A STRONG COMPETITIVE PEPTIDE-INHIBITOR FOR LEUCINE AMINOPEPTIDASE

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1. Introduction

Aminopeptidases from animal tissue as well as from various microorganisms are currently investigated in several laboratories with respect to catalytic and structural properties and with respect to the biological role of these enzymes [1–8]. Specific reversible inhibitors for this type of peptidase have never been described so far. Such ligands might facilitate kinetic and structural as well as biochemical studies of aminopeptidases considerably.

2. Materials and methods

Leucine aminopeptidase EC 3.4.1.1 was obtained from Worthington Biochemicals, Freehold, New Jersey, USA. Leucine *p*-nitroanilide was from Serva Chemicals, Heidelberg, Germany.

The peptides

$$\text{Z-Thr}(\text{Bu}^t)\text{–Phe–Pro-OH}^*$$

and

$$\text{H-Thr}(\text{Bu}^t)\text{–Phe–Pro–Gln–Thr}(\text{Bu}^t)\text{–Ala–Ile–Gly-OH}$$

were obtained from Drs. B. Riniker and W. Rittel, Ciba-Geigy Ltd, Basel, Switzerland. Both peptides are fragments of synthetic human calcitonin [10].

* Abbreviations according to the IUB–IUPAC rules, European J. Biochem. 1 (1967) 375.

The tripeptide

$$\text{H-Thr}(\text{Bu}^t)\text{–Phe–Pro-OH}$$

was obtained from the corresponding carbobenzoxy-derivative by catalytic hydrogenation (Pd/C) and subsequent recrystallisation of the product from isopropanol–diisopropyl ether.

The free tripeptide H-Thr–Phe–Pro-OH was prepared in our laboratory.

Proton NMR spectra were carried out at 220 mHz in DMSO-d6 at 24°. The sample concentration was 5×10^{-2} M.

Leucine aminopeptidase was activated by incubation at 40° for 3 hr in 50 mM Tris-buffer pH 8.5 in the presence of 5 mM $MgCl_2$ and 2 mM $MnCl_2$. The protein concentration in the activation mixture was about 0.5 mg/ml.

The activity of the preparation towards 1 mM leucine *p*-nitroanilide was in the order of 200 μmoles/min/mg at 30°. Inhibition experiments were carried out with Tris-buffered substrate solutions at pH 8.5, 5 mM $MgCl_2$ at 30°. The enzyme concentration was usually 2–4 μg/ml. We measured the enzyme catalyzed hydrolysis of leucine *p*-nitroanilide in a Zeiss spectrophotometer (P.M.Q. II) at 405 nm. Further details on inhibition tests have been reported elsewhere [9].

3. Results and discussion

The tripeptide H-Thr–Phe–Pro-OH was readily hydrolyzed by leucine aminopeptidase to yield free threonine and the dipeptide H-Phe–Pro-OH. The

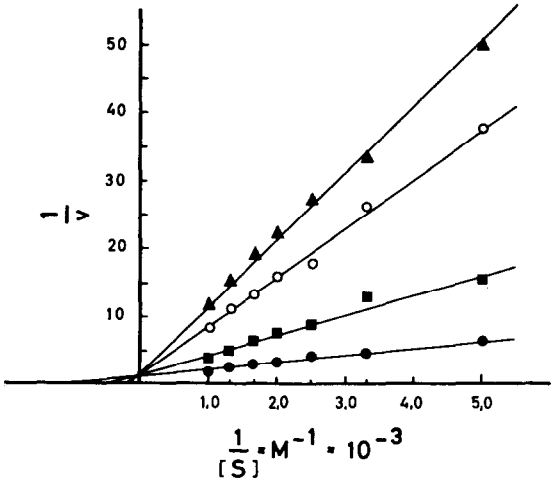

Fig. 1. Lineweaver–Burk plot for the leucine aminopeptidase catalyzed hydrolysis of leucine *p*-nitroanilide. Inhibition by $H\text{-}Thr(Bu^t)\text{–}Phe\text{–}Pro\text{-}OH$. (●–●–●): uninhibited reaction; (■–■–■): 0.01 mM inhibitor; (○–○–○): 0.05 mM inhibitor; (▲–▲–▲): 0.10 mM inhibitor. *v* is expressed in optical density units at 405 nm.

initial rate of the threonine release at maximal velocity was 35 μmoles/min/mg at 30°. The tripeptide was apparently a good substrate with respect to affinity as it's K_m value is about 5×10^{-4} M. Incubation of this peptide with very high amounts of enzyme yielded in addition to the products mentioned above small amounts of proline and phenylalanine.

