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Ca^{2+} and Mg^{2+} bind tetracycline with distinct stoichiometries and linked deprotonation

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Abstract

Tetracycline depends on divalent metal ions for its biological function, but its multiple ionization states, conformations, and tautomers at varying solution conditions complicate its ion-binding equilibria, and the stoichiometry of the biologically relevant Ca^{2+} or Mg^{2+} complexes has not been clear. Isothermal titration calorimetry was used in the present work to study Ca^{2+} and Mg^{2+} binding to tetracycline. The two metal ions bind with distinct stoichiometries, one Ca^{2+} per tetracycline and one Mg^{2+} per two tetracyclines, and with differing dependence on solution conditions, indicating that these two ions bind TC differently. An endothermic process accompanies ion binding that is proposed to reflect conformational changes in tetracycline. The results identify conditions that limit the distribution of species and may facilitate structural study.

Keywords: Isothermal titration calorimetry; Tetracycline metal ion interaction and stoichiometry; Linked deprotonation; Intrinsic binding enthalpy; Conformational change

1. Introduction

The tetracyclines are a group of broad-spectrum antibiotics that were commonly used in the treatment of bacterial infections before the emergence of widespread resistance [1,2]. Tetracyclines inhibit protein synthesis by binding to the small ribosomal subunit at the A site, where the acceptor tRNA binds [3,4]. Their antibiotic activity depends on the presence of divalent metal ions [5–7]. Recently, the tetracyclines have been reported to inhibit amyloidogenesis [8,9] and prion infectivity [10] associated with neurodegenerative disorders such as Alzheimer's disease and bovine spongiform encephalopathy, stimulating renewed interest in their clinical application. Their proposed mechanism of action is by direct interaction with hydrophobic regions of the prion protein [8,10]. The role of metal ions in the anti-amyloidogenic activities of the tetracyclines is presently unknown.

The metal–ion complexation of the tetracyclines has been the subject of numerous studies (for recent review see [11] and references therein), most of them focused on Ca^{2+} and Mg^{2+}, the physiologically relevant divalent metal ions. Affinity, stoichiometry, and locations of binding sites were investigated using a variety of spectroscopic and electrochemical methods. For both metal ions, stoichiometries of 1:1, 1:2, and 2:1 ions bound per tetracycline have all been reported in aqueous solutions between pH 6.5 and 8.5 [12–17]. No crystal or solution structures of Ca^{2+} and Mg^{2+} or other alkaline earth metal–tetracycline complexes are available that could address the stoichiometry or locations of bound ions. X-ray crystal structures of tetracycline (TC, Scheme 1), the parent compound of the tetracyclines, are available in complex with the bacterial tetracycline repressor [6,18] and at the ribosomal A site [19,20].

Abbreviations: TC, tetracycline; H_4Tc^+, H_3Tc, H_2Tc^-, HTc^{2-}, and Tc^{3-}, the different ionic forms of tetracycline; ITC, Isothermal titration calorimetry.

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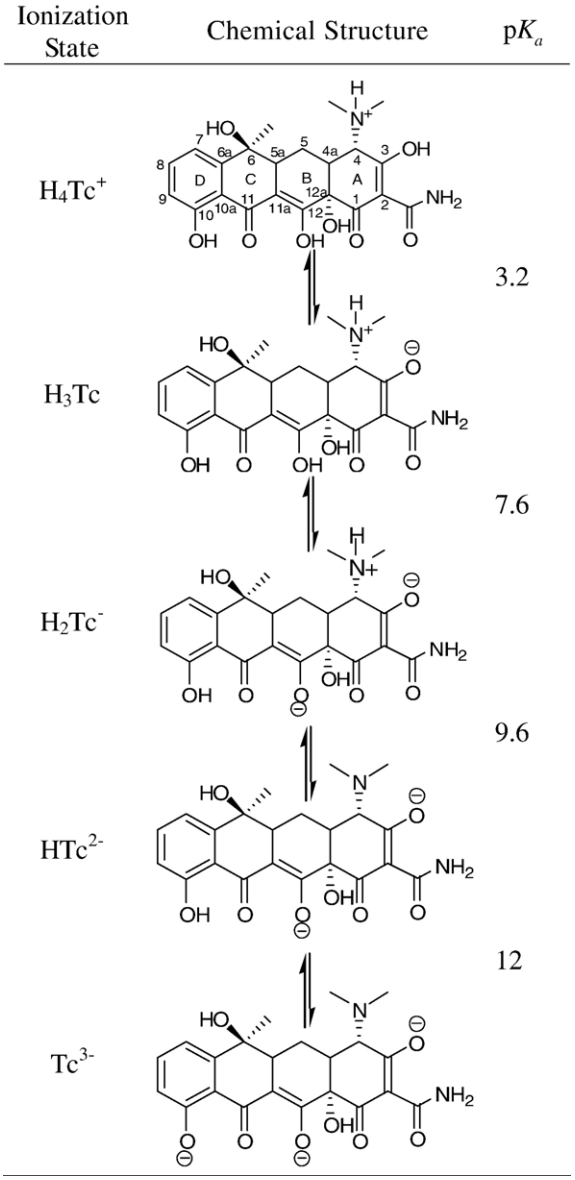

Scheme 1. Ionization states of tetracycline. Only one of several possible tautomers is shown for each ionization state.

Both structures reveal a TC bound to one Mg^{2+} that also interacts with the macromolecule.

The tetracyclines have four ionization equilibria (Scheme 1) with pK_as at approximate pH values of 3.2, 7.6, 9.6, and 12 [21,22] and five protonation states that can be represented by H_4Tc^+, H_3Tc, H_2Tc^-, $HTc^{2-}$and Tc^{3-}. Ion-binding studies over this entire pH range are limited by hydroxide precipitation of metal ions at the high end of the range and by TC solubility and ion affinity at the low end of the range. Metal ion binding is likely to alter the pK_a values of TC and thus the overall ionic state of the complex. Additionally, X-ray crystallographic studies of free tetracyclines [23–25] reveal significant variations in the conformation of the tetracyclines with protonation state that are likely to affect ion binding. The neutral zwitterionic TC (H_3Tc, Scheme 1) and OTC (oxytetracycline: OH replacing H at C5) assume an extended conformation, whereas the neutral non-ionized tautomer of OTC (proton at N4 transferred to C3) assumes a folded conformation (Fig. 1). Circular dichroism spectroscopy also reveals two classes of TC conformations above and below pH 8 [27–29], consistent with theoretical studies [22,30,31] that led some authors to conclude that TC is a highly adaptive molecule capable of structural response to its chemical environment [22]. Better understanding of TC–ion binding and conformational equilibria may shed light on biological modes of action of TCs, and stimulate design of novel derivatives for contemporary clinical applications.

Isothermal titration calorimetry (ITC) can be used to determine molar ratios of binding [32]. ITC also yields thermodynamic parameters for a binding process, and it provides a means to estimate the extent of deprotonation that may be coupled to binding events [33]. In the present work, ITC was used to complement the spectroscopic and electrochemical results already reported by others for Ca^{2+} and Mg^{2+} binding to TC, and to extend previous ITC studies at a single pH [34]. Binding studies were performed for Ca^{2+} in the pH range 6.5 to 11.7 and for Mg^{2+} in the pH range 6.5 to 9.5. We report distinct stoichiometry for each ion:TC complex ($1Ca^{2+}$:TC versus $1Mg^{2+}$:2TC), each of which is invariant with pH. Significant TC deprotonation is linked to binding of both ions. The distinct stoichiometries and ionic states of the Ca^{2+} and Mg^{2+} complexes, as well as the intrinsic thermodynamic parameters for their binding, all suggest that the two metal ions bind TC differently.

