the assumption that a single order parameter, S_{zz}, adequately describes molecular ordering, allowing the relatively simple eqs 1 and 2 to be applied in structural analysis. Having completed the order matrix approach, we wished to compare these results with those that are obtained if the more restrictive assumptions are employed. We chose to use the NMR–pseudoenergy method incorporating eqs 1 and 2 because it allows for minor adjustment of bond and dihedral parameters from idealized values—a biochemically probable situation. Sixteen starting conformers were used as starting structures, which were generated by four 90° increments of the glycosidic angles ϕ and ψ. The data were input as absolute value splittings with the $^{13}C-^{13}C$ 1 bond, quadrupolar, $^{13}C-^{13}C$ 2 and 3 bond, and $^{13}C-H$ data being weighted by relative factors of 1.85:1.0:0.5:0.004. The size of the weighting factors was such that the pseudoenergy term describing deviation of the experimental data from calculated splittings was generally about half of the total energy of the usual molecular mechanics force field terms. ^2H NMR data for the deuterons of the 1- and 2-positions of the dodecyl chain were included to allow the program to dictate chain geometry. One of the 16 starting structures converged upon a structure whose total real energy and pseudoenergy were, at most, only half of any other final structures and whose total real energy (16 kcal) was only 8 kcal more than that for the structure obtained by minimization in the absence of experimental constraints. Back calculation of the predicted quadrupolar and dipolar splittings yielded splittings of the correct absolute signs, even though this information was not input with the data. However, the agreement of calculated and observed splittings is clearly poorer (Table I) than results from the order matrix approach. This structure is displayed in Figure 9B and possesses an S_{mol} of 0.42 (see eqs 1 and 2). The orientation of the head group with respect to the director is nearly equivalent to that determined by the order matrix approach. Examination of the linkage conformation reveals ϕ and ψ of 37° and 172° with the next two dihedral angles going down the dodecyl group being 170° and −171°. This result suggests that, even for a structure which clearly deviates from simple cylindrical symmetry, utilization of equations such as (1) and (2) can lead to a nearly correct average orientation. While it is difficult to generalize from this one comparison, the calculation does lend support for the use of the simplified equations in cases where adequate orientational data are difficult to obtain.

Conclusions

The experiments described herein demonstrate that with ^{13}C labeling it is possible to generate a considerable body of dipolar coupling data for molecules associated with phospholipid bilayers. The utilization of these data allowed for a more complete description of the structure and dynamics of BDOG than had previously been accomplished for other glycolipids. The results so obtained are more satisfactory not only because of their exactness but because a description of molecular order allows a more explicit physical chemical rationalization of the motional restriction. The methodology employed to facilitate measurement of the dipolar splittings for BDOG should be readily extendable to more complicated membrane-associated glycoforms, although labeling may be restricted by resolution to only one or two of the sugar rings at a time unless more sophisticated NMR techniques are used. Nevertheless, this work certainly represents a logical first step in approaching more complex problems.

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NMR and Fluorescence Studies of Cyclodextrin Complexes with Guest Molecules Containing Both Phenyl and Naphthyl Units[1]

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Contribution from the FR Organische Chemie der Universität des Saarlandes, D-6600 Saarbrücken 11, FRG. *Received July 12, 1990*

Abstract: The complexation constants of α-, β-, and γ-cyclodextrin (α-, β-, γ-CD's) with 1-anilino-8-naphthalenesulfonate (ANS) and 2-toluidino-6-naphthalenesulfonate (TNS) are (re)evaluated on the basis of different complex and computational models based particularly on NMR titrations. The NMR shifts exerted by the CD's on protons of the guest molecules together with intermolecular NOE's taken from 2D-ROESY spectra give insight into the modes of intracavity inclusion in aqueous solution. In contrast to expectations based on the cavity width α-CD binds phenyl moieties only weakly, whereas β-CD and γ-CD show more or equal intracavity immersion of the smaller phenyl than of the larger naphthalene residues.

