Closely Related Ionophores Cezomycin and Calcimycin (A 23187): Cooperative Formation of the Transporting Species

Anne-Marie Albrecht-Gary,*,† Sylvie Blanc,† Lucien David,‡ and Georges Jeminet§

Laboratoire de Physico-chimie Bioinorganique, URA 405 CNRS, EHICS, 1, rue Blaise Pascal, 67000 Strasbourg, France, Laboratoire de Microbiologie, Université Blaise Pascal, 63177 Aubière Cedex, France, and Laboratoire de Chimie Organique Biologique, URA 485 CNRS, Université Blaise Pascal, 63177 Aubière Cedex, France

Received December 22, 1992®

Cezomycin, a biosynthetic analogue of calcimycin (A 23187), was studied to evaluate the effect of changes in structural features on the coordination and transport of calcium and magnesium. Its protonation and calcium and magnesium complexation properties were examined at equilibrium in pure methanol using potentiometric and spectrophotometric methods. Like calcimycin, cezomycin forms two complexes with calcium and magnesium, a neutral two-ligand A_2M complex and a charged species AM^+. The calcium species are slightly more stable than the corresponding magnesium species, and the cezomycin complexes are about 1 order of magnitude less stable than the corresponding calcimycin complexes. The dissociation kinetics of the complexes under acidic conditions showed no protonated kinetic intermediate complex, there being no secondary amine substituent on the benzoxazole moiety of the calcimycin analogue. Very similar thermodynamic and kinetic behaviours are observed for cezomycin and X 14885A, two calcimycin analogues with respectively no substituent and a hydroxyl group adjacent to the carboxyl function. As with calcimycin and X 14855A, a cooperative effect was observed at equilibrium for the formation of the neutral A_2M complexes and confirmed by the formation and dissociation mechanisms, postulated from kinetic investigations, which show that the rate-limiting steps involve the charged AM^+ complexes.

Introduction

Calcimycin or A 23187[1] (Figure 1a) and ionophore X 14885A[2,3] (Figure 1b), members of the polyether carboxylic acid class of antibiotics, are able to selectively transport divalent cations, in particular calcium. The biological activity of these compounds has been attributed to their ionophore properties.[4] Calcimycin has now become a privileged tool for biologists as a calcium probe in intracellular processes.[5,6] These carboxylic acids complex divalent cations as two carboxylate anions A^-. Their transporting species A_2M are uncharged, with the overall transport reaction producing an exchange of cation M^{2+} for two protons H^+. Rate constants for different steps in the diffusion process have been studied in model phospholipid membranes.[7] Previous work on calcimycin[8-13] and ionophore X 14885A[9] has shown that the calcium/magnesium selectivity is predominantly of kinetic origin.

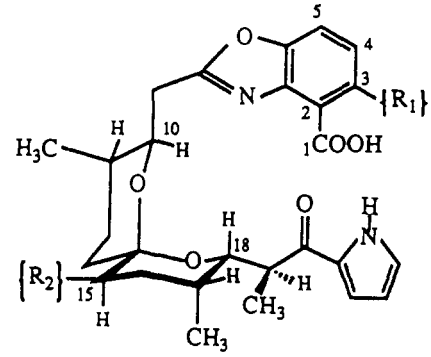

Figure 1. Chemical structures of (a) A 23187 or calcimycin,[1] R_1 = NHMe, R_2 = Me; (b) X 14885A,[2,3] R_1 = OH, R_2 = H; (c) *N*-methylcalcimycin,[14] R_1 = $N(Me)_2$, R_2 = Me; (d) *N*-acetylcalcimycin,[14] R_1 = NMe(COMe), R_2 = Me; (e) cezomycin,[16] R_1 = H, R_2 = Me.

The calcium and magnesium complexes of these antibiotics are very stable and their ionophoric properties are due to the sensitivity of the complexes over medium polarity and to the efficiency of the acid-catalyzed dissociation pathway in physiological conditions.

Thermodynamic studies carried out with natural or chemically modified antibiotics of the calcimycin family, X 14885A,[9] *N*-methylcalcimycin[14] (Figure 1c) and *N*-acetylcalcimycin[14] (Figure 1d) and "ab initio" computations[15] have indicated that an electron-donating substituent at the α-position, able to form a hydrogen bond with the carboxylic acid, is essential for the formation of stable neutral A_2M complexes. Accordingly, we decided to study the thermodynamic and kinetic properties of cezomycin[16] (Figure 1e), a natural analogue of calcimycin that

† EHICS.
‡ Laboratoire de Microbiologie, Université Blaise Pascal.
§ Laboratoire de Chimie Organique Biologique, Université Blaise Pascal.
• Abstract published in *Advance ACS Abstracts,* December 15, 1993.

(1) Hamill, R. L.; Gorman, M.; Gale, R. M.; Higgen, C. E.; Hoehn, M. M. *Proceedings of the 12th Interscience Conference on Antimicrobial Agents and Chemotherapy*; Atlantic City, NJ, 1972; p 32.
(2) Liu, C. M.; Chin, M.; Prosser, B la T.; Palleroni, N. J.; Westley, J. W.; Miller, P. A. *J. Antibiot.* **1983**, *36*, 1118-1122.
(3) Westley, J. W.; Liu, C. M.; Blount, J. F.; Sello, L. H.; Troupe, N.; Miller, P. A. *J. Antibiot.* **1983**, *36*, 1275-1278.
(4) Taylor, R. W.; Kauffman, R. F.; Pfeiffer, D. R. In *Polyether Antibiotics: Naturally Occurring Acid Ionophores*; Westley, J. W., Ed.; Dekker: New York, 1982; Vol. 1, pp 103-184.
(5) Samuelsson, B. *Angew. Chem., Int. Ed. Engl.* **1983**, *22*, 805-815.
(6) Johnson, H. M.; Torbes, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 5959-5962.
(7) Kolber, M. A.; Haynes, D. H. *Biophys. J.* **1981**, *36*, 369-391.
(8) Krause, G.; Grell, E.; Albrecht-Gary, A. M.; Boyd, D. W.; Schwing, J. P. In *Physical Chemistry of Transmembrane Ion Motions*; Spach, G., Ed.; Elsevier: Amsterdam, 1983; pp 255-263.
(9) Albrecht-Gary, A. M.; Blanc-Parasote, S.; Boyd, D. W.; Dauphin, G.; Jeminet, G.; Juillard, J.; Prudhomme, M.; Tissier, C. *J. Am. Chem. Soc.* **1989**, *111*, 8598-8609.
(10) Chapman, C. J.; Puri, A. K.; Taylor, R. W.; Pfeiffer, D. R. *Biochemistry* **1987**, *26*, 5009-5018.
(11) Chapman, C. J.; Puri, A. K.; Taylor, R. W.; Pfeiffer, D. R. *Arch. Bioch. Biophys.* **1990**, *281*, 44-57.
(12) Thomas, T. P.; Pfeiffer, D. R.; Taylor, R. W. *J. Am. Chem. Soc.* **1987**, *109*, 6670-6675.
(13) Tissier, C.; Juillard, J.; Boyd, D. W.; Albrecht-Gary, A. M. *J. Chim. Phys.* **1985**, *82*, 899-906.
(14) Juillard, J.; Tissier, C.; Albrecht-Gary, A. M.; Boyd, D. W.; Jeminet, G. *J. Chim. Phys.* **1987**, *84*, 935-939.
(15) Gresh, N. *Nouv. J. Chim.* **1986**, *10*, 201-204.

