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The Role of Strain Energy in Cycloamylose Substrate Complexation

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The cycloamyloses, a group of cyclic oligosaccharides composed of α-1,4-linked glucose units, provide an opportunity to study the driving forces responsible for enzyme–substrate binding. The cycloamylose substrate binding energy has been attributed to two sources: expulsion of high energy cavity water and release of conformational strain energy. Our studies have shown that release of strain energy plays only a minor role in overall energetics of binding.

INTRODUCTION

In recent years a great deal of effort has been invested in developing the cycloamyloses as enzyme active site models (*1*). The central theme in these studies has been to expand their spectrum as catalysts and to improve their catalytic ability by chemical modifications (*2–5*). Both of these endeavors have met with great success. However, little use has been made of these models for a study of the driving forces involved in enzyme–substrate binding. Not only can the cycloamylose substrate binding constants, as well as the thermodynamic parameters for inclusion, be easily measured (*6*), but it is now possible to modify these oligosaccharides and determine the effect of these structural changes on binding (*7*). Because of the symmetry of the cycloamylose, it is also possible to determine spectroscopically, with ease, changes that occur in the cavity when the molecule binds a substrate (*4*). These features render these cyclic oligosaccharides excellent candidates for enzyme–substrate binding studies.

BINDING FORCES

Although there has been a great deal of speculation, the driving forces for cycloamylose substrate complexation have not been quantitatively defined (*1*, *8*). A major problem is that any explanation of these forces must account for the large differences in binding constants observed for various cycloamylose substrate complexes. The differences in complex stability occur not only as the substrate changes but also as the host changes, and these differences can be as large as 6000 (Table 1) (*1*).

There are currently two popular explanations for the complexation driving force, one invoking relief of enthalpy rich water from the cycloamylose cavity (*1*), the other, relief of cycloamylose strain energy (*9*, *10*). These ideas are by no means mutually

TABLE 1

VARIATIONS WHICH OCCUR IN CYCLOAMYLOSE SUBSTRATE BINDING CONSTANTS

Cycloamylose	Substrate	Dissociation constant (M)
Cyclohexa-	Sodium propionate	$5.7 \pm 2.0 \times 10^{-1}$
	Methyl orange	1.1×10^{-4}
	p-Nitrophenyl acetate	1.2×10^{-2}
Cycloheptа-	*p*-Nitrophenyl acetate	6.1×10^{-3}

exclusive, and it is more likely that both factors are contributing to the overall driving force.

Bender has suggested that the water molecules associated with the cavity are enthalpy rich because they cannot have a full complement of hydrogen bonds and the inclusion of a substrate is favored by release of this high energy water. This downhill process would then be expected to be associated with a favorable enthalpy term. In fact, measurement of the thermodynamic parameters for the inclusion has shown this to be true (*1*).

The volumes of the cyclohexa-, cycloheptа-, and cyclooctaamylose cavities and thus the number of water molecules they can accommodate vary considerably. Assuming the cycloamyloses are shaped nearly like a cylinder and employing the molecular dimensions of Cramer (*6*), we have *approximated* the volumes and therefore the number of water molecules that could be contained by each cavity (Table 2).

TABLE 2

AN APPROXIMATION OF THE CYCLOAMYLOSES CAVITY VOLUME AND THE NUMBER OF WATER MOLECULES THEY COULD CONTAIN IN SOLUTION

Cycloamylose	Volume (Å^3)	Number of H_2O molecules
Cyclohexamylose	176	6
Cycloheptaamylose	346	11
Cyclooctamylose	510	17

From these calculations it is clear that expanding the cyclic oligosaccharide by one glucose unit at a time could substantially increase the volume of high energy cavity water as the cavity becomes larger and larger. However, the water displaced should approach bulk water in its energy content, and the driving force for complexation should disappear. It is unclear precisely how large the cavity must get before this begins to happen.

The fact that small substrates like sodium propionate (*1*), which would displace little cavity water, bind weakly and larger substrates like *p*-nitrophenylacetate (*1*), which would displace more cavity water, bind tightly is consistent with these enthalpy rich water arguments. Furthermore, that cycloheptaamylose binds a large number of phenyl esters more tightly than cyclohexaamylose but less effectively than cycloocta-

amylose is consistent with the idea that although the substrate fits into the cycloheptaamylose cavity more loosely it could displace more high energy water. In the case of cyclooctaamylose, the critical cavity volume has been reached and passed, the water displaced is becoming more like bulk water with the increasing cavity size, and the driving force begins to disappear. So it seems that, with anything larger than a 346 $Å^3$ cavity of the cycloamyloses' dimensions, the water molecules displaced energetically begin to approach bulk water. It is important to recognize that a single substrate would displace the same amount of water from each cycloamylose cavity only if each cavity is equally penetrated. Space filling models indicate that equivalent penetration would clearly not be the case.