Cyclodextrins are increasingly used for enzyme modeling, as catalytic systems, for chromatographic separations, for microencapsulation of drugs, and for many other applications.[2] It is of fundamental importance to develop better methods and a better understanding for the binding modes of substrates within the lipophilic cavities of these cycloamyloses in aqueous solution. Generally one assumes that the inner width of α-cyclodextrin (α-CD, n = 6 glucose units, ~4.5 Å) is suitable for taking up benzene derivatives, whereas β-cyclodextrin (β-CD, n = 7, ~7.0 Å) better accommodates the naphthalenes and γ-cyclodextrin (γ-CD, n = 8, ~8.5 Å) anthracene-type of substrates. The present paper demonstrates that the use of modern NMR techniques in combination with suitable fluorescence and calculational methods can lead to considerable modifications of these seemingly simple rules, if the substrate offers alternatively—or simultaneously—larger and smaller parts for intracavity immersion.

As substrates we have chosen two frequently used fluorescence dyes,[3] namely 1-anilino-8-naphthalenesulfonate (1,8-ANS) and

(1) Host–Guest Chemistry, paper 26. For paper 25, see: Schneider, H.-J.; Blatter, T.; Zimmermann, P. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 1161.

(2) (a) Bender, M. L.; Komiyama, M. *Cyclodextrin Chemistry*; Springer: Berlin, 1977. (b) Szejtli, J. *Cyclodextrin Technology*; Kluwer: Dordrecht; 1988. (c) Saenger, W. *Angew. Chem.* **1980**, *92*, 343; *Angew. Chem., Int. Ed. Engl.* **1980**, *21*, 344. (d) Komiyama, M.; Bender, M. L. In *The Chemistry of Enzyme Action*; Page, M. I., Ed.; Elsevier: Amsterdam, 1984; p 505ff. (e) Bergeron, R. J. In *Inclusion Compounds*; Atwood, J. L., Davies, J. E. D., MacNicol, D. D., Eds.; Academic Press: London, 1984; Vol. III, p 391ff. (f) Yamamoto, Y.; Inoue, Y. *J. Carbohydr. Chem.* **1989**, *8*, 29. These reviews also give detailed accounts on earlier NMR studies of cyclodextrins, although largely restricted to *p*-nitrophenolate complexes. (g) Matsui, Y.; Nishioka, T.; Fujita, T. *Top. Curr. Chem.* **1985**, *128*, 61.

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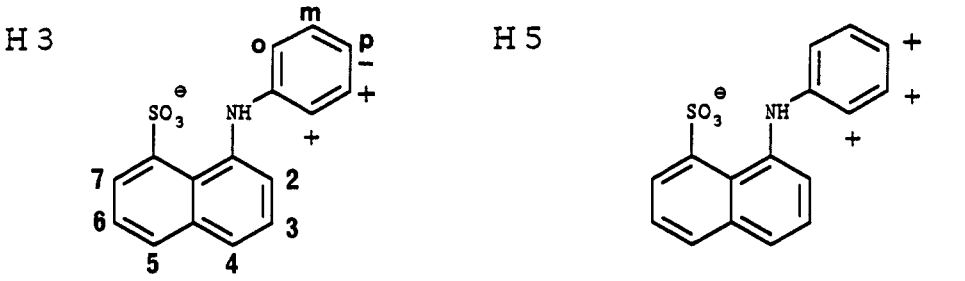
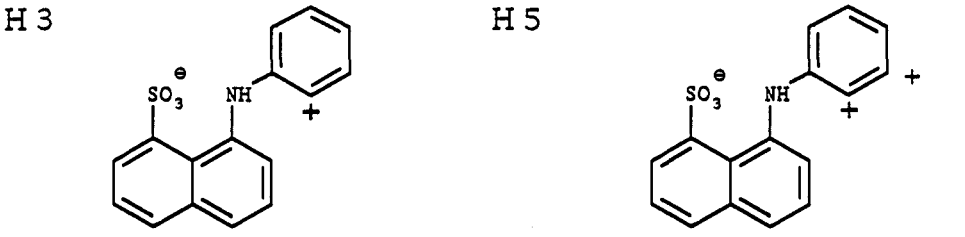
Scheme I. Intermolecular NOE Cross Peaks for ANS and Cyclodextrins[a,b]

a): α-CD



b): β-CD



c): γ-CD

[a] The number of + signs at the proton positions denote the increasing size of the cross peaks observed in the 2D ROESY spectra. [b] Because of signal overlapping the cross peaks from H2–H4 are not assigned.

