The Inhibition of Pepsin-Catalysed Reactions by Structural and Stereochemical Product Analogues

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1. The inhibition of pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine by products and product analogues was studied. 2. Inhibitors of the L-configuration give rise to linear non-competitive inhibition, whereas those of the D-configuration show linear competitive behaviour. 3. Non-competitive inhibition by the product *N*-acetyl-L-phenylalanine indicates an ordered release of products, which supports a common mechanism (involving an 'amino-enzyme') for pepsin-catalysed transpeptidation and hydrolysis reactions. 4. The differences in the types of inhibition caused by product analogues of the L- and D-series emphasize the stereospecificity of the binding of these inhibitors to free enzyme and to the putative amino-enzyme intermediate. 5. The results suggest that it is the anion of the acyl product that is released first in the hydrolytic reaction (see Kitson & Knowles, 1971).

The outline pathway of peptidolytic reactions catalysed by pepsin is expressed by eqn. (1) (for references, see Knowles *et al.* 1970):

According to this scheme, the rapid reversible formation of the enzyme-substrate complex is succeeded by a relatively slow reaction resulting in the formation of an 'amino-enzyme' (E-NH-Y) and the release of the carboxyl moiety of the substrate (X-CO₂H), followed by the hydrolysis of the amino-enzyme to release the other half of the substrate.

Originally, the evidence for this pathway came from studies of the products arising from transpeptidation rather than from hydrolysis. Thus from the work of Fruton, Fujii & Knappenberger (1961) and of Neumann, Levin, Berger & Katchalski (1959) it is apparent that in the pepsin-catalysed hydrolysis of a dipeptide substrate, X-CO-NH-Y, there is formed an intermediate 'amino-enzyme' (conveniently though not necessarily correctly formulated as a covalent intermediate, pepsin-NH-Y), and that this amino-enzyme lives long enough for X-CO₂H to be released, and (when transpeptidation occurs) X'-CO₂H to bind, with the catalysed formation of a new peptide X'-CO-NH-Y.

A substantial body of evidence now indicates

that in the pepsin-catalysed hydrolysis of small peptide substrates the rate-determining step of the catalytic reaction is that which immediately follows the formation of the Michaelis complex, and this fact effectively precludes the direct observation of any subsequent intermediates (Cornish-Bowden, Greenwell & Knowles, 1969). Classically, intermediates that follow the rate-determining step of a reaction can be detected by trapping experiments, where a reagent is added that reacts fast enough with the intermediate for one to observe product diversion with no change in overall reaction rate.

Although the transpeptidation reaction itself is strictly an example of such product diversion, it was found by Greenwell, Knowles & Sharp (1969) that analogues of acyl-amino acids that would have allowed the transpeptidation reaction to be followed directly did not act as acceptors. Thus it was found that *N*-acetyl-L-phenylalanine ethyl ester cannot act as an 'amino group' acceptor in a transpeptidation process. The same is true even for *N*-methanesulphonyl-L-phenylalanine methyl ester and methyl thiol ester. No methanethiol is liberated when the methyl thiol ester is present and inhibits the hydrolytic reaction, and the hope that such a thiol ester would more effectively trap the amino-enzyme was not fulfilled.

For an enzyme-catalysed process there is the additional possibility for the detection of intermediates, namely by studying the nature of inhibition of the reaction by a product or product analogue. This approach can be exemplified by the

work of Hsu, Cleland & Anderson (1966) on the inhibition of acid phosphomonoesterase. From the inhibition pattern these workers deduced that this enzyme operates by a mechanism involving the ordered release of products, and that two enzyme-phosphate complexes are involved in the catalytic reaction pathway.

The application of this approach to the case of pepsin concerns us here. The patterns of inhibition arising from the presence of acyl product and of chemical and stereochemical analogues of acyl product are reported. The results support and complement the preliminary studies of Greenwell *et al.* (1969), and, together with work reported by Inouye & Fruton (1968) on the inhibition of pepsin-catalysed reactions by the second type of product, support the pathway represented by eqn. (1) as operating for pepsin-catalysed hydrolysis, as well as for transpeptidation.

MATERIALS

Pepsin. This was obtained as described by Cornish-Bowden & Knowles (1969).

