Inorganica Chimica Acta 437 (2015) 152–158

Contents lists available at ScienceDirect

Inorganica Chimica Acta

journal homepage: www.elsevier.com/locate/ica

Calorimetric studies of ternary complexes of Ni(II) and Cu(II) nitrilotriacetic acid and N-acetyloligohistidines

Matthew R. Mehlenbacher [b], Fadi Bou-Abdallah [b,*], Xing Xin Liu [a], Artem Melman [a,*]

[a] Department of Chemistry & Biomolecular Science, Clarkson University, 8 Clarkson Ave. Potsdam, New York, USA
[b] Department of Chemistry, State University of New York at Potsdam, Potsdam, New York, USA

ARTICLE INFO

Article history:
Received 18 June 2015
Received in revised form 3 August 2015
Accepted 5 August 2015
Available online 29 August 2015

Keywords:
Isothermal titration calorimetry
Stoichiometry
Enthalpy change
Hexahistidine tag
Nickel
Copper

ABSTRACT

The thermodynamics of Cu^{2+}- and Ni^{2+}-NTA binding to polypeptides containing 2–6 histidine residues in neutral buffered aqueous solutions were characterized by isothermal titration calorimetry (ITC). The apparent dissociation constants of ternary complexes of N-acetylhexahistidine-Ni^{2+}-NTA and N-acetylhexahistidine-Cu^{2+}-NTA were 69.5 μM and 27.1 μM, respectively. Decreased number of histidine residues in the oligohistidine fragment caused gradual decrease in the stability of the mixed metal complexes and a change in the binding stoichiometry from ~1:1 for N-acetylhexahistidine and N-acetylpentahistidine to ~0.5:1 for N-acetyltetrahistidine and ~0.3:1 for N-acetyltrihistidine, N-acetyldihistidine and N-acetylhistidine ligands. In all cases, the binding interactions are shown to be enthalpically favored. Other metal cations formed less stable mixed metal complexes with stability constants in the order of $Cu^{2+} > Ni^{2+} > Mg^{2+}$, Co^{2+}, $Zn^{2+} >> Mn^{2+}$, Ca^{2+}. The significance of the data is discussed in terms of important practical applications.

1. Introduction

Formation of kinetically labile metal complexes is a commonly used approach in self-assembly and molecular recognition and is normally characterized by large changes in enthalpy [1,2]. Metal complexes with a single cation can easily achieve high stability that otherwise can only be obtained by simultaneous formation of weaker non-covalent interactions such as hydrogen bonds, salt bridges, dipole–dipole, or hydrophobic interactions [3]. This approach has yielded state-of-the-art coordination supramolecular architectures with a wide range of interest and applications including electronic [4,5], catalytic [6], and photophysical materials [6–9]. Coordination bonding has been used for self-assembly of metal oxide frameworks, coordination polymers [10–12], molecular grids [13,14], molecular cages and nanocapsules [1,15], helicates [16,17], topologically defined macrocycles [18,19], trigger type molecules [20], artificial base pairing in DNA [21], and other applications [22,23].

In contrast to the intrinsic complementarity of hydrogen bonding and other weak interactions [24,25], metal ions exposed to a mixture of different ligands in solution phase produce, in most cases, statistical mixtures of homo- and heteroleptic complexes [26]. A possible solution to induce selective formation of ternary complexes from two different chelate ligands involves using two ligands possessing different electric charges. Selective formation of heteroleptic ternary complexes has been observed for square planar complexes of copper(II) cation with α-amino acids and 2,2′-bipyridine [27], and for pentacoordinated copper(II) [28] and zinc(II) [29] cations with terpyridine and bipyridine ligands. Selective formation of ternary ligands around iron(III) cations has been achieved with a pair of complementary η^3-terdentate meridional binders (pincer ligands) both belonging to hydroxyamino-1,3,5-family [30]. This approach is commonly used in His-tag systems through formation of labile mixed complexes between a metal cation, most commonly Ni^{2+}, an aminocarboxylate chelate ligand such as iminodiacetate (IDA) or nitrilotriacetate (NTA), and an oligohistidine polypeptide sequence containing 6–10 histidine residues (Fig 1). This sequence is routinely introduced into the polypeptide chains of proteins using recombinant DNA methodology and the majority of recombinant proteins end up containing this oligohistidine polypeptide sequence.

The most common and practical application of ternary complexes involves Immobilized Metal-Affinity Chromatography (IMAC) [33–35]. Binding of the imidazole rings of His-tagged recombinant proteins to aminocarboxylate-Ni^{2+} functionalized stationary phase results in the immobilization and purification of His-tagged proteins which are subsequently recovered by elution

* Corresponding authors.
E-mail addresses: bouabdf@potsdam.edu (F. Bou-Abdallah), amelman@clarkson.edu (A. Melman).

http://dx.doi.org/10.1016/j.ica.2015.08.009
0020-1693/

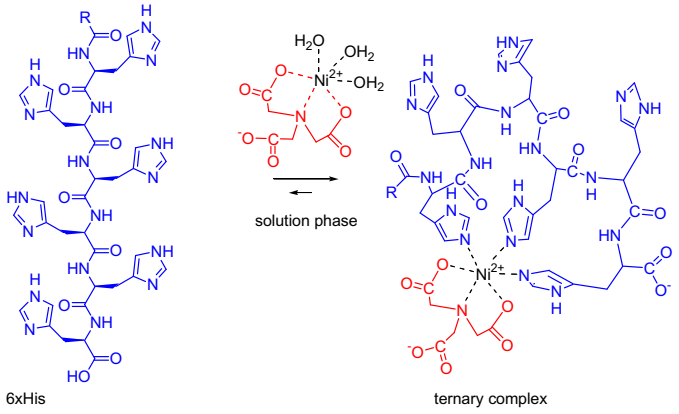

Fig. 1. Schematic representation of the formation of a ternary aminocarboxylate Ni^{2+} complex with the hexahistidine tag of a recombinant protein. The structure of the NTA-Ni^{2+} complex is adopted from [31,32].

with a solution of imidazole. Different aminocarboxylate functions such as iminodiacetate (IDA), nitrilotriacetate (NTA), and carboxymethylated aspartic acid with other metals such Cu^{2+}, Zn^{2+}, Co^{2+} have also been employed [36].

