Binding of Phosphates to Aminocyclodextrin Biomimetics

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Aminocyclodextrins (ACDs) in which the primary face is perfacially substituted with amino pendant groups provide three potential biomimetic binding domains: the hydrophobic cavity, the cationic annulus, and the corona formed by the pendant tendrils. Binding of phosphate monoester dianions and diester monoanions by ACDs and native CDs is compared to that of neutral guest molecules. Binding to ACDs can be understood in terms of cooperativity between binding domains in which electrostatic and hydrophobic forces may be additive or compromised. Unexpectedly, slow chemical exchange is observed, in particular for dianionic aryl ester guest molecules. The substantial kinetic barriers for dissociation of these pseudorotaxane complexes are explained by the unfavorable passage of the anionic phosphate headgroup through the relatively hydrophobic ACD cavity.

Introduction

The maltooligosaccharide cyclodextrins, αCD and *β*CD, have been prominent features in the field of host−guest molecular recognition.[1] The hydrophobic cavity has dominated the chemistry and applications of cyclodextrins, binding organic molecules of appropriate size and yielding inclusion complexes, which have utility in solubilization, encapsulation, and transport of small hydrophobic molecules including toxins and drugs.[2,3] Amino-CD (ACD), in which the primary face constitutes amine groups in place of the hydroxyls of CD itself, represents the parent molecule for a family of monofacially substituted ACD derivatives (ACDs).[4] We have shown that ACD derivatives show biological activity by binding glycosaminoglycan sulfates and thus hold significant potential for biomolecular recognition.[5] To better understand recognition by ACDs and to assess the potential of ACDs as biological probes and models, we have examined binding of simple guest molecules, including models of biologically important phosphate monoesters, diesters, and phospholipids, by a subset of the larger ACD family. The results show surprising differences between recognition by ACD derivatives and CD itself. The ACD cavity provides a significant hydrophobic contribution to binding, only when strong electrostatic binding at the cationic annulus is present. Furthermore, pseudorotaxanes are formed from complexation of ACDs with many phosphate guests, with the significant kinetic barriers attributed to passage of the anionic phosphate group through the hydrophobic cavity.

Table 1. Observed Binding Constants (K_b, M^{-1}) and Binding Energies ($-\Delta G_b$, kcal/mol) for Complexes between Substrates 1−7 and ACDs or Native CDs

	*β*CD pH = 7.3	*β*ACD pH = 7.3	αACD pH = 7.3	*β*eACD pH = 7.3	*β*eACD pH = 9
K_b					
1	2640	60	5	327	194
2	917	<5	<5	<5	
3	502	621	72	131	184
4	1200	591	48	896	217
5	698	1870	279	1770	564
6	<5	870	2800	920	0
7			155	1170	
$-\Delta G_b$					
1	4.7	2.4	<0.95	3.4	3.1
2	4.0	<0.95	<0.95	<0.95	
3	3.7	3.8	2.5	2.9	3.1
4	4.2	3.8	2.3	4.0	3.2
5	3.9	4.5	3.3	4.4	3.8
6	<0.95	4.0	4.7	4.0	
7			3.0	4.2	

[a] At 25 °C; 50 mM bisTris (pH 7.3); 50 mM Tris (pH 9); in 0.100 M KCl.

Results and Discussion

Hexakis-6-amino-6-deoxy-α-CD (**αACD**), heptakis-6-amino-6-deoxy-*β*CD (***β*ACD**), heptakis-6-(2-hydroxyethylamino)-6-deoxy-*β*CD (***β*eACD**), and ***β*CD** itself were examined as hosts for a selection of neutral and charged guest molecules (**1**−**6**), including the phosphatidyl-inositol analogue **3**.[6] Binding constants (K_b) were obtained using 4-isopropylphenyl derivatives as amplified probes of cavity binding. Branching of the alkyl ring substituent is known to strengthen the inclusion of arenes in *β*CDs while decreasing the binding to αCDs.[7] Thus use of 4-isopropylphenyl derivatives instead of simple phenyl

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(1) (a) Szejtli, J. *Cyclodextrins and their inclusion complexes*; Akademiai Kiado: Budapest, 1982. (b) Wenz, G. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 803.

