ELSEVIER

Journal of Chromatography A, 725 (1996) 225–235

JOURNAL OF
CHROMATOGRAPHY A

Multipoint binding in metal-affinity chromatography
II. Effect of pH and imidazole on chromatographic retention of engineered histidine-containing cytochromes *c*

Robert D. Johnson[1], Robert J. Todd[2], Frances H. Arnold*

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

Received 11 July 1995; revised 1 September 1995; accepted 1 September 1995

Abstract

Protein binding in immobilized metal affinity chromatography (IMAC) was studied using a set of *Saccharomyces cerevisiae* iso-1-cytochrome *c* variants which differed only in their histidine content and placement. Elution with an imidazole gradient enabled separation of cytochrome *c* variants based on their histidine multiplicity. Millimolar concentrations of imidazole dramatically decreased protein partitioning to the IMAC support as measured by the chromatographic capacity factors under isocratic conditions. Fitting the partitioning data to the "stoichiometric displacement" model indicates that cytochrome *c* variants containing from one to four surface histidines each displaced approximately three equivalents of imidazole upon adsorption. Therefore even a protein with a single surface histidine appears to coordinate to multiple copper sites on the IMAC support at neutral pH. The effect of pH on the capacity factors of these variants measured in the absence of imidazole further supports this interpretation. Although the presence of a surface histidine was required for retention at neutral pH, a variant with no surface histidines still partitioned strongly to the IMAC support at higher pH (pH>7.5). These results indicate the contribution of additional protein–metal-coordinating groups, presumably surface amines, to chromatographic retention in IMAC.

Keywords: Immobilized metal-affinity chromatography; Histidine–metal interactions; Protein–metal interactions; Retention mechanisms; Cytochrome *c*; Imidazole; Histidine; Proteins

1. Introduction

Immobilized metal affinity chromatography (IMAC) [1] has the potential to satisfy the need for novel separation processes which can recognize proteins with specificities approaching those of biological affinity chromatography, but also provide the durability, low operating expense, and versatility of traditional chromatographic techniques. Binding selectivity in IMAC is derived from the multiplicity and local environment of metal-coordinating residues [2], and as such can be tailored through the choice of metal ion, solvent conditions, or by modification of the target protein [3]. Two important advantages of IMAC over other chromatographic techniques [4] are the ability to perform the separation at high salt

*Corresponding author.

[1] Present address: Genentech, Inc., 460 Pt. San Bruno Bl., So. San Francisco, CA 94080, USA.

[2] Present address: Amgen, Inc., 3200 Walnut Ave., Boulder, CO 80301, USA.

0021-9673/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(95)00992-2

concentrations and the relatively high binding constant of individual metal–ligand interactions, enabling separation of proteins differing by a single amino acid [5]. Thus IMAC is effective for isolating proteins from crude mixtures [6] as well as for selective separations of closely related proteins [7,8].

We have previously probed the role of surface histidines in IMAC by measuring the equilibrium adsorption of a set of yeast cytochrome *c* variants differing only in surface histidine content [9,10]. Adding as few as three accessible histidines to the protein surface increased the binding affinity to the IMAC support by as much as a factor of 1000, the result of simultaneous coordination to more than one surface copper site. A simple model, which assumed that the copper sites were densely packed and that multiple sites could be blocked by protein adsorption, yielded binding constants for single-histidine proteins comparable to those for free imidazole and N-acetylhistidine. This interpretation allowed us to unify in one self-consistent framework our understanding of interactions between metal complexes and small histidine analogs in solution [11], results of solution studies of protein–metal interactions [12,13], and the protein adsorption data.

In ion-exchange chromatography (IEC), the effect of salt concentration on multivalent interactions between protein surface charged groups and complementary charged groups on the chromatographic support is generally described by "stoichiometric displacement" models for protein retention [14,15]. In this investigation we show the effect of imidazole concentration on chromatographic retention for the set of yeast cytochrome *c* variants differing in surface histidine content [9], and fit these data to a "stoichiometric displacement" model applicable to IMAC [16]. If, as predicted by our simple model, protein adsorption in IMAC is due to simultaneous coordination to more than one surface copper site, then the effect of changing protein surface histidines on protein retention in IMAC should be analogous to the effect of changing protein surface charged groups on protein retention in IEC [17]. These results demonstrate that even a protein with a single surface histidine displaces multiple equivalents of imidazole upon adsorption and therefore interacts with multiple immobilized metal ions. Such behavior cannot be explained by metal binding to surface histidines alone and must involve the coordination of additional protein functional groups. The pH behavior of these variants in IMAC suggests that the additional groups are most likely surface amino groups (amino terminus and/or some subset of the ca. fifteen surface lysines), which previously have been directly implicated in the binding of proteins and small peptides to IMAC supports under basic conditions [18–20].

2. Experimental

Variants of *Saccharomyces cerevisiae* iso-1-cytochrome *c* containing different distributions of histidine residues were expressed in yeast and purified as described previously [9,21]. Expression in a yeast strain lacking cytochrome *c* guarantees that each variant is biologically functional, which in turn ensures that a conformation very similar to the native one has been maintained. The structural integrity of each mutant was confirmed by UV–Vis spectroscopy, and the histidine content was confirmed by ^{1}H NMR spectroscopy. In contrast to other eukaryotic cytochromes *c*, the amino terminus of native *S. cerevisiae* iso-1-cytochrome *c* is not blocked by an α-N-acetyl group [22]. In each of the engineered cytochrome *c* variants, the lone cysteine at position 102 was replaced by a serine to prevent oxidative dimerization at the surface sulfhydryl group [23]. Additional surface amino acids were replaced to alter surface histidine content as described previously, and the variants will be referred to by the histidines present on the surface [9].