2. Experimental

2.1. Sample preparation

Experiments were conducted in 100 mM buffer with 0.15 M NaCl unless stated otherwise. Buffers used were sodium acetate at pH 5.0, Tris·HCl (tris (hydroxymethyl) aminomethane, pK_a 8.3) at pH 6.5, 7.5, and 8.5, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pK_a 7.55) at pH 8.5, triethanolamine·HCl (pK_a 7.8) at pH 7.5, and CAPS (3-(cyclohexylamino)-1-propanesulfonic acid, pK_a 10.4) at pH 9.5, 11.0, and 11.7. Tris·HCl was used at pH 6.5 because of the lack of a buffer of suitable pK_a that does not complex with or precipitate metal ions. The large buffer concentration (100 mM) in comparison to TC (1 mM) minimizes potential shifts in pH upon ion binding. The Ca^{2+} and Mg^{2+} solutions were prepared gravimetrically from $CaCl_2{\cdot}2H_2O$ and $MgCl_2{\cdot}6H_2O$ in buffer. Errors in metal ion concentrations were minimized by using fresh bottles of the hygroscopic salts and working quickly to avoid taking up water. Unintentional introduction of water would make actual concentrations lower than calculated and thus lead to over- rather than underestimation of fitted *m* (number of metal ions bound per TC) values. Metal ion concentrations thus obtained were within 2% of that determined from EDTA titration. Either no or very small pH shift (<0.02 pH unit for a 50 mM metal ion solution) was observed when the metal ion solutions were prepared in each of the above buffer solutions. Thus the buffer systems chosen for this work have no or negligible affinity for the metal ions. All solutions were prepared from Milli-Q water containing residual calcium or magnesium ion concentrations estimated at less than 1 μg/L

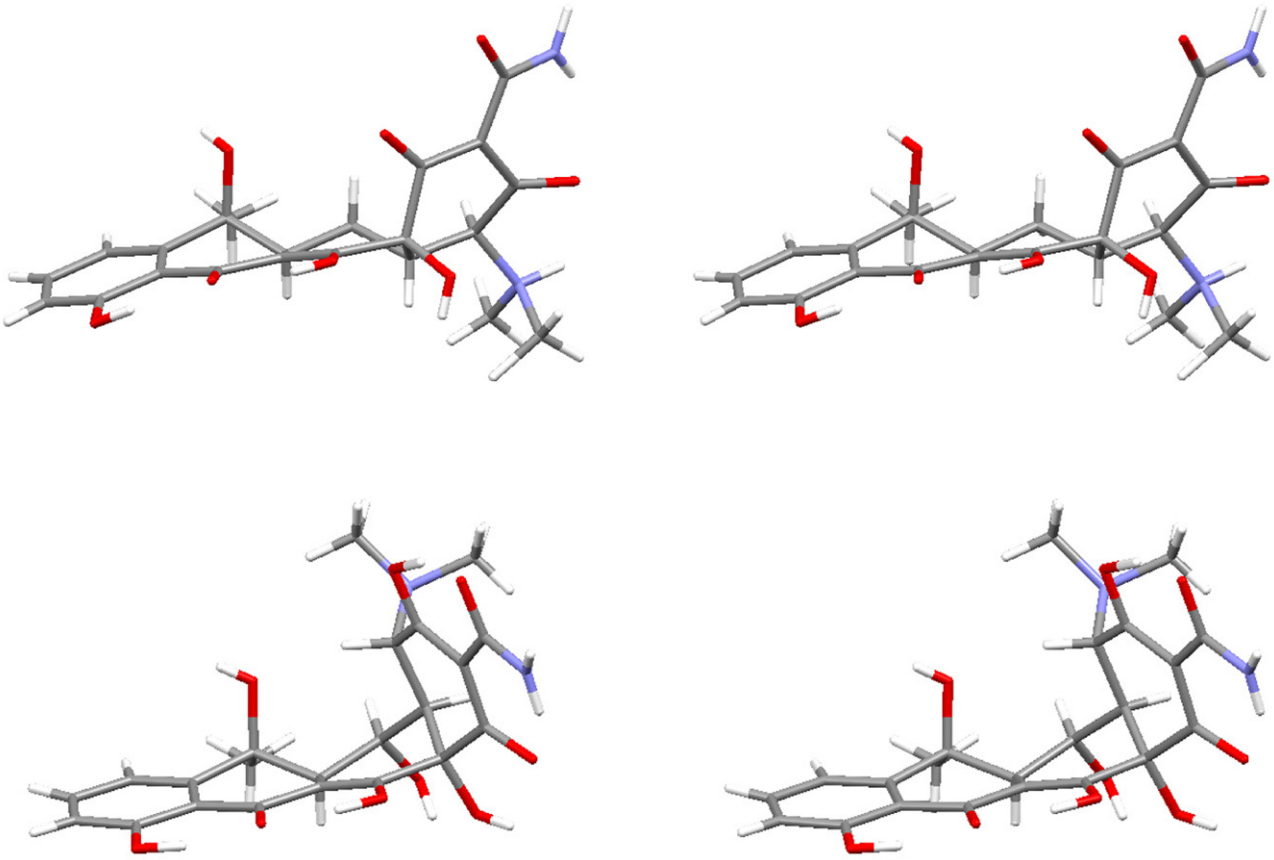

Fig. 1. Tetracycline conformations. Top: Stereo pair of neutral zwitterionic TC in extended conformation. Bottom: Stereo pair of neutral non-ionized OTC in folded conformation. Oxygen atoms are represented in red and nitrogen atoms in blue. Structural data are from reference [23] and the structures are generated using Mercury 1.4.1 [26].

(25 nM) (Millipore). Due to its instability in basic solution and its air- and light-sensitivity, TC was prepared immediately before each ITC experiment using fresh bottles of tetracycline hydrochloride (Sigma, >99%) dissolved in filtered and degassed buffer in tubes wrapped in dark cloth. When only fresh TC was used and extreme care was taken to minimize its exposure to air and light and the time between dissolving and titrating, the ITC results were reproducible (within ~4% of the mean reported values of ΔH°_{obs}) and apparently free from side reactions. The final pH of all stock solutions was adjusted to within ±0.02 unit of the stated pH.

2.2. Isothermal titration calorimetry (ITC)

Experiments were performed on a VP-ITC calorimeter (MicroCal, LLC) at 25 °C. Data were analyzed using Origin 7.2. The instrument was calibrated with an internal heat pulse. All solutions of titrant (in the injection syringe) and titrate (in the reaction cell, capacity ~1.4 mL) were fully degassed before loading. A typical titration experiment consisted of 28 injections of 10 μL each after a 3 μL first injection that was deleted before curve-fitting. Titrations of each metal ion (1.25–100 mM) into TC (0.25–2.5 mM) and of TC (1.25–10 mM) into each metal ion (0.25–0.5 mM) were carried out. Spacing between injections was set at 6 to 10 min as needed to allow full return of signal to baseline. Control experiments including titration of metal ion into buffer, buffer into TC, TC into buffer, and buffer into metal ion were performed and subtracted as appropriate. Baselines of raw data were manually adjusted. The binding isotherms (normalized heat in kcal per mole of titrant added as a function of titrant to titrate molar ratio) were fitted using the one-set-of-sites model provided by MicroCal, LLC with all parameters allowed to float. The uncertainties in parameters m, $K_{d,obs}$ and ΔH°_{obs} were minimized by the use of multiple titrate and titrant concentrations high enough to ensure accurate extraction of binding parameters from curve-fitting [35], and by conducting multiple independent replicates under each condition.