In addition to the purification of recombinant proteins, the His-tag binding system has been used in membrane separation of recombinant proteins [37], immobilization of recombinant proteins on interfaces [38,39] and on nanoparticles [40–42]. Other applications include the development of biosensors through self-assembly in solution phase [43] of hexahistidine tags with molecules containing two [44–46] or three [47–50] aminocarboxylate residues. Recently, several attempts for site specific functionalization of recombinant proteins using oligohistidine have been reported [51]. This approach involves the formation of a ternary metal complex between the oligohistidine tag of a recombinant protein and an alkylating reagent covalently attached to a nitrilotriacetate function. The resulting ternary complex serves as a template for subsequent alkylation of the non-metal binding imidazole groups on the oligohistidine tag. The alkylation of recombinant proteins with NTA tethered Baylis–Hillman esters [51] and other alkylating agents [52,53] is a particularly promising approach that could be used for covalent derivatization of His-tagged recombinant proteins. However, smaller sequences of 2–5 sequential histidine residues that appear in natural proteins much more frequently than hexahistidine sequences can potentially compete with this method of derivatization of recombinant proteins. Binding of M^{2+}-NTA by histidine molecules has been studied showing that histidine molecules can form stable ternary NTA-M^{2+}-His complexes coordinated via imidazole fragment [54].

Despite numerous applications of the NTA-Ni^{2+}-hexahistidine system, structural information using conventional X-ray diffraction methods are still lacking due to the inability of these heteroleptic complexes to form crystals. Furthermore, there is no in-solution thermodynamic data available on this system and the stability constants have only been indirectly determined using metal-chelating microscopy tip [55] and fluorescence titrations experiments [48,56–58]. Here, we provide the apparent values of all the thermodynamic parameters (binding affinity K_{ITC}, binding stoichiometry n, enthalpy change ΔH_{ITC}, entropy change ΔS and free energy change ΔG) for the binding interaction between divalent metals (mainly Ni^{2+} and Cu^{2+}) complexes of nitrilotriacetic acid with N-acetylhexahistidine and shorter oligohistidine sequences using isothermal titration calorimetry. This method has been previously employed in the study formation of metal complexes association with histidine [59] and histidine rich sequences [60,61] as well as for determination of binding constants in immobilized metal affinity chromatography [62].

2. Materials and methods

All chemicals, $ZnSO_4{\cdot}7H_2O$, and $NiSO_4{\cdot}7H_2O$ (Sigma–Aldrich), $CuSO_4{\cdot}5H_2O$, $MnSO_4{\cdot}H_2O$, $CaSO_4{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, and NaCl (Fisher Scientific), $CoSO_4{\cdot}H_2O$ (Alfa Aesar), 3-(N-Morpholino) propanesulfonic acid (Mops) (Research Organics), and nitrilotriacetic acid, NTA (Acros) were of reagent grade and were used without further purification. A 100 mM nitrilotriacetic acid stock solution in basic deionized H_2O at pH 12 was diluted down to 3 mM in 0.1 M Mops, 0.1 M NaCl, pH 7.0 buffer and then used to prepare the working solutions of all salts employed in this study. Unless otherwise mentioned, all stock salt solutions were initially prepared in deionized H_2O, pH 2.0 and then slowly added to the 3 mM NTA solution to give a final concentration of 1.5 mM metal ions and a ratio of 2 NTA:1 metal. Similar ITC results were obtained whether the stock solutions were prepared with 1 or 2 NTA molecules per metal ion. Under our experimental conditions, the M^{2+}-NTA complexes are shown to be essentially present as mononuclear species as confirmed by UV–Vis and ITC control titrations of metal ions into NTA solutions (data not shown). N-acetyloligohistidines were custom-synthesised by Biomatik (http://www.biomatik.com). Isothermal titration calorimetry experiments were carried out on a TA Instruments low volume Nano ITC with gold cells and an active cell volume of 185 μL. While the theoretical background and other practical aspects of ITC are discussed in detail elsewhere [63–69], it is worth noting that in a single ITC experiment the association constant (K_{ITC}), stoichiometry (n) and the enthalpy change (ΔH_{ITC}) of binding can be simultaneously determined. The Gibbs free energy of binding (ΔG) and the entropy change (ΔS) of the reaction can be calculated using the relationships $\Delta G = -RT\ln K_{ITC}$ and $\Delta G = \Delta H - T\Delta S$. We should mention that all reported thermodynamic quantities in this study are apparent values (i.e. experimentally measured values under non-standard conditions) which may include possible contributions to the overall equilibrium from different processes such as structural rearrangements of ligand–metal complexes upon interaction and ligands and buffer species in different states of protonation. Here, all titrations were performed at 25.00 °C in 0.1 M Mops, 0.1 M NaCl, pH 7.0 with a stirring rate of 250 rpm and a titrating syringe volume of 50 μL. Typically, an automated sequence of 16 injections, each of 3 μL NTA-metal titrant into the sample cell containing 0.2 mM oligohistidine, spaced at 5 min intervals to allow complete equilibration were performed with the equivalence point coming at the area midpoint of the titration. To show that polynuclear species were not formed between the ligands and the metal ions used in this study, ITC experiments were performed under higher concentrations (i.e. 1 mM ligands and 7.5 mM metal ions, respectively, panels H in Figs. 2 and 3). The results were similar to those reported under the lower concentrations of 0.2 mM ligands and 1.5 mM metal ions. The data were collected automatically and analyzed using the NanoAnalyze software from TA Instrument and a mathematical model involving one class of independent binding sites. All ITC experiments were repeated two to three times to ensure reproducibility with background heat corrections from NTA-metal ions titrations into buffer alone to account for the heat of dilution/mixing. Unless otherwise stated, the errors given in the tables from curve fitting are from replicate measurements. Conditions for each experiment are given in the figure captions.