2.1. IMAC experiments

A TSK Chelate-G6000XL (10-μm macroporous beads) HPLC column (7.5 cm×7.5 mm I.D.) from Tosohaas (08645) was used for all experiments. Experiments were performed at a flow-rate of 1 ml/min using a Hitachi Model L6200A high-pressure pump/low-pressure gradient former. The eluent was monitored by UV–Vis absorbance using a Kratos Spectraflow 783 variable-wavelength detector. The column was regenerated by first washing with 15 ml of 50 m*M* ethylenediaminetetraacetic acid (EDTA), pH 8.0, and then reloading with 15 ml of 50 m*M* $CuSO_4$ in water. The packed column

volume (v) is 3.31 ml and the void volume (V_0) is 2.3 ml [21]. Alterations in the flow-rate had no effect on measured retention volumes (the kinetics of the imidazole–Cu^{2+}–IDA interaction are very fast) and, therefore, local equilibrium conditions were assumed throughout. In addition, peaks were generally symmetrical, allowing us to approximate the center of mass by the maximum absorbance. In all cases, experiments were performed under analytical conditions, with less than 1% of the column capacity utilized for each injection.

To quantify saturation copper loading, the regenerated column was washed with 15 ml of 50 m*M* EDTA pH 8.0. The eluent was pooled and the concentration of CuEDTA determined by absorbance at 800 nm (ϵ_{800}=73.6 cm^{-1} M^{-1}). The copper loading was found to be 81 μmol of copper (24.5 μmol per ml of column), in agreement with values previously determined [21].

2.2. Imidazole gradient elution experiments

The regenerated TSK chelate column was equilibrated with 10 ml of 50 m*M* sodium phosphate, 0.5 *M* NaCl, 10 m*M* imidazole at pH 7.0 followed by 10 ml of the same buffer containing 1 m*M* imidazole. After injecting protein (20 μl of a 30 μ*M* cytochrome *c* solution in buffer containing 1 m*M* imidazole), the column was washed with buffer containing 1 m*M* imidazole for 6 min. The protein was then eluted in a linear gradient from 1 m*M* to 10 m*M* imidazole in 54 min and the eluent monitored by absorbance at 410 nm. The concentration of imidazole exiting the column was calculated from the elution volume of the peak absorbance maximum.

2.3. Isocratic elution experiments

For isocratic elution of cytochrome *c* variants in the presence of imidazole, the column was equilibrated (following regeneration) with 10 ml of 50 m*M* sodium phosphate, 0.5 *M* NaCl, pH 7.0 and 10 m*M* imidazole. Because imidazole binds strongly to the IMAC column (equilibrium dissociation constant of imidazole for immobilized CuIDA is approximately 0.2 m*M* [9]), equilibrating the column with imidazole-containing buffer should be considered a frontal elution experiment. Assuming local equilibrium conditions throughout the column [24], at low imidazole concentrations the elution front travels through the column at approximately the same rate as a pulse of imidazole (see Fig. 2 of Ref. [24]). Therefore at low imidazole concentrations (<1 m*M*) the column was equilibrated with 200 ml of elution buffer, twice the volume required to elute a pulse of N-acetylhistidine (see below). At high imidazole concentrations (>1 m*M*), it was sufficient to wash the column with excess imidazole (30 ml of elution buffer). After equilibration, 25 μl of 3 μ*M* cytochrome *c* dissolved in elution buffer was injected and the eluent monitored by absorbance at 410 nm. The elution time reported is that of the peak absorbance maximum.

For isocratic elution of cytochrome *c* variants and N-acetylhistidine (Ac-His) at varying pH in the absence of imidazole, the column was equilibrated (following regeneration) with 30 ml of the following buffer: 50 m*M* sodium acetate, 50 m*M* sodium phosphate, 50 m*M* boric acid, 0.5 *M* NaCl with pH adjusted in the range 6.0–9.0. For experiments with Ac-His, 100 μl of 1 m*M* Ac-His dissolved in elution buffer was injected and the eluent monitored by absorbance at 235 nm. For experiments with cytochrome *c* variants, 25 μl of 3 μ*M* cytochrome *c* dissolved in elution buffer was injected and the eluent monitored by absorbance at 410 nm. The elution time reported is that of the peak absorbance maximum.

2.4. NMR experiments

Samples were prepared using a modified version of the method of Pielak et al. [25]. Purified cytochrome *c* was diluted 20-fold into buffer (0.1 *M* sodium phosphate, 0.1 *M* NaCl, pH 4.0) and then concentrated to approximately 0.5 m*M* using an Amicon 8050 stirred ultrafiltration cell with a PM3 membrane. Samples were then lyophilized, resuspended in $^{2}H_2O$ and exchanged overnight at 37°C. Exchanged samples were lyophilized again and finally resuspended to a concentration of 0.5 m*M* in 99.9% percent $^{2}H_2O$ under nitrogen. The volume of each sample was 400 μl, and the final pH was adjusted to approximately 4.5 (actual reading) using 0.1 *M* sodium phosphate, 0.1 *M* NaCl, pH 3.0, in $^{2}H_2O$.

