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Energetics of Ca^{2+}–EDTA interactions: calorimetric study

Yuri V. Griko*

Department of Biology, The Johns Hopkins University, Baltimore, MD 21218, USA

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Abstract

The interaction between Ca^{2+} and EDTA has been studied using isothermal titration calorimetry to elucidate the detailed mechanism of complex formation and to relate the apparent thermodynamic parameters of calcium binding to the intrinsic effects of ionization. It has been shown that Ca^{2+} binding to EDTA is an exothermic process in the temperature range 5–48°C and is highly dependent on the buffer in which the reaction occurs. Calorimetric measurements along with pH-titration of EDTA under different solvent conditions shows that the apparent enthalpy effect of the binding is predominantly from the protonation of buffer. Subtraction of the ionization effect of buffer from the total enthalpy shows that the enthalpy of binding Ca^{2+} to EDTA is -0.56 kcal mol^{-1} at pH 7.5. The ΔH value strongly depends on solvent conditions as a result of the degree of ionization of the two amino groups in the EDTA molecule, but depends little on temperature, indicating that the heat capacity increment for metal binding is close to zero. At physiological pH values where the amino groups of EDTA with $pK_a = 6.16$ and $pK_a = 10.26$ are differently ionized, the coordination of the Ca^{2+} ion into the complex leads to release of one proton due to deprotonation of the amino group having $pK_a = 10.26$. Increasing the pH up to 11.2, where little or no ionization occurs, leads to elimination of the enthalpy component due to ionization, while its decrease to pH 2 leads to its increase, due to protonation of the two amino groups. The heat effect of Ca^{2+}/EDTA interactions, excluding the deprotonation enthalpy of the amino groups, i.e. that associated with the intrinsic enthalpy of binding, is higher in value ($\Delta_b H^o = -5.4$ kcal mol^{-1}) than the apparent enthalpy of binding. Thus, the large ΔG value for Ca^{2+} binding to EDTA arises not only from favorable entropic but also enthalpic changes, depending on the ionization state of the amino groups involved in coordination of the calcium. This explains the great variability in ΔH obtained in previous studies. The ionization enthalpy is always unfavorable, and therefore dramatically decreases Ca^{2+} affinity by reduction of the enthalpy term of the stability function. The origin of the enthalpy and entropy terms in the stability of the Ca^{2+}–EDTA complex is discussed.

Keywords: EDTA; Calcium; Thermodynamic parameters; Interactions

* Tel.: +1-410-516-6037; fax: +1-410-516-5213.

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1. Introduction

Ethylenediaminetetraacetate (EDTA), the polyamine polycarboxylate chelator [1] is known for its capacity to form complexes with metal ions. Characterization of the interaction of this chelating agent with divalent metal ions serves as a simple model to gain insight into both the mechanism and specificity of the process of interaction of metals with the specific metal binding site [2]. The growing applications of EDTA and its derivatives in biotechnology and in medicine as a therapeutic chelating agent raise specific interest in the energetics underlying the molecular mechanism of the chelating process [3].

Although the stability of EDTA complexes is mainly due to the entropies of metal binding, which are large and positive, the enthalpy terms make a contribution to the stability and there are significant variation in these. Previous studies have revealed that EDTA binds calcium with variable enthalpy: from -12 kcal mol^{-1} to $+1.9$ kcal mol^{-1} [4–7]. The broad spectrum of enthalpy values suggests that different solvent factors may contribute to the overall enthalpy of binding, masking the intrinsic binding effect. In order to describe the complete character of this biologically relevant interaction and gain insight into the mechanism of complex formation, we have obtained the intrinsic solution effects of Ca^{2+} binding under various conditions using precision titration calorimetry. The results of this study explain the observed variations in the measured thermodynamic parameters of Ca^{2+}–EDTA interactions.

2. Materials and methods

Reagent grade standard solutions of 1 M of $CaCl_2$ and EDTA were purchased from Ricca chemical company (Arlington, TX). Solutions of EDTA were prepared by dissolving the free acid in buffer solutions demetallized using Chelex-100 column and adjusting the pH to 7.5 with 1 M HCl. The calorimetric experiments were performed with the OMEGA titration calorimeter designed by MicroCal, Inc. (Northampton, MA). After degassing, the solutions were loaded into the calorimetric cell using a 2.5-ml syringe previously treated with 0.1 M HCl and washed with decalcionated buffer. Analysis of the calorimetric binding isotherm shows that the binding of Ca^{2+} to EDTA is a too cooperative process to apply a non-linear least-squares fitting algorithm for determination of the binding constant. In order to obtain an accurate K_b for such a tight binding reaction, ($K > 10^7$ M), the concentration of reactants was reduced. The concentration of EDTA (c_o) was 0.5 mM to keep the value of $c = K_b c_o$ within the range 5–500 [8]. Under these conditions the binding constant and thus ΔG, the enthalpy (ΔH) and the number of binding sites (n) can be determined from the calorimetric isotherm. The EDTA solution was titrated with sequential additions of 5 ml of 5 mM $CaCl_2$ solution. The heat effects associated with dilution of the EDTA were obtained by injection of the $CaCl_2$ into buffer and subtracted from the total heat effects. The ionization enthalpy of the buffers used in this study are: 11.35 kcal mol^{-1} for Tris, 8.76 kcal mol^{-1} for Imidazole, 5.32 kcal mol^{-1} for MOPS, and 2.74 kcal mol^{-1} for PIPES [9].

