BIOORGANIC CHEMISTRY 5, 425–436 (1976)

The Molecular Disposition of Sodium *p*-Nitrophenolate in the Cavities of Cycloheptaamylose and Cyclohexaamylose in Solution

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Received May 19, 1976

The extent to which sodium *p*-nitrophenolate penetrates the cycloheptaamylose and cyclohexaamylose cavities has been defined by nmr studies of the complexes in aqueous solution. Measurements of changes in the ^1H nmr spectra of both the sodium *p*-nitrophenolate guest and the cyclohexaamylose host, along with an intermolecular nuclear Overhauser effect, reveal that this guest only partially penetrates the cyclohexaamylose cavity and does so nitro end first. With cyclohepaamylose, sodium *p*-nitrophenolate penetrates more deeply, but the orientation may be less specific. These findings are in accord with the notion that *both* London dispersion forces and removal of high energy cavity water contribute to substrate binding.

INTRODUCTION

The cycloamyloses have received a great deal of attention in recent years as enzyme active-site models (*1–3*). The tack of most investigators has been to expand the spectrum of reactions the cycloamyloses catalyze (*4*) as well as to improve on their catalytic ability by chemical modification (*5–6*). However, there has been relatively little experimental effort invested in an explanation of the driving forces responsible for substrate complexation. A number of suggestions as to the origins of these forces has been made: release of high energy cavity water (*1*), London dispersion forces, and release of strain energy (*7*). It seems likely that all of these forces would contribute to the overall binding energy, although in a recent study on the role of strain energy in cycloamylose substrate complexation, we demonstrated that release of the alleged ring strain plays at most a small role in the complexation driving forces (*8*). This investigation left us feeling that either London dispersion forces or release of high energy cavity water on substrate complexation or both were responsible for inclusion. Verification of these ideas, of course, would require some knowledge of the host–guest disposition in solution. In this paper we report our efforts to determine the geometries in solution of the complexes of sodium *p*-nitrophenolate and cyclohexaamylose and cycloheptaamylose.

EXPERIMENTAL

Materials. The cycloamyloses were obtained from Aldrich Chemical Company and purified by the method of Cramer and Henglein (*9*). The *p*-nitrophenol was also obtained from Aldrich and was crystallized three times from chloroform. Deuterium oxide, 99.8%, was purchased from Merck and Co.

Sample preparation. The cycloamylose hydroxyl protons were exchanged for deuterium by lyophilizing 600 mg of the respective carbohydrates three times from 40 ml of D_2O. A stock solution of sodium *p*-nitrophenolate was prepared by adding *p*-nitrophenol to an equivalent weight of sodium hydroxide in deuterium oxide. This solution was freeze-dried, the resulting solid was dissolved in D_2O, and anhydrous trisodium phosphate was added. The pD was adjusted with deuterophosphoric acid to 11.0. Both the sodium *p*-nitrophenolate and the cycloamylose solutions were adjusted to pD 11.0 and ionic strength 0.5. Listed pD values were obtained by adding 0.4 to the pH meter reading, using a combination electrode which had been standardized with pH 10 buffer in H_2O, then rinsed with D_2O (*10*).

Nuclear magnetic resonance spectra. ^1H correlation mode nmr spectra (*11*), 250 MHz, were obtained at the NMR Facility for Biomedical Studies, Carnegie–Mellon University. Nuclear magnetic resonance spectra, 100.1-MHz ^1H pulsed Fourier transform, were obtained on the University of Maryland Varian XL-100 spectrometer. To eliminate interference of the residual proton resonance of the solvent with the anomeric proton resonance of the cycloamyloses, a WEFT pulse sequence was used for most of the 100.1-MHz spectra (*12*). The 250-MHz spectra were referenced to internal HDO, and a capillary of 0.005 *M* sodium formate was used as an external chemical shift reference for the 100.1-MHz spectra. ^1H homonuclear Overhauser enhancements (NOE's (*13*)) are reported as the percentage difference in integrated intensity of the resonance being observed when the second radiofrequency (rf) was applied first at the resonance frequency to be irradiated, and then set in a vacant region of the spectrum. In the 100.1-MHz NOE experiments, the irradiating rf was gated off during the acquisition of the free induction decay, but kept on during the long (10 sec) delay between pulses (*14*). Peak intensities were determined by planimetry. For 250-MHz spectra, the sample temperature was 28 ± 1°C; for 100.1⁻ MHz spectra, the sample temperature was 25 ± 1°C.