To check the accuracy of the measured enthalpies and validate the thermodynamic data, chemical (i.e. acid base titrations and/or Ba^{2+} titration into 18-Crown-6) and electrical (multiple 250 uJ

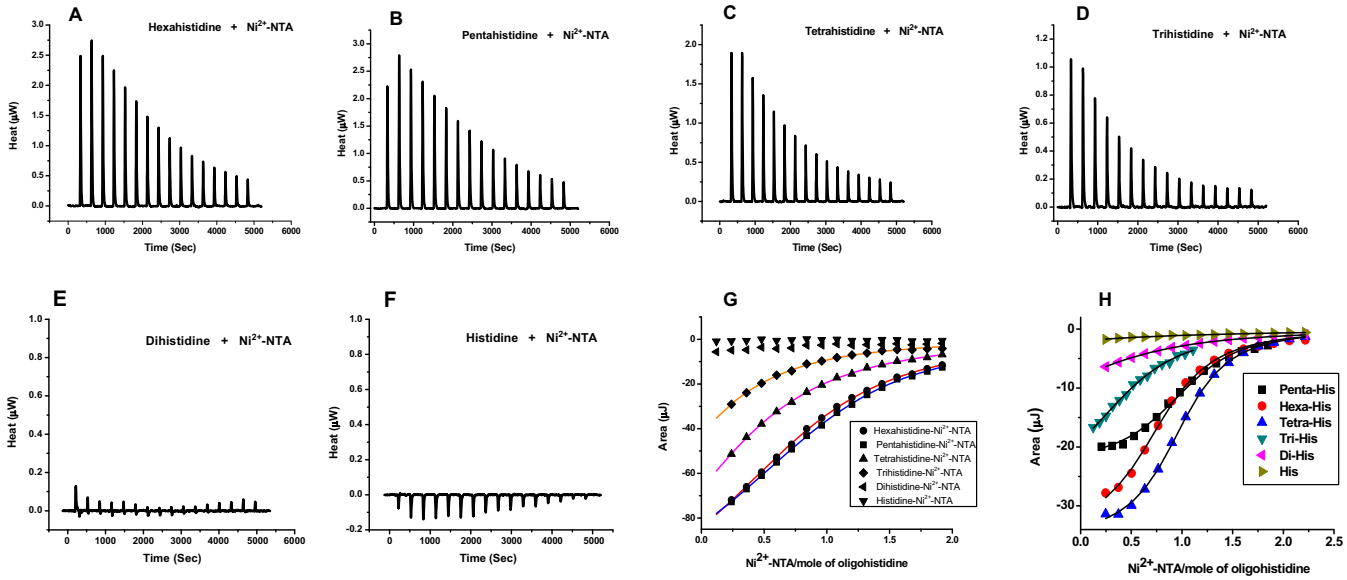

Fig. 2. Calorimetric titration of N-acetyloligohistidines with Ni^{2+}-NTA. (A, B, C, D, E and F) Raw ITC data; (G) Plots of the integrated heat vs. the Ni^{2+}-NTA/N-acetyloligohistidine ligand molar ratio. Conditions: 0.2 mM N-acetyloligohistidine titrated with 3 μL injections of 1.5 mM Ni^{2+}-NTA in 0.1 M Mops, 0.1 M NaCl, pH 7.0 and 25.00 °C. (H) Same as (G) but with 1 mM N-acetyloligohistidine and 7.5 mM Ni^{2+}-NTA. Note that the magnitude of the background dilution/mixing heat (0.2–0.3 μW) is very weak compared to the heats obtained when Cu^{2+}-NTA or Ni^{2+}-NTA solutions are titrated into oligohistidine solutions.

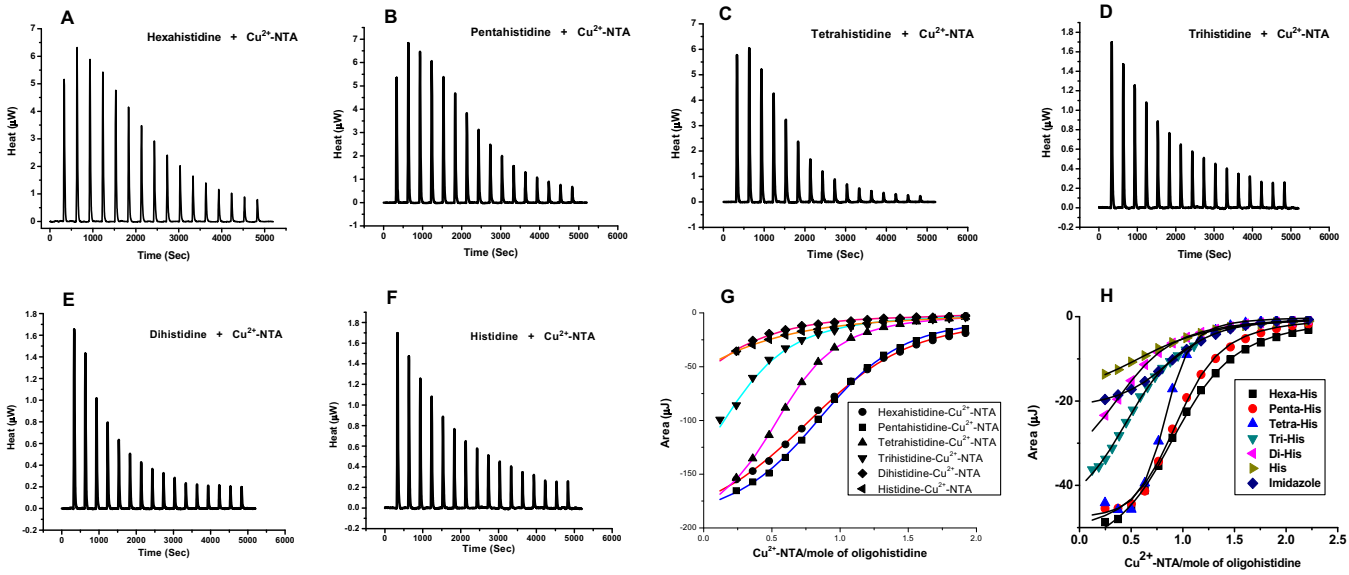

Fig. 3. Calorimetric titration of N-acetyloligohistidine with Cu^{2+}-NTA. (A, B, C, D, E and F) Raw ITC data; (G) plots of the integrated heat vs. the Cu^{2+}-NTA/histidine ligand molar ratio. Conditions: 0.2 mM N-acetyloligohistidine titrated with 3 μL injections of 1.5 mM Cu^{2+}-NTA in 0.1 M Mops, 0.1 M NaCl, pH 7.0 and 25.00 °C. (H) Same as (G) but with 1 mM N-acetyloligohistidine and 7.5 mM Cu^{2+}-NTA.

pulses) calibrations were performed according to the manufacturer recommendation and published protocols [70,71].

