Stability and Structure of Activated Macrocycles. Ligands with Biological Applications

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Single *p*-toluic acid pendant groups were attached to 1,4,7,10,13-pentaazacyclopentadecane (15aneN5) and 1,4,8,11-tetraazacyclotetradecane (cyclam) to prepare bifunctional reagents for radiolabeling monoclonal antibodies with ^{64,67}Cu. The ligands are 1,4,7,10,13-pentaazacyclopentadecane-1-(α-1,4-toluic acid) (PCBA) and 1,4,8,11-tetraazacyclotetradecane-1-(α-1,4-toluic acid) (CPTA). For the parent macrocycles and their pendant arm derivatives, the 1:1 Cu^{2+} complexes dissociate only below pH 2. At pH 0.0 and 25 °C the CPTA−Cu complex has a half-life toward complete dissociation of 24 days. A new approach was developed for the estimation of the Cu^{2+} stability constant for the kinetically robust CPTA. All other formation constants were determined at 25.0 °C with batch spectrophotometric techniques. Potentiometric titrations were used to determine the protonation constants of the macrocyclic ligands as well as of the metal chelates. The protonation constants, stability constants, and pM's are discussed in terms of both molecular mechanics calculations and the ligands' potential applicability as copper(II) radiopharmaceuticals.

Introduction

Monoclonal antibodies can be labeled with Cu radioisotopes for use in PET radioimmunodiagnosis (^{64}Cu) and radioimmunotherapy (^{67}Cu) of tumors.[1−9] The radiolabeled monoclonal antibodies can then be injected intravenously and should localize at a site in the body where the antigen (tumor) is present. Peptides can also be labeled with these copper radionuclides and have applications in both diagnosis and therapy.[10] It is important to form very stable copper chelates so that there is no dissociation *in vivo*. Examination of stability constant data[11] indicates that complexing agents based on the simple macrocycles 15aneN5, **1** (1,4,7,10,13-pentaazacyclopentadecane), and cyclam, **2** (1,4,8,11-tetraazacyclotetradecane), would qualify. In this work the two bifunctional macrocyclic derivatives PCBA, **3** (1,4,7,10,13-pentaazacyclopentadecane-1-(α-1,4-toluic acid)), and CPTA, **4** (1,4,8,11-tetraazacyclotetradecane-1-(α-1,4-toluic acid)), are considered as improvements, as they are easier to synthesize than the clinically used BAT (6-[*p*(-bromoacetamido)benzyl]-1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid) chelating ligand.[8,9] To this end, CPTA and PCBA were studied with Cu(II) and the stability constants of the Cu(II) complexes of the bifunctional ligands as well as of the parent compounds were determined. The ligand CPTA has been reported before, but its copper (II) stability constant was not reported.[12]

The efficacy of these complexes is primarily a function of pM (−log [M]) at physiological pH, conventionally set at pH 7.4 and 100% excess ligand. In this work, pM is computed from the equilibrium formation constants of the Cu(II) complexes of the ligands described above. In general, the higher the value of pM, the better the ligand is suited for complexation of metal ions under the conditions selected. However, it must be kept in mind that the pM for a given system does depend on the basicity of the ligand as measured by its protonation constants as well as on the magnitude of the stability constant. Of course, *in vivo*, a macrocyclic ligand also possess the advantage of kinetic inertness.

This work describes the preparation of the derivatives **3** and **4** Figure 1, and the determination of the stability constants of the Cu(II) complexes formed with **1**−**4**, as well as the

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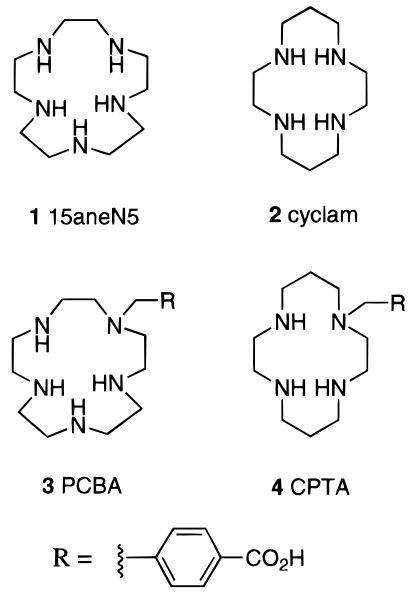

Figure 1. Structures of Ligands **1**−**4**.

protonation constants of **1**−**4**. In light of the extreme paucity of stability constant data in the literature on pairs of non- and monosubstituted macrocyclic ligands,[11] the technique of molecular mechanics was employed to further understand the relative stability constants obtained, as well as the unexpected and unusually slow Cu^{2+}−CPTA dissociation kinetics in strong acid solutions.

