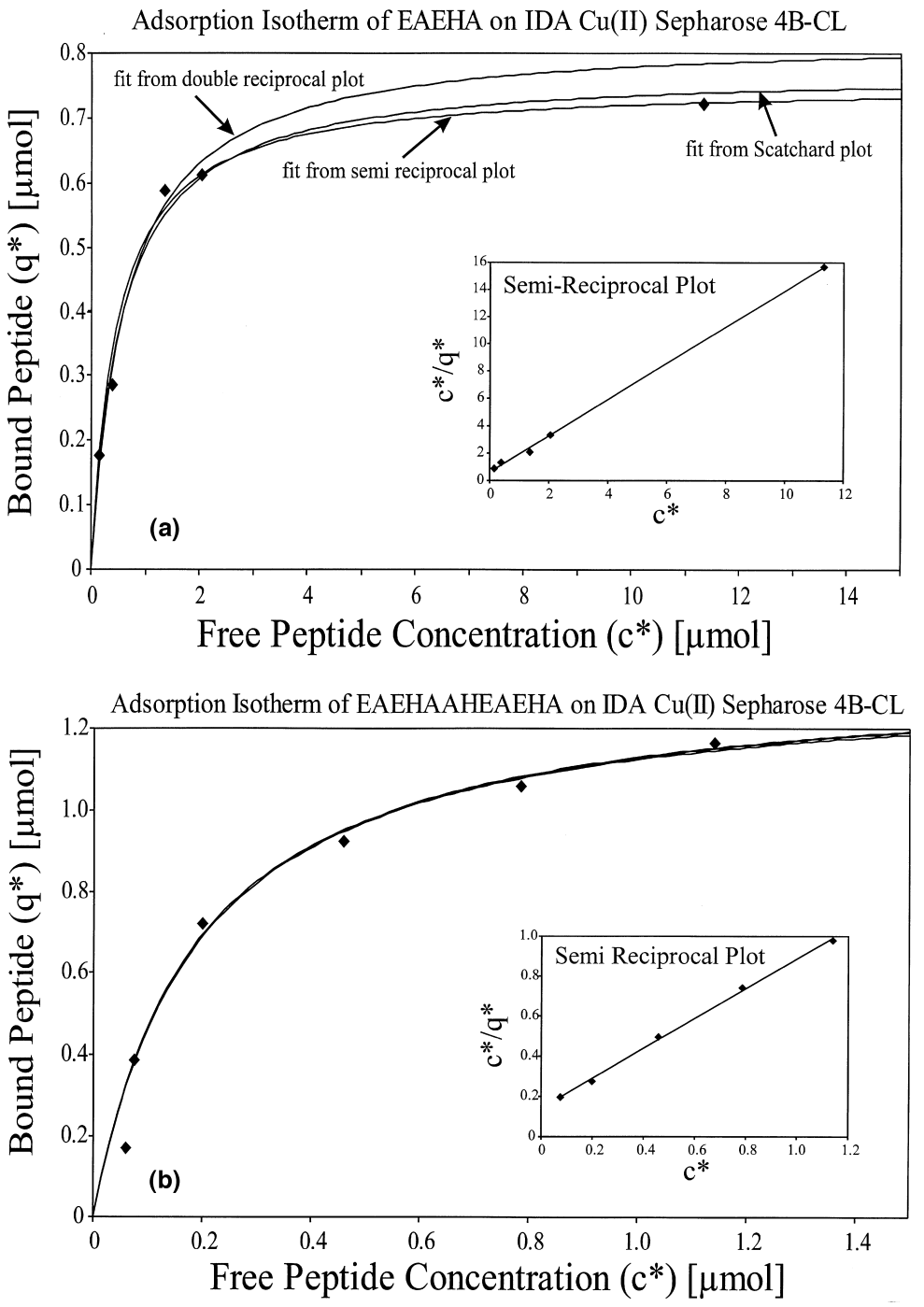

Fig. 3. Plots of the free adsorbate concentration (c^*) against the amount bound (q^*) for the peptides EAEHA (a) and EAEHAAHEAEHA (b) with the experimental data shown as ♦. The experimental errors for the measurement of the protein concentrations were typically $\leq \pm 5\%$ as a S.E.M. Also shown are the predicted plots based on double reciprocal ($1/c^*$ vs. $1/q^*$), semi-reciprocal (c^* vs. c^*/q^*) and Scatchard (q^* vs. q^*/c^*) analysis, assuming that the binding of these peptides to the immobilised Cu^{2+}–IDA complex can be approximated by a Langmuirean isothermal process.