3. Results and discussion

3.1. ITC parameters of N-acetyloligohistidine sequences binding to Ni^{2+}-NTA

The apparent thermodynamic parameters of N-acetylhexahistidine polypeptide binding to Ni^{2+}-nitrilotriacetic acid in buffered solution at pH 7.0 were obtained by isothermal titration calorimetry. Literature data indicate that the complex between Ni^{2+} and NTA is very stable with Log K near 11.50 [72]. In contrast, binding of Ni^{2+} by hexahistidine is considerably weaker [56,58]. As a result, NTA-bound Ni^{2+} cations in the presence of hexahistidine residues form ternary complexes of the type NTA-Ni^{2+}-His_6 rather than displacing NTA. The lack of NTA displacement is evident from the extensive use of NTA-Ni^{2+} on interfaces [47,48,73] where the presence of large excess of hexahistidine-tagged proteins does not result in the displacement of NTA by hexahistidine ligands with subsequent leaching of Ni^{2+} from interfaces into the solution phase. In homogeneous solutions, titration of analogous Ni^{2+}-iminodiacetate derivatized complex by hexahistidine-tagged proteins [43] revealed no displacement of iminodiacetate. Thus, it is reasonable to assume that the species present in appreciable amounts in

solutions containing $NiSO_4$, at least 1 equiv. of NTA and N-acetylhexahistidine are Ni^{2+}-NTA complexes and NTA-Ni^{2+}-N-acetylhexahistidine mixed complexes. To ensure that all Ni^{2+} cations initially exist as Ni^{2+}-NTA complexes, all titrations were performed at pH 7.0 in buffered solutions containing the Ni^{2+}-NTA complex in the titrating syringe (prepared using 2 equiv. NTA and 1 equiv. $NiSO_4$) and N-acetylhexahistidine in the ITC reaction cell.

Figs. 2A–F illustrate the raw ITC results for N-acetyloligohistidines titration with Ni^{2+}-NTA. The integrated heats (μW) for each injection versus the molar ratio of Ni^{2+}-NTA to N-acetylhexahistidine are shown in Fig. 2G after subtraction of the control heats due to dilution and mixing. The ITC upward positive peaks observed in Fig. 2 correspond to an exothermic reaction for Ni^{2+}-NTA binding to N-acetyloligohistidines. All experimental thermodynamic parameters obtained from curve fitting of the integrated heats are compiled in Table 1. The data show one class of binding sites with a stoichiometry of approximately one Ni^{2+}-NTA complex to one hexahistidine or one pentahistidine and dissociation constants $K_{d,\ ITC}$ on the order of 60–70 μM. Binding of Ni^{2+}-NTA complex to lower histidine sequences (i.e. tetrahistidine and trihistidine) showed higher dissociation constants of ~120 μM and ~140 μM and binding stoichiometries of ~0.5 and ~0.3 Ni^{2+}-NTA per tetrahistidine and trihistidine, respectively (Table 1). In contrast, no binding between Ni^{2+}-NTA and either dihistidine or histidine was observed under our experimental conditions.

Based on these results, we can write the net association reaction between oligohistidine and Ni^{2+}-NTA complex as follows:

$$Ac(His)_{1-6}COO^- + nNi^{2+}\text{-NTA} \rightarrow Ac(His)_{1-6}COO^-[Ni^{2+}\text{-NTA}]_n$$

The apparent $K_{d,\ ITC}$ value of ~70 μM obtained by ITC for the NTA-Ni^{2+}-N-acetylhexahistidine ternary complex (Table 1) is substantially higher than those obtained in earlier studies of ternary complex formation between nickel aminocarboxylates and hexahistidine. For instance, $K_{d,\ ITC}$ values of ~2–12 μM were reported for the binding of Ni^{2+} complexes of NTA functionalized polymerosomes [57] and diblock copolymers derivatives [58], or ~4 μM for NTA group attached to a lipid molecule [48]. This substantial difference might be expected given that all literature data were obtained on different types of interfaces where formation of polynuclear metal complexes between the hexahistidine tag and two or three Ni^{2+} cations is greatly favored. Our apparent $K_{d,\ ITC}$ value is, however, similar to a $K_{d,\ ITC}$ of 81 μM observed in Ni^{2+} complex of NTA with hexahistidine without an acetyl on the terminal amino group [62]. This similarity indicates that the terminal amino group of hexahistidine most likely does not participate in binding of Ni^{2+}. Very similar $K_{d,\ ITC}$ of 69 μM was also observed between Ni^{2+} complex of an N-alkyl iminodiacetate derivative (which has one less carboxylate group than our Ni^{2+}-NTA complex) and a hexahistidine tag suggesting that only two of the three NTA carboxylate residues bind Ni^{2+} [43] and that in these complexes NTA binds Ni^{2+} in a tridentate mode (Fig. 1).

The effect of shorter oligohistidine peptides on the formation and the strength of the ternary histidine polypeptides-Ni^{2+}-NTA complexes was also considerably different from existing literature values. For example, a comprehensive study of the binding of Ni^{2+}-NTA complexes immobilized on the surface of an SPR chip [35] with analogous oligohistidines reported much lower dissociation constants ($K_{d,\ ITC}$ ~0.014 μM for hexahistidine increasing to ~0.024 μM for pentahistidine and to ~0.313 μM for tetrahistidine). This significant discrepancy can only be explained by the dominant interaction between these oligohistidine sequences and multiple immobilized Ni^{2+}-NTA complexes.

In view of the lack of known crystal structure data on the NTA-Ni^{2+}-hexahistidine complexes in solution phase and obvious difficulties in determining the structures of the resulting paramagnetic Ni^{2+} and Cu^{2+} complexes by ^1H NMR and ESR (due to very fast isomerization equilibria), it is tempting to use the ITC results obtained here to shed light on the NTA-Ni^{2+}-hexahistidine complexation reaction despite the fact that thermodynamics does not provide direct structural information. The binding of Ni^{2+}-NTA complex by His residues of N-acetyloligohistidines can occur with 1,2- (two neighboring binding His residues), 1,3- (two binding residues separated by one non-binding residue), or 1,4- binding (two binding residues separated by two non-binding residues) modes. Because of the decrease in the binding stoichiometry between penta- and tetrahistidine (from ~1 Ni^{2+}-NTA:1 pentahistidine to ~0.5 Ni^{2+}-NTA:1 tetrahistidine, Table 1), it is reasonable to assume that three histidine residues in the pentahistidine oligomer bind octahedral Ni^{2+} while the remaining three coordinating sites of Ni^{2+} are occupied by NTA. With tetrahistidine, an analogous tridentate binding mode does not apply as evidenced by the ~0.5:1 stoichiometry. This difference can be explained assuming that a 1,2 mode of chelate binding of Ni^{2+} cation by histidine residues is energetically disfavored. Consequently, and in contrast to pentahistidine where two 1,3 binding modes take place, the tetrahistidine ligand can form only two stable coordination bonds with Ni^{2+} through either 1,3 or 1,4 binding modes while the remaining sixth coordination bond is formed by a histidine residue from another tetrahistidine molecule. However, the 1,4 binding mode is likely the preferred mode given that further decrease in the binding stoichiometry (down to ~0.3 Ni^{2+}-NTA molar equivalence) is observed with N-acetyltrihistidine (Table 1). In all cases,

Table 1
Best fits parameters for ITC measurements of Ni^{2+}-NTA and Cu^{2+}-NTA binding to N-acetyloligohistidines and imidazole. The experimental conditions are those of Figs. 2 and 3. *n* represents the number of moles of M^{2+}-NTA bound per oligohistidine.