RESULTS

Effect of sodium p-nitrophenolate on cycloamylose spectra. ^1H nmr spectra were obtained for a number of samples prepared with different ratios of *p*-nitrophenolate to cycloamylose. The spectrum of free cycloheptaamylose in D_2O was initially assigned by Demarco and Thakkar (*15*). These authors found that upon addition of any of a number of aromatic substrates, the H-3 and especially the H-5 resonances of cycloheptaamylose shift upfield because of the diamagnetic anisotropy (*20*) of the included benzenoid guests. We also observed this upfield shift of the two interior methine protons H-3 and H-5 of cycloheptaamylose upon addition of sodium *p*-nitrophenolate.

The ^1H nmr spectrum of cyclohexaamylose, shown in Fig. 1a, is almost identical to that of cycloheptaamylose. The assignments were confirmed in the present work by decoupling experiments at 250 MHz. However, the effect of *p*-nitrophenolate upon the cyclohexaamylose spectrum differs quite markedly from the situation with cycloheptaamylose. When sodium *p*-nitrophenolate is added to solutions of the cyclic hexamer, large changes occur in the chemical shift of H-3 only; the chemical shift of H-5 is affected very little. This result is illustrated in Fig. 1b, which shows the 250-MHz

spectrum of cyclohexaamylose (except for the anomeric proton resonance), which is 86% bound with *p*-nitrophenolate (assuming an association constant of the 1:1 complex of $4.5 \times 10^3\ M^{-1}$). Comparing this spectrum to the one above it, that of free cyclohexaamylose, it is seen that, while most of the cyclohexaamylose resonances have shifted somewhat upfield relative to the internal lock frequency (the HDO signal),

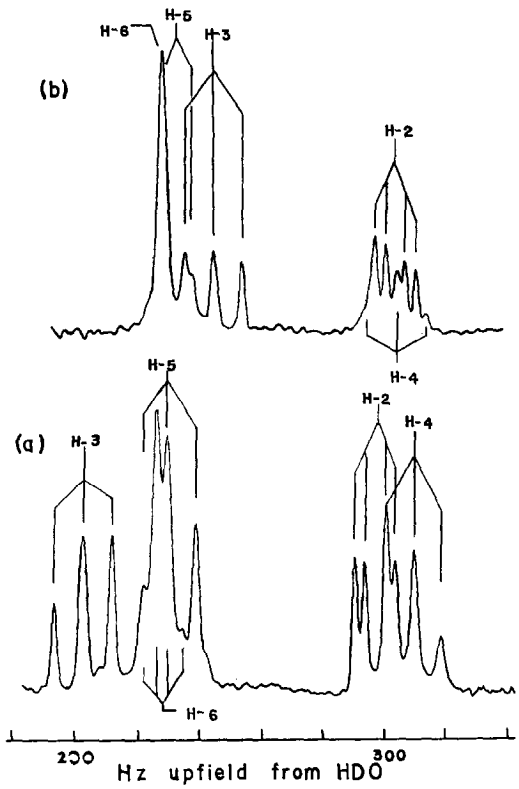

FIG. 1. ^{1}H correlation nmr spectra, 250 MHz, of (a) 0.005 *M* cyclohexaamylose and (b) 0.005 *M* cyclohexaamylose–0.005 *M* sodium *p*-nitrophenolate. Both samples were prepared using pD 11 phosphate buffer in D_2O, as described in the text. The resolution in both spectra was digitally enhanced; spectrum (b) is presented with a smaller vertical scaling factor.

only the resonance of H-3 has shifted more than 0.1 ppm. In particular, the resonance of H-5 has shifted only slightly, if at all.

Effect of cycloamyloses on the sodium p-nitrophenolate spectra. Figure 2 shows the effect of increasing the fraction of *p*-nitrophenolate bound to cycloamyloses upon the ^{1}H chemical shifts of *p*-nitrophenolate. Large downfield shifts are induced for the resonances of both sets of *p*-nitrophenolate protons by cyclohexaamylose. The *meta* proton resonance of *p*-nitrophenolate is shifted most drastically, approximately twice as much as is the *ortho* proton resonance for a given fraction of *p*-nitrophenolate bound to cyclohexaamylose. By comparison, the binding to cycloheptaamylose has relatively little effect on the chemical shifts of *p*-nitrophenolate. As shown in Fig. 2,

in this case the *ortho* proton resonance is shifted slightly downfield, while the *meta* proton resonance is shifted slightly upfield.