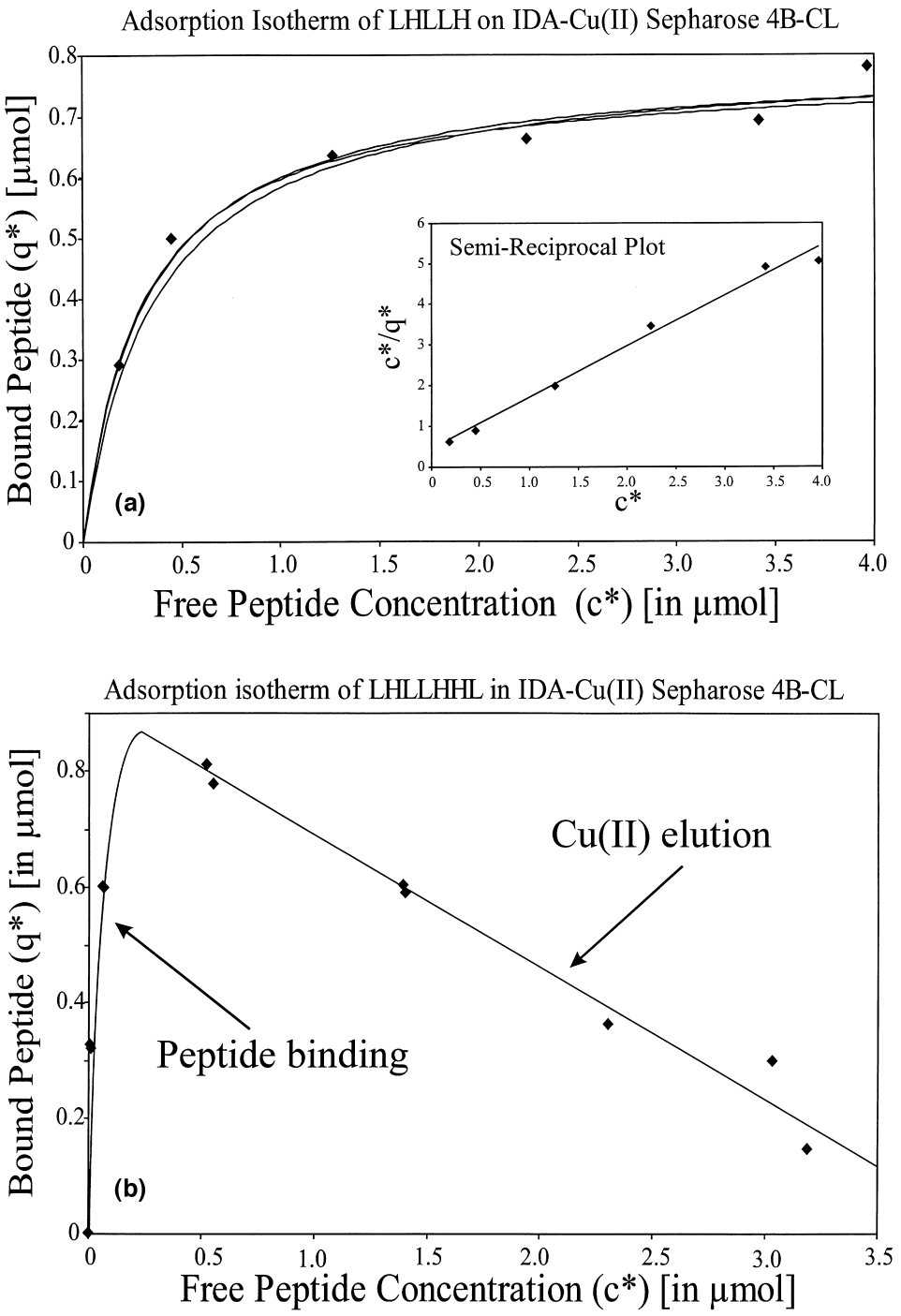

Fig. 4. Plots of the free adsorbate concentration (c^*) against the amount bound (q^*) for the peptides LHLLH (a), LHLLHHL (b) and LHLLHHLLH (c) with the experimental data shown as ♦. The experimental errors for the measurement of the protein concentrations were typically $\leq \pm 5\%$ as a S.E.M. Also shown are the predicted plots based on double reciprocal ($1/c^*$ vs. $1/q^*$), semi-reciprocal (c^* vs. c^*/q^*) and Scatchard (q^* vs. q^*/c^*) analysis, assuming that the binding of these peptides to the immobilised Cu^{2+}–IDA complex can be approximated as a Langmuirean isothermal process.

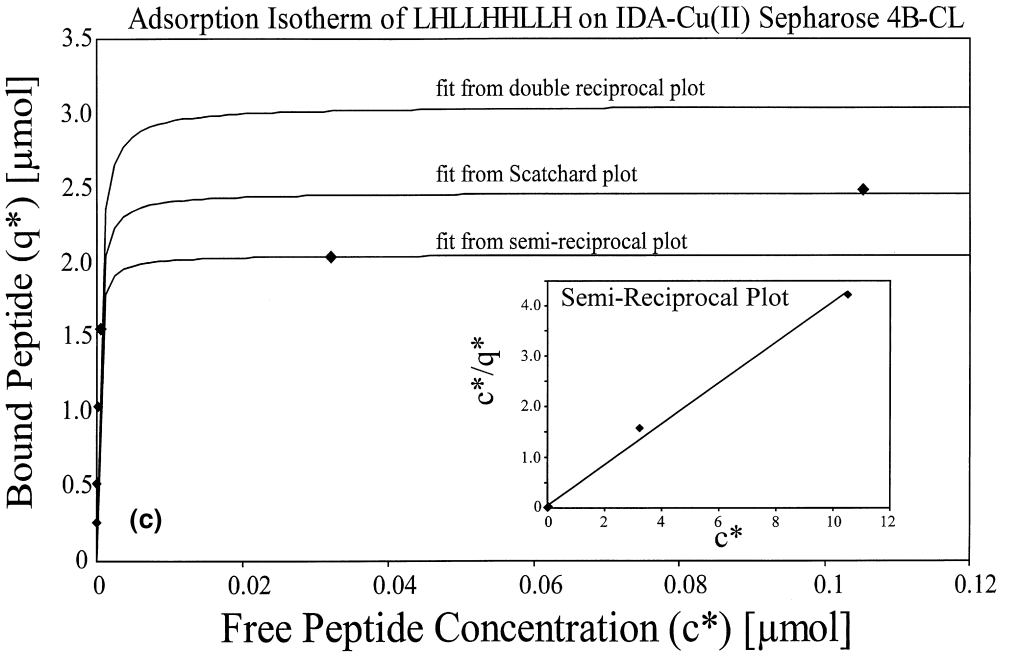

Fig. 4. (*continued*)

Firstly, comparable q_m values were obtained for the EAEHA-related peptide set and hexa-His, despite the differences in the histidine content, although approximately 100-fold differences in the K_d values were observed. These differences in K_d values for the EAEHA-related peptide set and hexa-His were reflected in the shape of the isothermal plots with the EAEHA-related peptides exhibiting more shallow profiles, although in all cases saturation of the sorbent to similar capacity values was achieved. Since the total binding capacities of the EAEHA-related peptides and hexa-His were similar under these batch equilibrium binding conditions, it can be concluded that similar ligate–adsorbate stoichiometry may apply with these different peptides, with little evidence for multi-layering or homodimerisation, typified as Temkin-like isotherms, that have been seen in the IMAC of proteins [33,38,50].

