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Effects of Cyclodextrins on Chymotrypsin Action

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Abstract. Inhibition by cyclodextrins of chymotrypsin-catalysed hydrolysis of *N*-acetyl-L-tyrosine ethyl ester (ATEE) and of *N*-succinyl-L-phenylalanine *p*-nitroanilide (SUPHEPA) was measured. Rates of proteolysis are reduced by a factor of three to four by a four-molar ratio of cyclodextrin to substrate, except for α-cyclodextrin and SUPHEPA where the rate reduction is much less. The kinetics of inhibition, as well as NMR and UV measurements, were used to measure association constants between the cyclodextrins and substrates. Agreement between these methods confirmed that inhibition by cyclodextrins is due to steric effects at the substrate, rather than direct interaction with the enzyme.

Key words: cyclodextrin, chymotrypsin, enzyme

1. Introduction

When cyclodextrins [1] act as host molecules in water towards reaction substrates, two functions may be observed for them. The cyclodextrin may act as a catalyst or 'artificial enzyme' [2] towards the included guest molecule, as in the hydrolysis of penicillins [3], or it may protect the guest from reactions such as reduction [4].

Among the many functions of the carbohydrate moiety of protein glycoconjugates is protection of proteins from enzymatic degradation while allowing them to be recognised by receptor sites [5]. Non-bonded interactions with the protein must also play a part in deciding extramolecular effects. Such a role for cyclodextrins is worth assessing, besides their more usual applications to solubilisation and transport for drug delivery.

Amino acids [6, 7] and dipeptides [8] have been shown by NMR spectroscopy to form complexes with cyclodextrins by inclusion of hydrophobic sidechains. The complexes formed between cyclodextrins and a peptide-like protease substrate would model a glycopeptide in which the carbohydrate moiety modifies the reactivity of the peptide towards proteolysis. Cyclodextrins are expected to complex with the hydrophobic phenylalanine sidechain and, in conflict with that, chymotrypsin is selective for aromatic aminoacids [9].

We have measured the inhibition of chymotrypsin-catalysed hydrolysis of *N*-acetyl-L-tyrosine ethyl ester (ATEE) [10, 11], and of *N*-succinyl-L-phenylalanine

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p-nitroanilide (SUPHEPA) [12] by α-cyclodextrin (cyclomaltohexaose) and by β-cyclodextrin (cyclomaltoheptaose). We also studied complexation between these cyclodextrins and the chymotrypsin substrates by NMR and UV spectroscopy.

2. Experimental

Cyclodextrins (Sigma) were dried at 100 °C, 100 pa for 48 h. ATEE and SUPHEPA (Aldrich) were of highly purified grade. Chymotrypsin (Aldrich Type II) had activity 40–60 units/mg. Buffers were of analytical grade. For NMR spectra (obtained at 25° on a Jeol 270 MHz instrument) TMS in $CDCl_3$ was used as external standard; guest concentrations were 1×10^{-3} M (ATEE) and 3.6×10^{-3} M (SUPHEPA). UV measurements were recorded at 25°; guest concentration was 3×10^{-5} M, which was within a range where no self-aggregation was detected by absorption measurements.

Hydrolysis of ATEE [10, 11] was measured at λ = 240 nm in 0.05 M phosphate buffer (pH 7.0) over 1 hour at 40°; hydrolysis of SUPHEPA [12] was measured at λ = 400 nm in Tris buffer (pH 8.5) at 25°. Each kinetic run was repeated three times. No correction was made for the effect of cyclodextrin complexation on absorbance.

3. Results and Discussion

3.1. MEASUREMENT OF ASSOCIATION CONSTANTS BY NMR

The ^{1}H chemical shift changes observed on inclusion of the substrates' aromatic rings by CD cavities were used to measure apparent association constants (K_a) [13]. Similar changes have been observed for example with *p*-nitrophenyl glycoside [14]. Double reciprocal plots [15], which compare the shifts observed for various concentrations of cyclodextrin, were used to examine the stoichiometry of complexation and determine apparent K_a values. The double reciprocal plots were straight lines ($r > 0.9$). The calculated K_a values were obtained from different sets of protons (except for the ATEE meta protons in the presence of β-CD where $\Delta\delta$ values were $<$1 ppm), and agreed to within 20%. Averaged values are given in Table I.