2.3. Extent of deprotonation, intrinsic binding enthalpy and entropy, and TC ionization enthalpy

To determine the extent of deprotonation (ionization) of TC upon metal ion binding, the observed enthalpy change (ΔH°_{obs}) at a given pH in two buffers of significantly different ionization enthalpy were used. Buffer pairs used were Tris·HCl and HEPES at pH 8.5, and Tris·HCl and triethanolamine·HCl at pH 7.5, all at 100 mM with 0.15 M NaCl. Ionization enthalpies are 47.3 kJ/mol for Tris·HCl [36], 21.0 kJ/mol for HEPES buffer [37], 33.6 kJ/mol for triethanolamine·HCl [37], and 48.5 kJ/mol for CAPS [38]. Enthalpy change determined from curve-fitting was used for all pHs. At pH 8.5, enthalpy change was also

obtained directly by titrations using high concentrations of TC and the metal ion. A total of 28 injections of 5 mM TC (10 μL each) was made into 100–500 mM Ca^{2+} or Mg^{2+} solution. Two controls, TC titration into buffer and buffer titration into Ca^{2+} or Mg^{2+} were performed and the data were subtracted from the TC into ion titrations; the derived average heat in kJ per mole TC was taken as the enthalpy change. This value was within 4% of the enthalpy change derived from curve-fitting. At pH 7.5, due to the weaker affinity and limited TC solubility at lower pH, direct measurement of enthalpy change was not possible. At pH 9.5 and above, due to the lack of a second buffer that does not interfere with Ca^{2+} and Mg^{2+} binding but has significantly different ionization enthalpy compared with either Tris·HCl or CAPS buffer, the extent of deprotonation was not determined the same way as at pH 7.5 and 8.5 but was estimated as detailed later.

For pH 7.5 and 8.5, ΔH°_{obs} at a given pH was plotted as a function of buffer ionization enthalpy (ΔH^{b}_{ion}). According to the relationship by Baker and Murphy [33],

$$\Delta H^{\circ}_{obs} = \Delta H^{\circ}_{0} + N_{H+}\Delta H^{b}_{ion} \quad (1)$$

the slope was N_{H+}, the number of TC protons removed (when negative) or added (when positive).

Binding of TC at a given ionic state (H_aTc^{a-3}) to a divalent metal ion (Mg^{2+}) can be viewed as the sum of three processes: TC deprotonation, buffer (B) protonation, and intrinsic binding of the deprotonated TC ($H_{a-n}Tc^{a-3-n}$) to the metal ion. For simplicity, n is used to represent $-N_{H+}$. The negative sign is used because by definition N_{H+} is negative if proton is removed from TC. The "a" in the formula for TC is dependent on pH and is approximately 2.5, 2, and 1.5 at pH 7.5, 8.5 and 9.5, respectively. The three processes and the overall binding reaction are listed below:

$$H_aTc^{a-3} + nH_2O \leftrightarrow nH_3O^{+} + H_{a-n}Tc^{a-3-n} \quad -N_{H+}\Delta H^{TC}_{ion} \quad (2)$$

$$nH_3O^{+} nB \leftrightarrow nBH^{+} + nH_2O \quad N_{H+}\Delta H^{b}_{ion} \quad (3)$$

$$H_{a-n}Tc^{a-3-n} + mM^{2+} \leftrightarrow mM^{2+}\cdot H_{a-n}Tc^{a-3-n} \quad \Delta H^{\circ}_{in} \quad (4)$$

$$H_aTc^{a-3} + mM^{2+} + nB \leftrightarrow mM^{2+}\cdot H_{a-n}Tc^{a-3-n} + nBH^{+} \quad \Delta H^{\circ}_{obs} \quad (5)$$

Listed to the right of each reaction is the corresponding enthalpy change. Similar terms can also be written for the entropy change. m is the number of metal ions bound per TC. ΔH^{TC}_{ion} is TC ionization enthalpy and it may differ for different ionizable protons on TC. The observed enthalpy change of binding, ΔH°_{obs}, can therefore be expressed as follows:

$$\Delta H^{\circ}_{obs} = \Delta H^{\circ}_{in} + N_{H+}\Delta H^{b}_{ion} - N_{H+}\Delta H^{TC}_{ion} \quad (6)$$

The sum of the intrinsic binding enthalpy (ΔH°_{in}) and TC deprotonation heat ($-N_{H+}\Delta H^{TC}_{ion}$) is equivalent to the ΔH°_{0} in Eq. (1). Note that intrinsic binding refers to binding of the metal ion to TC at the ionic state assumed following, rather than prior to, the metal ion-induced deprotonation. If no linked TC deprotonation occurs ($N_{H+}=0$), ΔH°_{obs} is equivalent to ΔH°_{in}. If buffer and TC have identical ionization heat ($\Delta H^{b}_{ion}=\Delta H^{TC}_{ion}$), ΔH°_{obs} will also be equal to ΔH°_{in}, irrespective of the extent of deprotonation. In either case, observed binding enthalpy will be independent of pH. Eq. (6) was used to determine ΔH°_{in} for Ca^{2+} and Mg^{2+} binding at pH 7.5 and 8.5. Eq. (6) was also

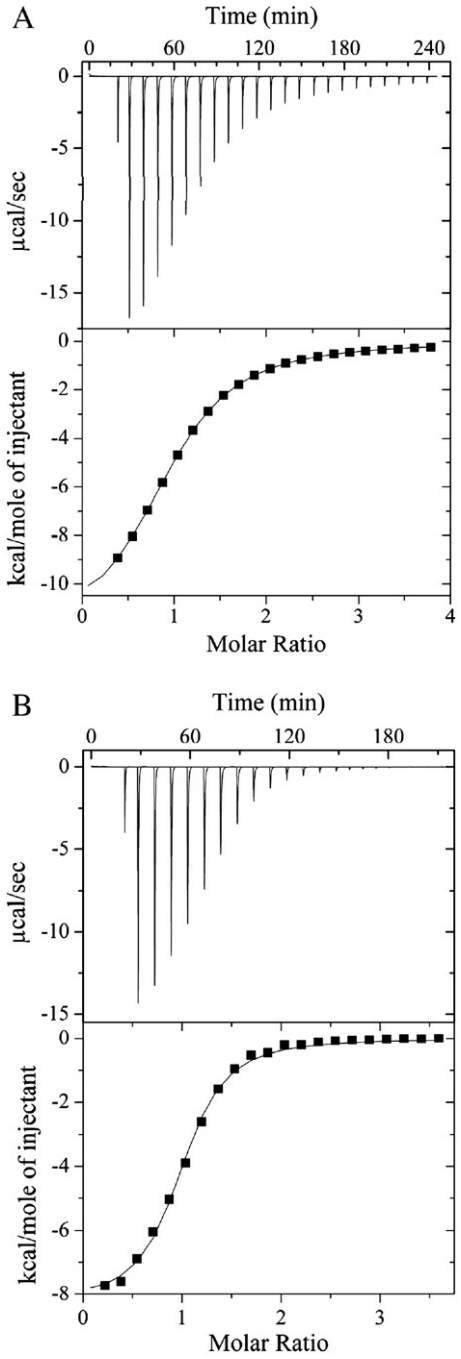

Fig. 2. ITC data and fitted binding isotherms for titration of 7.5 mM Ca^{2+} into 0.25 mM TC in A) 100 mM Tris·HCl buffer, pH 8.5 and B) 100 mM CAPS buffer, pH 11.7, both with 0.15 M NaCl. The first injection is 3 μL and the rest are 7.5 μL each. Molar ratio is ratio of Ca^{2+} to TC. Data are corrected for control heats of buffer into TC, Ca^{2+} into buffer, and water into water.

