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Cu(II)-Binding Properties of a Cytochrome c With a Synthetic Metal-Binding Site: His-X_3-His in an α-Helix

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ABSTRACT **A metal-binding site consisting of two histidines positioned His-X_3-His in an α-helix has been engineered into the surface of *Saccharomyces cerevisiae* iso-1-cytochrome c. The synthetic metal-binding cytochrome c retains its biological activity in vivo. Its ability to bind chelated Cu(II) has been characterized by partitioning in aqueous two-phase polymer systems containing a polymer-metal complex, Cu(II)IDA-PEG, and by metal-affinity chromatography. The stability constant for the complex formed between Cu(II)IDA-PEG and the cytochrome c His-X_3-His site is 5.3×10^4 M^{-1}, which corresponds to a chelate effect that contributes 1.5 kcal mol^{-1} to the binding energy. Incorporation of the His-X_3-His site yields a synthetic metal-binding protein whose metal affinity is sensitive to environmental conditions that alter helix structure or flexibility.**

Key words: synthetic metalloproteins, protein engineering, iso-1-cytochrome c, metal binding

INTRODUCTION

The ability of proteins to bind metal ions is widely exploited in biological systems to effect catalysis, stabilize folded conformations, and to contribute to the binding of other molecules. There is growing interest in engineering metal-binding sites into proteins for catalysis,[1,2] regulation of enzyme activity,[3,4] and protein recognition and purification.[5–7] Natural metal-binding proteins use a variety of metal-coordinating functional groups and elements of secondary structure to create specific binding sites for metal ions. Since these basic elements are common to nearly all proteins, proteins provide a convenient framework for the design of synthetic metal-binding agents. As part of an effort to more fully understand mechanisms of metal recognition in proteins and to explore the design of synthetic metal-binding sites, we have engineered a high-affinity site consisting of two histidines positioned His-X_3-His in an α-helix into the surface of *Saccharomyces cerevisiae* iso-1-cytochrome c. The His-X_3-His motif forms part of the metal-binding sites of several natural metalloproteins, including thermolysin[8] and "zinc-finger" DNA-binding proteins.[9,10] His-X_3-His sites have also been incorporated into synthetic metal-binding somatotropins[6] and a four-helix bundle protein.[11] The purpose of this study is to assess this site's contribution to copper ion binding and to identify structural features of the protein that affect the His-X_3-His site's affinity for the metal by studying the differential partitioning of cytochrome c variants in PEG/dextran two-phase systems containing small amounts of Cu(II)IDA-PEG. We have also investigated the engineered metal-binding protein's ability to bind Cu(II)IDA attached to a solid support in metal-affinity chromatography.

MATERIALS AND METHODS

Site-Directed Mutagenesis

A 2.5 kb BamH1-HindIII fragment (CYC1) that encodes iso-1-cytochrome c cloned into M13mp8 was the generous gift of Professor M. Smith (University of British Columbia). Cytochrome c genes containing the mutations for the base protein (C102S)[12] and H39Q;C102S were kindly provided by J. H. Richards, California Institute of Technology. Site-directed mutagenesis was performed by the method developed by Kunkel,[13] using the supplies and protocols from the Muta-Gene System (Bio-Rad). Reactions were carried out using the following 23-base oligonucleotides: 5′ AGCACCTTTATGAGCAGAACCGG 3′ for K4H and 5′ TCTTGAAAAGGTGAGCACCTTTC 3′ for T8H. After extension and ligation, the reaction was used to transform *E. coli* TG1 obtained from Amersham. The resulting plaques were screened using standard ^{32}P labelling techniques.[14] The DNA sequence was confirmed by the dideoxy method.[15]

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Abbreviations: Cu(II)IDA-PEG: poly(ethylene glycol) substituted with iminodiacetic acid and metallated with Cu(II); MPEG 5000: poly(ethylene glycol) monomethyl ether, M_r 5000.