0020-1669/94/1333-0518$04.50/0

has no substituent in the α-position, with calcium and magnesium in methanol. These new data on calcium and magnesium transport selectivity are compared with those obtained for calcimycin and antibiotic X 14885A and may afford a better understanding of the ionophore transport mechanism in biological systems.

Experimental Section

Samples of cezomycin were obtained by controlled biosynthesis.[16] Anhydrous methanol (Merck, Waterfree) containing less than 0.01% water was chosen as solvent. Our experiments were always carried out at 25.0 ± 0.1 °C and at a constant ionic strength of 0.1 M using tetrabutylammonium trifluoromethanesulfonate TBATFMS (Fluka, purum). We used perchloric acid (Merck, for analysis, 70%) and tetrabutylammonium hydroxide (Merck, 25% in methanol). Calcium cation was introduced as perchlorate ($Ca(CLO_4)_2 \cdot 4H_2O$, Fluka, purum), which was previously dried in a vacuum oven at 60 °C, and commercial magnesium trifluoromethanesulfonate ($Mg(CF_3SO_3)_2$), Fluka, purum) was used for magnesium cation. The solutions were continuously swept with solvent saturated argon to exclude atmospheric carbon dioxide without evaporation of the solvent.

Potentiometric and Spectrophotometric Measurements. The method employed in our study is based on the classical potentiometric method of hydrogen ion competition to determine stability constants.[9,13] Experiments were always carried out with the antibiotic in the presence or absence of either alkaline earth cation in an acid medium containing about 10^{-3} mol L^{-1} $HClO_4$. The solutions, placed in a jacketed cell maintained at 25.0 ± 0.1 °C by water flow from a HAAKE F1 thermostat, were titrated with tetrabutylammonium methoxide (Merck, 25% in methanol). These solutions were purged for 5–10 min beforehand with solvent-saturated argon. The hydrogen ion concentration was measured with a combined glass electrode (Tacussel, High Alkalinity) in which the reference electrode (Ag/AgCl) was filled with tetrabutylammonium perchlorate (5×10^{-2} mol L^{-1}, Fluka, purum) in methanol. Titrated perchloric acid solutions (~ 10^{-3} mol L^{-1}) and buffer solutions proposed by De Ligny et al.[17,18] were used for standardization in a large scale of protons concentrations. Potential differences were given by a Tacussel Isis 20,000 millivoltmeter. Small samples (0.5 mL) were then taken from the solution for a spectrum, and the spectrophotometric measurements were made in 0.2-cm HELMA quartz cuvettes with a KONTRON UVIKON 860 spectrophotometer. The cezomycin was titrated at a concentration of 2×10^{-4} mol L^{-1} over the $-\log [H^+]$ range 2–10. For the metal ion titrations, the cezomycin concentration, metal ion, concentration, and $-\log [H^+]$ range were as follows: for Ca^{2+}, 2×10^{-4} M L^{-1}, 1×10^{-4} mol L^{-1}, 3–11 and 1×10^{-4} mol L^{-1}, 1.7×10^{-2} mol L^{-1}, 3.5–11.5; for Mg^{2+}, 2×10^{-4} mol L^{-1}, 1×10^{-4} mol L^{-1}, 3–11 and 1×10^{-4} mol L^{-1}, 1×10^{-2} mol L^{-1}, 3.5–11.5. An example of the evolution of the absorption spectra as a function of hydrogen ion concentration is given in Figure 2a for the free antibiotic and in Figure 2b for cezomycin in presence of Ca^{2+}.

Kinetic Measurements. Complex formation and dissociation with cezomycin are fast reactions and were studied by a stopped flow method (Durrum Gibson spectrophotometer). The spectrophotometric data were stored using a DATALAB DL 905 transient recorder, and an on-line Apple II microcomputer was programmed to treat first order or pseudo-first-order kinetics.[19] From our thermodynamic studies and with the help of the HALTAFALL program,[20] it was possible to calculate experimental conditions providing complete formation and dissociation reactions for the kinetic measurements.

Due to the low solubility of a large excess of cation, the AM^+ complex alone could not be formed in methanol. Therefore, we only studied the dissociation kinetics of the neutral complexes of cezomycin with calcium and magnesium. These stable complexes in basic conditions (hydrogen ion concentration less than 10^{-8} mol L^{-1}) were totally formed with a 2/1 ionophore–metal concentration ratio. The same experimental conditions were chosen for calcium and magnesium (0.5×10^{-5} mol L^{-1}) with a concentration of cezomycin of 10^{-5} mol L^{-1}. Complete dissociation of the complexes was observed for hydrogen ion concentrations higher than 2×10^{-6} mol L^{-1}. For proton concentrations higher than 10^{-4} mol L^{-1}, perchloric acid solutions were used. At proton concentrations which were at least 10 times lower than the complex concentrations, a buffer (dichloroacetic acid, Laboratory Reagents/ tetrabutylammonium hydroxide, Merck, 25% in methanol) was employed. The values of the pseudo-first-order rate constants k_{obs} determined in these experimental conditions are presented in Table 1.

