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# Enkephalin-Degrading Dipeptidylaminopeptidase: Characterization of the Active Site and Selective Inhibition

P. CHÉROT, J. DEVIN, M. C. FOURNIÉ-ZALUSKI, and B. P. ROQUES
*Département de Chimie Organique, UA 498 CNRS-U 266 INSERM, UER des Sciences Pharmaceutiques et Biologiques, 75006 Paris, France*
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## SUMMARY

The enkephalins are degraded *in vitro* by three types of metallopeptidases including a dipeptidylaminopeptidase (DAP) which releases Tyr-Gly. In order to test the physiological significance of this enzyme in enkephalin metabolism, a membrane-bound DAP from porcine brain was purified. The structural characteristics of the active site of this enzyme were studied using several enkephalin-related fragments as substrates and various peptides as inhibitors. The active site possesses an anionic moiety able to interact with the ammonium group of the substrate and four hydrophobic subsites $S_2$, $S_1$, $S_1'$, $S_2'$ surrounding the metal-containing catalytic site. This porcine brain enzyme shows the same characteristics as a partially purified DAP from rat brain. Potent inhibitors of both enzymes were obtained with compounds interacting with either the $S_1'$, $S_2'$, or $S_1$, $S_2$ parts of the active site and containing a hydroxamate of an *N*-acyl-*N*-hydroxy amino group as the metal-chelating agent. The compounds synthesized to bind the $S_1'$, $S_2'$ subsites, such as $HN(OH)—CO—CH_2—CH(CH_2\phi)\text{-}CONH—CH(CH_2\phi)—COOH$, behave as highly potent and mixed inhibitors of both DAP ($K_i = 2.5$ nM) and enkephalinase ($K_i = 0.3$ nM). In contrast, Tyr-Phe-NHOH, designed to interact with $S_2$,$S_1$ subsites through its positively charged Tyr as the $P_2$ component, is a highly potent ($K_i = 9$ nM) and selective DAP inhibitor. The ability of Tyr-Phe-NHOH to protect, *in vivo*, endogenous enkephalins was evaluated using the hot plate test with mice. The intracerebroventricular injection of Tyr-Phe-NHOH alone (0.17 μmol, 60 μg) does not significantly modify the jump latency time as compared to the control. The effect of the association of Tyr-Phe-NHOH (0.17 μmol, 60 μg), bestatin (0.16 μmol, 50 μg) and thiorphan (0.20 μmol, 50 μg) is not statistically different from that produced by bestatin (0.16 μmol) plus thiorphan (0.20 μmol). Likewise, the effect of bestatin (50 μg) or thiorphan (25 μg) is not significantly modified by intracerebroventricular co-administration of increasing concentrations of Tyr-Phe-NHOH. These results seem to indicate that the DAP does not play a major role in enkephalin metabolism, at least at the supraspinal level.

The opioid peptides Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) possess most of the properties elicited by classical neurotransmitters (1). In the brain, they are rapidly metabolized by peptidases, which are probably localized close to the site of peptide action. *In vitro* the enkephalins are cleaved by three distinct enzymatic pathways, but the major process appears to be the release of the *N*-terminal tyrosine (2) by various membrane-bound APs, which can be blocked by the nonselective inhibitor bestatin (3). Direct injection of bestatin into rodent brain has been reported to induce a protection of endogenous Met-enkephalin (4) and a subsequent, although inconsistent, analgesic effect (5). *In vivo*, AP inactivation of enkephalins is associated with a second major degradation process involving the brain-neural endopeptidase (EC 3.4.24.11), often designated enkephalinase (6). This enzyme cleaves the $Gly^3$-$Phe^4$ bond of enkephalins, and its inhibition by potent and selective blocking agents such as thiorphan (7) or retro-thiorphan (8) leads to significant and reproducible antinociceptive responses. In addition to these two metabolic pathways, a third degradation process, involving a dipeptidylaminopeptidase (DAP), has been evidenced by the presence of Tyr-Gly in brain tissue incubated with enkephalins (9, 10). Nevertheless, the physiological relevance of this peptidase in the *in vivo* degradation of the opioid peptides has not yet been clearly established. This is essentially due to the lack of potent and specific inhibitors of this enzyme. Recently, bidentate-containing inhibitors (11) have been shown to inhibit the three enkephalin-degrading enzymes (12). The *in vitro* and *in vivo* protection of enkephalins by one of these bidentates, designated kelatorphan, confirms that enkephalinase, and a well defined "kelatorphan-sensitive" AP, resembling AP M, are physiologically involved in opioid peptide metabolism (13). Furthermore, the small but significantly higher analgesic potency of kelatorphan, as compared to the association of bestatin and thiorphan, could suggest a possible, although minor, addi-

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**ABBREVIATIONS:** AP, aminopeptidase; DAP, dipeptidylaminopeptidase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IVC, intracerebroventicular.

tional role for a DAP activity in enkephalin metabolism (14). In order to test this hypothesis, we have purified a membrane-bound DAP from porcine brain.[1] This metallopeptidase has biochemical properties similar to those of the partially purified rat brain membrane-bound enzyme designed enkephalinase B, which generates Tyr-Gly from enkephalins (10).

In the present paper, we describe: 1) the molecular requirements for a peptide to interact with the porcine brain enkephalin-degrading DAP; 2) the potent and competitive inhibition of DAP by dipeptide hydroxamate and *N*-hydroxyaminocarbonyl derivatives; and 3) a pharmacological study of a selective DAP inhibitor, alone or in combination with bestatin or thiorphan, on nociceptive stimuli in mouse.