The mutant CYC1 fragments were cloned into the yeast shuttle vector YEp213 (contains ampicillin and tetracycline resistance genes and the Leu2+ gene from *S. cerevisiae*),[16] which was used to transform *E. coli* HB101. Plasmid from amp^r tet^s colonies, confirmed by restriction digest and gel electrophoresis to contain the 2.5 kb fragment, was used to transform yeast strain GM-3C-2 by the LiCl method.[17] Transformants were selected by growth on leu^- plates. The recipient yeast strain GM-3C-2 (*alpha, leu2-3, leu2-112, trp1-1, his4-519, cyc1-1, cyp3-1*) contains a deletion mutation (cyc1-1) and a point mutation (cyp3-1), which eliminate and inactivate the genes for iso-1- and iso-2-cytochrome c, respectively, and cannot grow on glycerol as the sole carbon source.[18]

Protein Purification

Cytochrome c variants were purified according to a modified version of the procedure developed by Cutler and coworkers.[19] 1-L shake flask cultures of yeast were grown at 30°C in YPG media (1% yeast extract, 2% peptone, 4% glycerol) for 3 days. The cells were harvested by centrifugation and washed once with 0.9% NaCl. The cytochrome c is extracted by stirring with 0.5 volumes of 1 M NaCl, 0.1 M sodium phosphate, and 0.25 volumes ethyl acetate for 16–24 hours. Cell debris was removed by centrifugation, and the resulting supernatant dialyzed overnight against 0.1 M sodium phosphate, pH 7.2. The dialyzed cell extract was applied to a CM-Sepharose CL-6B cation exchange column equilibrated with 0.1 M sodium phosphate, pH 7.2, and the cytochrome c was eluted with 0.1 M sodium phosphate, pH 7.2, 1M NaCl. The purity was confirmed by metal-affinity chromatography. If necessary a final metal-affinity chromatography purification step was performed. The resulting cytochrome c was fully oxidized.

Metal-Affinity Partitioning

Cu(II)IDA-PEG was prepared from MPEG 5000 as described previously.[20] The two-phase systems contained 7% MPEG 5000, 4.4% dextran T500 (Pharmacia), 0.023 M sodium phosphate, 0.23 M NaCl (4.0 g total weight). For copper-containing systems, Cu(II)IDA-PEG varied from 0.5% (0.8×10^{-4} M) to 3% of the total PEG. The partitioning experiments were performed at room temperature, as described previously.[21]

Determination of Stability Constants

Stability constants for formation of a complex between the His-X_3-His site and Cu(II)IDA-PEG were obtained from partitioning data, as described in detail elsewhere.[6]

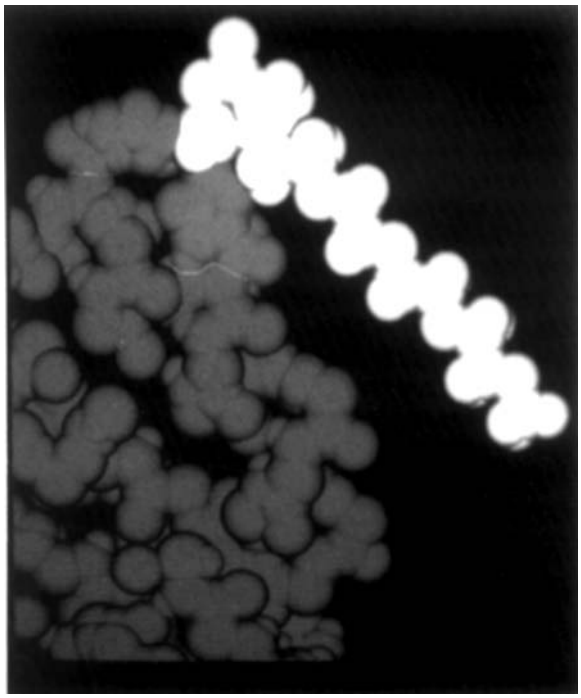

Fig. 1. Model of His-X_3-His site in N-terminal helix of *S. cerevisiae* iso-1-cytochrome c binding Cu(II)IDA-PEG (shows His side chains only).

