Cyclodextrins to limit substrate inhibition and alter substrate selectivity displayed by enzymes

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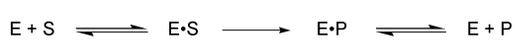
The substrate inhibition exhibited by carboxypeptidase A in catalysing the hydrolysis of (*S*)-2-*O*-(*N*-benzoylglycyl)-β-phenyllactate) is limited by addition of cyclodextrins. The cyclodextrins do not significantly change the maximum rate of reaction, but they increase the concentration of the substrate at which the maximum rate of reaction is observed, by more than an order of magnitude when 0.105 mol dm^{-3} hydroxypropyl-β-cyclodextrin is used. Cyclodextrins also alter the substrate selectivity of α-chymotrypsin in catalysing the hydrolysis of (*S*)-*N*-acetylleucine methyl ester and (*S*)-*N*-acetylphenylalanine methyl ester, in favour of reaction of the former. Calculations show that these effects are due to complexation of the substrates by the cyclodextrins. Thus the results establish that cyclodextrins can be used to manipulate the concentrations of enzyme substrates in free solution in a predictable manner.

Introduction

Enzymes are highly efficient biological catalysts (Scheme 1).



E, enzyme; S, substrate; P, product

Scheme 1

However, some of the important features of enzyme catalysis *in vivo* limit the utility of enzymes *in vitro*. In biological systems enzymes catalyse reactions of substrates that are present only at low concentrations, but for *in vitro* applications it is often desirable to have much higher substrate concentrations whereas the substrates often have only low aqueous solubility. This has been addressed using enzymes in organic solvents[1] and with surfactants.[2] Alternatively, cyclodextrins have been used to complex enzyme substrates and increase the total amount taken up in water, making them more accessible for reaction.[3,4]

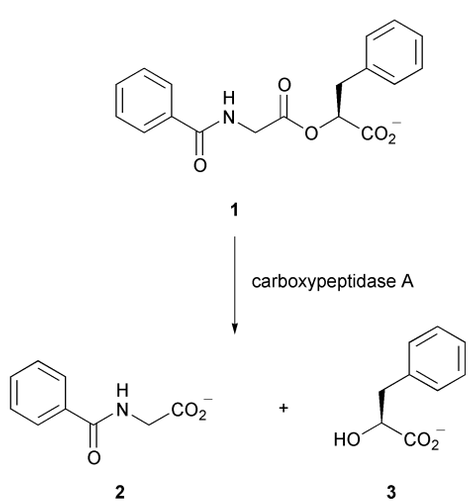
Enzyme catalysis *in vivo* is regulated by mechanisms which also limit the utility of enzymes *in vitro*. Product inhibition is one such form of regulation.[5] Biochemically this prevents wasteful metabolism of a substrate but *in vitro* it reduces the rate and extent of conversion to product. Previously we have shown that cyclodextrins can be used to reduce this inhibition, through selective product complexation.[3,6] Phenylalanine ammonia lyase shows product inhibition in catalysing the conversion of (*S*)-phenylalanine to (*E*)-cinnamate,[7] but the extent of that inhibition is reduced and the efficiency of the catalysis is increased by using cyclodextrins to selectively complex the cinnamate in the reaction mixture as it forms. Another form of *in vivo* regulation of enzyme catalysis is substrate inhibition, where high substrate concentrations decrease enzyme activity. Typically this is the result of a second substrate molecule binding to the enzyme (Scheme 2).[5] Again this prevents excessive substrate metabolism in a biological system but limits the *in vitro* utility of the process because low concentrations must be used. We now report the use of cyclodextrins to reduce substrate inhibition, as displayed by carboxypeptidase A.[8] We also discuss the application of cyclodextrins to alter the substrate selectivity shown by α-chymotrypsin.

