ACTIVATION OF THE ACTION OF PENICILLOPEPSIN ON LEUCYL-TYROSYL-AMIDE BY A NON-SUBSTRATE PEPTIDE AND EVIDENCE FOR A CONFORMATIONAL CHANGE ASSOCIATED WITH A SECONDARY BINDING SITE.*

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SUMMARY

The cleavage of L-leucyl-L-tyrosyl amide by penicillopepsin is activated about tenfold by L-leucyl-glycyl-L-leucine. The latter is not a substrate. The activator has no effect on K_M. An activation constant $K_A = 2.0 \pm 0.6$ mM has been calculated. Leucyl-glycyl-leucine also affects four bands of the circular dichroism spectrum of the enzyme. A dissociation constant of 2.4 mM has been calculated from a titration of the ellipticity changes. The results suggest that a conformational change caused by binding of the peptide is responsible for the increased catalytic activity.

Fruton (1) proposed that a secondary binding site played an important role in the action of porcine pepsin. Such sites are also important for the pepsin homologue penicillopepsin (2) and other acid proteinases (3,4). The evidence for the secondary sites comes from studies of the effect of increasing the chain length of peptide substrates. The large increases in activity found were mainly due to increases in k_{cat} with only small effects on K_M. It was concluded that binding of the extended peptide chains in secondary sites caused conformational changes which were responsible for the increased catalytic activity (1). No direct evidence is available at present for these conformationa changes. In an earlier paper (5) it was shown that Leu-Tyr amide is a substrate for penicillopepsin. In the present report we show that Leu-Gly-Leu which is not a substrate causes conformational changes and concomitantly activates the enzyme.

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MATERIALS AND METHODS

Penicillopepsin was prepared from Penicillium janthinellum essentially according to the method of Sodek and Hofmann (6). Leu-Tyr amide was from Cyclo Chemical Co.; Leu-Gly-Leu from Mann Research Laboratories and Research Plus Laboratories.

The action of penicillopepsin on Leu-Tyr amide was studied kinetically by measuring the initial rates of release of Tyr-amide on a Beckman Spinco Model 120C amino acid analyzer as described (5).

Circular dichroism spectra were recorded at room temperature with an ORD/CD-15 spectropolarimeter (Durrum/Japan Spectroscopic Co.) equipped with the SS-20 CD modification as described previously (7). The protein concentration was 1.5 mg/ml in a cell with a path length of 1.00 cm. Increasing amounts of solid Leu-Gly-Leu were added and the spectra recorded after each addition.

RESULTS AND DISCUSSION

The activating effect of Leu-Gly-Leu on the cleavage of Leu-Tyr amide as shown by the release of Tyr amide is shown in Fig. 1. The Lineweaver-Burk plots represent experiments carried out with 2 mM and 6 mM Leu-Gly-Leu (curves a and b) and with substrate only (c). It is clear that the activator has no

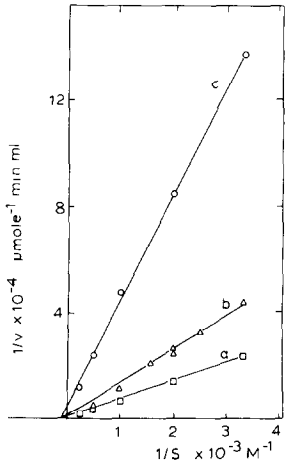
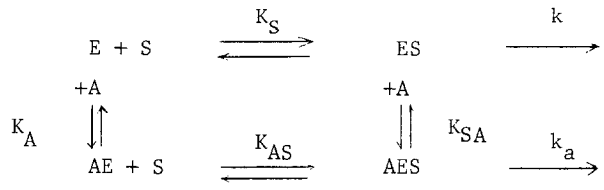


Figure 1. Lineweaver-Burk plots of effect of substrate concentration (Leu-Tyr amide) on initial velocity with and without Leu-Gly-Leu. a, 6 mM Leu-Gly-Leu; b, 2 mM Leu-Gly-Leu; c, no Leu-Gly-Leu. Assay Conditions: 0.05 M acetate, pH 3.4, 37°.

effect on K_M but increases k_{cat} by a factor of about ten. The reactions involved can be represented by the following scheme:

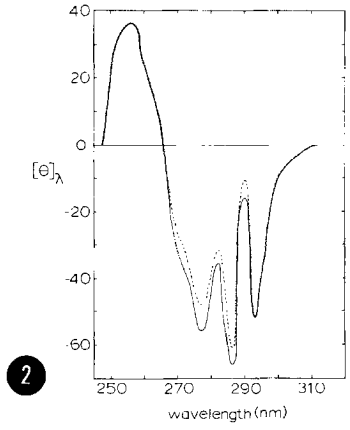


This is analogous to an enzyme system of non-competitive inhibition where the ternary complex has residual activity (Type IIb acc. to Dixon and Webb, ref. 8). The activation constant K_A can readily be calculated from the equation:

$$K_A = \frac{a(1-V'/V_a)}{V/V_a-1}$$

where a = activator concentration, V = maximum velocity in absence and V_a in presence of activator; and V' = maximum velocity in presence of saturating concentration of both substrate and activator. V' was obtained by extrapolation V_a to infinite activator concentrations (8). A value of K_A = 2.0 ± 0.6 mM was obtained. A similar value (2.1 mM) was also obtained from a plot of v_a/v, the ratio of observed velocities versus Leu-Gly-Leu concentrations at 2 mM substrate concentration.