Table 1
Binding parameters from Ca^{2+} titration into TC

pH	m (M:TC)	$K_{d,obs}$ (μM)	ΔG°_{obs} (kJ/mol)	ΔH°_{obs} (kJ/mol)	$T\Delta S^{\circ}_{obs}$ (kJ/mol)	$\Delta H^{\circ}_{obs} - \Delta H^{\ddagger}_{in}$ (kJ/mol)	$T\Delta S^{\circ}_{obs} - T\Delta S^{\circ}_{in}$ $^{\mp}$ (kJ/mol)
6.5[†]		1100±90	−17.4±0.2				
7.5	0.94±0.01	590±60	−18.5±0.3	−58±2	−40±2	−23±2	−32±3
8.5	0.95±0.04	48±4	−24.6±0.2	−51.0±0.4	−26.4±0.4	−16±2	−19±2
9.5	1.10±0.05	29±5	−25.9±0.2	−40.2±0.4	−14.2±0.4	−5±2	−7±2
11.0	0.98±0.04	15±1	−27.6±0.3	−35±2	−7.5±1.3	0±2	0±2
11.7	0.97±0.05	14±1	−27.7±0.2	−34.7±0.4	−7.1±0.4	0±2	0±2

Titrations were carried out at 25 °C in 100 mM Tris·HCl buffer (pH 6.5, 7.5, and 8.5) or CAPS buffer (pH 9.5, 11.0, and 11.7), all containing 0.15 M NaCl. Except for ΔH°_{in} and ΔS°_{in}, all values are averages ± S.D. from two or three experiments. All thermodynamic parameters are per mole of bound Ca^{2+}. m is the number of moles of Ca^{2+} bound per mole TC. [†]At pH 6.5, affinity is too low to yield accurate m and ΔH°_{obs} but $K_{d,obs}$ could be determined with confidence [35]. [‡]The difference between the observed and the intrinsic binding enthalpy. [∓]The difference between the observed and the intrinsic binding entropy multiplied by the temperature.

used to determine ΔH^{TC}_{ion} for the second and the third deprotonation of TC. These calculations used the ΔH°_{obs} and N_{H+} values determined for pH 7.5, 8.5 and 11.0. Given the ΔH°_{in} and ΔH^{TC}_{ion} values, Eq. (6) was also used to determine N_{H+} for Ca^{2+} and Mg^{2+} binding at pH 9.5 using data in one buffer (CAPS). The N_{H+} values were then used to calculate the net charge of ion-bound TC and the metal ion–TC complex at a given pH.

A similar equation can be written for the observed binding entropy:

$$\Delta S^{\circ}_{obs} = \Delta S^{\circ}_{in} + N_{H+}\Delta S^{b}_{ion} - N_{H+}\Delta S^{TC}_{ion} \quad (7)$$

where ΔS°_{in}, ΔS^{b}_{ion} and ΔS^{TC}_{ion} are intrinsic binding entropy of metal ion to TC, and buffer and TC ionization entropy, respectively. Tris·HCl and CAPS buffer ionization entropies are 5.0 and −36 J/mol·K [38], respectively. Intrinsic binding entropy for Ca^{2+} binding to TC was then calculated from Eq. (7) using data for pH 7.5, 8.5 and 9.5. However, ΔS°_{in} for Mg^{2+} binding could not be obtained because ΔS°_{in} likely differs at different pH because of the different ionic state of TC following Mg^{2+}-induced deprotonation.

3. Results

3.1. Ca^{2+} binding

To examine the effect of pH, and thus of the ionic state of TC, on Ca^{2+} binding, ITC experiments were carried out at pH 5.0, 6.5, 7.5, 8.5, 9.5, 11.0, and 11.7 at 25 °C in 100 mM buffers containing 0.15 M NaCl. Under these conditions TC exists as the following ionic species: mainly H_3Tc at pH 5.0 and 6.5; H_3Tc and H_2Tc^- in ~1:1 molar ratio at pH 7.5; mainly H_2Tc^- at pH 8.5; H_2Tc^- and HTc^{2-} (~1:1) at pH 9.5; mainly HTc^{2-} at pH 11; and HTc^{2-} and Tc^{3-} (~3:4) at pH 11.7. Titrations were carried out to determine $K_{d,obs}$, the observed equilibrium dissociation constant; ΔH°_{obs} per mole of metal ion; and m, the molar ratio of bound metal ion to TC (here also referred to as stoichiometry, see Discussion).

Ca^{2+} was first titrated into TC. At pH 5.0 no binding was detected with 0.5 mM TC up to a final Ca^{2+} concentration of 10 mM, putting a lower limit on $K_{d,obs}$ of ~100 mM. Representative titration data and isotherms at higher pHs are shown in Fig. 2, and binding parameters obtained from fitting with a one-set-of-sites model are listed in Table 1. Binding is apparently exothermic at all pHs, and apparent affinity increases as pH increases. At pH 6.5, binding is so weak that the maximum achievable c value (defined as the product of m, K_a and titrate (TC) concentration) allowed accurate determination of only $K_{d,obs}$ (=$1/K_a$) and not m or ΔH°_{obs} [35]. At higher pHs, increased affinity and higher TC solubility permitted optimal c values to be achieved and thus accurate recovery of all three binding parameters. The data are fit well by the one-set-of-sites model at pH values between 6.5 and 9.5 (Fig. 2A), and reasonably well at pH 11 and 11.7 with minor deviation in the pre-plateau region (Fig. 2B). Consistent with the good fit of the one-set-of-sites model, m values indicate a stoichiometry of one Ca^{2+} per TC throughout the pH range 7.5 to 11.7 using triethanolamine·HCl (pH 7.5), Tris·HCl (pH 7.5 through 9.5), or CAPS buffer (pH 9.5 through 11.7). The fitted $K_{d,obs}$ values are ~1 mM at pH 6.5, ~0.6 mM at pH 7.5 (Tris·HCl), and ~10 to ~50 μM at pH 8.5 to 11.7.

Throughout the pH range 7.5 to 11.7 using either Tris·HCl or CAPS buffer, the observed binding enthalpy changes sharply with pH from −58.2 kJ/mol at pH 7.5 to −35 kJ/mol at pH 11.0. Such pH-dependence suggests a shift of a proton equilibrium coupled to metal ion binding. The increasingly less exothermic ΔH°_{obs} is accompanied by increasingly less negative $T\Delta S^{\circ}_{obs}$ ranging from −40 kJ/mol at pH 7.5 to −7.5 kJ/mol at pH 11.0. Affinity approximately doubles from pH 6.5 to 7.5, from 8.5 to 9.5, and from pH 9.5 to 11.0. The largest increase (12-fold) in observed binding affinity ($1/K_{d,obs}$) per unit increase in pH is seen from pH 7.5 to 8.5. The observed binding parameters result

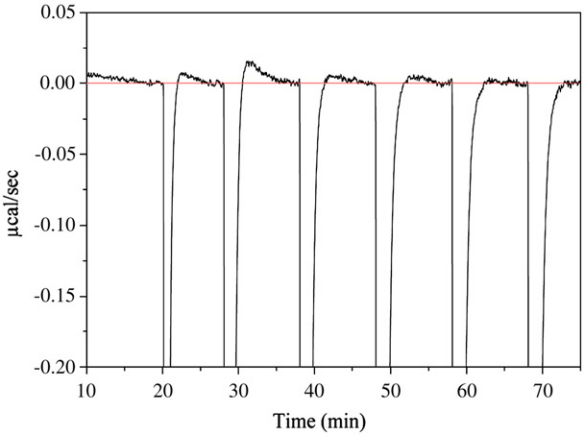

Fig. 3. A zoomed-in look at the first few injection peaks for 7.5 mM Ca^{2+} titration into 0.25 mM TC at pH 11.7. See Fig. 2B for complete ITC data.

from the combined effects of intrinsic metal ion binding to TC, TC deprotonation, and buffer protonation.

At pH 11.7 the first few injections show a small but distinct and slowly evolving endothermic component of the reaction heat (Fig. 3) in addition to the main exothermic heat flow. Because in all experiments heat power signals were far above zero, this endothermic component is not due to instrumental overshoot that can occur when heat signals fall below zero (Microcal, LLC). Increased stirring speed from 310 to 570 rpm reduces but does not eliminate the endothermic component, and does not affect the shape of the binding curve. The effect of stirring speed indicates that the endotherm is at least partly due to a diffusion-controlled process. The points most affected by the endothermic component, usually the first one or two full-volume injections under the titrate and titrant concentrations used here, deviate from the one-set-of-sites model. This slight deviation could reflect differences in binding of HTc^{2-} and Tc^{3-}, the two predominant ionic forms of TC at pH 11.7. However, binding parameters obtained at pH 11.0 and 11.7 are identical despite the different populations of HTc^{2-} and Tc^{3-}, suggesting their affinities for Ca^{2+} are similar. When the pH 11.7 data were fit with a two-sets-of-sites model, fitting parameters were hypersensitive to small variations in data points from replicate runs, and reliable parameters for the two ionic forms could not be obtained. This may be due partly to the large number of variables allowed to float during fitting (two sets of m, $K_{d,obs}$ and ΔH°_{obs}). When the points most affected by the endothermic component were deleted, data fit to a one-set-of-sites model reasonably well, with only minor deviations at the pre-plateau region. These results therefore suggest that a second process may accompany the binding event.