(2) (a) Komiyama, M. In *Comprehensive Supramolecular Chemistry*; Lehn, J. M., Ed.; Pergamon: New York, 1996; Vol. 3 and references therein.

(3) For example: Rudiger, V.; Eliseev, A.; Simova, S.; Schneider, H.-J.; Blandamer, M. J.; Cullis, P. M.; Meyer, A. J. *J. Chem. Soc., Perkin Trans. 2* **1996**, 2119.

(4) Vizitiu, D.; Walkinshaw, C. S.; Gorin, B. I.; Thatcher, G. R. J. *J. Org. Chem.* **1997**, *62*, 8760.

(5) Borrajo, A. M. P.; Gorin, B. I.; Dostaler, S. M.; Riopelle, R. J.; Thatcher, G. R. J. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1185.

(6) All ACDs are characterized as homogeneous compounds by ^{13}C NMR, elemental analysis, and FAB-MS: see, ref 4 and Gorin, B. I.; Riopelle, R. J.; Thatcher, G. R. J. *Tetrahedron Lett.* **1996**, *37*, 4647. All guest compounds were fully characterized.

(7) For example, the binding constant of 4-isopropyl benzene to *β*-CD is 1200 M^{-1}, but to α-CD is only 72 M^{-1}, purportedly due to the differing cavity volume, representing $\delta\Delta G_b \approx 1.7$ kcal/mol: (a) Kano, K.; Tamiya, Y.; Hashimoto, S. *J. Inclusion Phenom.* **1992**, *13*, 287. (b) Connors, K. A. *J. Pharm. Sci.* **1995**, *84*, 843.

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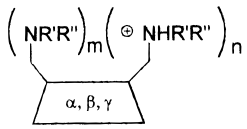
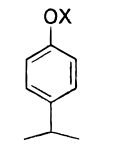
derivatives may yield more detailed information on inclusion of the aryl group within the CD cavity.

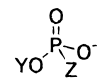
Binding studies were carried out at pH 7.3 and pH 9. pK_a values for ACD derivatives are depressed by up to 4 units relative to the parent amines, owing to cooperativity between the neighboring amine bases: pK_a(**αACD**) = 7.4−10.2; pK_a(**βACD**) = 7.0−10.2; pK_a(**βeACD**) = 5.5−8.8.[8] Nevertheless, at neutrality, all ACDs are fully or partially protonated, and for anionic substrates, strong binding is predicted at neutral pH where the host ACD amino groups are protonated and the guest is ionized.



α: m + n = 6; β: m + n = 7 , γ m + n = 8
α, βeACD: R' = $HO(CH_2)_2$; R'' = H
α, βMeACD: R' = $MeO(CH_2)_2$; R'' = H
βNMeACD: R' = $HO(CH_2)_2$; R'' = Me
α, βACD R' = H; R'' = H



1, X = -H
2, X = -$COCH_3$



3, Y = 4-iPrPh, Z = -1-*myo*-inositol
4, Y = 4-iPrPh, Z = -OCH_3
5, Y = 4-iPrPh, Z = -O^-
6, Y = -CH_3, Z = -O^-
7, Y = 4-NO_2Ph, Z = -O^-

The simple phenol, **1**, binds to **βCD** with a submillimolar dissociation constant, as expected, but binding of the acetylated phenol to **βCD** is diminished (Table 1). This is compatible with the proposed inclusion mode of phenols within the CD cavity, involving hydrogen bonding between the phenolic hydroxy group and the secondary face C2-OH of the host.[9,10] The acetate group of **2** hinders binding at the secondary annulus, lowering K_b. Therefore, the CD cavity provides 4 kcal/mol of hydrophobic binding energy, with an additional 0.7 kcal/mol available from hydrogen bonding.

