Use of cyclodextrins to limit product inhibition of (*S*)-phenylalanine ammonia lyase

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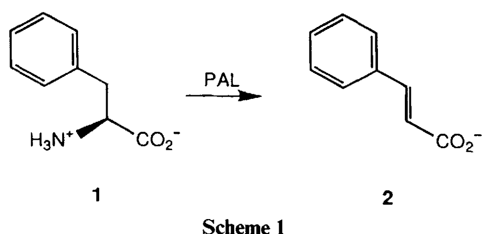
The extent of product inhibition of (*S*)-phenylalanine ammonia lyase, in catalysing the conversion of (*S*)-phenylalanine into *trans*-cinnamate, is reduced, and the efficiency of the reaction increased, through the addition of a cyclodextrin to sequester the cinnamate.

(*S*)-Phenylalanine ammonia lyase (PAL) catalyses the elimination of ammonia and a proton from (*S*)-phenylalanine **1**, to give *trans*-cinnamate **2** (Scheme 1)[1,2] which is a competitive



Scheme 1

inhibitor of the enzyme.[2] While product inhibition of this type is an important form of control of enzyme activity *in vivo*, it limits the utility of enzymes in organic synthesis. In this manuscript we report the use of α- and β-cyclodextrin to limit the effect of the cinnamate **2** on the catalytic activity of PAL, as an illustration of an approach to reduce product inhibition of enzymes.

Reactions of (*S*)-phenylalanine **1** catalysed by PAL (Grade 1 from *Rhodotorula glutinis*, purchased from Sigma Chemical Co.) were followed by monitoring changes in the UV absorbance at 268 nm accompanying formation of the cinnamate **2** (Fig. 1). Comparative experiments using the same quantity of enzyme were carried out with no cyclodextrin and with either α-cyclodextrin or β-cyclodextrin, in the presence and absence of the cinnamate **2**. Owing to its increased solubility in aqueous solutions compared to β-cyclodextrin,[3] it was possible to use α-cyclodextrin at higher concentration.

The results of the experiments show that the addition of the cinnamate **2** increases the extent of reaction over the first 1–3 min, but reduces the extent of reaction in the longer term. The initial increase can be attributed to the effect of the cinnamate **2** to bind competitively to the enzyme and thus slow the negative allosteric effect of the phenylalanine **1**.[4] The later reduction in the extent of each reaction with added cinnamate **2** is a clear illustration of the effect of the cinnamate **2** to inhibit the enzyme, an effect which is also apparent in the reduction in the rate of the reaction as each experiment proceeds and the cinnamate **2** is produced.

At the concentrations used, α- and β-cyclodextrin each marginally reduce the molar UV absorption of the cinnamate **2**.[5] Consequently, the effect of the cyclodextrins to increase the absorption of reaction mixtures clearly demonstrates that both α- and β-cyclodextrin increase the extent of reaction. The obvious interpretation of this effect is that the cyclodextrins complex the cinnamate **2**, irrespective of whether it is only

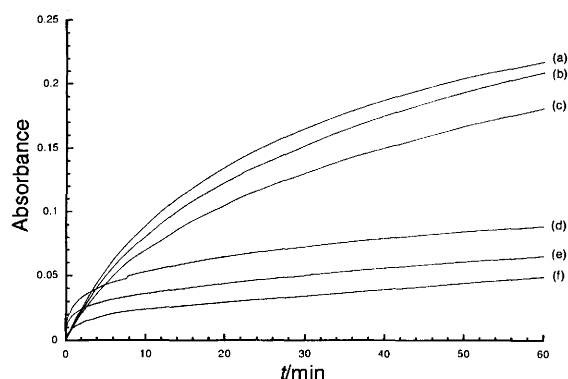


Fig. 1 Change in UV absorbance at 268 nm of solutions containing (*S*)-phenylalanine **1** (0.25×10^{-3} mol dm^{-3}), PAL (*ca.* 70 units dm^{-3}) and either (a) α-cyclodextrin (0.080 mol dm^{-3}), (b) β-cyclodextrin (6.9×10^{-3} mol dm^{-3}), (c) no cyclodextrin, (d) α-cyclodextrin (0.075 mol dm^{-3}) and the cinnamate **2** (0.26×10^{-3} mol dm^{-3}), (e) β-cyclodextrin (6.5×10^{-3} mol dm^{-3}) and the cinnamate **2** (0.26×10^{-3} mol dm^{-3}), or (f) the cinnamate **2** (0.26×10^{-3} mol dm^{-3}) but no cyclodextrin, in 0.05 mol dm^{-3} phosphate buffer at pH 6.9 and 303 K.

