Chelating efficiency and mechanisms of interaction of some toxic and biologically important cations with EDTA by isothermal titration calorimetry

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Abstract: Ethylene-diaminetetraacetic acid (EDTA) is the gold standard as a chelating agent in treatment of certain diseases as well as treatment of metal poisoning. Here, we used isothermal titration calorimetry (ITC) as a simple and rapid method for detecting the stoichiometry, binding affinities and mechanism of interactions of EDTA with several toxic and biologically important cations. The aspects of this work will clarify the differences in the interactions of various cations with EDTA. Mono-, bi- and trivalent cations were titrated into EDTA solution under isothermic conditions by using ITC. Weak or no binding patterns were observed with mono- and trivalent cations. Divalent cations can be classified into two groups, high affinity cations as Ca^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+} and $Pb^{2+}$and medium affinity cations as Ba^{2+} and Mg^{2+}. All EDTA-bound cations showed the profiles of tight binding as favorable enthalpic and entropic terms. In contrast, Mg^{2+} showed a different profile by adopting unfavorable enthalpic binding conditions. ITC allows graphical display of the EDTA-cations binding events, so that, the number of cations binding with EDTA, binding affinity and mechanism of interaction can be concluded by visual examination of the binding isotherms. By ITC, we show that EDTA adapts to the binding with cations under highly variable enthalpic and entropic conditions. The ITC experiment not only determines the number of cations interacting with one molecule of EDTA, but also, we can determine the mechanisms of interaction and full thermodynamic parameters in one experiment.

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1. Introduction

While a considerable bulk of research was devoted to EDTA and its associated cations binding, the direct and differential estimation of cations binding with EDTA is not precisely realized until the moment. There is a wide spread use and huge number of applications using the ion chelating properties of EDTA in many areas of life science including chemistry **(1)**, biochemistry **(2,3)**, pharmacology **(4-6)**, dentistry **(7)**, internal medicine **(8)** and other disciplines **(9-13)**. Above all, the premise of using EDTA is widespread in toxicological sciences especially dealing with heavy metal poisoning **(14-17)**. Within all of these verities of disciplines and applications it is widely accepted by custom knowledge that EDTA is a strong chelator. In spite of this inherently accepted statement about EDTA, no one can precisely describe the course of interaction of EDTA with its chelates. Furthermore, it is not well understood whether EDTA binds different cations with similar efficiency or there is variations among the different reactants with it. Moreover, the mechanism of recognition of different cations with EDTA is not well recognized. In this context, we provide here a reliable quantitation of the binding affinity between EDTA and various cations of toxicological, pharmacological and biological importance. Furthermore, we provide the mechanisms of interaction as well as the potential course of binding events during titration of EDTA with different mono-, bi- and trivalent cations. In this study, several questions has been raised to evaluate the exact molecular aspects of EDTA-cations binding. First, what is the binding stoichiometry (number of cations that can bind to one molecule of EDTA)? Second, what is the binding affinity of EDTA with various monovalent and divalent cations? Lastly, what is the proposed mechanism of the binding of EDTA with the tested material?

2.Materials and Methods

Materials:

All chemicals were purchased in highest available grades. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was purchased from Nacalaitesque (Kyoto, Japan). All other chemicals were purchased from Sigma-Adrich (USA).

Preparation of samples

EDTA was dissolved at 100 mM concentrations in a solution of 25 mM HEPES buffer pH 7 and kept at stock solution. Fractions of stock solution were diluted to the experimental concentration by using the same buffer. Substrate solution was made from the same buffer solution to minimize artifacts due to subtle differences in buffer composition. The binding partners of EDTA were dissolved in the same buffer at 2-10 mM concentrations. Stock solutions were kept at 4ºC and diluted immediately before use in ITC titrations to a final concentration of 100 µM EDTA and 2-10 mM of the ligand.

Isothermal titration microcalorimetry

Highly sensitive ITC conditions were set to calorimetric experiments by using VP-ITC (GE Healthcare, Uppsala, Sweden). Feedback-gain mode was set to high. The ITC solutions were thoroughly degassed and loaded into the sample cell. The substrate solution was loaded into the syringe and used to titrate EDTA solution by up to seventy sequential injections. Control experiments were performed by injecting the ligands into the running buffer to determine the heats of dilution. The apparent heat change after each injection was integrated and corrected for the heat of dilution.

