Calorimetric Studies of Metal Binding to Tetracycline. Role of Solvent Structure in Defining the Selectivity of Metal Ion−Drug Interactions†

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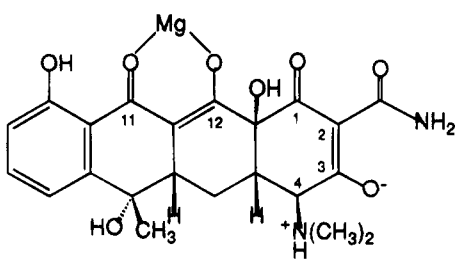
Received December 14, 1994®

Binding enthalpies (ΔH), entropies (ΔS), and free energies (ΔG) have been evaluated for complex formation of tetracycline with Mg^{2+}, Mn^{2+}, Ca^{2+}, and Sr^{2+}. From the temperature dependence of enthalpic and entropic components, the changes in heat capacities (ΔC_p) accompanying complex formation have been determined. The magnitudes of these thermodynamic parameters show a strong correlation with the charge-density of the metal ion, and reflect changes in the immediate hydration sphere of the metal−drug complex that are likely to be involved in defining the extent of any interactions with nucleic acids. The similar binding constants exhibited by the physiologically relevant Mg^{2+} and Ca^{2+} ions do not reflect the variation in entropic and enthalpic factors that underlie complex formation for each of these metal ions.

Introduction

Tetracycline (**1**) is a metal-dependent antibacterial agent that inhibits bacterial protein synthesis by binding to the ribosome.[1] In the extracellular form is likely to exist as either a Mg^{2+} or Ca^{2+} complex since both the binding affinities and extracellular concentrations of Mg^{2+} and Ca^{2+} are similar (1 mM vs 4 mM).[2] However, in the intracellular matrix the magnesium complex is the predominant form. NMR evidence suggests that Mg^{2+} is chelated by oxygen centers as indicated in structure **1**.[3]



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The factors that regulate metal selective binding by drug molecules, and binding of these complexes to cognate DNA sequences are of general interest.[4−6] A full evaluation of the chemical interactions underlying these binding reactions requires characterization of the complete thermodynamic binding profile, since enthalpy−entropy compensations can result in similar binding affinities even if the thermodynamic forces driving the reaction are distinct. Such compensatory relationships can reflect differing modes of binding contact or changes in solvation and counterion complexation and have been widely observed.[7,8] In this paper we evaluate the thermodynamic parameters (ΔG, ΔH, ΔS, ΔC_p) underlying magnesium binding to tetracycline. A comparison is made with the binding properties of Mn^{2+}, Ca^{2+}, and Sr^{2+}. These data reflect changes in the immediate hydration sphere of the metal-drug complex that are likely to be involved in defining the extent of any interactions with nucleic acids. These ions were selected on the basis of their biological relevance and/or distinct charge densities. To eliminate complications from ligand field terms, transition metal ions were not considered, with the single exception of high spin divalent Mn^{2+}.

Experimental Methods

Calorimetry Measurements. Experiments were performed on a Microcal OMEGA ultrasensitive titration calorimeter (MicroCal Inc.). The instrumentation and data acquisition/analysis software have been described elsewhere.[9a] Prior to use, the instrument was calibrated against an internal heat pulse, and the functional response was verifed by determination of the heat of dilution of a concentrated sucrose solution and comparison with the literature value.[9b] Data points were collected every 2 s. Each binding isotherm (Figure 1) was determined following 16 automatic injections from a 250-μL injection syringe (containing 5−10 mM Mg^{2+}) into the reaction cell (1.3415 mL) containing 0.5 mM tetracycline. Injection volumes (15 μl) were delivered over a 10-s time interval with 3−4 min between

† Supported by grants from the Petroleum Research Fund, administered by the American Chemical Society, and Procter and Gamble Company.

‡ J.A.C. is a Fellow of the Alfred P. Sloan Foundation, a Camille Dreyfus Teacher−Scholar, and a National Science Foundation Young Investigator.

® Abstract published in *Advance ACS Abstracts*, May 15, 1995.