pH-titration was performed at different constant temperatures using automatic syringes filled with 1 M HCl (downscale titration) and 1 M NaOH (upscale titration). The reagent is added by step injections of 10 μl at a rate of 1 μl min^{-1} to keep the system under equilibrium. The data were corrected for the volume change.

3. Results

3.1. Enthalpy of the Ca^{2+} binding to EDTA

Fig. 1 shows calorimetric titration of EDTA with $CaCl_2$ in a solution of 10 mM Tris–HCl at pH 7.5 at 25°C. The binding of Ca^{2+} to EDTA in this solution is characterized by an exothermic heat effect with enthalpy $\Delta_b H(\text{cal}) = -11.97$ kcal mol^{-1}. The molar ratio of EDTA/Ca^{2+} was nearly 1 at saturation indicating that there are no other effects that might interfere with direct binding to EDTA.

Fig. 2 shows titration isotherms obtained at

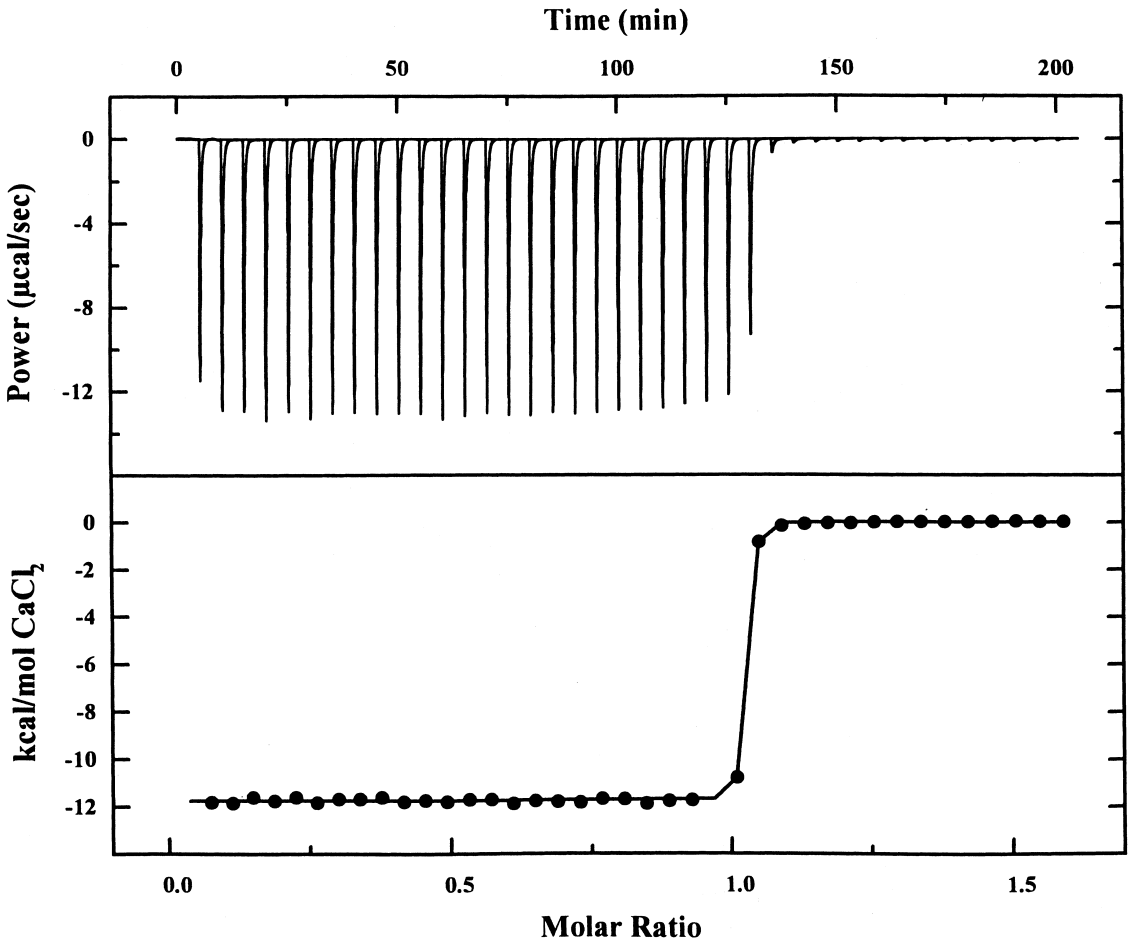

Fig. 1. Calorimetric titration isotherm of the binding interaction between EDTA and Ca^{2+} in 10 mM Tris–HCl buffer (pH 7.5) at 25°C. Forty 5-ml volume of 5 mM $CaCl_2$ were injected into the calorimeter cell containing 0.5 mM EDTA. The top panel shows power in mcal s^{-1} vs. time. The bottom panel shows the integrated areas corresponding to each of the injections, normalized per mole of $CaCl_2$ injected, vs. the molar ratio of EDTA in the cell after each injection.

