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DISAPPEARANCE OF ENERGY-TRANSFER AS A TOOL FOR FLUORIMETRIC STUDY OF THE DEGRADATION OF ENKEPHALINS BY AMINOPEPTIDASE ACTIVITY FROM MOUSE BRAIN

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SUMMARY :
The breakdown of the Tyr-Gly bond of enkephalins by aminopeptidases from mouse striatum can be studied by following the disappearance of energy transfer between the tyrosine residue and the dimethylaminonaphtalene sulfonyl group (DNS) in the biologicaly active fluorescent enkephalin, Tyr-Gly-Gly-Phe-Met-NH-$(CH_2)_2$-NH-DNS (Met-E-C_2-DNS). Such a process can be followed in a continuous mode yielding a simple method for the study of the effects of pH, temperature, etc... Furthermore, the enzymatic degradation of unsubstituted Met-E leads to several products, whereas Met-E-C_2-DNS gives only Gly-Gly-Phe-Met-NH-$(CH_2)_2$-DNS as the degradation product and behaves thus as a selective substrate in the study of aminopeptidases. This fluorimetric method should prove useful for studying the degradation of other neuropeptides or hormones containing one tyrosine or tryptophane residue in their sequence.

Fluorescent analogues of peptides are useful tools in biochemistry, especially for conformational studies, owing to the possibility of energy transfer between two different chromophores closely located into the same molecule (1). The cleavage of any of the chemical bonds in the chain linking the two chromophores will induce the disappearance of the enrgy transfer. We intended to make use of these properties for enzymatic studies and we checked the feasibility of the method by following the enzymatic degradation of Met-enkephalin (Met-E).

Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) are endogenous brain peptides which exhibit opiate-like activity (2). Their activity remains purely transient, even after intracerebral administration. This is due to their rapid inactivation by the enzymes present in the plasma and the brain. Several authors have observed that the degradation of enkephalins begins by a very fast hydrolysis of the Tyr-Gly peptide bond by aminopeptidases (3-9). For a better understanding of the regulation of enkephalin activity it is important to study such degradating enzymes.

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For this purpose, three methods have so far been used : thin layer chromatography (3, 5, 8, 9), high pressure liquid chromatography (6, 10) and adsorption to Porapack Q beads (7), but these techniques usually require radioactive compounds and do not allow a continuous monitoring of the enzymatic degradation.

We propose here a new method for the kinetic study of these enzymes, based on the continuous disappearance of the energy transfer between the tyrosine residue and the dimethylaminonaphtalene sulfonyl group (DNS) in Tyr-Gly-Gly-Phe-Met-NH-$(CH_2)_2$-NH-DNS (Met-E-C_2-DNS). As reported previously (11), this fluorescent analogue of enkephalin retains the biological properties of the parent compound, *in vivo* and *in vitro*, and is consequently a particularly suitable substrate.

MATERIAL AND METHOD.

Chemicals.

Met-E was purchased from Bachem (Bubendorf, Switzerland). Tyr-Gly-Gly-Phe-Met-NH-$(CH_2)_2$-NH-DNS, I, and the enzyme resistant analogue Tyr-D-Ala-Gly-Phe-Met-NH-$(CH_2)_2$-NH-DNS, II, both exhibiting opiate-like activity, have been synthesized according to FOURNIE-ZALUSKI et al. (11). A straight-forward modification of this method was used to synthesize Gly-Gly-Phe-Met-NH-$(CH_2)_2$-NH-DNS, III, by coupling the Boc-Gly-Gly-Phe-Met with NH_2-$(CH_2)_2$-NH-DNS using dicyclohexylcarbodiimide and hydroxybenzotriazole as reagents (12) followed by a deprotective step with trifluoroacetic acid. Rf_{III} = 0.47 in BuOH:AcOH:H_2O (4:1:1).

All the fluorescent analogues are used as trifluoroacetate derivatives and kept in a lyophylised form at 5°C protected from light. Stock solutions of the fluorescent probes were prepared by a preliminary dissolution of the peptide in ethanol, followed by a dilution with Tris-buffer 0.05 M, pH 7.4 to a final proportion of 1 % ethanol (V/V). Peptide concentrations were determined from their U.V. absorbance. Met-E : ε_{211} = 16,950, ε_{273} = 1,500 ; Met-E-C_2-DNS : ε_{245} = 14,700, ε_{327} = 4,350.

Bacitracine purchased from Sigma was diluted with Tris-buffer 0.05 M prepared from Trizma free base and Trizma HCl (Sigma). The pH was adjusted to 7.4 at the desired temperature as indicated in the Sigma tables.

Enzyme preparations.