Data analysis

The data was fitted to a single-binding site model by non-linear regression analysis to yield the thermodynamic parameters K_a, association constant; ΔH, enthalpy of binding; and n, the stoichiometry of binding. The affinity of the nucleotides to protein is given as the dissociation constant (K_d=1/K_a). The binding entropy contributions were calculated from the equation $\Delta G = \Delta H - T\Delta S$ and $\Delta G = -RT \ln K$, where K is the association constant, T is the absolute temperature, ΔS is the entropy change, and ΔG is Gibb`s free energy change. The theory of binding model and data analysis was previously described **(18,19)**.

3. Results and Discussion

ITC was initially developed as a specialist technique in biophysical studies. However, ITC is now revolved to be a standard binding technique in most fields of biological and molecular sciences. ITC has largely became the method of choice in determining the binding affinity of two interacting partners **(20-25)**. Furthermore, in one experiment we can get the full thermodynamic profile associated with binding. In this work, ITC was carried out for determining the binding parameters as well as the mechanisms of interaction of different cations with EDTA. The typical ITC titrations included addition of the EDTA in the cell and titrated with a cation solution in a time controlled process. So that, if a binding event is likely to occur, the initial titrations will give high exothermic or endothermic peaks. As the titrations proceed, the free EDTA binding sites will be gradually occupied by the ligand giving rise to smaller peaks. In the last injections, as the binding sites are completely occupied, the heat arising from injections are similar to the heat of background. By tracing the ITC exotherms, we can confirm the strength of binding as the free sites will be rapidly depleted by strong binders. In contrast medium interactions produces a characteristic sigmoid-shaped peak, which turns to hyperbolic shape in the presence of weaker cations.

Figures 1 and 2 shows the typical titration of EDTA with various cations. The right panel shows the raw calorimetric data referring to the amount of heat produced following each injection. The left panel shows the integrated amount of heat generated per injection as a function of the molar ratio of ligand to EDTA. The thermodynamic constants of interaction are given in Table 1.

The binding affinity

According to the strength of binding affinity, we can classify the cations into three groups. Group 1(strong EDTA binders) includes cations with dissociation constant (K_d, Table 1, Fig. 1) less than 500 nM and includes Ca^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+} and Pb^{2+}. Within this group, the binding of cations was on the following order Zn^{2+}>Cu^{2+}>Co^{2+}>Ca^{2+}>Pb^{2+}. The dissociation constant is a measure for possible displacement interactions of different cations at their binding sites of EDTA. In the presence of a mixture of the above cations, the major fraction of EDTA is likely to be found in combination with Zn^{2+}. Based on the measured binding affinity, the binding of EDTA with Pb^{2+} is expected to be adversely affected by higher affinity ligands as Zn^{2+}, Mn^{2+}, Co^{2+} or Ca^{2+}. Group 2 includes medium affinity cations as Ba^{2+} and Mg^{2+}. They have K_d value above 1 µM (Table 1, Fig. 2). Comparing the shapes of curves in figures 1 and 2 gives an insight into the strength of binding. The sigmoid character of Ba^{2+} and Mg^{2+} binding isotherms indicate their lower binding strength in comparison with the cations showed in Fig. 1. The third group includes cations showed low or no binding of EDTA as Ce^{3+}, Na^{+}, K^{+}, Mo^{2+}, Cs^{+} and Rb^{+}. These data underscores the role of EDTA in Ce^{3+}, Mo^{2+}, Cs^{+} and Rb^{+} toxicities. Furthermore, the non-detectible binding of EDTA with Na^{+} and K^{+} implies its safety in processes induced by Na^{+} and K^{+} as transcellular

cellular transmission, Na^+/K^+ pumps or neurotransmission. The binding isotherms of this group show flat featureless isotherms from which the binding parameters cannot be accurately measured (Fig. 3).Tracing the thermodynamic peaks during ITC experiments gives us an indication about the progress of interaction between EDTA and cations. A multistep peaks can be observed during titrations e.g. with Cu^{2+}. The first plateau is corresponding to the heat of binding, while the second plateau is corresponding to the vanishing heat of dilution.

Binding enthalpies

With exception of Mg^{2+}, a negative binding enthalpy (negative ΔH) was a regular observation in the measured binding events. The degree of negativity was correlated with the binding affinity as the lowest ΔH was observed with Ca^{2+} (-25.3 kj/mol). In contrast, Mg^{2+} binding showed a strong positive enthalpy of binding (ΔH = 19.3 kj/mol, Fig. 2).