To verify binding parameters obtained from Ca^{2+} titration into TC (hereafter referred to as forward titration), reverse titration of TC into Ca^{2+} was also carried out between pH 8.5 and 11.7. Reverse titration could not be performed at or below pH 7.5 due to lower TC solubility and weaker affinity and the need for higher TC concentration as titrant. At pH 9.5, 11.0, and 11.7, curve-fitting with the one-set-of-sites model was of the same good quality as for the forward titrations and yielded similar m values (~0.90 TC per Ca^{2+}). The slight deviation of m from the expected value of 1.0 may be due to a systematic error related to the relatively large exothermic control heat of TC titration into buffer that was subtracted from the reverse titration data. The same general trend of $K_{d,obs}$, ΔH°_{obs}, and $T\Delta S^{\circ}_{obs}$ as a function of pH was observed in the reverse as in the forward titrations, although exact parameter values recovered from the fits differ slightly.

Endothermic tailing was not observed at or above pH 9.5 under the conditions used to obtain the complete reverse binding isotherm. However, other signs of an endothermic component of the reaction heat were present in the first two injections, including faster return of signal to baseline compared to peaks that immediately follow, and narrower peak width at baseline. Presumably due to these effects, the first (or occasionally the first two) full-volume points deviate from the one-set-of-sites model. When these points are deleted, the rest of the data points are fit well by the one-set-of-sites model, suggesting that the endothermic component is not proportional to the extent of binding of the single metal ion. Due to the lower TC concentrations achievable at lower pH, reverse titrations of TC into Ca^{2+} at pH 8.5 were too incomplete to yield reliable binding parameters (Fig. 4). Nevertheless, relatively large endothermic tailing was observed for the first few injection peaks (Fig. 5 inset). The magnitude of the endothermic heat signal relative to the exothermic one in the first few peaks yields a binding isotherm with a pronounced trough (Fig. 4), rather than the sigmoid-shaped isotherm expected for a single binding event that would be consistent with the derived m value of ~0.9.

3.2. Mg^{2+} binding

Mg^{2+} binding was also studied as a function of pH in 100 mM buffers containing 0.15 M NaCl. Titrations of Mg^{2+} into TC could be performed at pH values of 5.0, 6.5, 7.5, 8.5, and 9.5. At pH 5.0, no binding was observed at concentrations as high as 0.5 mM TC and 10 mM Mg^{2+}. At pH 6.5, just as for Ca^{2+} binding, the weak affinity allowed determination of affinity only ($K_{d,obs}$ ~2.2 mM). At pH 7.5 and 8.5 in Tris·HCl buffer and at pH 9.5 in CAPS buffer, data were fit well by the one-set-of-sites model (Fig. 6), with binding parameters listed in Table 2. However, in contrast to Ca^{2+} binding, the molar ratio m was only 0.5 Mg^{2+} per TC, even when Mg^{2+} was present in up to 10-fold molar excess. The m value obtained in triethanolamine·HCl was 0.4, close to the value of 0.5 in Tris·HCl buffer at the same pH. Thus stoichiometry is constant over pH 7.5 to 9.5. Similarly as for Ca^{2+}, the observed binding enthalpy for Mg^{2+}

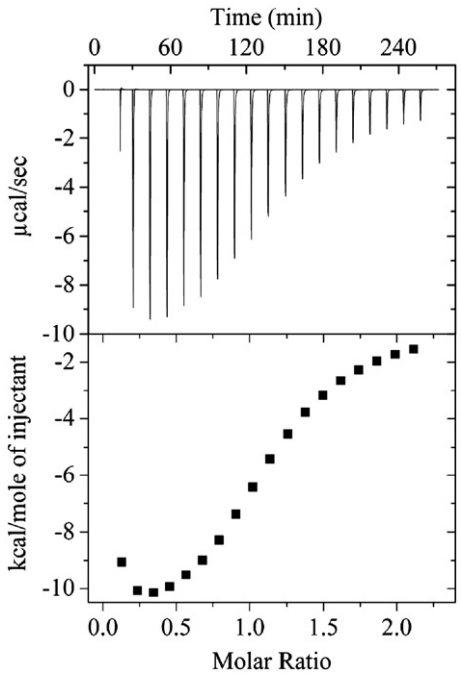

Fig. 4. ITC data and normalized heat in kcal per mole Ca^{2+} for titration of 2.5 mM TC into 0.25 mM Ca^{2+} in 100 mM Tris·HCl buffer, 0.15 M NaCl, pH 8.5. The first injection is 3 µL and the rest are 15 µL each. Control heats of buffer into Ca^{2+} and TC into buffer were subtracted from the isotherm. No fit is shown because data do not fit to one-set-of-sites model and the fit to two-sets-of-sites model is associated with excess error to yield meaningful m, $K_{d,obs}$ and ΔH°_{obs}.

Table 2
Binding parameters from Mg^{2+} titration into TC

pH	m (M:TC)	$K_{d,obs}$ (μM)	ΔG°_{obs} (kJ/mol)	ΔH°_{obs} (kJ/mol)	$T\Delta S^{\circ}_{obs}$ (kJ/mol)	$\Delta H^{\circ}_{obs} - \Delta H^{\circ\ddagger}_{in}$ (kJ/mol)
6.5[†]		2200±100	−19.1±0.1			
7.5	0.50±0.01	350±20	−21.2±0.2	−38.9±0.8	−19.2±0.8	−24±1
8.5	0.50±0.01	177±6	−21.4±0.1	−29.3±0.8	−7.9±0.8	−14±1
9.5	0.51±0.02	65±5	−23.9±0.2	−19.7±0.8	4.2±0.8	−5±1

Titrations at 25 °C in 100 mM Tris·HCl buffer (pH 6.5, 7.5 and 8.5) or CAPS buffer (pH 9.5), all containing 0.15 M NaCl. Except ΔH°_{in}, all values are averages ± S.D. from two or three experiments. All thermodynamic parameters are per mole of bound Mg^{2+}. m is the number of moles of Mg^{2+} bound per mole TC. [†]At pH 6.5, affinity is too low to yield accurate m and ΔH°_{obs} but $K_{d,obs}$ could be determined with confidence [35]. [‡]See Table 1 legend.

changes sharply with pH, suggesting shift of a proton equilibrium coupled to Mg^{2+} binding. The less favorable enthalpic contribution with increasing pH is more than offset by a less unfavorable entropic contribution, resulting in about a 2-fold increase in affinity from pH 8.5 to 9.5. At all pHs tested, neither endothermic tailing nor faster return of signal to baseline was observed for any injection peak in isotherms used to determine Mg^{2+} binding parameters.

Reverse titration of TC into Mg^{2+} was performed as well, but only at pH 8.5 and 9.5 due to even more severe limitations of the same kinds as for Ca^{2+}. At pH 9.5, binding parameters (not shown) obtained from the one-set-of-sites model were similar to those of the forward titration in Table 2. No endothermic tailing was evident, but faster return of signal to baseline was observed for the first two injections, consistent with the presence of an endothermic component of the reaction heat. Fitting with the one-set-of-sites model was reasonable after deleting the first full-volume point, and parameter values match well with those from forward titrations. At pH 8.5 only incomplete binding data could be obtained (not shown), and these displayed slight endothermic tailing and a trough in the binding isotherm similar to that for Ca^{2+} shown in Fig. 4.