The striata of 3 male Swiss mice (18-20g) were homogenized into 10 ml of 0.05 M Tris-HCl buffer pH 7.4 (3 x 5 sec at 1,500 rpm in a glass-teflon potter homogeniser, clearance 0.1, 0.15 mm). A pellet was obtained after centrifugation at 4°C (1,000 g x min) ; the supernatant was centrifuged (200,000 g x min) and the supernatant of the second centrifugation (S_2) was used for enzymatic studies. Quantitative determination of proteins was performed by the Lowry method (13). The final concentrations of proteins in S_2 fractions were about 40 µg/100 µl.

Thin Layer Chromatography (TLC).

In order to control the degradation of Met-E, I and II, the enzymatic reactions were chromatographed on Kieselgel 60F254 plastic sheets (Merck) using BuOH:AcOH:H_2O (4:1:1) as solvent. The spots were visualized with long-wave U.V. light (dansylated compounds) and ninhydrine or fluorescamine spray, according th VOGEL et al. (7) and the R_f was compared to those of the reference compounds.

Fluorescence measurements.

Fluorimetric measurements were performed on a Perkin-Elmer MPF 44 A spectrofluorometer fitted with a 150 W Xenon lamp and equipped with a thermostated cell-holder and a differential corrected Perkin-Elmer spectra unit, model 445. The transfer efficiencies were calculated according to SCHILLER (14).

Fluorimetric determination of the enzymatic degradation of Met-E-C_2-DNS.

General procedure : a freshly prepared stock solution of I was diluted to 2.9 ml with 0.05 M Tris-buffer pH 7.4, in order to obtain serial concentrations of I ranging from 10^{-6} to 5 x 10^{-5}M. After equilibration at the desired temperature, 0.1 ml of a freshly obtained enzyme preparation was added to it. The two solutions were mixed up immediately. The Tyr fluorescence intensity was recorded, for at least three different concentrations of I, as a function of time (λ_{ex} : 277 nm and λ_{em} : 305 nm) until a plateau was observed in the experimental curve. At this stage, the complete degradation of I was checked by TLC. It is thus possible to relate the increase of Tyr fluorescence intensity with the amount of hydrolysed substrate. Consequently, to determine the initial rate of the reaction for different concentrations of substrate only the linear part of the degradation curve was necessary.

RESULTS AND DISCUSSION.

Fluorescent properties of Met-E-C_2-DNS.

The energy transfer from tyrosine (donor) to dansyl (acceptor) is evidenced by a comparison between the corrected fluorescence emission spectra of I and III recorded at the excitation wavelength of tyrosine (270 nm) (figure 1). It can be observed that direct excitation of the dansyl group also occurs at 270 nm as clearly shown by the strong residual emission in Gly-Gly-Phe-Met-NH-$(CH_2)_2$-NH-DNS, III (figure 2).

The transfer efficiency can be calculated either from the donor fluorescence quantum yield or from the increase of the acceptor fluorescence (14). However, in the case of I, we observe that the transfer efficiency measured from the donor quantum yield (0.87) (figure 1) is about four times higher than that of the acceptor (0.26) (figure 2). This results from the occurrence of an additional quenching of the donor fluorescence due to the presence of the acceptor (14).

It follows, that the splitting of tyrosine from the rest of the molecule in Met-E-C_2-DNS leads to an increase of the tyrosine fluorescence quantum yield from 0.0038 in Met-E-C_2-DNS to 0.14 in free tyrosine. This increase is much higher than the corresponding decrease of the dansyl emission (which results only from the disappearance of energy transfer) (15). Furthermore, the decrease in the fluorescence emission of dansyl at 560 nm cannot be detected with I in the presence of the enzyme preparation at 37°. This is due to the enhancement of the intrinsic dansyl fluorescence which is greater in III than in I, at 37°C. These features might result from different conformational states of peptides I and III at this temperature. As already shown by NMR studies (16,17) modifications in the N or C-terminal part of enkephalins lead to different preferential conformations in solution.