The free energy of binding

The free energy change of binding (ΔG) showed marked differences among the tested cations (Table 1). Furthermore, the enthalpic and entropic components of the free energy shows marked distinctions. The negative value for ΔG indicates that the binding of cations with EDTA is favorable interaction. The negative free energy is also including the binding of Mg^{2+}, that is the positive enthalpic term accompanying Mg^{2+} binding did not alter the overall favorable interaction of Mg^{2+} with EDTA.

The binding entropy

Favorable entropic interactions were observed within most of the detected binding interactions. The binding of Ce^{3+} is accompanied by marked unfavorable entropy (negative sign of $T\Delta S$). Furthermore, the above mentioned unfavorable enthalpy produced by Mg^{2+} binding is accompanied with markedly high entropy, that is about 2-3 folds higher that noticed during the binding of the other cations with EDTA (Table 1).

Mechanism of interaction of different cations with EDTA

EDTA is a multivalent ligand with four carboxylates and two amino groups that shares in binding (Fig. 4). With exception of Mg^{2+}, all EDTA-bound cations produced negative interaction enthalpies. Hydrogen bonds and van der Waals interactions are often considered to be the major source for negative enthalpies. The analysis of thermodynamic profiles of EDTA-cations interactions reveals a characteristic feature of strong and stable complexes. The negative value of ΔH (favorable enthalpic changes) indicates the proper placement and formation of hydrogen bonding. Furthermore, the positive value of $T\Delta S$ means favorable entropic changes. The favorable enthalpic and entropic terms are indicating tight binding. Hydrophobic interactions are not expected to share in the EDTA-cations recognition as these interactions are usually associated with small favorable enthalpic changes and very high positive entropic changes. The mechanism by which EDTA binds metals is a subject of debate **(26)**. Several models discussed this mechanism with each author is either supporting the role enthalpy or entropic contributions in metal chelates mechanisms **(27,28)**. In this context, our data provides the flexibility of EDTA compounds to adapt the binding of several cations under variable enthalpic and entropic conditions. The findings provided here highlight the ability of EDTA to maintain complexes under variable entropic conditions (either favorable or unfavorable) as well as variable enthalpic conditions (either favorable or unfavorable).

Table 1. The thermodynamic constants obtained by isothermal titration calorimetry for the association of different cations with EDTA. The one set of sites binding model is used for fitting the data. (n=3)

Substrate	n	K_d	ΔH	ΔG	$T\Delta S$
		(nM)	(kj/mol)	(kj/mol)	(kj/mol)
Ca^{2+}	0.95 ± 0.1	100	-25.3± 2	-39.3	14 ± 1.1
Co^{2+}	0.95 ± 0.1	84	-15± 1.2	-39.7	25.2 ± 2.3
Zn^{2+}	0.95 ± 0.08	42	-18.6± 1.6	-41.4	22.8 ± 1.9
Mn^{2+}	1.1 ± 0.15	55	-0.52 ± 0.05	-4.8	5.3 ± 0.2
Pb^{2+}	1.1 ± 0.12	450	-10.8 ± 1	-34	23 ± 2.2
Ba^{2+}	1.07 ± 0.08	1082	-18 ± 1.5	-27.9	9.8 ± 0.5
Mg^{2+}	1.02 ± 0.05	1680	19.3 ± 1.8	-32.4	51.8 ± 4
Ce^{3+}	0.2 ± 0.1	504960	-1.75 ± 0.8	-18.5	-16 ±
Na^+, K^+, Mo^{2+}, Cs^+, Rb^+	ND	ND	ND	ND	ND

