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Multiple-site binding interactions in metal-affinity chromatography

I. Equilibrium binding of engineered histidine-containing cytochromes *c*

Robert J. Todd, Robert D. Johnson and Frances H. Arnold*

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125 (USA)

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ABSTRACT

Mechanisms of protein retention in immobilized metal-affinity chromatography (IMAC) have been probed using a set of *Saccharomyces cerevisiae* iso-1-cytochrome *c* histidine variants constructed by site-directed mutagenesis. Proteins containing a single accessible histidine exhibit Langmuir-type isotherms with maximum protein binding capacities between 5 and 10% of the maximum copper loading and the capacity of the support to bind imidazole. A simple model that assumes that the copper sites are densely packed and can be blocked by protein adsorption yields binding constants for single-histidine proteins that are similar to the binding constant for free imidazole. Proteins containing multiple accessible histidines do not exhibit simple Langmuir-type behavior; they appear to interact with the support by simultaneous coordination to more than one metal ion, the result of which is to increase the apparent binding affinity by as much as a factor of 1000. The protein binding constant depends on the availability of copper sites: binding is significantly weaker at low surface concentrations of copper that presumably cannot support multiple-site interactions. The protein binding capacity drops to zero at copper loadings less than one-half the maximum, indicating that immobilized iminodiacetic acid ligands are sufficiently close together that two can coordinate a single copper ion, which precludes its interaction with a protein. Protein adsorption via multiple-site coordination has important consequences for the optimization of IMAC separations and the design of new IMAC supports.

INTRODUCTION

Immobilized metal-affinity chromatography (IMAC) has proven to be a useful and versatile technique for the isolation and purification of proteins. As ligands for affinity separations, metal ion complexes offer important advantages over biological affinity agents such as inhibitors and antibodies [1]. Small, inexpensive metal complexes are stable under a wide range of conditions, can be recycled many times without loss of activity and can be formulated into very-high-capacity chromatographic supports. Elution can be effected under relatively mild conditions, and the columns can be cleaned and regenerated easily, without reduction in protein binding capacity. The selectivity of the separation can be tailored through the choice of metal ion, solvent conditions, or by modification of the target protein (*e.g.*, the addition of histidine-rich affinity "handles"). These advantageous features have driven the recent rapid growth in IMAC applications [2] (for a recent compendium of IMAC applications and methods, see ref. 3).

Rapidly reversible interactions with metal ions

* Corresponding author.

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immobilized on a hydrophilic chromatographic support (*e.g.*, Cu^{2+} chelated by an iminodiacetate-derivatized resin) result in the retention of proteins with metal-coordinating ligands on their surfaces, primarily histidine at neutral pH. Depending on the elution conditions, selectivity can be derived from the multiplicity or local environment of metal-coordinating residues [4,5]. Thus IMAC is effective for isolating proteins from crude mixtures; it can also be used to effect highly selective separations of closely related proteins.

Despite recent progress in elucidating IMAC adsorption mechanisms and quantifying protein binding behavior [6,7], the precise molecular mechanisms by which proteins are selectively retained on IMAC matrices are not well understood. Equilibrium binding studies have shown that a simple Langmuir-type model may not adequately describe protein binding to IMAC supports: binding heterogeneity as evidenced by non-linear Scatchard plots is often observed for tightly binding proteins [6,7]. Based on these observations, Hutchens *et al.* [6] proposed that protein binding may involve simultaneous interactions between multiple sites on the protein and the IMAC support. The nature of protein binding has important implications for the design of efficient separations as well as for the design of new materials for IMAC supports. Interactions at multiple sites can greatly enhance binding affinity and dramatically alter specificity for certain classes of proteins. This can affect retention profiles and lead to puzzling differences in chromatographic performance on different supports or under different conditions. Multiple-site binding can also provide a basis for the design of materials capable of selective protein recognition. It is possible to target individual molecules by matching the distribution of metal ions to a spatial distribution of metal-coordinating ligands [8], and efforts to prepare new metal-affinity chromatography supports capable of specific multiple-site binding to target molecules are underway [9,10].

Homologous proteins that differ in their histidine contents have proven useful for elucidating molecular bases of IMAC retention [4,11]. The ability to use protein engineering to add or delete specific amino acid residues, while leaving the remaining protein surface unchanged, allows a more complete and unambiguous study of the contributions of individual metal-coordinating sites [5]. In this study, we compare the equilibrium binding isotherms of variants of a small globular protein, iso-1-cytochrome *c* from *Saccharomyces cerevisiae*, on a TSK polymer matrix derivatized with Cu^{2+}–iminodiacetate (Cu^{2+}-IDA). Using these well-characterized protein variants, we are able to evaluate how the number and placement of surface histidines influences binding and IMAC separation. The engineered histidine-containing cytochrome *c* proteins are ideally suited to investigating the extent to which binding to IMAC supports involves coordination at multiple sites. This information is used to develop a simple but useful description of protein binding to IMAC supports that is consistent with our understanding of analogous metal ion complexes in solution.

MATERIALS AND METHODS

Variants of *S. cerevisiae* iso-1-cytochrome *c* ($M_r = 12\,588$) containing different distributions of histidine residues were constructed by site-directed mutagenesis, expressed in yeast and purified as described previously [12,13]. Cytochromes *c* from tuna heart ($M_r = 12\,170$) and horse heart ($M_r = 12\,384$) were purchased from Sigma and used without further purification.

Preparation of metal-affinity gel

TSK Guardgel Chelate-5PW (17-μm macroporous beads) was purchased from Tosohaas. The specific surface area of this polymer-based, hydrophilic support in the swollen state is not known; a pore size of *ca.* 1000 Å is reported by the manufacturer. The immobilizing ligand, iminodiacetic acid (IDA), is attached to the TSK matrix via a spacer. To prepare the support for use in binding studies, the TSK Chelate-5PW was packed into a column (10 cm × 1 cm) and washed extensively with 50 m*M* EDTA, pH 8.0. To obtain the maximum copper loading, the column was equilibrated with 50 m*M* $CuSO_4$. Excess copper was removed by washing with 0.1 *M* sodium acetate, pH 4.0. The gel was then

equilibrated in 50 m*M* sodium phosphate (NaP_i), 0.5 *M* NaCl, pH 7.0 for measurement of binding isotherms. For copper loading less than maximum, the column was equilibrated with 0.1 *M* sodium acetate, pH 4.0, and 2-ml aliquots of the gel were transferred to 50-ml centrifuge tubes. Different copper loadings were obtained by adding limiting amounts of $CuSO_4$ ($< 18.5 \cdot 10^{-3}$ mmol Cu per ml gel) dissolved in 40 ml acetate buffer, followed by rapid vortexing and subsequent mixing by inversion for at least 1 h. The gel was then washed three times with 40 ml of acetate buffer and three times with 40 ml phosphate buffer.

