Note

Cyclodextrin–ligand interaction as a simplified model of biospecific affinity chromatography

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Cyclodextrins (CDs) are generally considered to form inclusion complexes which mimic biological interactions, *e.g.*, the complex between an enzyme and substrate[1]. These molecular adducts of CDs have been extensively studied in solution[2] and by X-ray crystallography[3–5]. Matrix-bound CDs have manifold chromatographic applications in the purification of low-molecular-weight substances[6]. In contrast, the chromatography of CDs on chemically modified supports has not been studied. Formally this system closely resembles affinity chromatography and hence could serve as a model of it. Since CDs are small, non-ionic, commercially available pure substances, whose concentration can be varied over large ranges, experiments which cannot be carried out with other biomolecules are possible. The existence of three homologous forms of CDs, α-, β- and γ-, with variable complexing properties further facilitates analysis of this model.

We describe here an affinity system for CDs involving naphthoxyacetic acid bound to aminated Bio-Gel P-6 or to cellulose. These supports separated the three CD-forms. The effects of concentration, ligand, temperature and binding capacity were studied. Interactions of CDs between free and support-bound ligands are discussed.

EXPERIMENTAL

Preparation of the modified sorbents
Bio-Gel P-6 (200–400 mesh; Bio-Rad Labs., Richmond, CA, U.S.A.) was aminated and the concentration of the amino groups determined by normal acid–base titration[7]. (2-Naphthoxy)acetic acid (1–5 g; Aldrich, Milwaukee, WI, U.S.A.) was dissolved in aqueous 20% dimethyl sulphoxide and then 50 ml (a settled volume) of aqueous aminated Bio-Gel P-6 were added and the pH was adjusted to 4.7 with 1 M sodium hydroxide. The suspension was stirred at 20°C while N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (4 g in 20 ml of water at 0°C; Sigma, St. Louis, MO, U.S.A.) was added dropwise during 30 min while the pH was maintained at 4.7. Gentle stirring was continued for 4 h. The gel was washed with water, 50% ethanol, ethanol, 50% ethanol, water, 0.5 M sodium chloride and water, 500 ml of each.

A 10-g amount of cellulose MN 300 (Sigma) was suspended in water and swelled overnight. It was washed with 50 ml of 2 M sodium hydroxide at 0–5°C on a glass filter

and then with 100 ml of acetone at $-20°C$. The solvent was filtered off and the powder immediately transferred to a vial containing 3 g of cyanuric chloride (Aldrich) in 50 ml of acetone at $-20°C$. The suspension was continuously stirred while 50 ml of ice-cold water were added. Then the solution was allowed to warm to 20°C within about 10 min. The pH should then be 1–2. The derivative was washed on a glass filter with ice-cold acetic acid (20% in water), and acetone (each 200 ml). A solution of bis(aminopropyl)amine (0.5 M 250 ml) was adjusted to pH 10 with hydrochloric acid and cooled in an ice-bath. The triazine-activated cellulose was added and the pH was maintained at 10 with 5 M sodium hydroxide with stirring. When the consumption of the base had ceased, the aminated cellulose was washed with water, ethanol, acetone and water (each 500 ml). (2-Naphthoxy)acetic acid was coupled to the aminated cellulose as above.

Equipment

The chromatographic system usually consisted of a gel column (450 mm × 6 mm), a peristaltic pump (P-3; Pharmacia, Uppsala, Sweden) and a refractive index detector (Model 2142; LKB, Stockholm, Sweden). In typical experiments, 0.2 ml of 5 mM CD solutions (Sigma) were applied. The elution was carried out with distilled water.

In the studies at different temperatures, a jacketted column (118 mm × 10 mm; Pharmacia) and an high-precision, medium-pressure pump (of an amino acid analyzer Perkin-Elmer KLA-2, Japan) were used. The samples (0.2 ml; containing 3.33 mM of each CD) were applied with an applicator described earlier[8].

Determination of the ligand concentration

(2-Naphthoxy)acetic acid absorbs light at 326 nm in acidic aqueous dimethyl sulphoxide and the absorption is linearly proportional to the concentration of (2-naphthoxy)acetate up to 9 mM. The gel was brought into solutions as follows: 0.2 g of suction-dry moist gel were suspended in a mixture of 6 M hydrochloric acid (5 ml) and dimethyl sulphoxide (1 ml), and the suspension was kept in a bath of boiling water for 1 h. Occasional slight turbidity was removed by centrifugation (10 000 rpm, an Eppendorf centrifuge) and the absorbance of the solution was measured. (2-Naphthoxy)acetic acid (1–20 mg) served as the standard for aminated Bio-Gel P-6, while the mixture was treated as above. This method produced similar results to the acid–base titration of the unmasked amino groups.