We have studied the formation kinetics of the neutral A_2M magnesium and calcium cezomycin complexes at various proton concentrations. Because the reactions are too fast for the stopped-flow technique, we were unable to adopt pseudo-first-order conditions. A 2/1 (ionophore/

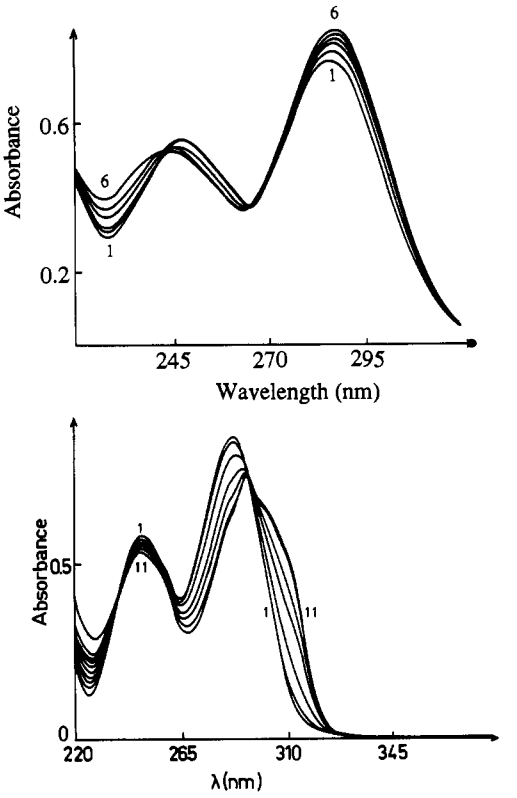

Figure 2. Experimental spectrophotometric study of protonation and calcium complexation of cezomycin. Solvent = methanol; T = (25.0 ± 0.1) °C; I = 0.1 M; l = 2 mm. (a) Top: Absorption spectra of free cezomycin. $-\log [H^+]$ = 4.25, 5.97, 7.55, 8.42, 8.80, and 9.80 for spectra 1–6, respectively. $[\text{cezomycin}]_0 = 2 \times 10^{-4}$ mol L^{-1}. (b) Bottom: Absorption spectra of calcium cezomycin complexes. $-\log [H^+]$ = 3.59, 4.78, 5.35, 5.86, 6.31, 7.19, 7.56, 8.60, 9.37, 9.90, and 10.69 for spectra 1–11, respectively. $[\text{cezomycin}]_0 = 2 \times 10^{-4}$ mol L^{-1}; $[Ca^{2+}]_0 = 10^{-4}$ mol L^{-1}.

Table 1. Variations of the Observed First-Order Rate Constant with Proton Concentrations for the Decomplexation of the Calcium and Magnesium A_2M Cezomycin Complexes[a]

$[H^+]_0$ (mol L^{-1})	$A_2Ca \rightarrow 2AH + Ca^{2+}$ $k_{obs} \pm 2\sigma$ (s^{-1})	$A_2Mg \rightarrow 2AH + Mg^{2+}$ $k_{obs} \pm 2\sigma$ (s^{-1})
2.1×10^{-6}	129 ± 4	0.89 ± 0.05
3.6×10^{-6}	141 ± 7	1.7 ± 0.1
4.3×10^{-6}		2.1 ± 0.1
4.6×10^{-6}		2.33 ± 0.03
7.4×10^{-6}	178 ± 10	2.7 ± 0.1
1.07×10^{-5}	...	3.4 ± 0.1
1.10×10^{-5}	212 ± 20	3.4 ± 0.1
1.27×10^{-5}	227 ± 30	
1.80×10^{-5}	293 ± 25	
2.40×10^{-5}	344 ± 34	7.1 ± 0.2
6.92×10^{-5}		21.0 ± 0.5
1.349×10^{-4}		41.5 ± 0.8
1.380×10^{-4}		42 ± 1
2.500×10^{-4}		65 ± 2
3.715×10^{-4}		107 ± 3
4.179×10^{-4}		123 ± 3
4.265×10^{-4}		135 ± 3
5.310×10^{-4}		164 ± 7
6.456×10^{-4}		190 ± 10

[a] Solvent = methanol; T = (25 ± 0.1) °C; I = 0.1 M.

(16) David, L.; Kergomard, A. *J. Antibiot.* **1982**, *35*, 1409–1411.
(17) De Ligny, C. L.; Luykx, P. F. M.; Rehbach, M.; Wieneke, A. A. *Recl. Trav. Chim. Pays Bas* **1960**, *79*, 699–726.
(18) Alfenaar, M.; De Ligny, C. L. *Recl. Trav. Chim. Pays Bas* **1967**, *86*, 1185–1190.
(19) Lagrange, J.; Lagrange, P. *J. Chim. Phys.* **1984**, *81*, 425–431.
(20) Ingri, N.; Kakolowics, W.; Sillen, L. G.; Warnqvist, B. *Talanta* **1969**, *14*, 1261–1286.

Table 2. Variations of the Experimental Second-Order Rate Constant with Proton Concentrations for the Formation of the Magnesium and Calcium A_2M Cezomycin Complexes[a]

$[H^+]_0$ (mol L^{-1})	$A^- + H^+ \rightleftarrows AH$ (K_1) % $[A^-]/[A]_{tot}$	% $[AH]/[A]_{tot}$	Ca^{2+} $10^{-6}(k_{obs} \pm 2\sigma)$ (mol^{-1} L s^{-1})	Mg^{2+} $10^{-5}(k_{obs} \pm 2\sigma)$ (mol^{-1} L s^{-1})
1.0×10^{-10}	98.0	2.0		40 ± 4
3.0×10^{-10}	96.0	4.0		38 ± 4
5.0×10^{-10}	90.0	10.0		35 ± 3
9.0×10^{-10}	84.0	16.0		33 ± 3
1.7×10^{-9}	76.9	23.1		28 ± 2
2.4×10^{-9}	67.7	32.3		25 ± 1
3.2×10^{-9}	65.0	35.0		22 ± 2
4.6×10^{-9}	56.0	44.0		18.5 ± 1
1.0×10^{-8}	34.5	65.5		11.2 ± 0.5
1.5×10^{-8}	24.9	75.1		8.2 ± 0.5
2.5×10^{-8}	17.3	82.7		5.7 ± 0.3
5.2×10^{-8}	10.0	90.0	43 ± 5	
7.9×10^{-8}	7.0	93.0	34 ± 5	
8.0×10^{-8}	6.0	94.0		2.6 ± 0.2
1.2×10^{-7}	4.5	95.5	22 ± 3	
1.3×10^{-7}	4.0	96.0	22 ± 2	

[a] Solvent: methanol; T = (25.0 ± 0.1) °C; I = 0.1.

