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An unusual type of enzyme inhibition

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SUMMARY

The inhibition of papain by benzoylamido acetonitrile with benzoylglycine methylester as substrate exhibits unusual kinetics. In the double reciprocal plot the lines of various inhibitor concentrations intersect in the first quadrant. It is tentatively explained by association between substrate and inhibitor.

Ordinary competitive inhibition was observed with papain when benzoylarginine ethylester was used as a substrate and benzoylamido acetonitrile as the inhibitor[1]. However, with benzoylglycine methylester (prepared according to the method of Lucas and Williams, ref. 2) as substrate, all other materials, methods and computations being equal, the lines in the $1/v$ *versus* $1/[S]$ plot corresponding to various inhibitor concentrations are found to intersect in the first quadrant (Fig. 1). The line corresponding to the highest inhibitor concentration even intersects the ordinate at a negative V value.

These data can be understood from a combination of competitive inhibition and association between this particular substrate and the inhibitor. The following equilibria and conservation equations are involved:

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k} E + P$$

$$E + I \overset{K_i}{\rightleftharpoons} EI \qquad e = [E] + [ES] + [EI]$$

$$S + I \overset{K_a}{\rightleftharpoons} SI \qquad i = [I] + [SI] + [EI] \qquad (1)$$

in which e and i are total enzyme and total inhibitor concentrations respectively. Since $i \gg e$, [EI] can be neglected in Eqn 1. Steady-state treatment then yields

$$\frac{k \cdot e}{v} = 1 + \frac{K_m}{[S]} \left\{ 1 + \frac{i}{K_i(1 + [S]/K_a)} \right\} \quad (2)$$

If $[S] < K_a$ Eqn 2 can be approximated by

$$\frac{k \cdot e}{v} = 1 + \frac{K_m}{[S]} \left\{ 1 + \frac{i}{K_i} \left(1 - \frac{[S]}{K_a} \right) \right\} = \left(1 - \frac{K_m \cdot i}{K_a \cdot K_i} \right) + \frac{K_m}{[S]} \left(1 + \frac{i}{K_i} \right) \quad (3)$$

The slope of the $1/v$ *versus* $1/[S]$ plot equals

$$\frac{K_m}{k \cdot e} \left(1 + \frac{i}{K_i} \right)$$

Hence K_i can be calculated from the ratio of the slopes of the lines with and without inhibitor. The values obtained from the four inhibition lines were $K_i = 0.150 \pm 0.006$ mM, virtually equal to the value of 0.14 mM observed when benzoylarginine ethylester was the substrate[1]. K_m is found as 16 mM.

Furthermore it follows from Eqn 3 that

$$R = \frac{1/V^0 - 1/V}{1/V^0} = \frac{K_m \cdot i}{K_a \cdot K_i} \quad (4)$$

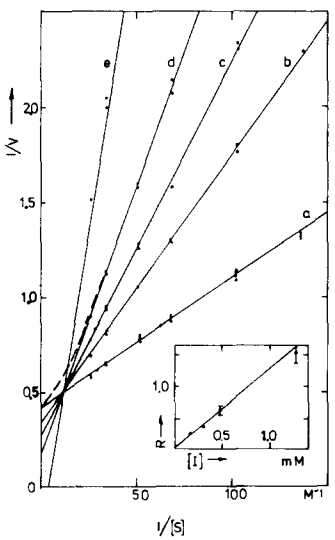

Fig. 1. Double reciprocal plot of the inhibition of papain by benzoylamido acetonitrile with benzoylglycine methylester as substrate: a, no inhibitor, b–e, 0.157, 0.293, 0.481 and 1.27 mM inhibitor, respectively. Only the lower experimental points of lines c, d and e are shown. Broken line: theoretical curve according to Eqn 2 with K_a = 51 mM, K_i = 0.152 mM and K_m = 16.3 mM. Inset: plot according to Eqn 4.

where V^0 is the maximum rate in the absence of inhibitor. Hence R should be proportional to i, as is indeed observed (Fig. 1, inset). From the slope a value of about 0.08 M is calculated for K_a.

Finally it can be shown from Eqn 3 that the intersection of the lines occurs at $1/[S] = 1/K_a$. This also yields $K_a = 0.08$ M.

Eqn 3 yields lines which are tangents at infinitely low substrate concentration, whereas the experimental points are located on those lower parts of the curves of Eqn 2 which are only virtually straight, within experimental error (standard deviation ± 3%). This has displaced the lines and hence the point of intersection to the left, *i.e.* to too high a value of K_a. In fact, one can derive a more accurate value using Eqn 2 by plotting $1/v$ *versus* i for several values of [S]. The slopes of the lines, s, are assessed. In a secondary plot $1/[S] \cdot s$ is plotted *versus* [S]. The intercept of the straight line of the latter plot at the [S]-axis equals $K_a = 51 \pm 8$ mM. A theoretical line can then be fitted to the experimental points using Eqn 2 (Fig. 1). At pH 8.4 both K_i (0.28 mM, *cf.* ref. 1) and K_m (33 mM) are found to be two times higher than at pH 6.0, whereas K_a, as derived from the point of intersection, is virtually unchanged (0.09 M). This fits in with the explanation; since both substrate and inhibitor are non-ionizing compounds their association is expected to be pH independent.

Further confirmation was attempted by calorimetry in the micro flów calorimeter of L.K.B. (Sweden) adjusted to 37 °C. When solutions of 60 mM substrate and 15 mM inhibitor are introduced into the flow cell, taking the proper controls into account, a small exothermic effect is indeed observed. Though the effect is too small to allow of an independent determination of K_a, it does provide a qualitative confirmation of association.

Lucas and Williams[2], examining the same system, did not observe the unusual kinetics. Therefore the experiments were repeated under their conditions, *i.e.* at pH 6.0, 35 °C and 10 vol.% acetonitrile. The intersection in the first quadrant is again observed, the parameters being $K_m = 34$ mM, $K_i = 0.73$ mM and $K_a = 0.18$ M. The discrepancy between our results and those of the previous workers is probably due in part to the higher value of K_a under their conditions, in part to a more limited range of substrate concentrations used by them (10–30 mM) than by us (7–40 mM at 25 °C, 10–100 mM at 35 °C). In fact, when their data (Fig. 2A of ref. 2) were processed using our computer programme, intersection in the first quadrant was observed and a value of $K_a = 0.2$ M was calculated, comparing favourably with our value of $K_a = 0.18$ M.

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