Measurement of copper loading

To quantify copper loading, the TSK gel was resuspended in the equilibration buffer (50 m*M* NaP_i, 0.5 *M* NaCl, pH 7.0; packed volume:total volume = 1:4). A 50 m*M* solution of EDTA, pH 8.0 (600 μl) was added to 400 μl of the gel suspension in a microcentrifuge tube. This suspension was equilibrated for 60 min by inversion and spun at 10 000 rpm (800 *g*) in a microcentrifuge for 5 min. The supernatant was transferred to a fresh tube, and the Cu^{2+}-EDTA concentration was determined, in quadruplicate, by measuring the visible absorbance at 800 nm in comparison with that of Cu^{2+}-EDTA, pH 7.0 ($\epsilon_{800} = 73.6$ cm^{-1} M^{-1}). The maximum copper loading (Cu_{max}) was found to be $18.5 \cdot 10^{-3}$ mmol per ml of packed gel, in agreement with values previously determined by repeated washing with EDTA [12].

Equilibrium binding isotherms

Equilibrium binding isotherms were measured at room temperature using a modified version of the procedure developed by Hutchens and coworkers [6,7]. The TSK gel was loaded with copper ions and resuspended in the equilibration buffer (50 m*M* NaP_i, 0.5 *M* NaCl, pH 7.0; packed volume:total volume = 1:4) as described above. The high ionic strength, sufficient to elute the net positively charged cytochromes *c* from CM-Sephadex cation-exchange resin [12,13], was used to minimize electrostatic interactions during IMAC. Cytochrome *c* (800 μl of 0.4–0.004 m*M* dilutions prepared in the same buffer) was added to 200 μl of the gel suspension in a microcentrifuge tube. This suspension was equilibrated by inversion for 30 min (after which time no further significant adsorption is observed) and spun at 10 000 rpm (800 *g*) in a microcentrifuge for 5 min. The supernatant was transferred to a fresh tube, and the protein concentration was determined, in duplicate, by measuring the visible absorbance at 550 nm ($\epsilon_{550} = 2.76 \cdot 10^4$ cm^{-1} M^{-1}) after reduction with sodium dithionite [14].

Capacities and binding constants from isotherms

Simple Langmuir binding is described by eqn. 1, where Q is the amount of protein adsorbed per ml of packed gel and C is the liquid-phase protein concentration, at equilibrium. The isotherm is fully described by two parameters, a binding constant K and the maximum capacity for the adsorbed protein, Q_{max}.

$$Q = \frac{Q_{max} K C}{1 + KC} \quad (1)$$

This simple model cannot describe the isotherms of the cytochrome *c* variants containing multiple histidines. The simplest model to which these isotherms can be fit is a bi-Langmuir isotherm,

$$Q = \frac{Q_{max,A} K_A C}{1 + K_A C} + \frac{Q_{max,B} K_B C}{1 + K_B C} \quad (2)$$

where K_A and K_B represent the binding constants to two types of binding sites, strong and weak, respectively. The corresponding maximum capacities for binding at these two types of sites are $Q_{max,A}$ and $Q_{max,B}$.

To describe the binding of imidazole to the IMAC support, it is necessary to include the possibility that more than one imidazole will coordinate with different affinities to a single metal. Assuming two imidazoles can coordinate to a single copper, the adsorption isotherm for imidazole to immobilized Cu^{2+}-IDA is described by

$$Q = \frac{Q_{max,A} K_A C + (Q_{max,A} + Q_{max,B}) K_A K_B C^2}{1 + K_A C + K_A K_B C^2} \quad (3)$$

where K_A refers to the first imidazole binding to the coordination sites of Cu^{2+}-IDA and K_B to a second imidazole binding to the remaining

coordination sites. The maximum capacity for imidazole is $Q_{max,A} + Q_{max,B}$.

RESULTS

Cytochrome c surface histidine variants

A series of *S. cerevisiae* cytochromes *c* were designed and constructed for these studies (Table I). The label assigned to each variant in Table I indicates the surface histidines. Mutations made to the wild-type protein and the total number of surface histidines are also listed. Amino acids 4 and 8 were chosen as sites for replacement with histidine, based on their high degree of surface accessibility. In addition, naturally occurring histidines at positions 26, 33 and 39 were replaced with other amino acids in order to determine the contributions these residues make to the protein's interaction with the IMAC matrix. All the natural surface histidines have been replaced by other amino acids in the H(−) control.

In each of the iso-1-cytochrome *c* variants, the lone cysteine at position 102 was replaced by a serine to prevent oxidative dimerization at the surface sulfhydryl group [15]. The structural integrity of each mutant was confirmed by UV–visible spectroscopy, and the histidine content was confirmed by ^{1}H NMR spectroscopy [12,16]. Expression of these variants in a strain of yeast lacking cytochrome *c* guarantees that each is biologically functional, which in turn ensures that a conformation very similar to the native one has been maintained [12,13].

Formation of the ternary IDA-Cu^{2+}–protein complex that leads to retention on the IMAC support is expected to depend on the accessibilities of individual surface histidines. The accessibilities of the ϵ-nitrogens of each histidine residue to a probe approximately the size of Cu^{2+}-IDA (1.93 Å radius) were determined using the coordinates of the crystal structure of *S. cerevisiae* cytochrome *c* [5,17] (Table II). Engineered histidines were incorporated into a modified cytochrome *c* structure using the molecular graphics software Insight (version 2.0). Based on this structure, the histidines at positions 4 and 8 are expected to be fully accessible to Cu^{2+}-IDA. The remaining histidines exhibit varying, lower degrees of accessibility, as indicated in Table II.

TABLE I

ENGINEERED VARIANTS OF *S. CEREVISIAE* ISO-1-CYTOCHROME *c* AND NATIVE CYTOCHROMES *c*

All engineered variants ($M_r \approx 12\,500$) contain the replacement of cysteine by serine at position 102 to prevent dimerization. Remaining mutations alter surface histidine content. Surface histidines of native cytochromes *c* from tuna ($M_r = 12\,170$) and horse ($M_r = 12\,384$) are listed for comparison. Number of surface-accessible histidines includes His 26. C = Cysteine; H = histidine; K = lysine; L = leucine; N = asparagine; Q = glutamine; S = serine; T = threonine.