N-acetyl oligo-histidine	Metal cation	K_{ITC} (M^{-1})	$K_{d,\ ITC}$ (μM)	ΔH_{ITC} (kJ/mol)	*n*	ΔG (kJ/mol)	ΔS ($J\ K^{-1}\ mol^{-1}$)
N-Ac 6xH	Ni^{2+}	$(1.44 \pm 0.53) \times 10^4$	(69.44 ± 0.26)	−28.53 ± 1.82	0.91 ± 0.02	−23.66 ± 0.93	−16.3 ± 3.7
	Cu^{2+}	$(3.69 \pm 1.62) \times 10^4$	(27.10 ± 0.12)	−47.23 ± 2.14	0.92 ± 0.02	−25.91 ± 1.08	−71.5 ± 4.4
N-Ac 5xH	Ni^{2+}	$(1.63 \pm 0.76) \times 10^4$	(61.35 ± 0.29)	−26.09 ± 0.71	0.87 ± 0.14	−23.87 ± 1.07	−7.4 ± 2.4
	Cu^{2+}	$(4.33 \pm 0.93) \times 10^4$	(23.09 ± 0.05)	−44.53 ± 0.09	1.15 ± 0.19	−26.43 ± 0.54	−60.7 ± 1.0
N-Ac 4xH	Ni^{2+}	$(8.39 \pm 1.40) \times 10^3$	(119.19 ± 0.02)	−32.28 ± 1.31	0.55 ± 0.02	−22.37 ± 0.39	−33.2 ± 2.5
	Cu^{2+}	$(4.85 \pm 0.68) \times 10^4$	(20.62 ± 0.03)	−45.24 ± 0.31	0.52 ± 0.08	−26.73 ± 0.34	−62.1 ± 0.8
N-Ac 3xH	Ni^{2+}	$(7.10 \pm 0.48) \times 10^3$	(140.84 ± 0.01)	−26.89 ± 1.19	0.30 ± 0.02	−21.98 ± 0.17	−16.5 ± 2.2
	Cu^{2+}	$(2.23 \pm 0.28) \times 10^4$	(44.84 ± 0.06)	−45.92 ± 2.20	0.31 ± 0.04	−24.81 ± 0.30	−70.8 ± 4.1
N-Ac 2xH	Ni^{2+}	————	————	————	————	————	————
	Cu^{2+}	$(9.98 \pm 1.87) \times 10^3$	(100.20 ± 0.02)	−31.97 ± 1.53	0.28 ± 0.04	−22.80 ± 0.47	−30.7 ± 2.9
N-Ac 1xH	Ni^{2+}	————	————	————	————	————	————
	Cu^{2+}	$(5.41 \pm 0.87) \times 10^3$	(184.84 ± 0.03)	−36.31 ± 1.04	0.31 ± 0.03	−21.29 ± 0.39	−50.4 ± 2.0
Imidazole	Ni^{2+}	————	————	————	————	————	————
	Cu^{2+}	$(1.22 \pm 0.25) \times 10^4$	(81.97 ± 0.17)	−29.65 ± 2.39	0.55 ± 0.07	−23.32 ± 0.21	−21.2 ± 4.4

the complexation reactions between NTA-Cu^{2+} (or NTA-Ni^{2+}) and oligohistidines are enthalpically favored (i.e. large negative ΔH) and accompanied by a decrease in system disorder (i.e. negative ΔS values could be associated with the conversion of flexible N-acetyloligohistidine chains to a more rigid cyclic structure upon metal ion complexation).

3.2. ITC parameters of N-acetyloligohistidine sequences binding to Cu^{2+}-NTA and other metal cations-NTA complexes

Because Cu^{2+} cations have been shown to bind histidine residues [51], analogous ITC measurements were conducted with NTA-Cu^{2+} complexes and N-acetylhexahistidines. As reported under Materials and Methods, similar results were obtained whether the stock solutions were prepared with 1 or 2 NTA molecules per metal ion suggesting that dinuclear complexes (if present) do not significantly influence the results of the complexation reaction. Fig. 3A–F show the raw ITC data of a series of titrations of Cu^{2+}-NTA complexes with different N-acetyloligohistidine polypeptides with the integrated heats for each injection shown in Fig. 3G following subtraction of control heats. In contrast to Ni^{2+}-NTA, alteration of the number of histidine residues did not completely eliminate the binding of Cu^{2+}-NTA to dihistidine and histidine but had a pronounced effect on the binding stoichiometry reducing it down to one-third of that of hexahistidine. However, the affinity remained pretty much constant for most histidine sequences with the exception of dihistidine and histidine which exhibited about 4-fold and 7-fold reduction, respectively (Table 1). Therefore, while Cu^{2+}-NTA showed good association with all polyhistidine peptides tested here, Ni^{2+}-NTA showed no measurable association with either dihistidine or histidine. The difference in stability constants for the polyhistidine-Cu^{2+}-NTA ternary complexes was on average 2- to 3-fold higher than polyhistidine-Ni^{2+}-NTA complexes. These results suggest a stronger association between Cu^{2+}-NTA and oligohistidines and are reflected in the higher values of the enthalpy change of the reaction (Table 1). Nonetheless, exothermic reactions were observed with all M^{2+}-NTA-oligohistidine complexes tested in this study and excellent fits of the data (Figs. 2G, 2F and 3G, 3F) are achieved using a model with one set of independent binding sites (Table 1).

The ITC binding data of Cu^{2+}-NTA to N-acetyloligohistidines is analogous to that observed with Ni^{2+}-NTA in that both hexa- and pentahistidine provided similar binding constants with 1:1 stoichiometry of ternary complexes. Transition from pentahistidine-Cu^{2+}-NTA complexes to tetra- and then trihistidine peptides resulted in identical reduction in binding stoichiometries from ~1:1 to ~0.5:1 to ~0.3:1 M^{2+}-NTA:oligohistidine, respectively. The higher stability constants of Cu^{2+} cations in comparison to Ni^{2+} cations allowed measurement of their NTA ternary complexes with N-acetyldihistidine and N-acetylhistidine (Table 1).