Determination of the binding capacity

The binding capacity of a gel containing 23 g naphthoxy ligands per litre of Bio-Gel P-6 was determined by using frontal analysis[9] of 5 mM CD solutions at 22°C with a flow-rate of 24 ml/h (gel column 450 mm × 6 mm).

RESULTS

The elution of α-, β-, and γ-CDs on naphtyl-derivatized Bio-Gel P-6 is illustrated in Fig. 1. The separation of pure CDs was complete on this gel, but on the naphthyl derivative of cellulose α- and β-CDs were not completely separated. This was probably due to an adsorption of α-CD on the non-modified amino ligands. The results on naphthyl-Bio-Gel are mainly dealt with here.
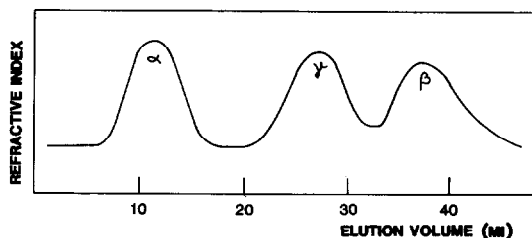
Fig. 1. Separation of CDs on (2-naphthoxy)acetate-liganded Bio-Gel P-6 (300 × 6 mm; 8 ml) with a flow-rate of 24 ml/h water. The ligand concentration of the gel was 12 g/l. The sample (0.2 ml) contained 5 mmol/l of each CD form. The elution volume of α-CD was 11.5 ml and those of glucose and ^2H_2O were 13.1 and 13.4 ml, respectively.

The elution volume of α-CD (Fig. 1) was less than that of glucose or ^2H_2O and hence α-CD was slightly excluded by the gel. The general appearance of the chromatogram shows broad peaks. A tailing of the peak of β-CD is also evident. The flow-rate of the mobile phase did not affect the elution volumes within the range applicable. While at high concentrations of CDs the elution volumes of β- and γ-CDs were shortened, at 5 mM they were eluted equally when chromatographed separately or as the mixture.

Effects of the substitution degree

Variation of the ligand concentration over a quite narrow range compared to the total content of the amino functions in the gel has dramatic effects on the elution behaviour of the CDs (Table I). While the elution of α-CD is independent of the ligand concentration, the elutions of β- and γ-CDs are strongly dependent on it. The limit of non-elution was met abruptly (Table I).

TABLE I

ELUTION VOLUMES OF CYCLODEXTRINS ON NAPHTHOYLATED BIO-GEL P-6

The aminated Bio-Gel P-6 contained 0.57 mol amino groups per litre of gel. The concentration of naphthyl functions is indicated. The column volume was about 13 ml and the sample volume was 0.2 ml. The CDs were chromatographed at 22°C with distilled water as the mobile phase. The flow-rate was measured by collecting the eluent in a graduated cylinder.

Ligand concentration (g/l of gel)	Elution volume (ml)			Flow-rate (ml/h)
	α-CD	β-CD	γ-CD	
4	15.2*	28.6*	22.6*	32.6*
	14.7	28.2	22.2	29.0–30.2
12	15.3*	36.0*	27.0*	13.1*
	14.8	36.8	26.0	12.6–17.0
20	15.3*	52.1*	36.6*	13.0*
	15.0	50.3	38.0	13.1–17.8
23	14.8*	**	**	14.0

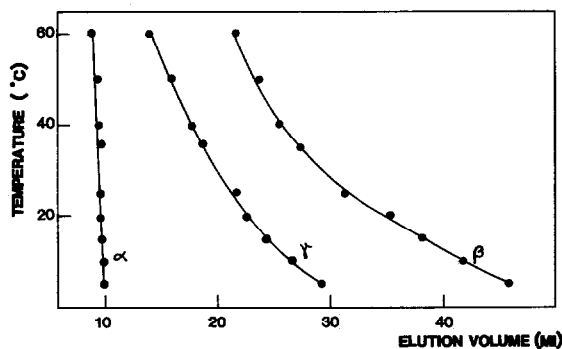* Chromatographed as the mixture of the CDs.
** Bound irreversibly.