Metal-Affinity Chromatography

A 5 mm × 25 mm column was packed with IDA-derivatized G6000PW (Toya Soda) and loaded with $CuSO_4$. Proteins are eluted with a gradient of imidazole (0–10 mM over 300 minutes) at a flow rate of 0.2 ml/min in 0.05 M sodium phosphate, 0.5 M NaCl, pH 7.0. The column was washed with EDTA (50 mM) and reloaded with $CuSO_4$ after each use.

RESULTS AND DISCUSSION

To construct the synthetic metal-binding site, Lys 4 and Thr 8 (higher eukaryotic cytochrome c numbering system) in the N-terminal α-helix of the cytochrome c were both replaced with histidine. In this particular configuration, the two histidines are located on adjacent turns of the helix, and the τ-nitrogens of the imidazole groups from both residues can coordinate a single metal (Fig. 1). Based on the notion of increased stability of multidentate protein-metal interactions in structurally rigid regions of proteins, simple modeling calculations were carried out on common secondary structural motifs (Haymore, B. L., Clare, M. C., unpublished results.) Chelating di-histidine interactions were found for α-helices (His-X_3-His), β-strands (His-X-His), and reverse β-turns (His-X_2-His). A subsequent survey of structural data showed that similar modes of metal-binding have been observed in natural metalloproteins: thermolysin contains a Zn-binding His-X_3-His

TABLE I. Metal-Affinity Partitioning of Native Heme Proteins and Oxidized *S. Cerevisiae* iso-1-Cytochrome c Variants in the Absence (K_0) and Presence (K) of 1.6×10^{-4} M Cu(II)IDA-PEG*

Protein	K_0	K	Accessible histidines
Tuna cytochrome c	0.56 ± 0.01	0.57 ± 0.01	0
C. krusei cytochrome c	0.61 ± 0.01	0.83 ± 0.01	2
Horse heart myoglobin	0.41 ± 0.01	0.72 ± 0.01	4
Sheep myoglobin	0.48 ± 0.01	0.97 ± 0.01	5
S. cerevisiae cytochromes c			
C102S	0.51 ± 0.01	0.74 ± 0.01	2 (33,39)†
H39Q;C102S	0.52 ± 0.01	0.63 ± 0.01	1 (33)
K4H;H39Q;C102S	0.60 ± 0.01	0.84 ± 0.02	2 (4,33)
T8H;H39Q;C102S	0.52 ± 0.01	0.67 ± 0.01	2 (8,33)
T8H;C102S	0.52 ± 0.01	0.79 ± 0.01	3 (8,33,39)
K4H:T8H:H39Q:C102S	0.57 ± 0.01	2.20 ± 0.05	3 (4.8.33)

*Two-phase systems contain 0.25 mg/ml protein, pH 7.6. All partitioning experiments were carried out in duplicate at room temperature.
†Accessible histidine positions, determined from crystal structures.[31]

site in an α-helix,[8] superoxide dismutase a His-X-His Cu-binding site in a β-strand,[22] carbonic anhydrase a His-X-His Zn-binding site in a β-strand,[23] among others. The chelating residues in these proteins are buried, and metal complexes such as Cu(II)IDA cannot bind to the apo-proteins. To bind to chelated metals such as Cu(II)IDA-PEG, the binding site must be accessible on the protein surface. Lys 4 and Thr 8 in the cytochrome c α-helix are both accessible to the solvent.[24]