The linewidths of the *p*-nitrophenolate resonances increased significantly with the fraction of *p*-nitrophenolate bound to cycloamylose. No internal linewidth standard was available, since the possibility existed that any small molecule added to the solutions to provide such a standard would compete with *p*-nitrophenolate in binding to the cycloamylose. Thus, no perfectly quantitative linewidth comparisons are possible from

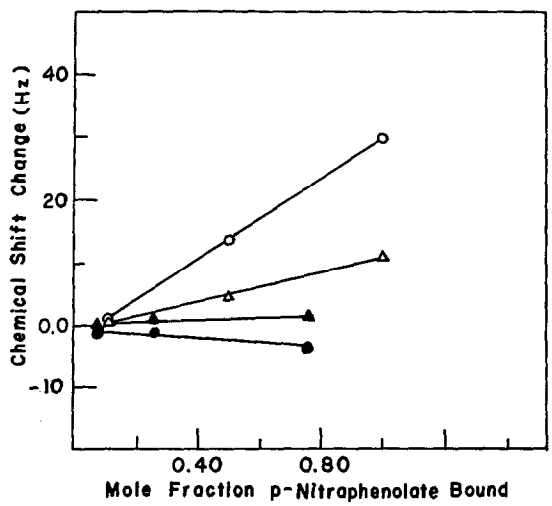

FIG. 2. Effects of cycloheptaamylose and cyclohexaamylose complexation of sodium *p*-nitrophenolate on the ^{1}H nmr of sodium *p*-nitrophenolate. Samples were prepared as described in the experimental section. The sodium *p*-nitrophenolate concentrations were varied between 0.005 and 0.050 *M* and the cycloamylose concentrations between 0.005 and 0.02 *M* for cyclohexamylose and 0.005 and 0.017 *M* for cycloheptaamylose. The cycloamylose-induced changes in chemical shifts of the sodium *p*-nitrophenolate are plotted relative to free sodium *p*-nitrophenolate. (△, ○) Cyclohexaamylose system; (▲, ●) cycloheptamylose system; (△, ▲) *ortho* protons of sodium *p*-nitrophenolate; (○, ●) *meta* protons of sodium *p*-nitrophenolate.

one sample to another. For the binding of *p*-nitrophenolate to cyclohexaamylose, however, it was clear that in each sample the *meta* proton resonance lines broadened significantly more than those of the *ortho* protons. For example, under the spectrometer conditions used, including an exponential weighting factor for the free induction decay of −1.0 sec, the linewidth for each major resonance line of the *meta* and *ortho* protons in free *p*-nitrophenolate was 1.0 Hz. Under the same conditions, the *p*-nitrophenolate linewidths for a sample 0.005 *M* in *p*-nitrophenolate and 0.02 *M* in cyclohexaamylose (i.e., with 99% of the *p*-nitrophenolate bound to cyclohexaamylose) were 3.0 Hz for the *meta* proton resonances and 2.7 Hz for the *ortho* proton resonances. Binding to cycloheptaamylose appeared to increase the *p*-nitrophenolate linewidths to about the same extent as did binding to cyclohexamylose for an equal fraction of *p*-nitrophenolate bound. In this case, however, both the *ortho* and *meta* proton resonances were broadened approximately equally in each sample.

Effect of sodium p-nitrophenolate concentration on its ^{1}H chemical shifts. In order to ascertain the effect of self-association on the ^{1}H chemical shifts of *p*-nitrophenolate in

D_2O, spectra were obtained for a series of samples of different concentrations of sodium *p*-nitrophenolate, always in pD 11 phosphate buffer. The set of concentrations of *p*-nitrophenolate (0.005 to 0.100 *M*) spanned the range of *p*-nitrophenolate concentrations used in the samples containing cycloamylose. Chemical shifts were measured relative to the signal from a concentric capillary of 0.005 *M* sodium formate. The chemical shifts of both *ortho* and *meta* protons of *p*-nitrophenolate moved upfield with increasing concentrations, as would be expected from the effect of self-association, but the magnitude of the upfield shift from 0.005 to 0.100 *M* was quite small: 0.04 ppm for the *meta* proton resonance, and 0.03 ppm for the *ortho* proton resonance.