produced during the reaction or also added initially. This reduces the concentration of the cinnamate **2** free in solution, thus limiting the inhibitory effect on the enzyme. The results indicate that each cyclodextrin binds the cinnamate **2** in preference to (*S*)-phenylalanine **1**. This is consistent with the reported stability constants of the complexes of α- and β-cyclodextrin with (*S*)-phenylalanine **1**, of 8 and 3 dm^3 mol^{-1}, respectively,[6] and with the cinnamate **2**, of 109 and 313 dm^3 mol^{-1}, respectively.[5] From these stability constants it can be calculated that a solution containing β-cyclodextrin (6.5×10^{-3} mol dm^{-3}) and either the cinnamate **2** (0.26×10^{-3} mol dm^{-3}) or (*S*)-phenylalanine **1** (0.25×10^{-3} mol dm^{-3}) would contain only 34% of the cinnamate **2** or 98% of the phenylalanine **1** free in solution, while in analogous solutions of α-cyclodextrin (0.075 mol dm^{-3}) the amount of the cinnamate **2** and (*S*)-phenylalanine **1** unbound would be 11 and 63%, respectively.

To confirm the above interpretation of the experiments illustrated in Fig. 1, and the effect of the cyclodextrins, the experiments beginning with (*S*)-phenylalanine **1** and the cinnamate **2**, with no cyclodextrin and with either α- or β-cyclodextrin, were repeated using >99% 2-^{13}C-labelled (*S*)-phenylalanine **1** and approximately double the concentration of PAL. After 1 h, each reaction mixture was acidified to pH 1 and extracted with chloroform, and the residue obtained from concentration of the organic extract was analysed by ^{1}H NMR spectroscopy (Fig. 2). In these experiments the unlabelled

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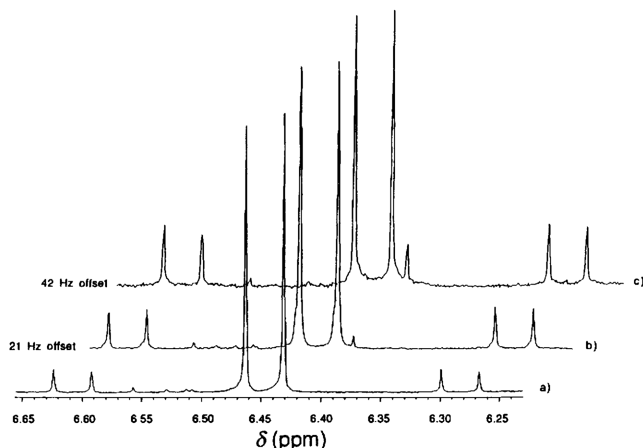

Fig. 2 ^1H NMR spectra (500 MHz, $CDCl_3$) of the material obtained by treatment of a solution of 2-^{13}C-labelled (*S*)-phenylalanine **1** (0.25×10^{-3} mol dm^{-3}) and the cinnamate **2** (0.26×10^{-3} mol dm^{-3}), containing either (a) no cyclodextrin, (b) β-cyclodextrin (6.5×10^{-3} mol dm^{-3}), or (c) α-cyclodextrin (0.075 mol dm^{-3}) in 0.05 mol dm^{-3} phosphate buffer at pH 6.9, with PAL at 303 K for 1 h.

cinnamate **2** is an internal standard and the different ratios of unlabelled to labelled cinnamate **2** isolated from the reaction mixtures are a measure of the relative extents of reactions. The ^1H NMR spectra show signals due to the ^{13}C-labelled cinnamate **2** produced during reaction, at δ 6.45 (dd, J_H 16 Hz, J_C 164 Hz), and due to the cinnamate **2** added initially to each reaction mixture, at δ 6.45 (d, J_H 16 Hz). Integration of these signals shows that whereas the reaction in the absence of a cyclodextrin proceeded to an extent of 16%, the reaction carried out under otherwise identical conditions, but in the presence of β-cyclodextrin had proceeded to an extent of 29%, while the analogous reaction in the presence of α-cyclodextrin had proceeded even further, to an extent of 41%. These results were confirmed by using gas chromatography–mass spectrometry to determine the ^{13}C-isotope content of the cinnamate **2** recovered from each of the reaction mixtures.