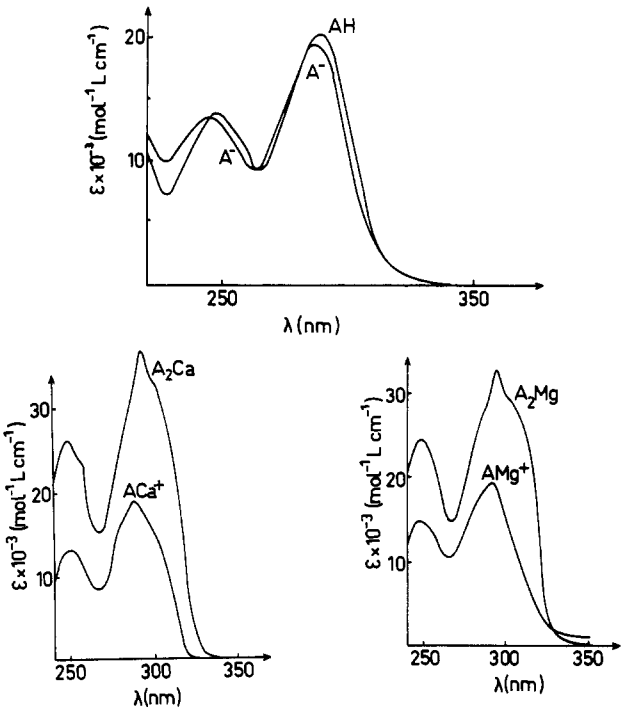

Figure 3. Calculated electronic spectra for various protolytic species of the free antibiotic cezomycin and its calcium and magnesium complexes: (a, top) deprotonated and monoprotonated free cezomycin species; (b, bottom left) calcium cezomycin complexes; (c, bottom right) magnesium cezomycin complex es. Solvent = methanol; T = (25.0 ± 0.1) °C; I = 0.1 M.

cation) concentration ratio was chosen with $[\text{cezomycin}]_0 = 2 \times 10^{-5}$ mol L^{-1}. The range of hydrogen ion concentrations studied was from 10^{-7} to 10^{-9} mol L^{-1}; an imidazole buffer was used under these conditions (pK = 7.92 ± 0.06).[21] The values of the second-order rate constants k determined under those experimental conditions are presented in Table 2.

Results

Thermodynamic and Spectrophotometric Study. The acid–base titration of the free antibiotic cezomycin has provided potentiometric and spectrophotometric data which were fitted by the LETAGROP SPEFO program[22] based on a nonlinear least-squares method. Two protonated species, AH and AH_2^+,

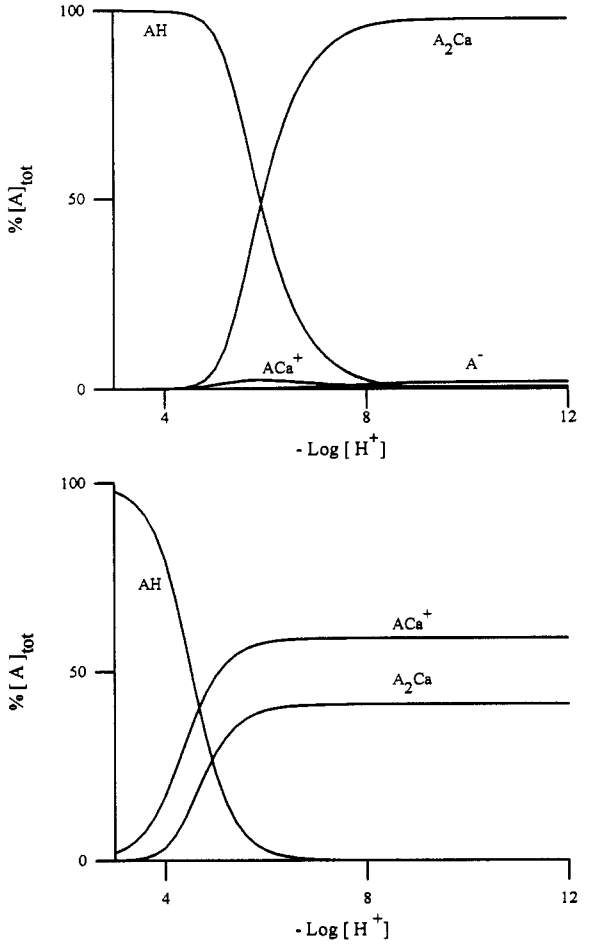

Figure 4. Formation curves of calcium cezomycin complexes under various acidic conditions: (a, top) $[A]_{tot} = 2 \times 10^{-4}$ mol L^{-1}, $[Ca^{2+}] = 10^{-4}$ mol L^{-1}; (b, bottom) $[A]_{tot} = 10^{-4}$ mol L^{-1}, $[Ca^{2+}] = 1.7 \times 10^{-2}$ mol L^{-1}. Solvent = methanol; T = (25.0 ± 0.1) °C; I = 0.1 M.

have been determined; the first protonation constant is log K_1 = 8.3 ± 0.1 and the corresponding calculated electronic spectra (220–350 nm) is presented in Figure 3a. It was not possible under our experimental conditions to calculate an accurate value of the thermodynamic constant related to the second protonation of cezomycin; therefore, the corresponding absorption spectrum of the species AH_2^+ could not be obtained.

The same program[22] was used to fit the experimental potentiometric and spectrophotometric data obtained in the presence of calcium or magnesium. Two complexes have been determined: a neutral complex A_2M and a charged species AM^+ with calcium and magnesium. The thermodynamic stability constants related to these two species are as follows: log β_{ACa^+} = 5.4 ± 0.3; log β_{AMg^+} = 5.2 ± 0.2; log β_{A_2Ca} = 12.8 ± 0.2; log β_{A_2Mg} = 11.8 ± 0.2. The corresponding electronic spectra (230–350 nm) are respectively presented in parts b and c of Figure 3.