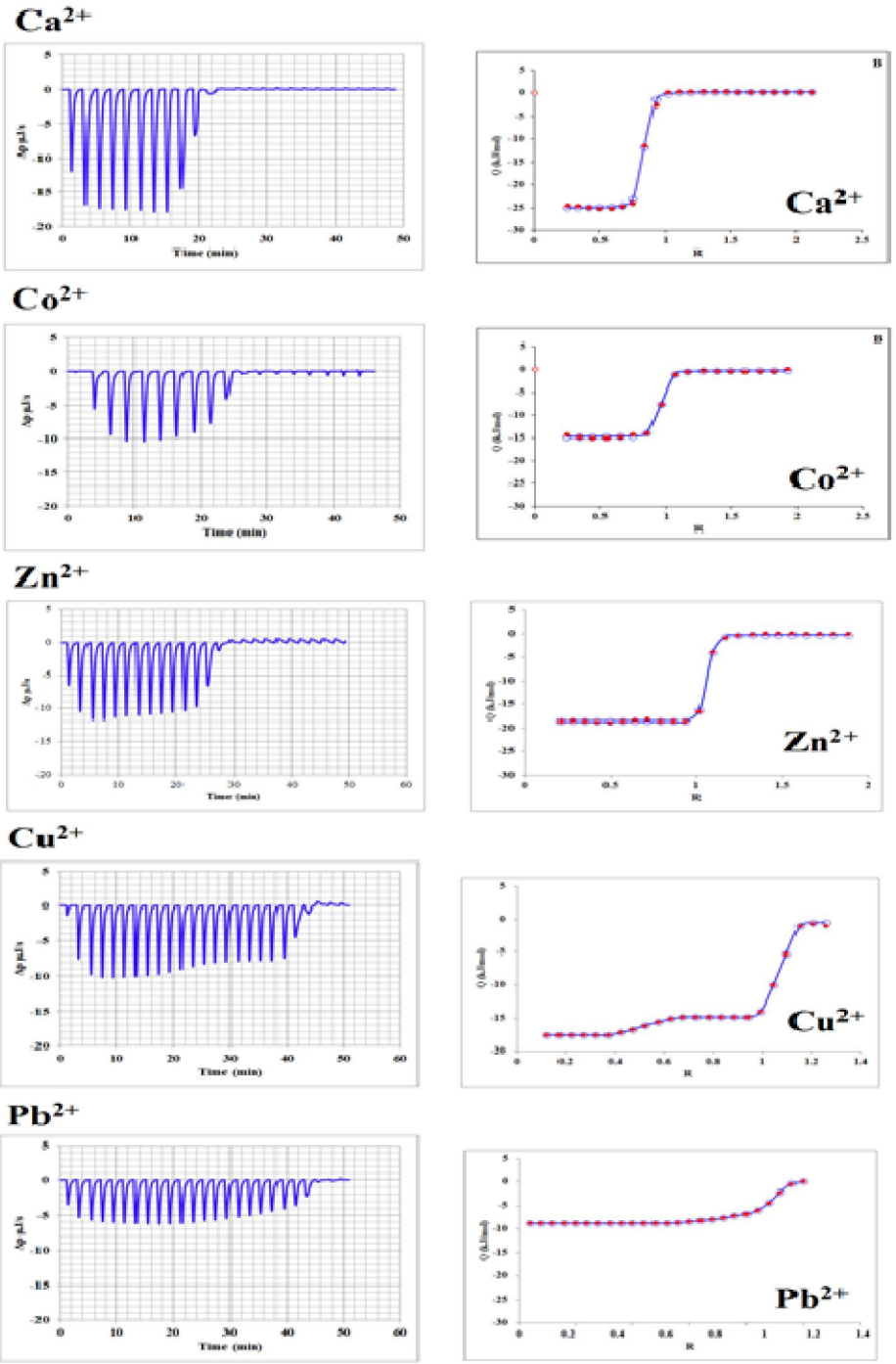

Fig. 1

Fig. 1: Representative ITC profiles of the binding of cations to free EDTA. The left panels show the raw data heat changes while The right panel shows the integrated binding isotherms as a function of the molar ratio of cation to EDTA.

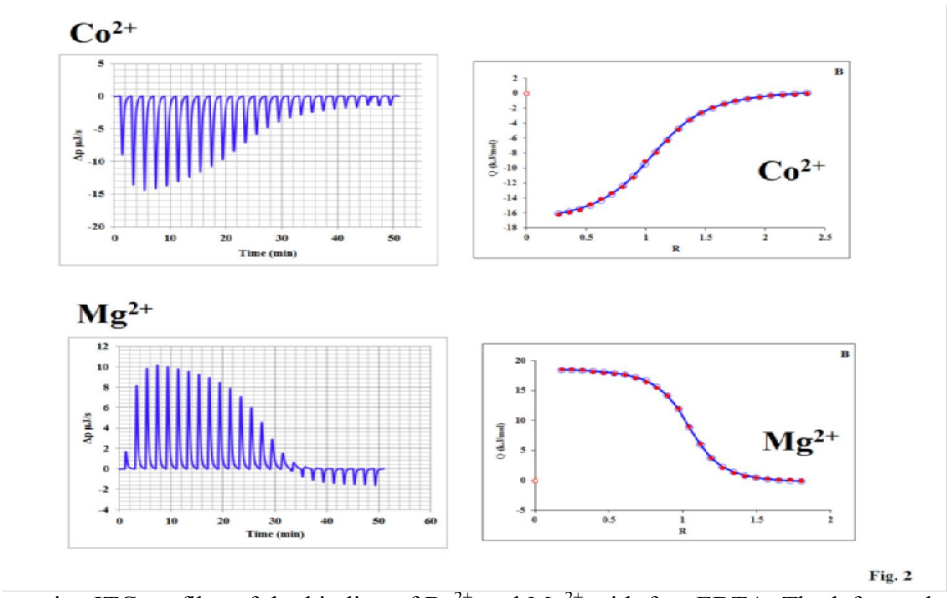

Fig. 2: Representative ITC profiles of the binding of Ba^{2+} and Mg^{2+} with free EDTA. The left panels show the raw data heat changes while The right panel shows the integrated binding isotherms as a function of the molar ratio of cation to EDTA.