Label	Amino acid substitutions	Number of surface histidines
H(−)	C102S, H39Q, H33N, H26N	0
H_{26}	C102S, H39Q, H33N	1
H_4	C102S, H39Q, H33N, H26N, K4H	1
H_8	C102S, H39Q, H33N, H26N, T8H	1
$H_{26}H_4$	C102S, H39Q, H33Q, K4H	2
$H_{26}H_8$	C102S, H39Q, H33Q, T8H	2
$H_{26}H_{33}$	C102S, H39Q	2
$H_{26}H_{33}H_{39}$	C102S	3
$H_{26}H_{33}H_4$	C102S, H39Q, K4H	3
$H_{26}H_{33}H_8$	C102S, H39Q, T8H	3
Tuna (H_{26})		1
Horse (H_{26},H_{33})		2

TABLE II

CALCULATED ACCESSIBLE SURFACE AREAS OF ISO-1-CYTOCHROME *c* SURFACE HISTIDINES

Calculations were performed using coordinates from the high-resolution crystal structure of *S. cerevisiae* iso-1-cytochrome *c* [17]. The surface area is reported as the total area at the δ- and ε-nitrogens accessible to a probe the size of Cu^{2+}-IDA (1.93 Å radius) [5]. The percent accessibility is the accessible surface area relative to an unhindered imidazole.

Histidine position	Accessible surface area (Å^2)	Percent accessibility
4	25.7	88
8	25.5	87
26	3.2	11
33	5.1	17
39	20.9	71

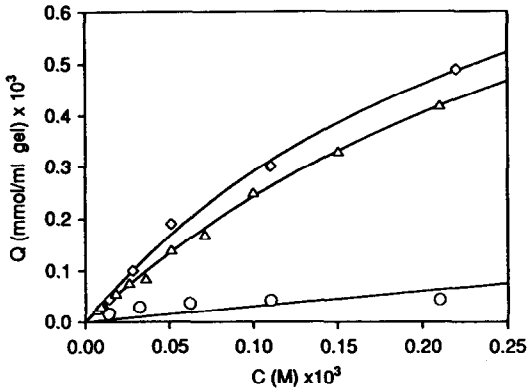

Fig. 1. Equilibrium binding isotherms of weakly binding cytochromes *c*. Solid lines represent fit of Langmuir isotherm (eqn. 1) with parameters given in Table III. (◇) H_{26}; (△) tuna; (○) H(−).

Binding isotherms for single-histidine proteins and imidazole at maximum copper loading

Protein binding isotherms were measured at pH 7.0, where surface histidines are largely unprotonated and free to coordinate to the metal[a]. Isotherms for the H(−) control, H_{26} and tuna cytochrome *c* are shown in Fig. 1. These proteins exhibit simple Langmuir-type binding behavior (linear Scatchard plots), and the capacities and binding constants obtained by fitting these data to the Langmuir isotherm (eqn. 1) are listed in Table III. The H(−) variant with no surface-accessible histidines exhibits almost no affinity for the Cu^{2+}-IDA matrix at pH 7.0. Tuna cytochrome *c* and the H_{26} *S. cerevisiae* variant, both of which have only histidine 26, exhibit similar binding isotherms with apparent binding constants of $2 \cdot 10^3$–$4 \cdot 10^3$ M^{-1}. Their maximum capacities (extrapolated from the data) are *ca.* $1.2 \cdot 10^{-3}$ mmol protein per ml of gel (*ca.* 15 mg/ml).

Equilibrium binding isotherms for the three single-histidine variants shown in Fig. 2 are also adequately described by a simple Langmuir equation. Variants with fully accessible histidines at positions 4 and 8 bind significantly more tightly than H_{26}, whose histidine 26 has less than 10% of the accessibility of histidines 4 and 8 (Table II).

Binding isotherms were also measured for imidazole (data not shown). While histidines at a protein surface are sterically excluded from occupying two coordination sites on one metal, two equivalents of imidazole can bind to matrix-bound Cu^{2+}-IDA. Maximum capacity ($Q_{max,A} + Q_{max,B}$) and binding constants for the first and second imidazoles (K_A and K_B) were obtained by fitting eqn. 3 to the experimental isotherms. The resulting values are reported in Table III. The binding constant K_A for the first imidazole is similar to the binding constant for imidazole to Cu^{2+}-IDA in solution[b] ($3.4 \cdot 10^3$ M^{-1} at 25°C) [18,19]. The observed maximum capacity for imidazole ($Q_{max,A} + Q_{max,B}$) equals twice the copper loading and is much higher than the capacities observed for the single-histidine proteins. In fact, while all of the copper sites are available to imidazole, only 5–10% can be occupied by cytochromes *c* with single histidines.

The apparent binding constants of the single-histidine proteins obtained by fitting eqn. 1 to the isotherms are almost ten times greater than

[a] The pK_a values for the individual surface histidines, measured by ^{1}H NMR spectroscopy, range from 5.4 to 6.5 [12,16].

[b] The reported binding constant of $2.2 \cdot 10^3$ M^{-1} (imidazole–Cu^{2+}-IDA at 35°C) [18] was extrapolated to 25°C using $\Delta H = -7.6$ kcal/mol (imidazole–Cu^{2+} at 25°C) [19].

TABLE III

LANGMUIR BINDING PARAMETERS FOR ISO-1-CYTOCHROME *c* VARIANTS, NATIVE CYTOCHROMES *c* AND IMIDAZOLE TO Cu^{2+}-IDA TSK 5PW

Adsorption isotherms of single-histidine proteins have been fit to the Langmuir model (eqn. 1). Isotherms of multiple-histidine proteins have been fit to the bi-Langmuir model (eqn. 2). Imidazole isotherms have been fit to eqn. 3.