Furthermore this idea has some basis in experimental fact. Earlier workers demonstrated that both the three and five methine protons of cycloheptaamylose were shielded on complexation of benzoid aromatic substrates (*7*). This of course implies that both of these protons are sitting in the magnetic field of the aromatic pi cloud and therefore that substrate penetration is complete. We have found that in the case of cyclohexaamylose only the three methine protons are shielded, suggesting substrate penetration is limited.[1] So, two factors here are at odds. As the cavity increases in size, the enthalpy rich water displaced becomes more like bulk water, but more of this lower energy water is released. In the case of cyclooctaamylose, even though penetration is maximal, the water displaced is of low energy, and thus there is less of a driving force for complexation.

Unfortunately, these trends in binding can just as easily be explained by release of strain energy. In a recent X-ray study it was shown that one of the glucose rings of cyclohexaamylose is orthogonal to the others (*9*, *10*). The 6-hydroxyl group of this ring is pointing into the cavity and thus serves as a hydrogen bond acceptor for cavity water. Extrapolating from these studies in the matrix to solution phenomena, Saenger has suggested that the strain energy associated with this orthogonal glucose ring is released on complexation of a substrate. Small substrates would, of course, be expected to be less effective in using this strain energy for binding than would larger substrates. The fact that cycloheptaamylose binds the same substrates more tightly than cyclohexaamylose but less tightly than cyclooctaamylose would have to mean its orthogonal form is of higher energy than that of the hexamer or octamer.

PARTITIONING OF DRIVING FORCES

It should be possible to get some idea of the extent to which the release of strain energy plays a role in the overall driving forces for complexation. Changing the 6-hydroxyl position, making it either more or less difficult for a glucose unit to attain its orthogonal position, should alter the ability of the cycloamylose to bind if strain energy is important. A comparison, then, of how well the modified cycloamylose and the unmodified cycloamylose bind to the same substrate should qualitate the importance of strain.

In order for the data from such an experiment to be meaningful, refunctionalization of the 6-hydroxyls must be complete. Failure to do this would mean that the energy

[1] Ray Bergeron and Robert Rowan, in preparation.

required for the unmodified glucose unit to attain orthogonality would be different from that required for the modified units. The source of strain energy would be unclear. Space filling models clearly indicate that methylation of all of the 6-hydroxyls, because of steric hindrance, would make it more difficult for any glucose unit to attain orthogonality. This is true no matter what the orientation of the methyls. However, it is likely that all of the methyl groups would be pointing into the center of the cavity optimizing the hydrophobic interaction between them (*5*) thus generating even more steric hindrance to attainment of orthogonality. The methyl groups are not sufficiently large, however, to completely prevent attainment of orthogonality. This means that if one of the 6-*O*-methylated rings is orthogonal to the others, at the expense of steric interaction, the methylated cycloamylose will be of higher energy, and tighter substrate binding should be observed if release of strain energy is important.

A comparison of the binding of *p*-nitrophenolate to cyclohexaamylose with its binding to dodecakis–2,6-*O*-methylcyclohexaamylose revealed the 2,6-*O*-methylated compound was only (2.9 ± 0.08) times more effective in binding. This number hardly fixed any great significance to strain energy. However, it was not clear at this point if methylation of the 2-hydroxyls rendered the results inaccurate. In particular, it has been shown by both X ray (*11*) and nmr (*12*) that the 3-hydroxyl hydrogens of the glucose units are strongly hydrogen bonded to the 2-hydroxyl oxygen of adjacent units. Further, Rao's (*13*) calculations have shown that hydrogen bonding plays a significant role in the potential energy of the cycloamyloses. For example, it lowers the energy of cyclohexaamylose by 20 kcal $mole^{-1}$ and the energy of cycloheptaamylose by 39 kcal $mole^{-1}$. If this hydrogen bonding is really important in aqueous solution, methylation of the 2-hydroxyl groups could easily effect the mobility of the glucose units by restricting rotation about the C-1-*O*-C-4′-glycosidic bonds. Any effect on such mobility would influence the strain energy and therefore the binding. To verify that this hydrogen bonding was insignificant and, therefore, the binding constant measurements meaningful, we completely removed the hydrogen bond donors, the 3-hydroxyls. This was accomplished by selective and complete 3-*O*-methylation.

We chose to examine several cycloheptaamylose systems and see if this complete 3-*O*-methylation had any effect on the binding ability of these oligosaccharides. Cycloheptaamylose and its analogs were chosen because of their synthetic accessibility and because of the alleged importance of hydrogen bonding in the parent compound. The heptakis-3-*O*-methyl cycloheptaamylose was generated in a four step sequence (*14*). The 2- and 6-hydroxyls were protected by exhaustive allylation with 3-bromopropene in dimethyl sulfoxide and dimethyl formamide with barium oxide and barium hydroxide octahydrate as base. The tetradecakis-2,6-*O*-allylcycloheptaamylose was then methylated in dimethyl formamide with methyl iodide (sodium hydride as base), and the resulting tetradecakis-2,6-*O*-allylheptakis-3-*O*-methylcycloheptaamylose was isomerized to the corresponding vinyl ether. This ether was cleaved under neutral conditions with mercuric chloride and mercuric oxide in a water–acetone mixture, providing the desired product. Because of the fact that hydrogen bonding is more important in the structure of cycloheptaamylose by 19 kcal $mole^{-1}$ than in cyclohexaamylose, it seemed that, if there were going to be any importance to this hydrogen bonding in binding a substrate in aqueous solution, the heptamer would be the place to look for it. Our results are summarized in Table 3.