Two of the cytochrome c variants constructed, K4H;H39Q;C102S and T8H;H39Q;C102S contain the histidine substitutions Lys→His 4 and Thr→His 8, respectively, whereas a third, K4H;T8H;H39Q; C102S, contains histidines at both positions. In all three variants the accessible histidine at position 39 was replaced with glutamine. A fourth mutant, T8H;C102S, contains the original histidine at 39 as well as the Thr→His 8 substitution. The histidine at position 33 in the native cytochrome c was left unaltered in all four mutants. To simplify analysis of the metal-binding properties of the mutant cytochromes c, all mutations were made on a base protein in which the cysteine at position 102 had been replaced by serine (C102S). Cysteine 102 has been implicated in the formation of intermolecular dimers and complications in spectroelectrochemical measurements[19] and leads to precipitation in the presence of copper. The cytochromes c were expressed in a yeast host strain (GM-3C-2) that does not produce a functional cytochrome c. In each case the plasmid-encoded cytochrome c was able to confer upon this yeast host the ability to grow on a nonfermentable carbon source and, therefore, retained its biological activity.[18]

Following purification, the (fully oxidized) cytochrome c variants were characterized by metal-affinity partitioning in a PEG/dextran two-phase system.[21] Addition of a small amount of Cu(II)IDA-PEG to a two-phase system formed from aqueous solutions of PEG and dextran alters the partitioning of proteins that contain surface-accessible histidines. Partitioning experiments can be used to obtain fundamental information on complexes formed between proteins and metal chelates: binding constants, number of binding sites, and pK_a's of binding sites. From the partitioning of a series of heme-containing proteins in PEG/dextran two-phase systems, we have found that the average association constant for the complex formed between Cu(II)IDA-PEG and an accessible histidine is 4.5×10^3 M^{-1} in the dextran-rich phase.[21] This association constant is very similar to that for the complex formed between N-methyl-IDA-Cu(II) and N-acetyl-histidine methyl ester, measured using conventional potentiometric techniques (log K = 3.6; Haymore, B. L., Stover, F. S., unpublished results). The binding is slightly weaker in the PEG-rich phase: 2.2×10^3 M^{-1}.

Partition coefficients of the cytochrome c variants and of native proteins containing different numbers of exposed histidines are presented in Table I. In the absence of Cu(II), the partition coefficients (K_0) do not vary greatly among the cytochrome c variants. Addition of Cu(II)IDA-PEG increases the partitioning of proteins that contain accessible histidines into the top (PEG-rich) phase and dramatically alters the partitioning of the cytochrome c that contains the histidines placed His-X_3-His in an α-helix. When the histidines bind the polymer-bound metal independently of one another (no chelating interaction), the difference between the logarithms of the partition coefficients in the presence (K) and absence (K_0) of metal-chelating polymer is proportional to the number of exposed histidines.[21] In (K/K_0) for natural histidine-containing proteins and the cytochrome c variants are plotted versus the number of surface-accessible histidines in Figure 2. These natural histidine-containing proteins define a calibra-

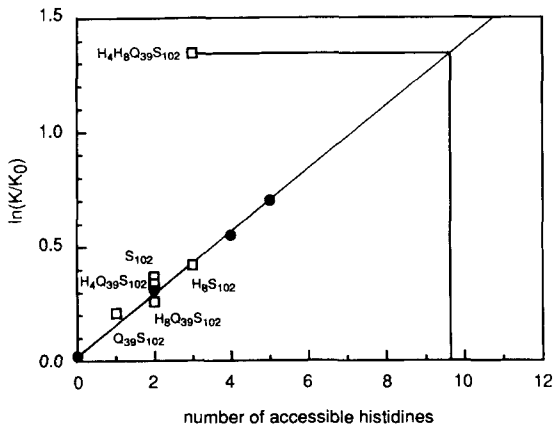

Fig. 2. Partitioning of native (•) and variant (□) proteins in PEG/dextran aqueous two-phase systems at room temperature, pH 7.6 (see Table I). Partitioning of native proteins defines a calibration for the effect of independent, accessible histidines on $\ln(K/K_0)$. Addition of two histidines in the K4H;T8H;H39Q;C102S His-X_3-His variant increases $\ln(K/K_0)$ to a value which corresponds to a hypothetical protein with 9–10 accessible histidines.

