SELECTIVITY OF CATION CHELATION TO TETRACYCLINES: EVIDENCE FOR SPECIAL CONFORMATION OF CALCIUM CHELATE

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SUMMARY: Tetracycline antibiotics in apolar solvents chelate to Ca in a different conformation from that of the Mg chelate. Evidence for this different conformation is adduced from the fluorescence, absorption and circular dichroism spectra of the antibiotic bound to Ca and Mg. The conformation of the antibiotic chelated to Ca is a high affinity form. Only those divalent cations of a size similar to or greater than that of Ca are able to induce this conformation. It is tentatively proposed that liganding occurs between both the A ring and the BCD ring conjugated system.

Tetracycline antibiotics show a propensity for chelation with divalent cations. Several authors have determined the selectivity of chelation with a range of cations in aqueous solution and it has been considered that this chelation plays a determining role in the bacteriostatic properties of the antibiotic (1,2,3). Chelation of the antibiotic also determines its uptake, body distribution and deleterious action on bone and tooth growth (4).

The demonstration by Caswell and Hutchison (5) that tetracyclines bind preferentially to cations on membrane surfaces and the propensity of the chelate to bind to serum albumin suggest that the chelation properties and mechanism of action of the antibiotic might most readily be understood by determination of the conformation in apolar media. The present paper describes the marked alterations of chelation properties of the antibiotic as the medium polarity is reduced and the appearance of a specific conformation in the presence of Ca^{++} associated with high affinity to the cation.

Materials and Methods

Tetracycline antibiotics were obtained as follows: chlorotetracycline and tetracycline from Nutritional Biochemicals Corp.; Doxytetracycline from Pfizer Laboratories; Minocycline from Lederle Laboratories.

Fluorescence spectra were observed using a Hitachi-Perkin-Elmer MPF-2A Spectrofluorometer. Correction of the spectra for variation in lamp intensity with excitation wavelength was effected using ferrioxalate actinometry in situ and determining the lamp output as a function of wavelength. Absorption spectra were determined with a Perkin-Elmer Coleman 124 spectrophotometer and fixed wavelength absorption with a Beckman DU2 spectrophotometer. Circular dichroism spectra were run on a Cary 60 spectropolarimeter.

Results and Discussion

Bathochromic shifts in the UV spectra of tetracyclines induced by additions of divalent and trivalent cations in aqueous solution have been reported by various authors (1,2). The effect of cations on tetracycline fluorescence has also been reported (5,6). Mitscher et al (7) observed alterations in the CD spectra of tetracyclines on addition of cations in aqueous solution. No material difference has been reported between the spectra of the Ca chelate and the Mg chelate. Our results confirm that any difference between the Ca and Mg chelate in aqueous solution is slight. Conversely in 90% methanol we observe a dramatic alteration in the spectra of the Ca chelate as compared with the Mg chelate or compared with the chelates of both cations in aqueous solution. This difference covered the fluorescence excitation and emission spectra, the UV absorption spectrum and the CD spectrum. These spectra are shown in Fig. 1.

Both the fluorescence excitation spectrum and the UV absorption spectrum show a bathochromic shift of the near UV peak of about 8nm of the Ca^{++} chelate compared with the Mg chelate (Fig. 1A and C). In the far UV region a more extensive alteration is reflected by the appearance of an absorption maximum at 290nm for the Ca complex which is absent or masked in the Mg complex; also the Ca complex shows a diminution in the peak at 270nm. These modifications in the UV absorption and excitation spectra are not observed if a purely aqueous solution is employed. The fluorescence emission spectrum of Ca-chlorotetracycline also differs from that

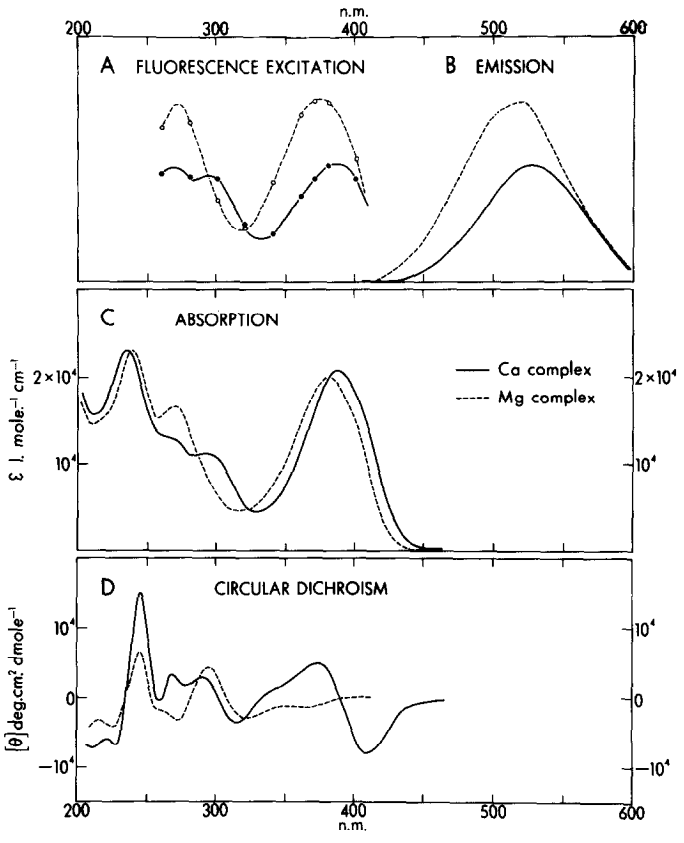
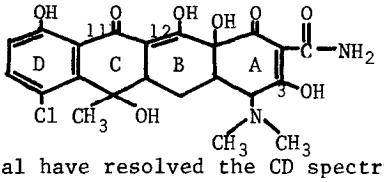


Fig. 1. Alterations of chlorotetracycline chromophores on binding to divalent cations. All spectra were run in 90% methanol buffered with Tris Cl, pH 7.4. Cations were added as 2mM cation chloride. The points on the fluorescence excitation spectra represent the wavelengths at which actinometer readings were made and corrections for light intensity variation carried out. The emission spectra are uncorrected. Solid traces represent the Ca complex and dashed traces the Mg complex.

of Mg-chlorotetracycline in 90% methanol solution. Fig. 1B shows that the emission spectrum of the Mg chelate has a peak at 520nm and is skewed while that of the Ca-chelate is at 530nm and is more nearly gaussian.