25°C in three buffers with different ionization enthalpy (Tris, MOPS and PIPES). The binding enthalpy at constant ionic strength, temperature and pH = 7.5 was found to vary depending on the buffer. The enthalpy was always exothermic and becomes more negative as the buffer ionization enthalpy increases (Tris > MOPS > PIPES). Values of the thermodynamic parameters obtained under different solvent conditions are listed in Table 1.

Fig. 3 presents plots of the calorimetrically determined apparent binding enthalpy against the ionization enthalpy of the buffers at different pH values. The intercept gives the enthalpy of binding $\Delta_b H'^o = -0.56$ kcal mol^{-1} corrected for the ionization enthalpy of the buffers. This value represents the enthalpy of Ca^{2+} binding which includes the ionization enthalpy of EDTA, but excludes the buffer contribution. The slope of the linear regression that reveals the number of protons released or absorbed upon binding, shows that approximately one proton ($n = 1.17$) is released by EDTA and taken up by the buffer at pH 7.5. Decreasing the pH from 7.5 to 6.2 leads

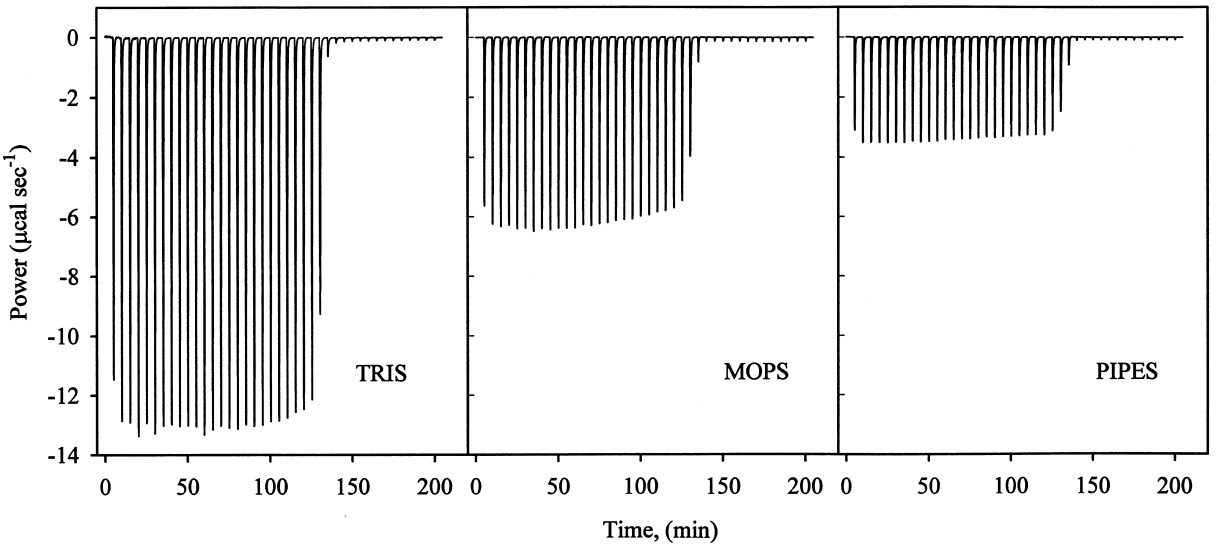

Fig. 2. EDTA/Ca^{2+} titration isotherms in buffers with different ionization enthalpies (10 mM Tris–HCl; 10 mM MOPS; 10 mM PIPES) at 25°C, pH 7.5.

to an increase in the degree of ionization, indicating that an additional site on EDTA was partially deprotonated on Ca^{2+} binding.