The phosphates, **3**−**5**, are good hydrogen bond acceptors, but these more hydrophilic molecules have higher aqueous solvation energies than **1** and **2**. Since K_b for **βCD** is largely a measure of the competition between aqueous solvation and hydrophobic cavity-binding (i.e., desolvation), observed K_b values are reduced for the hydrophilic phosphates. The influence of hydrophobic binding can be demonstrated by observing binding of **1** and **3** to **βCD** in DMSO/water mixtures, in which an exponential decrease in K_b is observed (Figure 1). The organic cosolvent is likely functioning as a competitive substrate for **βCD**, displacing the arene substrate from the cavity.[11] The hydrophilic inositol phosphate headgroup of the PI analogue, **3**, clearly contributes to the observed K_b depression. Taking account of hydrogen bonding contributions to binding of **3**−**5**, the hydrophobic binding energy for the aryl phosphates in the CD cavity can be very approximately estimated as reduced to 3 kcal/mol. Not unexpectedly, the hydrophobic binding energy due to the methyl group of **6** in **βCD** is too small to observe (<1 kcal/mol).

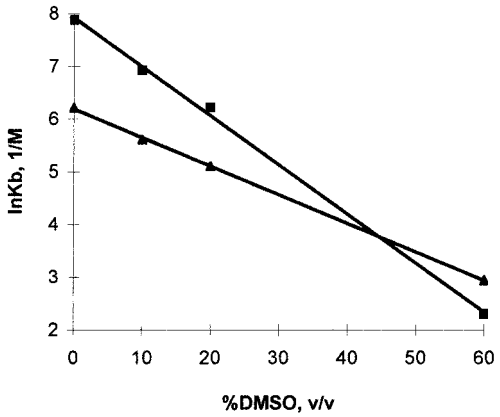


Figure 1. Organic cosolvent influence on binding of neutral and anionic guests to native **βCD**: **1** (■) and **3** (▲).

The hydrophobic cavity would be predicted to be little disturbed by the conservative mutation of the **βCD** primary face hydroxyls to the amino groups of **βACD**. However, remarkably, binding of the phenol, **1**, is largely lost in **βACD**, being reduced almost to the low level expected and observed for **αACD**. Indeed, no binding of the acetate **2** was observed with any ACD. The simplest explanation for this surprising observation is that hydrophobic binding within a CD is driven, in part, by the displacement of high-energy water molecules included within the cavity.[12] Water molecules in the **βCD** cavity lack the complement of stabilizing hydrogen bonds that would be available to them in the bulk solvent phase. Conversely, the protonated amino groups of the **βACD** annulus stabilize the included water molecules through a strong hydrogen bonding network. Again taking account of hydrogen bonding contributions, a hydrophobic binding contribution of 1−1.5 kcal/mol for **βACD** and <1 kcal/mol for **αACD** can be estimated from these data.

Binding of the phosphate monoanions by ACDs shows very different behavior. The electrostatic stabilization between the cationic annulus of **βACD** and the phosphate monoanions (≈ 2 kcal/mol) offsets the loss of the hydrophobic component (reduced from 3 kcal/mol in **βCD** to less than 1.5 kcal/mol in **βACD**). Binding to **αACD** is 10-fold weaker than to **βACD**, clearly indicating that cavity inclusion of the phenyl groups is occurring. Binding of the monoanions to **βeACD** at pH 7.3 should reflect a slightly decreased electrostatic, but increased hydrophobic, contribution and a potential influence of the ethanolamine tendrils. This is reflected in the low K_b for **3**, which is clearly influenced by steric hindrance between the inositol headgroup and the tendrils.[13] The similar binding constants for **1**, **3**, and **4** to **βeACD** at pH 9 (ΔG_b ≈ 3 kcal/mol) indicate that modest secondary face binding

(8) Martell, A. E.; Motekaitis, R. J. *Determination and Use of Stability Constants*; VCH Publishers: Weinheim.

(9) Gillet, B.; Nicole, D. J.; Delpuech, J. J. *Tetrahedron Lett.* **1982**, *23*, 65.

(10) An induced fit mechanism has also been postulated: Lichtenthaler, F. W.; Immel, S. *Starch/Starke* **1996**, *48*, 145.

(11) (a) Fornasier, R.; Parmagnani, M.; Tonellato, U. *J. Inclusion Phenom. Mol. Recognit. Chem.* **1991**, *11*, 225−231. (b) Breslow, R.; Halfon, S. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6916−6918.

(12) (a) van Etten, R. L.; Sebastian, J. F.; Clowes, G. A.; Bender, M. L. *J. Am. Chem. Soc.* **1967**, *89*, 3242. (b) Chacko, K. K.; Saenger, W. *J. Am. Chem. Soc.* **1981**, *103*, 1708.