Experimental Section

Instrumentation. Samples for UV−vis spectra were studied in matched 10.00 mm quartz cells mounted in a thermostated holder connected to a temperature-adjustable VWR-constant temperature bath on a Perkin-Elmer Model 553 spectrophotometer connected to a Perkin-Elmer Model 500 chart recorder. A Corning Model 150 digital pH meter was used for potentiometric titrations and was interfaced to a Mime 2A terminal for storing timed pH readings. A Metrohm 10 mL capacity piston buret was used for precise standard 0.100 M KOH delivery. The meter was calibrated in terms of $-\log [H^+]$ with a standard very dilute HCl solution at $\mu = 0.1$ (KCl) and 25.0 °C, thus avoiding activity coefficient conversions. In this paper "pH" refers to the negative logarithm of the hydrogen ion concentration. ^{1}H and ^{13}C NMR were obtained on a Varian Gemini 300 (300 MHz, ^{1}H; 75 MHz, ^{13}C) spectrometer.

Reagents and Solutions. All commercial reagents were analytical grade substances. The macrocycle cyclam was obtained from Sigma Chemical Co. and Aldrich Chemical Co. and 15aneN5 was purchased from Parish Chemical Co. (Vineland, Utah), while the derivatives of these ligands were synthesized as described below. KCl was weighed directly to make a 1.000 M stock solution. Approximately 1.0 g of $CuCl_2 \cdot 2H_2O$ (MC&B) was diluted to 500 mL, and the resulting solution was standardized using Dowex cation exchanger 50W-X8.

Syntheses. (a) 1,4,7,10,13-Pentaazacyclopentadecane-1-(α-1,4-toluic acid) (PCBA). 1,4,7,10,13-Pentaazacyclotetradecane pentahydrochloride (1.00 g, 2.52 mmol) was dissolved in 7 mL of H_2O, and the pH was raised to 8 with the addition of ∼200 mg of LiOH. To this solution was added 15 mL of EtOH, followed by α-bromo-*p*-toluic acid (90.3 mg, 0.42 mmol), and the reaction mixture was stirred at 60 °C for 3 h. The EtOH was removed by rotary evaporation and the aqueous phase exhaustively extracted with $CHCl_3$ (7 × 10 mL). The aqueous phase was purified by reverse-phase HPLC, Vydac C-18 201HS1022 (22 × 250 mm) was used on a Spectra-Physics SP8700XR pump, and a SP4400 integrator was run with a gradient of 10% acetonitrile with 0.1% TFA in water to 90% acetonitrile and a flow rate of 2.0 mL/min, to give the desired product as the tetrakis-(trifluoroacetic acid) salt with a retention time of 6.81 min (60 mg, 26.9%). ^{1}H NMR (D_2O, pH = 1.0, reference TSP-d_4): δ 7.932 (d, 2H, J = 8.1 Hz), 7.490 (d, 2H, J = 8.1 Hz), 4.028 (s, 2H), 3.616 (s, 12H), 3.445 (m, 4H), 3.152 (m, 4H). ^{13}C NMR (D_2O, pH =1.0, reference TSP-d_4): δ 172.813, 141.045, 133.610, 132.992, 132.701, 59.267, 52.027, 47.137, 45.889, 45.670, 45.515. Electrospray MS *m*/*e* $(M + H)^+$ = 350.20, calcd = 350.49. Anal. Calcd for $C_{26}H_{35}N_5O_{10}F_{12} \cdot H_2O$: C, 37.92; H, 4.53; N, 8.51. Found: C, 37.54; H, 4.54; N, 8.66.

(b) 1,4,8,11-Tetraazacyclotetradecane-1-(α-1,4-toluic acid) (CPTA). CPTA was synthesized according to the procedure of Studer and Kaden.[12] The precipitate was collected and dried to give the product as the tetrahydrochloride salt (1.309 g, 58.1%). ^{1}H NMR (D_2O, reference TSP): δ 8.236 (d, 2H, J = 8.1 Hz), 7.816 (d, 2H, J = 8.1 Hz), 5.002 (s, 2H), 3.944−3.767 (m, 8H), 3.680−3.513 (m, 8H), 2.359−2.306 (m, 4H). Electrospray MS *m*/*e* $(M + H)^+ = 335.10$, calcd = 335.47. Anal. Calcd for $C_{18}H_{34}N_4O_2Cl_4 \cdot H_2O$: C, 43.39; H, 7.28; N, 11.24. Found: C, 43.01; H, 7.14; N, 10.78.

Potentiometric Titrations. (a) Ligands. A quantity of each ligand corresponding to 0.100 mmol was weighed out directly, and each test solution was made up by the addition of 5.000 mL of 1.000 M KCl and 45.00 mL doubly-distilled water. Excess known acid was added as standard 0.100 M HCl. When preneutralization was required, a corresponding adjustment to the water added was made. Forward titrations were made with 0.100 M standard KOH solution added incrementally while $-\log[H^+]$ was monitored. At least 10 data points were obtained for each neutralization step. From the 50−60 total points for each curve the ligand protonation constants were determined. Single titrations were employed for pK_a determinations.

(b) Metal−Ligands. A basic solution of each copper−ligand combination was prepared by adding exactly 0.100 mmol of standard copper to the alkaline solution obtained from the ligand titration described above and after 24 h back-titrating with standard HCl while $-\log [H^+]$ of the solution was monitored. These titrations were done for the determination of chelate protonation constants. The resulting acid solutions were stored as stock solutions for spectrophotometric work.