## Materials and Methods

[Tyrosyl-3,5-$^3$H]Leu$^5$-enkephalin (35 Ci/mmol) was from the Centre de l'Energie Atomique, Saclay, France. Tyr-Gly, Tyr-Gly-Gly, Gly-Gly, Met-enkephalin, and (Des-Tyr)-Leu-enkephalin were from Sigma. Gly-Phe, D-Ala-Phe, and Ala-Phe were from Serva (Heidelberg, FRG); Tyr-Glu, Tyr-Lys, Tyr-Phe, Tyr-Tyr, Tyr-Gly-NH$_2$, and Boc-enkephalin were from Bachem (Switzerland). Bestatin was a generous gift from Roger-Bellon Laboratories. *N*-Acetyl-Leu-enkephalin was prepared as already described (15). All other peptides and inhibitors, including thiorphan, were synthesized in our laboratory. The reagents used in this work were of analytical grade (Janssen, Prolabo, Merck).

**Enzyme preparation.** The purification of porcine brain DAP was carried out as previously described in detail,[2] starting from a crude mitochondrial extract of porcine brain. The extract was solubilized with 0.5% Triton X-100 and was applied onto a DEAE column in 50 mM Tris-HCl buffer containing 0.1% Triton X-100. DAP and AP activities coeluted as a symmetrical peak with a linear gradient of 0–0.5 M NaCl. The pooled active fractions were supplemented with ammonium sulfate (40% saturation, w/v) and the soluble activities were applied on a phenyl-Sepharose column. DAP and AP were separated by AH-Sepharose chromatography using a linear gradient of 0.15–0.5 M NaCl in Tris-HCl, 10 mM (pH 7.0). DAP was finally purified to homogeneity by Mono P HR 5/20 chromatography followed by chromatofocusing on the same column.

In order to determine the subcellular localization of DAP, the crude extract was distributed on a Ficoll-sucrose gradient. Two peaks of enzymatic activity sedimented at densities corresponding to the distribution of synaptosomes. After elimination of Ficoll by washing, the pooled active fractions were submitted to osmotic shock and by lysed membranes were applied to a sucrose gradient. The DAP activity sedimented in the lighter fractions corresponded to 0.7 M sucrose.

The DAP activity from rat brain was purified as described (11). Although not homogeneous, this preparation was shown to cleave enkephalin only at the Gly$^2$-Gly$^3$ level.

The rabbit kidney enkephalinase was purified to homogeneity according to the method of Almenoff and Orlowski (16).

**Standard assay of DAP.** The assay for DAP measured the release of [$^3$H]Tyr-Gly from [$^3$H]Leu-enkephalin. Before use, the [$^3$H]Leu-enkephalin was purified on a Sep-Pak C$_{18}$ cartridge (Waters Associates) previously washed with 10 ml of ethanol, followed by 10 ml of H$_2$O. Ten µl of radioactive solution were applied to the cartridge, impurities were eluted by washing with 10 ml of H$_2$O, and [$^3$H]Leu-enkephalin was eluted with 2 ml of ethanol.

The standard reaction mixture contained, in a final volume of 200 µl, 2 pmol of [$^3$H]Leu-enkephalin and the required concentration of inhibitor in 0.05 M Hepes/KOH (pH 7.0) buffer. The mixture was incubated at 25° for 15 min and the reaction was stopped by heating for 3 min at 80°. Aliquots (150 µl) were then applied to Sep-Pak columns, previously prepared as described above, [$^3$H]Tyr-Gly was eluted directly into scintillation vials with three times distilled 2-ml H$_2$O, and [$^3$H]Leu-enkephalin was eluted with 2 ml of ethanol. This gave a quantitative separation of tritiated peptide fragments from native enkephalin, >99% of the latter being retained on the resin.

The radioactivity eluted with water was determined by liquid scintillation counting using Beckman Ready solvant MP as scintillation liquid. Under these conditions the assay was linear with both time and enzyme concentration, in the range of 0.08–0.70 pmol of hydrolyzed substrate. One unit of DAP was defined as the amount of enzyme releasing 1 pmol of [$^3$H]Tyr-Gly from [$^3$H]Leu-enkephalin in 1 min. Enkephalinase activity was tested using [$^3$H]Leu-enkephalin as substrate, as described in detail (17).

**High performance liquid chromatographic analysis of peptide hydrolysis by DAP.** The identification of enzyme products was performed using a Waters Associates high performance liquid chromatograph equipped with a C$_{18}$ µ-Bondapak column with a particle size of 9 µm (0.39 × 30 cm). Enzyme preparation (0.65 unit) was incubated at 25° in 50 mM potassium phosphate, pH 7.0, with 1 mM synthetic peptide in a final volume of 200 µl for 240 min. The reaction was stopped by heating for 3 min at 80°. An aliquot of the sample was applied to the C$_{18}$ column and eluted, at ambient temperature, with a mixture of acetonitrile and 0.1 M phosphate buffer, pH 3.0, at a flow rate of 2 ml min$^{-1}$. The peptides were measured by UV absorption at 210 nm. Various proportions of acetonitrile and phosphate buffer (1:99, 5:95, and 20:80) were used to separate the different products of enzymatic cleavage.

The reaction velocity of the first cleavage was measured by integration of peak areas and comparison with authentic standard peptides.

**Analgesic tests.** Analgesic effects were evaluated on the hot-plate test at 55 ± 0.2° according to the method of Eddy and Leimbach (18), using male Swiss mice (20–22 g, Charles River breeding Laboratories). The jump latency time (cut-off time: 240 sec) was measured 10 min after ICV drug administration (10-µl volume) using a Hamilton syringe according to the method of Haley and McCormick (19). Naloxone was injected subcutaneously 10 min before ICV administration of the drugs.

