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Isothermal titration calorimetry of Ni(II) binding to histidine and to N-2-aminoethylglycine

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ABSTRACT

Isothermal titration calorimetry (ITC) is used to study the complexation thermodynamics of Ni(II) with histidine (His) and with N-2-aminoethylglycine (EDMA). The titrations were performed in HEPES and Tris buffers at various ionic strengths and pH values around 8. The results show the influence of the experimental conditions on the shape and fitting parameters of the calorimetric curves. For the studied systems, the main reaction is concomitant with a number of side reactions which contribute to the global energy measured. From the calorimetric data measured, the formation constants for the species $NiHEPES^+$, $Tris^+His^-$, $TrisNiHis^+$ and $[Ni(EDMA)_2OH]^-$ have been evaluated for the first time and the values obtained properly validated.

1. Introduction

An emerging and powerful technique to evaluate experimentally the thermodynamic quantities involved in chemical interaction processes is the isothermal titration calorimetry (ITC). This technique is mainly used in the study of bimolecular biological interactions, which can be very complex and often include several binding sites involving a number of chemical interactions and molecular changes [1]. It has also been demonstrated that ITC is a relevant tool to study the interactions of metal ions with biological macromolecules [2–4]. The calorimetric curves obtained allow the assignation of the enthalpy variation and binding constant associated to each binding site by means of any appropriate software package. Nevertheless, these quantities include the contribution of all side reactions and molecular changes (i.e. reactions involving the buffer species, effect of folding when protein ligands are involved and others) and it is not an easy task to isolate each one of these contributions.

On the other hand, simpler chemical interactions, such as the complexation reactions between any inorganic cation and an organic ligand [5,6], the acidic dissociation of small molecules like the L-cysteine [7], the protonation of polybases such as the phytate ion [8] or the protonation of monoprotic bases in hydroalcoholic media [9] were also studied. In some cases, these simpler systems allowed the comparison of ITC results with those derived from measurements carried out through a reference technique, mainly potentiometry, and, hence, the critical evaluation of the ITC results. The agreement of results obtained from both approaches calorimetric and potentiometric, is good for the acid–base reactions [7–9] but just fair for the complexation processes [5]. This is because the acid–base reactions are very simple chemical systems and it is easy to select the best experimental conditions to isolate the main reaction to be studied. However, many side reactions are involved in ordinary complexation processes (i.e. protonation or deprotonation of reactants or products, the complexation of the metal ion with the basic buffer species and others) and, very often, the constants associated to these side reactions are unknown. Since ITC measures the global heat variation, the final results include the contributions of all reactions involved in the process and the obtained values can differ significantly of those corresponding to the isolated main reaction. Consequently, the thermochemical quantities determined show a significant degree of variability depending on the experimental conditions which significantly determine the nature and extension of the side reactions. Nevertheless, the complexity of the metal ion complexation processes is intermediate between the simplest acid–base reactions and the biological interactions and could be a good model to evaluate the ITC ability to measure the contribution of single interactions involved in a global

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process and, therefore, to open the door to the reaction discrimination.

In this work, ITC is used to determine the complexation enthalpies and reaction constants of some pattern reactions between Ni(II) and two different amino acids with the aim to explore the effect of the experimental conditions in the final results. Most constants for the reactions involved in the complexation processes selected were determined by potentiometry and included in the Stability Constants Data Base [10] and they have been used as the reference values. The comparison of the calorimetric results for the interactions in the chemical systems selected (the main reaction is accompanied of several side reactions some of them with unknown equilibrium constants) under a variety of experimental conditions (aqueous solutions with different buffering agents at several ionic strengths and pH) with those obtained from a reference technique allows us a critical evaluation about the quality of the values achieved by ITC. The conclusions achieved in this work can be useful in further studies about interactions between metal ions and molecules of biological significance such as the proteins.

1.1. Theory

When working at neutral or slightly basic solutions, Ni(II) binds to histidine to form 1:1 and 1:2 complexes according to the following equilibria. The interactions between Ni(II) and N-2-aminoethylglycine are properly described by equivalent stepwise reactions [10]

$$Ni^{2+} + His^{-} \Leftrightarrow NiHis^{+} \quad (1)$$

$$His^{-} + NiHis^{+} \Leftrightarrow Ni(His)_2 \quad (2)$$

The stepwise thermodynamic formation constants of these equilibria, K_1 and K_2, are related to their concentration constants, K_1^c and K_2^C, by means of

$$K_1 = \frac{a_{NiHis^+}}{a_{Ni^{2+}} a_{His^-}} = K_1^c \frac{\gamma_{NiHis^+}}{\gamma_{Ni^{2+}} \gamma_{His^-}} \quad (3)$$

$$K_2 = \frac{a_{Ni(His)_2}}{a_{NiHis^+} a_{His^-}} = K_2^c \frac{\gamma_{Ni(His)_2}}{\gamma_{NiHis^+} \gamma_{His^-}} \quad (4)$$

where a_i is the activity of the species shown in the subscripts and γ_i is the activity coefficient which can be computed through the Debye–Hückel equation (Eq. (5))

$$\log \gamma = -\frac{Az^2 I^{1/2}}{(1 + a_0 B I^{1/2})} \quad (5)$$

where I is the ionic strength, z is the charge of the species and A and a_0B are 0.514 and 1.501, respectively [11].

Another way to express the interactions between Ni(II) and histidine is through the global equilibria. In this case, the overall concentration formation constants, β_1^c and β_2^c, and their relation with the stepwise concentration constants are defined as

$$\beta_1^c = \frac{[NiHis^+]}{[Ni^{2+}][His^-]} = K_1^c \quad (6)$$

$$\beta_2^c = \frac{[Ni(His)_2]}{[Ni^{2+}][His^-]^2} = K_1^c K_2^c \quad (7)$$

The thermodynamic value of the overall formation constants, β_i, will be achieved after correcting, β_i^c values for the ionic strength through the activity coefficients.