Compared to hexa-His, the peptide LHLLH exhibited a lower binding capacity, and a K_d value similar to that observed for the EAEHA-related peptides. For the peptide LHLLHHLLH, a higher binding capacity was observed with a K_d value that was ca. 1000-fold smaller than that determined for the other synthetic peptides and 10-fold smaller than hexa-His (Table 1). Since this peptide has the potential to present an amphipathic face to the external solvent, the high q_m value may be due to peptide self association [33,38,50] at the IMAC sorbent surface. Consistent with this conclusion, the peptide LHLLHHL also exhibited anomalous adsorption behaviour. At low concentrations, this peptide bound very strongly to the immobilised Cu^{2+}–IDA sorbent as evident from the very steep slope in the initial part of the isotherm, but at higher concentrations the peptide LHLLHHL eluted the Cu^{2+} ions from the IMAC sorbent. This metal ion stripping effect was immediately apparent from a discolouration of the particles, as well as from the generation of a blue coloured supernatant. Accordingly, the concentration of peptide bound to the sorbent decreased with increasing peptide concentration as evident from the isothermal plot shown as Fig. 4b. As a consequence, reliable K_d and q_m values for the peptide LHLLHHL could not be determined from the first order double reciprocal, semi-reciprocal and Scatchard plots transformations of these experimental data. Other peptides (e.g., LHLLH and

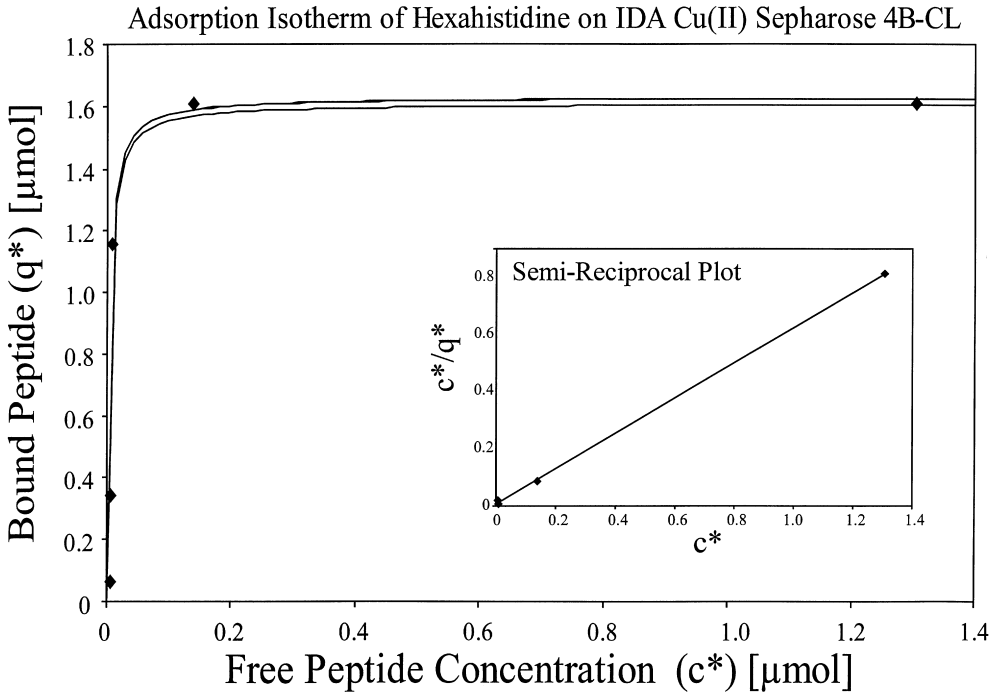

Fig. 5. Plots of the free adsorbate concentration (c^*) against the amount bound (q^*) for hexa-His with the experimental data shown as ♦. The experimental errors for the measurement of the protein concentrations were typically ≤±5% as a S.E.M. Also shown are the predicted plots based on double reciprocal ($1/c^*$ vs. $1/q^*$), semi-reciprocal (c^* vs. c^*/q^*) and Scatchard (q^* vs. q^*/c^*) analysis, assuming that the binding of these peptides to the immobilised Cu^{2+}–IDA complex can be approximated as a Langmuirean isothermal process.