	K_A (M^{-1})	$Q_{max,A}$ (mmol/ml gel) × 10^3	K_B (M^{-1})	$Q_{max,B}$ (mmol/ml gel)x10^3	Initial slope (ml/ml gel)
Imidazole	$5.7 \pm 0.7 \cdot 10^3$	18.0 ± 0.9	$4.0 \pm 3.0 \cdot 10^1$	18.8 ± 0.6	$1.0 \pm 0.1 \cdot 10^2$
H(−)	N.D.[a]	N.D.[a]	–	–	0.3 ± 0.1
Tuna	$2.4 \pm 0.1 \cdot 10^3$	1.25 ± 0.3	–	–	3.0 ± 0.2
H_{26}	$3.5 \pm 0.6 \cdot 10^3$	1.12 ± 0.13	–	–	4.0 ± 0.3
H_4	$5.6 \pm 0.3 \cdot 10^4$	1.15 ± 0.02	–	–	$6.4 \pm 0.2 \cdot 10^1$
H_8	$4.9 \pm 0.5 \cdot 10^4$	1.03 ± 0.04	–	–	$5.0 \pm 0.4 \cdot 10^1$
Horse	$3.8 \pm 0.6 \cdot 10^5$	0.17 ± 0.02	$0.7 \pm 0.3 \cdot 10^4$	1.33 ± 0.34	$7.6 \pm 0.5 \cdot 10^1$
$H_{26}H_4$	$9.0 \pm 1.0 \cdot 10^5$	1.10 ± 0.10	$1.9 \pm 1.0 \cdot 10^4$	0.29 ± 0.10	$1.0 \pm 0.2 \cdot 10^3$
$H_{26}H_8$	$1.0 \pm 0.1 \cdot 10^6$	0.78 ± 0.06	$1.8 \pm 1.0 \cdot 10^4$	0.56 ± 0.09	$7.8 \pm 1.4 \cdot 10^2$
$H_{26}H_{33}$	$7.4 \pm 0.7 \cdot 10^5$	0.52 ± 0.03	$2.4 \pm 0.5 \cdot 10^4$	0.90 ± 0.02	$4.3 \pm 0.4 \cdot 10^2$
$H_{26}H_{33}H_4$	$5.6 \pm 0.8 \cdot 10^6$	1.33 ± 0.13	$4.8 \pm 2.0 \cdot 10^4$	1.08 ± 0.13	$7.6 \pm 1.3 \cdot 10^3$
$H_{26}H_{33}H_8$	$3.6 \pm 0.7 \cdot 10^6$	1.32 ± 0.10	$1.5 \pm 0.8 \cdot 10^4$	0.95 ± 0.11	$4.8 \pm 1.0 \cdot 10^3$
$H_{26}H_{33}H_{39}$	$4.3 \pm 0.4 \cdot 10^6$	1.27 ± 0.04	$2.9 \pm 0.5 \cdot 10^4$	0.96 ± 0.04	$5.3 \pm 0.5 \cdot 10^3$

[a] K and Q_{max} could not be determined independently.

that of imidazole (*e.g.*, $5 \cdot 10^4$ M^{-1} for H_8 *versus* $6 \cdot 10^3$ M^{-1} for imidazole). Similarly low capacities and high apparent binding constants have been reported for lysozyme and ovalbumins binding Cu^{2+}-IDA–Sepharose and Cu^{2+}-IDA–TSK gels [6,20].

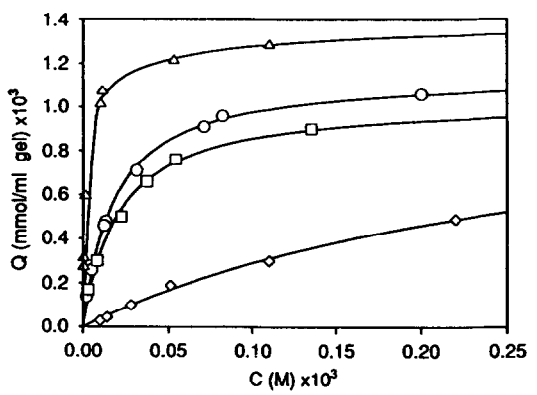

Fig. 2. Equilibrium binding isotherms of iso-1-cytochrome *c* variants. Solid lines through data for H_{26} (◇), H_4 (□) and H_8 (○) variants represent fit of Langmuir isotherm (eqn. 1) with parameters given in Table III. Solid line through data for $H_{26}H_4$ variant (△) represents the fit to a bi-Langmuir model (eqn. 2) using the parameters given in Table III. The initial slope of the isotherm for the two-histidine variant ($H_{26}H_4$) is >10 times larger than the initial slopes of the single-histidine isotherms.

Binding isotherms for multiple-histidine proteins at maximum copper loading

Proteins with multiple exposed histidines bind significantly more tightly and have slightly higher maximum capacities than single-histidine proteins, as shown in Fig. 2 for the $H_{26}H_4$ variant. The sole difference between $H_{26}H_4$ and the H_4 variant, a second histidine only partially exposed on the surface of the protein (histidine 26), increases the initial slope of the binding isotherm by more than a factor of 10 (Table III). Scatchard plots of the isotherm data for the $H_{26}H_4$ and $H_{26}H_8$ two-histidine variants, shown in Fig. 3, indicate at least two modes of binding. Binding data for all the multiple-histidine proteins were fit to the bi-Langmuir isotherm (eqn. 2), and the resulting binding constants and capacities are given in Table III. The first binding constants for the two-histidine variants are approximately 10^6 M^{-1}, while the second binding constants (weak site) are similar to those of

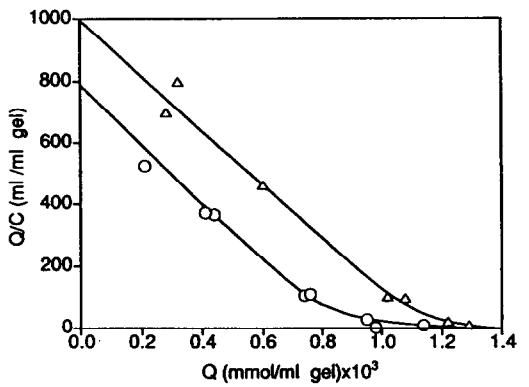

Fig. 3. Scatchard plot of $H_{26}H_4$ (△) and $H_{26}H_8$ (○) equilibrium binding data fit to bi-Langmuir binding isotherm (eqn. 2).

the single-histidine variants (*ca.* 10^4 M^{-1}). The total capacities for the proteins with two surface histidines are approximately 30% greater than the capacities for single-histidine cytochrome *c* variants.

Isotherms of the three-histidine variants also indicate multiple binding modes (data not shown), one of which corresponds to a very strong interaction [12]. Fitting the bi-Langmuir model to the isotherms yields values for the first binding constant of $5 \cdot 10^6$–$10 \cdot 10^6$ M^{-1} and capacities that are approximately twice those of the single-histidine proteins (Table III).

Binding isotherms for a multiple-histidine protein at decreased copper loading

Equilibrium binding isotherms were measured for horse cytochrome *c* on the TSK Guardgel Chelate 5PW at copper loading varying from $18.5 \cdot 10^{-3}$ mmol/ml (Cu_{max}) to $6.1 \cdot 10^{-3}$ mmol/ml (data not shown). At high copper loading ($14.5 \cdot 10^{-3}$–$18.5 \cdot 10^{-3}$ mmol/ml) the adsorption isotherms of this two-histidine protein display multiple binding modes. These data were analyzed using the bi-Langmuir isotherm (eqn. 2), and the binding parameters are presented in Table IV. At lower copper loading ($6.1 \cdot 10^{-3}$–$12.3 \cdot 10^{-3}$ mmol/ml), analysis of the data using the bi-Langmuir model resulted in negligible ($< 2 \cdot 10^{-5}$ mmol/ml) values for $Q_{max,A}$, the strong binding site capacity. The data at these lower loadings were adequately described by the single Langmuir isotherm (eqn. 1).