Usually, the main equilibria, Eqs. (1) and (2), are concomitant with different side reactions involved in stepwise complexation. Conditional or effective concentration formation constants, $K_{i,\text{eff}}$, are defined to describe the Ni/His system taking into account the involved side equilibria [11,12].

$$K_{1,\text{eff}} = \frac{[NiHis^+]'}{[Ni^{2+}]'[His^-]'} \quad (8)$$

$$K_{2,\text{eff}} = \frac{[Ni(His)_2]'}{[His^-]'[Ni(His)^+]'} \quad (9)$$

where the symbol "prime" stands for the conditional concentration of the species in brackets, that is the sum of the concentration of the free species plus the concentration of this species involved in the side reactions.

The coefficient α_i relates the conditional concentration of the species in subscript with its free concentration in the medium, $[i]$, and can be expressed as [11,12]

$$\alpha_{i(Y)} = \frac{[i]'}{[i]} = 1 + \beta_{1(Y)}^c [Y] + \beta_{2(Y)}^c [Y]^2 + \ldots + \beta_{n(Y)}^c [Y]^n \quad (10)$$

where Y stands for a side ligand present in the medium and $\beta_{1(Y)}^c$, $\beta_{2(Y)}^c$, etc. refer to the overall concentration formation constants of the side reactions. When the species i takes part in more than one side reaction, with $Y, Z, \ldots$ ligands, the following expression should be used

$$\alpha_{i(Y,Z,\ldots)} = \alpha_{i(Y)} + \alpha_{i(Z)} + \ldots + (1 - \text{number of side reaction ligands}) \quad (11)$$

Including Eq. (10) in Eqs. (8) and (9)

$$K_{1,\text{eff}} = K_1^c \frac{\alpha_{NiHis^+}}{\alpha_{Ni^{2+}} \alpha_{His^-}} \quad (12)$$

$$K_{2,\text{eff}} = K_2^c \frac{\alpha_{Ni(His)_2}}{\alpha_{His^-} \alpha_{NiHis^+}} \quad (13)$$

achieving in this way, expressions that relate the experimental formation constants, $K_{1,\text{eff}}$ and $K_{2,\text{eff}}$, with the concentration constants, K_1^c and K_2^c, which in turn are related to the thermodynamic formation constants through Eqs. (3) and (4), respectively.

The thermodynamic formation constants are the values of interest in this work since they allow the comparison with those published and determined by a reference method. However, K_1 and K_2 can only be obtained if all the side reaction coefficients, α_i can be calculated, that is, if the overall formation constants of all the side reactions are known.

2. Experimental

2.1. Instruments

- The ITC experiments were carried out in a VP-ITC microcalorimeter (MicroCal, LCC) equipped with a 1.4047 mL cell. ITC instrument was supplied with the ThermoVac accessory, a device for thermostating and degassing. The generated ITC data were collected automatically and subsequently analyzed by the software also supplied by MicroCal.
- pH measurements were taken with a Ross combination electrode Orion 8102 (glass electrode and a reference electrode with a 3.0 M KCl solution in water as a salt bridge) in a Crison micropH 2002 potentiometer with a precision of ±0.1 mV.

2.2. Chemicals

- Nickel(II) nitrate hexahydrate was obtained from Merck, L(+)-histidine (His) from J.T. Baker and N-2-aminoethylglycine (EDMA)

from Sigma–Aldrich; all of them show a purity higher than 99% and they were used as received.

- Tris [Tris(hydroxymethyl)aminomethane] (>99.8%, A.C.S., J.T. Baker) and HEPES [N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)] (Fluka >99%) were used to prepare buffer solutions. Hydrochloric acid 0.5 M (Titrisol Merck) and sodium hydroxide 1 M (Titirsol Merck) were used to adjust the desired pH.
- Water purified by a Milli-Q plus system from Millipore with a resistance higher than 18 MΩ were used to prepare buffer solutions and to clean the microcalorimeter.

2.3. Procedures

- HEPES buffer solutions were prepared at approximately pH 8 with various ionic strengths of 20, 25, 50 and 100 mM. The HEPES buffer was prepared forming the sodium salt form, Na^+Hepes^-, and adding the required amount of HCl to adjust the desired pH value. Working in this way, the concentration of buffer was equal to the ionic strength of the buffer provided that the ionic strength was calculated assuming that zwitterions do not contribute to it [13].
- Tris buffer was prepared at several pH values around 8 and ionic strength 25, 50 and 100 mM neutralizing the half of the added Tris in the solution with HCl. Operating in this way, the concentration of buffer is double of the ionic strength. On the other hand, Tris buffer at pH around 8.0 and ionic strength 50 and 100 mM was prepared forming the chloride form, $TrisH^+Cl^-$, and adding the required amount of NaOH to adjust the desired pH value. Therefore, the concentration of buffer is equal to its ionic strength.
- Titrant and sample solutions were prepared from the same stock buffer solution. Ni(II) solution was placed in the cell and His or EDMA were the titrant in the syringe. Both titrant and sample solutions were degassed for 2 min before each titration. In the titration of Ni(II) with His, His concentration was 1.125 mM and Ni(II) concentration was in the range from 0.055 mM to 0.065 mM. In the titrations of Ni(II) with EDMA, the concentrations were 1.441 mM of EDMA and 0.059 mM of Ni(II) when HEPES was the buffer and 2.110 mM of EDMA and 0.085 mM of Ni(II) when the solutions were buffered by Tris.
- ITC measurements were carried out at 25.0 ± 0.2 °C. The solution in the cell was stirred at 290 rpm by the syringe to ensure rapid mixing. Typically, 7.5–10 μL of titrant was delivered in the cell over 20 s under control into a known volume of sample in the cell. The number of additions was from 30 to 40 (depending on the volume of each addition) with an adequate interval of 240 s between injections to allow complete equilibrations. Titrations continued until the molar ratio of ligand vs. sample in the cell was approximately 3–4 to ensure that no additional complexes were formed in excess of titrant. A background titration, consisting of the identical titrant solution but with the sample cell filled just by the buffer solution was carried out to determine the background heat to be subtracted of the main experiment. Each assay was repeated three or more times.