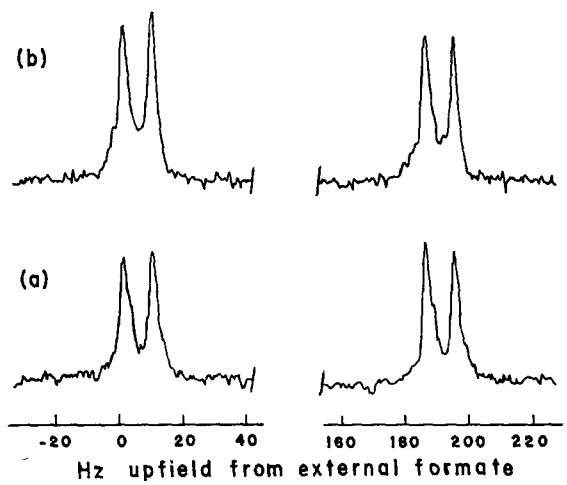

FIG. 3. A ^1H homonuclear intermolecular NOE experiment on sodium *p*-nitrophenolate and cyclohexaamylose: (a) second radiofrequency far from any resonances of sample; (b) second radiofrequency saturating cyclohexaamylose C–H resonances. In each spectrum, left-hand doublet is due to *p*-nitrophenolate *meta* protons, and right-hand doublet to *p*-nitrophenolate *ortho* protons. Sample was prepared using pD 11 phosphate buffer as described in the text, 0.020 *M* in cyclohexaamylose, and 0.005 *M* in *p*-nitrophenolate. Both spectra were obtained at 100.1 MHz in Fourier transform mode, using 65 pulses and the procedure described in the text.

Intermolecular nuclear Overhauser enhancement. A ^1H homonuclear Overhauser experiment was done on samples of *p*-nitrophenolate plus cyclohexaamylose at both 100.1 and 250 MHz. In both cases, substantial enhancement of the *meta* proton resonance of *p*-nitrophenolate was observed upon saturation of the cyclohexaamylose resonances upfield of the HDO resonance. In these experiments there was no significant effect on the intensity of the *ortho* proton resonance of *p*-nitrophenolate. In Fig. 3 are shown 100.1-MHz spectra from such an NOE measurement, for a sample in which the *p*-nitrophenolate was 99% bound to cyclohexaamylose; the measured enhancement was 34% for the *meta* proton resonance and 1% for the *ortho* resonance. At 250 MHz, the greater separation of the peaks made it possible to saturate particular individual cyclohexaamylose resonances. As shown in Table 1, irradiation of the H-3 resonance of cyclohexaamylose produced the largest enhancement of the *p*-nitrophenolate resonances. For each reported NOE, the precision is estimated to be ±3%.

TABLE 1

250-MHz Intermolecular Nuclear Overhauser Enhancements

m[a]	Cyclohexaamylose Proton(s) irradiated[b]	p-Nitrophenolate NOE's[c]	
		meta	*ortho*
0.86[d]	H-3	8	−2
0.99[e]	H-3	9	2
0.99[e]	H-5	3	0
0.99[e]	H-2, H-4	0	0

[a] Mole fraction of p-nitrophenolate bound to cyclohexaamylose.

[b] Second radiofrequency was centered at the resonance position of the indicated proton, using the minimum power level necessary to saturate that resonance.

[c] Enhancements in percents; estimated accuracy ±3%.

[d] Sample composition: cyclohexaamylose, 0.005 M; p-nitrophenolate, 0.005 M; solvent, pD 11 buffer as described in text.

[e] Sample composition: cyclohexaamylose, 0.020 M; p-nitrophenolate, 0.005 M; solvent, pD 11 buffer as described in text.

DISCUSSION

In this section we first interpret all of the nmr results in terms of the orientation and depth of penetration of p-nitrophenolate when bound to each of the cycloamyloses. Then we discuss the implications of the molecular disposition of p-nitrophenolate in the respective cycloamylose cavities with regard to theories of the driving forces for complexation.