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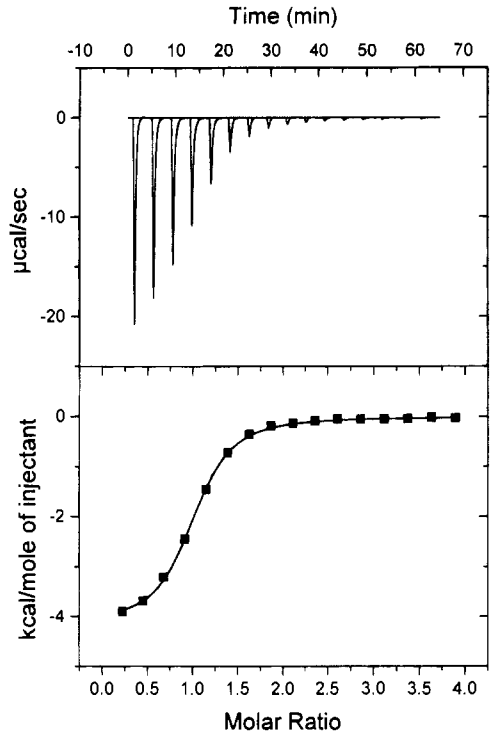

Figure 1. Calorimetric titration of tetracycline (0.5 mM) with 16 × 15 μL injections of Mg^{2+} (10 mM) at 288 K in 20 mM Tris buffer, pH 9.5. The raw data are shown above, and the integrated heats are shown below. The optimal fit to the data gave n = 0.943 binding sites and $K \sim 4.53 \times 10^4\ M^{-1}$.

Table 1. Thermodynamic Parameters for Magnesium Binding to Tetracycline (pH 9.5)

temp (K)	10^4K (M^{-1})	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal K^{-1} mol^{-1})
277.8	2.77 ± 0.14	−5.67 ± 0.03	−5.03 ± 0.05	2.3 ± 0.2
287.6	4.53 ± 0.27	−6.13 ± 0.03	−3.98 ± 0.04	7.4 ± 0.2
297.5	9.74 ± 1.80	−6.8 ± 0.1	−2.81 ± 0.06	13.4 ± 0.4
308.1	6.70 ± 0.62	−6.80 ± 0.06	−2.38 ± 0.04	14.4 ± 0.2

injections to allow complete equilibration. Background buffer was the same for both solutions (20 mM Tris) to minimize heat changes from mixing. Adjustments to solution pH were made by addition of small aliquots of hydrochloric acid or sodium hydroxide. To overcome solubility problems for Mn^{2+} at higher pH, a solution of Mn^{2+} (pH ~ 7.5) was added to an alkaline solution of tetracycline (pH ~ 9.5). The resulting solution pH lay in the range between 8 and 9, in the window between the published ligand pK_a's of approximately 7.5 and 9.6.[3] For the other metal ions the pH of both solutions was 9.5. Control experiments in the absence of tetracycline were used to determine and, if necessary, correct for background heats of dilution or (de)protonation of the Tris buffer. All solutions were thoroughly degassed under vacuum prior to data aquisition to obtain better baseline stability. The data curves were obtained using the resting baseline determined by the software. During data fitting, all parameters were floating: including, heat of binding (ΔH), binding constant (K), and the number of binding sites (n).

Determination of Thermodynamic Parameters. Titration calorimetry affords a convenient method for complete characterization of the binding thermodynamics of metal-ligand interactions.[10] The net heat change provides a direct measurement of reaction enthalpy (ΔH), while the titration method (as

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Table 2. Thermodynamic Parameters for Calcium Binding to Tetracycline (pH 9.5)

temp (K)	10^4K (M^{-1})	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal K^{-1} mol^{-1})
277.8	10.8 ± 1.3	−6.40 ± 0.07	−13.1 ± 0.2	−24.1 ± 0.7
287.6	11.5 ± 2.7	−6.7 ± 0.1	−11.0 ± 0.2	−15 ± 1
297.5	11.4 ± 1.9	−6.9 ± 0.1	−10.0 ± 0.2	−10.6 ± 0.6
308.1	12.9 ± 1.8	−7.20 ± 0.08	−8.8 ± 0.2	−5.3 ± 0.6

Table 3. Thermodynamic Parameters for Manganese Binding to Tetracycline (pH 9.0)

temp (K)	10^4K (M^{-1})	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal K^{-1} mol^{-1})
277.8	5.93 ± 0.26	−6.07 ± 0.02	−4.67 ± 0.04	5.0 ± 0.2
287.6	6.13 ± 0.57	−6.31 ± 0.05	−4.09 ± 0.08	7.7 ± 0.3
297.5	6.55 ± 0.85	−6.57 ± 0.08	−3.21 ± 0.08	11.3 ± 0.4
308.1	24.9 ± 7.5	−7.6 ± 0.2	−2.43 ± 0.08	16.8 ± 0.6