(13) The observed data fit 2:1 binding for the PI analogue at ratios of **βeACD**:**3** greater than 8:1. The simplest explanation has 1:1 binding via aryl inclusion at the secondary face of **βeACD** with the second **βeACD** providing weak electrostatic binding.

of the isopropyl phenyl moiety becomes dominant on loss of the cationic annulus at higher pH.[14]

Binding constants for the aryl monoester dianions **5** and **7**, at pH 7.3, are significantly larger than those for the monoanions (Table 1). Contributions to binding of methyl phosphate (**6**) must be largely electrostatic in nature. Interestingly, binding of **6** by **αACD** is stronger than that by ***β*ACD** (Table 1). One explanation, supported by molecular modeling, is that the smaller annulus is more complementary to the PO_3^{2-} moiety, optimizing electrostatic interactions. At pH 9, as expected, no binding of methyl phosphate (**6**) by ***β*eACD** is observed. From the data for **6**, in aqueous solution at physiological pH, the electrostatic contribution to binding of a phosphate anion by the cationic annulus of ACD is estimated as 2−2.2 kcal/mol per negative charge for **αACD** and 1.8−2 kcal/mol for ***β*ACD**. Eliseev and Schneider have studied the binding of nucleotides to one *N*-methyl-*β*ACD derivative by potentiometric titration and find that each salt bridge contributes an electrostatic stabilization of 1.0 ± 0.2 kcal/mol in accord with other non-CD receptors.[15] The electrostatic contribution to phosphate dianion binding to the *β*ACDs is compatible with four salt bridges at pH 7.3.

Having estimated both hydrophobic and electrostatic contributions to binding, from data on substrates **1**−**4** and **6**, prediction of the ACD binding energy for the aryl dianions **4** and **6** is possible. Optimal electrostatic stabilization and hydrophobic binding would yield ΔG_b for **αACD** and ***β*ACD** of approximately 5.0 and 5.5 kcal/mol, respectively. However, the observed binding is 1−1.5 kcal/mol weaker than predicted. It is tempting to conclude that there is no hydrophobic contribution, and therefore no driving force for inclusion. However, there are three unambiguous observations that require aryl group inclusion: (1) the ^{1}H NMR of the substrates show clear chemical shifts of the aryl and isopropyl protons ($\Delta\delta \approx 0.15$ ppm) on binding, even in the smaller **αACD** cavity; (2) binding to **αACD** is weaker than to ***β*ACD** as expected for isopropylphenyl group inclusion; (3) discrete NMR signals are observed for bound and unbound substrate (vide infra), requiring a substantial barrier to dissociation of the ACD complex, which is not easily explained without aryl group inclusion. The lower than predicted binding of aryl dianions is instead explained by the shallow depth of the CD torus. The large electrostatic interactions between the cationic annulus and the phosphate dianion, when optimized, serve to pull the substrate deeper into the ACD cavity pushing the isopropylphenyl group out of the secondary face into bulk aqueous solution (Figure 2). Thus the geometry of the complex does not allow both optimal electrostatic and optimal hydrophobic binding, compromising both contributions. The relative rigidity of the **αACD** binding domains exacerbates this situation.

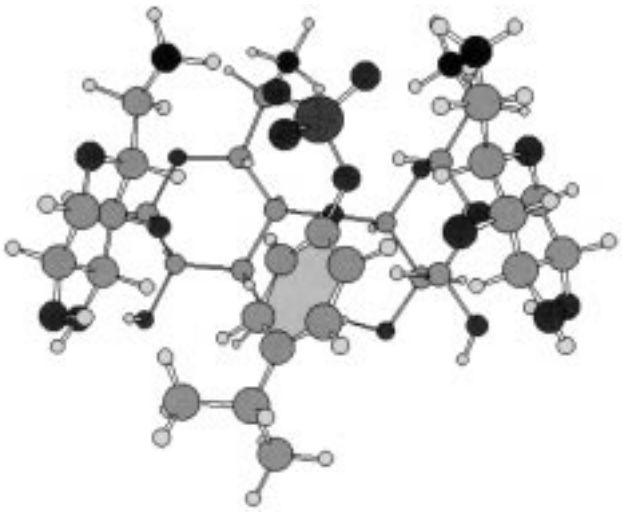


Figure 2. Structure of complex obtained from molecular mechanics force field geometry optimization on **αACD·5**, using option to optimize electrostatic interactions, but negelect van der Waals interactions, showing protrusion of aryl group from the secondary face (the front of the ACD torus has been cut out for clarity).