Sodium p-nitrophenolate binding to cyclohexaamylose. From the observed changes in the chemical shifts for both the p-nitrophenolate guest and the cyclohexaamylose host as the host:guest ratio was varied, as well as from the known forward and reverse rate constants for the association equilibrium ($5.2 \times 10^8\ M^{-1}\text{sec}^{-1}$ and $1.3 \times 10^5\ \text{sec}^{-1}$, respectively) (*2*), it is clear that the system is in the nmr chemical shift fast-exchange limit. (*17*) That is, the p-nitrophenolate resonances appear at the average of the chemical shift of free p-nitrophenolate and the chemical shift of p-nitrophenolate bound in each possible orientation to cyclohexaamylose, weighted by the fractional population of p-nitrophenolate molecules in each environment. Likewise, each of the cyclohexaamylose resonances occurs at its fast-exchange position, weighted by the fraction of empty cyclohexaamylose molecules and the fraction of cyclohexaamylose molecules which have p-nitrophenolate guests.

Considering first the observation that only the resonance of H-3 of cyclohexaamylose shifts significantly upfield when p-nitrophenolate is present in the solution, we conclude that p-nitrophenolate only partially penetrates the cyclohexaamylose cavity. If full penetration had occurred, the resonance of H-5 of cyclohexaamylose would also be shifted upfield at least to some extent, and this was not observed. However, it is clear from the shift of H-3 that it is indeed the "open" face of cyclohexaamylose which is the

site of inclusion of *p*-nitrophenolate. The conclusion that only partial penetration of the cyclohexaamylose cavity by *p*-nitrophenolate occurs is consistent with model building, which indicates that, although possible, complete penetration of the cyclohexaamylose cavity by *p*-nitrophenolate is unlikely. The substrate simply will not fit entirely into the cavity without considerable distortion of the cyclic oligomer. Furthermore, only two general orientations of the *p*-nitrophenolate with respect to the cavity allow any penetration at all, namely, the orientations which have either the hydroxyl or the nitro end of the guest projecting into the cavity.

Turning next to consideration of the changes in the *p*-nitrophenolate spectra caused by binding to the cyclohexaamylose, we must first account for the marked downfield shifts observed for each of the *p*-nitrophenolate resonances and then for the fact that the resonances of the *meta* protons are shifted about twice as much as those of the *ortho* protons. Similar downfield shifts were reported recently by MacNicol (*18*) for the *ortho* and *meta* resonances of an aqueous solution of *p*-cymene and cyclohexaamylose, although no assignments of the aromatic peaks were made. Downfield ^1H magnetic resonance shifts can be induced in the spectrum of one molecule when binding to another by several physical mechanisms: diamagnetic anisotropy of particular bonds or regions of the host (*19*), Van der Waals shifts (*16*), or steric perturbation (*21*). From the limited amount of experimental data available, it is not possible to assign the observed downfield shifts in *p*-nitrophenolate and *p*-cymene caused by complexation to cyclohexaamylose to a particular mechanism, but two conclusions are inescapable. First, whatever the mechanism, there must be intimate contact between the *p*-nitrophenolate protons whose resonances are being shifted and an area of the host molecule which is causing the downfield shift. Second, and more important, is the implication that the complexation of *p*-nitrophenolate to cyclohexaamylose is not random but has a strong orientational preference. For, if the complexation occurred randomly between the two possible orientations, both *ortho* and *meta* protons of *p*-nitrophenolate should exhibit approximately equal shifts. Since the *meta* protons, however, are shifted much more than the *ortho* protons, then presumably it is the *nitro* end of *p*-nitrophenolate which preferentially enters the cyclohexaamylose cavity.

Next, we consider the differential line-broadening observed in *p*-nitrophenolate in the presence of cyclohexaamylose. The *meta* proton resonance lines, which had the largest downfield shift, also exhibited significantly greater broadening than did the resonance lines of the *ortho* protons. Also, in the spectrum shown by MacNicol, the lines of the more-downfield-shifted doublet of *p*-cymene were clearly broader than the upfield lines. In the case of *p*-nitrophenolate, two factors could be contributing to the added linewidth of the most downfield *meta* proton resonances of *p*-nitrophenolate. First, there could be exchange broadening, a contribution to the linewidth caused by the process of chemical exchange itself (*17*). This contribution would be predicted to be larger for the *meta* proton lines than for the *ortho* proton resonances, because the shift upon binding of the *meta* resonances is larger. Second, the differential broadening could be due to a greater transverse relaxation rate of the *meta* protons of *p*-nitrophenolate than of the *ortho* protons, while *p*-nitrophenolate is bound to cyclohexaamylose. This could occur, for example, if, in the complex, the orientation of *p*-nitrophenolate were such that the *meta* protons of *p*-nitrophenolate are held in closer juxtaposition to particular protons of the cyclohexaamylose, causing the *meta p*-nitrophenolate protons

to experience enhanced nuclear dipole–dipole relaxation relative to the relaxation rate of the *ortho* protons. In MacNicol's (*18*) aqueous *p*-cymene–cyclohexaamylose complex, one cannot exclude the possibility that there are different contributions of unresolved spin–spin coupling from the protons of the methyl and isopropyl side groups to the observed linewidths of the *ortho* and *meta* proton resonances.