Spectroscopic Determinations: General Procedure. A 5.00 mL aliquot of the metal chelate solution described above was diluted to a volume of 50.0 mL after the appropriate addition of 1.00 M standard HCl and 1.0 M KCl if the pH was higher than 1.00. The pH was calculated stoichiometrically in order to avoid low-pH errors when the values were determined potentiometrically. Each test solution was placed in a thermostated cuvette at 25.0, 70, 80, or 90 °C, and the decrease in the spectral absorption corresponding to the dissociation of the complex was monitored at 270 nm with time. The lifetimes of all the complexes in acid were sufficiently short to complete the measurements for various degrees of dissociation equilibria except in the case of CPTA−Cu. This complex was studied at several pH's at the higher temperatures listed, and then the results were extrapolated to room temperature.

Computations. Protonation constants were calculated from the forward titration data with BEST.[13] Stability constants were determined from equilibrium spectrophotometric data with extinction coefficients for ML (or MHL) and protonated ligand determined in separate experiments. In each case, mass balance and total absorbance equations were set up and solved algebraically for a proton-displacement equilibrium constant of the form

$$M + H_nL \rightleftharpoons ML + nH \qquad K = [ML][H]^n/[M][H_nL]$$

with M being Cu^{2+} and the understanding that L embodies a protonated carboxylate on the pendant-arm ligands and the term *n* does not include this proton. The values of this constant were then converted into normal stability constants with the help of protonation constants of the ligands and the protonation constants of the chelates.

The constant *K* measured for the kinetically inert CPTA−Cu at high temperatures was extrapolated to room temperature using the linear plot of log *K* *vs* 1/*T*, thus avoiding the need to evaluate ΔH for protonation constants. Only after the extrapolation was the constant converted to standard form as described above.

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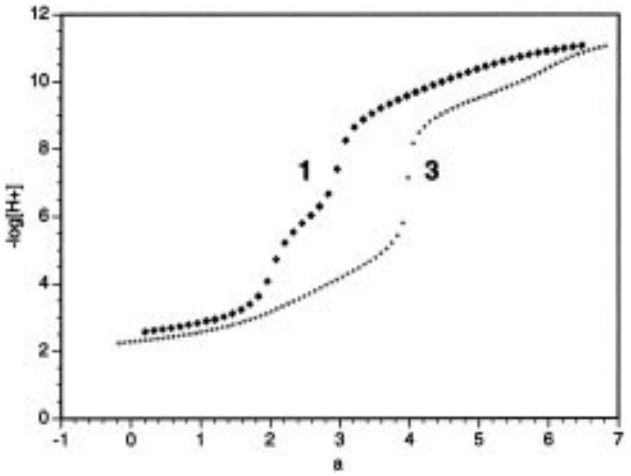

Figure 2. Potentiometric equilibrium curves for 15aneN5·5HCl, **1**, and its *p*-toluic acid derivative PCBA·5HCl , **3**, at 25 °C, $\mu = 0.100$ M (KCl), plotted as a function of *a* values, where *a* is the moles of 0.100 M standard KOH added per mol of ligand present.

Molecular Modeling. Molecular mechanics studies were done on a CAChe workstation (version 3.6).[14] This software utilizes an extended version of the MM2 force field,[15] capable of performing calculations on square planar, trigonal bipyramidal, and octahedral atoms. In all of the complexes, copper was given a charge of +2, the carboxylic acid was left protonated, and dative bonds between copper and nitrogen atoms were utilized. The starting structure of Cu^{2+}−cyclam was the conformation found to be the most stable in a study by Hancock and co-workers.[16] The copper was removed and the ligand subjected to molecular dynamics; the five lowest energy conformations were then minimized with the lowest assumed to be the preferred conformation.

The starting structure of Cu^{2+}−15aneN5 was derived from the X-ray structure of Cu^{2+}−py15aneN5.[17] The lowest energy conformation of 15aneN5 was found in a fashion similar to that described for cyclam.

The initial structures of the copper complexes of CPTA and PCBA were derived from the parent complexes. Numerous conformations were built and examined for each complex; the lowest energy complexes were then used as starting points for a sequential search of the dihedral angles defining the orientation of the benzoic acid moiety. The two dihedrals were searched in 15° increments. The five lowest energy conformations found in each search were then selected and reminimized to give the final structure. The energies of the free ligands were found as previously described for cyclam.

Results and Discussion

Protonation Reactions. Figure 2 is a comparison of the potentiometric titration curves obtained for 15aneN5 and its *p*-toluic acid derivative normalized to a pentahydrochloride stoichiometry. The parent macrocycle is characterized by two strongly acidic deprotonations followed by a deprotonation reaction centered near pH 6 and after a steep rise at $a = 3$ two further high deprotonations for a total of five. The PCBA molecule is decidedly less basic, because all of its corresponding deprotonation reactions occur at lower pH values. Thus the initial break is absent due to overlap of the lowered third nitrogen deprotonation as well as the presence of the carboxylate group itself. Additionally, the final two N-based deprotonation reactions occur at $a = 4$, 1 equiv beyond the $a = 3$ observed for the parent curve, indicating that the carboxylate group has been neutralized. The protonation constants calculated are listed in Table 1.