The extensive differences between the optical absorption and fluorescence spectra of Mg and Ca complexes in 90% methanol are indicative of a conformational difference between the two chelates. Circular dichroism is responsive to the asymmetry of the chromophore of the molecule and so this technique was employed in further study of the conformation of the chelate. Two chromophore regions exist in the tetracycline molecule asso-

ciated respectively with the A ring and the BCD ring.



chlorotetracycline

Mitscher et al have resolved the CD spectrum into the two chromophores through their conclusion that the CD peak at 262nm belongs to the A ring chromophore. Our CD spectra in methanol correspond to those of Mitscher et al (8) in aqueous solution except that we are able clearly to resolve the 260nm region negative peak into two separate peaks located at 262nm and 273nm in the spectrum of the Mg complex. The spectrum of the Ca complex is materially different from that of the Mg complex. Large CD signals appear in the near UV-visible region at 375 and 415nm which are absent or negligible in the Mg spectrum. The far UV spectra are altered in intensity and a positive peak at 268nm replaces the negative peak at 273. It is not possible to resolve a difference in the peak at 262nm. The CD alterations are diagnostic of a conformational difference between the Ca and the Mg complex. Participation of the A ring in the complex with Ca is implied by the alteration of the 273nm peak; however, it is necessary to clarify the issue as to whether this peak should be ascribed to the A ring spectrum.

Other tetracyclines have been examined to determine whether the specific Ca conformation is a general property of the antibiotic. Tetracycline, doxycycline and minocycline all shows UV spectra corresponding to the existence of a special Ca conformation.

The special conformation of the Ca chelate implies that this should be associated with an alteration in affinity of Ca for the tetracycline. If this conformation is associated with a molecular fit of the Ca^{++} ion into a crevice in the tetracycline molecule, then the transition from the aqueous configuration of the Ca chelate to the thermodynamically more stable apolar conformation should be associated with an extensive increase in

affinity. The K_Ds of the cations to chlorotetracycline in the aqueous phase (Table I) are those observed when the conformation of the chelate is similar in each case. However in 70% methanol the full Ca conformation is obtained for Ca while the Mg chelate remains in the same conformation as in water. The order of affinities in the aqueous phase is Zn>Sr>Mg>Ca. In the 70% methanol phase the order is altered to Zn>Ca>Mg>Sr. In the third column the ratio of the O.D. at 305 to that at 270nm is shown which is considered as a criterion for the formation of the Ca conformation. The ratio of the K_D of Ca in methanol compared with water phase is .020 while that for Mg is .093, indicating that the Ca conformation is a high affinity form. To test the hypothesis that the Ca conformation is determined by a molecular fit, a range of cations have been examined for formation of the

TABLE I

Affinities of cations for chlorotetracycline in aqueous and methanolic solutions.

Cation	K_D aqueous μM	K_D 70% methanol μM	OD $\frac{305}{270}$nm.	ionic radius Å
Mg^{++}	267	24.7	.354	.65
Zn^{++}	42	7.0	.420	.74
Co^{++}			.482	.74
Mn^{++}			.447	.80
Cd^{++}			.535	.97
Ca^{++}	440	9.0	.745	.99
Sr^{++}	65	44.4	.557	1.13
Ba^{++}			.526	1.35

K_D estimations were made through fluorescence titrations as described previously (5). 1:1 complexes were formed in all cases. The OD estimates were carried out in 70% methanol with 25μM chlorotetracycline, 1mM divalent cation chloride, 1mM Tris Cl pH 7.4.

Ca conformation. Only those cations similar to or larger than Ca show a material increase in the absorption at 305nm, i.e. Cd^{++}, Sr^{++} and Ba^{++}, while Ca^{++} shows the strongest appearance of absorption at 305nm (Table I).

The existence of a 1:1 complex between cation and chlorotetracycline in aqueous and 70% methanol phase shown by Table I requires that liganding to Ca^{++} occur through a single tetracycline molecule. The dramatic effect on the K_D of Ca^{++} caused by reducing the water concentration implies that chelation involves ligand coordination with extensive cation dehydration. CPK molecular models show that such an optimum coordination could best occur through bending of the A ring back towards the B and C rings so that oxygen atoms at positions 11 and 12 in conjunction with those at the 3 and 2 (carboxamide) positions form a coordination site within which the cation fits. Molecular models show that considerable strain would be involved in liganding to a small cation.

The finding that tetracyclines exhibit a selectivity for binding to Ca in apolar media should be significant in understanding their mode of action. It is intriguing to consider that tetracycline antibiotics bind to divalent cations with a selectivity reminiscent of that imposed by molecular fit of monovalent cations to transport inducing antibiotics. The special optical and fluorescence properties of the Ca conformation enhance the value of tetracyclines as fluorescent probes of cations bound to biological membranes.

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