Ditopic binding of nucleotides by heptakis(6-hydroxyethylamino-6-deoxy)-β-cyclodextrin

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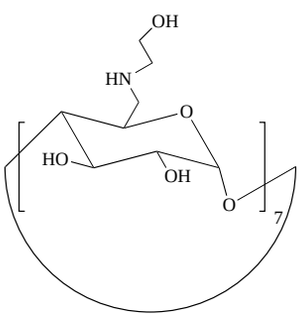
The potential of heptakis(6-hydroxyethylamino-6-deoxy)-β-cyclodextrin (1) as a ditopic receptor capable of electrostatic interaction and hydrogen bonding by its amino side-chains, together with inclusion in its hydrophobic cavity, has been assessed for nucleotides as guest molecules. The interaction of 1 with adenosine-5′-triphosphate (ATP) and adenosine-5′-monophosphate (AMP) has been studied by potentiometry in water. Exceptionally strong binding is observed for fully protonated 1 with ATP^{4-} ($K = 10^{10}$ M^{-1}) relative to AMP^{2-} ($K = 10^{6}$ M^{-1}), consistent with stronger electrostatic interaction with the former. Binding of AMP and *p*-nitrophenyl phosphate is of the same order of magnitude, while the complexes formed with ribose-5-phosphate are much weaker. The NMR changes observed upon addition of the nucleotides to 1 confirm interaction with the amino sites, together with inclusion in the cyclodextrin cavity.

Introduction

Natural cyclodextrins[1] have been the subject of much redesigning for purposes such as improving the binding characteristics of these host molecules,[2] the solubility of their inclusion complexes[3,4] and their versatility as drug delivery systems.[5]

Heptakis(6-hydroxyethylamino-6-deoxy)-β-cyclodextrin (HEA-CD) (**1**) is a highly branched cyclodextrin in which the



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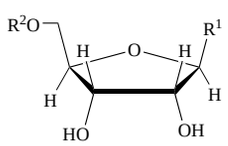

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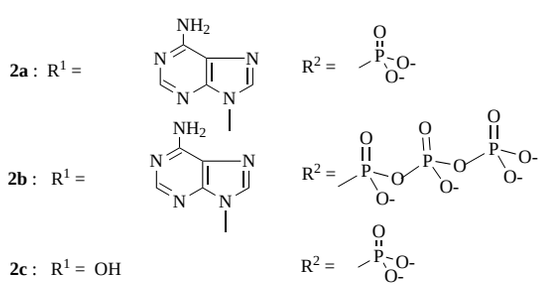

chains act as an extension of the more hydrophobic primary-hydroxy side of the cavity. A fluorescence study has demonstrated the effects of the branches on binding of anilinonaphthalene sulfonate probes.[6] The host showed selectivity towards guests and pH-dependent binding, consistent with polar interactions between guest sulfonate anions and the protonated amino groups, together with inclusion of the guest into the hydrophobic cavity of the CD. In spite of the extended cavity, this cyclodextrin is much more soluble in water than natural cyclodextrin, and has also found use as a mobile-phase additive for the separation of enantiomers by capillary zone electrophoresis.[7]

We have now assessed HEA-CD for its ability to bind and transport nucleotides. While aminocyclodextrins have previously been exploited for selective hydrolysis of nucleotides,[8,9] we wished to develop the potential of such host molecules for the supramolecular chemistry associated with gene therapy. The use of polycationic liposomes[10] for this purpose has been for some time an area of great interest. While HEA-CD in itself does not have pronounced amphiphilic character, its incorporation as headgroup into an amphiphilic cyclodextrin structure would be expected to give an overall structure with properties which have been specified for transfection systems.[11] Cationic liposomes form complexes with the negatively-charged DNA *via* charge interactions, and optimal transfection by the liposome–DNA complex requires the presence of up to 10 times excess cationic charge. This is to avoid neutralisation by negatively-charged serum protein and for an efficient interaction with the negatively charged cell membrane. A colloidally stable structure is also required, and amphiphilic cyclodextrins, as oligomers of monosaccharide amphiphiles, have been shown to form particularly stable structures.[12]

