Vol. 143, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 27, 1987 Pages 58-66

NEW SUBSTRATES FOR ENKEPHALINASE (NEUTRAL ENDOPEPTIDASE) BASED ON FLUORESCENCE ENERGY TRANSFER

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Received January 8, 1987

Novel fluorescent substrates for enkephalinase (neutral endopeptidase; EC 3.4.24.11) have been developed. These new assays are based on the disappearance of energy transfer between a tryptophan or a tyrosine residue and the 5-dimethylaminonaphthalene-1-sulfonyl group (dansyl) in the substrates dansyl-Gly-Trp-Gly or dansyl-Gly-Tyr-Gly upon hydrolysis of their Gly-Trp or Gly-Tyr amide bond by enkephalinase. No significant difference in Km or kcat values were found for dansyl-Gly-Trp-Gly and dansyl-Gly-Tyr-Gly, indicating that, in contrast to thermolysin, the active site of enkephalinase easily accommodates tryptophan residues. Both tryptophan and tyrosine-containing substrates can be used for continuous recording of enkephalinase activity and should prove useful for detailed study of the substrate specificity of this enzyme.

The study of the hydrolysis of enkephalins in brain provided evidence that the cleavage of their Gly^3-Phe^4 amide bond is a major pathway for their inactivation *in vivo* (1-5). The enzyme responsible for this hydrolysis, enkephalinase, is a membrane-bound metallopeptidase. Its distribution in brain closely parallels that of the enkephalins (6). It is also widely distributed in many peripheral organs (7), most notably in the kidney where its activity is greatest. It was soon realized (8,9) that enkephalinase is identical to a known peptidase, neutral endopeptidase (EC 3.4.24.11), identified and purified from the kidney by Kerr and Kenny (10) using the B chain of insulin as substrate.

Although capable of hydrolyzing many biological peptides *in vitro* (11-13), *in vivo* enkephalinase has as yet only been implicated in the hydrolysis of endogenous enkephalins when released in brain (3,5,14,15). However, its widespread distribution would suggest that, at least in the periphery, the enzyme might have many other endogenous substrates.

The substrate specificity of enkephalinase has been studied using the enzyme purified from rat and human kidney (16-18). These studies showed

0006-291X/87 $1.50

that enkephalinase preferentially hydrolyses peptide bonds comprising the amino group of a hydophobic residue, shows a marked preference for short peptides, and is most efficient when it acts as a dipeptidyl carboxypeptidase, thus releasing a carboxy-terminal dipeptide (9,19).

Enkephalinase activity has been measured using many different substrates, including tritiated enkephalins or analogs (1,3,6) and fluorescent substrates in two-step assays (18,20-22). Florentin et al (23) recently designed a novel fluorescent substrate that allows continuous recording of enkephalinase activity. This assay is based on the occurrence of intramolecular quenching of dansyl fluorescence by a nitrophenyl group. In this paper, we describe novel fluorescent enkephalinase substrates based on energy transfer that also allow continuous recording of the enzyme activity.

MATERIALS AND METHODS

Purification of rat kidney enkephalinase: Enkephalinase was purified from rat kidneys as previously described (16). The specific activity of the purified enzyme was 27 nmoles/mg protein/min when assayed using 20 nM ^{3}H-(DAla^2, Leu^5) enkephalin as the substrate at 25° C, in 50 mM, pH 7.4 HEPES buffer containing 0.1% Tween 20 and proteins measured by the method of Bradford (24) using bovine serum albumin as a standard.

Synthesis of substrates: The peptides were synthesized via solid phase methodology (25). The dansyl group was introduced with dansyl chloride before the cleavage of the peptide from the resin support. After removal of the peptides from the support, purification was accomplished via preparative HPLC. Peptides were characterized by amino acid analysis and NMR.