## Results

**Structural requirements for porcine brain DAP activity.** The three-dimensional structure of DAP being still unknown, the interaction of peptides and inhibitors with the enzyme is interpreted using the classical active site model previously used in the development of potent inhibitors of metallopeptidases such as angiotensin-converting enzyme (20) or enkephalinase (7, 8, 17). The nomenclature used for the characterization of the individual amino acids (P$_2$, P$_1$, P$_1'$, P$'_2$, etc.) of a substrate or an inhibitor and of the corresponding subsites (S$_2$, S$_1$, S$_1'$, S$_2'$, etc.) of the enzyme is that of Schechter and Berger (21). The peptides studied were assumed to interact with their N-terminal group located in the S$_2$ subsite because this arrangement would optimize the binding of both the ammonium group, with a putative negatively charged acceptor group, and the carboxyl or carbonyl groups, with the metal atom of the catalytic site.

The degradation rate and the number of cleavage sites of various enkephalin-related peptides are reported in Table 1. Leu-enkephalin and the tetrapeptide fragment Tyr-Gly-Gly-Phe were shown to be cleaved at the Gly$^2$-Gly$^3$ bond, with the same efficiency. Gly-Gly-Phe-Leu was also degraded by DAP, but its rate of hydrolysis was 10-fold slower than that of Leu-enkephalin. In contrast, the N-terminal and C-terminal fragments of Leu-enkephalin, Tyr-Gly-Gly, and Gly-Phe-Leu did not act as DAP substrates. This seems to indicate that a tetrapeptide fragment is the minimal enkephalin sequence for

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[1] P. Chérot, M. C. Fournié-Zaluski, and J. Laval, submitted for publication.

[2] P. Chérot, M. C. Fournié-Zaluski, and J. Laval, submitted for publication.

TABLE 1

**Rate of degradation of various peptide substrates by a porcine brain dipeptidylaminopeptidase**

The rate of the first cleavage and the identification of cleavage sites in enkephalins and enkephalin-related peptides were determined by HPLC analysis with a $C_{18}$ $\mu$-Bondapak 9-$\mu$m column. Cleavage sites are indicated by an arrow and the rates of the first cleavage are in pmol/min$^{-1}$ of formed dipeptide.

| $S_2$ $S_1$ $S_1'$ $S_2'$ | Number of cleavage sites | V |
|---|---|---|
| | | *pmol · min$^{-1}$* |
| Tyr–Gly–Gly | 0 | 0 |
| Tyr–Gly-↓Gly–Phe | 1 | 31 |
| Tyr–Gly-↓Gly–Phe-Leu | 1 | 32 |
| Tyr–Gly-↓Gly–Phe↓Leu-Arg | 2 | 3.1 |
| Tyr–Gly-↓Gly–Phe↓Met-Arg-Phe | 2 | 1.6 |
| Tyr–Gly–Gly–Phe-Met-Arg-Gly-Leu | 0 | 0 |
| Gly–Phe–Leu | 0 | 0 |
| Gly–Gly-↓Phe-Leu | 1 | 3.1 |
| Tyr–Phe-↓Phe | 1 | 1 |
| Ac-Tyr–Gly–Gly-Phe-Leu | 0 | 0 |
| Tyr–D·Ala-Gly-Phe-Met-$NH_2$ | 0 | 0 |

TABLE 2

**Inhibitory potency of various peptides on porcine brain dipeptidylaminopeptidases**

| $S_2$ $S_1$ $S_1'$ $S_2'$ | $K_i$[a] |
|---|---|
| | *μM* |
| Tyr–Gly–Gly–Phe–Leu | 2.5 ± 0.2 |
| Ac-Tyr–Gly–Gly–Phe–Leu | >1000 |
| Boc-Tyr–Gly–Gly–Phe–Leu | >1000 |
| Tyr–Gly–Gly–Phe–Met | 4.5 ± 0.2 |
| Tyr-D.Ala-Gly–Phe–Leu | >1000 |
| Gly–Gly | >1000 |
| Leu–Gly | >1000 |
| Tyr–Gly | 90.0 ± 5.0 |
| Tyr–Phe | 0.6 ± 0.05 |
| Tyr–Tyr | 2.0 ± 0.2 |
| Tyr–Glu | 20.0 ± 1.0 |
| Tyr–Lys | 70.0 ± 4.0 |
| Gly–Phe | >1000 |
| L·Ala–Phe | 40.0 ± 2.0 |
| D·Ala–Phe | >1000 |
| Tyr–Gly–$NH_2$ | >1000 |
| Tyr–Gly–Gly | >1000 |
| Tyr–Phe–Ala | 4.0 ± 0.5 |
| Tyr–Phe-Phe | 0.9 ± 0.1 |
| Tyr–Phe–Asp | 0.7 ± 0.01 |
| Tyr–Gly–Gly–Phe | 8.0 ± 0.5 |
| Tyr–Gly–Phe–Phe | 0.4 ± 0.05 |
| Tyr–Met-Gly–Trp | 0.7 ± 0.01 |

[a] $K_i$ values were determined using the Cheng-Prusoff transformation. The enzyme was preincubated with the inhibitor for 15 min, in Hepes-KOH, 50 mM, pH 7.0, 25°, and the activity of porcine brain DAP was determined with [$^3$H]Leu-enkephalin (10 nM) as substrate. The values are the means ± standard errors from triplicate experiments.

enzyme recognition. However, it is interesting to note that this rule does not apply to strongly hydrophobic tripeptides such as Tyr-Phe-Phe.