Table 1. Protonation Constants of the Hydrochlorides of Ligands **1**−**4** Determined at 25.0 °C and $\mu = 0.100$ M (KCl) by Potentiometry

	log K^H_n			
step	15aneN5 (H_5L)	PCBA (H_6L)	cyclam (H_4L)	CPTA (H_5L)
K^H_1	10.01	9.76	11.29	10.63
K^H_2	9.28	9.31	10.19	8.87
K^H_3	5.87	4.67	1.61	3.71
K^H_4	1.84	3.60	1.91	1.88
K^H_5				1.51
σ_{fit}[a]	0.009	0.004	0.004	0.010

[a] $\sigma_{fit} = (U/N)^{1/2}$, where $U = \sum_{i=1}^{N} w_i(pH_{obs_i} - pH_{calc_i})^2$; $w = 1/(pH_{i+1} - pH_{i-1})^2$.

It is seen in Table 1 that the pentaprotonated species could not be determined for either 15aneN5 or PCBA. This is reasonable in terms of electrostatic considerations, as positive charges are crowded into closer proximity with successive protonation steps. From the observed trend on successively protonating 15aneN5, it can be estimated that the fifth log (protonation constant) should be less than −3. The PCBA molecule has lower first and third protonation constants, but the fourth is decidedly larger relative to 15aneN5. This is the carboxylic acid pK_a and is in line with the protonation constant for 4-methylbenzoic acid of 4.2 ($\mu = 0.0$, 25 °C)[11] when one considers the effect of a positively charged moiety at the *para* position in the macrocycle. While it could not be determined by potentiometry, a similar extrapolation of the log (protonation constant) of the fourth nitrogen by subtracting 4 log units from 4.67 might predict it to be about 0.6.

The protonation constants for cyclam and CPTA were computed from their respective titration curves (not shown). If the cyclam structure were to be considered as two independent ethylenediamine moieties connected by two trimethylene bridges, then it becomes readily apparent that the 1.1 log value difference in the first two protonations can be interpreted in terms of just such a model. Of further interest is that as the structure of H_2L^{2+} is changed through the addition of a third proton, affinity for the fourth proton is then enhanced, as seen from the inversion of the usual decreasing order of successive protonation constants. If configurational factors were not predominant here, electrostatic considerations alone would predict the fourth protonation constant of cyclam to be very small.

The CPTA molecule is less basic than cyclam for the same reasons given above for the 15aneN5−PCBA pair. The *p*-toluic acid side arm has a protonation constant of 3.71, very similar to the corresponding log K^H_4 of PCBA, 3.60. The protonation steps 4 and 5 are not inverted, but are very close, much closer than electrostatic effects on the relative values would predict. Thus the conformational effects on the relative values of K^H_3 and K^H_4 of cyclam apply here too.

In the literature there is some variation in the magnitude of the protonation constants of 15aneN5. The first report was by Kimura in 1978, who measured mixed pK_a's at 25 °C, $\mu =$ 0.20 ($NaClO_4$): 10.85, 9.65, 6.00, 1.74, 1.16 log units. The other report gives concentration pK_a's by Jackels in 1988 at 25 °C, $\mu = 0.20$ (KBr): 10.39, 9.36, 6.06 log units. The other literature report is a temperature study involving only the third protonation constant at 25 °C, $\mu = 0.20$ (KCl): 6.033 log units. Considering the widely different conditions and constant definitions, it is seen that the constants obtained in this work are consistent with the previously published data. The 10.85 value for the first protonation constant seems high and may be a reflection of the experimental uncertainties in the characterization of the ligand by the Kimura group.

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Table 2. Stability Constants Determined at 25 0 °C and $\mu = 0.100$ M (KCl) by Spectrophotometry

	log *K*			
equilibrium quotient[a]	15aneN5	PCBA	cyclam	CPTA
[ML]/[M][L]	26.73(3)	24.27(2)	28.09(3)	23.6(1)
[MHL]/[M][HL]	17.68(3)	18.30(2)		16.8(1)
[MHL]/[ML][H]	0.96(5)	3.79(2)		3.82(3)

[a] The charges of species are omitted for simplicity.

Table 3. UV and vis Spectroscopic Maxima and Extinction Coefficients for the Copper(II) Complexes at 25 °C and $\mu = 0.1$ M (KCl)

species	λ_{max}, nm	ϵ, $cm^{-1}\,M^{-1}$	λ_{max}, nm	ϵ, $cm^{-1}\,M^{-1}$
Cyclam, ML^{2+}	258	6340	515	112
CPTA, MHL^{+}	272	9406	518	155
15aneN5, ML^{2+}	270	4700	585	181
PCBA, MHL^{+}	275	6530	578	186

A detailed discussion of cyclam protonation constants may be found in the literature.[18] The protonation constants of cyclam correlate well with computed strain energies for cyclam. There is only a small decrease in strain energy on the first deprotonation but a very large decrease on the second deprotonation. These strain energies are related to "exo" *vs* "endo" nitrogen conformations. For details see ref 18. The protonation constants for CPTA reported at 25 °C and $\mu = 0.5$ (KNO_3) by Studer and Kaden[12] (11.69, 9.91, 3.84, <2, <2) are much higher than the ones reported herein. As the protonation constants of cyclam in this reference are also reported to be about 0.5 log unit higher than the standard value determined by numerous other workers, there is good reason to doubt the accuracy of this reference. The experimental description of the automated potentiometry is insufficient to speculate on the real causes of this comparison.