Fluorometric determination of the hydrolysis of dansylated peptides: Fluorescence measurements were made using a Perkin Elmer, model 650-10S spectrofluorometer, equipped with a temperature controlled cell-holder, maintained at 37° C. Two procedures were used: a) a 50 ul solution of dansylated peptide was added to 50 ul of purifed rat kidney enkephalinase in 50 mM, pH 7.4 HEPES buffer, containing 0.1% Tween 20, yielding a substrate concentration from 1 uM to 1 mM. After 1 h at 37° C, 500 ul of 0.1M EDTA were added to the tubes. The 600 ul mixture was transfered into a quartz cuvette, and fluorescence measured. For dansyl-Gly-Trp-Gly, the excitation wavelength was set at 280 nm and emission at 360 nm. For the two tyrosine containing peptides, the excitation wavelength was at 277 nm and emission at 315 nm. Both slits were set at 2 nm. Known amounts of Trp-Gly and Tyr-Gly, diluted in the same mixture (100 ul HEPES, Tween 20, 500 ul EDTA), were run in parallel as standards. The fluorescence in the incubation media could thus be related to the amounts of hydrolyzed substrates. b) A second procedure allowed continuous monitoring of the hydrolysis of the dansylated peptides. A 500 ul solution of substrate at appropriate concentration, in 50 mM, pH 7.4 HEPES buffer containing 0.1% Tween 20 was pipetted in a quartz cuvette, and allowed to equilibrate in the cell holder of the fluorimeter at 37° C. Ten ul of purified rat kidney enkephalinase was added and the increase in tryptophan or tyrosine fluorescence was continuously monitored (excitation = 280 nm, emission = 360 nm for tryptophan, excitation 277, emission 315 for tyrosine). To minimize oxidation of tryptophan the intensity of excitation was reduced by adjusting the fluorimeter shutter so that a 10^{-4}M solution of tryptophan in water gave a fluorescence of 20.0 with excitation set at 280 nm, emission at 360 nm, both slits at 2 nm, and

sensitivity at 0.1. Under these conditions, tryptophan fluorescence was stable for over 2 h.

RESULTS

When incubated with purified rat kidney enkephalinase, the peptides Gly-Trp-Gly, N-acetyl-Gly-Trp-Gly and dansyl-Gly-Trp-Gly were all hydrolysed at the Gly-Trp amide bond (Fig 1). The unmodified tripeptide Gly-Trp-Gly was hydrolysed at a much lower rate than the two N-terminally substituted tripeptides. HPLC analysis demonstrated that the two peptides dansyl-Gly-Tyr-Gly and dansyl-Gly-Tyr-Gly-NH2 were hydrolysed at the Gly-Tyr amide bond (not shown). The hydrolysis of all substrates was completely inhibited when thiorphan was added in the reaction media (Figure 1).

The fluorescence spectrum of dansyl-Gly-Trp-Gly was dramatically modified when the Gly-Trp amide bond was hydrolysed. Upon excitation at 280