With the well known impurity of the commercial leucine aminopeptidase preparation in mind, the release of a small amount of proline by a very high concentration of the protein, is not necessarily an activity of leucine aminopeptidase [11].

In striking contrast to the results with the free tripeptide we found that the tertiary butyl derivative

$H\text{-}Thr(Bu^t)\text{–}Phe\text{–}Pro\text{-}OH$

was not at all affected by the enzyme preparation. Drastic digestion with up to 100 μg of enzyme-protein in the incubation mixture and high peptide concentration (10–50 mM) failed to yield any detectable amino acids released from this peptide. When the digestion mixture was applied to a Dowex-50 column and assayed on the amino acid analyzer, the peptide was found to be unchanged and no additional peaks appeared.

A very similar result was obtained from digestion experiments with the octapeptide

$H\text{-}Thr(Bu^t)\text{–}Phe\text{–}Pro\text{–}Gln\text{–}Thr(Bu^t)\text{–}Ala\text{–}Ile\text{–}Gly\text{-}OH.$

Extended incubation for 1 hr or longer revealed, in this case, traces of amino acids identified as

$H\text{-}Thr(Bu^t)\text{-}OH$

and phenylalanine. The amount of free amino acids after 1 hr of incubation with 100 μg of leucine aminopeptidase might account for 0.3–0.5% of hydrolysis of this peptide.

Both resistant peptides compete effectively with leucine *p*-nitroanilide for the active site of leucine aminopeptidase. The enzyme activity was markedly affected by 10^{-6} M of tripeptide in an incubation mixture which contained 1 mM of leucine *p*-nitroanilide was 2–4 μg of enzyme. At a concentration of the tripeptide of 10^{-5} M, the inhibition produced was 50%. The same effect was obtained with 10^{-4} M of the octapeptide.

The inhibition was reversible as shown by dilution experiments which followed prolonged incubation of the enzyme with the inhibitors in the absence of substrate. In the diluted samples the enzyme activity was determined and compared to a control sample in which the enzyme had been preincubated in buffer only and was subsequently diluted in the same manner. We found that the percentage of inhibition produced was clearly related to the dilution rate and an enzyme inactivation can be excluded.

The influence of the substrate concentration on the velocity of the reaction was studied with various concentrations of inhibitor present. Reciprocal plots according to Lineweaver–Burk indicated with both peptides a competitive type of inhibition (fig. 1). The inhibition constants were derived from Dixon graphs [12] and the values of 10^{-5} M for the tripeptide

$H\text{-}Thr(Bu^t)\text{–}Phe\text{–}Pro\text{-}OH$

and 10^{-4} M for the octapeptide were obtained (fig. 2).

Our results indicate that the tripeptide H-Thr–Phe–Pro-OH which is a substrate of rather high affinity of leucine aminopeptidase, can be transformed chemically into an inhibitor of this enzyme with a con-

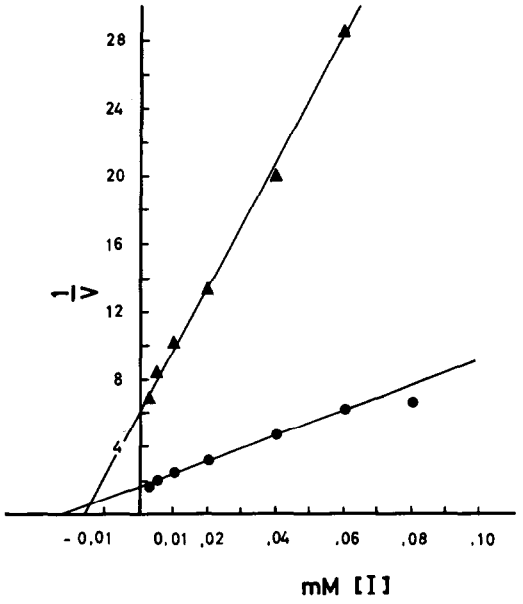

Fig. 2. Dixon-plot for the leucine aminopeptidase catalyzed hydrolysis of leucine *p*-nitroanilide. Inhibition by

Bu^t
H-Thr–Phe–Pro-OH

(●–●–●): 1.0 mM substrate; (▲–▲–▲): 0.2 mM substrate.