Purified DAP was also incubated with C-terminally extended enkephalin sequences corresponding to larger natural opioid peptides. In the case of Leu$^5$-enkephalyl-Arg$^6$ and Met$^5$-enkephalyl-Arg$^6$-Phe$^7$, two successive cleavages occurred at Gly$^2$-Gly$^3$ and Phe$^4$-Leu$^5$ (or Phe$^4$-Met$^5$), respectively. Interestingly, the octapeptide Met$^5$-enkephalyl-Arg$^6$-Gly$^7$-Leu$^8$ was not cleaved under the same conditions.

Furthermore, as expected for an exopeptidase such as DAP, neither an N-protected derivative of Leu-enkephalin (Ac-Tyr-Gly-Gly-Phe-Leu) nor an analog with a D-residue in position 2 were cleaved, indicating that a free ammonium group and a well defined stereochemistry of the residues interacting at the active site are essential for DAP activity. Inhibitory potency of some enkephalin analogs (Table 2) on DAP activity confirms that Met-E and Leu-E inhibit the enzymatic activity with an $IC_{50}$ in the $\mu$M range, whereas *N*-protected analogs (*N*-acetyl and *N*-Boc) as well as D-Ala$^2$-Leu-E show no inhibition.

These results led to the conclusion that the porcine brain DAP requires the presence of a positively charged amino acid as the $P_2$ component and contains a relatively large active site with $S_2$, $S_1$, $S_1'$, and $S_2'$ subsites interacting preferentially with hydrophobic side chains.

This preliminary investigation proved to be essential for the design of selective DAP inhibitors.

**Study of the active site of porcine brain DAP.** The determination of cleavage sites and hydrolysis rates for some enkephalin-related peptides gave an indication of the selectivity of porcine brain DAP. Therefore, only a limited series of peptides was used for a more accurate determination of the specificity of the $S_2'$-$S_2$ subsites. As shown in Table 2, an aromatic ring, namely Tyr, is preferred at the $S_2$ level. Interestingly, the same requirement seems to exist for $S_1$ since Tyr-Phe and Tyr-Tyr behave as the most potent dipeptide inhibitors.

The high affinity of Ala-Phe for DAP, contrasting with the lack of potency of Gly-Phe and D-Ala-Phe, suggests that the important ionic interaction involving the positively charged ammonium group is hindered by the glycine flexibility in Gly-Phe and by an unfavorable spatial orientation in D-Ala-Phe. The importance of the binding to the metal located in the catalytic site is clearly demonstrated by the lack of potency following amidation of the carboxylate group of Tyr-Gly. Despite the presence of an amide carbonyl group which theoretically should be in the correct orientation for interaction with the metal atom of DAP, the tripeptide Tyr-Gly-Gly is inactive. This is probably due to the flexibility of the two successive glycine residues coupled with their very weak hydrophobicity.

The $S_1'$ and $S_2'$ subsites of DAP do not exhibit a formal specificity, although hydrophobic moieties, especially of the aromatic type, appear to be preferred.

**Inhibitory potency of various bidentates on DAP and enkephalinase.** Owing to their ability to form bidentate complexes with the metal located in the metallopeptidase catalytic site, hydroxamic acid derivatives are among the most potent inhibitors of such enzymes (11). A series of dipeptide analogues has recently been designed (13) to inhibit the three metallopeptidases involved in the *in vitro* enkephalin degradation. Their inhibitory potency on DAP is reported in Table 3 and compared to their previously determined activities against enkephalinase (13).

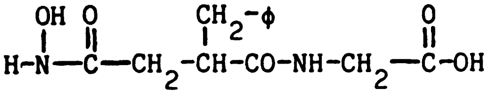
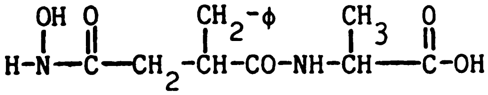
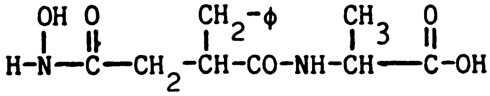
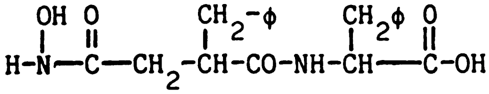
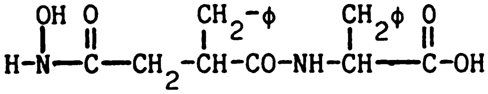
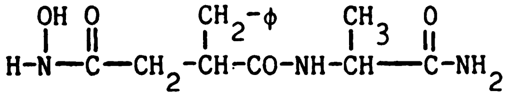
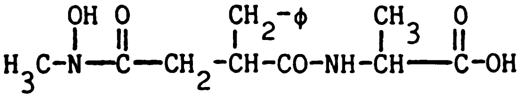
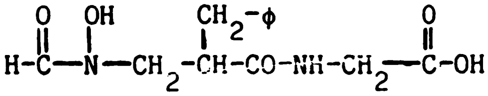
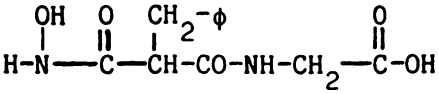
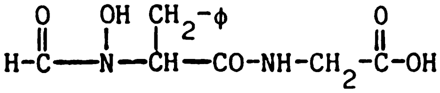
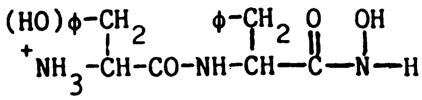
These compounds are characterized by a benzyl moiety in the $P_1'$ position, various aminoacids in the $P_2'$ position, and a hydroxamate or an *N*-acyl,*N*-hydroxy amino group (compounds **1**–**10**).