We have assessed the ability of HEA-CD to form ditopic complexes with AMP (**2a**), ATP (**2b**), ribose-5-phosphate (**2c**) and *p*-nitrophenyl phosphate (*p*-NPP), by potentiometric titration and NMR spectroscopy. These methods have shown this multibranched cyclodextrin to have advantages over previous polyaminomacrocycles,[13,14] and in comparison with the simpler methylaminocyclodextrin.[8,9]

Results and discussion

Potentiometric titrations

The logarithms of the stepwise protonation constants for hosts and guests are given in Table 1. The pK_a values of all seven protonated forms were determined. The range of values for the structurally identical amino groups of the cyclodextrin (from 5.49 to 8.75) is probably due to interactions such as hydrogen bonding between amino functions on adjacent anhydroglucose units. The distribution of the successive protonated species is shown in Fig. 1. The percentage of formation of these species ranges from 30 to 65%, with the highest for LH_6. Around neutral pH, the region in which interaction of the receptor with the

Table 1 Logarithms of the protonation constants β_i and K_i for HEA-CD and phosphonucleotides (I = 0.1 M NaCl, 25 °C)

Reaction	HEA-CD	AMP	ATP
$L + H \rightleftharpoons LH$	8.75(4)[a]	6.22(1)	6.66(1)
$L + 2H \rightleftharpoons LH_2$	16.74(4)	10.12(1)	10.90(1)
$L + 3H \rightleftharpoons LH_3$	24.20(7)		
$L + 4H \rightleftharpoons LH_4$	31.38(6)		
$L + 5H \rightleftharpoons LH_5$	38.03(9)		
$L + 6H \rightleftharpoons LH_6$	44.64(8)		
$L + 7H \rightleftharpoons LH_7$	50.1(1)		
$LH + H \rightleftharpoons LH_2$	7.98(2)	3.90(1)	4.24(1)
$LH_2 + H \rightleftharpoons LH_3$	7.46(5)		
$LH_3 + H \rightleftharpoons LH_4$	7.19(5)		
$LH_4 + H \rightleftharpoons LH_5$	6.64(7)		
$LH_5 + H \rightleftharpoons LH_6$	6.62(6)		
$LH_6 + H \rightleftharpoons LH_7$	5.49(8)		

[a] The values in parentheses correspond to $\pm\sigma_{N-1}$, the standard deviations of the mean values ($N \geqslant 4$). Charges are omitted for simplicity.

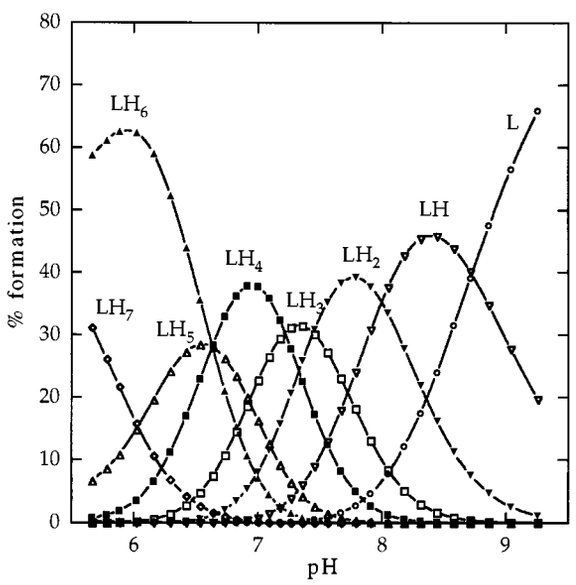

Fig. 1 Distribution curves of the protonated species of HEA-CD (8.35×10^{-4} M) *versus* pH

nucleotide is important for biological purposes, mainly LH_3, LH_4 and LH_5 coexist. ATP is present as ATP^{4-} and $HATP^{3-}$, and AMP as AMP^{2-} and AMP^{-}, and the overall basicity of HEA-CD is high enough to allow the formation of a large amount of highly protonated ligand, thus fulfilling the conditions required for nucleotide transport.