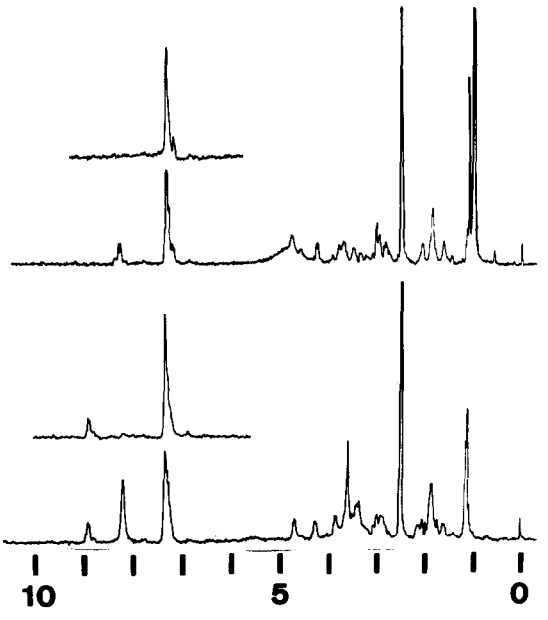

Fig. 3. Proton NMR spectra of free H-Thr–Phe–Pro-OH (bottom) and its tertiary butyl derivative

Bu^t
H-Thr–Phe–Pro-OH

(at the top). The upper trace in each spectrum was obtained after 5 min exposure to 10% D_2O. Scale: ppm selective to internal TMS. 1.0 ppm: Thr $(-CH_3)_3$ tertiary butyl function; 1.1 ppm: Thr $(-CH_3)$ free side chain; 1.5–2.2 ppm: Pro (beta and gamma protons); 2.5 ppm: solvent (DMSO); 7.3 ppm: Phe (aromatic protons); 8.2 ppm: Phe (amide proton) in the tertiary butyl derivative; 8.2 ppm: NH_3^+ of the free peptide; 8.8 ppm: Phe (amide proton) in the free peptide.

siderably increased affinity. The chemical modification consists in the introduction of a bulky hydrophobic group (tertiary butyl-) into the hydroxyl function of the threonine side chain. This difference between the free and the substituted threonine side chain could be easily demonstrated in the proton NMR spectra of the two tripeptides (fig. 3). The two spectra showed in addition to this another difference with respect to the amide proton of the phenylalanine residue, which was clearly shifted in one peptide relative to the other. This proton exchanged completely with D_2O in 5 min in the tertiary butyl derivative, whereas no exchange was observed after 5 min exposure to 10% D_2O in the free tripeptide.

We concluded that in the latter case the amide proton might be engaged in intramolecular hydrogen bonding, thus being stabilized against exchange with the solvent. The rapid rate of D_2O exchange in the tertiary butyl derivative indicated that the proton is strongly exposed to the solvent in this peptide.

Whether these observations are directly connected to the behaviour of the two peptides in their function as ligands (substrate or inhibitor) of leucine aminopeptidase cannot be decided at the moment. Presently no details are available on the accomodation of a peptide substrate in the active center of leucine aminopeptidase.

The well known preference of this enzyme for hydrophobic side chains in the amino-terminal residue is impressively illustrated by the high affinity of the inhibitor tripeptide. If the same sequence of amino acids is enlarged towards the carboxyl end by another 5 amino acid residues, as in the case of our octapeptide, the affinity of this ligand is decreased for about one order of magnitude. In addition to

this, a slight catalytic action of the enzyme is observed. It seems therefore that the interactions of the first three residues and those of the more distant residues from the amino end of the peptide are opposed to each other. The high affinity of the tripeptide and its clearly competitive interaction with substrate binding makes it an interesting tool in future studies of the active center of leucine aminopeptidase by affinity labeling.

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