3.3. Effect of Na^+ ion

To determine the effect of Na^+ ion on Ca^{2+} and Mg^{2+} binding, titrations were also carried out without Na^+ ion between pH 7.5 and 9.5 in Tris·HCl buffer. At higher pHs where CAPS is used, the complete absence of salt ion in buffer is not possible because CAPS free acid must be titrated with sodium hydroxide to adjust pH. For Ca^{2+} binding, absence of Na^+ did not change stoichiometry or ΔH°_{obs} but had a slight effect on affinity, an increase of up to two-fold depending on the pH. Mg^{2+} affinity is also slightly higher in the absence of Na^+ by up to two-fold depending on the pH, but stoichiometry was altered: absence of Na^+ ion had no effect at pH 7.5, a slight increase in m from 0.50 to 0.55 at pH 8.5, and a larger increase to 0.75 at pH 9.5. These differences in m are not simply a consequence of covariance between ΔH°_{obs} and m in data fitting, as no other physically reasonable molar ratios fit the data acceptably.

3.4. Proton ionization, intrinsic binding enthalpy and entropy, and TC ionization enthalpy

The strong pH-dependence of ΔH°_{obs} suggests a shift in proton equilibria coupled to ion binding. The number of protons, N_{H+}, removed or added to TC upon ion binding was determined at pH 7.5 and 8.5 by measuring ΔH°_{obs} in two buffers of very different ionization enthalpy. The resulting data were analyzed according to the method of Baker and Murphy [33]. For Ca^{2+} binding, 1.6±0.1 and 1.20±0.04 protons dissociate from TC at pH 7.5 and 8.5, respectively. Given the ionic state of free TC at these two pH values (Scheme 1), these results imply that the charge on TC when bound to Ca^{2+} is approximately −2.1 at pH 7.5 and −2.2 at pH 8.5. Thus, even though free TC exists as mostly the H_3Tc and H_2Tc^- ionic states at pH 7.5 to 8.5, Ca^{2+}-bound TC assumes predominantly the HTc^{2-} ionic state, due to TC deprotonation linked to binding.

At pH 6.5, N_{H+} was not determined because weak affinity prevents accurate measurement of enthalpy change, and at pH 9.5, 11.0 and 11.7, N_{H+} was not determined directly for lack of a suitable second buffer. However, ΔH°_{obs} values for ion binding were identical at pH 11.0 and 11.7 in CAPS buffer even though the fourth pK_a of TC is ~12. These results imply the absence of a further shift in proton equilibrium from pH 11.0 to 11.7. Thus the observed binding enthalpy at pH 11 and 11.7, −35±2 kJ/mol, is taken to be the intrinsic enthalpy change (ΔH°_{in}) of Ca^{2+} binding to both HTc^{2-} and HTc^{3-}. This value is in good agreement with ΔH°_{in} values determined from the relationship of ΔH°_{obs} with N_{H+}, ΔH°_{in}, ΔH^{TC}_{ion} and ΔH^{b}_{ion} at pH 7.5 and 8.5 (−36±2 and −34±2 kJ/mol, respectively), further supporting the conclusion that no shift in protonation equilibrium is linked to binding at pH 11.0 or 11.7. Since the ionic states of TC at pH 7.5 and 8.5 following Ca^{2+} binding are both HTc^{2-}, intrinsic

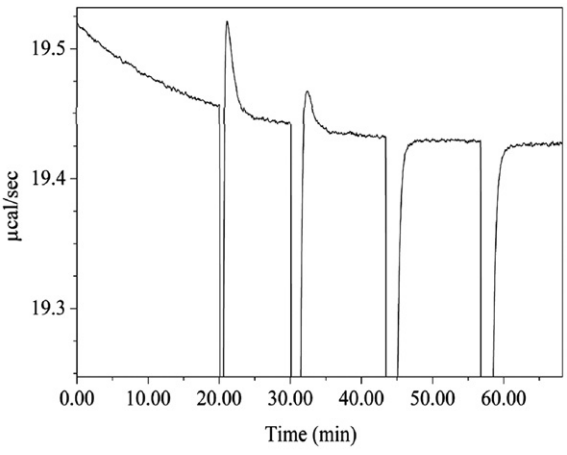

Fig. 5. A zoomed-in look at the first few injection peaks for 2.5 mM TC titration into 0.25 mM Ca^{2+} at pH 8.5. See Fig. 4 for complete ITC data.

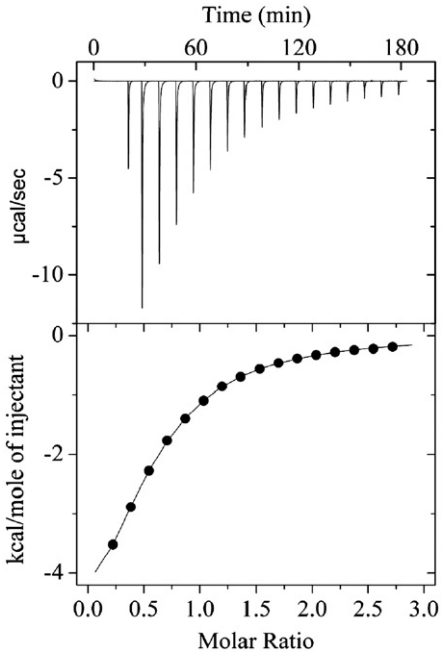

Fig. 6. ITC data and fitted binding isotherms for titration of 15 mM Mg^{2+} into 0.5 mM TC in 100 mM Tris·HCl buffer, 0.15 M NaCl, pH 8.5. The first injection is 3 μL and the rest are 7.5 μL each. Control heats of buffer into TC and Mg^{2+} into buffer were subtracted.

binding of Ca^{2+} at these pHs is therefore to the HTc^{2-} ionic state. The ionization enthalpies, ΔH^{TC}_{ion}, for the deprotonation of the second and the third ionizable protons, were also calculated using the same relationship at pH 7.5 and 8.5. The values are 31±2 and 34±2 kJ/mol at pH 7.5 and 8.5, respectively.

At pH 9.5 N_{H+} could not be determined directly, but ΔH°_{obs} in CAPS buffer was more exothermic than at pH 11 and 11.7, suggesting the presence of linked deprotonation. Given the ΔH°_{obs} in CAPS buffer and ΔH°_{in} for HTc^{2-} and ΔH^{TC}_{ion} determined above, N_{H+} was calculated to be −0.3±0.1 for Ca^{2+} binding to TC at pH 9.5, and Ca^{2+}-bound TC carries approximately −1.8 charges, similar as at pH 7.5 and 8.5. Taken together, the results for linked deprotonation of TC upon Ca^{2+} binding indicate that the 1:1 Ca^{2+}:TC complex carries a net charge of approximately −0.1,−0.2,+0.2 and 0 at pH 7.5, 8.5, 9.5 and 11.0. Thus, the Ca^{2+}:TC complex is nearly neutral over this entire pH range where the charge on free TC changes from −0.5 to −2.0.

For formation of the Mg^{2+}:2TC complex at pH 7.5 and 8.5, respectively, approximately 0.75±0.05 and 0.55±0.05 protons dissociate from each TC. Thus each Mg^{2+}-bound TC carries approximately −1.3 and −1.6 charges and exists predominantly as H_2Tc^- and HTc^{2-} in approximately 2:1 and 1:1 molar ratio at pH 7.5 and 8.5, respectively. The resulting Mg^{2+}:2TC complex thus carries a net charge of −0.6 and −1.2 at pH 7.5 and 8.5, respectively. Similarly as for Ca^{2+} binding, N_{H+} at pH 9.5 cannot be obtained directly. However, using the ΔH^{TC}_{ion} values for H_2Tc^- and HTc^{2-} and the ΔH°_{obs} values, the ΔH°_{in} values of Mg^{2+} binding at pH 7.5 and 8.5 were calculated to be −15±2 kJ/mol and equal to each other. Using this value and ΔH°_{obs} at pH 9.5 in CAPS buffer, it was estimated that approximately 0.15±0.05 proton was removed from each TC upon Mg^{2+} binding at this pH. Thus at pH 9.5 Mg^{2+}-bound TC carries −1.7 charges and the Mg^{2+}:2TC complex carries a net charge of −1.4. Thus, for both Ca^{2+} and Mg^{2+}, ΔH°_{in} is exothermic and independent of pH. The difference between the observed binding enthalpy and the constant intrinsic binding enthalpy, $\Delta H^{\circ}_{obs}-\Delta H^{\circ}_{in}$, is a result of the linked TC deprotonation (and the subsequent buffer protonation). For both metal ions, this difference becomes smaller with increasing pH (Tables 1 and 2), a result of the decreasing extent of TC deprotonation.