2-toluidino-6-naphthalenesulfonate (2,6-TNS). Both compounds have been the subject of spectroscopic measurements with the cyclodextrins,[4-8] which, however, showed partially quite conflicting results. Thus, equilibrium constants K (always in M^{-1}) in water for β-CD/ANS were reported to be 64[7] or 11,[6] for γ-CD/ANS 280[6] vs 1260,[12] for γ-CD/TNS 1500[4] vs 425.[6] It has[4] and will be shown in the present work that the calculational consideration of other models than of one simple 1:1 complex between CD and the substrate can lead already to modified constants, which, however, on the basis of fluorescence measurements alone give no reliable numbers. NMR shift titrations, which to our knowledge have not yet been applied to cyclodextrin complexes, provide independent information on the underlying equilibria.

Methods

The basis for the NMR spectroscopic equilibrium titrations are the shifts observed on suitable cyclodextrin protons—mostly H5 and H3 or H6—upon addition of aromatic substrates.[9] The (100%) complexation-induced shifts (CIS values) and the corresponding equilibrium constants K were obtained by nonlinear least-squares fitting techniques as described earlier;[10] the presence of non-1:1 complexes, however, required modifications of the

(3) For most recent investigations of cyclodextrins with fluorescence dyes and leading references, see: (a) Jobe, D. J.; Verral, R. E.; Palepu, R.; Reinsborough, V. C. *J. Phys. Chem.* **1988**, *92*, 3582. (b) Patonay, G.; Shapira, A.; Diamond, P.; Warner, I. M. *Ibid.* **1986**, *90*, 1963. (c) Nelson, G.; Patonay, G.; Warner, I. M. *Anal. Chem.* **1988**, *60*, 274. (d) Hamai, S. *J. Am. Chem. Soc.* **1989**, *111*, 3954. (e) Catena, G. C.; Bright, F. V. *Anal. Chem.* **1989**, *61*, 905. (f) Bright, F. V.; Catena, G. C.; Huang, J. *J. Am. Chem. Soc.* **1990**, *112*, 1343.

(4) Kendo, K.; Nakatami, H.; Hivomi, K. *J. Biochem.* **1976**, *79*, 393.

(5) Harada, H.; Furure, M.; Mazakura, S. *Polymer J.* **1980**, *12*, 29.

(6) Crescenci, V.; Gamini, A.; Palleshi, A.; Rizzo, R. *Gazz. Chim. Ital.* **1986**, *116*, 435.

(7) Franke, J.; Merz, F.; Lorensky, H. W.; Müller, W. M.; Werner, W.; Vögtle, F. *J. Incl. Phenom.* **1985**, *3*, 471.

(8) For related complexation measurements with cyclodextrins, see ref 2 and the following: (a) Hamai, S. *J. Phys. Chem.* **1988**, *92*, 6140. (b) Buvari, A.; Barcza, L. *J. Chem. Soc., Perkin Trans. II* **1988**, 543. (c) Sonemasa, I.; Akamine, Y. *Bull. Soc Chem. Japn.* **1987**, *60*, 2059. (d) Ueda, H.; Nagai, T. *Chem. Pharm. Bull.* **1980**, *28*, 1415 and references cited therein.

(9) Komiyama, M.; Hirai, H. *Bull. Soc. Chem. Jpn.* **1981**, *54*, 828; *Polym. J.* **1981**, *13*, 171. See also ref 2d.

(10) Schneider, H.-J.; Kramer, R.; Simova, S.; Schneider, U. *J. Am. Chem. Soc.* **1988**, *110*, 6442.

calculational approach (see below). If the equilibrium constants are known from these or from similarly evaluated[9] fluorescence titrations, one can also obtain the CIS NMR values on the *substrate* signals from measurements with known substrate and comparable, or higher, host (CD) concentrations. These substrate CIS values—other than the fluorescence data—can then be used to indicate which substrate parts are immersed in the CD cavity.