To obtain information about the ionizable groups that may affect the energetics of Ca^{2+} binding to EDTA, pH titration of EDTA has been performed in a range of pH values from 2 to 11.2. Fig. 4 shows that the three main regions of pH where the changes in the degree of protonation occur can be separated. In the range of pH from 2 to 3 the significant changes in protonation are presumably due to titration of the carboxyl groups of EDTA ($pK_1 = 1.15$; $pK_2 = 1.15$; $pK_3 = 2.12$; $pK_4 = 2.57$) [6]. There are also measurable changes in ionization over the pH ranges 5–7 and 9.5–11.5, which are related to ionization of the two ethylendiamine groups in EDTA.

In order to estimate the intrinsic enthalpy of binding, the calorimetric measurements have also been performed at pH 11.2, where all the groups of EDTA involved in Ca^{2+} binding are ionized and do not contribute to the ionization enthalpy. The heat effect of calcium binding related to the intrinsic enthalpy of binding is found also to be exothermic but higher in value $\Delta_b H^\circ = -5.4$ kcal mol^{-1} than the apparent enthalpy of binding ($\Delta_b H'^\circ = -0.56$ kcal mol^{-1}). This means that the ionization enthalpy of the amino groups of

Table 1
Thermodynamic parameters of Ca^{2+} binding to EDTA in different buffer solutions at 25°C

Buffer	pH	ΔH (cal) (kcal mol^{-1})	K'_b (M^{-1})	n
TRIS	7.5	-11.97 ± 0.15	$(1.98 \pm 0.6) \times 10^8$	1.0
MOPS	6.25	-6.38 ± 0.15	$(2.26 \pm 0.4) \times 10^6$	1.01
	7.5	-5.73 ± 0.15	$(2.35 \pm 0.6) \times 10^8$	0.99
PIPES	6.25	-3.50 ± 0.15	$(3.12 \pm 0.4) \times 10^6$	0.95
	7.5	-3.32 ± 0.15	$(1.04 \pm 0.6) \times 10^8$	1.01
Imidazole	6.25	-11.15 ± 0.15	$(1.84 \pm 0.4) \times 10^6$	0.93

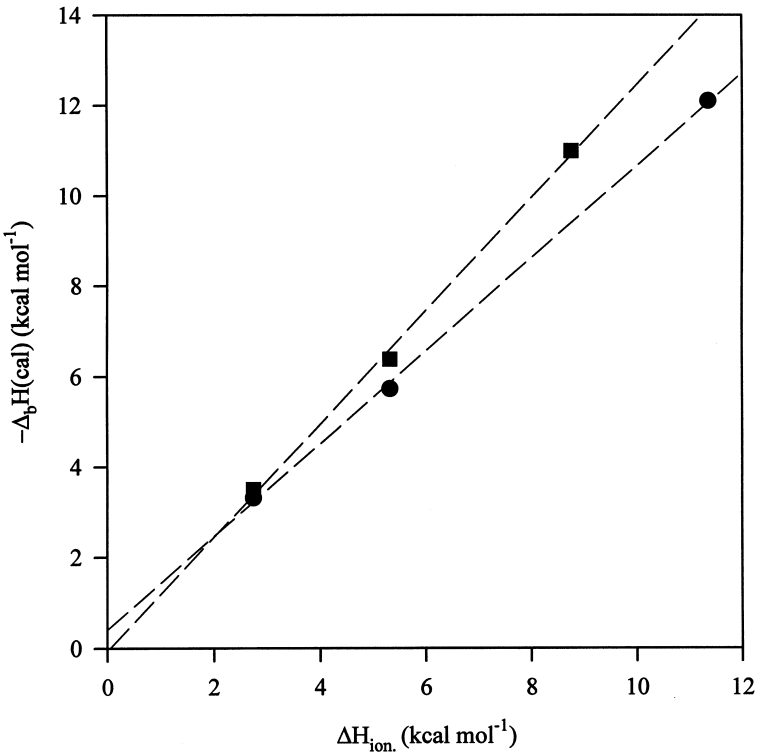

Fig. 3. Enthalpy change on binding vs. the ionization enthalpy of the reaction in different buffers at pH 6.2 (—■—) and pH 7.5 (—●—). The points represent titration in 10 mM Tris–HCl; MOPS; PIPES; Imidazole. The ionization enthalpy of the buffers used are: 11.35 kcal mol^{-1} for Tris, 5.32 kcal mol^{-1} for MOPS, 2.74 kcal mol^{-1} for PIPES, and 8.76 kcal mol^{-1} for Imidazole [9]. The dashed lines are linear regressions of the data.

EDTA which ionize upon calcium binding contribute positively to the binding enthalpy, being close to 4.84 kcal mol^{-1}.

Thus, the presence of protonatable groups in EDTA gives a significant contribution to the binding energetics. Because the pK_a values of these groups are generally a function of the structural state and local environment in metal binding site, changes in pH, ionic strength, and temperature will also effect the thermodynamic parameters of Ca^{2+}–EDTA interaction.