ΔS°_{in} for Ca^{2+} binding was also calculated. ΔS°_{in} is −50±2 J/mol·K and thus $T\Delta S^{\circ}_{in}$ is −15±1 kJ/mol at 25 °C for Ca^{2+} binding to HTc^{2-}. This value, however, is twice the value of $T\Delta S^{\circ}_{obs}$ obtained at pH 11.0 (−7.5 kJ/mol) (Table 1). The latter is equivalent to the $T\Delta S^{\circ}_{in}$ value for binding to HTc^{2-} because no deprotonation is linked at pH 11.0. Since the Ca^{2+}-induced ionic state of TC at pH 7.5 and 8.5 is HTc^{2-}, the same as at pH 11.0 in the absence of deprotonation, the large difference between the $T\Delta S^{\circ}_{in}$ values of pH 11.0 and 7.5 or 8.5 suggests that there may be an additional process that contributes to the observed binding entropy at the two lower pHs. This additional process may be a conformational change associated with TC deprotonation. Therefore, the difference between the observed and the intrinsic binding entropy, $\Delta S^{\circ}_{obs}-\Delta S^{\circ}_{in}$, is the net entropy change of TC deprotonation and buffer protonation, the former including contributions of deprotonation and possibly also TC conformational change. When −7.5 kJ/mol is used as $T\Delta S^{\circ}_{in}$, values of $T\Delta S^{\circ}_{obs}-T\Delta S^{\circ}_{in}$ become less negative with increasing pH (Table 1). This net unfavorable entropic contribution as a result of TC deprotonation (and subsequent buffer protonation) more than offsets the favorable enthalpic contribution ($\Delta H^{\circ}_{obs}-\Delta H^{\circ}_{in}$), giving rise to the observed decreasing affinity with decreasing pH. The intrinsic binding affinity is the same throughout the pH range 7.5 to 11.7. The decrease in affinity from pH 8.5 to 7.5 is particularly large (12-fold), suggesting an unusually large entropic cost of deprotonation at pH 7.5. Finally, unlike in Ca^{2+} binding, Mg^{2+}-bound TC assumes different ionic state at different pH and thus likely has different ΔS°_{in} values. Therefore, no reliable ΔS°_{in} for Mg^{2+} binding could be calculated.

4. Discussion

ITC has two well-known strengths: its ability to determine molar ratios of interacting components in systems that permit suitable experimental conditions to be achieved, and its ability to quantify protonation events that accompany binding by analyzing the variation of binding enthalpy change in buffers of different ionization enthalpy. Furthermore, endothermic tailing of exothermic injection peaks in ITC, a feature that might be considered a nuisance to data analysis and thus to be avoided, can sometimes point to hidden processes, including conformational changes, that may be otherwise difficult to detect. These features were exploited in the present work in an effort to elucidate the molar ratios and influence of protonation on binding of Ca^{2+} and Mg^{2+} ions to tetracycline. The results underscore the importance of determining thermodynamic

quantities under a variety of solution conditions [39], such as pH, buffer, and salt concentration in the present work.

Molar ratios for TC ion binding have been persistently difficult to define with confidence, with previous reports ranging from 1:2 to 2:1 ions per tetracycline. The many titratable groups on tetracycline lead to charge variation with pH, and the different charge states may bind with varying molar ratios. In the present work, molar ratios were determined with confidence over the pH range 7.5 to 11.7 for Ca^{2+} and 7.5 to 9.5 for Mg^{2+}. Over these pH ranges and in the presence of NaCl, molar ratios were constant despite the fact that the negative charge on free TC increases from approximately −0.5 at pH 7.5 to −2.0 at pH 11.0 and −2.5 at pH 11.7. The present results therefore indicate that charge variation on TC does not in itself account for the previous reports of varying molar ratio of its ion complexes.

Nevertheless, molar ratios were found in the present work to be distinct for the two ions, and were not simply a consequence of covariance between ΔH°_{obs} and m in data fitting. Ca^{2+}:TC molar ratio is 1:1 over the entire pH range in Tris·HCl, triethanolamine·HCl or CAPS buffers with or without Na^{+}. For Mg^{2+}, one ion is bound per two TC at pH 7.5 in Tris·HCl and triethanolamine·HCl, at pH 8.5 in Tris·HCl, and at pH 9.5 in CAPS. Although Tris has slight and equivalent affinity for Ca^{2+} and Mg^{2+}(K_d=0.2 M) [40], this should affect TC–ion affinity, not stoichiometry, and it cannot account for the differing stoichiometries in the other buffers, which have even lower ion affinities. The absence of Na^{+} ion led to non-integral molar ratios of Mg^{2+}:TC binding at pH 9.5, which could suggest the coexistence of 1:2 and 1:1 binding modes. Na^{+} does not affect the stoichiometry of Ca^{2+} binding. The differential effect of Na^{+} on Ca^{2+} and Mg^{2+} binding may be a result of their different binding mode. It is known from NMR study that Na^{+} ion competes for and shifts the site of lanthanide coordination [41]. Competition from Na^{+} could explain the observed small decrease in Ca^{2+} and Mg^{2+} affinity for TC in the presence of Na^{+}.

The molar ratio of one Mg^{2+} per two TC was derived from datasets that displayed excellent fit to a one-set-of-sites model. This is a self-contradictory result, yet the fits to this model were excellent. This contradiction can be resolved if pre-existing dimers of TC are responsible for Mg^{2+} binding. NMR evidence [42] supports the possibility that free TC is not monomeric, as specific chemical shift changes accompany TC dilution. The possibility that TC pre-exists in multimeric forms further implies that even the apparent 1:1 molar ratio found for Ca^{2+} binding in most conditions might correspond to a true stoichiometry of 2:2. This possibility cannot be directly addressed by the data, but it would also be consistent with the good fit of the Ca^{2+} data to the one-set-of-sites model. In any case, the molar ratios found here for Ca^{2+} and Mg^{2+} imply fundamental differences in binding mode between the two ions. Although this conclusion may appear improbable, it is consistent with many previous reports.

The second strength of ITC, the ability to quantify protonation effects, also proved essential to clarifying the binding modes of Ca^{2+} and Mg^{2+}. Over the pH range studied, the net charge on free TC ranges from approximately −0.5 to −2.5. Such large variation in charge is expected to strongly influence the binding of divalent cations. Yet for each ion over its accessible pH range the molar ratio of binding is constant. This result indicates that the basis for complex formation is more specific than simple charge compensation. This interpretation is also supported by the fact that the two ions bind with different molar ratios despite their common +2 charge.

Ion binding might also be expected to influence the protonation states of TC. The findings of the present work confirm this expectation. In fact, the net charge in the Ca^{2+}:TC complex is nearly neutral, and is approximately the same at all pH values below 11 despite large variation in the charge on free TC. At pH 11 and higher where free TC carries two or more negative charges, the additional negative charge does not increase ion affinity, and no further shift in proton equilibrium occurs upon Ca^{2+} binding. The −2 charge on Ca^{2+}-bound TC between pH 7.5 and 9.5 results from deprotonation of the second and third ionizable protons on TC. Binding of Ca^{2+} has thus altered the pK_a values of these two protons. Unlike for Ca^{2+}, the charge on the Mg^{2+}-bound TC varies from −1.3 to −1.7, giving rise to Mg^{2+}:2TC complexes with a total charge of −0.6 to −1.4 between pH 7.5 and 9.5. This result also suggests a distinct binding mode of Mg^{2+} as compared to Ca^{2+}.