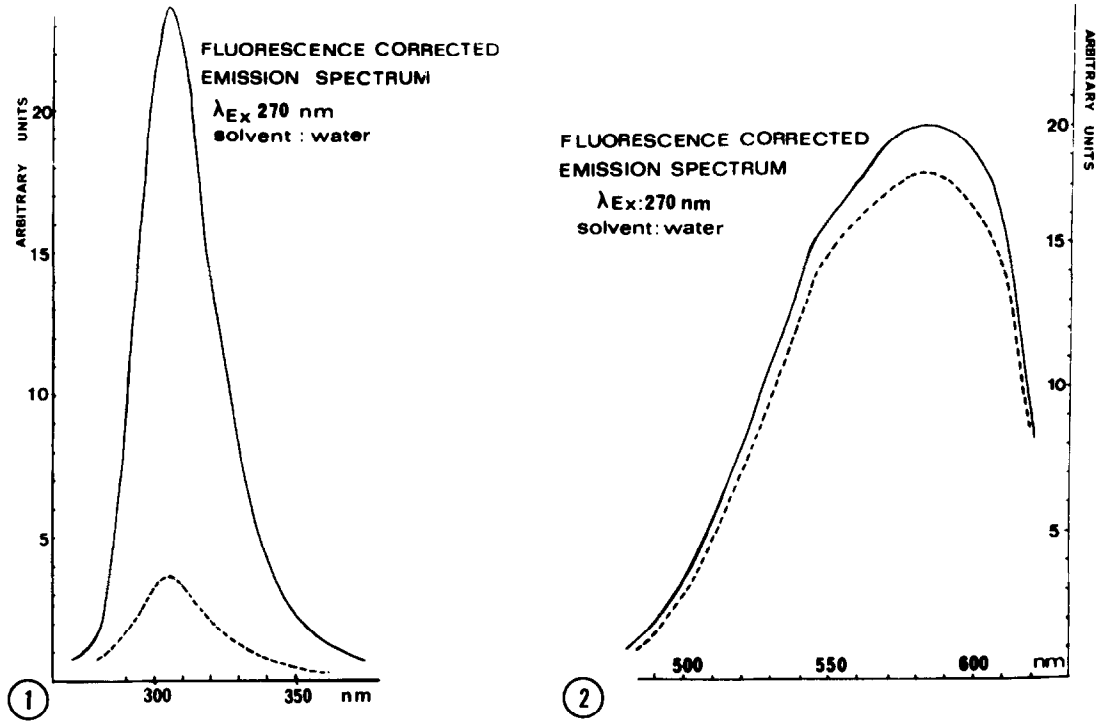

Figure 1. Comparison between the corrected emission intensity of Tyr upon excitation at 270 nm for Met-E (—) and Met-E-C_2-DNS (--) at the same concentration (4 x 10^{-5} M) in 0.05 M Tris-HCl buffer, pH 7.4.

Figure 2. Comparison between the corrected emission intensity of DNS upon excitation at 270 nm for Met-E-C_2-DNS (—) and Gly-Gly-Phe-Met-NH-$(CH_2)_2$-NH-DNS (--) at the same concentration (4 x 10^{-5}M) in 0.05 M Tris-HCl buffer, pH 7.4.

Enzymatic studies.

Accordingly, the increase in tyrosine fluorescence has been recorded at 305 nm with excitation at 277 nm (figures 3 A-B). The time course of this process recorded in a continuous mode is shown in figure 3 A. At the plateau the completion of the reaction has been controlled by TLC : only one spot corresponding to Gly-Gly-Phe-Met-NH-$(CH_2)_2$-NH-DNS, III, could be visualized. Consequently, the increase of Tyr fluorescence can be related to the amount of degraded Met-E-C_2-DNS ; this permits the evaluation of the initial rate of the reaction.

The relation between the increase of Tyr fluorescence and the enzymatic degradation is clearly confirmed by the lack of fluorescence change when using a fluorescent analogue II of D-Ala_2-Met-E, a peptide known for its resistance to the enzymatic degradation (4). The rate of hydrolysis is proportional to the concentration of the enzyme and depends on the concentration of the substrate ; as shown in table I, the enzymatic reaction is saturable. These pro-

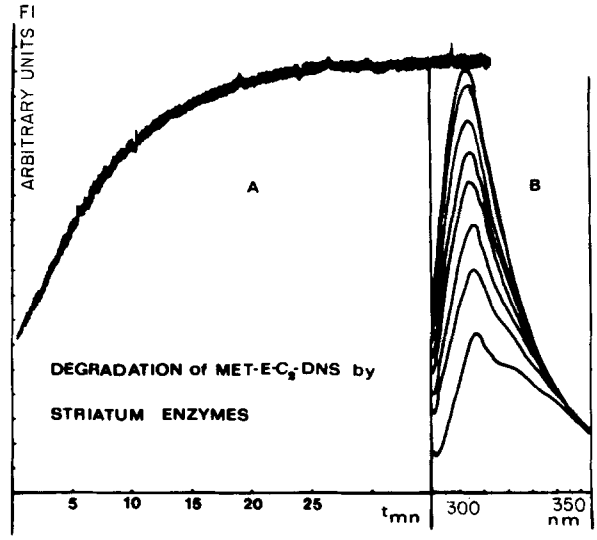

Figure 3. Degradation of Met-E-C_2-DNS, I, by striatal enzymes. The sample cell equilibrated at 37°C contained 2.9 ml of a solution of I, (1.7 x 10^{-5}M) in Tris-HCl buffer 0.05 M, pH 7.4. At time t=0, 0.1 ml of a solution of the enzymatic preparation is added and the reaction is recorded : A) by continuous recording of the Tyr fluorescence increase (ratio mode) λex = 277 nm, λem =305 nm B) by recording the emission spectra of Tyr at different times.

perties are consistent with a specific enzymatic hydrolysis and the corresponding kinetic parameters have been evaluated as follows : K_m = 6.5 x 10^{-6}M and V_{max} = 2.9 µmole/mg Prot/h (averages of 8 and 3 determinations for K_m and V_{max}, respectively).