3.2. Temperature dependence of $\Delta_b H'^o$ and K'_b

The enthalpy of binding was measured at different temperatures and buffer conditions to evaluate the heat capacity change upon binding and stability of the Ca^{2+}/EDTA complex. Fig. 5a demonstrates that binding enthalpy is nearly temperature independent over the temperature range 5–50°C. Linear regression of the experimental data indicate that formation of the EDTA/Ca^{2+} complex is characterized by a small if not zero $\Delta_b C_p$ that varies slightly when measured in different buffers (0.013 kcal K^{-1}mol^{-1} in Tris; 0.015 kcal K^{-1}mol^{-1} in MOPS; ~0 kcal K^{-1}mol^{-1} in PIPES). The temperature dependence of the apparent affinity constant (K'_b) obtained from ITC titration curves measured at different temperatures (Table 2, Fig. 5b) is in good agreement with that estimated using the heat capacity increment and the affinity constant of the binding determined at $T_o = 25$°C according to the following equation:

$$\ln K'_b(T) = \ln K'_b(T_o) - \frac{\Delta_b C_p}{R \cdot \ln\left(\frac{T_o}{T}\right)}$$

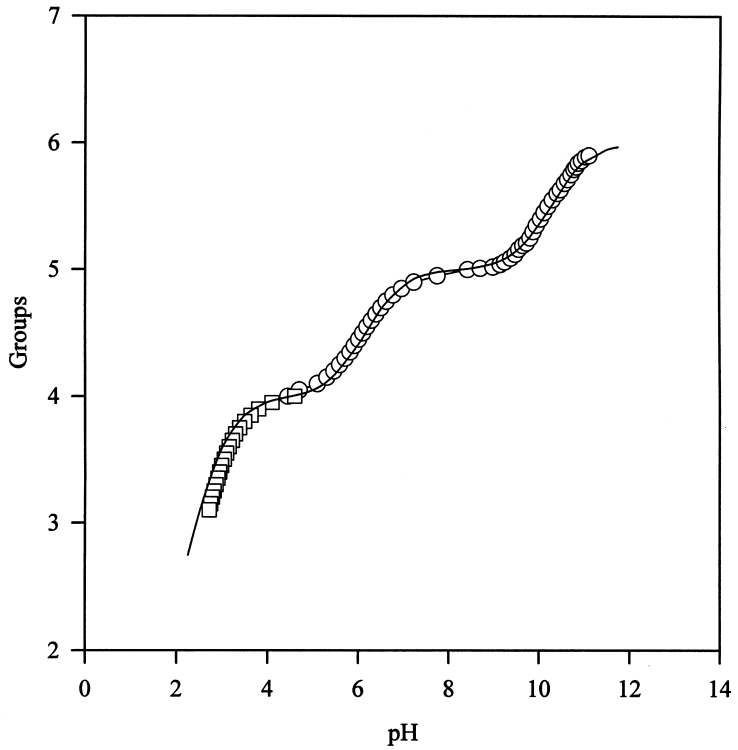

Fig. 4. pH-titration curve of EDTA at 25°C corrected for the titration of water. The square symbols (—□—) present down-scale titration with 1 M HCl. The circle symbols (—○—) show up-scale titration with 1 M NaOH. The points have been obtained by step injections of 10 ml of 1 M NaOH/HCl. EDTA concentration is 10 mM. The solid lines calculated for the pK values given by Marini et al. [6].

$$- \frac{\left(\Delta_b H'^{o}(T_o) - T_o \cdot \Delta_b C_p\right)}{R} \cdot \times \left(\frac{1}{T} - \frac{1}{T_o}\right) \quad (1)$$

The temperature dependence of the change in Gibbs free energy for the binding reaction $\Delta_b G'^{o} = -RT \ln K'_b$ is given as follows

$$\Delta_b G'^{o}(T) = \Delta_b G'^{o}(T_o) - \Delta_b C_p \cdot T \cdot \ln\left(\frac{T}{T_o}\right) + \frac{\Delta_b C_p - \Delta_b S'^{o}(T_o)}{T - T_o} \quad (2)$$

where $\Delta_b G'^{o}(T_o)$ is at $T_o = 293.15$ K, $\Delta_b C_p \sim 0$ kcal $K^{-1}mol^{-1}$ is constant, and $\Delta_b S = -\frac{\partial(\Delta_b G'^{o})}{\partial T}$.

4. Discussion

The aim of this study was to identify the key factors that contribute to the enthalpy term and cause the variability of the measured thermodynamic parameters of the calcium binding to EDTA. The results obtained show that the reason for the variation in these ΔH values is that formation of the complex takes place through proton displacement from EDTA and absorbance of this proton by buffer.