Significantly, while the decrease in binding affinity (from $\sim 2 \times 10^4$ to $\sim 1 \times 10^4$ to $\sim 0.5 \times 10^4$ M^{-1} for trihistidine, dihistidine and histidine, respectively) is likely due to the decreased number of total histidine residues (from 3 in N-acetyltrihistidine to one in N-acetylhistidine), the binding stoichiometry of the Cu^{2+}-ternary complexes remained at 3 histidine ligands per 1 Cu^{2+}-NTA (Table 1). The higher binding constant obtained for imidazole (which is in good agreement with the literature data [74]) in comparison to N-acetylhistidine ($\sim 1.2 \times 10^4$ M^{-1} versus $\sim 5 \times 10^3$ M^{-1}) can be attributed to lower steric congestion in the case of imidazole. Nonetheless, our ITC results with Cu^{2+} and Ni^{2+} cations suggest similar binding modes between the N-acetyloligohistidines and M^{2+}-NTA.

The ITC data show large negative ΔS° values of ternary complex formation particularly with Cu^{2+} cations. This negative entropy can be attributed to the complexation reaction between M^{2+}-NTA and N-acetyloligohistidines and the conformational changes resulting from the conversion of flexible oligohistidine peptides in the free N-acetyloligohistidines in solution to more rigid cyclic structures upon ternary complex formation (Fig. 1). These negative entropy changes are partially offset by an increase in entropy due to the release of water molecules from the inner coordination sphere of NTA-M^{2+}-$(H_2O)_{n=1-3}$ upon binding to N-acetyloligohistidine ligands. The observed differences between Cu^{2+}-NTA and Ni^{2+}-NTA can be ascribed to the different coordination chemistries of the two cations as Cu^{2+} commonly forms 4- and 5-coordinate complexes compared to the predominantly hexacoordinated Ni^{2+}. Consequently, 4-coordinate NTA-Cu^{2+}-H_2O complexes would release only up to one water molecule upon ternary complex formation in comparison to up 3 water molecules that could be released from hexacoordinated NTA-Ni^{2+}-$(H_2O)_{n=2-3}$ complex.

The small differences in the binding constants between N-acetylhexahistidines and shorter N-acetyloligohistidines observed here for both Ni^{2+} and Cu^{2+} complexes have important practical applications. The common rationale for using hexahistidine tags for affinity purification is a combination of the rarity of the HHHHHH motif in natural proteins with significant differences in binding of aminocarboxylate-Ni^{2+} on interfaces. As suggested by the ITC results in Table 1, in homogeneous solutions the difference in binding affinity between Ni^{2+}-NTA or Cu^{2+}-NTA and hexahistidine (or pentahistidine) is relatively small, and non-specific binding of aminocarboxylate complexes of Ni^{2+} or Cu^{2+} to native proteins containing different histidine-rich sequences will probably occur in a similar way to that observed here with hexahistidine. The importance of this study lies in the potential use of these complexes as tools to create probes for protein labeling and to design novel strategies for bio-imaging of target proteins [43,56].

To investigate the possibility of ternary complex formation between N-acetyloligohistidines and other metal cations-NTA complexes, ITC experiments using NTA complexes of several common bivalent metal cations (i.e. Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+}) found in living cells, were performed under conditions identical to those employed with Cu^{2+}- and Ni^{2+}-NTA. All of these cations are known to form stable complexes with NTA [72]. The results of their association with different oligohistidine sequences are compiled in Table 2. In contrast to Cu^{2+} and Ni^{2+}, NTA complexes of Ca^{2+} and Mn^{2+} did not exhibit a measurable enthalpy change upon complexation with N-acetylhexahistidine. This lack of heat of binding does not allow us to characterize the formation of ternary complexes (if any) with these metal ions under our experimental conditions. However, relatively weak binding is observed with

Table 2
Best fit parameters for ITC measurements of M^{2+}-NTA binding to N-acetyloligohistidines. Conditions: 0.2–0.3 mM N-acetyloligohistidine titrated with 3 μL injections of 1.5–3.0 mM M^{2+}-NTA in 0.1 M Mops, 0.1 M NaCl, pH 7.0 and 25.00 °C. *n* represents the number of moles of M^{2+}-NTA bound per oligohistidine.

N-acetyloligohistidine (mM)	[M^{2+}-NTA] (1:2)	K_{ITC} (M^{-1})	ΔH_{ITC} (kJ/mol)	*n*	ΔG (kJ/mol)	ΔS (J K^{-1} mol^{-1})
0.2	1.5 mM Mg^{2+}	$(2.45 \pm 1.03) \times 10^3$	−19.03 ± 3.31	0.92 ± 0.13	−19.34 ± 1.04	1.1 ± 6.3
0.2	1.5 mM Co^{2+}	$(2.29 \pm 0.14) \times 10^3$	−14.91 ± 0.92	1.02 ± 0.17	−19.17 ± 0.15	14.3 ± 1.7
0.2	1.5 mM Mn^{2+}	Very weak binding	————	————	————	————
0.2	1.5 mM Ca^{2+}	Very weak binding	————	————	————	————
0.3	3.0 mM Zn^{2+}	$(2.55 \pm 0.65) \times 10^3$	−10.65 ± 0.59	1.12 ± 0.14	−19.44 ± 0.63	29.5 ± 1.6

Mg^{2+}, Co^{2+} and Zn^{2+} cations with affinity constant 6 times and 15 times lower than with Ni^{2+} and Cu^{2+} cations, respectively. For these M^{2+}-NTA binding to N-acetyloligohistidines (where $M^{2+} = Mg^{2+}$, Co^{2+}, Zn^{2+}), the complexation reactions are shown to be mainly driven by favorable enthalpy changes with minor increases in the entropy of the system (Table 2).

These observations are generally in line with the known order of retention times observed in chromatography of hexahistidine-tagged proteins on iminodiacetate functionalized affinity chromatography columns [33]. No appreciable binding was anticipated with Mg^{2+} due to its small size, strong preference to anionic oxygen ligands, and low coordination number. However, the binding affinity of Mg^{2+}-NTA to N-acetylhexahistidine in aqueous solution was similar to that of Co^{2+} or Zn^{2+}. To the best of our knowledge, there exist no literature data on the stability of aminocarboxylate-Mg^{2+}-hexahistidine ternary complexes, or retention times in Mg^{2+} charged aminocarboxylate affinity chromatography columns. Further studies are needed to address this question and the possibility that Mg^{2+} cations might interfere with the separation of hexahistidine-tagged proteins by affinity chromatography.