It can be observed that a continuous increase in the hydrophobicity of the $P_2'$ residue (compounds **1**, **2**, and **4**) led to a 200-fold enhancement in inhibitory potency.

A comparison of the $K_i$ values for compounds **1**, **8**, **9**, and **10** indicates that the most active derivatives (**1** and **8**) correspond to molecules containing a methylene spacer between the benzyl group and the metal-chelating agent.

TABLE 3

**Inhibitory potency of various bidentate-containing dipeptides on DAP and enkephalinase[a]**

| | | | $K_i$[a] | |
|---|---|---|---|---|
| | | | DAP | Enkephalinase |
| | | | nM | |
| 1 |  $\mathrm{H{-}\overset{OH}{N}{-}\overset{O}{C}{-}CH_2{-}\overset{CH_2{-}\phi}{C}H{-}CO{-}NH{-}CH_2{-}\overset{O}{C}{-}OH}$ | (*R*+*S*)[b] | 500 | 2.2 |
| 2 |  $\mathrm{H{-}\overset{OH}{N}{-}\overset{O}{C}{-}CH_2{-}\overset{CH_2{-}\phi}{C}H{-}CO{-}NH{-}\overset{CH_3}{C}H{-}\overset{O}{C}{-}OH}$ | (*R*,*S*)[c] | 9 | 2.5 |
| 3 |  $\mathrm{H{-}\overset{OH}{N}{-}\overset{O}{C}{-}CH_2{-}\overset{CH_2{-}\phi}{C}H{-}CO{-}NH{-}\overset{CH_3}{C}H{-}\overset{O}{C}{-}OH}$ | (*S*,*S*)[c] | 900 | 1.2 |
| 4 |  $\mathrm{H{-}\overset{OH}{N}{-}\overset{O}{C}{-}CH_2{-}\overset{CH_2{-}\phi}{C}H{-}CO{-}NH{-}\overset{CH_2\phi}{C}H{-}\overset{O}{C}{-}OH}$ | (*R*,*S*)[c] | 2.5 | 0.34 |
| 5 |  $\mathrm{H{-}\overset{OH}{N}{-}\overset{O}{C}{-}CH_2{-}\overset{CH_2{-}\phi}{C}H{-}CO{-}NH{-}\overset{CH_2\phi}{C}H{-}\overset{O}{C}{-}OH}$ | (*S*,*S*)[c] | 350 | 0.56 |
| 6 |  $\mathrm{H{-}\overset{OH}{N}{-}\overset{O}{C}{-}CH_2{-}\overset{CH_2{-}\phi}{C}H{-}CO{-}NH{-}\overset{CH_3}{C}H{-}\overset{O}{C}{-}NH_2}$ | (*R*,*S*+*S*,*S*)[d] | 3,500 | 36 |
| 7 |  $\mathrm{H_3C{-}\overset{OH}{N}{-}\overset{O}{C}{-}CH_2{-}\overset{CH_2{-}\phi}{C}H{-}CO{-}NH{-}\overset{CH_3}{C}H{-}\overset{O}{C}{-}OH}$ | (*R*,*S*+*S*,*S*)[d] | 5 × 10$^5$ | 70,000 |
| 8 |  $\mathrm{H{-}\overset{O}{C}{-}\overset{OH}{N}{-}CH_2{-}\overset{CH_2{-}\phi}{C}H{-}CO{-}NH{-}CH_2{-}\overset{O}{C}{-}OH}$ | (*R*+*S*)[b] | 6,000 | 15 |
| 9 |  $\mathrm{H{-}\overset{OH}{N}{-}\overset{O}{C}{-}\overset{CH_2{-}\phi}{C}H{-}CO{-}NH{-}CH_2{-}\overset{O}{C}{-}OH}$ | (*R*+*S*)[b] | >10$^6$ | 12 |
| 10 |  $\mathrm{H{-}\overset{O}{C}{-}\overset{OH}{N}{-}\overset{CH_2{-}\phi}{C}H{-}CO{-}NH{-}CH_2{-}\overset{O}{C}{-}OH}$ | (*R*+*S*)[b] | >10$^6$ | 1,500 |
| 11 |  $\mathrm{{}^{+}NH_3{-}\overset{(HO)\phi{-}CH_2}{C}H{-}CO{-}NH{-}\overset{\phi{-}CH_2}{C}H{-}\overset{O}{C}{-}\overset{OH}{N}{-}H}$ | | 9 | 28,000 |

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[a] The inhibitory potency of each compound was determined from $IC_{50}$ values using the Cheng-Prusoff transformation. Briefly, a given inhibitor was preincubated with DAP or enkephalinase for 15 min in 50 nM Krebs-KOH, pH 7.0, at 25°, and the reaction was initiated by addition of [$^3$H]Leu-enkephalin (10 nM) as substrate. The values are the means (standard error <15%) from triplicate experiments.

[b] These compounds were used as a racemic mixture of *R* and *S* isomers.

[c] These compounds are pure (*R*,*S*) or (*S*,*S*) isomers.

[d] These compounds are a diastereoisomeric mixture of (*R*,*S*) and (*S*,*S*) isomers.

However, the structure of the bidentate ligand plays an important role, since the replacement of the *N*-hydroxyamino carbonyl group (in compound **1**) by the *N*-formyl-*N*-hydroxyamino group (compound **8**) leads to a 12-fold decrease in inhibitory potency. Moreover, a large decrease in the enzyme recognition is induced by methylation of the bidentate group, since the $K_i$ ratio between **7** (*RS* + *SS*) and a mixture of **2** and **3** can be estimated to be higher than 25,000. Finally, the amidification of the C-terminal carboxylate group in compound **6** leads to a significant loss of activity. Interestingly, all these general requirements for optimal DAP recognition were also valid for enkephalinase (13, 17), indicating the presence of important analogies in the active sites of both enzymes.