The binding reaction, which involves ionization, may be presented as:

$$EDTA^x + Ca^{2+} \Leftrightarrow EDTA^y \cdot Ca^{2+} + \Delta n \cdot H^+ \quad (3)$$

for the ionization upon complex formation, and

$$Buffer + \Delta n \cdot H^+ \Leftrightarrow Buffer \cdot \Delta n \cdot H^+ \quad (4)$$

Table 2
Enthalpy of Ca^{2+} binding to EDTA measured by ITC at different temperatures and buffer solutions[a]

Buf.\Tem.	5°C	15°C	25°C	48°C
TRIS	11.76 ± 0.15	11.9 ± 0.15	12.1 ± 0.15	12.4 ± 0.15
MOPS	5.2 ± 0.15	5.4 ± 0.15	5.7 ± 0.15	5.9 ± 0.15
PIPES	3.24 ± 0.15	3.24 ± 0.15	3.24 ± 0.15	3.2 ± 0.15

[a] $\Delta_b H$ (cal) is in kcal · mol^{-1}

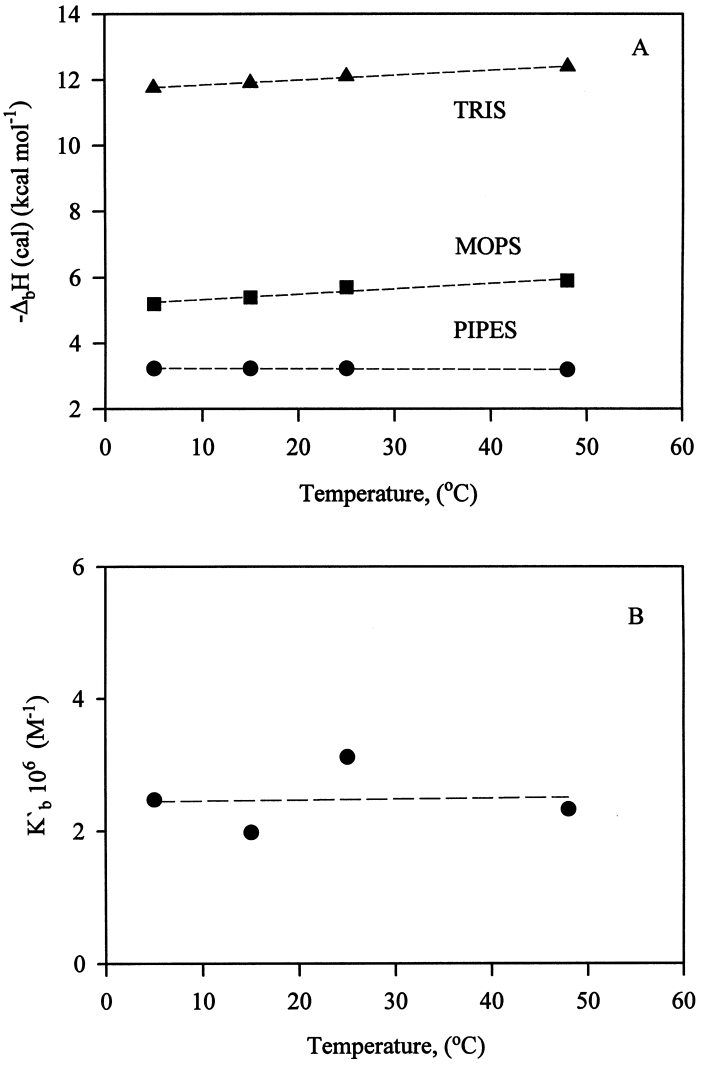

Fig. 5. (a) Plot of ΔH vs. temperature for Ca^{2+}–EDTA binding in different buffer solutions. The dashed lines represent the linear regression of the data. The slope of the best-fit line gives the binding heat capacity change of $\Delta C_p = 0.013$ kcal mol^{-1} · K in 10 mM Tris; $\Delta C_p = 0.015$ kcal mol^{-1} · K in MOPS; $\Delta C_p \sim 0$ kcal mol^{-1} · K in PIPES. (b) Temperature dependence of the apparent affinity of Ca^{2+} to EDTA obtained from ITC titration curve, measured at 25°C in 10 mM PIPES buffer (pH 6.25).