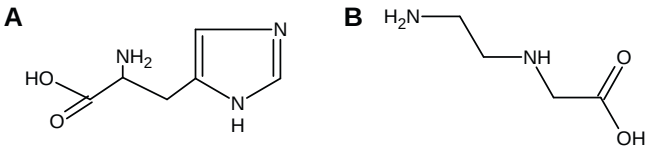

Fig. 1. Studied ligands: (A) histidine (His) and (B) N-2-aminoethylglycine (EDMA).

2.4. Calculations

ITC data were collected automatically and subsequently analyzed with the two-site binding model by the Windows based Origin software package supplied by MicroCal. The Origin software uses a nonlinear least-squares algorithm (minimization of χ^2) and the concentration of the titrant and the sample to fit the heat flow per injection to an equilibrium binding equation, providing best fit values of the stoichiometry (n), change in enthalpy ($\Delta H_{(ITC)}$), and formation constant ($K_{b(ITC)}$). The data were fit with the constraint that $n_1 = n_2$, since it was well known that Ni(II) binds to the ligands His or EDMA forming 1:1 and 1:2 stepwise complexes and, in both instances, the successive formation constants, $\log \beta_1$ and $\log \beta_2$, differ in about seven units, as shown in Table 1 [10].

3. Results and discussion

In this work, the thermodynamic quantities related to the binding interactions of Ni(II) (represented by Ni) to Histidine (His) and to N-2-aminoethylglycine (EDMA) have been determined from calorimetric data. The selected amino acids (Fig. 1) bind to Ni in 1:1 and 1:2 complexes. The thermodynamic formation constants are given in Table 1 [10,14–16] which shows that the formation constants for the system Ni/EDMA are slightly higher than those for the Ni/His system. Fig. 2 represents all the species involved in the Ni/His system complexation equilibria according to the literature data.

3.1. Titration curves

Fig. 3 shows the ITC titration curves of Ni with His in HEPES buffer and pH around 8. The top panel shows exothermic heat produced from 30 to 40 injections of 7–10 μL of ligand to the known concentration of Ni(II). Integration of the peaks with respect to time allows the calculation of ΔH for each injection as shown in the

Table 1
Formation constants, $\log \beta_i$ or $\log K_i$, for the interactions Ni/His and Ni/EDMA and for the side reactions.

Main reaction: M/L	$\log \beta_i$ and $\log K_i$ (I = 100 mM)[a]				$\log \beta_i$ and $\log K_i$ (I = 0)			
	ML/M·L	ML_2/M·L^2	ML_2/ML·L	ML_3/ML_2·L	ML/M·L	ML_2/M·L^2	ML_2/ML·L	ML_3/ML_2·L
Ni/His	8.66	15.52			9.10[b]/8.68[15]/8.90[16]	16.18[b]/15.51[15]/16.1[16]	7.06[b]/6.83[15]/7.20[16]	
Ni/EDMA	10.44	16.78			10.88[b]	17.44[b]	6.56[b]	
His/H	9.09		6.02	1.70	9.16[15]/9.18[16]		6.09[15]/5.97[16]	2.05[15]/1.77[16]
EDMA/H	9.84		6.67	1.80				
NiHis/H	3.62							
$Ni(His)_2$/H	5.03							
Ni/OH					4.10[10]	9.00[10]		12.00[10]
Ni/Tris	2.63	4.60						
Tris/H					8,07[10,14]			
HEPES/H					7.55[10,14]			

[a] Overall formation constants determined potentiometrically at ionic strength 100 mM and 25 °C published in the Stability Constants Data Base [10].
[b] Thermodynamic formation constants (I = 0) computed from the values published in [10] and Eq. (5).

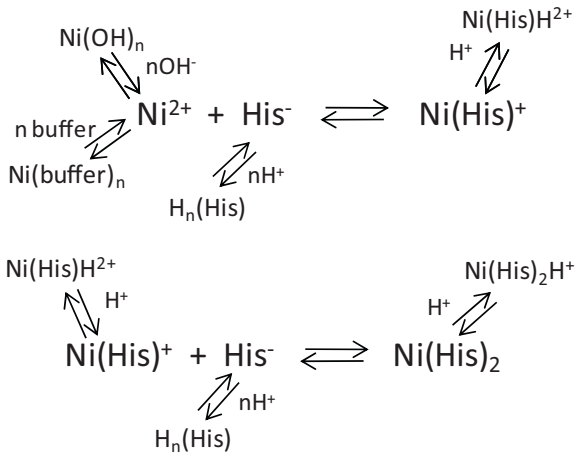

Fig. 2. Side equilibria closely related to the main equilibrium between Ni (II) and His.

lower panel. Peaks of top go down showing an exothermic process. The heat flow shows two different tendencies as adding His in the cell which agree with the fact that Ni binds to His in 1:1 and 1:2 ratios. At the beginning of the titration, the heat involved increases when adding His in the cell whereas, after a molar ratio higher than 1, the heat flow diminishes suddenly and a high jump is observed. The shapes of the curves are similar working at different ionic strength.

The Ni/His system was also studied at similar pH achieved with Tris buffer and various ionic strengths and two tendencies are observed too. Fig. 4 shows that the heat flow always diminishes as adding His in the cell, firstly the slope is very small and then the heat flow diminishes suddenly. Comparing Figs. 3 and 4 (plots on the bottom), it is observed that the shapes of the curves are different showing that the buffer strongly influences the titration process.

Titrations of Ni with EDMA were carried out in HEPES and Tris buffers (Fig. 5). As expected, the shapes of the titration curves show also two tendencies. The comparison of the assays carried out in Tris and HEPES buffers for both complexation processes (Figs. 3–5) shows that the heat involved using Tris buffer is always significantly higher than in HEPES buffer.