The changes in the titration curve of the host, in the presence of a 1:1 ratio of guest (Fig. 2) indicate that interaction takes place over the whole range of pH studied. HEA-CD forms with ATP and AMP a series of 1:1 complexes, LGH_i, with various degrees of protonation. The logarithms of their overall stability constants β_{xyz}, characterising equilibrium (1), are given in Table 2. These complexes can contain 0–8 protons. In the case of LGH_8, at least one proton has to be located on a phosphate group of the nucleotide, and the deprotonation reaction, $LGH_8 \rightleftharpoons LGH_7 + H^+$, can take place in theory either on the receptor or the nucleotide. Thus LGH_7 could be expressed as LH_6-GH, rather than LH_7-G, but calculation of the corresponding constants predict that the latter situation is more likely [equilibria (2) and (3)].

$$xL^{n-} + yG^{m+} + zH^+ \rightleftharpoons L_xG_yH_z^{(my - nx + z)} \quad (1)$$

$$LH_6 + GH \rightleftharpoons LGH_7 \quad \log K = 8.4 \quad (2)$$

$$LH_7 + G \rightleftharpoons LGH_7 \quad \log K = 9.56 \text{ (for ATP)} \quad (3)$$

Table 2 Logarithms of the overall (β) and stepwise (K) stability constants for the complexes of HEA-CD(L) with phosphonucleotides (G) (I = 0.1 M NaCl, 25 °C)

Reaction	AMP	ATP	5-RP[a]	*p*-NPP[b]
$L + G \rightleftharpoons LG$	4.09(4)			
$L + G + H \rightleftharpoons LGH$	13.15(7)	12.44(3)		
$L + G + 3H \rightleftharpoons LGH_3$	29.00(5)			27.73(7)
$L + G + 4H \rightleftharpoons LGH_4$				35.88(3)
$L + G + 5H \rightleftharpoons LGH_5$	43.28(4)		41.69(1)	43.30(2)
$L + G + 6H \rightleftharpoons LGH_6$	49.88(3)	52.97(3)	48.46(1)	50.17(2)
$L + G + 7H \rightleftharpoons LGH_7$	56.00(3)	59.70(1)	54.39(1)	55.97(2)
$L + G + 8H \rightleftharpoons LGH_8$	60.43(3)	63.66(2)		
$LH + G \rightleftharpoons LGH$	4.40	3.69		
$LH_3 + G \rightleftharpoons LGH_3$	4.80			3.53
$LH_4 + G \rightleftharpoons LGH_4$				4.50
$LH_5 + G \rightleftharpoons LGH_5$	5.25		3.66	5.27
$LH_6 + G \rightleftharpoons LGH_6$	5.24	8.33	3.82	5.53
$LH_7 + G \rightleftharpoons LGH_7$	5.86	9.56	4.25	5.83
$LH_7 + GH \rightleftharpoons LGH_8$	4.11	6.9		
$LH_6 + GH \rightleftharpoons LGH_7$	5.14	8.4		

[a] Ribose-5-phosphate. [b] *p*-Nitrophenyl phosphate.