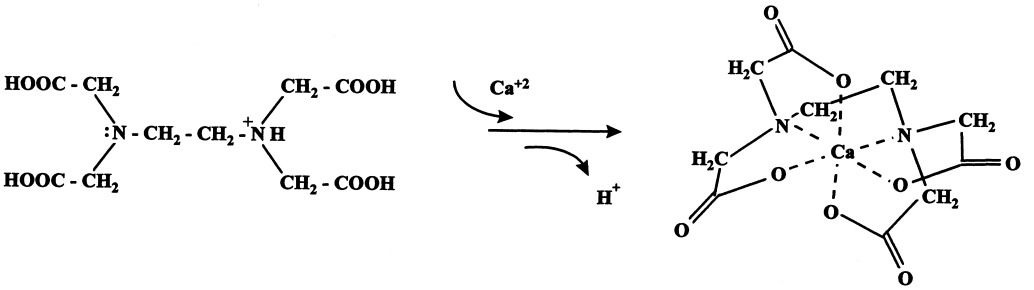

Fig. 6. Schematic presentation of the rearrangement in EDTA structure induced by calcium binding through proton displacement from the nitrogen site.

for the buffer; x and y are the number of charged ions; Δn is the change in protonation ($\Delta n = x - y$).

The apparent enthalpy [$\Delta_b H$(cal)] as obtained from ITC experiments includes the intrinsic enthalpy of binding ($\Delta_b H^o$) and the enthalpy associated with ionization of EDTA (ΔH^{ion}_{EDTA}) and buffer (ΔH^{ion}_{buf}):

$$\Delta_b H(\text{cal}) = \Delta_b H^o + \Delta H^{ion} \cdot F \quad (5)$$

where $\Delta H^{ion} = \Delta H^{ion}_{EDTA} + \Delta H^{ion}_{buf}$ is the ionization enthalpy, and $F = \frac{K \cdot a}{1 + K \cdot a}$ is its fractional degree of saturation. Thus the total ionization enthalpy is the difference between the ionization enthalpy of the EDTA and the ionization enthalpy of buffer. Thus the thermodynamic parameters of calcium binding obtained in buffers with high ionization enthalpy have to be corrected to obtain intrinsic binding characteristics.

Fig. 6 presents schematically the formation of the Ca^{2+}–EDTA complex. The EDTA anion can be considered as a hexadentate ligand consisting of six atoms available for coordination: each of the acetic groups can give up a proton, yielding an anion with net charge (-4). In addition, each of the two nitrogens has a pair of non-bonding electrons. In the complex EDTA acts as a hexadentate unit by wrapping itself around the metal ion with four oxygen atoms and two nitrogen atoms arranged approximately octahedrally. The pK_a values of the four carboxyl and two amino groups for EDTA are $pK_1 = 1.15$, $pK_2 = 1.15$, $pK_3 = 2.12$, $pK_4 = 2.57$, $pK_5 = 6.16$, $pK_6 = 10.26$ [6,10], so at neutral pH, all carboxyl groups are deprotonated, as is the one amino group with pK_a 6.16. Only the second amino group remains protonated. Since in EDTA all donor groups are generally coordinated to the metal ion, formation of the complex takes place through proton displacement from the nitrogen site resulting in a significant increase in the stability of the complex. It is obvious that release of a single proton observed upon complex formation in solutions with pH 7.5 is associated with the ionization the amino group with $pK_a = 10.26$.

The maximum affinity for Ca^{2+} is when all six groups involved in its coordination in the complex are ionized. The association constant under these conditions (K) was found to be 3.89×10^{10} M^{-1} ($\log K = 10.59$) [10]. The binding enthalpy under such solvent conditions represents the intrinsic enthalpy of Ca^{2+}–EDTA interactions (ΔH^o). Its negative value (-5.4 kcal mol^{-1}) means that this process is slightly favorable enthalpically: thus under conditions where amino groups involved in coordination of calcium ion in Ca^{2+}–EDTA complex are ionized, binding is significantly affected by enthalpic parameters. At pH 7.5 where only a small proportion of the total EDTA will be present as LH^{6-}, its apparent affinity will be greatly reduced and will decrease as the solution is made more acidic. The ratio of the true (K) and apparent binding constants (K') which is a function of pH and ionic strength is given according to [17] by:

$$\frac{[K]}{[K']} = 1 + K_1[H^+] + K_1K_2[H^+]^2$$

$$+K_1K_2K_3[H^+]^3 + K_1K_2K_3K_4[H^+]^4 + K_1K_2K_3K_4K_5[H^+]^5 + K_1K_2K_3K_4K_5K_6[H^+]^6 = \frac{[Ca^{2+}_{unc}]}{[EDTA^{6-}]} \quad (6)$$

where K_1, K_2, K_3, K_4, K_5, K_6 are stepwise protonation constants of EDTA.

The apparent affinity of calcium obtained from calorimetric measurements in different buffers at pH 7.5 was found to be $(1.98–5.0)\cdot 10^8$ M^{-1}, which appears to be in good agreement with the previously obtained value of $\sim 10^{7.7}$ M^{-1} [10,18]. The affinity drops to $K' = 10^6$ M^{-1} when the pH decreases to 6.25 because the contribution from the fifth term becomes significant. The average value of the K calculated from the measured values of K' is nearly 4.9×10^{10} M^{-1}.