K_m values reported in the literature for Met-E = 2 x 10^{-5}M (7), 5.5 x 10^{-5}M (18), 8 x 10^{-5}M (5,6) are higher than for III. These discrepancies can easily be explained by the different experimental conditions used in each study (temperature, Leu-E instead of Met-E as substrate, different sources of enzymatic preparations etc...). Moreover, Met-E-C_2-DNS, I, is a substrate differing slightly from Met-E and its increased affinity might be attributed to additional hydrophobic interactions with the enzyme.

In addition, it must be underlined that the enzymatic degradation of unsubs tituted Met-E leads to several products including Tyr and Gly-Gly-Phe-Met (3-9) as well as Tyr-Gly-Gly (18) whereas I gives only Gly-Gly-Phe-Met-NH-$(CH_2)_2$-NH-

Table 1. *Effect of Met-E-C_2-DNS concentration on the rate of degradation (V).*

Met-E-C_2-DNS conc. (10^{-6}M)	20	10	8	6	4
V (nM/mn)	1.89	1.84	1.73	1.14	0.85

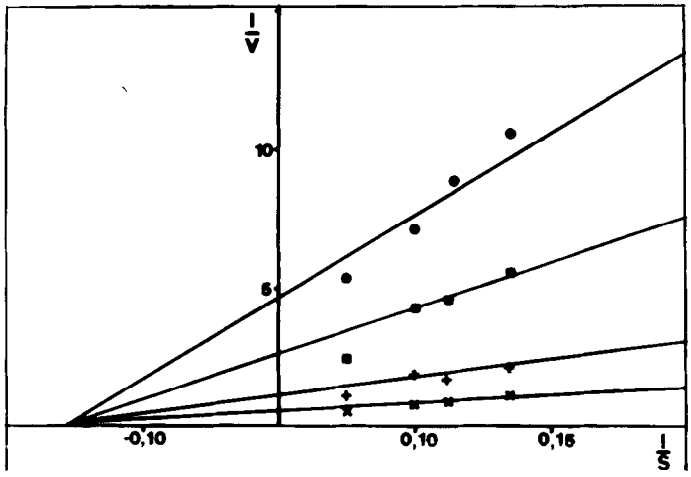

Figure 4. Lineweaver-Burk plots of the enzymatic degradation of Met-E-C_2-DNS, I, by striatal enzymes and its inhibition by bacitracine at the concentration indicated. The experiments were performed in a way similar to that described in the experimental section, with a preincubation (10 mn) of the bacitracine and Met-E-C_2-DNS before the addition of 100 µl of enzymatic preparation. Slopes of the lines were determined by a least squares method. × no bacitracine ; + 10^{-6}M bacitracine ; ✱ 5 x 10^{-6}M bacitracine ; ● 10^{-5}M bacitracine.

DNS, III, as degradation product. Hence it appears that the fluorescent enkephalin used in this study is a selective sustrate for aminopeptidases ; this property should be related to the inhibition of endopeptidases and carboxypeptidases resulting to the steric effect of the N-terminal dansylated group.

As an example of the possible application of the method, we have studied the enzymatic inhibition by bacitracine, a compound previously shown to inhibit the breakdown of a variety of peptides (19) including enkephalins (20). As shown in figure 4, the inhibition is not competitive, K_i = 1.0 x 10^{-6}M.

CONCLUSION

It is currently admitted that neuropeptides represent a new class of neurotransmitters or neuromodulators in the nervous system (21,22). Such a role involves their eventual formation by break down of polypeptidic precursors and their inactivation by degradation enzymes (18). These processes have been widely studied in the case of hormones (21) and, more recently, of the endogenous opioid peptides Met-E and Leu-E, using both radiolabeled compounds and chromatographic techniques (3-9,18).

As a great number of hormones and neuropeptides (23) contains only one tyrosine or tryptophane residue in their sequence, the introduction in such peptides of a fluorescent acceptor like a DNS group will lead to a substrate in which the degradation can be followed by energy transfer experiments, provided that the break down occurs between the two chromophores. Although the sen-

sitivity of this method is significantly lower than techniques using radioactive compountd, its advantages, as illustrated by the degradation of a biologically active fluorescent enkephalin, lie in the use of non-radioactive compounds and continous recordings of the enzymatic degradation. It is therefore possible to study easily and accurately the effects of ions, pH, temperature, inhibitors etc... Moreover in the case of enkephalins, the introduction of a dansyl group leads to a more specific substrate for aminopeptidases than Met-E itself, allowing accurate studies of these enzymes in biological fluids.

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