In order to illustrate the thermodynamic protonation and calcium complexation properties of cezomycin in methanol under various acidic conditions in a 1/2 calcium/cezomycin concentration ratio and with a large excess of calcium, we present in Figure 4 the formation curves which were calculated using the HALTAFALL[20] program and the previous data.

Kinetic Study. Dissociation Kinetics. The decomplexation reaction of neutral cezomycin magnesium and calcium A_2M complexes under acidic conditions followed first-order kinetics with respect to the complex, the hydrogen ion concentration being kept constant during the reaction either by using a sufficient excess or by buffering the reaction medium. The corresponding rate law can be written

$$v = k_{obs}[A_2M] \qquad (1)$$

with k_{obs} = observed first-order rate constant (s^{-1}).

(21) Boyd, D. W. Thèse d'Etat, Université Louis Pasteur, Strasbourg, France, 1982.
(22) Sillen, L. G.; Warnqvist, B. *Ark. Kemi* **1968**, *31*, 377–390.
(23) Pfeil, A.; Lehn, J. M. *J. Chem. Soc., Chem. Commun.* **1992**, 838–840.

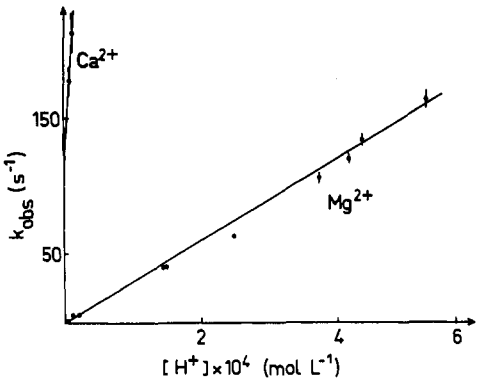

Figure 5. Dissociation of the cezomycin calcium and magnesium A_2M complexes: variations of the pseudo-first order rate constants with proton concentrations. Solvent = methanol; T = (25.0 ± 0.1) °C; I = 0.1 M.

A linear variation of the k_{obs} values (Table 1) with the proton concentrations was observed (Figure 5) for both calcium and magnesium complexes:

$$k_{obs} = k_D + k_H[H^+]_0 \quad (2)$$

with k_D = direct dissociation rate constant (s^{-1}) and k_H = proton assisted dissociation rate constant (mol^{-1} L s^{-1}). The direct decomplexation pathway (k_D) occurs in the absence of protons and is related to the thermodynamic stability of the complexes under our experimental conditions and the rate law becomes:

$$v = (k_D + k_H[H^+])[A_2M] \quad (3)$$

The values of the rate constants k_D and k_H were calculated by the linear regression method and are as follows: for Ca^{2+}, k_D = 100 ± 20 s^{-1}, k_H = (1.00 ± 0.02) × 10^7 mol^{-1} L s^{-1}; for Mg^{2+}, k_D = 0.05 ± 0.05 s^{-1}, k_H = (2.98 ± 0.04) × 10^5 mol^{-1} L s^{-1}.

In agreement with the experimental observations of a single rate-limiting step and of a significant loss of spectrophotometric amplitude during the dead time (~5 ms) of the stopped-flow method and by similarity with results obtained for other carboxylic antibiotics of the same family, namely calcimycin and X 14885A,[9] we propose that the loss of the first antibiotic in the neutral A_2M complex under acidic conditions is faster than the dissociation of the second ligand in the charged complex AM^+:

$$A_2M + 2H^+ \underset{\text{fast}}{\rightarrow} AM^+ + AH + H^+ \xrightarrow{k_H} 2AH + M^{2+} \quad (4)$$

hydrogen ion assisted decomplexation

$$AM^+ \xrightarrow{k_D} A^- + M^{2+} \quad (5)$$

direct decomplexation

A parallel dissociation reaction of the species AM^+ formed by the faster step takes place in methanol which is consistent with the ordinate at the origin determined for the linear variation of k_{obs} with proton concentrations (Figure 5).

Formation Kinetics. The overall reaction for the formation of the neutral calcium and magnesium complexes with cezomycin can be written

$$2A^-_{tot} + M^{2+} \xrightarrow{k} A_2M \quad (6)$$

To reduce the reaction rate, the initial concentration ratio of the reactants was chosen according to the stoichiometry of the reaction (two ligands to one cation). If the reaction is first order with respect to the ionophore and the metal, the spectrophotometric detection of the reaction kinetics yields the following equation:

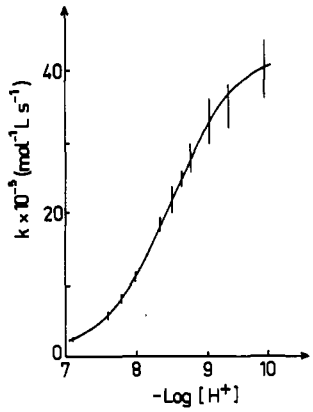

Figure 6. Formation of the neutral magnesium cezomycin A_2Mg complex: variations of the second-order rate constants with proton concentrations. Solvent = methanol; T = (25.0 ± 0.1) °C; I = 0.1 M.

$$\frac{A - A_0}{A_\infty - A} = 2[A^-]_0 kt \quad (7)$$

Here, k = second order rate constant (mol^{-1} L s^{-1}), A_0, A, and A_∞ = the initial, the instantaneous, and the final optical density, respectively, and $[A^-]_0$ = the total initial concentration of ionophore.