3.2. Calorimetric fitting parameters

Since the studied complexation processes involve two successive steps, the titration curves were fitted to the two-binding site model. Table 2 collects the fit parameters for titrations carried out for the systems Ni/His and Ni/EDMA obtained in different experimental conditions. In all instances, the values of n close to 1 indicate that the chosen mathematical model is suitable to fit the curves. From the ITC assays, the conditional formation constants ($K_{1,\mathrm{eff}}$ and $K_{2,\mathrm{eff}}$) are directly determined. However, these constants are usually named $K_{\mathrm{b1(ITC)}}$ and $K_{\mathrm{b2(ITC)}}$ when working with ITC and these have been the notations used henceforth in this work. As expected from the shape of the curves, the precision for $K_{\mathrm{b1(ITC)}}$ is lower than for $K_{\mathrm{b2(ITC)}}$ in both, HEPES and Tris buffers assays. $\Delta H^{o}{}_{1(\mathrm{ITC})}$ and $\Delta H^{o}{}_{2(\mathrm{ITC})}$ are also experimental values that embody all the interactions involved in the heat flow and, therefore, the values are different when working with HEPES and Tris buffers. In both systems, $\Delta H^{o}{}_{1(\mathrm{ITC})}$ and $\Delta H^{o}{}_{2(\mathrm{ITC})}$ are more negative in Tris than in HEPES buffer.

3.3. Ni/His system

Table 3 shows the mean and standard deviation values for the enthalpies and formation constants derived from the assays carried out for the system Ni/His. In HEPES buffer, $\Delta H^{o}{}_{1(\mathrm{ITC})}$ values are constant whereas $\Delta H^{o}{}_{2(\mathrm{ITC})}$ becomes slightly more negative with the increase of the HEPES concentration. Regarding the experimental formation constants, $\log K_{\mathrm{b1(ITC)}}$ values become slightly lower when increasing the HEPES concentration and $\log K_{\mathrm{b2(ITC)}}$ remains constant. In contrast, in Tris buffer, the most negative $\Delta H^{o}{}_{1(\mathrm{ITC})}$ value is achieved in the assay carried out at ionic strength 25 mM whereas varying the ionic strength from 50 to 100 mM, $\Delta H^{o}{}_{1(\mathrm{ITC})}$ values are

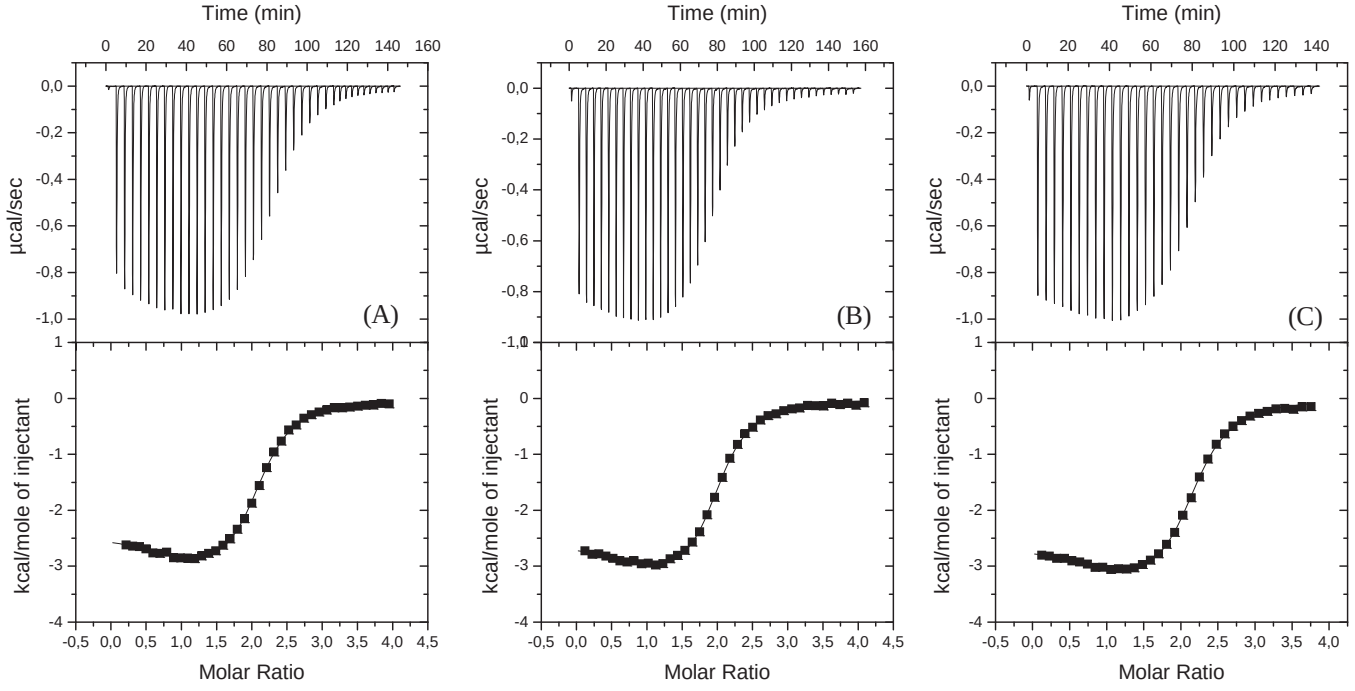

Fig. 3. ITC curves obtained from titration of 0.065 mM of Ni (II) with 1.125 mM of His in HEPES buffer: (A) I = 25 mM, pH 7.85; (B) I = 50 mM, pH 8.04; and (C) I = 100 mM, pH 8.15.