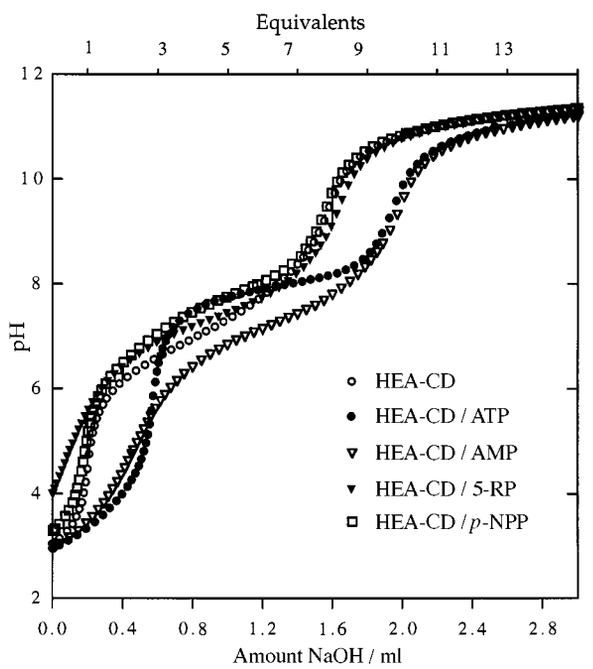

Fig. 2 Titration curves for HEA-CD (8.35×10^{-4} M), alone and in the presence of nucleotide at 1:1 molar ratio

As expected, the strongest complexes, independently of the guest, are formed with the fully protonated state of the ligand. The most stable complexes are formed between ATP, which possesses the strongest anionic character, and the most highly protonated forms of HEA-CD (LH_5, LH_6, LH_7). The complexes of AMP, although less stable than those of ATP, are more numerous and cover nearly the whole range of protonated states of HEA-CD. The distribution of the complexes of ATP and AMP is shown in Fig. 3.

Comparison of the stability of the complexes of AMP with those of ribose-5-phosphate and *p*-nitrophenyl phosphate shows that inclusion of the hydrophobic moiety of AMP or *p*-NPP into the cavity of the CD increases the stability of the complexes: log β values for AMP and *p*-NPP are very similar, whereas those for ribose-5-phosphate are two log units smaller. This suggests that for the last either the ribose moiety is not included within the cavity and the electrostatic interaction takes place externally, or there is inclusion of the ribose but with an unfavourable effect on the stability of the complex. Eliseev and Schneider, in their study of the complexes of ATP and AMP with methylaminocyclodextrin, have shown by NMR

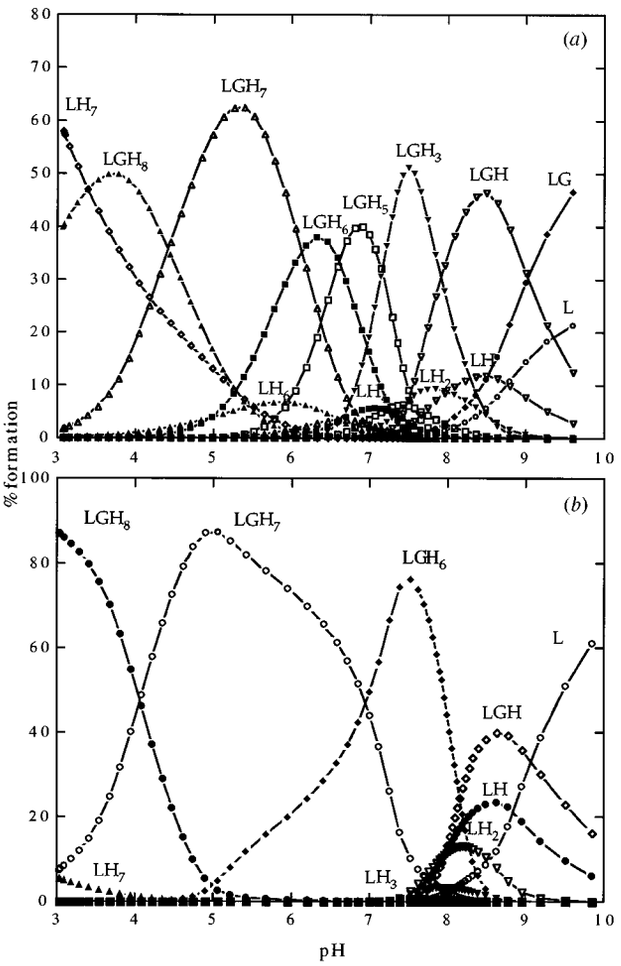

Fig. 3 Distribution curves of the complexes *versus* pH: (*a*) HEA-CD/AMP and (*b*) HEA-CD/ATP (concentrations 8.35×10^{-4} M)