Copper−Ligand Equilibria. (a) Potentiometric Determinations. It was found that the equilibrium constants could not be determined by direct titration for any of the systems studied because at equilibrium the Cu(II) ion complexes are already 100% formed at and above pH 2. Also, in acidic solution the rates of formation of the complexes are so slow that titrations are rendered totally impractical, even for the determination of the protonation constants on the pendant-arm carboxylate. For these reasons, back-titrations were performed from alkaline solution, where complexation was complete within several hours. However, a period of 24 h was allowed in each case for the formation of the complex, prior to back-titration.

The protonation constants for the carboxylate on the PCBA and CPTA complexes of copper are 3.79 and 3.82, respectively. These values are similar to those of the respective unchelated ligands. All of the other constants in Table 2 were obtained by spectrophotometry.

(b) Spectrophotometric Determinations. The electronic spectra of the copper complexes are characterized by two dominant peaks: a broad band above 500 nm and a much stronger, sharper band in the UV region below 300 nm. Their exact intensities and positions are summarized in Table 3. The visible absorbances of the N5 complexes appear at markedly longer wavelengths with considerable intensity enhancement relative to the N4 complexes. The relative UV band positions for these complexes were less markedly affected in going from N4 to N5. It was the more intense UV band that was used for stability constant measurements.

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Table 4. log (Proton Displacement Formation Constants) for CPTA as a Function of Temperature: log *K* ($Cu^{2+} + H_5L^{4+} \rightleftharpoons CuHL^{2+} + 4H^{+}$)

temp, °C	log *K* (av dev)	temp, °C	log *K* (av dev)
90	1.30(11)	70	1.17(4)
80	1.18(6)	25	0.80(7)

During the determinations of the absorption spectra of the Cu−15aneN5 system, the nonisosbestic behavior found indicated the presence of an unexpected MHL complex. Therefore, in the course of the calculations MHL was included, and the formation constants of both ML and MHL were thus determined. The results of these calculations are listed in Table 2. This protonated complex must be nitrogen based. All other acidic equilibrations possessed a single isosbestic point, indicating only simple equilibria. Thus the other chelate protonation constants belong to the carboxylate function and occurred at too high a pH to overlap with metal ion formation, which generally occurred near pH 1.

The stability constant of the CPTA complex could not be determined directly at room temperature because at pH 0 the half-life for its dissociation was 28 days. This dissociation rate was even slower as the pH was raised. To solve this problem, a procedure was devised whereby equilibrations were done at several initial pH values and at 70, 80, and 90 °C. The proton displacement constants so obtained were averaged for each temperature and then extrapolated to room temperature. The temperature dependence of the protonation constants was not specifically invoked, because the calculated equilibrium expression was written as a proton displacement reaction: $Cu^{2+} + H_5L^{4+} \rightleftharpoons CuHL^{2+} + 4H^{+}$. This reaction has a temperature dependence of its own, implicitly including the temperature dependence of the overall protonation constant of CPTA. The strength of the method also lies in the fact that it is not necessary to know the specific degree of ligand deprotonation at these elevated temperatures. A drawback of the method is that the extrapolation relies on the linear behavior of the logarithm of this equilibrium constant with inverse absolute temperature over quite a wide temperature range. However, this seems to be the best method available under the circumstances. The error in the extrapolation is mitigated by the moderate slope, so that the resulting extrapolated value is not very different in magnitude from the higher temperature values. The high-temperature values and the extrapolated room-temperature value are listed in Table 4. This extrapolated value (0.80) was converted with the help of ligand protonation constants and the chelate protonation constant to the standard form shown in Table 2.

Some generalizations may be made regarding the constants shown in Table 2. The addition of a noncoordinating substituent reduces the stability constants of the Cu(II) complex. However, the pH values at which complex formation occurs remain about the same because substitution also reduces the basicity of the ligand. Since the carboxylic acid group on the pendant arm has a pK_a of about 3.8, at physiological pH the formal charge on the Cu(II) complex is +1 for PCBA and CPTA.