Detailed studies with α-CD and a series of substituted benzenes combined with NOE measurements[11] and the present investigation have established that encapsulation of neutral aromatic substrates into cyclodextrin cavities is invariably accompanied by up to −0.25 ppm shielding of a fully immersed aromatic proton. In an earlier investigation[8d] *de*shielding of substrate protons upon complexation with β-CD was reported; it was, however, overlooked that these complexations were performed with partially deprotonated CD anions due to the basic reaction conditions used (0.2 N NaOD, pH ~13). Corresponding ^{13}C-NMR CIS values[2d,9] show a quite irregular pattern[11], in line with related cyclophane host–guest investigations.[12]

Complementary information on the immersion mode are accessible from intermolecular NOE data, which—other than in earlier investigations of cyclodextrin complexes[2a,c,9,13]—were obtained in this work from spin-lock rotating frame NOE experiments (ROESY).[14] This technique allows to circumvent the problem of extremely small NOE's resulting from the unfavorable correlation times of host–guest complexes with a molecular weight around 1000, particularly if one wants to use the high magnetic fields necessary for the dispersion of the complex signals. Although our current instrumentation precluded accurate integration of the NOE signals, the relative intensities of the 2D cross peaks—denoted by + or ++ in the structure diagrams—furnished a pattern for the immersion mode in the CD's which is generally consistent with the observed ^{1}H CIS values.

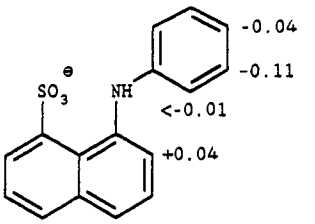
ANS Complexes

The complexation constants of K = 64 M^{-1} for β-CD and of K = 1260 M^{-1} for γ-CD determined by Vögtle[7] et al., or by Catena and Bright[3e] for β-CD (110 M^{-1}), with fluorescence titrations were found to be in general accord with ^{1}H NMR effects of ANS on all six CD signals, which yielded the same CIS values within usually ±0.01 ppm for three different mixtures for each CD between 20% and 70% complexation (see supplementary material, Table V). The errors estimated on this basis correspond to K = (65 ± 7) M^{-1} for β-CD and to K = (1260 ± 180) M^{-1} for γ-CD and clearly rule out other literature[6] results. The reported[7] value of K = "0" for α-CD, however, is at variance with both the observation of—although small—shielding effects on H3 and H5 of α-CD as well as with cross peaks in the ROESY spectra (see below). On the basis of the typical CIS values observed with benzene derivatives in α-CD as well as with naphthalenes in β-CD (upper limit 0.12 ppm for H3 and 0.25 ppm (for H5)) and the small shifts measured in α-CD upon addition of concentrated ANS (3.6×10^{-2}), which amount to −0.02 ppm for H3 and −0.04 for H5, one can estimate a constant of $K \simeq 5$ M^{-1} for the ANS/α-CD complex. A similar value ($K \simeq 2$ M^{-1}) was obtained from the ANS fluorescence emission shift of only $\Delta\lambda$ = 3 nm observed upon addition of α-CD (10^{-1} M) compared to typical shifts of $\Delta\lambda$ = 30 to 50 nm observed for ANS fully complexed by β-CD or γ-CD.

The geometry information obtained from the ROESY peaks (Scheme I) as well as from the CIS values of ANS are as much consistent as surprising: in β-CD only the phenyl ring of ANS as the *smaller* component shows cross peaks *and* upfield CIS (Scheme II), although the larger naphthalene moiety is generally assumed to fit better. Furthermore, NOE and CIS (Schemes I and II) indicate, even for the still wider γ-CD cavity, inclusion of *both* the phenyl and naphthyl part, presumably in the form of two different complexes. Finally, the ROESY peaks observed with α-CD demonstrate that the phenyl part is indeed immersed in α-CD but in view of the small equilibrium constants (see above) and the absence of visible CIS only to a very small degree, in spite of the expected suitability of α-CD for benzene derivatives. The observed NOE and CIS values allow only limited conclusions with respect to the detailed conformations in the complexes; they generally agree with the approach of ANS from the wider side of the torus-shaped cavity, with the nonencapsulated part on this side, and the immersed part closer to H3 than to H5 of the cycloamylose.