N-Acetyl-L-phenylalanyl-L-phenylalanylglycine. This was synthesized as described by Cornish-Bowden & Knowles (1969).

N-Acetyl-L-phenylalanine. This was prepared as described by Greenwell *et al.* (1969); it had m.p. 168–171°C and $[\alpha]_D^{20} +46.1^\circ$ (c 2 in ethanol). Coffy, Green & Kenner (1959) give m.p. 169–170°C and $[\alpha]_D^{20} +47.1^\circ$ (c 2 in ethanol).

N-Acetyl-D-phenylalanine. This compound was prepared in the same way as the L-enantiomer; it had m.p. 168–170°C and $[\alpha]_D^{20} -46.0^\circ$ (c 2 in ethanol).

N-Acetyl-L-phenylalanine ethyl ester. This compound was prepared from L-phenylalanine ethyl ester hydrochloride, which was in turn prepared by the method of Curtius & Goebel (1888).

L-Phenylalanine ethyl ester hydrochloride (2.9 g, 12.6 mmol) was suspended in dry chloroform and the mixture cooled to 0°C. Triethylamine (1.27 g, 12.6 mmol) was dissolved in the same amount of chloroform, cooled to 0°C and added to the suspension. During 45 min acetic anhydride (1.29 g, 12.6 mmol) was added in portions. The reaction mixture was then washed successively with water, 0.2M-HCl, water, 0.2M-KHCO₃ and water. The

chloroform solution was dried over MgSO₄, filtered and evaporated to dryness. The resulting solid was recrystallized from ethanol–water, giving fine white needles (2.25 g; 75% yield); it had m.p. 89.5–90.5°C and $[\alpha]_D^{20} +13.8^\circ$ (c 2 in ethanol). Caplow & Jencks (1964) give m.p. 89.5–91°C and Bergel, Johnson & Wade (1962) give $[\alpha]_D^{20} -13.1^\circ$ (c 2.6 in ethanol) for the D-enantiomer.

N-Acetyl-D-phenylalanine ethyl ester. This compound was prepared in the same way as the L-enantiomer; it had m.p. 90–91°C and $[\alpha]_D^{20} -13.9^\circ$ (c 2 in ethanol).

N-Acetyl-L-phenylalanine amide. N-Acetyl-L-phenylalanine ethyl ester (1.4 g, 5.96 mmol) was dissolved in dry methanol that had been previously saturated with NH₃ and cooled to 0°C. After standing at 0°C for 3 days the solution was evaporated to dryness and the resulting solid recrystallized from ethanol–light petroleum (b.p. 40–60°C) as white needles (0.73 g; 65% yield); it had m.p. 180–182°C and $[\alpha]_D^{20} +27.2^\circ$ (c 1 in methanol). Huang, Foster & Niemann (1952) give m.p. 176–177°C and $[\alpha]_D^{25} -27.0^\circ$ (c 1 in methanol) for the D-enantiomer.

N-Acetyl-D-phenylalanine amide. This compound was prepared in the same way as the L-enantiomer; it had m.p. 180–182°C and $[\alpha]_D^{20} -27.2^\circ$ (c 1 in methanol).

METHODS

The nature of the inhibition produced by the compounds studied and the corresponding inhibition constants were determined by using the continuous automatic ninhydrin method described by Cornish-Bowden & Knowles (1969).

The least-squares lines of plots of $1/v_0$ versus $1/[S_0]$ were evaluated by computer. Plots of the vertical intercepts of these lines against inhibitor concentration show whether the intercepts are constant within experimental error (i.e. competitive inhibition), or there is a regular dependence of intercept on inhibitor concentration (i.e. non-competitive inhibition; see, e.g., Fig. 4).

RESULTS

The nature of the inhibition produced by the compounds listed in Table 1, and the corresponding inhibition constants, were determined against N-acetyl-L-phenylalanyl-L-phenylalanylglycine as substrate. In all cases the reactions were carried out at 37°C and at pH 2.1. Inhibitors of the L-configuration give rise to linear non-competitive

Table 1. Inhibition of pepsin-catalysed hydrolysis by a product and product analogues

Inhibition was tested against N-acetyl-L-phenylalanyl-L-phenylalanylglycine as substrate, the concentration of which was 0.17–1.1 mM. The temperature was 37°C and the pH was 2.1. The terms for the type of inhibition are as defined by Hsu *et al.* (1966).