Fig. 2. Temperature dependence of the retention of CDs on (2-naphthoxy)acetic acid-derivatized Bio-Gel P-6 (jacketted column, 118 mm × 10 mm; Pharmacia) with a flow-rate of 30 ml/h. The gel contained 4 g naphthoxy ligands per litre. Samples (0.2 ml) contained 3.33 mmol/l of individual CDs and the elution was isocratic with water.

Operational capacity of the sorbent

The operational CD-binding capacities of naphthoylated supports were determined by using a frontal analysis method[9] with separate 5 mM CD solutions. For naphthyl-cellulose the capacities were 0.3, 1.5 and 0.5 g/l of packed support with α-, β- and γ-CD, respectively. With the Bio-Gel derivative the capacity was negligible for α-CD, whereas with β-CD it was 14.1 g/l of packed gel. The capacity was not studied for γ-CD. With a benzoylated Bio-Gel[7] the effect of temperature was also tested for β-CD. Surprisingly, the capacity was higher at 22°C (8.3 g/l) than at 0°C (7.3 g/l).
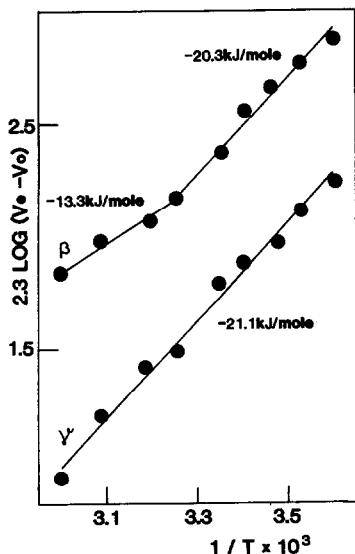

Fig. 3. The Van 't Hoff plots obtained from the data in Fig. 2 for the interaction of β- and γ-CDs with naphthoxylated Bio-Gel P-6. The enthalpy values are shown in the figure.

Effects of the temperature

Fig. 2 shows the elution volumes of separately chromatographed α-, β- and γ-CDs on the naphthyl-Bio-Gel at different temperatures. The elution of α-CD slightly decreases at higher temperatures and the dependence is linear whereas the elutions of β- and γ-CDs are logarithmic functions. Their resolution shows little improvement at low temperatures even though the separation from α-CD considerably increases.

Fig. 2 much resembles the one obtained previously by us for affinity chromatography of alanine aminotransferase[10]. The linear dependence of α-CD on the temperature can be ascribed to a slight effect of temperature on the size-exclusion term of the separation with a close analogy to the behaviour of the non-binding reference protein described previously[10]. Although β- and γ-CDs have slightly higher molecular weights than that of α-CD, their elution in the absence of specific ligands should be nearly equal to that of α-CD. Evidence in supports of this was provided by results obtained on chemically derivatized gels which did not separate the CDs (data not shown). On this basis the difference between the line for α-CD and the curve for β- and γ-CDs (Fig. 2) reflects the term due to specific adsorption. Therefore the heat of adsorption is obtained from this difference. Fig. 3 shows that the Van 't Hoff plots for

TABLE II

INTERACTION OF α-, β- AND γ-CDs WITH NAPHTHYL LIGANDS IN SOLUTION

Compound	CD form	K (M^{-1})	Conditions	Refs.
Naphthalene*	β	685	Fluorescence, 25°C, water	16
2-Naphthol	α	127	Fluorescence, water	12
	β	625		
	γ	53		
	β	32	Spectrophotometry, potassium chloride–hydrochloric acid, $I = 0.06$, pH 2.2, 25°C	17
1-Bromonaphthalene**	β	≈10 000	Phosphorescence, water, 15°C	18
2-Methoxynaphthalene***	β	630	Fluorescence, water, 25°C	16
1-Naphthoylacetic acid	β	714	Fluorescence, 0.05 M borate, pH 10, 25°C	19
2-Naphthoyltrifluoroacetone§	α	73	Gel chromatography, 10 mM	13
	β	1320	sodium acetate, pH 4.3, 25°C	
	γ	142		
1-Anilino-8-naphthalene-sulphonate	β	77	Fluorescence, 0.05 M borate, pH 11.0, 25°C	19
2-p-Toluidinonaphthalene-6-sulphonate	β	4000	Fluorescence, 0.1 M phosphate, pH 5.9, 25°C	20
	α	18	Fluorescence, 0.08 M sodium	21
	β	1538	acetate, pH 5.3, 25°C	
	γ	1515		
1,3-Di-α-naphthylpropane	α	≈0	Solubility, water, 25°C	15
	β	840		
	γ	5800		