However, an important difference between DAP and enkephalinase is related to the influence of the absolute configuration of the $P_1'$ moiety on the inhibition of both enzymes. Indeed, an *R* configuration of the $P_1'$ residue corresponding to the stereochemistry of a natural amino acid is crucial for DAP recognition, as shown by the increased $K_i$ values (by 2 orders of magnitude) of compounds **3** and **5**, as compared to com-

TABLE 4

**Analgesic effects of peptidase inhibitors on mice determined by the hot-plate test**

The jump latency time was measured 10 min after intracerebroventricular administration to mice of 10 $\mu l$ of inhibitor or a mixture of them at the dose indicated. The results are the means ± standard error of 10 experiments.

| Inhibitors | Hot-plate test:<br>Jump latency time |
|---|---|
| | *sec* |
| Saline | 59 ± 6 |
| Thiorphan (50 $\mu g$) (TH) | 108 ± $8^{a,b}$ |
| Bestatin (50 $\mu g$) (BE) | 83 ± 8,$^{a}$ NS$^{c}$ |
| Tyr-Phe-NHOH (60 $\mu g$) (YFNHOH) | 82 ± 12,$^{a}$ NS |
| TH (50 $\mu g$) + BE (50 $\mu g$) | 169 ± $13^{a,d}$ |
| TH (50 $\mu g$) + BE (50 $\mu g$) + YFNHOH (60 $\mu g$) | 188 ± 10,$^{e}$ NS |
| TH (50 $\mu g$) + BE (50 $\mu g$) + YFNHOH (60 $\mu g$) + naloxone (2.5 mg/kg) | 49 ± 8 |

[a] Compared to saline.
[b] $p < 0.05$ (Student's *t* test).
[c] NS, not significant.
[d] $p < 0.001$ (Student's *t* test).
[e] Compared to bestatin + thiorphan.

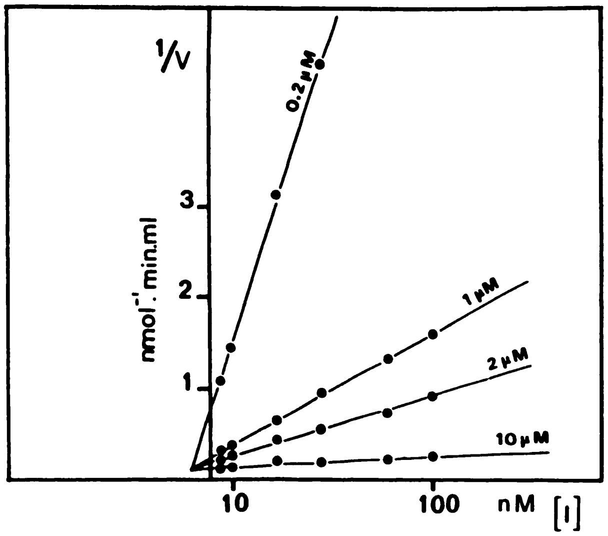

**Fig. 1.** Dixon plots of the inhibition of a porcine brain DAP by compound **11** using a mixture of [$^3$H]Leu-enkephalin and Leu-enkephalin at the indicated concentrations. Each *point* represents the mean of triplicate experiments.

pounds **2** and **4**. In contrast, enkephalinase is similarly inhibited by compounds containing either an "*R*" or "*S*" asymmetric center in the $P_1'$ position (22). These results suggest that the most efficient way to obtain selective inhibitors of DAP would be to design compounds interacting with the $S_2$,$S_1$ subsites. For this purpose, a hydroxamate group was introduced on the C-terminus of the potent, hydrophobic dipeptide Tyr-Phe. As shown in Fig. 1, this new compound **11** inhibited DAP with a $K_i$ value of 9 nM; Dixon plots showed the competitive character of this inhibition. As reported in Table 3, only compounds **2, 4** and **11** inhibit DAP with $K_i$ values in the nM range. The activities of these two latter derivatives on the two other enzymes involved in enkephalin metabolism were also tested to determine their selectivity.

Compound **4** inhibits enkephalinase, purified from rabbit kidney and the membrane-bound AP M, with $K_i$ values of 3.4 × $10^{-10}$ M and 7.2 × $10^{-7}$ M, respectively. Compound **11** has a $K_i$ value of 2.8 × $10^{-5}$ M for enkephalinase, 5 × $10^{-4}$ M for angiotensin-converting enzyme, and up to $10^{-3}$ M for AP M. Tyr-Phe-NHOH behaves, therefore, as the most selective inhibitor of DAP and may be used to investigate the importance of this enzyme in the physiological degradation of enkephalins.

The same kinds of structure-activity relationship, including the stereochemical requirements, was found with the DAP purified from rat brain (13). However, the various bidentates were slightly more potent on the rat peptidase (11), whereas Tyr-Phe-NHOH was 8-fold less active ($K_i = 6 \times 10^{-8}$ M).

In the absence of a specific radioimmunoassay for Tyr-Gly, the *in vivo* efficiency of Tyr-Phe-NHOH to protect the endogenous enkephalins from DAP activity cannot be evaluated. However, as already shown for kelatorphan (14), Tyr-Phe-NHOH completely inhibits the formation of [$^3$H]Tyr-Gly from [$^3$H]Leu-enkephalin incubated with rat striatal slices (data not shown), illustrating the efficiency of this inhibitor in conditions where the integrity of nerve terminals and peptides is preserved.