3.2. MEASUREMENT OF ASSOCIATION CONSTANTS BY UV ABSORPTION

The double reciprocal plots for UV-absorption by ATEE in the presence of varying amounts of CD are also linear, however the corresponding plots of absorbance changes for SUPHEPA show pronounced deviation from linearity at high concentrations of CD. In comparison with the conditions for NMR measurements, much greater molar ratios of CDs are obtainable below their solubility limit, and this deviation from linearity indicates 2 : 1 (CD : SUPHEPA) complexation [15] as expected for this bicyclic substrate. Values of K_a were estimated from the linear

Table I. Apparent association constants K_a (±20%) for complexation of cyclodextrins with ATEE and SUPHEPA as measured by NMR, UV and kinetic methods

	$K_a \times 10^{-3}$ (M^{-1})		
	NMR	UV	Kinetic
ATEE			
α-CD	0.7	0.5	0.9
β-CD	0.8	0.9	1.5
SUPHEPA			
α-CD	0.6	0.1	0.2
β-CD	0.1	0.1	0.1

part of these plots obtained at the lower CD concentrations, making the approximation that the apparent values represent the sums of those for the two possible ring binding sites. Red shifts were observed, as for complexed L-phenylalanine [7].

3.3. MEASUREMENT OF ASSOCIATION CONSTANTS BY KINETICS OF PROTEOLYSIS

Considering the system CD/enzyme/ATEE (or SUPHEPA) as a competitive complexation equilibrium, and applying the literature K_m values for enzyme-substrate complexation [11, 12], it can be calculated [15] that less than 2% of the enzyme is complexed with substrate (only) at the highest concentrations used for the kinetics experiments. In considering the effect of the CDs therefore, we are dealing essentially with enzymolysis of a substrate that is complexed with CD. Ebel et al. [4] showed that the kinetic method for measuring association constants is reliable even where (as in their case of reduction of CD-complexed nitroxide by ascorbate) the reactivity of the included molecule is reduced by a factor of only two compared with the free molecule. In view of this small reactivity difference, they also concluded that electron transfer rather than H-atom transfer was involved in the ascorbate reaction, since the latter would be more sensitive to the steric protection afforded by cyclodextrin.

The presence of α- or β-cyclodextrin lowers the rate of proteolysis of ATEE (Figure 1) and in each case the pseudo-first-order rate constant is lowered by a factor of about three with the four-molar ratio of cyclodextrin to substrate. Saturation is reached more rapidly with β-cyclodextrin. The K_a values calculated from kinetics, using a double reciprocal plot, also show stronger binding by β- than

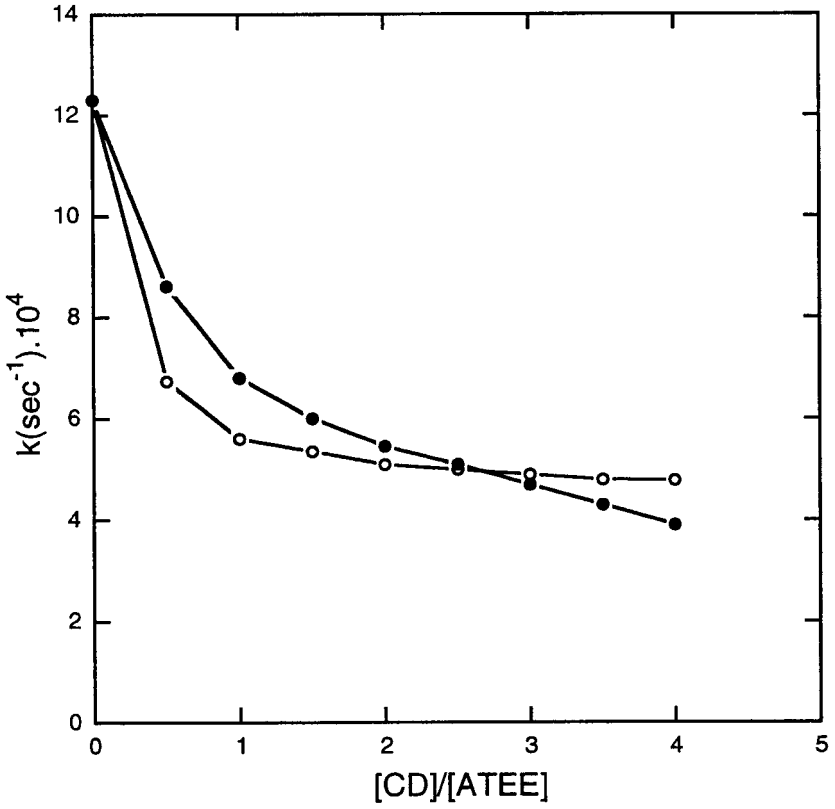

Figure 1. Effect of the molar ratio of α-cyclodextrin (●) and of β-cyclodextrin (○) to substrate on the rate of chymotrypsin-catalysed hydrolysis of ATEE.

by α-cyclodextrin (Table I). These results conform with our previous results for complexation of *p*-nitrophenyl glucoside [14].