As the copper loading is decreased, the first binding constant (strong site) of horse cytochrome *c* for the IMAC support decreases rapidly at first, from $4 \cdot 10^5$ M^{-1} at maximum loading to $5 \cdot 10^3$ M^{-1} at two-thirds loading ($12 \cdot 10^{-3}$

TABLE IV

LANGMUIR BINDING PARAMETERS FOR HORSE HEART CYTOCHROME *c* AT DECREASING COPPER LOADING

Adsorption isotherms for horse cytochrome *c* on IDA-TSK 5PW at high copper loading ($14.8 \cdot 10^{-3}$–$18.5 \cdot 10^{-3}$ mmol/ml gel) have been fit to the bi-Langmuir model (eqn. 2). Isotherms at low copper loading have been fit to the simple Langmuir model (eqn. 1).

Cu (mmol/ml gel) × 10^3	K_A (M^{-1})	$Q_{max,A}$ (mmol/ml gel) × 10^3	K_B (M^{-1})	$Q_{max,B}$ (mmol/ml gel) × 10^3	Initial slope (ml/ml gel)
18.5 ± 0.4	$3.8 \pm 0.6 \cdot 10^5$	0.17 ± 0.02	$7.3 \pm 3.0 \cdot 10^3$	1.33 ± 0.34	$7.6 \pm 0.5 \cdot 10^1$
15.6 ± 0.1	$2.1 \pm 0.4 \cdot 10^5$	0.23 ± 0.05	$7.4 \pm 4.9 \cdot 10^3$	0.99 ± 0.35	$5.8 \pm 0.3 \cdot 10^1$
14.8 ± 0.1	$1.2 \pm 0.4 \cdot 10^5$	0.19 ± 0.08	$3.8 \pm 1.9 \cdot 10^3$	1.19 ± 0.18	$2.5 \pm 0.2 \cdot 10^1$
12.3 ± 0.2	$5.0 \pm 1.4 \cdot 10^3$	0.97 ± 0.25	–	–	5.0 ± 0.2
12.0 ± 0.1	$5.3 \pm 0.9 \cdot 10^3$	0.80 ± 0.12	–	–	4.3 ± 0.2
10.5 ± 0.1	$4.2 \pm 1.3 \cdot 10^3$	0.53 ± 0.15	–	–	2.3 ± 0.2
9.5 ± 0.1	$4.0 \pm 3.1 \cdot 10^3$	0.43 ± 0.31	–	–	1.8 ± 0.3
7.6 ± 0.4	$4.3 \pm 2.8 \cdot 10^3$	0.41 ± 0.15	–	–	2.0 ± 0.4
6.1 ± 0.3	N.D.[a]	N.D.[a]	–	–	0.4 ± 0.1

[a] K and Q_{max} could not be determined independently.

mmol/ml). The extrapolated maximum capacity ($Q_{max,A} + Q_{max,B}$) decreases steadily with copper loading, until there is negligible adsorption at one-third the maximum copper loading ($6.1 \cdot 10^{-3}$ mmol/ml).

DISCUSSION

Single-site protein binding

Comparing the isotherms for the H(−) and H_{26} variants (Fig. 1) indicates that a surface histidine is required for any significant binding of cytochrome *c* to the Cu^{2+}-IDA support at pH 7.0. In the absence of accessible histidines, the contributions from the remaining surface residues and the amino terminus in H(−) result in minimal protein adsorption. This observation mirrors those reported for other proteins and other IMAC supports at neutral pH [4,5,8,21]. The free cysteine (C102) on the surface of native yeast cytochrome *c* has been replaced by serine in the variants studied here. When C102 is present, metal affinity chromatography on Cu^{2+}-IDA yields significant amounts of a second protein form which co-elutes with variants containing twice the surface histidine content of the monomer (data not shown). This second form is most likely the disulfide-cross-linked dimer commonly observed for this protein.

Once there is a histidine accessible for coordination to the immobilized copper ions, protein binding is assured. However, the binding behavior of histidine-containing proteins differs from that of imidazole (Table III). While all the immobilized copper ions appear to be accessible to imidazole, only a small fraction (< 10%) can be involved in protein binding. Furthermore, cytochrome *c* variants with fully accessible histidines bind with apparent affinities ten times greater than imidazole. What is the source of this high binding affinity?

A simple model which assumes that protein adsorption blocks access to multiple metal ion sites at the surface of the chromatographic support can explain both the large apparent binding constants for single-histidine proteins and the low protein binding capacities. For a protein with a single histidine, the interaction illustrated in Fig. 4a is described by

$$P + nCu \rightleftharpoons PCu + [(n-1)Cu] \quad K_1 = \frac{[PCu]}{[P][Cu]}$$

where the protein (P) blocks $n-1$ additional copper sites from further interactions (blocked sites designated by brackets) upon coordinating to a metal ion (Cu) on the chromatographic support. This model also results in a Langmuir-type isotherm,

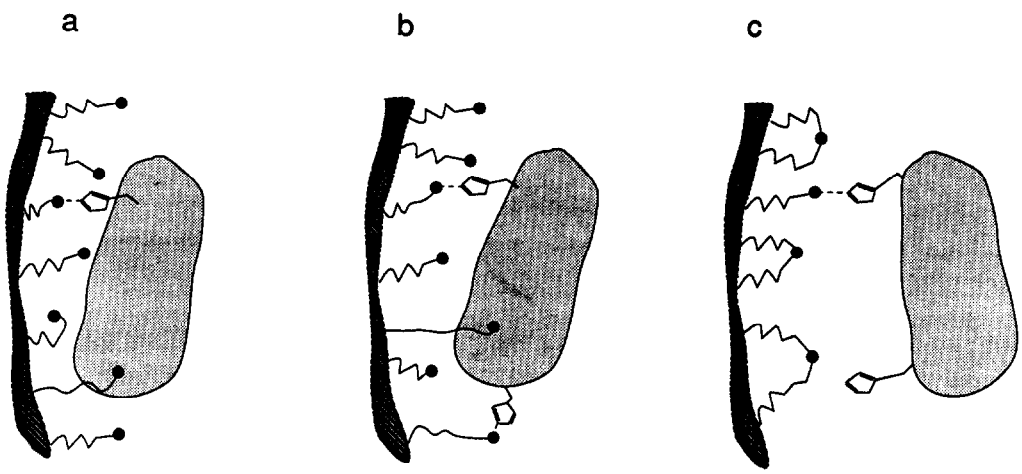

Fig. 4. Proposed binding interactions in IMAC. (a) Protein binds IMAC surface at a single copper site and blocks access to other sites (by protein). (b) Multiple-site binding to IMAC surface: two histidines simultaneously coordinate to separate copper sites. (c) Multiple-histidine protein exhibiting single-site binding to IMAC surface at reduced copper loading. Adjacent immobilizing ligands simultaneously chelate a single metal, preventing interaction with histidine (and other ligands).

$$Q = \frac{Cu_{acc} K_1 C}{1 + nK_1 C} \quad (4)$$

where Cu_{acc} is the total concentration of immobilized copper accessible for protein binding and K_1 is the binding constant for a protein bound to a single copper site via a single histidine. At saturation copper loading, the concentration of accessible copper is assumed equal to the total concentration of immobilized copper ($Cu_{acc} = Cu = Cu_{max}$). Parameters obtained by fitting the isotherms to eqn. 4 are given in Table V. This analysis indicates that adsorption of cytochromes *c* with single histidines blocks *ca.* 16–18 copper sites.