Table 4. Thermodynamic Parameters for Strontium Binding to Tetracycline (pH 9.5)

temp (K)	10^4K (M^{-1})	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal K^{-1} mol^{-1})
277.8	6.16 ± 0.09	−6.10 ± 0.01	−13.57 ± 0.04	−26.8 ± 0.1
287.6	6.45 ± 0.18	−6.33 ± 0.02	−12.2 ± 0.07	−20.4 ± 0.2
297.5	6.41 ± 0.35	−6.54 ± 0.03	−10.8 ± 0.1	−14.2 ± 0.4
308.1	6.01 ± 0.37	−6.73 ± 0.04	−8.6 ± 0.1	−5.9 ± 0.4

distinct from batch calorimetry) gives the binding constant, the reaction free energy (ΔG), and by difference, the reaction entropy (ΔS). Calorimetric plots (such as the one shown in Figure 1) for the reaction summarized in eq 1 were obtained at

$$M^{2+}(aq) + tet^{-}(aq) \rightleftharpoons [M\text{-}tet]^{+}(aq) \quad (1a)$$

$$\Delta H = \Delta H_f\{[M\text{-}tet]^{+}(aq)\} - [\Delta H_f\{[M]^{2+}(aq)\} + \Delta H_f\{tet^{-}(aq)\}] \quad (1b)$$

$$\Delta S = \Delta S_f\{[M\text{-}tet]^{+}(aq)\} - [\Delta S_f\{[M]^{2+}(aq)\} + \Delta S_f\{tet^{-}(aq)\}] \quad (1c)$$

$$\Delta\Delta H = \Delta C_p \Delta T \quad (2)$$

$$\Delta\Delta S = \Delta C_v \Delta(\ln T) \quad (3)$$

four temperatures, and changes in heat capacity were obtained by fitting to eqs 2 and 3, where all the symbols have their normal meanings. Protonation of O12 at the metal binding site (see illustration **1**) is significant at solution pH's below 7.5, and so experiments were carried out at a pH of 9.5, in the window between the published ligand pK_a's of approximately 7.5 and 9.6,[3] to ensure complex formation between the specific solution species indicated by eq 1 and shown in illustration **1**. Independent pH titration studies indicated that the protonation state of N4 had no influence on the binding constant of magnesium ion.

Results

For each metal complex studied we have evaluated each of the principal thermodynamic parameters (ΔG, ΔH, ΔS, ΔC_p) by direct titration calorimetric methods. Changes in heat capacity (ΔC_p) accompanying complex formation were independently evaluated from the temperature dependence of ΔH and ΔS parameters as defined by eqs 2 and 3. Tables 1−5 summarize the data obtained for Mg^{2+}, Mn^{2+}, Ca^{2+} and Sr^{2+} binding, and Figure 2 shows typical plots of ΔH or ΔS vs T or ln T, respectively. Under conditions of constant pressure and

Table 5. Changes in Heat Capacities for Metal Binding to Tetracycline

	Mg^{2+}	Ca^{2+}	Sr^{2+}	Mn^{2+}
charge density $(q/r)^a$	2.56	1.89	1.57	2.2
ΔC_p (cal K^{-1} mol^{-1})^b	107 ± 17	159 ± 20	187 ● 20	95 ± 19

[a] Evaluated assuming q = integral charge and taking r = ionic radius in Å according to Goldschmidt.[16] [b] Average from the temperature dependence of ΔH and ΔS (Figure 2).