Inhibitor	Type of inhibition	K_i (mM)	$[I_0]$ range (mM)
N-Acetyl-L-phenylalanine	Linear non-competitive	27	0–15.3
N-Acetyl-L-phenylalanine ethyl ester	Linear non-competitive	17	0–12.0
N-Acetyl-L-phenylalanine amide	Linear non-competitive	47	0–18.3
N-Acetyl-D-phenylalanine	Linear competitive	28	0–21.6
N-Acetyl-D-phenylalanine ethyl ester	Linear competitive	12	0–12.8
N-Acetyl-D-phenylalanine amide	Linear competitive	46	0–16.4

Fig. 1. Lineweaver-Burk plot for the inhibition of the pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine by *N*-acetyl-L-phenylalanine at pH 2.1 and at 37°C. ○, Uninhibited; ●, [I] 5.1 mM; □, [I] 10.2 mM; ■, [I] 15.3 mM.

Fig. 3. Plot of the gradients of the lines of the Lineweaver-Burk plot for inhibition by *N*-acetyl-L-phenylalanine (Fig. 1) against inhibitor concentration.

Fig. 2. Lineweaver-Burk plot for the inhibition of the pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine by *N*-acetyl-D-phenylalanine at pH 2.1 and at 37°C. ○, Uninhibited; ●, [I] 5.4 mM; □, [I] 10.8 mM; ■, [I] 16.2 mM; △, [I] 21.6 mM.

Fig. 4. Plot of the vertical intercepts of the lines of the Lineweaver-Burk plot for inhibition by *N*-acetyl-L-phenylalanine (Fig. 1) against inhibitor concentration.

inhibition, and those of the D-configuration show linear competitive behaviour (Figs. 1 and 2). For all the inhibitors a similar plot to that of Fig. 3 (for the particular case of *N*-acetyl-L-phenylalanine)

was obtained, showing that in all cases the inhibition has linear kinetics. The other inhibitors of the L-configuration behave similarly to *N*-acetyl-L-phenylalanine in exhibiting a dependence of vertical

* The vertical intercept is linear in $[X]$, and the horizontal intercept is non-linear in $[X]$.

though only a single inhibitor concentration was investigated. The discrepancies both in the magnitude of K_i and in the nature of the inhibition demanded a more careful study of this reaction. As Table 1 and Fig. 1 show, the inhibition is indeed non-competitive, with K_i 27 mM.

In the simplest case the product of an enzymic reaction can inhibit the forward reaction in three ways: (i) by competing only with substrate for a single active site on the free enzyme (Table 2, pathway *b* with $X \equiv I$), (ii) by combining only with that enzyme complex with which it would react as a substrate in the reverse reaction (Table 2, pathway *e*) (or, possibly, by combining non-productively with this enzyme complex, which is equivalent to Table 2, pathway *c* with $X \equiv I$) or (iii) by combining both with free enzyme and with the enzyme complex (Table 2, pathway *f*) (or again, if the combination with enzyme complex is non-productive, see Table 2, pathway *d* with $X \equiv I$). As is obvious from Table 2, the inhibition patterns for these three cases differ, and in principle allow one to decide on the nature of the pathway of the catalysed reaction and of the interaction of the product with the various enzyme species.

Since the inhibition by acyl product (here *N*-acetyl-L-phenylalanine) is non-competitive (see Fig. 1), only pathways (*d*), (*e*) and (*f*) concern us. When the gradients of the lines of Fig. 1 are plotted against inhibitor concentration (Fig. 3: this is equivalent to a replot of vertical intercept versus $[I_0]$), a straight line is obtained, consistent with pathways (*d*) or (*e*), or, if the predicted non-linearity is very slight, pathway (*f*). Which, then, of pathways (*d*), (*e*) and (*f*) represents the true situation? We can immediately see that pathway (*e*) (although it would give rise to the observed inhibition) is the least probable, since it takes no account of possible binding between the acyl product and the free enzyme. Since it appears from the results that all the other inhibitors studied bind to the free enzyme (see below), it seems unlikely that *N*-acetyl-L-phenylalanine itself should be unable to do so.