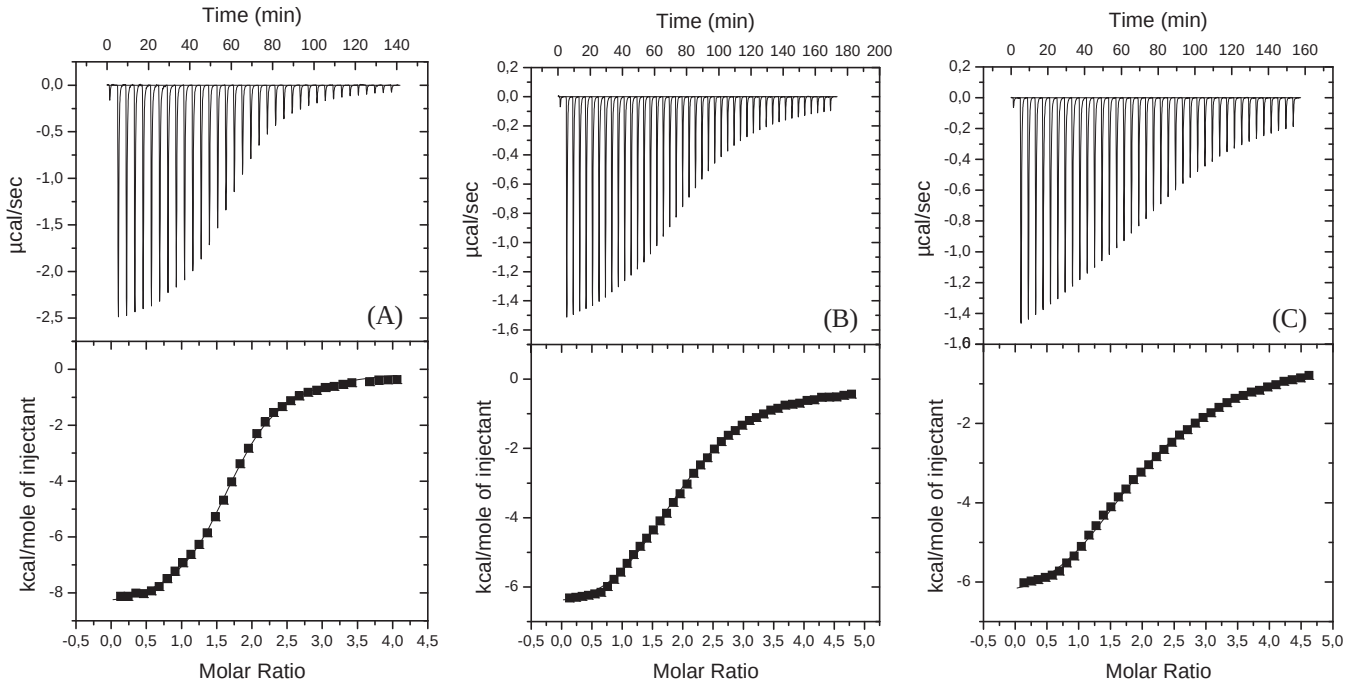

Fig. 4. ITC curves obtained from titration of Ni (II) with 1.125 mM His in Tris buffer: (A) I = 25 mM, pH 8.24 and [Ni] = 0.065 mM; (B) I = 50 mM, pH 8.32 and [Ni] = 0.055 mM; and (C) I = 100 mM, pH 8.32 and [Ni] = 0.055 mM.

almost constant. On the other hand, at the same ionic strength, $\Delta H^{o}_{(ITC)}$ value is slightly more negative when the concentration of Tris is equal to the ionic strength than when the concentration of Tris is double. Regarding the experimental formation constants, $\log K_{b1(ITC)}$ and $\log K_{b2(ITC)}$, the obtained values are similar when working at the same Tris concentration independently on the ionic strength. This shows that the influence of the concentration of buffer in the medium is more significant than the ionic strength on the fitting parameters. Moreover, $\log K_{b1(ITC)}$ and $\log K_{b2(ITC)}$ values diminish when the buffer concentration increases.

The system Ni/His was previously studied by Zhang et al. [5] by means of ITC under slightly different experimental conditions

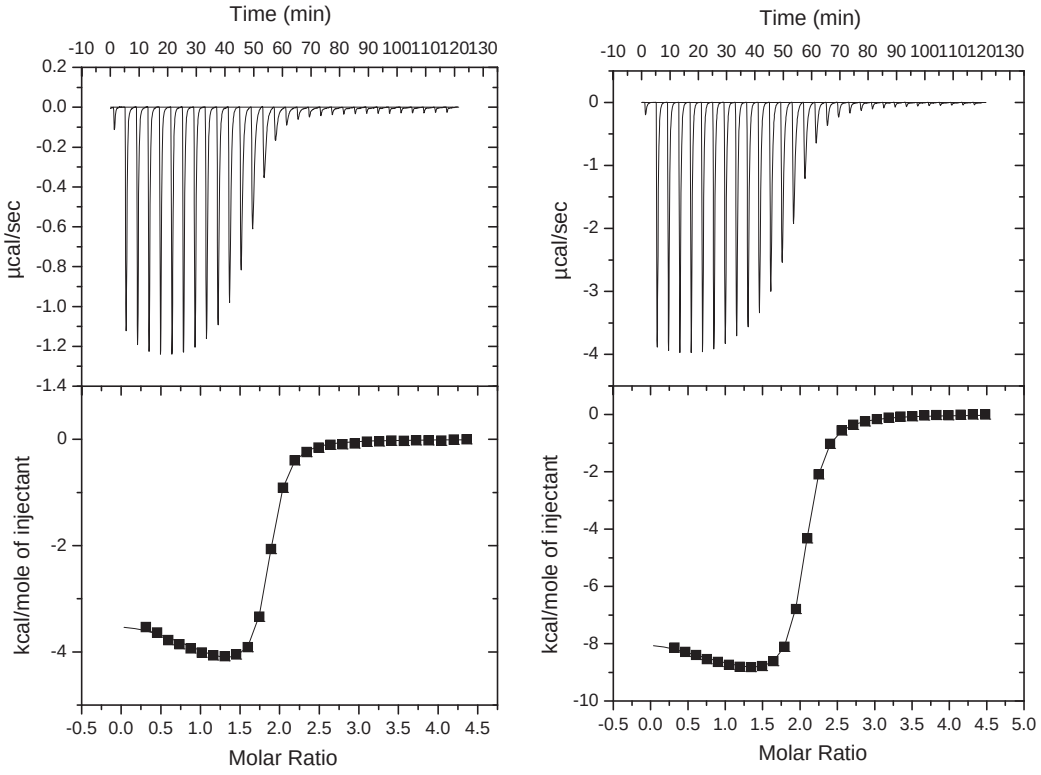

Fig. 5. ITC curves obtained from titration of Ni (II) with EDMA working at I = 50 mM: (A) HEPES buffer, pH 7.97; [Ni] = 0.059 mM; [EDMA] = 1.441 mM; (B) Tris buffer, pH 8.00; [Ni] = 0.085 mM; [EDMA] = 2.110 mM.