(c) pM. The most convenient measure of thermodynamic efficacy for medicinal applications is the concept of pM, defined herein as $-\log [Cu^{2+}]$ at pH 7.4 and in the presence of 100% excess ligand. Thus pM is effectively a measure of strength of metal binding under the conditions selected. The higher the pM, the more effective is the ligand. On the one hand, for Cu−cyclam, the pM so calculated is 21.4 and decreases to 20.2 upon derivatization to CPTA. On the other hand, for 15aneN5, the pM is 22.2 and decreases to 19.9 upon derivatization. The differences of 1 and 2 log units between log *K* and pM in this pair of macrocycles cannot be ascribed to any single cause

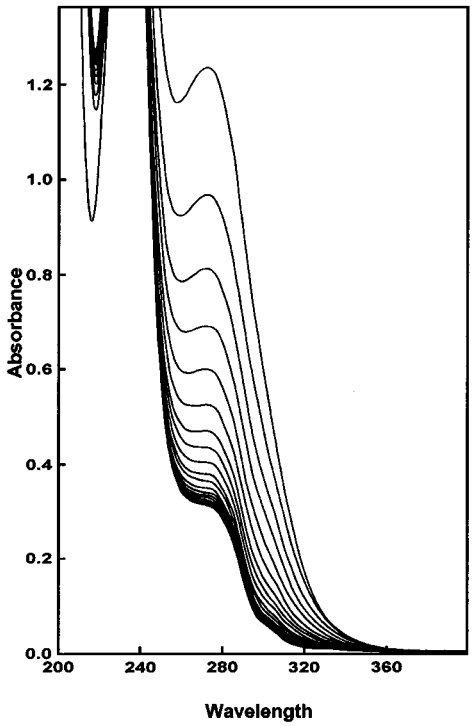

Figure 3. Successive (top to bottom) UV spectra of the acid dissociation of CuCPTA at 1×10^{-4} M, pH 0.018, and 90 °C. Spectra were taken at 10.0 min intervals.

because the pM observed at pH 7.4 is a multiple function of log *K* and ligand basicity and reflects contributions from differences in charge distribution, coordination numbers, and the actual values of the macroscopic protonation constants as well as steric effects in the resulting complexes. Thus if this comparison were made at a higher pH, such as 12, where the acid dissociation of the free ligand is almost complete, for the cyclam pair the pM's would be 28.0 and 24.9 and for the 15aneN5 pair they would be 26.7 and 24.2, respectively. If the same comparisons were made at low pH, such as 2, then for the cyclam pair the parent pM would be reduced from 10.1 to 9.0 upon derivatization and similarly the 15aneN5 pair would change from 8.0 to 7.0.

In addition to thermodynamic considerations, the cyclam structure especially has the characteristic property of a slow rate of ligand−metal dissociation at low pH. For obvious reasons, this dissociation rate could not be measured at pH 7.4, but is expected to persist at least to some degree, giving rise to very low ligand exchange rates.

Kinetics of Copper Displacement by Protons. The slow equilibria, even at 90 °C, provided an opportunity to obtain pseudo-first-order rate constants for the dissociation reaction

$$\mathrm{CuHL} + n\mathrm{H}^+ \rightarrow \mathrm{Cu}^{2+} + \mathrm{H}_{n+1}\mathrm{L}$$

Figure 3 shows a typical spectrophotometric kinetic run where the complex MHL dissociates to form free Cu^{2+} and H_5L^{4+}. The strong peak at 272 nm is that of the MHL copper complex, and the weak final peak, quite coincidentally located at the same wavelength, is that of the protonated ligand alone. The conditions shown are highly favorable for dissociation: high temperature and pH 0. Nineteen scans were measured at 10 min intervals revealing that even under these conditions the half-life of the complex approaches $^1/_2$ h. Such slow Cu(II) complex rate behavior may seem quite unusual but is a manifestation of the macrocyclic effect[19] of a small, tightly fitting tetranitrogen macrocyclic complex coupled with the noncoordinating pendant arm, which probably offers steric resistance to the inversion necessary to expel the Cu^{2+} ion upon protonation. This and additional runs at three temperatures and higher pH values are summarized in Table 5.

Table 5. Pseudo-First-Order Rate Constants for Dissociation of the Protonated Cu(II)−CPTA Complexes, $CuHL^{3+}$, as a Function of Temperature and pH

temp °C	pH[a]	k_{obs} (min^{-1})	$t_{1/2}$ (min)
90	0.018	0.028	24
80	0.018	0.011	63
70	0.018	0.0038	180
90	0.249	0.011	63
80	0.249	0.0058	120
90	0.654	0.0043	160
80	0.654	0.0010	670
90	0.770	0.0038	180
80	0.770	0.0012	590
70	0.770	0.00035	2000

[a] At 25 °C.