Before consideration of pathways (*d*) and (*f*) in greater detail it is important to consider the inhibition produced by the other inhibitors studied and to discuss the significance of the K_i values.

Inhibition by *N*-acyl-L-amino acid ethyl ester and amide. Consideration of Table 2 shows that the pathways that would account for the observed linear non-competitive inhibition by *N*-acetyl-L-phenylalanine ethyl ester and the corresponding amide are again (*d*), (*e*) and (*f*). However, pathways (*e*) and (*f*) predict that these compounds would act as amino group acceptors with the concomitant release of ethanol and ammonia respectively. That this does not occur has been demonstrated both for the ester (Greenwell *et al.* 1969) and for the amide (Kitson &

Knowles, 1971). Thus it appears that pathway (*d*) applies, and that these two compounds inhibit the forward reaction both by competing with substrate for the active site of the free enzyme and also by combining with the intermediate amino-enzyme in a non-productive mode, presumably blocking that part of the active site previously vacated by the first product *X*.

Inhibition by the *D*-series. Table 2 further suggests that the three compounds, *N*-acetyl-D-phenylalanine and the corresponding ester and amide, inhibit the forward reaction merely by binding to the free enzyme (Table 2, pathway *b*). Only this would give rise to the observed linear competitive inhibition. It seems that the *D*-molecules cannot bind to the amino-enzyme, even though they bind readily to the free enzyme, presumably because the amino moiety of the substrate restricts the available space (and may also have caused a conformational change in the protein molecule), thus allowing binding of only those molecules with the 'correct' L-configuration.

Magnitude of the K_i values. Table 1 shows that, although the nature of the inhibition differs, the magnitude of K_i for a particular inhibitor of the L-series is very similar to that for the corresponding *D*-compound. What is the reason for this correlation? The K_i values were calculated from the slopes of the Lineweaver-Burk plots by using the expression:

$$K_i = \frac{[I]}{(\text{slope})_i / (\text{slope})_{i=0} - 1} \quad (2)$$

It is also possible to derive an expression for K_i in terms of the rate constants for a particular reaction pathway. Consider the case of pathway (*d*) of Table 2 (Scheme 1), which, as we have agreed above, applies to the L-ester and L-amide. It can be shown that, for this scheme, eqn. (2) reduces to:

$$K_i = k_{-5}/k_{+5}$$

The same expression is also derived (more obviously in this case) from pathway (*b*) of Table 2, which applies to the *D*-acid, *D*-ester and *D*-amide. Thus, even though these two pairs of inhibitors show different types of inhibition, we may expect the K_i values (calculated from the slopes) to depend only on the binding to free enzyme, and to be similar numerically, as observed experimentally.

Scheme 1.

[This is, of course, only true if the interaction of only one or two of the substituents on the asymmetric carbon atom with their complementary loci on the enzyme dominates the binding constant. Moreover, interactions at the remaining two (or three) loci must be permitted, in order to see the same K_i values for L- and D-compounds. This is not unusual, however, and is most completely documented for α -chymotrypsin (see, e.g., Knowles, 1965).] Further, it is the near identity of the K_i values for the L-acid and the D-acid that allows a distinction to be drawn between the applicabilities of pathways (d) and (f) to the case of the L-acid (see below).

Inhibition by N-acetyl-L-phenylalanine. It must first be mentioned that pathway (f) of Table 2 requires that the horizontal intercepts of the Lineweaver-Burk plot for inhibition by N-acetyl-L-phenylalanine should vary in a non-linear manner with [X]. Inspection of Fig. 5, however, shows that, within experimental error, the horizontal intercept is invariant with [X]. For pathway (f) of Table 2 (and where the rate constants are designated analogously to eqn. 3) the K_i value derived from the slopes of the double-reciprocal plots is:

$$K_i = \frac{k_{+3}k_{-5}(k_{-1} + k_{+2})}{k_{+3}k_{+5}(k_{-1} + k_{+2}) + k_{-1}k_{-2}(k_{+5}[X] + k_{-5})} \quad (4)$$

This would result in a K_i value different from that for the corresponding D-acid, for which (from Table 2, pathway b):

$$K_i = k_{-5}/k_{+5}$$

Yet experimentally these values show no significant difference (Table 1).