The most remarkable observation on the ACD complexes is the observation of discrete ^{31}P and ^{1}H NMR signals for both **5** and **7** corresponding to free and ACD-bound substrate at room temperature. These observations are indicative of slow chemical exchange and formation of pseudorotaxanes.[16] For example, at a ratio of **5**/***β*eACD** of ≈2:1, discrete signals are observed for **5** and for the pseudorotaxane complex ***β*eACD·5**, showing roughly equal populations. The observed coalescence temperature of 43 °C yields a barrier to complex dissociation ($\Delta G^{\ddagger}_b$) of 14.4 kcal/mol. The calculated dissociation rate constant at this temperature is 7×10^3 s^{-1}. Dissociation from **αACD**, of all ACDs, shows the highest barrier. The coalescence temperature for **αACD·5** can be extrapolated from experimental data to approximately 100 °C, yielding $\Delta G^{\ddagger}_b \approx 16.8$ kcal/mol. Furthermore, binding is clearly reversible, since when the pH of a solution of ***β*eACD·5** complex is raised from 7.3 to 9.0, the phosphate is seen to dissociate by ^{31}P NMR. Energy barriers resulting from either passage of the anionic headgroup through the somewhat hydrophobic cavity or passage of the hydrophobic aryl moiety through the smaller cationic annulus could be envisioned. Binding data and molecular mechanics force field calculations are compatible with the lowest energy pathway being inclusion via the wider secondary face, with the kinetic barrier deriving from passage of the phosphate through the hydrophobic cavity.

Summary

In comparison to the plethora of studies on CDs, little work on ACD derivatives has been reported.[17] In this work, ACD derivatives are seen as excellent models for studies on fundamental binding and recognition interac-

(14) ***β*ACD** is insoluble at mildly alkaline pH and cannot be used for study of pH dependence.

(15) (a) Eliseev, A. V.; Schneider, H.-J. *J. Am. Chem. Soc.* **1994**, *116*, 6081. (b) Schwinte, P.; Darcy, R.; O'Keeffe, F. *J. Chem. Soc., Perkin Trans. 2* **1998**, 805.

(16) Potentiometric titrations have indicated high binding constants for phosphate nucleotides to two ACD derivatives [ref 15], but high barriers to dissociation and pseudorotaxane formation have not been reported. Pseudorotaxane formation between ACDs and nucleotides has been observed in our labs [Borrajo and Thatcher, unpublished data]. A large literature exists on pseudorotaxane formation with native CDs, in which metal ions or aqueous solvation spheres serve to cap the included thread [Hermann, W.; Keller, B.; Wenz, G. *Macromolecules* **1997**, *30*, 4966. Smith, A. C.; Macartney, D. F. *J. Org. Chem.* **1998**, *63*, 9243].

(17) (a) Breslow, R.; Czarniecki, M. F.; Emerrt, J.; Hamaguchi, H. *J. Am.. Chem. Soc.* **1980**, *102*, 762. (b) Yatsimirsky, A. K.; Eliseev, A. V. *J. Chem. Soc., Perkin Trans. 2* **1991**, 1769. (c) Matsui, Y.; Tanemura, E.; Nonomura, T. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 2827.

tions of significance in biochemical processes, providing at physiological pH and in aqueous solution cationic ammonium sites, free amine groups, and a hydrophobic binding site. Cooperativity is clearly observed between these sites, and variation of the amino pendant group can be used to control the protonation state at neutral pH and provide added binding interactions. The defined stereochemistry and relative rigidity of the annulus provide advantages over macrocyclic polyamines,[18] including observation of pseudorotaxanes and the ability to study binding kinetics, of relevance, for example, to ion channels.[19]