The intermolecular nuclear Overhauser experiments described above in the Results section and listed in Table 1 identify the cause of the differential line broadening of the *p*-nitrophenolate resonances and the nature of the preferred orientation of *p*-nitrophenolate in its complex with cyclohexaamylose. The theory of intermolecular NOE's in rapidly exchanging systems has been presented by Noggle and Schirmer (*13*). Balaram *et al.* (*22*) have considered the specific case of observing the resonances of a small molecule exchanging between its environment free in solution and a position bound to a macromolecule, while saturating resonances of the macromolecule. As with intramolecular NOE's, the magnitude of the observed effect depends upon the relative extent to which the nucleus whose resonance is being observed is relaxed by the nucleus whose resonance is being saturated. While quantitative interpretation of intermolecular NOE's in exchanging systems may in general be quite complex, the situation is simplified when, as in the present case, the chemical shift fast-exchange approximation holds. The most important conditions for the validity of the interpretation which follows are: first, that the bound lifetime of the species observed in the NOE experiment be long enough for significant intermolecular relaxation to occur; and second, that the mole fraction of the observed species in the bound environment be large enough that the NOE intensity changes will be seen in the averaged (free plus bound) spectrum. The most complete discussion of intermolecular NOE's in bimolecular systems in equilibrium has been presented by Bothner-By and Gassend (*26*).

Since the resonances of the *meta* protons of *p*-nitrophenolate show the larger enhancements when the cyclohexaamylose resonances were saturated, the cycloamylose protons contribute substantially more to the relaxation of the *meta* protons than of the *ortho p*-nitrophenolate protons. This also establishes that it is indeed increased intermolecular relaxation of the *meta p*-nitrophenolate protons, and not exchange broadening, which causes the observed greater increase in the linewidth of these resonances in the presence of cyclohexaamylose. It is well known that internuclear dipole–dipole relaxation rates have a $1/r^6$ dependence on the distance r between the relaxed nucleus and the nucleus causing relaxation. Thus, the preferred orientation of *p*-nitrophenolate in the bound complex is firmly established as the one having the nitro group pointing into the cavity, for only in this orientation can the differential line broadening and NOE's be explained.

The NOE's at 250 MHz listed in Table 1 also offer further confirmation of the proposal that the depth of penetration of *p*-nitrophenolate into the cyclohexaamylose cavity is not enough to bring the protons of *p*-nitrophenolate close to H-5 of the cyclohexaamylose. Irradiation of H-5 caused significantly smaller enhancements of the *p*-nitrophenolate resonances than did saturation of H-3 of cyclohexaamylose, implying a substantially greater distance of H-5 from the *p*-nitrophenolate protons. Furthermore, nonspecific contact between the exterior of cyclohexaamylose and the protons of *p*-nitrophenolate can be eliminated as causing relaxation of *p*-nitrophenolate, since saturation of H-4 and H-2 caused no enhancement of *p*-nitrophenolate at all.

Balaram *et al.* (*22*) showed in the case of small molecules binding to macromolecules that as the quantity $2\pi\nu_0\tau_c$ increases, where τ_c is the rotational correlation time of the macromolecular complex and ν_0 is the resonance frequency, the NOE would be diminished and eventually become negative; i.e., the signal of the nucleus being observed would *decrease* in intensity when resonances of the macromolecule were saturated. The molecular weight of the cyclohexaamylose *p*-nitrophenolate complex (about 1100) is such that in aqueous solution τ_c of the complex is calculated from microviscosity theory to be about 1.1×10^{-10} sec, assuming the solution is sufficiently dilute that the viscosity is not greatly increased above that of water, so $2\pi\nu_0\tau_c \simeq 0.18$ for $\nu_0 = 250$ MHz, and $2\pi\nu_0\tau_c \simeq 0.07$ for $\nu_0 = 100$ MHz. This would account for the substantially lower NOE's observed at the higher frequency. This variation in magnitude of the NOE's observed at the two resonance frequencies, combined with the large downfield shift of the *p*-nitrophenolate resonances upon complexation, enables an additional comment about the nature of the bound complex to be made. These data suggest the tentative conclusion that *p*-nitrophenolate binds quite rigidly to cyclohexaamylose, in the sense that, during the lifetime of an individual bimolecular complex molecule, little or no rotation of the *p*-nitrophenolate within the cavity occurs. This conclusion could be verified by measuring the extent to which the rotational correlation time of *p*-nitrophenolate changes going from its solution environment to the bound complex. ^{13}C nmr relaxation time measurements, which will enable the change in correlation time to be determined, are presently in progress in this laboratory.