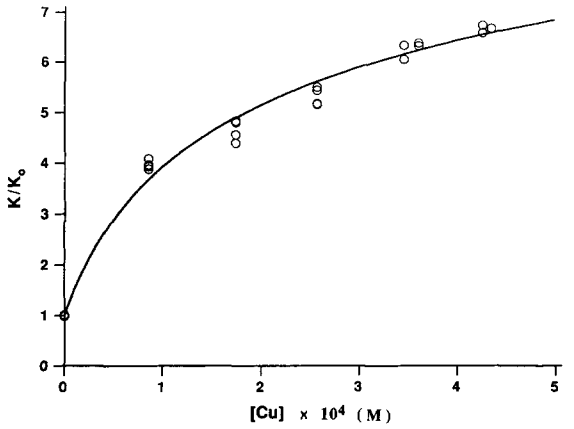

Fig. 3. Effect of Cu(II)IDA-PEG concentration on partitioning (K/K_0) of *S. cerevisiae* iso-1-cytochrome c His-X_3-His variant (K4H;T8H;H39Q;C102S) in PEG/dextran two-phase systems, pH 7.9, 0.05–0.07 mg/ml protein. The solid line represents the best fit of Eq. 2, ref. 6, to the partitioning data, using parameters determined previously for identical phase systems:[21] $K_H = 3.1 \times 10^6$, $R = 2.52$, $K_M = 3.61$, $K_a' = 2.2 \times 10^3$ and $K_a'' = 4.5 \times 10^3$.

tion curve for the influence of (non-interacting) accessible histidines on partitioning.

The cytochrome c variants K4H;H39Q;C102S and T8H;H39Q;C102S, both of which contain two accessible histidines, partition with apparent metal affinities comparable to other two-histidine proteins (C102S or cytochrome c from *Candida krusei*). The variations in $\ln(K/K_0)$ values observed for these four, very similar two-histidine proteins are indicative of the degree to which the secondary contributions from other amino acids can affect apparent metal affinity. Nearby side chains can interfere with the accessibility of the histidyl nitrogens or influence the histidyl's pK_a and therefore its availability for coordination to the metal. A third surface histidine in T8H;C102S increases the partition coefficient by an amount expected for an additional independent histidine.

The two new histidines in the His-X_3-His variant, K4H;T8H;H39Q;C102S, increase the partition coefficient dramatically, far more than can be accounted for by the addition to the protein surface of two noninteracting histidines. In fact, the partitioning behavior of this variant is equivalent to that of a hypothetical protein with nine or ten independent surface-accessible histidines (Fig. 2).

To determine stability constants for formation of the complex between the His-X_3-His site and the Cu(II)IDA-PEG, partitioning experiments were performed at a series of different concentrations of Cu(II)IDA-PEG (Fig. 3). The value of the stability constant derived from these experiments is $5.3 \times 10^4\ M^{-1}$ for the PEG-rich phase ($3.2 \times 10^4\ M^{-1}$ for the dextran-rich phase). This stability constant is 24 times the binding constant for interaction of Cu(II)IDA-PEG with a single histidine. The difference between binding at the His-X_3-His site ($5.3 \times 10^4\ M^{-1}$) and binding to two surface histidines that are not interacting with one another to form a chelating site ($2 \times 2.2 \times 10^3\ M^{-1}$) corresponds to a chelating contribution to the binding free energy in the PEG-rich phase of 1.5 kcal mol^{-1}.