NMR data were collected at 37°C using AM

500-MHz and AMX 500-MHz Bruker spectrometers. Although previous studies have been performed at 25°C [26–28], it was necessary to perform these experiments at 37°C to fully resolve the C_2 proton resonances for each of the histidines. Titration curves were prepared by taking NMR spectra at intervals of approximately 0.3 pH units from pH 4.5 to 8.0. The pH was adjusted by the addition of 0.1 *M* sodium phosphate, 0.1 *M* NaCl, pH 12 in ^2H_2O. All samples contained approximately 100 m*M* of TSP (Na-3-trimethylsilylpropionate [2,2,3,3-d_4]), and chemical shifts are reported in ppm against this standard. Titration data were analyzed by the method of Markley [29].

3. Results and discussion

3.1. Imidazole gradient elution

Imidazole gradient elution is perhaps the most versatile of IMAC separation techniques. As shown in Fig. 1, elution with an imidazole gradient from the TSK-IDACu column can separate closely related cytochrome *c* variants differing only in the number and/or placement of surface histidines. The order of elution follows that of increasing binding affinity previously measured in equilibrium adsorption experiments [9,10]. Here, the imidazole gradient was started at 1 m*M*, which is sufficient to elute zero- and single-histidine variants (equilibrium binding constant of Temkin model $K_T < 1 \times 10^5\ M^{-1}$) in the void volume. In the gradient from 1 to 10 m*M* imidazole, the remaining variants, containing from two to four surface histidines (Temkin model binding constant $K_T = 10^5 - 10^8\ M^{-1}$), separate into groups according to their histidine multiplicity. Similar results have been observed in other protein systems [5,7].

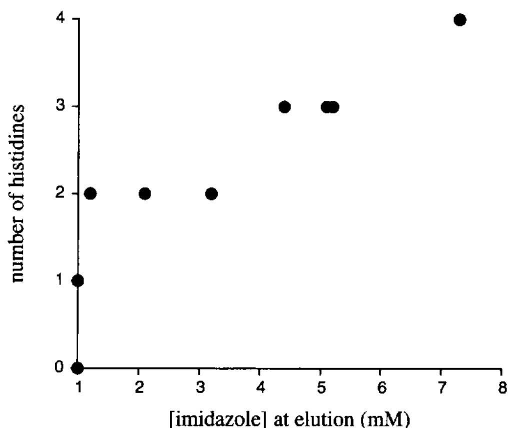

Fig. 1. Imidazole gradient elution in IMAC. Selected *S. cerevisiae* iso-1-cytochrome *c* variants were eluted from a TSK-IDACu column by a linear gradient in imidazole from 1 to 10 m*M*. Zero-histidine (H(−)) and single-histidine variants (H_4, H_8, H_{26}) were not retained in the 1 m*M* loading buffer. Multiple-histidine variants (left to right: $H_{26}H_{33}$, H_8H_{26}, H_4H_{26}, $H_8H_{26}H_{33}$, $H_{26}H_{33}H_{39}$, $H_4H_{26}H_{33}$, $H_8H_{26}H_{33}H_{39}$) are eluted in groups according to the histidine multiplicity and in accordance with the binding affinities determined from their equilibrium binding isotherms. (●) Imidazole concentration at maximum absorbance of protein elution peak.

3.2. Effect of imidazole concentration in IMAC

It is clear from the gradient elution results that imidazole (which binds to the TSK-IDACu support with $K \sim 10^4\ M^{-1}$ [9]) functions as a very effective competitor for proteins, although the proteins have equilibrium binding affinities orders of magnitude stronger. To further evaluate this competition, we measured the chromatographic retention on the TSK-IDACu column of five *S. cerevisiae* iso-1-cytochrome *c* variants, ranging from zero (H(−)) to four ($H_8H_{26}H_{33}H_{39}$) surface histidines at pH 7.0 for imidazole concentrations ranging from 0 to 7 m*M* (isocratic elution).

Protein retention in chromatography is usually measured by the capacity factor (k', ml eluent/ml column) which describes the partitioning of the protein between the mobile phase (aqueous solution) and the stationary phase (column material, in this case TSK-IDACu). The capacity factor is calculated from the volume of eluent required for the protein to travel the length of the chromatography column under isocratic conditions:

$$k' = \frac{V_R - V_o}{v} \quad (1)$$

where V_R is mean retention volume of the protein (ml eluent), V_o is the column void volume (ml eluent), and v is the total column volume (ml TSK). As shown in Fig. 2, the variants containing surface histidines partition strongly to the column, as evidenced by their large capacity factors ($k' \gg 1$).