The diameter of hydrated cytochrome *c* is 34 Å [17]. Assuming adsorption does not involve large conformational changes, bound proteins could be expected to block further access to $34^2 = 1156$ Å^2 of surface per molecule. This area corresponds to 16 copper sites at an average spacing of *ca.* 8.5 Å between metal ions. A conceptually similar analysis applied to ion exchange indicates that adsorption of similarly sized proteins can block access to *ca.* 30 salt ions [22].

If protein adsorption only involves coordination of the imidazole ring of a single histidine residue to an immobilized copper ion, then the intrinsic binding constants should not be greater than the binding constant of free imidazole to the IMAC support. If the solvation effects involved in a protein histidine–Cu interaction are the same as for an imidazole–Cu interaction, then free imidazole should in fact provide an upper bound for the contribution of the first fully accessible histidine to protein binding. Binding constants for the H_4 and H_8 variants with fully accessible histidines become comparable to the binding constant for imidazole when the protein adsorption isotherms are fit using eqn. 4, as shown in Table V. The relatively low binding constant for H_{26} can once again be attributed to the low accessibility of its histidine.

A lower bound for the contribution of a fully accessible histidine can be estimated by comparing the binding of the H_4 and H_8 variants to the same protein without surface histidines, H(−). The binding constant of an essentially non-binding protein, H(−), can be estimated from the initial slope of its adsorption isotherm, $Cu_{acc}K_1$. The single histidines in the H_4 and H_8 variants contribute more than a factor of 200 to the binding constant, relative to the H(−) variant (Table V), and >3 kcal mol^{-1} (1 cal = 4.1868 J) to the binding free energy. For comparison, the free energy of binding of a single imidazole to aqueous Cu^{2+}-IDA is 4.8 kcal mol^{-1} at 25°C [18,19].

This simple "site exclusion" model can also explain the seemingly surprising result reported by Hutchens and Yip [20] that free copper does

TABLE V

BINDING PARAMETERS FOR SELECTED ISO-1-CYTOCHROME *c* VARIANTS BASED UPON "SITE-EXCLUSION" MODEL

Adsorption isotherms for single-histidine variants have been fit using eqn. 4. Isotherms for two-histidine proteins have been fit to eqn. 6, using K_1 from analogous single-histidine variants. Total copper loading (Cu_{acc}) is $18.5 \cdot 10^{-3}$ mmol/ml gel.

Variant	K_1 (M^{-1})	*n*	K_2	θ
H(−)	$1.6 \pm 0.4 \cdot 10^1$	N.D.[a]	–	–
H_{26}	$2.2 \pm 0.2 \cdot 10^2$	16 ± 2.2	–	–
H_4	$3.5 \pm 0.1 \cdot 10^3$	16 ± 0.2	–	–
H_8	$2.7 \pm 0.2 \cdot 10^3$	18 ± 0.7	–	–
$H_{26}H_4$	$3.5 \cdot 10^3$	14 ± 0.3	22 ± 3	0.76 ± 0.05
$H_{26}H_8$	$2.7 \cdot 10^3$	15 ± 0.4	27 ± 4	0.58 ± 0.05

[a] *n* cannot be determined.

not influence protein binding to an IMAC support and therefore must differ from immobilized copper in how it interacts with the protein. Free copper at the low concentrations used in those experiments (10–100 μM) would in fact not be expected to influence binding if the intrinsic protein-to-immobilized copper binding constants are only *ca.* $3 \cdot 10^3$ M^{-1} instead of ten times larger. Although free copper may be able to bind at sites not accessible to immobilized copper, its affinity for cytochrome *c* surface histidines is immediately apparent in ^{1}H NMR spectra [23] and is well documented for other proteins [24].

The possibility that protein binding blocks access to other copper sites was first discussed by Belew *et al.* [25], who rejected it in favor of invoking multipoint attachment of the protein to the IMAC matrix via undefined histidine, cysteine or tryptophan residues to explain the apparent high affinities. The cytochromes *c* used in the current studies do not have surface cysteine residues, and the lone surface tryptophan (33) in tuna cytochrome *c* is not present in the proteins from *S. cerevisiae* or horse. While one cannot absolutely rule out other sites (*e.g.* lysines or a free N-terminus) for multipoint attachment of the cytochromes *c* containing only one accessible histidine, the contribution of all the other surface residues is certainly small compared to that of even a poorly accessible histidine. Therefore, we favor the "site-exclusion" explanation, which yields intrinsic protein binding constants that are consistent with analogous IDA-Cu^{2+}–imidazole complexes, both immobilized and in solution. As will be shown below, this framework is also consistent with binding data for multiple-histidine proteins and is further supported by evidence of a high surface density of immobilized metal sites.

Distribution of immobilized copper sites

The Cu^{2+}-IDA complexes must be quite densely packed at the surface of this IMAC support for adsorption of cytochrome *c* to block 16 copper ions. Protein adsorption behavior at decreased copper loading provides strong evidence for this close packing of metal sites. Consider a pair of immobilized IDA ligands, L. Under copper-limited conditions, two ligands will chelate a single copper ion, provided they can both reach the metal. The L–Cu–L complex completely cages the copper, leaving no vacant coordination sites for protein binding:

$$\text{L–Cu} + \text{L} \rightleftharpoons \text{L–Cu–L} \nrightarrow \text{L–Cu–L–P}$$

If the immobilized IDA ligands are close enough together to form L–Cu–L complexes, then the total protein binding capacity should decrease rapidly at copper loadings less than maximum.

Because the metal can coordinate two IDA ligands, a distinction must be made between the concentration of immobilized copper accessible to protein (Cu_{acc}) and the total concentration of immobilized copper (Cu). Assuming equilibrium binding constants of immobilized IDA for copper that are comparable to formation constants for aqueous Cu-IDA species (*ca.* 10^{11} M^{-1} for aqueous Cu^{2+}-IDA and *ca.* 10^{17} M^{-2} for Cu^{2+}-$(IDA)_2$ [19]), all the immobilized copper will be coordinated by a single IDA group at saturation loading ($Cu_{acc} = Cu = Cu_{max}$). At lower copper loading, however, some fraction of the immobilized copper will be in the form of L–Cu–L complexes, precluded from protein interactions ($Cu_{acc} < Cu$). There is in fact no measurable adsorption of horse cytochrome *c* to the TSK IMAC gel at low concentrations of copper ($Cu:Cu_{max} < 1:3$) (Table IV). Significant residual protein binding capacity would be expected if the metal ions were isolated and could remain accessible.