We observed for calcium and magnesium cezomycin complexes a single rate limiting step with no more loss of spectrophotometric amplitude than that expected from the dead time of the stopped-flow technique used. The kinetic data fitted eq 7 with an excellent statistical confidence. These results agree with the following rate law:

$$v = k[A^-]_{tot}[M^{2+}] \quad (8)$$

Here, $[A^-]_{tot}$ and $[M^{2+}]$ = instantaneous concentration of total ionophore and cation, respectively. Our experimental observations, which agree with our previous results obtained for calcium and X 14885A,[9] suggest that the rate-limiting step of the overall formation of the neutral A_2M cezomycin complexes is the formation of the charged ligand:

$$2A^-_{tot} + M^{2+} \underset{\text{rate limiting step}}{\xrightarrow{k}} AM^+ + A^- \underset{\text{fast}}{\rightarrow} A_2M \quad (9)$$

The variation of the second order rate constants with proton concentrations is presented in Table 2 for cezomycin magnesium and calcium complexes. Because more experimental kinetic data are available for the magnesium species than for the calcium complex, due to slower formation kinetics of the magnesium complexes, we have chosen to show the variations of the rate constants k with proton concentrations for the magnesium complex in Figure 6. The sigmoidal shape of the curve $k = f(-\log [H^+])$ suggests two competing reactions via the deprotonated species A^- and via the monoprotonated AH of the free ionophore. The following mathematical expression, eq 10, was fitted by a statistical nonlinear least-squares method for the different values of k measured at different concentrations of protons:

$$k = \frac{a + b[H^+]}{1 + c[H^+]} \quad (10)$$

If we write the chemical equations involved in the proposed mechanism, we have to consider the acid–base equilibrium of the free cezomycin

$$A^- + H^+ \overset{K_1}{\rightleftarrows} AH \quad K_1 = \frac{[AH]}{[A^-][H^+]} \quad (11)$$

Table 3. Thermodynamic Constants of Protolytic Species and of Calcium and Magnesium Cezomycin Complexes and Comparison with Calcimycin and Closely Related Ionophores[a]

equilibrium	$A^- + H^+ \rightleftarrows AH$	$AH + H^+ \rightleftarrows AH_2^+$	$A^- + M^{2+} \rightleftarrows AM^+$		$2A^- + M^{2+} \rightleftarrows A_2M$	
	$\log K_1$	$\log K_2$	$\log \beta_{ACa^+}$	$\log \beta_{AMg^+}$	$\log \beta_{A_2Ca}$	$\log \beta_{A_2Mg}$
cezomycin	8.3 ± 0.1	<1.5	5.4 ± 0.3	5.2 ± 0.2	12.8 ± 0.2	11.8 ± 0.2
calcimycin[9]	10.7 ± 0.1	3.3 ± 0.1	6.4 ± 0.1	6.2 ± 0.1	14.1 ± 0.1	13.8 ± 0.2
X 14885A[9]	7.6 ± 0.1	2.3 ± 0.2	6.0 ± 0.1	5.2 ± 0.1	13.8 ± 0.2	12.2 ± 0.2
N-methylcalcimycin[13,14]	9.5 ± 0.2	3.8 ± 0.4	4.5 ± 0.3	4.5 ± 0.1	8.5 ± 0.6	9.5 ± 0.2

[a] The errors indicated in this table are equal to $\pm 3\sigma$. Solvent = methanol; T = (25.0 ± 0.1) °C; I = 0.1 M.

and the two rate-limiting steps of complexation

$$A^- + M^{2+} \xrightarrow{k_A} AM^+ \quad (12)$$

$$AH + M^{2+} \xrightarrow{k_{AH}} AM^+ + H^+ \quad (13)$$

The corresponding rate law can be written

$$v = k[A^-]_{tot}[M^{2+}] = k_{AH}[AH][M^{2+}] + k_A[A^-][M^{2+}] \quad (14)$$

The expression of the second-order rate constant k with $[H^+]$ is

$$k = \frac{k_A + k_{AH}K_1[H^+]}{1 + K_1[H^+]} \quad (15)$$

The overall mechanism is presented here:

$$\begin{array}{l} 2A^- + M^{2+} \underset{k_D}{\overset{k_A}{\rightleftarrows}} AM^+ + A^- \xrightarrow[\text{fast}]{} A_2M \\ + \\ 2H^+ \\ K_1 \downarrow\uparrow \\ 2AH + M^{2+} \xrightarrow{k_{AH}} AM^+ + A^- \xrightarrow[\text{fast}]{} A_2M + 2H^+ \end{array} \quad (16)$$

The thermodynamic K_1 value related to the protonation of the free antibiotic cezomycin and the kinetic parameters k_A and k_{AH} related to the formation of the charged complex AM^+, respectively, via the deprotonated species of cezomycin A^- and the monoprotonated AH, were determined from the kinetic measurements. They are, for calcium, $k_A = (9 \pm 1) \times 10^8$ mol^{-1} L s^{-1} and $k_{AH} = (4 \pm 3) \times 10^6$ mol^{-1} L s^{-1} and, for magnesium, $k_A = (4.21 \pm 0.02) \times 10^6$ mol^{-1} L s^{-1} and $k_{AH} = (9 \pm 2) \times 10^4$ mol^{-1} L s^{-1}.

Discussion

The thermodynamic K_1 and K_2 values determined for the formation of the protonated species of the free ionophore cezomycin in pure methanol are shown in Table 3. These data are compared with the corresponding parameters obtained in the same conditions for calcimycin,[8] X 14885A,[9] and *N*-methylcalcimycin, a chemically[13,14] modified analogue.

We observed a decrease of about 2 orders of magnitude in the protonation constant K_1 of cezomycin compared with calcimycin and a similar K_1 value for the two ionophores cezomycin and X 14885A (Table 3). The possible hydrogen bonds[9] in the monoprotonated species AH, formed by protonation of the carboxylic group, of calcimycin and of three closely related antibiotics are represented in Figure 7. Clearly, the presence of an amine substituent at the α-position of the carboxylic acid (calcimycin, *N*-methylcalcimycin) leads to higher protonation constants for the carboxyl function. A less important implication for the acidic properties of the carboxyl function is the possibility of hydrogen bonding with the α-substituent. A decrease of 3 orders of magnitude was observed for X 14885A compared with calcimycin. These two ionophores can have hydrogen bonds between the α-substituent and the carboxyl group. Due to the low value of the second protonation constant K_2, we were unable to obtain precise spectrophotometric data for the diprotonated free cezomycin, but by comparison with X 14885A,[9] the K_2 value could be assigned to the oxazole nitrogen of the molecule.