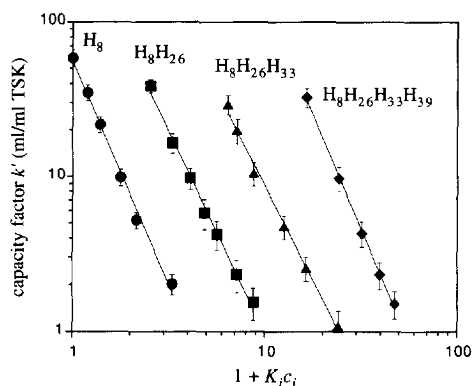

Fig. 2. Effect of imidazole on isocratic zonal elution of cytochrome *c* variants. The chromatographic capacity factor for TSK-IDACu is measured at pH 7.0 in the presence of imidazole for *S. cerevisiae* iso-1-cytochrome *c* histidine variants (●) H_8, (■) H_8H_{26}, (▲) $H_8H_{26}H_{33}$, (◇) $H_8H_{26}H_{33}H_{39}$. Partitioning of the variant with no surface histidines (H(-)) is negligible ($k' \ll 1$) under these conditions. Solid line shows best fit through data calculated using parameters of Table 1.

Furthermore, the partitioning of these proteins is strongly dependent on the concentration of imidazole in the running buffer.

The effect of imidazole on protein partitioning can be interpreted using a modified "stoichiometric displacement" model [14,16]. As illustrated in Fig. 3, this model considers a protein to displace z equivalents of imidazole upon adsorption to the TSK-IDACu. Each imidazole binds to vacant immobilized copper sites with an equilibrium binding constant K_i ($K_i \sim 10^4\ M^{-1}$). Therefore the fraction of copper sites occupied by imidazole at equilibrium (θ_i) is:

$$\theta_i = N_i/N_t = K_i c_i \theta_v \quad (2)$$

where N_i (mol/ml TSK) is the surface concentration of immobilized copper sites occupied by imidazole at equilibrium, N_t is the total surface concentration of immobilized copper sites (mol/ml TSK), c_i is the solution concentration of imidazole (M), and θ_v is the fraction of copper sites that are vacant. In the absence of protein, this expression results in the Langmuir isotherm for imidazole adsorption [9]. Although we have previously shown that the immobilized copper can weakly coordinate a second imidazole ($K \sim 10\ M^{-1}$) [9], this effect is negligible at the concentrations used in these experiments ($c_i < 10$ mM).

A mass action expression [30] for protein attachment to z vacant immobilized copper sites with an overall equilibrium binding constant K_p (M^{-1}) results in:

$$\theta_p = (z + \sigma)N_p/N_t = K_p c_p [\theta_v]^z \quad (3)$$

where N_p is the surface concentration of bound protein molecules (mol/ml TSK), c_p is the protein solution concentration (M), and θ_p is the fraction of immobilized copper sites occupied by protein. We have included a steric factor (σ) to indicate some number of surface sites blocked from additional protein binding [9,16], but its value is not of consequence to this investigation.

It should be noted that experimental evidence shows protein binding to chromatographic materials to be inherently heterogeneous [10,31–33]. Therefore a range of binding affinities and/or stoichiometries should be considered in Eq. 3. However, protein loading in the current experiments is orders of magnitude below our estimates of monolayer coverage ($\theta_p \ll 1$). We have demonstrated elsewhere on statistical thermodynamic grounds [34] that under these conditions the parameters K_p and z of Eq. 3 refer only to the subset of surface sites that are occupied at very low protein loading.

At this point we should consider the thermodynamic implications of the mass action expression of Eq. 3. First, we have included adsorbed species in the mass action expression in terms of the fractional coverage (θ) and *not* in terms of the surface concentration. This necessary departure from previous mass action derivations [14,15] results from the fact that the activity of adsorbed species (and vacant sites) is related to the mole fraction of total surface sites. Therefore the equilibrium binding constant (K_p) has consistent units (M^{-1}) regardless of the value of the parameter z. Second, we have implicitly assumed a protein solution standard state corresponding to unit activity at 1 M. Because the mass action expression is of first order in protein solution concentration, this has the effect of scaling all equilibrium binding constants by a constant factor (presuming that similar proteins have the same