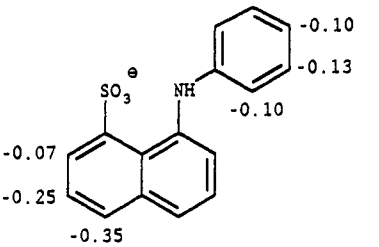
Scheme II. Complexation Induced ^{1}H NMR Shifts (CIS, in ppm) at 100% Complexation for ANS with β- and γ-CD[a,b]

a): β-CD



All other CIS values smaller than - 0.02 ppm.

b): γ-CD



[a] For part (a) all other CIS values smaller than −0.02 ppm. [b] Due to signal overlapping no CIS values are detectable for H2–H4; the signal however is shifted by >−0.15 ppm.

TNS Complexes

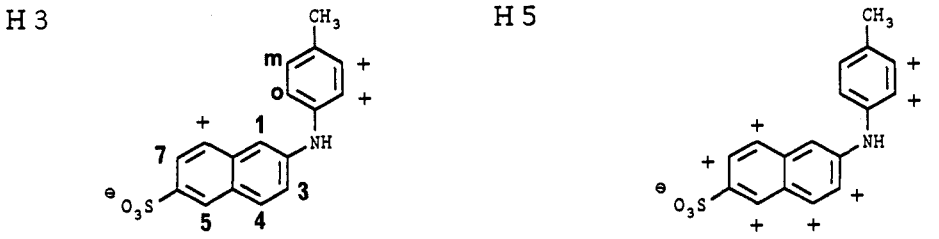
The fluorimetrically determined literature[4] value of K = 18 M^{-1} for the α-CD/TNS complex cannot be checked by NMR titration due to the low solubility of TNS (<5×10^{-3} M). Although TNS binding to α-CD is stronger than ANS, *no* intermolecular cross peaks could be observed in the ROESY spectra in spite of several attempts which did show the expected *intra*molecular NOE's. However, α-CD does generate very small upfield ^{1}H NMR shifts at the naphthalene moiety of TNS as well as larger deshielding at the phenyl ring, the latter being reminiscent of the only deshielding observed with ANS upon addition of α-CD (Scheme II). The shielding effects point to very weak complexation of the naphthalene part, which due to the 2,6-substitution in TNS can immerse into an α-CD cavity better than ANS (1,8-substituents). Another complex seems to be present in which the methyl group at the phenyl substituent—which is absent in ANS—approaches the cavity from the top side.

Fluorimetric titrations of TNS with β-CD already have been described in the literature on the basis of 1:1 complexes (K_{11} = 2000[3e] or 1500[4] or 4000[6] M^{-1}) in addition to (β-CD)$_2$. TNS (2:1) complexes with K_{21} = 600[3e] or 14[4] or 20[7] M^{-2}. Indeed, we find a poor fit *and* a strong dependence of the obtained K values on

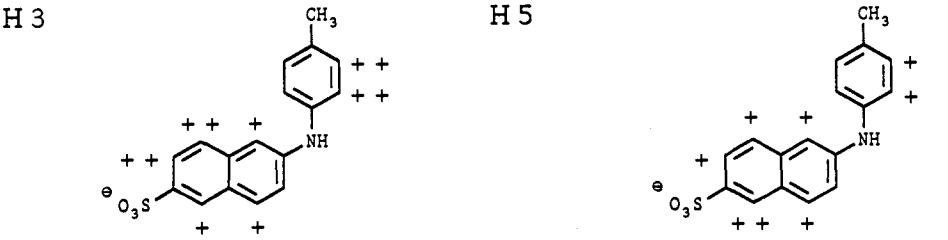
(11) Schneider, H.-J.; Simova, S. Unpublished results.
(12) Kumar, S.; Schneider, H.-J. *J. Chem. Soc., Perkin Trans. II* **1989**, 245.
(13) Bergeron, R.; Channing, M. A.; McGovern, K. A. *J. Am. Chem. Soc.* **1978**, *100*, 2878.
(14) Bothner-By, A. A.; Stephens, R. L.; Lee, J.-M.; Warren, C. D.; Jeanloz, R. W. *J. Am. Chem. Soc.* **1984**, *106*, 811.