TABLE 3

Cycloamylose	*p*-Nitrophenolate dissociation constant[a] (M)	Dissociation constant ratio[b]
Cyclohexaamylose[c]	$4.0 \pm 0.8 \times 10^{-4}$	
Dodecakis-2,6-*O*-methylcyclohexaamylose	$1.4 \pm 0.2 \times 10^{-4}$	2.9 ± 0.08
Cycloheptaamylose	$1.4 \pm 0.3 \times 10^{-3}$	
Tetradecakis-2,6,*O*-methylcycloheptaamylose	$1.4 \pm 0.5 \times 10^{-3}$	1.0 ± 0.41
Heptakis-3-*O*-methylcycloheptaamylose	$1.6 \pm 0.4 \times 10^{-3}$	1.0 ± 0.36

[a] All cycloamylose values determined spectrospecifically at 25°C, $I = 0.5$, pH = 11.0.
[b] Ratio of the dissociation constants of modified to unmodified cycloamyloses.
[c] Literature value 3.55×10^{-4} *M*.

Comparison of the binding of *p*-nitrophenolate to cycloheptaamylose with its binding to tetradecakis-2,6-*O*-methyl cycloheptaamylose and with this binding to heptakis-3-*O*-methyl cycloheptaamylose reveals only small differences. Tetradecakis-2,6-*O*-methyl cycloheptaamylose binds the substrate about as well as cycloheptaamylose itself but slightly more effectively than heptakis-3-*O*-methylcycloheptaamylose. It is true that unlike 2,6-*O*-alkylated cyclohexaamylose, the 2,6-alkylated heptamer binds the same as the unalkylated analog, but what is of concern here is how the 3-*O*-alkylated case compares with the parent oligosaccharide. The 3-*O*-methylated case binds *p*-nitrophenolate only slightly less effectively than the unmodified oligosaccharide. If strain energy is at all important, this implies that it is easier for a ring to attain orthogonality and therefore that the overall system would have less strain energy. However, this difference is particularly small and hardly enough to disqualify the earlier findings with the dodecakis-2,6-*O*-methyl cyclohexaamylose if strain energy is very important in the overall driving force for complexation.

CONCLUSION

Our findings indicate that, although release of strain energy may play a role in binding, it is not the major driving force in complexation. Although it is possible that our analysis of the direction of the effect of 6-*O*-methylation is incorrect, it is clear that if relief of ring strain is of great importance in the inclusion driving forces, a change in binding constants in some direction should be observed on 6-*O*-methylation of the cycloamyloses. This change we would expect to be substantially larger than the one we observed. It is much more likely that a variety of factors are at work in the overall energetics of inclusion: high energy water, London Dispersion forces, and possibly relief of ring strain. We are currently engaged in investigating these forces.

EXPERIMENTAL

Cyclohexaamylose and cycloheptaamylose were obtained from Sigma Chemical Co. and Nutritional Biochemicals Corp., respectively. They were purified according to

the method of Cramer and Henglein (*15*). All other substituted cyclodextrins were purified as previously described (*14*). *p*-Nitrophenol, obtained from Aldrich Chemical Co., was recrystallized from chloroform. All solutions were made up in phosphate buffer (pH = 11, $I = 0.5$).

Determination of dissociation constant by spectrophotometric methods. The change in absorbance of *p*-nitrophenolate was measured as a function of changing concentrations of cycloamylose, using a Carey Model 14 recording spectrophotometer with the cell compartment thermostated at 25°C. From four to six points were obtained at cycloamylose concentrations spanning two orders of magnitude in the K_{diss} range (10^{-5} up to 10^{-2} *M*). The substrate concentration was held constant at 5.0×10^{-5} *M*. The various cycloamylose–*p*-nitrophenolate solutions were run against reference solutions containing an equal concentration of the cyclic oligosaccharide. The final dissociation constants recorded in Table 3 represent no less than five determinations.

The data were treated according to the Hildebrand–Benesi procedure (*16*), plotting $(C_0 S_0/\Delta\text{Abs})$ vs $(C_0 + S_0)$, providing a slope of $1/\Delta\varepsilon$ and an intercept of $K_{diss}/\Delta\varepsilon$. All data were analyzed with a least-squares regression program, and only K_{diss} values with high residual ratio values were accepted.

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