Table 1
Dissociation constant (K_d) and binding capacity (q_m) data for the synthetic peptide sets[a]

	Double reciprocal plot	Semi-reciprocal plot	Scatchard plot
EAEHA			
q_m	$1.77\cdot10^{-6}$	$1.85\cdot10^{-6}$	$1.89\cdot10^{-6}$
K_d	$3.68\cdot10^{-7}$	$3.91\cdot10^{-7}$	$4.19\cdot10^{-7}$
EAEHAAHEAEHA			
q_m	$1.36\cdot10^{-6}$	$1.36\cdot10^{-6}$	$1.35\cdot10^{-6}$
K_d	$1.89\cdot10^{-7}$	$1.92\cdot10^{-7}$	$1.85\cdot10^{-7}$
LHLLH			
q_m	$7.74\cdot10^{-7}$	$7.97\cdot10^{-6}$	$7.87\cdot10^{-6}$
K_d	$2.94\cdot10^{-7}$	$3.65\cdot10^{-7}$	$3.12\cdot10^{-7}$
LHLLHHLLH			
q_m	$3.05\cdot10^{-6}$	$2.05\cdot10^{-6}$	$2.47\cdot10^{-6}$
K_d	$3.47\cdot10^{-10}$	$3.68\cdot10^{-10}$	$2.45\cdot10^{-10}$
HHHHHH			
q_m	$1.63\cdot10^{-6}$	$1.61\cdot10^{-6}$	$1.63\cdot10^{-6}$
K_d	$3.45\cdot10^{-9}$	$3.52\cdot10^{-9}$	$3.46\cdot10^{-9}$

[a] The experimental data for the peptide LHLLHHL could not be fitted to a simple Langmuir-type isotherm due to the displacement of the Cu^{2+} ion at higher peptide concentrations, whilst the corresponding adsorption isotherm for the peptide LHLLHHLLHHLLH could not be determined due to the insolubility of this peptide under the adsorption equilibration conditions.

LHLLHHLLH) showed more conventional isothermal behaviour, although in the case of LHLLHHLLH it was clear from the divergences evident between the double reciprocal, semi-reciprocal and Scatchard plots that the adsorption data of this peptide correlates less well to Langmuirean behaviour. The binding behaviour of LHLLHHL to Cu^{2+} ions can be explained by individual peptide molecules preferentially binding at low peptide concentrations to the Cu^{2+}–IDA complex. Once the concentration of the peptide exceeded the Cu^{2+}–IDA concentration (i.e., ≥ca. 34 μmol/g gel) then competition between the N–O–O groups of IDA forming the tridentate coordinate structure of the Cu^{2+}–IDA–peptide complex and the peptide molecules per se for the Cu^{2+} ions results in the Cu^{2+} ions being progressively stripped from the IMAC sorbent. A similar observation, although to a far lesser extent, was found with hexa-His, where at the highest peptide concentration a slight blue colouration of the supernatant was similarly evident. Metal ion stripping has been recognised [3,34] previously as one of the limiting features of IDA-based sorbents and related tridentating IMAC systems, necessitating the development of chelating ligates with higher metal ion stability constants, such as the NTA or the Tacn-related ligates [15,35,38,51].

The above results comparing the adsorption behaviour of the EAEHA- and HLLH-related peptides to the hexa-His, clearly indicate that the magnitude of the binding affinity between the peptide and Cu(II) does not necessarily increase with the number of histidines present in the molecule. Since the synthetic peptides examined in the present investigation contain repeat structures, it is also interesting to note that a limit to the repeat size is dictated on the one hand by the geometry of the coordination structure of the immobilised Cu^{2+}–IDA complex itself, and on the other by the relative solubility (or propensity to self-associate) of the peptide construct. For example, a ca. two-fold difference in the binding affinity occurred between EAEHA and EAEHAAHEAEHA, consistent with a greater stability for the larger peptide. Moreover, these investigations have demonstrated that peptides with the potential to form amphipathic α-helical conformations on interaction with IMAC ligates can exhibited binding characteristics with affinity constants that exceed the binding strength of hexa-His. As a consequence, representative examples of these and other peptides generated from combinatorial synthetic procedures could provide useful alternatives for use in the purification of recombinant proteins via the peptide tag approach. In associated studies [52–54], we have examined the suitability of such peptide inserts to aid the purification of recombinant proteins, as well as examined their binding behaviour with new classes of chelating ligates that have higher metal ion stability constants for Cu(II) or other borderline metal ions than IDA, and thus avoid the problem of metal ion leakage from the IMAC sorbent.

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