Table 2
Fitting parameters for the ITC curves shown in Figs. 3–5. The two-binding site model with the constrain $n_1 = n_2$ is used.

Ni/His in HEPES buffer	I = 25 mM; pH 7.85	I = 50 mM; pH 8.04	I = 100 mM; pH 8.15
n_1	1.02 ± 0.01	0.98 ± 0.01	1.06 ± 0.01
$\Delta H^o_{1(ITC)}$[a]	−2.43 ± 0.17	−2.35 ± 0.47	−2.23 ± 0.68
$K_{b1(ITC)}$	(1.52 ± 0.79) × 10^6	(1.21 ± 0.71) × 10^6	(1.07 ± 0.58) × 10^6
n_2	1.02 ± 0.01	0.98 ± 0.01	1.06 ± 0.01
$\Delta H^o_{2(ITC)}$[a]	−3.51 ± 0.21	−3.67 ± 0.50	−3.90 ± 0.71
$K_{b2(ITC)}$	(3.04 ± 0.36) × 10^5	(4.36 ± 0.97) × 10^5	(4.46 ± 1.07) × 10^5
Ni/His in Tris buffer[b]	I = 25 mM; pH 8.24	I = 50 mM; pH 8.32	I = 100 mM; pH 8.32
n_1	0.98 ± 0.02	1.15 ± 0.04	0.97 ± 0.02
$\Delta H^o_{1(ITC)}$[a]	−8.13 ± 0.13	−6.49 ±0.09	−6.18 ± 0.11
$K_{b1(ITC)}$	(1.33 ± 0.37) × 10^6	(5.56 ± 0.81) × 10^5	(1.48 ± 0.41) × 10^5
n_2	0.98 ± 0.02	1.15 ± 0.04	0.97 ± 0.02
$\Delta H^o_{2(ITC)}$[a]	−6.064 ± 0.15	−5.51 ± 0.23	−8.6 ± 2.38
$K_{b2(ITC)}$	(1.43 ± 0.08) × 10^5	(5.38 ± 0.31) × 10^4	(2.64 ± 0.22) × 10^4

Ni/EDMA in two different buffers	HEPES I = 50 mM; pH 7.97	Tris[c] I = 50mM; pH 8.00
n_1	0.91 ± 0.09	0.99 ± 0.01
$\Delta H^o_{1(ITC)}$[a]	−3.77 ± 0.17	−7.47 ± 0.43
$K_{b1(ITC)}$	(1.07 ± 0.68) × 10^7	(3.40 ± 1.20) × 10^6
n_2	0.91 ± 0.09	0.99 ± 0.01
$\Delta H^o_{2(ITC)}$[a]	−4.63 ± 0.26	−10.36 ± 0.45
$K_{b2(ITC)}$	(1.64 ± 0.19) × 10^6	(9.15 ± 0.84) × 10^5

[a] kcal/mol.
[b] Concentration of Tris is double to the ionic strength.
[c] Concentration of Tris is equal to the ionic strength.

than those assayed in this work. One of the assays was carried out in 100 mM HEPES buffer, ionic strength 50 mM and pH 7.6. Comparing with our results, obtained from 50 mM HEPES, ionic strength 50 mM and pH 8.07, the reported formation constants show a higher $\log K_{b1(ITC)}$ value and a lower $\log K_{b2(ITC)}$ than those achieved in this work, probably due to the differences in the experimental conditions. Other reported assay was performed in 100 mM of Tris buffer, ionic strength 50 mM and pH 8.1 and, in this instance, the $\log K_{b2(ITC)}$ reported value agrees with that obtained in this work whereas $\log K_{b1(ITC)}$ is 0.8 units higher. Other ITC assays were carried out at lower buffer concentration and added KNO_3 to keep the ionic strength constant at 100 mM [5].

3.4. Ni/EDMA system

This complexation process has been studied under a lower variety of experimental conditions than the more relevant Ni/His system as shown in Table 3. However, the results reveal that

Table 3
Experimental enthalpies, binding constants and derived thermodynamic formation constants for the interactions of Ni(II) with His and EDMA.

Ni/His (HEPES)			$\Delta H^o_{i(ITC)}$[a]		$\log K_{bi(ITC)}$		α_i[b]				$\log K_i$[c]	
I (mM)	c_{HEPES}	pH	i = 1	i = 2	i = 1	i = 2	His-H	NiHis-H	Ni-OH	$NiHis_2$-H	i = 1	i = 2
25	25	7.85	−2.41 ± 0.05	−3.51 ± 0.05	6.17 ± 0.02	5.49 ± 0.03	26.36	1.00	1.01	1.00	7.85	7.04
50	50	8.04	−2.30 ± 0.07	−3.70 ± 0.19	6.10 ± 0.02	5.67 ± 0.03	16.47	1.00	1.01	1.00	7.67	7.06
50	50	8.12	−2.34 ± 0.20	−3.88 ± 0.05	6.06 ± 0.03	5.62 ± 0.02	13.78	1.00	1.01	1.00	7.57	6.96
100	100	8.15	−2.36 ± 0.21	−4.07 ± 0.18	5.95 ± 0.06	5.60 ± 0.07	12.41	1.00	1.01	1.00	7.49	6.96