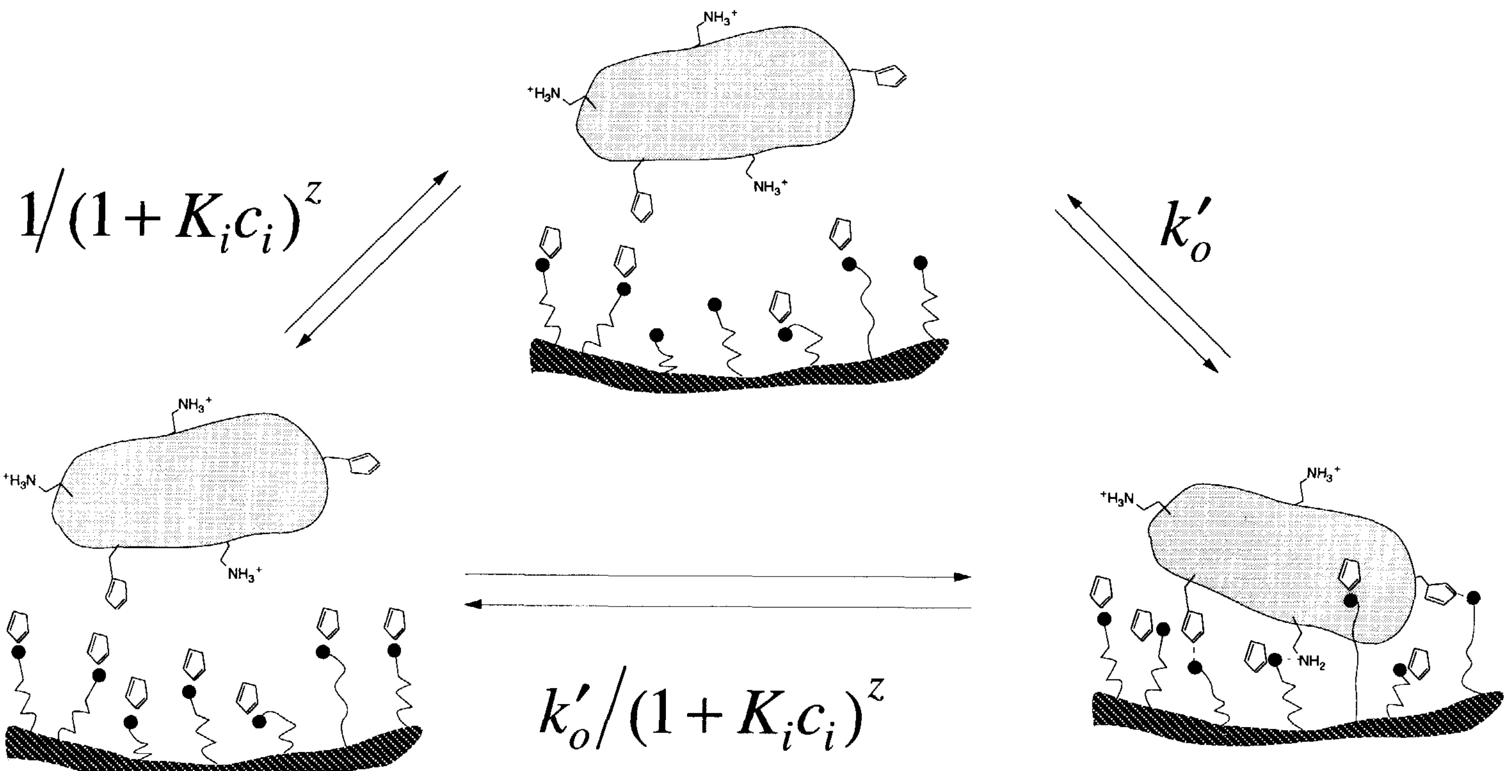

Fig. 3. Displacement model for imidazole competition in IMAC. Imidazole adsorption (k') is described by the Langmuir model with equilibrium binding constant K_i. Protein adsorption is described by the mass action approach in which z molecules of imidazole are displaced per molecule of protein. Protein partitioning in the absence of imidazole is described by the capacity factor k'_o.

standard state concentration). Therefore relative protein binding energies could be calculated directly from the ratio of the equilibrium binding constants.

The fraction of vacant surface sites is related to the imidazole and protein occupancy by:

$$\theta_v = 1 - \theta_i - \theta_p \quad (4)$$

Assuming that protein adsorption is in local equilibrium [24], the chromatographic capacity factor at low loading is given by the initial slope of the equilibrium adsorption isotherm ($N_p(c_p)$),

$$k' = \frac{dN_p}{dc_p}_{c_p=0} = \frac{K_p N_t/(z + \sigma)}{(1 + K_i c_i)^z} \quad (5)$$

This expression can be linearized by taking the logarithm of both sides:

$$\ln(k') = \ln(k'_o) - z \ln(1 + K_i c_i) \quad (6)$$

where k'_o (ml/ml TSK) is the capacity factor observed in the absence of imidazole,

$$k'_o = K_p N_t/(z + \sigma) \quad (7)$$

and corresponds to the vertical intercept ($c_i=0$) of a plot of log (k') vs. log ($1+K_i c_i$).

Table 1 summarizes the parameters of best fit for regression of the cytochrome *c* chromatographic retention data to Eq. 5. As anticipated from Eq. 5, there is good correspondence between the chromatographic capacity factors extrapolated to zero imidazole concentration (k'_o) and the initial slope of the binding isotherm previously calculated from equilibrium adsorption experiments [9,10]. Surprisingly, however, all four cytochrome *c* variants in this series appear to displace the *same* number of imidazoles upon adsorption (z=3.0±0.1), regardless of the number of surface histidines.

To displace multiple imidazoles, the protein must be contributing multiple coordination ligands. Additional surface groups must therefore be coordinating to immobilized copper ions, particularly in the case of the single-histidine variant (H_8). The additional groups are most likely primary amines (e.g., lysine and amino-terminus), which have been demonstrated to play a role in the adsorption of small peptides to IMAC supports at alkaline pH [18–20]. The equilibrium binding constant of a deprotonated amine for Cu^{2+} is comparable to that of imidazole (e.g., $10^{3.8}$ M^{-1} for glycine methyl ester versus $10^{4.2}$ M^{-1} for N-acetylhistidine) [35]. These groups generally had been presumed to be of little importance in IMAC; however, because of their relatively high pK (>7.5 for amine groups [36] versus 5.5–6.5 for histidines; see below).