It is useful to compare these nmr measurements defining the disposition of *p*-nitrophenolate in the cyclohexaamylose cavity in solution, with recent X-ray crystallographic determinations of the geometry of complexation in the solid state between *p*-iodoaniline and cyclohexaamylose (*23*). In the crystal, *p*-iodoaniline was found with the hydrophobic iodine deeply buried in the cyclohexaamylose cavity. The nmr results discussed above indicate that *p*-nitrophenolate binds with the uncharged and, thus, less hydrophilic nitro group buried in the cavity. Because of the absence of a significant upfield shift of the cyclohexaamlyose C-5 methine proton resonance, we concluded that *p*-nitrophenolate does not penetrate as deeply into the cyclohexaamylose cavity in solution as did *p*-iodoaniline in the crystal. The differences in the depth of penetration could easily be attributed to the differences between the two guest molecules, or to packing forces in the crystal, or both.

A recent ^{13}C nmr and ^{2}H nmr relaxation study by Behr and Lehn of complexes between cyclohexaamylose and three substituted benzenoid aromatic guests, including *p*-methyl cinnamate, addressed the question of the extent to which such guests reorient (presumably about the six-fold axis of the host) while bound within the cyclohexaamylose cavity (*24*). These authors concluded that, while the rotational correlation time of the guests they studied increased by a factor of 4 upon complexation with cyclohexaamylose, there was significant motion of the substrate relative to the host, within the bound complex. For this substrate, in describing the dynamic rigidity of its complex with cyclohexaamylose, Behr and Lehn found a "dynamic coupling coefficient" of only 0.16 between the motion of guest and host. This would seem to be evidence against our tentative conclusion that cyclohexaamylose binds *p*-nitrophenolate rigidly in the dynamic sense, but one should note that the association equilibrium constant in the case of *p*-nitrophenolate and cyclohexaamylose ($2.50 \times 10^3\ M^{-1}$) is approximately a factor of

10 greater than that found by Behr and Lehn for *p*-methyl cinnamate binding to cyclohexaamylose. It is interesting to note that, from the decrease in internal rotation of the monodeuteromethyl group of *p*-methyl cinnamate, these authors tentatively concluded that this *para*-substituted guest, like *p*-iodoaniline in the crystal and *p*-nitrophenolate in the present work, binds with the more hydrophobic, less easily solvated (methyl) substituent pointing into the cavity.

Sodium p-nitrophenolate binding to cycloheptaamylose. For reasons identical to those discussed above in the complexation of *p*-nitrophenolate to cyclohexaamylose, and as pointed out by Demarco and Thakkar (*15*), it is evident that the association equilibrium between *p*-nitrophenolate and cycloheptaamylose is also in the nmr chemical shift fast-exchange limit. The fact that the resonances of both interior methine protons of cycloheptaamylose, H-5 and H-3, are shifted upfield upon addition of *p*-nitrophenolate is strong evidence that, with this cycloamylose, *p*-nitrophenolate fully penetrates the cavity. Furthermore, the fact that both *ortho* and *meta* proton resonances of *p*-nitrophenolate are broadened equally indicates that the protons in both positions on the aromatic ring of the guest molecule experience equal intermolecular dipolar relaxation in the complex. However, since no large shifts in the *p*-nitrophenolate spectra are observed upon complexation with cycloheptaamylose, we infer that in this case the contact between guest and host is not as close as it was in the complex of *p*-nitrophenolate with cyclohexaamylose. Also, from the data plotted in Fig. 2, it is not evident that there is a strong orientational preference for *p*-nitrophenolate in the cycloheptaamylose cavity.