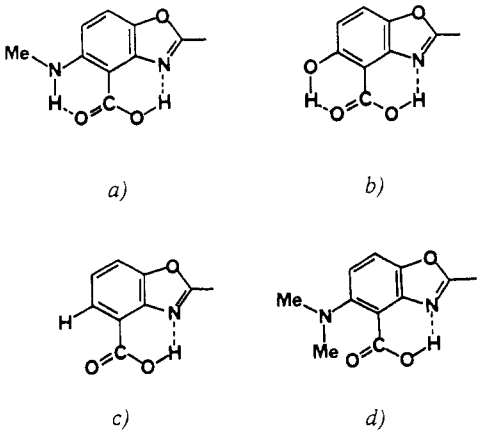

Figure 7. Hydrogen bonds in monoprotonated species of calcimycin and closely related ionophores: (a) calcimycin; (b) X 14885A; (c) cezomycin; (d) *N*-methylcalcimycin.

The coordination properties of cezomycin and X 14885A with magnesium are very similar; they form magnesium complexes which are about 1 order of magnitude less stable than the corresponding calcimycin species (Table 3). The cezomycin calcium complexes are significantly weaker than the calcium complexes formed with calcimycin and X 14885A. The decrease in the volume of the α-substituent from calcimycin to cezomycin (Figure 1) and then the reduction of the steric hindrance lead to a strong enhancement of the positive cooperative effect in the formation of the neutral calcium cezomycin complex.[24,25] If we consider the successive complex formation equilibria

$$A^- + M^{2+} \overset{\beta_{AM^+}}{\rightleftarrows} AM^+ \quad (17)$$

$$AM^+ + A^- \overset{K_{A_2M}}{\rightleftarrows} A_2M \quad (18)$$

with

$$\beta_{A_2M} = \beta_{AM^+} \times K_{A_2M} \quad (19)$$

a higher thermodynamic stability of the neutral A_2M complex compared with the charged complex is observed for the natural antibiotics:

$$\log K_{A_2Ca} - \log \beta_{ACa^+} = 2.0 \text{ (for cezomycin)}$$

$$\log K_{A_2Mg} - \log \beta_{AMg^+} = 1.4 \text{ (for cezomycin)}$$

$$\log K_{A_2Ca} - \log \beta_{ACa^+} = 1.3 \text{ (for calcimycin)}$$

$$\log K_{A_2Mg} - \log \beta_{AMg^+} = 1.4 \text{ (for calcimycin)}$$

$$\log K_{A_2Ca} - \log \beta_{ACa^+} = 1.8 \text{ for X 14885A}$$

$$\log K_{A_2Mg} - \log \beta_{AMg^+} = 1.8 \text{ for X 14885A}$$

The strongest cooperative effects appear for cezomycin with calcium and for X 14885A with calcium and magnesium. The

(24) Perlmutter-Hayman, B. *Acc. Chem. Res.* **1986**, *19*, 90–96.
(25) Wilkins, R. G. In *The Study of Kinetics and Mechanism of Reactions of Transition Metal Complexes*; Ed.-Allyn and Bacon: Boston, MA, 1974; Chapter 4, pp 181–184.

Table 4. Cezomycin and Closely Related Antibiotics of the Calcimycin Family: Calcium and Magnesium Coordination Properties and Thermodynamic and Kinetic Parameters[a]

		kinetic params					thermodynamic params	
complexes		$k_D \pm 2\sigma$ (s^{-1})	$k'_H K_{AMH^{2+}} \pm 4\sigma$ (mol^{-1} L s^{-1})	$k_H \pm 2\sigma$ (mol^{-1} L s^{-1})	$k_A \pm 3\sigma$ (mol^{-1} L s^{-1})	$k_{AH} \pm 3\sigma$ (mol^{-1} L s^{-1})	log $(k_A/k_D) \pm 5\sigma$	log $K_{AM^+} \pm 3\sigma$
cezomycin	Ca^{2+}	100 ± 20		$(1.0 \pm 0.2) \times 10^7$	$(9 \pm 1) \times 10^8$	$(4 \pm 3) \times 10^6$	7.0 ± 0.2	5.4 ± 0.3
	Mg^{2+}	0.05 ± 0.05		$(2.98 \pm 0.04) \times 10^5$	$(4.21 \pm 0.02) \times 10^6$	$(9 \pm 2) \times 10^4$	6.6 ± 0.5	5.2 ± 0.3
calcimycin[9]	Ca^{2+}	2 ± 1	$(1.1 \pm 0.6) \times 10^8$		$> 5 \times 10^7$	$(9 \pm 3) \times 10^5$	> 7	6.4 ± 0.1
	Mg^{2+}	0.3 ± 0.1	$(3.3 \pm 0.7) \times 10^6$		$(4.6 \pm 0.1) \times 10^6$	$(1.0 \pm 0.6) \times 10^5$	7.2 ± 0.5	6.2 ± 0.1
X 14885A[9]	Ca^{2+}	7 ± 3		$(2.6 \pm 0.1) \times 10^5$	$> 10^8$	$(1.2 \pm 0.4) \times 10^6$	> 7	6.0 ± 0.1
	Mg^{2+}	3.0 ± 0.8		$(1.15 \pm 0.03) \times 10^4$	$(1.1 \pm 0.2) \times 10^7$	$(1.4 \pm 0.7) \times 10^5$	6.6 ± 0.3	5.2 ± 0.1

[a] Solvent = methanol; $T = (25 \pm 0.1)$ °C; $I = 0.1$ M.

cooperative effect disappears with a bulky α-substituent, such as the tertiary amine of *N*-methylcalcimycin (Table 3).

The formation and dissociation mechanisms proposed for the calcium and magnesium cezomycin complexes show that the rate-limiting steps concern the formation or the dissociation of the charged complex AM^+, the steps which involve the neutral A_2M complex being always faster. These mechanisms completely confirm a positive cooperative formation of the neutral complex, the transporting species in biological membranes.

Dissociation kinetics in acidic medium of cezomycin complexes show, as for calcimycin and antibiotic X 14885A,[8,9] a selectivity for release of calcium compared with magnesium (Table 4). For cezomycin and ionophore X 14885A, the attack of the proton is a second-order rate reaction while a protonated complex AMH^{2+} appears in the dissociation of A 23187 complexes. However, in the presence of H^+, calcium and magnesium complexes of calcimycin dissociate respectively 400 and 10 times faster than those of cezomycin. In the absence of protons, the results suggest that the dissociation constant k_D can be attributed to the dissociation of the charged AM^+ complexes. We observe that the loss of the second ligand for the calcium complexes is for calcimycin 50 times slower than for cezomycin and 3 times slower than for X 14885A. However, this is not so for magnesium, while X 14885A complexes are 10 times more labile than those of calcimycin and 100 times more than those of cezomycin. This indicates that in the absence of protons more effective release of calcium is obtained with cezomycin and of magnesium with X 14885A. Taking now into account the acid catalysis which could take place at the membrane interface, it is noteworthy to observe that the calcium complexes of all these antibiotics are more sensitive to the presence of protons than the corresponding magnesium complexes and that calcimycin complexes dissociate more rapidly (Table 4).