* $\Delta H^0 = -19$ kJ/mol, $\Delta S^0 = -10$ J/mol · K.
** $\Delta H^0 = -96$ kJ/mol, $\Delta S^0 = -60$ J/mol · K.
*** $\Delta H^0 = -17$ kJ/mol, $\Delta S^0 = -5$ J/mol · K.
§ $\Delta H^0 = -38$ kJ/mol, $\Delta S^0 = -67$ J/mol · K.

the CDs are reasonably linear for the calculation of adsorption enthalpy. However, the line for β-CD has an inflection point which may indicate a change of the complexation mode (Fig. 3).

DISCUSSION

The order of elution of the individual CD forms is in reasonable accord with the equilibrium constants between them and the naphthyl group in solution (Table II). However, an exact correlation is difficult because the various methods for measuring the equilibria show considerable deviation[11]. On the other hand, even small differences in the substituents of naphthalene may affect the equilibria; the situation is related to the properties of the spacer arm in affinity chromatography.

In some studies (Table II), 2-substituted naphthalene has been found to form a complex with α-CD[12,13]. The present results clearly show that α-CD does not bind with the naphthyl group, at least in the absence of a polar "introductory" function (Table I, Fig. 1). This is probably not due to any special property of the support-bound naphthyl group since the complex of β- and γ-CDs forms normally and rapidly on the time-scale of chromatography. Therefore the naphthyl ring can, at most, penetrate α-CD to only a limited extent.

The samples (0.2 ml) for the chromatographic experiments were usually 5 mM, while the concentrations of naphthyl groups were from 17 to 115 mM (Table I). Supposing a "plate volume" of 0.2 ml, the maximum molar amount of the solute in the stationary phase was 4–30% of that of the ligands. On the other hand, by neglecting the volume taken by the gel and considering the operational capacity of the affinity gel for β-CD of 14 g/l (with the gel containing 89 mM ligand), at breakthrough conditions the column contains 12 mM solute in the stationary phase (5 mM in the mobile phase) which means a 13% complexation of the ligands. Supposing an even distribution of the ligands in the column volume, and a 1:1 complexation mode, an equilibrium constant of 31 M^{-1} is calculable. Compared with the results of solutions studies, this value is at least one order of magnitude too small. More exact analysis will readily be achieved by frontal analysis of CDs at different concentrations[14].

In solution, the complexation degree of CDs is strongly dependent on the temperature[11]. While the elution volumes indicate the same behaviour on the naphthyl gel (Fig. 2), the binding capacity of 5 mM β-CD was slightly less at 0 than at 22°C with the benzoylated Bio-Gel. Thus the supports should have ligands whose availability is independent of the temperature and these ligands interact with CDs as in solution. Possibly a considerable heterogeneity of the ligands exists and some clusters of them are dissolved at higher temperatures or at higher concentrations of CDs. A consequence of this is that the capacity for zone elution may be relatively high from more concentrated solutions which allows practical preparation of analytical grade CDs by affinity chromatography. Preliminary evidence for this has been obtained.

The strong and relatively abrupt increase in the binding strength as a function of ligand concentration in the case of γ-CD compared with β-CD suggests that when the average distance between naphthyl groups reaches a certain value on the matrix, the binding mode changes from 1:1 to 1:2 (CD:ligand). Evidence in support of this is provided by the very strong forces between 1,3-di-α-naphthylpropane and γ-cyclodextrin (Table II, ref. 15).

Despite relatively complex ligand–ligand or ligand–gel interactions probably occur in the systems studied, the interactions should be equal for β- and γ-CDs. Hence the two-phase Hill plot (Fig. 3) for β-CD may not originate from the support itself. The ΔH^0 values for β- and γ-CDs are in a good accord with previous values in solution (see footnotes to Table II).

Although this study is far from a detailed analysis of the system, it shows many chromatographically interesting aspects which can be illustrative in the context of affinity chromatography. The benefit of the present model lies in the fact that cross-studies of zone and broad-zone elution concomitantly with batch studies can be carried out to obtain thermodynamic and other parameters through independent routes. Theoretical development of the affinity method is especially desirable to improve its predictability.

ACKNOWLEDGEMENT

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