With SUPHEPA, the rate of proteolysis is reduced by a factor of four with a four-molar ratio of β-cyclodextrin (Figure 2). The effect of α-cyclodextrin under the same conditions is much less. A possible reason is the difference in size of the macrocycles if inhibition is due to steric hindrance at the site of proteolysis. Considering the two possible 1 : 1 complexes present during reaction, β-CD complexed with the phenyl ring may be more effective in providing steric hindrance to proteolysis at the *p*-nitroacetanilide ring. There could be partial inclusion of both rings simultaneously into the β-cyclodextrin cavity, and deeper inclusion.

In Table I, results for association constants calculated from spectroscopic measurements and from kinetics of enzymolysis are compared. There is good agreement even though the UV results for SUPHEPA are estimated and the kinetic inhibition effect for α-CD with this substrate also is small.

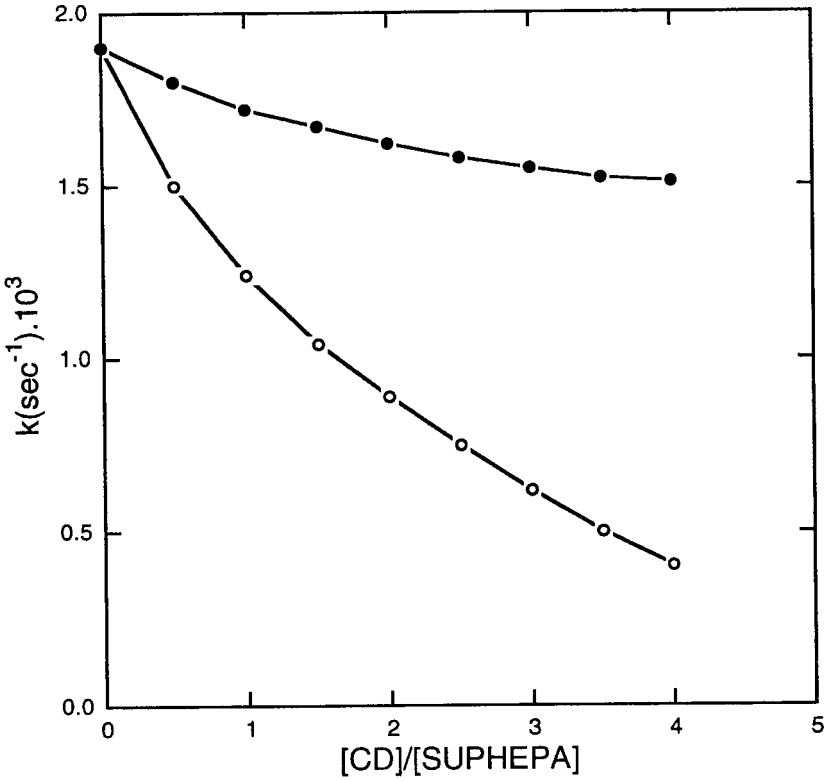

Figure 2. Effect of the molar ratio of α-cyclodextrin (●) and of β-cyclodextrin (○) to substrate on the rate of chymotrypsin-catalysed hydrolysis of SUPHEPA.

4. Conclusions

To our knowledge, the only previous study on inhibition of an enzyme by cyclodextrins has been that on the inhibition of α-amylases, where the cyclodextrin is similar to the enzyme's substrate, and where it was concluded that inhibition was pure classic noncompetitive (noncovalent interaction with both enzyme and the normal enzyme-substrate complex) [16].

The good agreement between spectroscopic and kinetic methods for measuring K_a indicates that the cyclodextrins are not reducing the rate of enzymolysis by deactivating the enzyme, since if this were so, the kinetic method would yield significantly higher K_a values. It is very unlikely that denaturation of the enzyme is involved, since it has been shown that significant protein denaturation by CDs comes about only above 50 °C [17]. The direct relationship between inhibition and complexing ability confirms that steric effect at the CD-complexed substrate slows proteolysis.

Inhibition of degradation is particularly important for therapeutic peptides. Although the inhibitory effect of cyclodextrins on protease as observed here is small,

there is potential for its development, for example by use of modified cyclodextrins or peptide-cyclodextrin prodrugs in which covalent attachment of the peptide aids inclusion. Other applications may be possible, such as modification of the action of enzymes in biotransformations.

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