E, enzyme; S, substrate; P, product; CD, cyclodextrin

Scheme 2

Results and discussion

Carboxypeptidase A catalyses the hydrolysis of (*S*)-2-*O*-(*N*-benzoylglycyl)-β-phenyllactate (**1**) (Scheme 3).[8] The rate of



Scheme 3

formation of the products **2** and **3** is a function of the concentration of the substrate **1**, as shown in Fig. 1a. This profile is characteristic of substrate inhibition of enzyme catalysis. At low concentrations of the substrate **1** (below 10^{-4} mol dm^{-3}) the reaction efficiency is determined primarily by the extent of binding of the substrate **1** to the enzyme, to form the binary complex E·S (Scheme 2). At higher concentrations, additional

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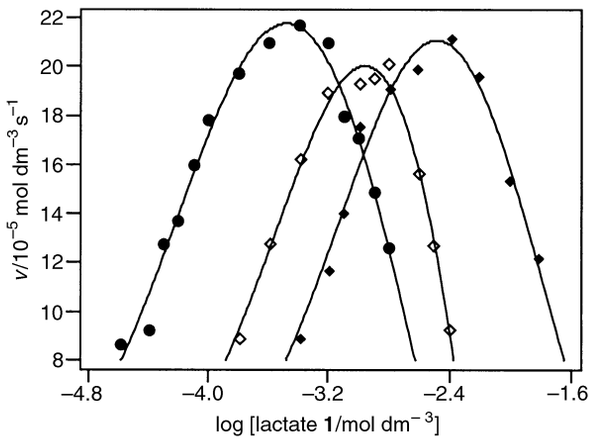


Fig. 1 Rate of hydrolysis of the lactate **1** catalysed by carboxypeptidase A (20 units dm^{-3}) in 0.05 mol dm^{-3} Tris buffer ($I = 0.5$ mol dm^{-3}, KCl), at pH 7.5 and 298 K, containing either a) no cyclodextrin (●), b) β-cyclodextrin (9.96×10^{-3} mol dm^{-3}) (◇) or c) hydroxypropyl-β-cyclodextrin (0.105 mol dm^{-3}) (◆).

binding of the substrate **1** occurs to give the catalytically inactive ternary complex $E{\cdot}S_2$, so the rate of reaction decreases.

When β-cyclodextrin and hydroxypropyl-β-cyclodextrin are added to the reaction mixtures, bell-shaped logarithm of substrate **1** concentration *versus* reactivity profiles are observed (Figs. 1b and 1c), similar to that seen in the absence of a cyclodextrin. The maximum rate of reaction does not change significantly. However, the concentration of the substrate **1** giving rise to the maximum rate of catalysis is larger, by a factor of *ca.* 3.5 for the reaction in the presence of β-cyclodextrin (9.96×10^{-3} mol dm^{-3}) and *ca.* 10 when hydroxypropyl-β-cyclodextrin (0.105 mol dm^{-3}) is used. In practical terms, the latter result means that the scale of the reaction can be decreased by at least an order of magnitude without adversely affecting the rate.

The effects of the cyclodextrins can be attributed to their complexation of the substrate **1** and the effects of this complexation on the interactions of the substrate **1** with the enzyme, in accord with Scheme 2. At low concentrations of the substrate **1**, its complexation by the cyclodextrins decreases the extent of formation of the binary complex E·S, thereby reducing the rate of reaction. At higher concentrations, cyclodextrin complexation of the substrate **1** decreases the extent of formation of the ternary complex $E{\cdot}S_2$ in favour of the binary complex E·S, thus increasing the rate of reaction.

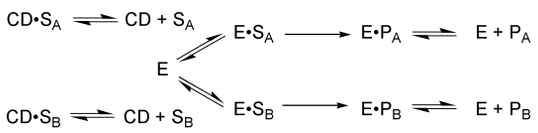
Hydroxypropyl-β-cyclodextrin and other modified cyclodextrins are often used because they have higher affinities for particular guests and therefore display more marked effects.[9] However, that was not the case in this work. Using ^{1}H NMR spectroscopy and the changes in chemical shift of signals of the substrate **1** on complexation with the cyclodextrins,[10] the association constants with β-cyclodextrin and hydroxypropyl-β-cyclodextrin were calculated to be 250 ± 60 and 120 ± 20 dm^3 mol^{-1}, respectively. Instead, in this study the greater effect of the hydroxypropylcyclodextrin relative to β-cyclodextrin on the enzyme catalysis reflects the use of a larger amount of the former. The solubility of β-cyclodextrin in water is limited to less than 0.02 mol dm^{-3} but more of the hydroxypropylcyclodextrin could be used because it is much more soluble.[11]