3.3. Effect of pH in IMAC

Because primary amino groups usually have much higher pK values than surface histidines, we can expect to evaluate their role by taking a closer look

Table 1
"Stoichiometric displacement" model and equilibrium adsorption parameters

Variant	k'_o (ml/ml TSK)	z	Initial slope (ml/ml TSK)
H(−)	<1	N.D.	<1
Horse	$2.5 \pm 0.1 \cdot 10^1$	3.03±0.11	$3.2 \pm 1.2 \cdot 10^1$
H_8	$5.9 \pm 0.1 \cdot 10^1$	3.06±0.54	$8.5 \pm 2.1 \cdot 10^1$
H_8H_{26}	$6.0 \pm 1.4 \cdot 10^2$	2.95±0.22	$1.6 \pm 0.4 \cdot 10^3$
$H_8H_{26}H_{33}$	$7.6 \pm 3.7 \cdot 10^3$	3.01±0.17	$2.5 \pm 0.8 \cdot 10^4$
$H_8H_{26}H_{33}H_{39}$	$1.8 \pm 0.7 \cdot 10^5$	3.08±0.13	N.D.

Stoichiometric displacement parameters (k'_o and z) determined by nonlinear regression of Eq. 5 to isocratic partitioning data for five *S. cerevisiae* iso-1-cytochrome *c* variants and horse heart cytochrome *c*. For comparison, the initial slope of the equilibrium adsorption isotherms (measured in the absence of imidazole [9,10]) are presented to show relationship between equilibrium adsorption and chromatographic partitioning behavior.
N.D.=not determined.

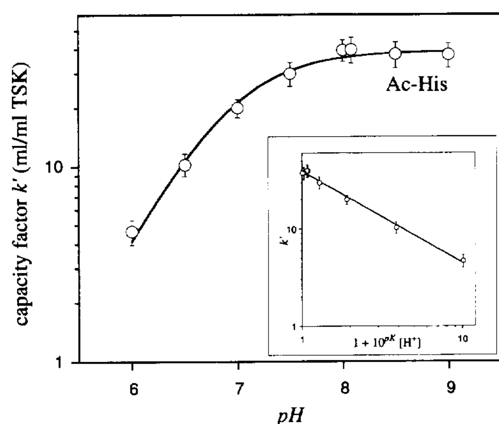

Fig. 4. Effect of pH on isocratic zonal elution of N-acetylhistidine (Ac-His). The chromatographic capacity factor (○) of Ac-His for TSK-IDACu is measured at different pH in the absence of imidazole (error bars represent peak width at half-maximum absorbance). Solid line represents best fit of N-acetylhistidine data to single protonation equilibrium (Eq. 5 with $z=1$, $K_i = 10^{pK}$, $c_i = [H^+]$, $k'_0 = 40$ ml eluent/ml TSK) using reported imidazole pK of 6.96 [35]. Inset presents data in a form analogous to Fig. 2.

at the effect of pH upon protein partitioning in IMAC. Chromatographic capacity factors of a simple single-histidine analog (N-acetylhistidine, or Ac-His) and two *S. cerevisiae* iso-1-cytochrome *c* variants, one with a single surface histidine (H_8) and one with none (H(−)), were measured on the TSK-IDACu column. These experiments were performed under isocratic conditions, in the absence of imidazole over the pH range 5.0 to 9.0. As shown in Fig. 4, the observed Ac-His capacity factors (k') increase from pH 5 to 7, reflecting deprotonation of the imidazole nitrogen, but reach a plateau at pH 7.5 as the lone imidazole group becomes completely deprotonated. In fact, the pH dependence of the Ac-His in IMAC can be described completely by the protonation of the lone imidazole with the reported pK of 6.96 [35], also shown in Fig. 4.

It is therefore necessary to know the pK of the individual surface histidines in order to evaluate the effect of pH on chromatographic protein retention. These values, reported in Table 2, were determined by monitoring the effect of pH on the ^{1}H NMR chemical shift of the imidazole ring protons [29] for all the histidine-containing cytochrome *c* variants shown in Fig. 1. It should be noted that the pK values for histidines 33 and 39 reported here (6.37 and 6.54, respectively) are approximately 0.3 pH units lower than those reported in the literature for native *S. cerevisiae* iso-1-cytochrome *c* at 25°C [26–28], consistent with the different NMR acquisition temperature (37°C) [21]. As the chromatographic experiments were also performed at 25°C, the pK values listed in Table 2 are likely to be approximately 0.3 pH units lower than the pK value in the chromatographic experiments.

Table 2
Surface histidine pK values for *S. cerevisiae* iso-1-cytochrome *c* variants measured by ^{1}H NMR at 37°C

Variant	His 33	His 39	His 4		His 8	
	pK	pK	pK	n	pK	n
H_4	–	–	5.82	0.59	–	–
H_8	–	–	–	–	5.30	0.62
H_4H_{26}	–	–	5.86	0.66	–	–
H_8H_{26}	–	–	–	–	5.32	0.57
$H_{26}H_{33}$	6.48	–	–	–	–	–
$H_4H_{26}H_{33}$	6.52	–	5.85	0.57	–	–
$H_8H_{26}H_{33}$	6.50	–	–	–	5.44	0.68
$H_{26}H_{33}H_{39}$	6.37	6.54	–	–	–	–
$H_8H_{26}H_{33}H_{39}$	6.44	6.53	–	–	5.48	0.72

Variants are labeled by the histidines present on the surface [9]. Histidine at position 26 was verified by the non-titrating proton resonance at 7.71 ppm. Due to its involvement in hydrogen bonding, histidine 26 does not titrate in the pH range studied (in fact, the pK of histidine 26 has been reported to be less than 3.0 [26]). Histidines 4 and 8 exhibited non-standard pH titrations, and are described by a Hill coefficient n less than 1 [29].