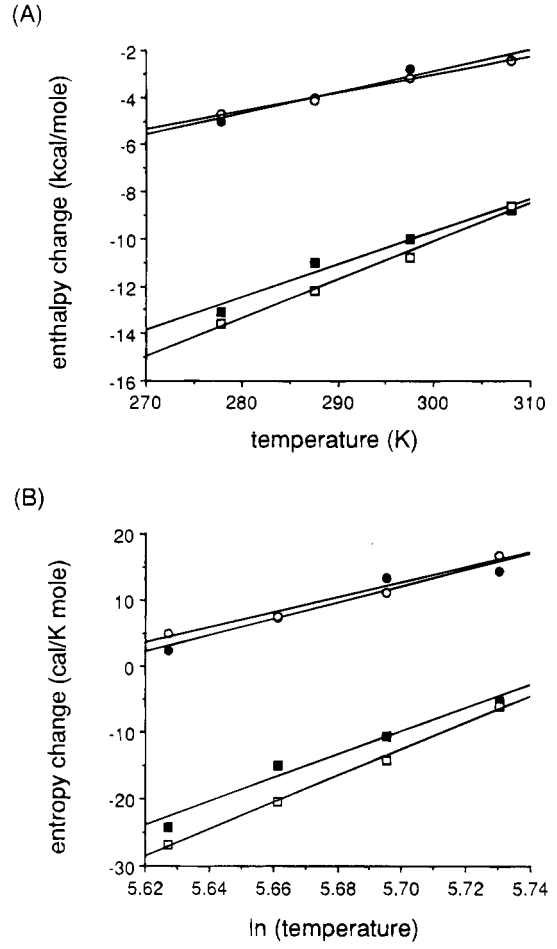

Figure 2. Plots of the variation of enthalpy (A) and entropy (B) change as a function of temperature for Mg^{2+} (●), Mn^{2+} (○), Ca^{2+} (■), and Sr^{2+} (□) binding to tetracycline. Data taken from Tables 1–4.

volume $\Delta C_p \sim \Delta C_v$, and was demonstrated to be within the limits of experimental error (Table 5). The heat capacity data determined from variable temperature experiments (Table 5 and Figure 2) show a general correlation with the charge densities of the cations, with a minor deviation observed for Mn^{2+}. For all data sets, the R-factors after fitting by linear regression were always better than 0.97, and so the quality of the fits was extremely good in all cases.

Of particular interest was the effect of the charge density of the metal cation on the binding parameters. For this reason a comparison was made of the binding chemistry of Mg^{2+} and Mn^{2+} relative to that of the larger ions Ca^{2+} and Sr^{2+}. All efforts to obtain data with trivalent ions (Al^{3+}, Fe^{3+}) failed as a result of precipitation problems. A relatively low pH (<5) was required to maintain solubility of the metal cation, however, binding to tetracycline was inhibited by protonation of the ring. Solubility problems were also noted with Mn^{2+}; however, this was overcome by adding a neutral solution of Mn^{2+} (pH ~ 7.5) to an alkaline solution of tetracycline (pH ~ 9.5). Subtraction of appropriate control data provided a workable system. Unfortunately this strategy was unsuccessful when applied to the trivalent ions.

When experiments were performed as a function of pH, the number of binding sites (n) was found to decrease as the pH was lowered, reflecting protonation of the metal binding site. The pH dependence of the number of sites (n) yielded a pK_a for the coordination site on the tetracycline ligand that is in good agreement with published values ($pK_a \sim 7.5$).[3] Moreover, independent pH titration studies indicated that the protonation state of N4 (illustration **1**) had no influence on the binding properties of magnesium ion in the pH range employed in this study. It should also be noted that the binding constants reported here cannot be reasonably compared with previous estimates since literature values were obtained under a variety of solution temperatures, pH, and ionic strength.[1]

Discussion

Enthalpic (ΔH) and Entropic (ΔS) Terms. It is important to note that despite the large number of potential metal ion binding sites around the tetracycline ring, in all cases studied the coordination site has been shown to be that illustrated in **1** for Mg^{2+}, irrespective of the metal ion, and so thermodynamic data obtained from a range of divalent metal ions can be reasonably compared. Binding constants and free energies have previously been reported over a narrow range of temperatures,[11-14] however, the measurements were made in distinct laboratories and no entropic or enthalpic values were reported. Also, the clear temperature dependence of ΔH and ΔS (Tables 1–4, and Figure 2) dictates that only average values of these parameters can be determined from van't Hoff plots of ln K vs $1/T$. By calorimetric methods the binding enthalpies and entropies can be directly determined. Comparison of these binding parameters for the various cations studied reveals that the binding of Mg^{2+} and Mn^{2+} is both enthalpically and entropically driven but is only enthalpy driven for Ca^{2+} and Sr^{2+} (Tables 1–4). The similarity in binding constants arises from a compensatory adjustment of entropic and enthalpic values. The change in sign of ΔS reflects the distinct hydration environments for each ion. The higher charge density of Mg^{2+} and Mn^{2+} promotes longer range ordering of solvent waters relative to Ca^{2+} and Sr^{2+}. After chelation by the tetracycline anion, the hydration sphere of Mg^{2+} and Mn^{2+} is most influenced by the decrease in local charge and the hydrophobicity of the tetracycline ring. Consequently, the release of waters from the solvation shell results in a significant increase in ΔS. The negative ΔS value noted for Ca^{2+} and Sr^{2+} most likely reflects the structure-forming contribution from water molecules around the hydrophobic tetracycline ring.[15] Such an interaction is also present in the magnesium complex but is hidden by the more extensive changes in hydration of Mg^{2+} and Mn^{2+} as described earlier.