The association constants of the complexes of the cyclodextrins with the lactate **1** were calculated using eqn. (1). Based

$$K = \frac{[\text{complex}]}{[\text{guest}][\text{cyclodextrin}]} \qquad (1)$$

on this formula, in the experiments discussed above β-cyclodextrin and the hydroxypropylcyclodextrin would be expected to reduce the concentration of the substrate **1** free in solution by factors of 3.5 and 13.5, respectively, across the range of substrate concentrations used. This correlates well with the experimentally determined effects of the cyclodextrins (Fig. 1) and confirms that they are adequately described by Scheme 2.

The principle described above, of using cyclodextrins to manipulate the concentration of an enzyme substrate free in solution, was also applied to alter the substrate selectivity displayed by α-chymotrypsin. Some enzymes catalyse reactions of mixtures of substrates (Scheme 4). It was envisaged that

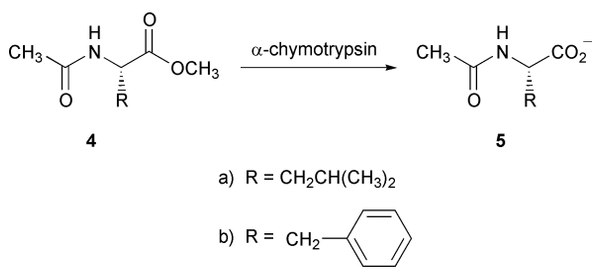


E, enzyme; S_A,S_B, substrates; P_A,P_B, products; CD, cyclodextrin

Scheme 4

through selective complexation, cyclodextrins could be used to change the ratio of the substrates free in solution, thereby affecting the relative rates at which the reactions of the substrates were catalysed.

α-Chymotrypsin catalyses the hydrolysis of *N*-acetylamino acid methyl esters (Scheme 5). When a *ca.* 10 : 1 mixture of



Scheme 5

(*S*)-*N*-acetylleucine methyl ester (**4a**) (1.01×10^{-2} mol dm^{-3}) and (*S*)-*N*-acetylphenylalanine methyl ester (**4b**) (1.00×10^{-3} mol dm^{-3}) was treated with α-chymotrypsin, the ratio of formation of the acids **5a** and **5b** was 1 : 3.9 when 25% of the phenylalanine derivative **4b** had been processed. This ratio decreased as the extent of reaction increased, consistent with the changing ratio of the residual substrates **4a** and **4b**. When 40% of the phenylalanine derivative **4b** had been processed, the ratio of the product acids **5a** and **5b** was 1 : 2.7. On repeating the reaction in the presence of hydroxypropyl-β-cyclodextrin (0.097 mol dm^{-3}), 6^A-amino-6^A-deoxy-β-cyclodextrin (0.12 mol dm^{-3}) and α-cyclodextrin (0.056 mol dm^{-3}), the respective ratios of the product acids **5a** and **5b** were 1 : 1.0, 1 : 1.0 and 1 : 0.6, when approximately 25% of the phenylalanine derivative **4b** had been consumed, and 1 : 0.9, 1 : 0.9 and 1 : 0.4, after approximately 40% reaction of the ester **4b**.

Each of the cyclodextrins changes the ratio of formation of the products **5a** and **5b**, this effect being greatest with α-cyclodextrin. To determine if these effects are adequately accounted for by the impact of cyclodextrin complexation as represented in Scheme 4, it was necessary to know the association constants for the cyclodextrin complexes. The value for the complex of the leucine derivative **4a** with α-cyclodextrin was determined to be 30 ± 29 dm^3 mol^{-1}, using ^{1}H NMR spectroscopy. However, despite considerable effort and using a variety of methods,[9] it was not feasible to determine the corresponding value in the case of the phenylalanine derivative **4b**. Using ^{1}H NMR and ultraviolet spectroscopy, no significant changes were observed on adding α-cyclodextrin to solutions of the amino acid derivative **4b**. With circular dichroism and fluorescence spectroscopy, the latter being used to study displacement of 6-(*p*-toluidino)-naphthalene-2-sulfonate from the cyclodextrin cavity, practical