Experimental Section

Spectrophotometric binding titrations were carried out at 25 °C on a Beckman DU7400 spectrometer, in a modified magnetically stirred cell holder: stirring being essential for accurate measurements. Direct UV measurements were employed for the determination of the binding constants in all cases where binding could be monitored by a significant absorbance change (>0.1 unit). UV absorbance readings (λ = 200−350 nm) were recorded 2 min after addition of each aliquot of (CD + substrate) solution to the cuvette containing the substrate solution. A plot of ΔA against CD concentration was drawn using data at the wavelength showing the maximum absorbance shift. Buffers used were 50mM bisTris for solutions at pH 7.3 and 50mM Tris for solutions at pH 9, both in 0.100 M KCl. Under our experimental conditions, no precipitation nor turbidity was observed in any ACD complex solution.

A 1:1 stoichiometry was assumed for the calculations of all binding constants, but the presence of 2:1 binding was also examined and observed in one case. Iterative, nonlinear, least squares regression was performed with GraphPad Prism software to analyze the data, solving for $y = B_{max}L/(K_d + L)$ and $L = (L_t - S_t - K_d)/2 + \sqrt{[(L_t - S_t - K_d)^2 + 4L_tK_d]}/2$, where L = concentration of free CD (M^{-1}), L_t = total concentration of ligand (M), K_d = dissociation constant of the CD·S complex (M), B_{max} = the maximal binding measurement, and S_t = total concentration of substrate (M). Errors for K_b values were determined to be <10% at 40 < K_b < 1200 and <20% outside this interval.

^{31}P NMR (162 MHz) titration was used to determine the binding constants of methyl phosphate as well as to confirm and reinforce the K_b values obtained spectrophotometrically for other phosphates, under the same conditions as used for UV-spectrophotometric titration. Cavity inclusion was tested by monitoring ^{1}H NMR (400 MHz) chemical shifts of aromatic and *i*-Pr group protons on titration in D_2O.

For all aryl phosphates studied by NMR, either discrete pairs of signals or signal broadening accompanies complexation due to slow chemical exchange. Further, with a small excess of ACD over guest, line sharpness is recovered. The possibility of signal from reaction products was dismissed, since at 37 °C, pH 6, with 25× molar excess of ACD, no reaction was detected after12 h with any substrate. ^{31}P NMR variable temperature coalescence experiments on a 1:2 mixture of **βeACD** and **5** (10mM) in 50 mM bisTris buffer, 0.1 M KCl, pH 7.3, were analyzed using the approximation: $\Delta G_b^{\ddagger} = 4.57\ T_c[9.97 + \log T_c/dw]$, where T_c is the coalescence temperature and dw is the chemical shift difference. The ratio of substrate to ACD was chosen to give equal populations of the two states (bound and unbound) as required by this approximation. Similar coalescence experiments were caried out for **αACD** and **5** to determine T_c and dw.

Potentiometric titrations were performed at 25.0 ± 0.2 °C on a thermostated Radiometer Copenhagen PHM82/TTT80/ABU80 pH Stat system. All solutions were flushed with Ar and maintained at 0.100 M (KNO_3). Titration solutions of KOH and HCl were rigorously calibrated against potassium hydrogen phthalate. The pK_a intervals were determined by triplicate titrations of the per-protonated ACDs (∼0.1 mM) with a stoichiometric number of molar equivalents of KOH and analyzed using the programs BEST or PKAS.[20]

Molecular modeling of ACD complexes was performed using Sculpt 2.5 from Interactive Simulations, San Diego, CA. This molecular mechanics based program allows docking of substrates with optional inclusion of either Coulombic/electrostatic or non-Coulombic/van der Waals interactions. Starting structures of ACDs were based upon native CD crystal structures. Modeling was used to visualize included complexes and the effects of competing electrostatic and van der Waals interactions, not to derive quantitative data.

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(18) Fenniri, H.; Hosseini, M. W.; Lehn, J. M. *Helv. Chim. Acta* **1997**, *80*, 786 and references therein.

(19) Dietrich, B. *Pure Appl. Chem.* **1993**, *65*, 1457 and references therein.

(20) Martell, A. E.; Motekaitis, R. J. *Determination and Use of Stability Constants*; VCH Publishers: Weinheim.