measurements that inclusion of the ribose moiety does in fact take place with these.[8,9]

The binding constants K referring to the formation of LGH_i from LH_i and G were calculated from the values of β_{xyz}, and range from log $K = 4.49$ (LGH) to 5.85 (LGH_7) for AMP, and from 3.71 (LGH) to 9.57 (LGH_7) for ATP. The last value is considerably higher than the highest value previously reported for cyclodextrin–nucleotide complexation, which was 6.5, for the complex of heptakis(6-methylamino-6-deoxy)-β-cyclodextrin with ATP.[8,9] The main structural difference here is the extended cavity created by the hydroxyethylamino side-chains. This extension of the more hydrophobic side of the natural cyclodextrin cavity apparently creates an environment within which the polar binding can better operate.

NMR measurements

In the presence of *p*-nitrophenyl phosphate, broadening of the two side-chain methylene peaks of **1** was observed. The NCH_2 peak was also shifted downfield by 0.02 ppm. *p*-NPP fits into the cavity of HEA-CD, threading through to allow the electrostatic interaction, and causing the observed broadening of peaks.

No broadening of the methylene peaks was observed in the presence of ribose-5-phosphate; there were small downfield shifts of the side-chain methylene protons. Inclusion of the ribose moiety into the hydrophobic cavity is generally thought to be unfavourable unless it is part of a nucleotide, and electrostatic interaction with the amino groups of HEA-CD may occur externally.

There was a significant downfield chemical shift change for both side-chain peaks upon addition of AMP. The NCH_2 peak was moved downfield by 0.17 ppm and the OCH_2 peak likewise, by 0.06 ppm. Broadening of both peaks was observed and inclusion of the guest in the cavity was shown by the partial separation of the overlapping H-5 and H-3 peaks. Addition of ATP resulted in extreme broadening of the OCH_2 peak. Such chemical shift changes for intracavity H-5 and H-3 protons are normal inclusion phenomena, yet are unknown for side-chain protons, with a few exceptions.[15] The broadening effects have been attributed to complexation of different nucleotide conformers,[9] and in the case of the side-chains to differentiation of the diastereotopic methylene protons.[15] These results show that the side-chains, as well as the cavity, take part in binding the nucleotides.

Conclusions

Previous examples of polyaminocyclodextrins have been considered for their potential as selective ribonucleases, and have been shown to have strong binding constants for ditopic inclusion of nucleotides.[8,9] While even stronger binding might be expected to act against such potential selectivity in catalysis,[2] very strong binding (greater than 10^7) is required for transport.[16] This is because the transporting host must compete with natural binders such as lipoproteins in biological systems. At the same time any cyclodextrin headgroup which is to be used in an amphiphile design must have high external polarity for solubilisation of the final macromolecular aggregate. A third requirement is the presence of multiple amino groups with pK_a values in the physiological range. Heptakis(6-hydroxyethylamino-6-deoxy)-β-cyclodextrin (**1**) appears to fulfil all of these requirements. The hydroxyethylamino side-chain provides an extension of the cavity within which the polar interactions between protonated amino groups and the phosphate anion are improved by partial exclusion of water. This is achieved with an increase in the external polarity, and increased solubility. HEA-CD and similar amino-modified cyclodextrins therefore show potential for incorporation into macro-amphiphiles and use in nucleotide drug delivery.