**Comparison of the analgesic properties of specific inhibitors of enkephalinase, and DAP.** The analgesic effect induced by potent inhibitors of the various enkephalin-degrading enzymes is a simple and rapid test to investigate the physiological implication of these peptidases in enkephalin metabolism. Accordingly, the specific DAP inhibitor Tyr-Phe-NHOH was studied on the mouse hot-plate test and compared with thiorphan (enkephalinase inhibitor) and bestatin (non-specific AP inhibitor) and their association.

The intracerebroventricular (ICV), administration in mice of 10 $\mu g$ (0.03 $\mu$mol), 30 $\mu g$ (0.09 $\mu$mol), and 60 $\mu g$ (0.17 $\mu$mol) of Tyr-Phe-NHOH alone induced at the highest dose studied (0.17 $\mu$mole) some increase in the jump latency time, but this effect was too small to reach the critical level of significance. Under the same conditions, bestatin (50 $\mu g$, 0.16 $\mu$mol) was also found to be inactive, whereas thiorphan (50 $\mu g$, 0.20 $\mu$mol) produced a statistically significant antinociceptive response. Due to its borderline analgesic effect, bestatin has been reported to be alternately inactive (4) or active (5) in the hot-plate test. By contrast, the well established ability of bestatin to potentiate thiorphan-induced analgesia (23, 24) was confirmed in the present study. The association of increased doses (up to 75 $\mu g$/mouse) of Tyr-Phe-NHOH with a single dose of thiorphan (25 $\mu g$) or bestatin (50 $\mu g$) dose not significantly potentiate the effect of these inhibitors (Fig. 2, A and B). Furthermore, Tyr-Phe-NHOH does not statistically potentiate the analgesia induced by the association of thiorphan and bestatin. In all these experiments the antinoceptive effects induced by the various inhibitors were antagonized by prior administration of naloxone, accounting for a stimulation of opioid receptors by the endogenous enkephalins protected from degrading enzymes.

## Discussion

As previously reported for monkey brain (25) and calf brain DAP (26), the DAP purified from porcine brain and cleaving the Gly$^2$-Gly$^3$ bond of enkephalins is a metallopeptidase.[3] The presence of this DAP activity in synaptosomal fractions suggested its possible involvement in the *in vivo* termination of enkephalinergic activity and hypothesis which could be tested using a highly specific inhibitor of the enzyme. As shown by cristallographic studies on carboxypeptidase A (27) or thermolysin (28), the specificity of metallopeptidases is ensured by

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[3] P. Chérot, M. C. Fournié-Zaluski, and J. Laval, submitted for publication.

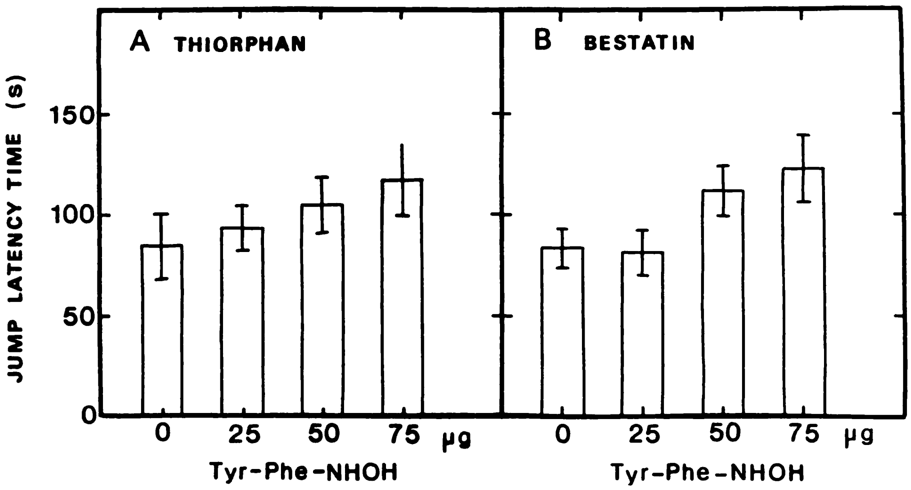

**Fig. 2.** The jump latency time was measured 10 min after ICV administration to mice of 10 μl of a mixture of inhibitors: A, 25 μg (0.10 μmol) of thiorphan and increasing doses of Tyr-Phe-NHOH [25 μg (0.075 μmol), 50 μg (0.15 μmol), 75 μg (0.22 μmol)] and B, 50 μg (0.16 μmol) of bestatin and increasing doses of Tyr-Phe-NHOH [25 μg (0.075 μmol), 50 μg (0.15 μmol), 75 μg (0.22 μmol)].

specific interactions between side chains of substrates or inhibitors and well defined subsites of these proteases.

For the DAP, the subsite specificity was determined using various peptides as substrates or inhibitors. The results indicated that a tetrapeptide, or at least a highly hydrophobic tripeptide, is the minimal sequence to be cleaved, and that the DAP active site probably contains four successive hydrophobic subsites $S_2$, $S_1$, $S_1'$, and $S_2'$. The same type of subsite selectivity was shown to occur in the partially purified membrane-bound (25) and cytosolic (26) DAPs from rat brain, indicating that these peptidases and the porcine brain DAP are probably closely related. Furthermore, the very low potency of *N*-protected enkephalins as substrates or inhibitors shows that an ionic interaction between a negatively charged group of the enzyme and the ammonium group of the interacting molecule is crucially involved in DAP recognition. In Fig. 3, a schematic representation of the putative DAP active site has been proposed. This model is expected to facilitate the design of highly potent and selective inhibitors. Such compounds should be obtained by introduction of a metal-chelating group on structures able to recognize the four hydrophobic subsites surrounding the catalytic site. However, as already shown in the case of angiotensin-converting enzyme (20) and enkephalinase (17), highly potent inhibitors may be obtained with compounds interacting with only a part of the active site of the metallopeptidase.