Binding forces. The cycloamylose systems are appropriate as models for substrate–enzyme binding for a number of experimental reasons. There is now a variety of simple and precise ways to monitor cycloamylose–substrate binding (*1*). Furthermore, with the new synthetic techniques we and others have developed for cycloamylose modification (*8*), binding constants can be measured as a function of both cycloamylose and substrate structural changes (*5*). In addition, the symmetry of the cycloamyloses (e.g., sixfold symmetry in cyclohexaamylose and sevenfold symmetry in cycloheptaamylose) greatly simplified their nmr spectra.

Any explanation of inclusion driving forces must, of course, be consistent with the large differences in dissociation constants observed for various cycloamylose–substrate complexes. These differences range over four orders of magnitude (*1*). We do not contend that only one phenomenon is responsible for complexation, to the exclusion of all others, but rather that several physical mechanisms contribute to the overall free energy of inclusion. Nor are the respective contributions of the different mechanisms necessarily the same with different cycloamyloses and different substrates. The data in Table 2 suggest that both the size and charge of the substrate are important in binding.

Of particular interest is the fact that sodium *p*-nitrophenolate binds more tightly to cyclohexaamylose than does neutral *p*-nitrophenyl acetate. This could be interpreted as solely an increase in the dipole-induced dipole interactions, the London dispersion forces between the guest and host, caused by an increase in the substrate's dipole moment as its charge increases. This explanation would be consistent with the difference in binding between cycloheptaamylose and *p*-nitrophenyl acetate and sodium *p*-nitrophenolate. However, there are discrepancies when comparing the binding of the

TABLE 2

CYCLOAMYLOSE–SUBSTRATE DISSOCIATION CONSTANTS[a]

Cycloamylose	Substrate	Dissociation constant (M)
Cyclohexaamylose	Sodium propionate	$5.7 \pm 2.0 \times 10^{-1}$
	p-Nitrophenyl acetate	1.2×10^{-2}
	Sodium *p*-Nitrophenolate	$4.0 \pm 0.8 \times 10^{-4}$[b]
Cycloheptaamylose	*p*-Nitrophenylacetate	6.1×10^{-3}
	Sodium *p*-Nitrophenolate	1.4×10^{-3}

[a] Reference (*1*).
[b] Reference (*25*).

same substrate to different cavities; for example, *p*-nitrophenyl acetate binds more tightly to cycloheptaamylose than to cyclohexaamylose, while sodium *p*-nitrophenolate binds more effectively to cyclohexaamylose than to cycloheptaamylose. An explanation which disposes of those apparent anomalies calls on *both* London dispersion forces and expulsion of high-energy water from the cycloamylose cavities. However, the validity of such an explanation depends entirely on an understanding and consideration of where the substrates are located in the respective cycloamylose cavities in solution.

The picture that emerges is the following. First, London dispersion forces favorably influence the binding of *p*-nitrophenolate to both cycloamyloses. Second, because of the magnitude of the shifts induced in the *p*-nitrophenolate spectra upon binding to cyclohexaamylose, and because of the magnitude of the observed intermolecular nuclear Overhauser enhancements, we conclude that the guest and host in this case are in intimate contact. Moreover, upon binding of *p*-nitrophenolate less water is excluded from the cyclohexaamylose cavity than from the cycloheptaamylose cavity, since penetration in the former case is substantially less complete.

If, as we suggested in an earlier paper (*25*), the cavity water inside cycloheptaamylose is more like bulk solvent than is the cavity water in cyclohexaamylose, then equal substrate penetration of both cavities would imply tighter binding to cyclohexaamylose. On the other hand, if a great deal more water were displaced from the heptamer's pore than from the hexamer's pore, i.e., if there were more extensive penetration into cycloheptaamylose, tighter substrate binding to cycloheptaamylose might be expected. Thus, to explain the observation that *p*-nitrophenyl acetate binds more tightly to cycloheptaamylose than does sodium *p*-nitrophenolate, penetration of the cycloheptaamylose cavity would have to be extensive enough that the acetate participated in the removal of cavity water. In conclusion, our experimental data regarding the directionality and depth of penetration of *p*-nitrophenolate in the two cycloamylose cavities are consistent with the notion that both removal of high-energy water and London dispersion forces play an important energetic role in the formation of both complexes.

ACKNOWLEDGMENTS

Acknowledgment is made to the Donors of The Petroleum Research Fund, administered by the American Chemical Society, for support (PRF 3127 G12) of this research.

The authors also gratefully acknowledge the financial support of NIH Grant RR-00292 to the NMR Facility for Biomedical Studies, Carnegie–Mellon University.

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