The effect of Leu-Gly-Leu on the circular dichroism spectrum of penicillopepsin between 250 and 320 nm is shown in Fig. 2. The enzyme itself shows a series of well resolved transitions which may be attributed to tryptophan (e.g. at 293, 290 and 286) or to tyrosine (e.g. 276 nm). Addition of increasing amounts of Leu-Gly-Leu caused progressive changes in all transitions with the notable exceptions of 293 nm and 256 nm. The changes in ellipticity in the various transitions are plotted as a function of Leu-Gly-Leu concentrations in Fig. 3. They are expressed as a percentage of the changes observed at 32 mM Leu-Gly-Leu. The changes seen in bands at 290, 286 and 276 nm show identical dependency on the activator concentration. From these data the dissociation constant estimated from that activator concentration which shows



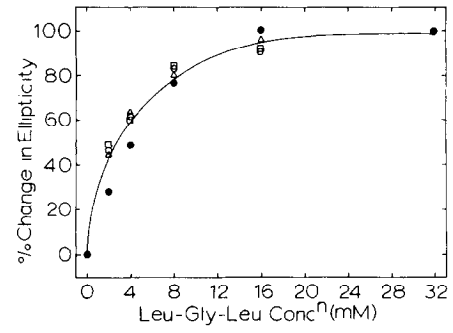


Figure 2. Circular dichroism spectra, between 250 and 320 mm, of penicillopepsin alone (——) and in the presence of 2 mM (----) and 32 mM (....) L-leucyl-glycyl-L-leucine. The mean residue ellipticity, [θ], is given in degrees cm^2. $decimole^{-1}$.

Figure 3. The percent change in ellipticity at 290 (Δ), 286 (O), 282 (●) and 276 (□) nm as a function of L-leucyl-glycyl-L-leucine concentration. At each wavelength the change observed in the presence of 32 mM Leu-Gly-Leu was taken as 100 percent.

a 50% change was 2.4 mM. This is close to the activator constant measured kinetically and provides strong evidence that the conformational change suggested by the circular dichroism data is directly related to the enzyme activation. The changes in the 282 nm band are anomalous and apparently give a higher dissociation constant. This anomaly might be accounted for if the apparent band at 282 nm were in fact a trough between the bands at 286 and 276 nm.

The simplest interpretation of these results is that Leu-Gly-Leu binds at the secondary binding site postulated for penicillopepsin (2), a site which is analogous to that observed for porcine pepsin (1). Evidence for this is the fact that Leu-Gly-Leu inhibits penicillopepsin when a large substrate (trypsinogen) is used. Fruton and his collaborators (1) have clearly shown that the increase of the peptide chain of substrates greatly increases the k_{cat} values for peptide band cleavage and postulate that a conformational

change due to binding of the extended chain in a secondary site is responsible for the increase in catalytic efficiency. Evidence has been presented in this laboratory that similar effects are found with the homologous penicillopepsin (2). The experiments described here show clearly the correlation between a conformational change and increased catalytic efficiency due to binding in a secondary site and lend strong support to Fruton's postulate.

Whether the conformational changes caused by Leu-Gly-Leu are localized or are of a more generalized nature, cannot readily be evaluated. The peptide band absorption region which could yield such information cannot be examined because of the absorption and optical activity of the activator peptide. In preliminary experiments it has been shown that Leu-Gly-Leu greatly increases the heat stability of the enzyme (Wang and Hofmann, to be published). This suggests that the conformational change due to Leu-Gly-Leu could involve a considerable part of the molecule and does not represent only a localized change. The conformational state induced by Leu-Gly-Leu probably involves increased hydrophobic interactions as shown by that fact that the transpeptidation reaction (formation of Leu-Leu) is increased to a considerably larger extent than the hydrolysis of the substrate. These latter observations which are being further studied probably have important implications on the mechanism of penicillopepsin and indirectly also that of porcine pepsin.

In conclusion it may be said that the experiments shown here are the first demonstration in the family of pepsin enzymes of a conformational change associated with increased catalytic efficiency due to binding of a peptide in a secondary site.

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