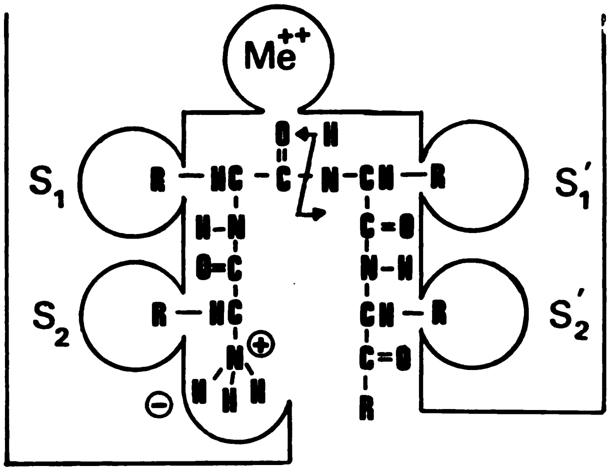

**Fig. 3.** Schematic active site model of a porcine brain DAP. The model represents the putative interaction of the enzyme with a potential substrate.

Accordingly, two types of DAP inhibitors were designed: 1) dipeptide-derived structures interacting with $S_1'$-$S_2'$ subsites and bearing the metal-chelating group at the N-terminus, and 2) dipeptide-derived structures interacting with $S_1$-$S_2$ subsites and bearing the coordinating agent at the C-terminus.

A large number of derivatives corresponding to the first series with a hydroxamate or an *N*-hydroxy-*N*-acyl group were tested on the purified DAP. As expected from the active site investigation, the best DAP inhibitor is the bidentate **4** which contains two aromatic side chains in $P_1'$ and $P_2'$ positions and a methylene spacer between the hydroxamate group and the $P_1'$ residue. However, this compound also behaves as the best enkephalinase inhibitor in the series. This result suggests an important analogy between the $S_1'$ and $S_2'$ subsites of both enzymes.

Therefore, the dipeptide analogue L-Tyr-L-Phe-NHOH, from the second series of putative inhibitors, was synthesized taking into account that enkephalinase (endopeptidase) and DAP (exopeptidase) belong to different classes of metallopeptidases. In Tyr-Phe-NHOH, the strong affinity and high selectivity for DAP is due to the simultaneous recognition of the enzyme anionic component by the ammonium group, the interaction of the two aromatic side chains with the hydrophobic $S_1$ and $S_2$ subsites, and the coordination of the metal atom by the hydroxamate group.

These three types of interactions cannot be satisfied simultaneously with enkephalinase even if the inhibitor was bound to the enzyme in a reverse manner, i.e., with the L-Phe and L-Tyr side chains in the $S_1'$ and $S_2'$ subsites, respectively. This unusual binding mode, allowing the interaction of the hydroxamate group with the metal, should lead to a relatively potent inhibitor since the recognition of the $S_2'$-$S_2'$ region in enkephalinase is not significantly affected either by the stereochemistry of the $P_2'$ and $P_2'$ components or the retro-inversion of the amide bond (8, 22). However, such a complex is probably disallowed by a strong adverse interaction which should occur between the ammonium group of the inhibitor and the guanidinium function of an Arg residue present in the enkephalinase active site (29).

Owing to the potency and relatively high selectivity of Tyr-Phe-NHOH for both the porcine brain DAP and the rat brain membrane-bound DAP, it was of great interest to test its analgesic properties.

The most useful method to evaluate the analgesic effect of enkephalin-degrading enzyme inhibitors is the measurement of the jump latency time of mice subjected to the hot-plate nociceptive stimuli (7, 13). It is generally agreed that the rat and mouse are very closely related species allowing the unambiguous comparison of the analgesic efficiency of a compound in mice with its inhibitory potency against rat peptidases.

The absence of any significant effect of Tyr-Phe-NHOH alone, or at various concentrations in combination with thiorphan and/or bestatin, suggests that the DAP is not crucially involved in *in vivo* enkephalin metabolism, at least in the brain. This is in accordance with the lack of enhancement of the metabolite [$^3$H]Tyr-Gly, after inhibition by the association of thiorphan and bestatin of [$^3$H]Leu-enkephalin degradation by rat striatal slices (14). It is very unlikely that the lack of analgesic effect of Tyr-Phe-NHOH is due to unfavorable pharmacokinetic properties. Indeed, the structure and the solubility in water or in lipophilic solvents of this inhibitor and very similar to those of the bidentates **2** and **4** which elicit strong antinociceptive effects after i.c.v. administration in mice (13).

The porcine brain DAP that we used to measure the inhibitory potency of synthetic compounds has been purified starting from a membrane extract, and this enzyme co-sediments with synaptosomes. In rat brain, a DAP with comparable biochemical properties is associated with membranes possessing opioid receptors at their surface. It has been reported elsewhere that the majority of the enkephalin-degrading DAP activity was present in the neuronal rather than in glial cultures (30), but about 90% of this activity could correspond to a cytosolic DAP. The physiological role of this or these enzymes remains to be determined.

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**Send reprint requests to:** Dr. B. P. Roques, Département de Chimie Organique, UA 498 CNRS-U 266 INSERM, UER des Sciences Pharmaceutiques et Biologiques, 4 avenue de l'Observatoire, 75006 Paris, France.