Ni/His (Tris)			$\Delta H^o_{i(ITC)}$[a]		$\log K_{bi(ITC)}$		α_i[b]					$\log K_i$[c]	
I (mM)	C_{Tris}	pH	i = 1	i = 2	i = 1	i = 2	His-H	Ni-Tris	NiHis-H	Ni-OH	$NiHis_2$-H	i = 1	i = 2
25	50	8.24	−8.19 ± 0.10	−6.87 ± 0.56	6.14 ± 0.03	5.15 ± 0.01	12.43	40.20	1.00	1.01	1.00	9.09	6.35
50	50	8.07	−6.54 ± 0.15	−5.38 ± 0.52	6.15 ± 0.01	5.07 ± 0.08	15.42	30.63	1.00	1.01	1.00	9.17	6.43
50	100	8.32	−6.47 ± 0.07	−5.18 ± 0.29	5.76 ± 0.02	4.75 ± 0.02	9.17	164.14	1.00	1.02	1.00	9.28	5.89
100	100	8.20	−6.63 ± 0.12	−5.23 ± 0.65	5.72 ± 0.02	4.73 ± 0.02	11.09	126.15	1.00	1.01	1.00	9.30	6.00
100	200	8.32	−6.15 ± 0.21	−8.37 ± 1.32	5.30 ± 0.21	4.40 ± 0.03	8.66	581.41	1.00	1.01	1.00	9.45	5.56

Ni/EDMA (HEPES)			$\Delta H^o_{i(ITC)}$[a]		$\log K_{bi(ITC)}$		α_i[b]		$\log K_i$[c]	
I (mM)	c_{HEPES}	pH	i = 1	i = 2	i = 1	i = 2	EDMA-H	Ni-OH	i = 1	i = 2
20	20	8.08	−2.99 ± 0.72	−3.38 ± 0.82	7.60 ± 0.18	6.20 ± 0.02	87.99	1.01	9.79	8.27
50	50	7.97	−3.46 ± 0.13	−4.61 ± 0.21	7.05 ± 0.03	6.20 ± 0.02	107.67	1.01	9.43	8.40

Ni/EDMA (Tris)			$\Delta H^o_{i(ITC)}$[a]		$\log K_{bi(ITC)}$		α_i[b]			$\log K_i$[c]	
I (mM)	C_{Tris}	pH	i = 1	i = 2	i = 1	i = 2	EDMA-H	Ni-Tris	Ni-OH	i = 1	i = 2
50	50	8.00	−7.47 ± 0.30	−9.65 ± 0.09	6.66 ± 0.19	6.00 ± 0.18	100.41	26.48	1.01	10.43	8.17

[a] kcal/mol.
[b] Values computed from $\log \beta_i$ (Table 1) and Eq. (10).
[c] Values computed from $\log K_{bi(ITC)}$ and Eqs. (12)–(13) and corrected for ionic strength.

Table 4
Overall formation constants determined in this work, β_i, for several side reactions involved in Ni/His and Ni/EDMA complexation systems.

log β_i values		Validation of the β_i values			
Working system	log β_i (I = 0)	Validation system	log K_{bi} (ITC)	log K_i	log $K_{i\ lit}$
Ni/His (HEPES)	log $\beta_{NiHepes}$ = 3.19 ± 0.07	[a]Ni/His (HEPES) (K_1) (this work)	5.91	9.20	9.10
		[b]Ni/His (HEPES) (K_1) (this work)	6.31	8.94	9.10
		[c]Ni/His (HEPES) (K_1) (Ref. [5])	6.19	9.20	9.10
		[d]Ni/EDMA (HEPES) (K_1) (this work)	7.60	10.94	10.88
		[d]Ni/EDMA (HEPES) (K_1) (this work)	7.05	10.87	10.88
Ni/His (Tris)	log $\beta_{Tris+His-}$ = 2.88 ± 0.08	[e]Ni/His (Tris) (K_1) (Ref. [5])	6.38	8.91	9.10
	log $\beta_{TrisNiHis+}$ = 1.74 ± 0.09	[e]Ni/His (Tris) (K_2) (Ref. [5])	5.27	6.93	7.06
Ni/EDMA(HEPES)	log $\beta_{[Ni(EDMA)_2OH]^-}$ = 7.67 ± 0.13	[d]Ni/EDMA (Tris)(K_2) (this work)	6.00	6.40	6.56

[a] pH 7.63, [HEPES] = 50 mM, I = 50 mM.
[b] pH 8.06, [HEPES] = 20 mM, I = 20 mM.
[c] pH 7.50, [HEPES] = 20 mM, I = 100 mM (KNO_3).
[d] See Table 3.
[e] pH 8.23, [Tris] = 25 mM, I = 100 mM (KNO_3).

enthalpies involved are more negative working in Tris buffer than in HEPES buffer and no dependence is observed in log $K_{b2(ITC)}$ with the HEPES concentration whereas log $K_{b1(ITC)}$ diminishes with the increase of buffer concentration.

3.5. Thermodynamic binding constants

To evaluate the quality of the formation constants obtained by ITC, the values achieved in different experimental conditions, $K_{bi(ITC)}$, have been corrected for side reactions (Eqs. (12)–(13)) and ionic strength (Eqs. (3)–(4)). However, the resulting thermodynamic formation constants can be compared with the published reference values only if all the coefficients of side reactions, α_i, can be computed, i.e. when the overall formation constants involved in the secondary equilibria are known (see Table 1).

3.6. Ni/His system

For the assays performed in HEPES buffer, Table 3 shows the α_i values calculated from the data given in Table 1 as well as the thermodynamic formation constants, K_1 and K_2, computed from $K_{b1(ITC)}$ and $K_{b2(ITC)}$ and the suitable α_i values. There is only one significant α value, which corresponds to the interaction of His to H^+, as shown in Table 3. Calculated log K_2 values agree between them and with the reference one, 7.06, shown in Table 1. However, log K_1 values slightly decrease with the increase of the buffer concentration and they are approximately 1.5 units lower than the reference value, 9.10. This difference is attributed to an unquantified interaction between Ni and HEPES buffer, which would affect the first equilibrium between Ni and His (Eq. (1)) but not the second one (Eq. (2)). This interaction has not been reported before but it is not surprising since it is well known that Ni strongly interacts with other amino buffers, such as Tris (see Table 1) and others [12,17,18].