The condition for the observed equality of the K_i values is that the term $k_{-1}k_{-2}(k_{+5}[X] + k_{-5})$ in eqn. (4) should be much smaller than the other term in the denominator. Very small values of k_{+5} and k_{-5} would not necessarily make this term small compared with $k_{+3}k_{+5}(k_{-1} + k_{+2})$ and we do not expect k_{-1} (the rate of dissociation of the Michaelis complex) to be very small. A very small value for k_{-2} (the rate of the reverse reaction between amino-enzyme and product X), however, would cause the apparent equality of the K_i values. It can also be shown that the expression for the vertical intercept of the Lineweaver-Burk plot is (for pathway f):

$$\frac{1}{v_0} = \frac{k_{-2}[X] + k_{+2} + k_{+3}}{k_{+2}k_{+3}[E]} \quad (5)$$

A very small relative value for k_{-2} would therefore result in a vertical intercept independent of [X] (i.e. competitive inhibition, which experiment shows to be certainly not the case; see Fig. 4). The conclusion here is that pathway (f) cannot simultaneously explain both the observed non-competitive inhibi-

tion by N-acetyl-L-phenylalanine and the observed equality of the K_i value for this compound and that for its D-isomer.

Pathway (d), however, with $I \equiv X$, would give directly the observed linear non-competitive inhibition and the observed equality of the K_i values, since this scheme gives rise to the following expressions for the vertical intercept and the inhibition constant:

$$\frac{1}{v_0} = \frac{k_{+2}k_{+4}[X] + k_{+2}k_{-4} + k_{+3}k_{-4}}{k_{+2}k_{+3}k_{-4}[E]}$$

and

$$K_i = k_{-5}/k_{+5}$$

This pathway requires that N-acetyl-L-phenylalanine binds to the free enzyme, and to the amino-enzyme unproductively. Why does X bind to the amino-enzyme unproductively? The simplest explanation is if the inhibitor is in the wrong ionization state, i.e. if the actual first product of the hydrolytic reaction is X^- (in this case, the anion of N-acetyl-L-phenylalanine). It is then reasonable to suppose that only the anion X^- binds in a productive manner (which would amount to pathway f), and since at pH 2 we are dealing primarily with the un-ionized molecule (the pK_a of N-acetyl-L-phenylalanine is 3.6) it is not surprising that we see unproductive binding (pathway d).

If the substrate for the formation of E·XY from EY is the anion X^- , then microscopic reversibility demands that the X moiety of the substrate that is released first be released as its anion. This important point is discussed more fully in the following paper (Kitson & Knowles, 1971), but it can be said here that this superficially surprising phenomenon is required by the mechanism for pepsin proposed by Knowles *et al.* (1970). The postulate does not affect the explanation given above for the inhibition by the other compounds studied, and indeed neatly explains the failure to detect product diversion by any neutral species such as N-methanesulphonyl-L-phenylalanine methyl thiol ester, N-acetyl-L-phenylalanine ethyl ester (Greenwell *et al.* 1969) and N-acetyl-L-phenylalanine amide (Kitson & Knowles, 1971).

Inhibition by the second product. The method for following the rate of pepsin-catalysed hydrolysis that we have used here does not allow a study of inhibition by the second product (L-phenylalanyl-glycine), since the necessary concentrations of this material would produce an unacceptable background ninhydrin colour. However, using a spectrophotometric method, Inouye & Fruton (1968) have found that the inhibition of the pepsin-catalysed hydrolysis of N-benzyloxycarbonyl-L-histidyl-p-nitro-L-phenylalanyl-L-phenylalanine methyl ester by the 'amino' product (L-phenylalanine methyl

ester) is of the linear competitive type as expected (Table 2, pathway *a* with added Y). [If the products X and Y were released in random order, the step immediately following formation of the Michaelis complex is rate-determining (the latter certainly applies for pepsin-catalysed peptidolytic reactions) and the release of products is very rapid the steps for release of products will be nearly at equilibrium, and under such conditions both X and Y will be competitive inhibitors.] The data of Inouye & Fruton (1968) therefore nicely complement the experiments reported here, and allow a consistent picture of the inhibition of pepsin-catalysed peptidolytic reactions to be drawn.

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