4. Conclusion

Here, we investigated the binding thermodynamics of short oligohistidine polypeptides to complexes of nitrilotriacetate with bivalent metal cations using isothermal titration calorimetry. Our results indicate that binding of N-acetylhexahistidine to Ni^{2+}-NTA complexes in aqueous solutions proceeds with 1:1 stoichiometry and a $K_{d,\ ITC}$ of ~70 μM, a value significantly lower than that reported for analogous binding interactions on solid interfaces. The binding thermodynamics of other metal cations-NTA binary complexes showed a spectrum of binding affinities to N-acetylhexahistidine ranging from non-measurable/very weak affinities in the case of Ca^{2+} and Mn^{2+}, to weak association in the case of Co^{2+}, Zn^{2+} and Mg^{2+}. The overall order of binding affinities for the metal cations-NTA-N-acetylhexahistidine ternary complexes was $Cu^{2+} > Ni^{2+} > Mg^{2+}$, Co^{2+}, $Zn^{2+} >> Mn^{2+}$, Ca^{2+}. Shorter oligohistidines exhibited weaker association and lower binding stoichiometries. The small differences in the binding constants between N-acetylhexahistidine and shorter N-acetyloligohistidines reported here for both Ni^{2+} and Cu^{2+} complexes have important practical applications. The common rationale for using hexahistidine tags for affinity purification is a combination of the rarity of the HHHHHH motif in natural proteins with significant differences in binding of aminocarboxylate-Ni^{2+} on interfaces. This notion is no longer applicable to homogeneous solutions where binding of proteins containing different dihistidine and trihistidine motifs might be significant.

Acknowledgments

This work was supported by NSF – Major Research Instrumentation Program (NSF MRI Award # 0921364) (FBA) and NSF award CHE 1150768 (AM). We thank the Kilmer Undergraduate Research Apprenticeship Program at SUNY Potsdam and the Collegiate Science and Technology Entry Program (CSTEP) for funding MRM.