The affinity of the synthetic metal-binding cytochrome c for Cu(II)IDA-PEG increases with pH from 5.5 to 8.0, as indicated in Figure 4. The solid line represents the predicted pH dependence (Eq. (2), ref. 6), using the simple assumption that the pK_a's of the two histidines in the chelating site are equal. The increase in K/K_0 with pH reflects imidazole deprotonation and, therefore, a larger concentration of available metal-binding sites. Above pH 8.0, however, the copper-binding affinity of the His-X_3-His cytochrome c decreases rapidly. Bovine somatotropin variants containing His-X_3-His sites engineered into constrained internal α-helices do not exhibit this behavior: K/K_0 increases monotonically up to pH 9.0.[6]

Although the protein remains folded up to pH 12, yeast cytochrome c undergoes its alkaline transition at approximately pH 8.5,[25] which roughly corresponds to the pH at which affinity for the copper complex is lost. It is possible that accompanying the alkaline transition there is a structural rearrangement of the N-terminal region of the protein which interferes with metal chelation. Alternatively, the reduced metal affinity at high pH may reflect flexibility in the N-terminus of cytochrome c: without a stable α-helix to provide the rigid base for formation of the chelate complex, coordination to both histidines would result in a large conformational entropy loss. Both NMR[26] and X-ray crystal structure data[24] on iso-1-cytochrome c suggest that the N-ter-

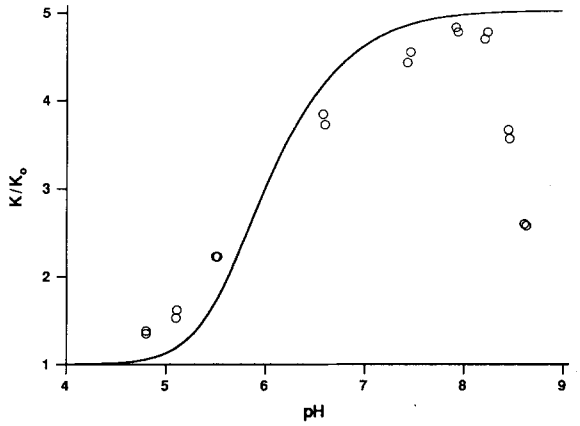

Fig. 4. Effect of pH on partitioning (K/K_0) of *S. cerevisiae* iso-1-cytochrome c His-X_3-His variant (K4H;T8H;H39Q;C102S) in PEG/dextran two-phase systems. [Cu(II)IDA-PEG] = 1.8×10^{-4} M, 0.05–0.07 mg/ml protein. (Solid line represents behavior predicted by Eq. 2, ref. 6, assuming $pK_a = 5.9$ for His 4 and His 8.)

minal α-helix that begins at residue 2 or 3 is quite rigid in the reduced protein. However, the oxidized protein may be less rigid: for example, oxidized horse cytochrome c is substantially more flexible than the reduced form.[27] Furthermore, the histidine substitutions at positions 4 and 8 may have increased the flexibility of the N-terminal region.

Ghadiri and Choi recently reported an association constant of 1.5×10^4 M^{-1} for the binding of free Cu^{2+} to a synthetic α-helical peptide with the His-X_3-His sequence.[28] This is significantly smaller than the stability constants measured for Cu(II)IDA binding by His-X_3-His sites in cytochrome c and bovine somatotropin (1.6×10^6 M^{-1}).[6] It is likely that the low stability of the peptide helix compared to helices in proteins accounts for the reduced copper affinity of the His-X_3-His site in the peptide.

The affinity of the His-X_3-His site for Cu(II) can be exploited in a class of separations that has proven very useful for isolating and purifying proteins which contain metal-coordinating amino acid residues on their surfaces. These purification techniques include immobilized metal-affinity chromatography,[29,30] extraction with metal-chelating polymers,[31] and metal-affinity precipitation.[20] Proteins are retained on a Cu(II)IDA column in an imidazole gradient according to the number of accessible histidines.[30] The concentrations of imidazole required to elute the cytochrome c variants from a column of Cu(II)IDA-G6000PW are reported in Table II. As with the metal-affinity partitioning, the retention of the Cu(II)IDA chromatography column of natural proteins and the cytochrome c variants containing non-interacting histidines parallel their surface histidine content. The chelating K4H;T8H;H39Q;C102S variant requires a substantially higher concentration of imidazole for elution than does T8H;C102S, even though both proteins contain the same number of accessible histidines (three). However, K4H;T8H;H39Q;C102S requires less imidazole than does horse heart myoglobin, which has five histidines that participate in retention on the Cu(II)IDA support (7.3 versus 8.7 mM imidazole). A small, but measurable chelate effect is evident for K4H;T8H;H39Q;C102S in metal-affinity chromatography.