Experimental

Materials

Heptakis(6-hydroxyethylamino-6-deoxy)-β-cyclodextrin was synthesised as previously described.[6] ATP, AMP, ribose-5-phosphate and *p*-nitrophenyl phosphate (from Aldrich) were used without further purification. HCl and carbonate-free NaOH solutions were prepared from Titrisol solutions.

Potentiometric titrations

Potentiometric titrations of HEA-CD alone, and in the presence of the phosphonucleotides adenosine-5′-triphosphate (5′-ATP) and adenosine-5′-monophosphate (5′-AMP), as well as the reference compounds *p*-nitrophenyl phosphate and D-ribose-5-phosphate, were conducted in water at 25 °C and $I = 0.1$ M NaCl, under argon, using a Mettler DL25 potentiometric titrator. The concentration of free hydrogen ions, $[H^+]$, was measured with a combined glass electrode (Orion 9103SC) connected to the titrator. The standard filling solution of the electrode external reference was replaced by a 0.1 M aqueous NaCl solution saturated with AgCl. The electrode was standardised in concentrations at pH 2 with a solution of 0.01 M HCl (NaCl = 0.09 M). As the junction potentials vary exponentially with $-\log[H^+]$, the correction relationship given in eqns. (4) and (5) was used.[17] Factors a and b were determined by

$$-\log[H^+]_{real} = -\log[H^+]_{meas} + a + b[H^+]_{meas} \quad (4)$$

or

$$pH_{real} = pH_{meas} + a + b \times 10^{-pH_{meas}} \quad (5)$$

measuring the pH of a 10^{-3} M HCl solution (NaCl = 9.9×10^{-2} M, $pH_{real} = 3$, $I = 0.1$ M). Mean values were $a = 0.087$ and

$b = -8.947$. The autoprotolysis constant of water at 25 °C and $I = 0.1$ M NaCl was determined by Gran titration of HCl with NaOH, pK_w being equal to $2 \times V_E$ (volume at neutrality). The mean value was 13.78. In a standard run, a solution of HEA-CD (10 ml, 10^{-3} M, $I = 0.1$ M NaCl), acidified with HCl, was titrated with NaOH (4×10^{-2} M). At least four titrations were performed. Alternatively, a 1 : 1 ratio mixture of HEA-CD and guest was titrated under the same conditions. The pH values of the titration curves were corrected and the data treated by the programme Hyperquad.[18]

Protonation of the ligand LH_n is described by equilibrium (6) and characterised, for particular conditions of temperature

$$L^{n-} + iH^+ \rightleftharpoons LH_i^{(i-n)} \quad (6)$$

and ionic strength, by overall protonation constants β_i [eqn. (7)], where $1 \leqslant i \leqslant n$. The stepwise constants K_{a_i} corre-

$$\beta_i = [LH_i^{(i-n)}]/[L^{n-}]\,[H^+]^i \quad (7)$$

sponding to successive protonation steps are directly related to the pK_{a_i} of the ligand, and are obtained from β_i [equilibrium (8), eqns. (9)–(11)].

$$LH_{i-1}^{(i-n-1)} + H^+ \rightleftharpoons LH_i^{(i-n)} \quad (8)$$

$$K_i = [LH_i^{(i-n)}]/[LH_{i-1}^{(i-n-1)}]\,[H^+] \quad (9)$$

$$\beta_n = \Pi_{i=1}^{n} K_{a_i} \text{ or } \log \beta_n = \Sigma_{i=1}^{n} \log K_{a_i} \quad (10)$$

$$pK_{a_1} = \log K_{a_n},\ pK_{a_n} = \log K_{a_1},\ pK_{a_i} = \log K_{a_{n-i+1}} \quad (11)$$

NMR measurements

NMR studies were performed with a JEOL GX270 spectrometer in D_2O solutions at pD7, using TMS/CCl_4 as an external standard, the guest being in one-molar excess.

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