Table 5 clearly shows that the reaction rates are a function of acid concentration and also of temperature. For a given temperature, the rate seems to be approximately inverse second order in hydrogen ion concentration, implying that two protons must be added to form the active intermediate in complex dissociation. Slow reactions of cyclam with Cu^{2+} are not unusual[20] and are well documented for certain cyclam derivatives.[21] It is very interesting that in water these half-lives are on the order of hours and days. In DMF, in the absence of water the half-life is only several minutes for the formation of Cu−tetramethylcyclam.[22,23]

Molecular Mechanics (MM). Some insight regarding the extreme slowness of CPTA−Cu to undergo proton-assisted demetalation might be obtained from MM calculations. Figures 4 and 5 show dramatic differences of minimal strain energy conformations for the two derivatives. These structures were generated using the extended MM2 force field utilized by CAChe. In the CPTA−Cu case, the pendant arm is seen to prefer to hover over the metal center, providing a steric barrier for the outgoing Cu^{2+} ion. On the other hand, the Cu−PCBA structure prefers the arm swung away from the metal center and is therefore free from such steric inhibition. MM minimization was likewise performed on the Cu-free ligands. Both ligands showed structural behavior parallel to their Cu-complexed counterparts. Thus CPTA alone was found to possess similar folded-over behavior of the pendant arm, while in the uncomplexed PCBA molecule this arm was found to be extended outside the macrocycle.

The Cu(II) environment in the simulated structure of Cu−CPTA (Figure 5) is definitely 4-coordinate planar with calculated *trans* angles N1−Cu−N3 and N2−Cu−N4 both being 178° and the ethylene *cis* angles N1−Cu−N2 and N3−Cu−N4 being 85 and 84°, respectively. The trimethylene-bridged *cis* angles N1−Cu−N4 and N2−Cu−N3 are 95 and 96°, respectively.

The geometry of the coordination sphere around the simulated Cu−PCBA complex (Figure 4) can be stated only in terms of being pentacoordinate. It is neither trigonal bipyramidal nor square pyramidal. These differences in the coordination spheres

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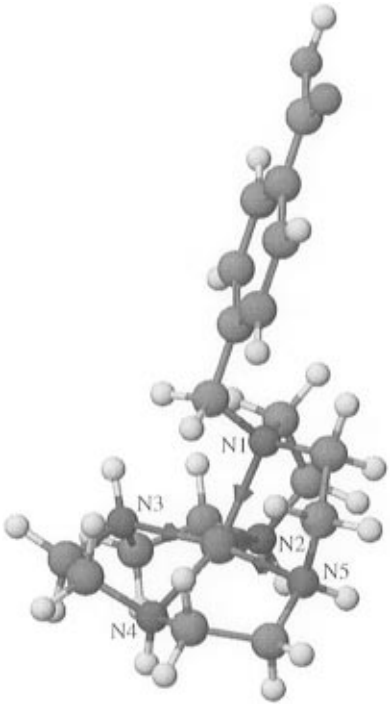

Figure 4. MM-generated drawing of the Cu^{2+}−PCBA complex at low pH.

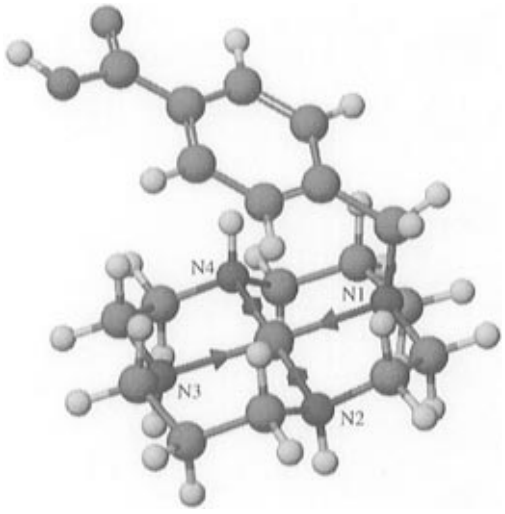

Figure 5. MM-generated drawing of the Cu^{2+}−CPTA complex at low pH.

are reflected in the large differences observed in the electronic spectra summarized in Table 3.

Other Work. The formation constants of both cyclam[24−28] and 15aneN5[24] have already been determined for Cu^{2+}. The literature values[11] for cyclam are 27.2 (25 °C, $\mu = 0.1$) and 26.2 (25 °C, $\mu = 0.5$). In addition to the difference in ionic strengths, this wide disagreement could be partly due to the observed differences in the overall basicity of cyclam in the two media as measured by the sum of the log (protonation constants). The ligand is more basic by 0.6 log units at $\mu = 0.1$ in the early work. However, in the present work it is shown

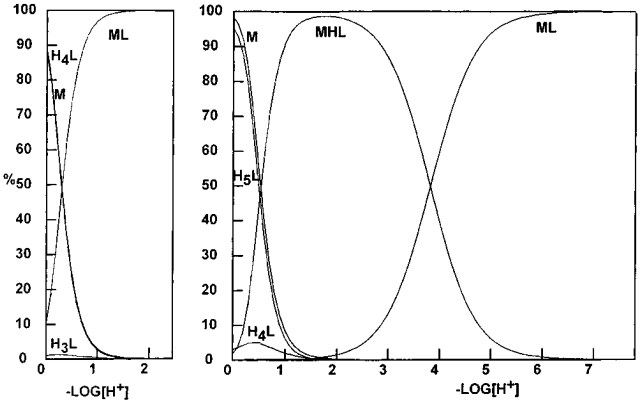

Figure 6. Equilibrium species−pH diagram for cyclam (left) and CPTA (right) with Cu^{2+} (1:1) at 1×10^{-4} M where M = Cu^{2+}, and L = ligand in its deprotonated form.