Scheme III. Intermolecular NOE for TNS

a): β-CD

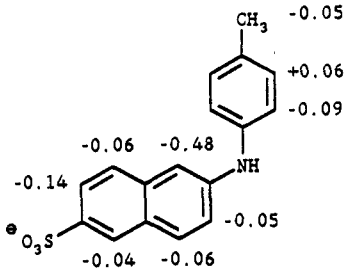


b): γ-CD



[a] For explanation see Scheme I.

Scheme IV. CIS Values for TNS from β-CD Calculated for a 1:1 Complex with $K_{11} \approx 2400\ M^{-1}$ [a]



[a] For explanation see Scheme II.

the emission wavelength if we evaluate the fluorescence titration on the basis of only 1:1 complexes (Table I, plot 1, supplementary material). The evaluation based on additional CD_2·TNS 2:1 complex formation ($\triangleq K_{21}$) leads to an excellent fit (plot 2, supplementary material) with $K_{11} = 6.65 \times 10^3\ M^{-1}$ and $K_{21} = 3.1 \times 10^2\ M^{-2}$, with intrinsic (relative) emission intensities of ϵ_{11} = 1.6E5 and ϵ_{21} = 4.8E5. In view of the parameter identification problem involved here with four unknowns the good fit does not preclude other either (a) 1:1 + 2:1 combinations (as described in the literature[4,7]) or, alternatively, (b) formation of two different 1:1 complexes. Subsequent titration of β-CD with TNS by observation of TNS ^1H NMR shifts leads to a slightly better fit for model (b) (plot 3, supplementary material) with $K_{11} = 2040 \pm 260$ and $K_{11'} = 1520 \pm 300$. Model (a) with $K_{21} = 1.4$ (from fluorescence titrations) and $K_{11} = 2400$ (from NMR) (Table IV, plot 4, supplementary material) can be made less likely based on the rationalization of NOE and CIS values (Schemes III and IV): while both NOE and CIS values would allow formation of (a) 1:1 + 2:1 or (b) two different 1:1 complexes (one with the naphthyl, the other with the phenyl part in the cavity), model (b) furnishes better agreement between the single K' values obtained from the good computer fit of four NMR signals (Table II, supplementary material).

TNS with γ-CD showed intermolecular NOE's as well as ^1H NMR shielding effects (Schemes III and IV) indicative of complexation at the naphthyl but also at the phenyl moiety, in spite of the large cavity. Although a fluorescence titration yielded a good fit on the basis of a single 1:1 complex (plot 5, supplementary material), similar and virtually undistinguishable agreement was obtained for three other models comprising (b) two different 1:1, (c) 2:1 (γ-CD_2·TNS) + 1:1, and (d) 1:1 + 1:2 (γ-CD·TNS_2) complexes (Table III, plots 6a–d, supplementary material). NMR titrations, on the other hand, yielded *no* satisfactory fit with *any* of the single combinations (a)–(d) or showed convergence only with definitely unrealistic CIS values as artifacts of the computer simulations. In view of the then too large number of unknown parameters (≥6 instead of 4), no attempt was made to fit the NMR data to more complex models than (b)–(d).

Conclusions

NMR shift titrations provide a generally better basis for the evaluation of cyclodextrin complexes than fluorescence titrations, which should at least be checked for consistency at different emission wavelengths. The shifts generated by the cyclodextrins on different protons of the guest molecule allow discrimination between the possible modes of encapsulation and, together with intermolecular NOE's, represent a superior method to prove intracavity inclusion in solution. Bright et al.[3f] have most recently shown, by nanosecond fluorescence spectroscopy, that even structures which appear homogeneous in time-averaged NMR spectra—such as ANS–β-CD complexes—coexist in discrete species with slightly different geometries and lifetimes.