The intrinsic enthalpy of formation of the Ca^{2+}–EDTA complex, which does not include the ionization enthalpy of EDTA, may be considered as primarily due to the formation of the coordinated bonds and a dehydration effect. Substitution of water binding to the amine and carboxyl groups is driven by the stronger Ca^{2+}–N and Ca^{2+}–O bonds. The strength of the bond is determined by the positions of the atoms in EDTA that are optimized relative to the centered metal ion [1].

Solvation effects represent interactions between the metal ion and water molecules which are displaced when the complex is formed. The largest contribution to the enthalpy term is expected from the dehydration of the calcium ion and the acetate groups [1]. The dehydration enthalpy of calcium estimated from comparison of heat effect of dissolving of its two forms: dehydrated $CaCl_2$ form, and hydrated one ($6H_2O\cdot CaCl_2$), gives $\Delta H = 25$ kcal mol^{-1}. However the enthalpy value is much higher ($-324.7 \div -589.5$ kcal mol^{-1}), when calculated using theoretical models [1,11]. To estimate the contribution of the carboxyl and amino groups to the stability of the complex, it is interesting to compare ΔH values for the EDTA complexes with those for the ethylene diamine chelates, which only have two coordinating amino groups. The results obtained for these two chelates show that EDTA enthalpies for metals of the first transition series is only slightly less than the ethylenediamine values [12,7,13]. This suggests that the contribution to the total enthalpy of reaction from acetate groups is expected to be small and endothermic, approximately 1 kcal mol^{-1} [14]. At the same time much larger entropy changes for the EDTA complexes (42–57 e.u. against 3–6 e.u.) as compared to the ethylenediamine complexes indicate that dehydration of the acetate groups in the EDTA ion (and formation of coordinated bonds) is considerable.

It has been shown that hydration/dehydration of different atoms in interacting molecules is closely associated with the heat capacity change upon binding [15,19]. ΔC_p can be estimated from enthalpy of calcium binding to EDTA measured at different temperatures, as well as from the differences in solvent-accessible surface area upon binding calculated from the crystal structure of the complex and its components. The latter is not presented in this study. It is expected that the accessible surface area (ASA) in the complex, when the charged groups are involved in formation of coordinated bonds with Ca^{2+}, and thereby dehydrated, is less then in the free state when they are largely exposed to solvent. The heat capacity of polar hydration is in general negative and small in value [15]. The results presented here for the Ca^{2+}/EDTA complex, where most of the change is contributed by the polar groups, clearly show that its formation is accompanied by a small, almost zero change in heat capacity. It is known that the enthalpy and entropy of polar group hydration is large and negative [15]. Both these terms will contribute positive to the stability of the complex as result of dehydration of the EDTA and Ca^{2+} molecules. The low and negative enthalpy of the binding reaction found in this study means that the unfavorable enthalpy of dehydration is completely canceled by favorable formation of the coordinated bonds in the complex. Since no significant contribution from nonpolar groups can be expected, the small heat capacity increment of binding means that nega-

tive enthalpy of dehydration of both reactants is canceled upon complex formation or they were initially small.

The increase in entropy upon complexation is not obvious since binding leads to greater order in complex and both components must be dehydrated. While the expected large enthalpy of dehydration may be canceled by favorable formation of the coordinated bonds in the complex, it is not the same for the entropy term. Four major sources of the large entropy change can be expected: (a) dehydration; (b) solvent ordering effect; (c) formation of coordination bonds; (d) potential deformation in the EDTA; and (e) the cratic entropy. The metal ions interact strongly with solvent molecules in aqueous solution to form aquo complexes: Ca^{2+} ion in aqueous solution is surrounded by six octahedrally disposed water molecules. This interaction may result in loss of the ordered outer-sphere water molecules on complexation of the metal ion with EDTA and may account for the positive entropy change. The importance of the solvent ordering effect has been demonstrated in experiments with differently charged ligands [16].

4.1. Proposed mechanism of Ca^{2+} binding to EDTA

The fact that the enthalpy and affinity of the Ca^{2+}–EDTA complex depend on the ionization state of nitrogen atoms of the EDTA anion, allow to suggest that the initial interactions upon formation of the Ca^{2+}/EDTA complex may be through the interaction with the two carboxyl groups that are close to the nitrogen atom having $pK_a = 6.16$. At pH 7.5 this amino group is mostly ionized and available for formation of the third coordinated bond with Ca^{2+}. It would be energetically much more unfavorable to initiate interaction with coordination of only the two acetate groups that are closely located to the protonated amino group. After the three metal coordinated bonds have formed, the probability of deprotonation of the amino groups with $pK = 10.26$ is increased, resulting in stepwise formation of the last coordinated bonds. For this step, metal complexion is only modestly affected by the degree of protonation.

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