TABLE II. Immobilized Metal-Affinity Chromatography of *S. Cerevisiae* Cytochrome c Variants and Horse Heart Myoglobin on Cu(II)IDA-G6000PW

Protein	[Imidazole]* (mM)	Accessible histidines†
C102S	4.7	2
H39Q;C102S	2.5	1
K4H;H39Q;C102S	3.7	2
T8H;H39Q;C102S	3.5	2
T8H;C102S	5.6	3
K4H;T8H;H39Q;C102S	7.3	3
Horse myoglobin	8.7	5

*Concentration of imidazole at elution.

†It should be noted that the histidine content for horse myoglobin listed in this table is five, whereas it is only four for the partitioning experiments. The two accessible histidines at positions 113 and 116 in the myoglobins are too closely spaced to both coordinate separate IDA-bound copper atoms. For this reason, only one of these two histidines is likely to contribute to increasing partitioning, which involves the simultaneous binding of several Cu(II)IDA-PEG molecules at histidines distributed over the protein surface. In contrast, interaction with a solid support involves binding at a single histidine, and the chromatographic retention time is proportional to the probability of finding a site. Therefore both histidines 113 and 116 are likely to contribute to chromatographic retention, whereas only one contributes to partitioning.

The elution order of the cytochrome c variants containing non-interacting histidines mirrors the order of $\ln(K/K_0)$ values in partitioning; there appears to be a close correspondence between the binding mechanisms for these proteins interacting with soluble and insoluble Cu(II)IDA. In contrast, the K4H;T8H;H39Q;C102S His-X_3-His variant exhibits an affinity for the soluble Cu(II)IDA-PEG complex that is comparable to a 10-histidine protein, whereas its affinity for the insoluble Cu(II)IDA-G6000PW is reduced to that of a four-histidine protein. The size and disparity of the chelate effects observed for K4H;T8H;H39Q;C102S binding to soluble Cu(II)IDA and Cu(II)IDA immobilized on a solid support may also reflect the fact that the metal-binding site was built into an α-helix that is potentially flexible. Significant conformational changes are known to accompany protein adsorption to surfaces.[32] These distortions are generally considered to be small for proteins interacting with hydrophilic chromatography matrices, yet may be large enough to overcome the small chelating contribution (1.5 kcal mol^{-1}) to the free energy of binding to

soluble Cu(II)IDA-PEG. Internal His-X_3-His sites in bovine somatotropin exhibit significantly higher affinities for both Cu(II)IDA-PEG and Cu(II)IDA chromatography supports.[6]

This work has demonstrated that two histidines arranged His-X_3-His in the N-terminal α-helix of iso-1-cytochrome c complexes Cu(II) that is bound to IDA-PEG 24 times tighter than does a single histidine. Furthermore, incorporation of the His-X_3-His site into a potentially flexible N-terminal region yields a synthetic metal-binding protein whose affinity for metal ions is sensitive to environmental conditions which affect helix structure or stability. In contrast to other proteins containing engineered His-X_3-His metal-binding sites,[33] this cytochrome c variant is expressed in the yeast cells in its biologically active form. Therefore, incorporation of an accessible metal-binding site does not necessarily interfere with biological activity, nor does it interfere with expression of the properly folded protein. Similar metal-binding sites may be useful in purification, enhancing crystallization of recombinant proteins, and when combined with additional metal-coordinating ligands, the design of synthetic enzymes and protein-based metal-recognition agents.

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