The popular view of supramolecular complexes—nowadays underscored by computer molecular modeling—generally is that a guest molecule will seek the closest contact, or fit, to the host cavity. Our results demonstrate again that this view is too simplistic at least for cyclodextrin complexes in water. The need to revise seemingly straightforward interpretations of cyclodextrins as enzyme models along these lines is emphasized by the recent finding[15] that the well-known acceleration of *p*-nitrophenylacetate cleavage by α-cyclodextrin is *not* due to intracavity inclusion of the substrate phenyl ring. The obvious trend found with all complexes investigated in the present paper is that the binding is more effective if there is enough room for high mobility of lipophilic guest moieties within the cavity. This is in accordance

(15) Tee, O. S.; Hoeven, J. J. *J. Am. Chem. Soc.* **1989**, *111*, 8318.

with large entropic contributions to hydrophobic binding in cyclodextrins.[2] Furthermore it is sufficient that the few enthalpy rich water molecules within the cavity, which lack several stabilizing hydrogen bonds, are replaced by a still highly mobile lipophilic substrate part. Similar reasoning has been applied earlier for explaining the often stronger binding of phenyl derivatives to β-CD in comparison to α-CD.[2a,e]

Experimental and Computational Details

α- and β-Cyclodextrins are commercially available (Fluka); γ-cyclodextrin was a gift of Professor Szejtli, Budapest. The cycloamyloses were dried prior to use at 100 °C over phosphorus pentoxide in vacuum and found to be at least 95% pure, based on ^{1}H-NMR signal comparison with added methanol as internal reference of known concentration. Only commercial γ-cyclodextrin from other sources showed other signals due to contaminations. ANS in the form of its ammonium salt (Sigma) was used as purchased, the TNS potassium salt (Serva) was dried at 100 °C prior to use. Equilibrium titrations were carried out similarly as described earlier[10] by using stock solutions of suitable concentrations in reagent grade solvents.

In a typical NMR or fluorescence titration 0.5 mL of a CD stock solution (e.g., 10^{-3} M) was added in eight steps to a solution of the substrate dye (for fluorescence, 2 mL, 1.00×10^{-4} M; for NMR, 0.5 mL, 10^{-3} M), leading to complexation degrees from ~20% to ~80%.

Fluorescence measurements were carried out with a Hitachi instrument F-3000 at ambient temperature at concentrations adjusted to the expected equilibrium constants and at emission wavelength values given in the supplementary material. All solutions were deoxygenated by purging with nitrogen for 5 min before measurements. The excitation wavelength for ANS and TNS was 375 or 366 nm, respectively.

NMR spectra were recorded on a Bruker AM400/Aspect 3000 system at 295 ± 2 K and 400 MHz. The ROESY spectra were measured without degassing by using the pulse sequence proposed by Griesinger and Ernst.[16] A DANTE (12) sequence of 4000 pulses of 12 μs (60°) with a delay of 63 μs was used for spin-locking on the transmitter channel. The spin-locking field was at the beginning approximately 2 kHz and then dropped continuously to 1.6 kHz. Typical measuring conditions were as follows: sweep width 5000 (2500) Hz; data size 2 K/1 K in 2/1 direction, n/3 shifted squared sine bell in both directions, delay between the scans 2 s; 8 or 16 scans.

Calculations were performed for 1:1 complexes as described earlier[10] for the NMR technique. For fluorescence titrations requiring adjustment of the emissions to the change of volumes and concentrations as well as for evaluation of more complicated equilibria, a program CHEMSIM written by R. Kramer for ATARI 1040 was used, which allows curve fitting for multistep equilibria by numerical integration by using a Newton zero search in several dimensions. Due to the alternating use of Simplex and Newton-Raphson procedures[17] the program shows usually fast convergence at the *same* set of parameters (equilibrium constants and "intrinsic" NMR CIS or fluorescence intensities) independent of different starting values for these variables.