Reducing product inhibition of an enzyme in this manner may be achieved if the cyclodextrins complex a reaction product in preference to a substrate. In a similar manner it may be possible to manipulate enzyme-catalysed equilibrations, or the substrate selectivity in enzyme-catalysed reactions, by selectively complexing components from mixtures. Studies to this effect are underway in our laboratories.

Experimental

Procedures for assaying the effect of cyclodextrins on the catalytic activity of PAL

For UV spectrophotometric studies, aliquots of stock solutions of (*S*)-phenylalanine **1** (5.0×10^{-3} mol dm^{-3} solution in 0.05 mol dm^{-3} pH 6.9 sodium phosphate buffer; 1.0×10^{-5} dm^3) and the cinnamate **2** (5.2×10^{-3} mol dm^{-3} solution in 0.05 mol dm^{-3} pH 6.9 sodium phosphate buffer; 1.0×10^{-5} dm^3), as appropriate, were diluted to 1.6×10^{-4} dm^3 with 0.05 mol dm^{-3} pH 6.9 sodium phosphate buffer containing either no cyclodextrin, α-cyclodextrin (0.107 mol dm^{-3}) or β-cyclodextrin (9.26×10^{-3} mol dm^{-3}). The resulting solutions were equilibrated at 303 K for 10 min and then a thermally pre-equilibrated solution of PAL (4.0×10^{-5} dm^3 of a 30% glycerol, 0.025 mol dm^{-3} pH 6.9 sodium phosphate buffer solution) was added to each one. These mixtures were prepared in a 1 mm path-length cell, and monitored for change in UV absorbance at 268 nm using a Cary 1E spectrophotometer, with the cell-holder thermostatted at 303 K.

For product studies, solutions were prepared as described above, except that >99% 2-^{13}C-labelled (*S*)-phenylalanine **1** was used and the scale of the reactions was increased 50-fold. After incubation at 303 K for 1 h, the solutions were each acidified to pH 1 with concentrated HCl and extracted with $CHCl_3$ (8×0.040 dm^3). For each reaction mixture, the combined extracts were dried ($MgSO_4$) and concentrated under reduced pressure, and the residue was analysed using ^1H NMR spectroscopy and mass spectrometry.

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References

1 E. A. Havir and K. R. Hanson, *Biochemistry*, 1968, **7**, 1896; K. R. Hanson and E. A. Havir, *Arch. Biochem. Biophys.*, 1970, **141**, 1; E. A. Havir, P. D. Reid and H. V. Marsh, *Plant Physiol.*, 1971, **48**, 130; E. A. Havir and K. R. Hanson, *Biochemistry*, 1975, **14**, 1620; D. S. Hodgins, *J. Biol. Chem.*, 1971, **266**, 2977; J. R. Parkhurst and D. S. Hodgins, *Arch. Biochem. Biophys.*, 1972, **152**, 597; C. Walsh, *Enzymatic Reaction Mechanisms*, W. H. Freeman and Co., San Francisco, 1979, pp. 563–567; K. R. Hanson and E. A. Havir, in *The Enzymes*, 3rd edn., P. D. Boyer, ed., Academic Press, New York, 1972, vol. 7, pp. 75–166; K. R. Hanson and E. A. Havir, in *The Biochemistry of Plants*, P. K. Stumpf and E. E. Conn, eds., Academic Press, New York, 1981, vol. 7, pp. 577–625.
2 E. A. Havir and K. R. Hanson, *Biochemistry*, 1968, **7**, 1904.
3 M. L. Bender and M. Komiyama, *Cyclodextrin Chemistry*, Springer, New York, 1977.
4 S. E. Iredale and H. Smith, *Phytochemistry*, 1974, **13**, 575.
5 K. Uekama, M. Otagiri, Y. Kanie, S. Tanaka and K. Ikeda, *Chem. Pharm. Bull.*, 1975, **23**, 1421.
6 M. V. Rekharsky, F. P. Schwarz, Y. B. Tewari and R. N. Goldberg, *J. Phys. Chem.*, 1994, **98**, 10282.

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