limitations resulted in very large experimental errors. As a result, reliable association constants could not be measured in these ways. Using ^{1}H NMR spectroscopy, the association constants of the complexes of the amino acid derivatives **4a** and **4b** with hydroxypropyl-β-cyclodextrin were calculated to be 30 ± 20 and 84 ± 15 dm^3 mol^{-1}, respectively. The large errors result from the small changes in chemical shift of signals of the guests **4a** and **4b** on complexation by the cyclodextrin. Taking the association constants at face value, it follows from eqn. (1) that under the conditions used in the experiment discussed above the cyclodextrin will complex *ca.* 75% of the leucine derivative **4a** and *ca.* 90% of the phenylalanine derivative **4b**, changing the ratio of the substrates **4a** and **4b** free in solution by a factor of *ca.* 2.5. Selective complexation of the phenylalanine derivative **4b** by the cyclodextrin is consistent with biasing the enzyme catalysis in favour of reaction of the leucine derivative **4a**, according to Scheme 4.

In summary, the results discussed above provide further examples of the use of cyclodextrins to increase the efficiency of enzymes *in vitro*[3] and they define parameters that should be considered in order to predict the effect of a cyclodextrin on an enzyme-catalysed process. They show that cyclodextrins can be used to manipulate the concentrations of enzyme substrates free in solution, to reduce substrate inhibition and alter substrate selectivity in manners which may be rationalised according to Schemes 2 and 4, respectively. In each system, it is reasonable to expect that a substrate complexed by a cyclodextrin will readily dissociate to maintain equilibrium with the free substrate as it is consumed through reaction. Studies[9] have shown that dissociation rate constants of cyclodextrin complexes are typically much greater than 1 s^{-1}. Therefore the cyclodextrins may be regarded as substrate reservoirs.

Experimental

Ultraviolet spectra were recorded on a Shimadzu UV-2101 PC spectrophotometer coupled to a Shimadzu CPS temperature controller. High performance liquid chromatography (HPLC) was carried out using Waters™ 510 HPLC pumps, a Waters™ 717plus Autosampler, a Waters™ Tunable Absorbance Detector and a Waters™ 410 Differential Refractometer, on a Waters™ Symmetry® column, eluting with acetonitrile–water (1 : 4, v/v) containing 0.01% TFA. ^{1}H NMR spectra were recorded at 500 MHz on a Varian Inova 500S spectrometer.

Carboxypeptidase A (type I from bovine pancreas), α-chymotrypsin (type II from bovine pancreas) and the racemate of the sodium salt of 2-*O*-(*N*-benzoylglycyl)-β-phenyllactate (**1**)† were purchased from Sigma Chemical Co. (*S*)-*N*-Acetylleucine methyl ester (**4a**) and (*S*)-*N*-acetylphenylalanine methyl ester (**4b**) were prepared using standard methods. α-Cyclodextrin and β-cyclodextrin were obtained as generous gifts from Nihon Shokuhin Kako Co. Hydroxypropyl-β-cyclodextrin was purchased from Cyclolab Ltd., Hungary. The ^{1}H NMR spectrum of this material showed an average of 3.5 hydroxypropyl groups per cyclodextrin moiety, giving the material an effective molecular weight of 1347. 6^A-Amino-6^A-deoxy-β-cyclodextrin was prepared as reported previously.[12] All cyclodextrins were dried to constant weight *in vacuo* over phosphorus pentaoxide prior to use.

† Racemic material was used in all experiments, in the knowledge that (*R*)-2-*O*-(*N*-benzoylglycyl)-β-phenyllactate is not hydrolysed by carboxypeptidase A, and does not affect the interaction of the (*S*)-enantiomer **1** with the enzyme.[13,14] The cyclodextrins were generally used in large excess, so their association complexes formed with (*R*)-2-*O*-(*N*-benzoylglycyl)-β-phenyllactate do not substantially affect their complexation of the (*S*)-enantiomer **1**. The ^{1}H NMR studies showed that complexation of 2-*O*-(*N*-benzoylglycyl)-β-phenyllactate by the cyclodextrins is not stereoselective. Quantities reported in the Experimental section refer to the (*S*)-enantiomer **1** only.