If adsorption of a single-histidine protein is due solely to metal coordination of the single surface histidine, then the pH profile of the H_8 variant should resemble that of Ac-His, only shifted, due to the lower pK of the surface histidine compared to Ac-His. As shown in Fig. 5, under acidic conditions (pH 5–6), the chromatographic retention of the H_8 variant is consistent with protein adsorption by the single fully accessible histidine at position 8. At neutral to slightly basic conditions (pH 6.5–7.5), however, the capacity factor increases much more rapidly with pH, suggesting metal coordination by additional protein surface groups. Indeed, even the H(−) variant, with no surface histidines, is retained strongly by the IMAC column at moderately basic conditions (pH~8). The behavior of both variants can be explained by including contributions from approximately three surface metal-coordinating groups each with pK~8 [12]. In fact, potentiometric titration of wild-type *S. cerevisiae* iso-1-cytochrome *c* shows approximately five groups titrating in the pH range of 5.0–9.0 [37], only two of which correspond to surface histidines (positions 33 and 39). Therefore the additional metal-coordinating groups most likely consist of the amino terminus and/or some subset of the ca. 15 surface lysines of yeast cytochrome *c*.

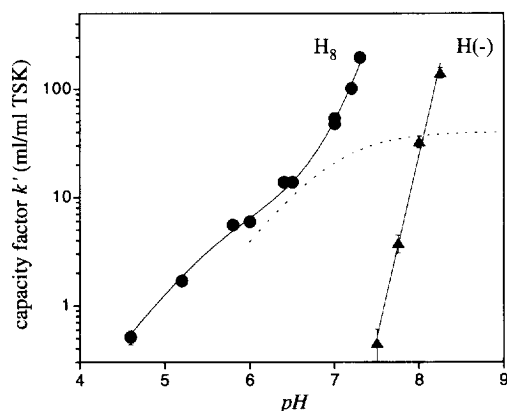

Fig. 5. Effect of pH on isocratic zonal elution of cytochrome *c* variants. The chromatographic capacity factor for TSK-IDACu is measured at different pH in the absence of imidazole for *S. cerevisiae* iso-1-cytochrome *c* histidine variants (●) H_8 and (▲) H(−). Error bars represent peak width at half-maximum absorbance. Best fit of Ac-His data from Fig. 4 (dashed line) is shown for comparison.

3.4. Implications to protein recognition

It has previously been demonstrated that processes which manipulate the extent to which multivalent binding can occur (e.g., metal-ion loading [9]) represent powerful parameters to tailor IMAC separations for particular target proteins. The wide range of pK values spanned by imidazoles and amines provides the opportunity to use pH to more specifically control the number of protein groups binding the IMAC support. The additional role of deprotonated amine groups in multivalent binding in IMAC therefore has important implications for the design of efficient separations as well as for the design of new materials for IMAC supports.

Because surface amines also contribute to binding, the binding mode of a single-histidine protein in IMAC can be strongly modulated simply by changing the pH. Under acidic conditions, metal coordination would be limited exclusively to surface histidines. In this regime, IMAC displays sequence specificity comparable to biological affinity separations. In contrast, under basic conditions, any of a large number deprotonated nitrogen side chains (e.g., histidines and lysines) can contribute to protein adsorption via metal coordination, generalizing protein binding based on metal-to-ligand coordination to a much wider array of proteins. In this regime, IMAC behavior is more typical of ion-exchange separations in that protein adsorption is driven by interactions with multiple sites on the support.

Multipoint binding provides the opportunity to target individual protein molecules by matching the distribution of metal ions to the spatial distribution of metal-coordinating imidazoles and amines [11,38]. The additional contribution of a common amino acid such as lysine may therefore provide a mechanism to distinguish among single-histidine proteins by the secondary effects of additional metal-coordinating groups. For example, template polymerization [38] at higher pH should correctly position the metal ions to complement the arrangement of protein surface imidazoles and amines. In this case, specific high-affinity binding sites would be created which match the arrangement of metal ligands on the surface to the distribution of histidines and lysines on a target protein. The ensuing protein recognition could be

performed at a lower pH, making surface histidines a prerequisite to adsorption, but also placing a premium on those proteins with appropriate patterns of surface lysines. However, with such a gain of generality comes a loss of specificity: lysine is a much more common amino acid (lysine is ca. 10% of globular proteins versus ca. 2% for histidine).

4. Conclusions

At the low coverages typical of chromatography, protein adsorption in IMAC is biased to those surface sites which accommodate multipoint binding of protein–metal-coordinating groups. Interactions at multiple sites can greatly enhance the binding affinity and dramatically alter specificity for certain classes of proteins. One consequence of this is a significant contribution from metal-coordinating residues such as lysines to protein binding, although surface histidines remain a prerequisite to binding at neutral pH. Simultaneous coordination of multiple copper sites in IMAC, particularly at low protein coverage and elevated pH, is therefore not limited to those proteins with multiple surface histidines. While the additional role of surface amine groups in IMAC complicates a molecular description of protein adsorption, it generalizes the phenomenon of multivalent binding to a wider class of proteins.