The more favorable enthalpic component for the Ca^{2+} and Sr^{2+} complexes reflects facile coordination of this larger cation by the tetracycline chelate. Coordination of the smaller Mg^{2+} and Mn^{2+} ions most likely introduces strain on the metal–ligand bonds that form the six-membered ring chelate complex. Hancock has documented strong evidence that six-membered

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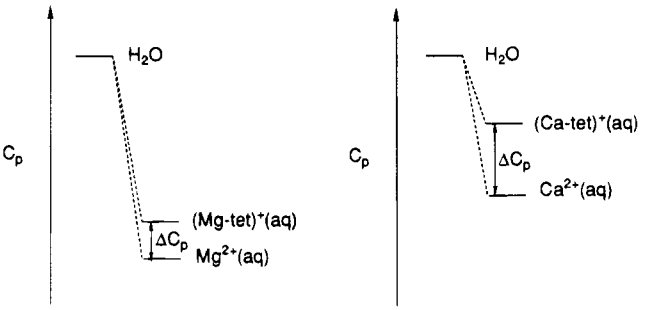
ring chelates favor smaller cations:[17] however, this analysis was applied to ligands based on aliphatic chains and invoked the steric requirements of cycloalkane-like structures. The analysis invokes the conformational preferences of cyclohexane-like structures in terms of prefered torsional angles and strain energy. We rationalize the apparent dichotomy by pointing out that the chelate ring in tetracycline possesses a higher degree of unsaturation that enforces a planar geometry, and so a direct comparison is probably not possible. Finally, we note that the data in Tables 1−4 reflect loss of hydration energy through partial loss of the solvent shell surrounding Mg^{2+} and Mn^{2+}, as described earlier.

Heat Capacities (ΔC_p). In aqueous solution the heat capacity of a complex depends on its influence on the structure of solvent water. The heat capacity of pure water is intrinsically large (C_p = 76 J mol^{-1} K^{-1})[15a] as a result of the internal ordering arising from extensive hydrogen bonding. Ionic solutes disrupt the organized structure of liquid water and, as a consequence, lower C_p. For example, the heat capacity of a solution of KBr decreases linearly as $[KBr]^{1/2}$.[15b] By analogy, the relative ΔC_p values for aqueous solutions of a metal complex and the free solvated metal ion can be understood in terms of the influence of each species on solvent structure. The change in heat capacity accompanying the complexation reaction described by eq 1 can be written in the form of eq 4. Inasmuch as any

$$\Delta C_p = C_p\{[\text{M-tet}]^+(\text{aq})\} - \{C_p\{[\text{M}]^{2+}(\text{aq})\} + C_p\{\text{tet}^-(\text{aq})\}] \quad (4)$$

contribution from the tetracycline ligand, $C_p\{\text{tet}^-(\text{aq})\}$, will be approximately constant, attention can be focused on the metal ion terms, $C_p\{[\text{M-tet}]^+(\text{aq})\}$ and $C_p\{[\text{M}]^{2+}(\text{aq})\}$. Ions with higher charge density possess larger solvation volumes in aqueous solutions, since they promote ordering in both inner and outer coordination spheres. In turn this results in a more extensive breakdown in solvent structure. Subsequent binding

Scheme 1



of these ions by tetracycline reduces the local charge density and results in a partial reordering of solvent molecules with an increase in the heat capacity of solvent water, although this will remain smaller than the heat capacity for pure water. These effects are similar to the factors controlling the sign and magnitudes of the enthalpic and entropic terms, as described earlier. The values obtained for ΔC_p in Table 5, therefore, reflect the extent by which C_p for pure water is lowered by M^{2+}(aq) relative to $[\text{M-tet}]^+$(aq). Scheme 1 presents a comparison of the situation for Mg^{2+} vs Ca^{2+}. A decrease in C_p is most favorable for cations of higher charge density, and the magnitude of ΔC_p can be understood in these general terms. The magnesium ion, bearing a high charge density, lowers C_p significantly for both the solvated and complexed ions. The effect is less pronounced for the larger calcium ion, and the tetracycline ligand is more effective in mollifying the influence of the divalent charge. However, comparison of data for Mg^{2+} and Mn^{2+} in Table 5 illustrates the fine balance for metal ions of similar charge densities.

Inasmuch as the interaction of metal-drug complexes with nucleic acids is intimately connected to displacement of solvent molecules and counterions from the latter, a quantitative understanding of the solution chemistry and solvent ordering (or disordering) propensities of metal ion−drug complexes is pertinent to the rationalization of their mechanism of action.

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