In free TC, the second and the third ionizable protons are generally assigned to the β diketone moiety (C11–C12) of the BC rings and the C4 dimethylammonium of the A ring, respectively (Scheme 1). Both groups have been implicated in direct interaction with Mg^{2+} and Ca^{2+} ions [16,27]. It is thus reasonable to expect their pK_a values to be altered upon Mg^{2+} and Ca^{2+} binding. Deprotonation of the dimethylammonium group has been linked to a change in free TC conformation from a folded to an extended form [26–28] (Fig. 1). This shift is the result of removing the steric crowding between the bulky positively charged dimethylammonium and the $C12_a$ hydroxyl group and formation of a hydrogen bond between the two groups following deprotonation. Ca^{2+}-induced deprotonation demonstrated in this work may thus shift TC conformation from the folded to the extended form, most significantly at pH 7.5 and 8.5 where one full proton is removed upon binding. Conformational change to the extended form upon Ca^{2+} binding has also been inferred in multiple spectroscopic studies at pH 8 [16]. Unlike for Ca^{2+}, Mg^{2+} binding has been shown spectroscopically to stabilize the folded conformation [16]. This is attributed to the different binding site for the two metal ions, i.e., Mg^{2+} coordinating to the dimethylamino-O3 site and Ca^{2+} coordinating to O10-O11 and O12-O1 sites [16]. Shift of the folded to the extended conformation thus may not occur upon Mg^{2+} binding, consistent with the more limited deprotonation found here, 0.3, 0.6, and 0.2 protons per TC at pH 7,5, 8.5, and 9.5, respectively.

Determination of the extent of deprotonation as a function of pH also permitted estimation of TC ionization enthalpy for the second and the third ionizable protons. TC ionization enthalpy has been reported only for the first and second protons from direct calorimetric measurements [43]. The value estimated here for the second deprotonation, 31 ± 2 kJ/mol, agrees well with the reported value of 34.5 kJ/mol [43]. Both values of TC ionization enthalpy are significantly smaller than the ionization enthalpies of Tris·HCl (47.3 kJ/mol) and CAPS (48.5 kJ/mol). This difference accounts for the pH-dependence of ΔH°_{obs}. Use of

buffers having the same ionization enthalpy as TC would have left the metal ion-induced TC deprotonation undetected, underscoring the importance of buffer choice and the benefit of using multiple buffers.

Determination of the extent of deprotonation as a function of pH also led to the estimation of the intrinsic binding enthalpies (ΔH°_{in}) of Ca^{2+} and Mg^{2+} to TC, specifically, to TC carrying −2 and −2.5 charges in the 1:1 complex with Ca^{2+} and to TC carrying −1.3 to −1.6 charges in the 1:2 complex with Mg^{2+}. Intrinsic binding enthlapy for both metal ions is exothermic at these ionic states. The ΔH°_{in} values for Ca^{2+} binding to the −2 and −2.5 ionic species are identical, −35±2 kJ/mol. The ΔH°_{in} values for Mg^{2+} binding to the −1.3 and −1.6 ionic species are also the same, −15 kJ/mol. The fact that the ΔH°_{in} values for the two metal ions are very different indicates that the identity of the metal ion, not the ionic state of TC, determines intrinsic binding enthalpy. The much less exothermic ΔH°_{in} for Mg^{2+} versus Ca^{2+} binding may reflect a higher extent of dehydration of Mg^{2+} and/or a more unfavorable enthalpic contribution per water removed from the hydration shell of Mg^{2+}, consistent with the higher charge density of Mg^{2+}. The number of waters removed from each Mg^{2+} is expected to be higher because one Mg^{2+} binds two TCs unlike Ca^{2+} which binds one TC. Consistent with an increased loss of water molecules, the observed binding entropy for Mg^{2+} is less negative than for Ca^{2+} at a given pH.

Although ΔH°_{in} is constant for Ca^{2+} throughout the pH range 7.5 to 11.0 and for Mg^{2+} from pH 7.5 to 9.5, ΔH°_{obs} varies greatly with pH. As the ionization enthalpy of either Tris·HCl or CAPS is more endothermic than that of TC, deprotonation of TC and the subsequent protonation of buffer yielded a net exothermic heat. The increasingly less exothermic ΔH°_{obs} with increasing pH is a result of decreasing extent of deprotonation. Although the enthalpic contribution of deprotonation becomes less favorable with increasing pH, the affinity of both ions for TC increases with increasing pH. This is a result of the increasingly less unfavorable ΔS°_{obs}. The change in ΔS°_{obs} is likely due to the less unfavorable overall entropic contribution of TC deprotonation and buffer protonation with increasing pH which more than compensates the increasingly less favorable enthalpic contribution. The change in affinity per unit change in pH, however, is not proportional to the difference in the number of protons removed. The particularly large (12-fold) increase in affinity from pH 7.5 to 8.5 for Ca^{2+} binding, as opposed to the smaller (2-fold) increase from pH 8.5 to 9.5 and 9.5 to 11, is a result of a much more unfavorable entropic contribution relative to the favorable overall enthalpic contribution associated with TC deprotonation and buffer protonation at pH 7.5. This may reflect an entropic cost of a conformational change upon deprotonation at pH 7.5. This suggests that removal of the second ionizable proton may be associated with a more unfavorable entropic contribution.

Endothermic tailing of the exothermic injection peaks accompanies the binding of Ca^{2+} and, to a lesser extent, Mg^{2+}, in both forward and reverse titrations under certain titrant and titrate concentrations at pH 8.5 and above. The increasing presence of this tailing with increasing pH is due in part to the decreasing exothermic binding enthalpy that is less able to mask the endothermic component of each injection heat. At any given pH endothermic tailing was not observed for Mg^{2+} binding in the forward titration or less pronounced in the reverse titration than for Ca^{2+} binding. The effect of this tailing on curve-fitting and on the derived values of binding parameters was minimized in titrations at relatively high concentrations that limited its effect to the first injection points. Artifactual sources of the endothermic component of the reaction heat were ruled out through a series of control experiments. Thus, it is necessary to consider that the endothermic component of the reaction heat may represent a process that is inherently associated with metal ion binding but is not proportional to the extent of ion binding.

Deprotonation of TC, excluding the possible coupled conformational change, though endothermic, is ruled out as a source of the endothermic tailing because at pH 11.0 and 11.7 where the endothermic tailing was most significant there is no evidence for the presence of TC deprotonation. The endothermic process may reflect an equilibrium associated with the metal ion, perhaps an equilibrium between the free metal ion and its complex with the hydroxide ion, even though no metal ion hydroxide precipitate is visible. However, endothermic tailing is less pronounced for Mg^{2+} than Ca^{2+} at a given pH, even though K_{sp} for magnesium hydroxide is orders of magnitude smaller than for calcium hydroxide. Conformational change of TC could also be linked to metal ion binding as a distinct process that can cause the endotherm. As discussed above, conformational changes likely occur as a result of Ca^{2+}- or Mg^{2+}-induced TC deprotonation at and below pH 9.5. Additionally, metal ion coordination may cause a conformational change in TC, separate from that induced by deprotonation. Indeed, theoretical studies reveal a diversity of tautomeric species that assume either the folded or the extended conformation that coexist at the same pH [22]. Depending on the coordination site of the metal ion, the chemical perturbation resulting from Ca^{2+} and Mg^{2+} binding could shift the equilibria among the different tautomers and the different conformations [22], contributing to the observed binding enthalpy. The different binding mode, both elucidated in various spectroscopic studies and also revealed in the present study, may explain the presence to different extent of the endothermic process in Mg^{2+} and Ca^{2+} binding.

5. Conclusions

TC–metal ion binding in solution has been studied extensively in the past using spectroscopic methods, but absorbance, fluorescence, or CD changes due to metal ion binding cannot be separated from spectroscopic changes that may result from protonation or conformational equilibria. ITC can shed light on protonation equilibria and sometimes on conformational changes coupled to binding. The present work identifies solution conditions in which contributions from conformational processes are minimal, and unique ion:TC stoichiometries can be achieved. These conditions may facilitate structural studies by reducing competition from multiple conformations, and thus enable analysis of metal ion–TC complexes that could shed light on their biological modes of action. The results also suggest that theoretical calculations involving TC with bound Ca^{2+} ions should consider the HTc^{2-} ionic state.

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