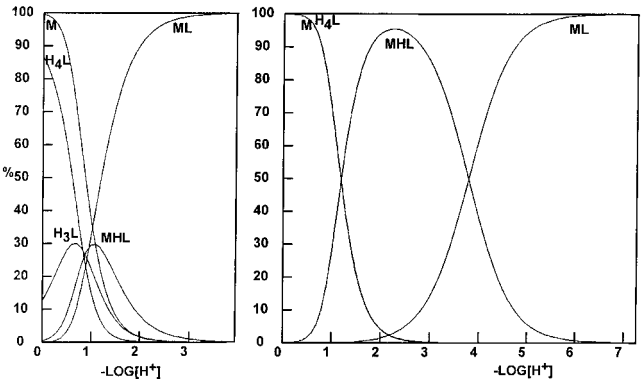

Figure 7. Equilibrium species distribution as a function of pH for 15aneN5 (left) and PCBA (right) with Cu^{2+} (1:1) at 1×10^{-4} M and 25 °C where H = H^+, M = Cu^{2+}, and L = deprotonated form of the ligands.

that the total basicity of cyclam (at 25 °C and $\mu = 0.1$) is 0.6 log unit higher yet than the accepted value shown in the standard reference.[11] This is mostly a result of the two reversed low-pH protonation constants. In this work the value for the log of the stability constant is 28.1 (25 °C, $\mu = 0.1$), and in view of this discussion, this value is justified as being the more accurate at $\mu = 0.1$.

The equilibrium results for both Cu−cyclam and Cu−CPTA are compared in Figure 6. The formation of the Cu−cyclam chelate begins at approximately pH 0 and is virtually complete by pH 1. There are no protonated metal chelate species. The formation of Cu−CPTA is also virtually complete by pH 1, but the Cu−CPTA−H complex that initially forms becomes completely deprotonated only at pH 6 and above. Thus at physiological pH the charge on this chelate is 1+.

The only determination of the normal Cu^{2+} stability constant of 15aneN5 was measured by polarography.[24] The same author also proposed a chelate protonation constant near pH 6. A published titration curve of 15aneN5 in the presence of Cu^{2+} clearly refutes the presence of such weakly acidic chelate species.[28] The stability constant found in this study is considered accurate and should replace the earlier one. In this work a weak protonation constant for the 15aneN5−Cu^{2+} chelate was found, but it is very low (log K^{H}_{MHL} ~1.0) and overlaps somewhat the formation of the normal (unprotonated) chelate.

In Figure 7 the equilibrium results for both Cu−15aneN5 and Cu−PCBA are compared. As the pH is increased, the formation of the Cu−15aneN5 chelate ML^{2+} begins just above pH 0 and is virtually complete by pH 3. An acidic protonated metal ion species MHL^{3+} forms as an intermediate, which

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reaches its maximum concentration near pH 1 and is completely converted to ML^{2+} by pH 3. The formation of Cu−PCBA is virtually complete by pH 2 and the Cu−PCBA−H complex that initially forms becomes completely deprotonated only at pH 6 and above. At physiological pH, the charge on this chelate is 1+. The absence of a MH_2L species at low pH for Cu−PCBA apparently indicates a decrease in basicity of the nitrogen substituted by the attached benzyl substituent.

Biological Significance. The bifunctional chelating agent 6-[*p*(-bromoacetamido)benzyl]-1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (BAT) has been shown to form stable complexes with copper[24] and is used in clinical trials with ^{64}Cu for tumor detection.[8] However, the difficult synthesis of this ligand has limited its use. The CPTA and PCBA ligands discussed in this paper have straightforward one-step syntheses and they have thermodynamic stability constants of $10^{23.6}$ and $10^{24.27}$, respectively, when complexed with copper. These constants are both larger than the $10^{21.87}$ value reported for 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA), the macrocyclic portion of BAT.[29]

Smith-Jones *et al*. have shown the copper complex of CPTA to be stable for greater than 24 h both in 0.05 M nitric acid and in human serum.[30] Smith-Jones also has conjugated CPTA with a monoclonal antibody, labeled the conjugate with ^{67}Cu, and shown it to have a higher tumor uptake than the ^{125}I-labeled antibody in tumor-bearing mice.[30] Anderson *et al*. have also shown that a ^{64}Cu-labeled CPTA−monoclonal antibody has good tumor uptake in hamsters;[31] however, this study also shows a larger uptake of activity in nontarget organs with the CPTA conjugate than with the BAT conjugate. CPTA conjugated to the peptide octreotide shows very high target uptake in the adrenal gland of rats; the uptake is much greater than with the corresponding BAT complex.[10]

The ease of synthesis of these two chelates and their binding with copper to form stable complexes makes them attractive for use as ^{64,67}Cu radiopharmaceuticals. Previous studies have shown that ^{64,67}Cu−CPTA−monoclonal antibodies have potential as radiopharmaceuticals;[30,31] however, additional information about chelate charge, lipophilicity, and linkages formed with antibodies must also be considered for determining the chelates' ultimate usefulness.

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