References

[1] M. Fujita, Chem. Soc. Rev. 27 (1998) 417.
[2] B. Linton, A.D. Hamilton, Chem. Rev. 97 (1997) 1669.
[3] H.J. Schneider, A.K. Yatsimirsky, Chem. Soc. Rev. 37 (2008) 263.
[4] G.S. Papaefstathiou, L.R. MacGillivray, Coord. Chem. Rev. 246 (2003) 169.
[5] J.C. Noveron, M.S. Lah, R.E. Del Sesto, A.M. Arif, J.S. Miller, P.J. Stang, J. Am. Chem. Soc. 124 (2002) 6613.
[6] D. Fiedler, D.H. Leung, R.G. Bergman, K.N. Raymond, Acc. Chem. Res. 38 (2005) 349.
[7] V. Balzani, G. Bergamini, F. Marchioni, P. Ceroni, Coord. Chem. Rev. 250 (2006) 1254.
[8] C. Giansante, P. Ceroni, V. Balzani, F. Vogtle, Angew. Chem., Int. Ed. 47 (2008) 5422.
[9] C.F. Chow, S. Fujii, J.M. Lehn, Angew. Chem., Int. Ed. 46 (2007) 5007.
[10] M. Eddaoudi, D.B. Moler, H.L. Li, B.L. Chen, T.M. Reineke, M. O'Keeffe, O.M. Yaghi, Acc. Chem. Res. 34 (2001) 319.
[11] S. Kitagawa, R. Kitaura, S. Noro, Angew. Chem., Int. Ed. 43 (2004) 2334.
[12] B.H. Ye, M.L. Tong, X.M. Chen, Coord. Chem. Rev. 249 (2005) 545.
[13] P. Broekmann, K.H. Dotz, C.A. Schalley, Templates in chemistry III, Topics in Current Chemistry, vol. 287, Springer-Verlag Berlin Heidelberg, 2009. p. 1.
[14] T. Kudernac, S.B. Lei, J. Elemans, S. De Feyter, Chem. Soc. Rev. 38 (2009) 402.
[15] C.Y. Su, Y.P. Cai, C.L. Chen, M.D. Smith, W. Kaim, J. Am. Chem. Soc. 125 (2003) 8595.
[16] C. Piguet, G. Bernardinelli, G. Hopfgartner, Chem. Rev. 97 (1997) 2005.
[17] C. Piguet, M. Borkovec, J. Hamacek, K. Zeckert, Coord. Chem. Rev. 249 (2005) 705.
[18] S. Leininger, B. Olenyuk, P.J. Stang, Chem. Rev. 100 (2000) 853.
[19] K.S. Chichak, S.J. Cantrill, A.R. Pease, S.H. Chiu, G.W.V. Cave, J.L. Atwood, J.F. Stoddart, Science 304 (2004) 1308.
[20] L. Zelikovich, J. Libman, A. Shanzer, Nature 374 (1995) 790.
[21] H. Weizman, Y. Tor, J. Am. Chem. Soc. 123 (2001) 3375.
[22] A.J. Blake, N.R. Champness, P. Hubberstey, W.S. Li, M.A. Withersby, M. Schroder, Coord. Chem. Rev. 183 (1999) 117.
[23] D.L. Long, E. Burkholder, L. Cronin, Chem. Soc. Rev. 36 (2007) 105.
[24] G. Cooke, V.M. Rotello, Chem. Soc. Rev. 31 (2002) 275.
[25] K.E. Feldman, M.J. Kade, T.F.A. de Greef, E.W. Meijer, E.J. Kramer, C.J. Hawker, Macromolecules 41 (2008) 4694.
[26] Z. Grote, R. Scopelliti, K. Severin, Angew. Chem., Int. Ed. 42 (2003) 3821.
[27] F. Turecek, Mass Spectrom. Rev. 26 (2007) 563.
[28] G. Arena, R.P. Bonomo, S. Musumeci, R. Purrello, E. Rizzarelli, S. Sammartano, J. Chem. Soc., Dalton Trans. (1983) 1279.
[29] J.R. Li, H.C. Zhou, Nat. Chem. 2 (2010) 893.
[30] G. Melman, P. Vimal, A. Melman, Inorg. Chem. 48 (2009) 8662.
[31] P.A. Petrenko, M. Gdaniec, Y.A. Simonov, V.G. Stavila, A.P. Gulea, Russ. J. Coord. Chem. 30 (2004) 813.
[32] M. Kruppa, D. Frank, H. Leffler-Schuster, Inorg. Chim. Acta 359 (2006) 1159.
[33] V. Gaberc-Porekar, V. Menart, J. Biochem. Biophys. Methods 49 (2001) 335.
[34] V. Gaberc-Porekar, V. Menart, Chem. Eng. Technol. 28 (2005) 1306.
[35] S. Knecht, D. Ricklin, A.N. Eberle, B. Ernst, J. Mol. Recognit. 22 (2009) 270.
[36] I.T.L. Bresolin, M. Borsoi-Ribeiro, W. Tamashiro, E.F.P. Augusto, M.A. Vijayalakshmi, S.M.A. Bueno, Appl. Biochem. Biotech. 160 (2010) 2148.
[37] T. Maruyama, S. Tabayashi, T. Honjo, K. Hoe, T. Tanaka, J. Shimada, M. Goto, H. Matsuyama, RSC Adv. 2 (2012) 125.
[38] G.B. Sigal, C. Bamdad, A. Barberis, J. Strominger, G.M. Whitesides, Anal. Chem. 68 (1996) 490.
[39] D. Kroger, M. Liley, W. Schiweck, A. Skerra, H. Vogel, Biosens. Bioelectron. 14 (1999) 155.
[40] J.D. Swartz, C.P. Gulka, F.R. Haselton, D.W. Wright, Langmuir 27 (2011) 15330.
[41] M. Ganesana, G. Istarnboulie, J.L. Marty, T. Noguer, S. Andreescu, Biosens. Bioelectron. 30 (2011) 43.
[42] J. Kim, H.Y. Park, J. Ryu, D.Y. Kwon, R. Grailhe, R. Song, Chem. Commun. (2008) 1910.
[43] S. Hutschenreiter, L. Neumann, U. Radler, L. Schmitt, R. Tampe, ChemBioChem 4 (2003) 1340.
[44] E.G. Guignet, R. Hovius, H. Vogel, Nat. Biotechnol. 22 (2004) 440.
[45] L.W. Miller, V.W. Cornish, Curr. Opin. Chem. Biol. 9 (2005) 56.
[46] A.N. Kapanidis, Y.W. Ebright, R.H. Ebright, J. Am. Chem. Soc. 123 (2001) 12123.
[47] S. Lata, A. Reichel, R. Brock, R. Tampe, J. Piehler, J. Am. Chem. Soc. 127 (2005) 10205.
[48] I.T. Dorn, K.R. Neumaier, R. Tampe, J. Am. Chem. Soc. 120 (1998) 2753.
[49] C.R. Goldsmith, J. Jaworski, M. Sheng, S.J. Lippard, J. Am. Chem. Soc. 128 (2006) 418.
[50] C.J. You, J. Piehler, Anal. Bioanal. Chem. 406 (2014) 3345.
[51] X.X. Liu, A. Melman, Chem. Commun. 49 (2013) 9042.
[52] S. Uchinomiya, H. Nonaka, S. Fujishima, S. Tsukiji, A. Ojida, I. Hamachi, Chem. Commun. (2009) 5880.
[53] M. Hintersteiner, T. Weidemann, T. Kimmerlin, N. Filiz, C. Buehler, M. Auer, ChemBioChem 9 (2008) 1391.
[54] I. Kiseleva, D. Pyreu, T. Krivonogikh, M. Bazanova, T. Hochenkova, E. Kozlovskii, Polyhedron 51 (2013) 10.
[55] L. Schmitt, M. Ludwig, H.E. Gaub, R. Tampe, Biophys. J. 78 (2000) 3275.
[56] A. Murata, S. Arai, S.I. Yoon, M. Takabayashi, M. Ozaki, S. Takeoka, Bioorg. Med. Chem. Lett. 20 (2010) 6905.
[57] P. Tanner, M. Ezhevskaya, R. Nehring, S. Van Doorslaer, W. Meier, C. Palivan, J. Phys. Chem. B 116 (2012) 10113.
[58] R. Nehring, C.G. Palivan, O. Casse, P. Tanner, J. Tuxen, W. Meier, Langmuir 25 (2009) 1122.
[59] Y. Zhang, S. Akilesh, D.E. Wilcox, Inorg. Chem. 39 (2000) 3057.
[60] R.G. Ge, Y. Zhang, X.S. Sun, R.M. Watt, Q.Y. He, J.D. Huang, D.E. Wilcox, H.Z. Sun, J. Am. Chem. Soc. 128 (2006) 11330.
[61] N.E. Grossoehme, S. Akilesh, M.L. Guerinot, D.E. Wilcox, Inorg. Chem. 45 (2006) 8500.
[62] W.R. Kirk, Protein Expr. Purif. 95 (2014) 1.
[63] E. Freire, O.L. Mayorga, M. Straume, Anal. Chem. 62 (1990) 950A.
[64] A. Cooper, in: J.E. Ladbury, B.Z. Chowdhry (Eds.), Biocalorimetry: Applications of Calorimetry in the Biological Sciences, Wiley, 1998, p. 103.
[65] R. O'Brien, I. Haq, in: J.E. Ladbury, M.L. Doyle (Eds.), Biocalorimetry 2: Applications of Calorimetry in the Biological Sciences, Wiley, 2005, p. 3.

[66] J.A. Thomson, J.E. Ladbury, in: J.E. Ladbury, M.L. Doyle (Eds.), Biocalorimetry 2: Applications of Calorimetry in the Biological Sciences, Wiley, 2005, p. 37.
[67] F. Bou-Abdallah, T.R. Terpstra, BBA Gen. Sub. 2012 (1820) 318.
[68] F. Bou-Abdallah, P. Arosio, S. Levi, C. Janus-Chandler, N.D. Chasteen, J. Biol. Inorg. Chem. 8 (2003) 489.
[69] F. Bou-Abdallah, P. Arosio, P. Santambrogio, X. Yang, C. Janus-Chandler, N.D. Chasteen, Biochemistry 41 (2002) 11184.
[70] N.A. Demarse, C.F. Quinn, D.L. Eggett, D.J. Russell, L.D. Hansen, Anal. Biochem. 417 (2011) 247.
[71] L. Baranauskiene, V. Petrikaite, J. Matuliene, D. Matulis, Int. J. Mol. Sci. 10 (2009) 2752.
[72] A.E. Martell, R.M. Smith, Critical Stability Constants, Plenum Press, 1975.
[73] R.E. Baltus, K.S. Carmon, L.A. Luck, Langmuir 23 (2007) 3880.
[74] D. Banerjea, T.A. Kaden, H. Sigel, Inorg. Chem. 20 (1981) 2586.