Multiple-site protein binding

The existence of multiple coordinating sites on a protein surface can influence binding affinity in various ways. If two histidines bind *independently* (only one at a time), the apparent binding constant of a two-histidine protein would be twice that of a protein containing only one histidine of comparable accessibility, reflecting the increased availability of binding sites (a statistical effect). If, on the other hand, two histidines (or a histidine and another functional group) can bind *simultaneously* to distinct copper sites on the matrix (two-site binding), the binding constant could be much larger.

Two-site binding, illustrated in Fig. 4b, can be analyzed in a manner analogous to chelation in

small metal ion complexes. The increase in the binding constant for the two-histidine protein compared to a single-histidine protein (Fig. 4a) will depend on the enthalpy gained from forming the second histidine–Cu^{2+}-IDA interaction relative to the entropy lost by further constraining the protein and the metal ion complexes at the support surface. If the entropic losses and the strain introduced upon binding are negligible, the increase in the apparent binding constant can be as large as the increase in affinity of a single-histidine protein over the protein with no histidines: a factor of 10^2–10^3.

Multiple-site binding to a support whose distribution of ligands is essentially random requires that the metal ion sites be densely packed in regions of the matrix accessible to protein. Our observations that *ca.* 16 immobilized copper ions are blocked by adsorption of cytochrome *c* and that the immobilized IDA ligands are close enough to form L–Cu–L complexes indicate a site density on this TSK gel that could support multiple-site binding.

For a protein with two potential binding sites, the reaction represented by eqn. 4 can be extended to include the second interaction,

$$P + nCu \rightleftharpoons PCu + [(n-1)Cu] \quad K_1 = \frac{[PCu]}{[P][Cu]}$$

$$PCu + [(n-1]Cu] \rightleftharpoons PCu_2 + [(n-2)Cu]$$

$$K_2 = \frac{[PCu_2]}{[PCu]}$$

where PCu_2 represents a protein bound to the matrix at two copper sites. As before, n represents the number of copper ions blocked by protein binding. The second binding constant K_2 represents the apparent binding affinity of the second (histidine) side chain for a nearby copper site on the surface, a reaction of zero order in protein concentration. These equilibria yield the modified isotherm of eqn. 5,

$$Q = \frac{Cu_{acc}K_1(1+K_2)C}{1+nK_1(1+K_2)C} \quad (5)$$

Because the distribution of copper ions on the support surface is not uniform, not all proteins adsorbed via a single histidine will find appropriate sites for a second interaction. Assuming that only some fraction θ of the surface sites can accommodate strong two-site binding ($K_2 \gg 1$), while the remainder of the sites can accommodate only single-site binding ($K_2 \approx 0$), eqn. 5 can be extended to a bi-Langmuir-type equation,

$$Q = \frac{\theta Cu_{acc}K_1K_2C}{1+nK_1K_2C} + \frac{(1-\theta)Cu_{acc}K_1C}{1+nK_1C} \quad (6)$$

K_1K_2 is the apparent binding constant for protein binding via two-site interactions.

The binding data for the $H_{26}H_4$ and $H_{26}H_8$ variants can now be analyzed using eqn. 6, where Cu_{acc} equals the total copper loading ($18.5 \cdot 10^{-3}$ mmol/ml) and the binding constants K_1 for fully accessible histidines 4 and 8 are already known from the single-histidine variants H_4 and H_8. The values of K_2, θ and n obtained from the isotherms for the multiple-histidine proteins are given in Table V. Adding histidine 26 to either histidine 4 or 8 results in a 20-fold or greater increase in the apparent binding constant, relative to single-site binding, for 60% of the surface sites. This increase matches the 20-fold increase that histidine 26 provides the H_{26} variant over H(−). Similarly, the 200-fold increase histidines 4 or 8 offer the $H_{26}H_4$ and $H_{26}H_8$ variant over H_{26} is consistent with the increase of at least 200-fold these histidines offer the H_4 and H_8 variants over H(−). Thus a second, fully accessible histidine adds >3 kcal mol^{-1} to the binding energy, compared to a single-histidine protein. The values of n obtained by this analysis are also consistent with those for the single-histidine variants: *ca.* 15 copper sites are blocked by each protein molecule.

Addition of histidine 33 in $H_{26}H_{33}H_4$ variant increases the apparent initial slope by a factor of 8 compared to $H_{26}H_4$ (Table III), indicating the possibility of three-site binding. Although the analysis represented by eqn. 6 could be extended to include simultaneous three-site binding, fitting the isotherms for the three-histidine cytochrome *c* variants leaves indeterminate the three-site binding parameters analogous to θ and K_2 for two-site binding. Because of the high affinity expected for simultaneous three-site binding (*ca.* 10^7 M^{-1}), the initial slope of the adsorption isotherm must be measured accurately at im-

practically low protein concentrations ($<10^{-7}$ M) to determine these parameters. Nonetheless, the apparent initial slope calculated from the first point on the adsorption isotherm represents a lower bound for the true initial slope. Thus simultaneous three-site binding is estimated to be at least an order of magnitude stronger than two-site binding.

The increased maximum capacity of the three-histidine variants correspondingly decreases the parameter n, the number of copper sites blocked by protein binding, from 16–18 for single-histidine proteins to only 8–10 for three-histidine proteins. The overly simplified model presented here cannot provide a satisfactory explanation for this result. The increased capacity for tighter-binding proteins could reflect a heterogeneous population of sites with different binding affinities, or, possibly, conformational rearrangement (compaction) of proteins adsorbed via high-affinity three-site interactions compared to lower-affinity single-site binding.

Binding mode changes with decreasing copper loading

We would expect binding via multiple-site interactions to depend strongly on the density and distribution of available copper ions at the support surface. While multiple-site binding might occur on a densely-derivatized support, binding should occur via single-site interactions on a support with a low concentration of available metal ions. This can be achieved easily by decreasing the copper loading (Fig. 4c). If the immobilized IDA groups are close enough to simultaneously coordinate a single metal, a decrease in copper loading results in a significant portion of the total immobilized copper sequestered in L–Cu–L complexes. This leads to a more rapid decrease in the concentration of immobilized copper accessible to protein surface groups ($Cu_{acc} < Cu$).