The formation kinetics of cezomycin, calcimycin, and X 14885A magnesium and calcium complexes occur via the deprotonated form of the free ligand A^- and the monoprotonated one AH. Using an Eigen–Winkler model[25] and typical values available in the literature[26] for the desolvation rates of calcium and magnesium in methanol (5×10^8 s^{-1} for Ca^{2+} and 8×10^5 s^{-1} for Mg^{2+}) and for the stability in the same solvent of an outer-sphere intermediate species between a divalent cation and a charged ligand (K_{os} = 10.01 mol L^{-1}) or a neutral ligand (K_{os} = 0.54 mol L^{-1}), we were able to calculate the expected values of the kinetic parameters:

$$k_A(Ca^{2+})_{calc} = 5 \times 10^9 \text{ mol}^{-1} \text{ L s}^{-1}$$

$$k_{AH}(Ca^{2+})_{calc} = 3 \times 10^8 \text{ mol}^{-1} \text{ L s}^{-1}$$

$$k_A(Mg^{2+})_{calc} = 8 \times 10^6 \text{ mol}^{-1} \text{ L s}^{-1}$$

$$k_{AH}(Mg^{2+})_{calc} = 5 \times 10^5 \text{ mol}^{-1} \text{ L s}^{-1}$$

$$k_f = K_{os} k^{M-S} \quad (20)$$

K_{os} (mol L^{-1}) is the equilibrium constant of an external sphere complex, and k^{M-S} (s^{-1}) is the desolvation rate constant of the cation.

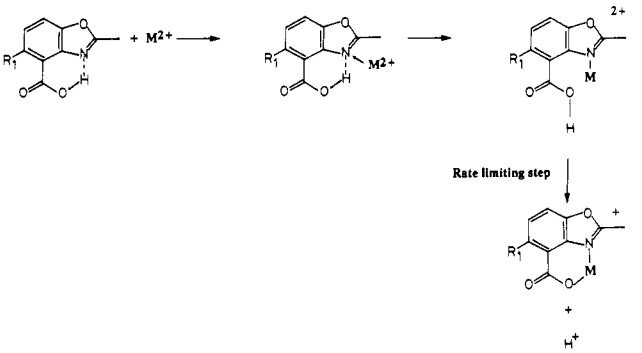

Figure 8. Formation mechanism of calcium and magnesium cezomycin complexes from monoprotonated ionophore.

The experimental and calculated values of k_A for magnesium and the three antibiotics considered are almost identical (Table 4). We can deduce that deprotonated cezomycin, calcimycin, and X 14885A desolvate the magnesium cation step by step. However it is different for calcium, where the calculated values are higher than the experimental ones (five times for cezomycin). This is certainly due to a multistep desolvation. Calcium (r = 0.99 Å), which is bigger than magnesium (r = 0.66 Å), must lose several solvent molecules to enter the cavity of the antibiotic,[9] and the formation rate falls. The experimental and calculated kinetic formation rate constants of complexes via the monoprotonated ligand AH are very different (Table 4). We can explain this by the presence of an internal hydrogen bond which blocks the reaction site.[27-30] Like the other antibiotics previously studied, cezomycin forms a hydrogen bond between the nitrogen of the benzoxazole moiety and the hydrogen of the carboxylic group. As the rate constant k_{AH} changes with the cation, the formation step is not related to the cleavage of the hydrogen bond, which is independent of the cation. According to the results obtained by Diebler et al.[31] for salicylate complexes, the rate-limiting step for the formation of complexes via the protonated species AH could be the cyclization rate between the two sites of coordination of the antibiotic benzoxazole moiety and the cation. The proposed mechanism is given in Figure 8.

A significant, systematic difference (1–2 orders of magnitude) has been observed between the thermodynamic and kinetic results (Table 4). The kinetic results (k_A/k_D) lead to stronger AM^+ complexes as expected from the thermodynamic measurements. The attribution of the rate constant k_D determined in the absence of protons to the rate-limiting dissociation of the charged complex AM^+ by similarity with the acidic dissociation step could be the reason for these differences. If there is any participation of the A_2M complex to the value of k_D, the ratio k_A/k_D does not equal K_{AM^+}.

In conclusion, our thermodynamic and kinetic results confirm the role of a hydrogen bond between the carboxyl group and the

(26) Winkler, R. *Struct. Bonding* **1972**, *10*, 1–24.
(27) Johnson, W. A.; Wilkins, R. G. *Inorg. Chem.* **1970**, *8*, 1917–1921.
(28) Onodera, T.; Fujimoto, M. *Bull. Chem. Soc. Jpn.* **1971**, *44*, 2003.
(29) Perlmutter-Hayman, B.; Shinar, R. *Inorg. Chem.* **1976**, *15*, 2932–2934.
(30) Chopra, S.; Jordan, R. B. *Inorg. Chem.* **1983**, *22*, 1708–1712.
(31) Diebler, H.; Secco, F.; Venturini, M. *J. Phys. Chem.* **1988**, *93*, 1691–1694.

substituent on the α position, while calcium and magnesium complexes of cezomycin are less stable than those of calcimycin and X 14885A. But the absence of the α substituent does not seriously affect the formation and dissociation kinetics of either calcium or magnesium complexes. An effective cooperative formation of the neutral complexes A_2M was observed at equilibrium and confirmed by the kinetic data. This positive cooperative effect increases with a lack of α substituent in cezomycin and is still present with a hydroxyl substituent in X 14885A or with a secondary amine in calcimycin, but does not exist anymore with a bulky group giving a steric effect on the carboxyl function, such as a tertiary amine in *N*-methylcalcimycin. This illustrates how well designed are the bacterial ligands for their ionophore properties.