Carboxypeptidase A catalysed hydrolysis of (*S*)-2-*O*-(*N*-benzoylglycyl)-β-phenyllactate (1), in the presence and absence of cyclodextrins

Solutions of (*S*)-2-*O*-(*N*-benzoylglycyl)-β-phenyllactate (**1**) (from 2.5 × 10^{-5} to 2.5 × 10^{-2} mol dm^{-3}) and carboxypeptidase A (*ca.* 20 units dm^{-3}) in 0.05 mol dm^{-3} Tris buffer (*I* = 0.5 mol dm^{-3}, KCl), at pH 7.5 and 298 K, containing either no cyclodextrin, β-cyclodextrin (9.96 × 10^{-3} mol dm^{-3}) or hydroxypropyl-β-cyclodextrin (0.105 mol dm^{-3}), were monitored for changes in absorbance at 250–290 nm.[13] Reaction rates were calculated from the spectra using the UV-2101 PC Kinetics software package. The results are illustrated in Fig. 1.

α-Chymotrypsin catalysed competitive hydrolysis of (*S*)-*N*-acetylleucine methyl ester (4a) and (*S*)-*N*-acetylphenylalanine methyl ester (4b), in the presence and absence of cyclodextrins

Solutions of (*S*)-*N*-acetylleucine methyl ester (**4a**) (1.01 × 10^{-2} mol dm^{-3}), (*S*)-*N*-acetylphenylalanine methyl ester (**4b**) (1.0 × 10^{-3} mol dm^{-3}) and α-chymotrypsin (*ca.* 600 units dm^{-3}) in 0.02 mol dm^{-3} Tris buffer, at pH 7.9 and 310 K, containing either no cyclodextrin, hydroxypropyl-β-cyclodextrin (0.097 mol dm^{-3}), 6^A-amino-6^A-deoxy-β-cyclodextrin (0.12 mol dm^{-3}) or α-cyclodextrin (0.056 mol dm^{-3}), were analysed at 15 min intervals using HPLC. The retention times of the esters **4a** and **4b**, and the acids **5a** and **5b** were 7.4, 9.8, 3.5 and 4.6 min, respectively.

Association constants of cyclodextrin complexes

Solutions of (*S*)-2-*O*-(*N*-benzoylglycyl)-β-phenyllactate (**1**) (1.0 × 10^{-3} mol dm^{-3}) in 0.05 mol dm^{-3} pH 7.5 Tris buffer (*I* = 0.5 mol dm^{-3}, KCl), comprising 10% deuterium oxide and containing either β-cyclodextrin (0–10 mole equivalents) or hydroxypropyl-β-cyclodextrin (0–100 mole equivalents), were analysed at 298 K using ^{1}H NMR spectroscopy. Changes in the difference between the chemical shifts of the signals due to the *ortho*- and *meta*-protons of the benzoyl group of the lactate **1** as a function of cyclodextrin concentration were analysed by non-linear regression to give association constants for 1 : 1 complexes of the lactate **1** with β-cyclodextrin and hydroxypropyl-β-cyclodextrin of 250 ± 60 and 120 ± 20 dm^3 mol^{-1}, respectively. The association constants of 30 ± 29, 30 ± 20 and 84 ± 15 dm^3 mol^{-1}, for the 1 : 1 complexes of the ester **4a** with α-cyclodextrin, and the esters **4a** and **4b** with hydroxypropyl-β-cyclodextrin, respectively, were measured in a similar manner, except that the experiments were conducted in 0.02 mol dm^{-3} pH 7.9 Tris buffer at 310 K, and complexation was monitored using changes in the difference between the chemical shifts of the signals due to acetyl protons and side chain protons of the esters **4a** and **4b** as a function of cyclodextrin concentration.

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