Acknowledgment. This work was financially supported by the Deutsche Forschungsgemeinschaft, Bonn, and the Fonds der Chemischen Industrie, Frankfurt. We thank the referees for valuable suggestions.

Supplementary Material Available: Tables I-V with equilibrium constants and spectral parameters and plots 1-6 with graphical representation of selected titrations (8 pages). Ordering information is given on any current masthead page.

(16) Griesinger, C.; Ernst, R. R. *J. Magn. Res.* **1987**, *75*, 261.
(17) Cf.: Press, W. H.; Flannery, B. P.; Teukolsky, S. A.; Vetterling, W. T. *Numerical Recipes*; Cambridge University Press: Cambridge, 1986; pp 254, 269, 289, 312ff.

Fluorescence Decay Kinetics of the Tryptophyl Residues of Myoglobin Single Crystals†

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Contribution from the Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6. *Received August 28, 1990*

Abstract: Picosecond time-resolved fluorescence measurements on protein single crystals are described. Fluorescence spectroscopy is used to probe long-range (20 Å) intramolecular interactions, between tryptophyl residues and heme, in the solution and crystalline states of myoglobin. The fluorescence of single crystals of sperm whale myoglobin decays with double exponential kinetics ($\tau_1 \approx 15$ ps, $\tau_2 \approx 70$ ps), while that of crystals of yellow fin tuna myoglobin obeys single exponential kinetics ($\tau \approx 24$ ps). These data are shown to be consistent with a protein structure, at atomic resolution, that is independent of sample state.

X-ray diffraction can provide high-resolution structural information on proteins in the crystalline state. Spectroscopic techniques including NMR,[1] EPR,[2] Raman,[3] and XAFS[4] have been employed to directly probe differences and/or similarities between crystal and solution protein structures. These techniques generally only report on the immediate environment of an atom or bond. Fluorescence spectroscopy has the advantages of high sensitivity and that long-range interactions can potentially be probed. Time-resolved fluorescence measurements on proteins in solution have provided considerable information on protein structure, function, and dynamics.[5] Clearly it would be of interest to study proteins in the crystalline state with this technique.[6]

The individual tryptophyl residues of myoglobin in solution exhibit well-characterized monoexponential decay kinetics with picosecond lifetimes.[7] The simple decay kinetics and short lifetimes are a consequence of the highly efficient energy transfer from the tryptophyl residues to the heme group.[7,8] A further consequence of the efficient energy transfer is that the fluorescence

† Issued as N.R.C.C. Publication No. 31932.

(1) Rothgeb, T. M.; Oldfield, E. *J. Biol. Chem.* **1981**, *256*, 1432-1446.
(2) Fiamingo, F. G.; Brill, A. S.; Hampton, D. A.; Thorkildsen, R. *Biophys. J.* **1989**, *55*, 67-77.
(3) Morikis, D.; Sage, J. T.; Rizos, A. K.; Champion, P. M. *J. Am. Chem. Soc.* **1988**, *110*, 6341-6342.
(4) Lin, S.-L.; Stern, E. A.; Kalb, A. J.; Zhang, Y. *Biochemistry* **1990**, *29*, 3599-3603.
(5) Beechem, J. M.; Brand, L. *Annu. Rev. Biochem.* **1985**, *54*, 43-71.
(6) Time-resolved fluorescence measurements on protein powders have been reported. Desie, G.; Boens, N.; DeSchryver, F. C. *Biochemistry* **1986**, *25*, 8301-8308 and references cited therein.
(7) Willis, K. J.; Szabo, A. G.; Zuker, M.; Ridgeway, J.; Alpert, B. *Biochemistry* **1990**, *26*, 5270-5275.
(8) (a) Hochstrasser, R. M.; Negus, D. K. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 4399-4403. (b) Janes, S. M.; Holton, G.; Ascenzi, P.; Brunori, M.; Hochstrasser, R. M. *Biophys. J.* **1987**, *51*, 653-660.

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