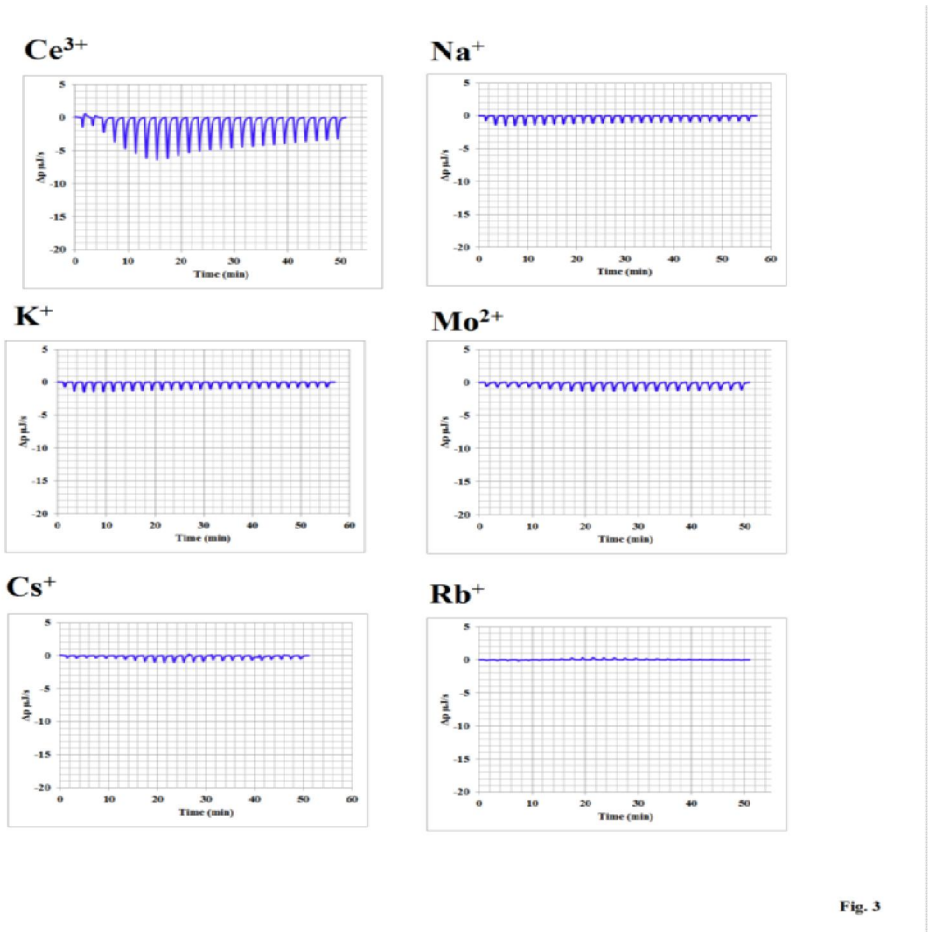

Fig. 3: The raw data heat changes associated with cations showing weak binding with EDTA

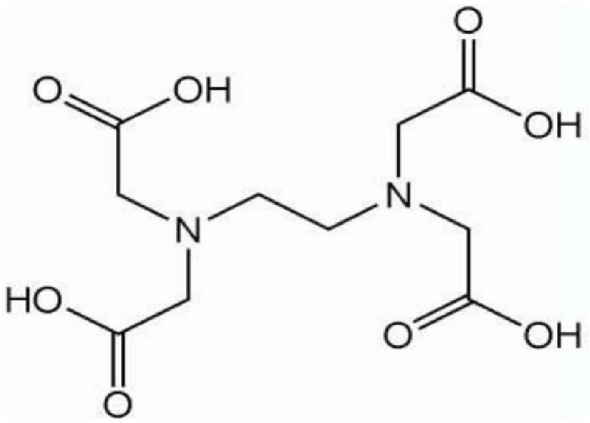

Fig. 4: Structure of EDTA

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