As shown in Fig. 5, the initial slope of the binding isotherm of a two-histidine protein decreases sharply with decreasing copper loading near the maximum ($Cu/Cu_{max} \approx 1$). At these high loadings the protein displays high-affinity binding characteristics of simultaneous, multiple-site interactions (Table IV). According to eqn. 6

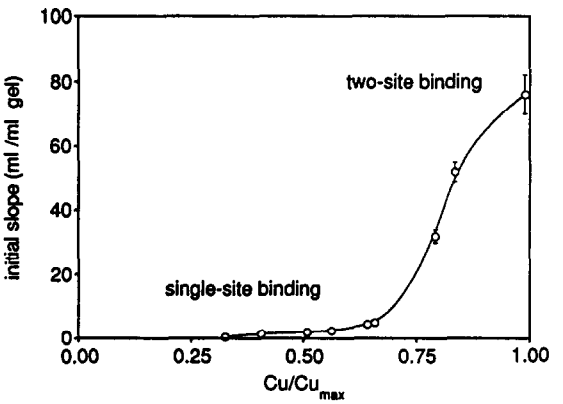

Fig. 5. Initial slopes of adsorption isotherms ($K_A Q_{max,A} + K_B Q_{max,B}$) of horse cytochrome c as a function of the fractional copper loading (Cu/Cu_{max}).

the initial slope of the adsorption isotherm is given by $\theta K_1 K_2 Cu_{acc}$. (This is proportional to protein retention in isocratic chromatography under analytical conditions ($Q \ll \theta Q_{max}/n$).) Decreasing the copper loading lowers the concentration of accessible copper sites (Cu_{acc}), which also affects the value of θK_2.

At lower copper loading ($Cu/Cu_{max} \approx 1/2$), accessible metal sites are much less densely packed, which decreases the likelihood that the surface can support high-affinity two-site binding. At these loadings, this two-histidine protein displays simple isotherms typical of single-histidine proteins (Table IV). The apparent binding constant remains roughly constant with copper concentration and is comparable to that of imidazole ($4 \cdot 10^3$ M^{-1}), suggesting that adsorption is due primarily to single-site binding to isolated copper sites. If so, the initial slope of the adsorption isotherm is $K_1 Cu_{acc}$, where K_1 does not depend on copper concentration. As expected, the initial slope of the horse cytochrome c adsorption isotherm is much less sensitive to the copper concentration in this range.

Implications of changing the binding mode from single to multiple-site interactions

The possibility that proteins can bind at multiple copper sites simultaneously has important implications for the use of existing chromatographic supports as well as the design of new ones. The non-uniform distribution of metals

results in binding sites characterized by a distribution of binding affinities, which can cause peak broadening and reduce the concentration range where linear behavior is observed.

If a protein can interact with the support at more than one metal site, the availability of metals on a particular chromatographic surface will dramatically influence the retention and separation that can be achieved. For example, retention during analytical isocratic chromatography of two proteins is governed by the ratio of the initial slopes of their respective binding isotherms. The initial slope of the isotherm is dominated by multiple-site interactions, as seen for the yeast cytochrome *c* variants with different surface histidines. The extent to which multiple-site interactions can occur is governed in turn by the density and accessibility of immobilized copper. Thus it may be possible to tailor IMAC supports for specific separations of multiple-histidine proteins by controlling the concentration of metal and how it is linked to the support.

For example, to separate a protein containing a single, high-affinity binding site (*e.g.*, a metal-chelating site [1]) from other proteins containing multiple, low-affinity binding sites (*e.g.*, non-chelating histidines), multiple-site interactions should be avoided. Multiple-site interactions are minimized at low loadings, where the metal ions are isolated. Under these conditions protein adsorption would be governed by the sum of single-site binding constants, which favor the single high-affinity site. A support with a low density of immobilized copper ions would efficiently separate a protein containing a dihistidine metal-binding site from other proteins with multiple surface histidines [13]. Because only a small fraction of the metal ions are actually involved in coordinating a protein, this could conceivably be accomplished without a drastic reduction in protein binding capacity.

Conversely, to optimize the separation of two-histidine proteins from those with single histidines, two-site interactions (θK_2) should be maximized relative to single-site interactions (K_1). The product θK_2 is sensitive to the density of copper sites, while the binding constant K_1 for a single histidine protein is not. The high density of immobilized copper sites of this TSK matrix provides for relatively large values of θ and K_2, which translate into efficient separations [16].

A logical extension of this concept is the creation of specific high-affinity binding sites by distributing metals on the chromatographic surface to match the distribution of histidines on a target protein. For example, receptor complexes that position two metals at a fixed distance selectively bind their complementary bis-imidazole "protein analogues" in the presence of other bis-imidazoles [8]. Selective chromatographic supports could be prepared by immobilizing appropriate bis-metal complexes. An alternative approach to preparing such "patterned" supports is template polymerization, in which the target molecule itself acts as a template to correctly position the metal ions in the polymer matrix during synthesis [9].

CONCLUSIONS

Equilibrium binding data for a series of homologous proteins with specifically engineered surface histidines can be interpreted within a framework that yields binding constants consistent with the behavior of analogous imidazole–copper complexes in solution and on IMAC supports. Equilibrium binding of multiple-histidine proteins is significantly tighter than expected based on statistical effects because it involves simultaneous interactions with more than one immobilized metal ion. A consequence of multiple-site interactions is a significant increase in the binding affinity that strongly depends on the metal ion distribution. This principle is not limited to metal-affinity chromatography, but should be applicable to any separation in which multiple-site interactions can dominate binding (*e.g.*, ion exchange [26]). The ability to control multiple-site interactions provides a unique opportunity to tailor IMAC and other chromatographic supports for specific protein separations.

SYMBOLS

C	protein concentration in the liquid phase (M)

K	apparent equilibrium binding constant for protein adsorption (M^{-1})
K_A	apparent equilibrium binding constant for protein adsorption (strong site) (M^{-1})
K_B	apparent equilibrium binding constant for protein adsorption (weak site) (M^{-1})
K_1	equilibrium binding constant for protein adsorption to a single copper site via a single protein binding site (M^{-1})
K_2	equilibrium binding constant to a second copper site via a second protein binding site of an adsorbed protein
n	number of copper sites blocked by protein adsorption
θ	fraction of copper sites that will support simultaneous two-site binding via two protein binding sites
Cu	concentration of immobilized copper (mmol/ml gel)
Cu_{max}	concentration of immobilized copper at saturation copper loading (mmol/ml gel)
Cu_{acc}	concentration of immobilized copper accessible to protein binding (mmol/ml gel)
Q_{max}	maximum capacity for adsorbed protein (mmol/ml gel)
$Q_{max,A}$	maximum capacity for adsorbed protein (strong sites) (mmol/ml gel)
$Q_{max,B}$	maximum capacity for adsorbed protein (weak sites) (mmol/ml gel)

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