Since only one unknown side reaction is postulated, the coefficient $\alpha_{Ni(Hepes)}$ can be computed from Eq. (12) using the K_1^c value calculated from the thermodynamic constant (Table 1) according to the experimental conditions of the assay carried out using ITC. Thus, the calculated $\alpha_{Ni(Hepes)}$ values were 17.0 ± 0.8, 27.8 ± 1.2 and 44.7 ± 3.6 at HEPES concentration of 25, 50 and 100 mM, respectively. Assuming that Ni binds to HEPES forming only one complex, $NiHEPES^+$, Eq. (10) allows us the calculation of the overall formation constant, log $\beta_{NiHEPES}$, which results in 3.19 ± 0.07. To validate this value, the formation constants obtained in HEPES buffer (in different conditions than those used to derive the log $\beta_{NiHEPES}$ value) have been recalculated embodying the side reaction between Ni and HEPES. The resulting log K_1 values are consistent with the reported value, as shown in Table 4. Moreover, the results obtained in HEPES buffer by Zhang et al. [5] have also been corrected in the same way. Table 4 shows that the log K_1 values computed agree with the one given in the literature.

For the titrations carried out in Tris buffer, apparently, the side reactions of all the species involved in the main equilibria are known. The highest α_i is for the interaction of Ni with Tris which react forming two successive complexes, but also the interaction of His with H^+ is significant. However, as shown in Table 3, the calculated thermodynamic formation constants do not agree between them. The increase in Tris concentration led to an increase in log K_1 but a decrease in log K_2 values. Similar trend is observed from assays using Tris buffer solution reported by Zhang et al. [5]. Comparing the results achieved in this work with those calculated from Ref. [5] there is a good consistency between log K_1 and log K_2 values obtained from both sources when the experimental working conditions are similar (log K_{b1} and log K_{b2} at pH 8.23 and 25 mM of Tris are 6.378 and 5.270, respectively [5]).

The strong dependence of the calculated log K_1 and log K_2 values on the buffer concentration suggests the presence of more than one unquantified side reaction involving the Tris buffer and affecting both equilibria. The simplest hypothesis is the postulation of two concomitant side reactions. The first interaction is the formation of the ionic pair between the protonated Tris and the anionic form of His. The second one is the adduct formation between the $NiHis^+$ and neutral Tris as previously pointed out by Zhang et al. [5] or as suggested for the TAPSO buffer (3-[N-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid) [18]. In the way explained before, the $\alpha_{Tris+His-}$ and $\alpha_{TrisNiHis+}$ values have been computed resulting in 15.64 ± 1.33 and 2.25 ± 0.19, 20.38 ± 1.06 and 4.64 ± 0.23 and 30.37 ± 1.0 and 7.33 ± 0.43 for concentration of Tris of 50, 100 and 200 mM, respectively. Therefore, the $\beta_{Tris+His-}$ and $\beta_{TrisNiHis+}$ values calculated according to equation [10] are 2.88 ± 0.08 and 1.74 ± 0.09, respectively. To validate these values, the Ni-His formation constants given by Zhang et al. [5] have been corrected including the postulated side reactions and the final results are consistent with the reference values as shown in Table 4.

3.7. Ni/EDMA system

A similar study was carried out for the Ni/EDMA system. Table 3 shows the enthalpies, the binding constants obtained in HEPES and in Tris buffers and the stepwise formation constants corrected according to the suitable α_i values (Table 1). In Tris buffer the log K_1 agrees with the reference one, 10.88, but log K_2 is about 2 units higher. In HEPES buffer the results for log K_1 have been corrected according to the Ni-HEPES interaction already evaluated and the calculated log K_1 agrees with the reference value validating again the derived log $\beta_{NiHEPES}$ value (Table 4). However, calculated log K_2

(8.46 and 8.50 for HEPES concentrations of 20 and 50 mM, respectively) are 2 units higher than the expected value, 6.56 (Table 1). This means that there is a side reaction affecting the second equilibrium but not the first one. This fact can be explained only if the species involved in the side reaction is $Ni(EDMA)_2$. As the effect is the same in HEPES than in Tris buffers, the interaction of $Ni(EDMA)_2$ should be related to a species independent of the buffer such as the hydroxyl ion. In the way already described, the coefficient of side reaction for the interaction of $Ni(EDMA)_2$ with OH^-, $\alpha_{[NiEDMA_2OH]^-}$, was calculated from the data obtained in HEPES buffer and the results were 79.5 ± 5.10 and 86.8 ± 5.55 at ionic strengths of 20 and 50 mM, respectively. Thus, assuming the formation of only one species, $[Ni(EDMA)_2OH]^-$, Eq. (10) allows the calculation of $\log \beta_{[NiEDMA_2OH]}$, which is 7.71 ± 0.03. To test the quality of this value, it was used to recalculate the $\log K_2$ value from the assays carried out in Tris buffer at pH 8.0 and ionic strength 50 mM and it results in 6.53. This value confirms the suitability of the calculation approach (Table 4).

4. Conclusions

The ITC derived data for the studied complexation systems, Ni/His and Ni/EDMA, clearly show that the main reaction is closely related to a number of side reactions. These reactions strongly affect the calorimetric quantities resulting from the fitting of the experimental data to the two binding site model, which has been selected according to the nature of the main interaction to be studied. The energetic contribution of the mentioned side reactions can be successfully evaluated when the thermodynamic reference values for several reactions involved in the global process are known. In this work, the global formation constants for the species $NiHEPES^+$, $Tris^+His^-$, $TrisNiHis^+$ and $[Ni(EDMA)_2OH]^-$ have been evaluated for the first time and the values obtained properly validated. Despite the ITC technique leads to the measurement of a non-specific property, the global heat involved in a process, the approach used in this work shows the capability of ITC to face processes of medium complexity, such as those studied here, to evaluate thermodynamic quantities related to the side reactions involved in the global process.

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