Acknowledgments

This research is supported by the National Science Foundation. F.H.A. acknowledges an NSF PYI award and a fellowship from the David and Lucile Packard Foundation. R.D.J. and R.J.T. acknowledge predoctoral training fellowships from the U.S. National Institute of General Medical Sciences, Pharmacology Sciences Program.

References

[1] J. Porath, J. Carlsson, I. Olsson and G. Belfrage, Nature, 258 (1975) 598.
[2] R.D. Johnson and F.H. Arnold, Biotechnol. Bioeng., 48 (1995) 437.
[3] F.H. Arnold (Editor), Methods: A Companion to Methods in Enzymology, 4 (1992) 1–108.
[4] S.R. Narayanan, J. Chromatogr. A, 658 (1994) 237.
[5] N.T. Mrabet, Biochemistry, 31 (1992) 2690.
[6] P.A. Feldman, P.I. Bradbury, J.D. Williams, G.E. Sims, J.W. McPhee, M.A. Pinnell, L. Harris, G.I. Crombie and D.R. Evans, Blood Coagul. Fibrinol., 5 (1994) 939.
[7] Y. Zhao, E. Sulkowski and J. Porath, Eur. J. Biochem., 202 (1991) 1115.
[8] A. Otto and G. Birkenmeier, J. Chromatogr., 644 (1993) 25.
[9] R.J. Todd, R.D. Johnson and F.H. Arnold, J. Chromatogr. A, 662 (1994) 13.
[10] R.D. Johnson and F.H. Arnold, Biochim. Biophys. Acta, 1247 (1995) 293.
[11] S. Mallik, R.D. Johnson and F.H. Arnold, J. Am. Chem. Soc., 116 (1994) 8902.
[12] R.D. Johnson, Ph.D. Thesis, California Institute of Technology, Pasadena, CA, 1995.
[13] C.D. Moore, O.N. Al-Misky and J.T.J. Lecomte, Biochemistry, 30 (1991) 8357.
[14] A. Velayudhan and C. Horvath, J. Chromatogr. A, 663 (1994) 1.
[15] J.A. Gerstner, J.A. Bell and S.M. Cramer, Biophys. Chem., 52 (1994) 97.
[16] S. Vunnum, S. Gallant, Y. Kim and S.M. Cramer, Chem. Eng. Sci., 50 (1995) 1785.
[17] D.J. Roush, D.S. Gill and R.C. Willson, J. Chromatogr. A, 704 (1995) 339–349.
[18] L. Andersson and E. Sulkowski, J. Chromatogr., 604 (1992) 13.
[19] P. Hansen and G. Lindeberg, J. Chromatogr. A, 662 (1994) 235.
[20] P. Hansen and G. Lindeberg, J. Chromatogr. A, 690 (1995) 155.
[21] R.J. Todd, Ph.D. Thesis, California Institute of Technology, Pasadena, CA, 1993.
[22] S.L. Mayo, W.R. Ellis, R.J. Crutchley and H.B. Gray, Science, 233 (1986) 948.
[23] G. Louie, W. Hutcheon and G. Brayer, J. Mol. Biol., 199 (1988) 295.
[24] F.H. Arnold, S.A. Schofield and H.W. Blanch, J. Chromatogr., 355 (1986) 1.
[25] G.J. Pielak, J. Boyd, G.R. Moore and R.J.P. Williams, Eur. J. Biochem., 117 (1988) 167.
[26] G.R. Moore and G.W. Pettigrew, in Cytochrome c: Evolutionary, Structural and Physicochemical Aspects, Springer-Verlag, Berlin, 1990.
[27] R.L. Cutler, A.M. Davies, S. Creighton, A. Warshel, G.R. Moore, M. Smith and A.G. Mauk, Biochemistry, 28 (1989) 3188.
[28] M.N. Robinson, A.P. Boswell, Z. Huang, C.G.S. Eley and G.R. Moore, Biochem. J., 213 (1983) 687.
[29] J. Markley, Acc. Chem. Res., 8 (1975) 70.
[30] H.L. Frisch, M.Y. Hellman and J.L. Lundberg, J. Polym. Sci., 38 (1959) 441.
[31] H.P. Jennissen, J. Chromatogr., 159 (1978) 71.
[32] D.S. Gill, D.J. Roush and R.C. Willson, J. Colloid Interface Sci., 167 (1994) 1.

[33] V. Dowd and R.J. Yon, J. Chromatogr. A, 693 (1995) 15.
[34] R.D. Johnson, Z.-G. Wang and F.H. Arnold, J. Phys. Chem., in press.
[35] A.E. Martell and R.M. Smith, Critical Stability Constants, Vols. 1–6, Plenum Press, New York, 1975.
[36] B.L. Haymore, G.S. Bild, W.J. Salsgiver, N.R. Staten and G.G. Krivi, Methods: A Companion to Methods in Enzymology, 4 (1992) 25–40.
[37] P.L. Barker, M.R. Mauk and A.G. Mauk, Biochemistry, 30 (1991) 2377.
[38] S. Mallik, S. Plunkett, P.K. Dhal, R.D. Johnson, D. Pack, D. Shnek and F.H. Arnold, New J. Chem., 18 (1994) 299.