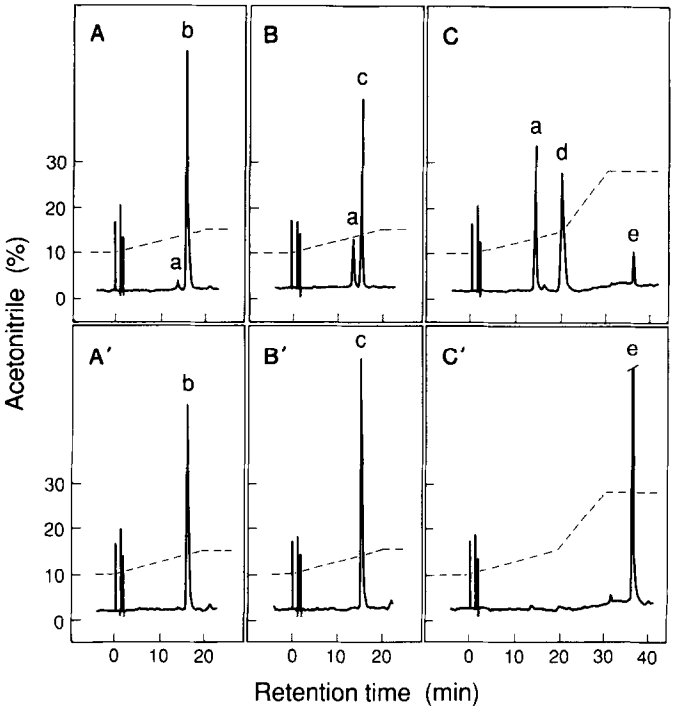

Figure 1. HPLC analysis of the hydrolysis of tryptophan-containing peptides by purified rat kidney enkephalinase

The peptides (1mM) were incubated for 1h at 37° C with 5 ng purified rat kidney enkephalinase, without (A, B, C) and with (A', B', C') 100 nM thiorphan. The substrates and fragments were resolved by reverse phase HPLC using a gradient of acetonitrile in 0.1% trifluoroacetic acid (dotted line). A and A', Gly-Trp-Gly; B and B', N-Acetyl-Gly-Trp-Gly; C and C', dansyl-Gly-Trp-Gly. Peptides eluted are: a,Trp-Gly; b, Gly-Trp-Gly; c, N-Acetyl-Gly-Trp-Gly; d, dansyl-Gly; and e, dansyl-Gly-Trp-Gly.

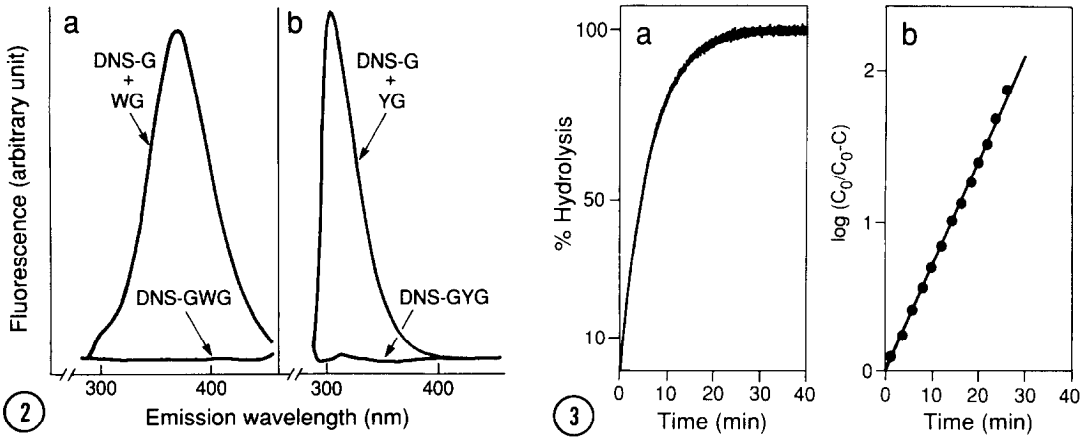

Figure 2. Characterization of the fluorescence transfer between tryptophan and dansyl in dansyl-Gly-Trp-Gly and tyrosine and dansyl in dansyl-Gly-Tyr-Gly

Fluorescence spectra were obtained using 10^{-4}M peptides in water at 37° C. Excitation was set at 280 nm. Slits were 2nm for both excitation and emission. The sensitivity of the fluorimeter was ten times higher for the tyrosine containing peptides than for tryptophan.

Figure 3. Continuous fluorescence recording of the hydrolysis of dansyl-Gly-Trp-Gly by purified rat kidney enkephalinase.

Purified rat kidney enkephalinase (350 ng) was added to 500 ul of dansyl-Gly-Trp-Gly (5.10^{-6}M) in 50 mM HEPES buffer, pH 7.4, containing 0.1% Tween 20. Fluorescence (excitation 280 nm, emission 350 nm, slits set at 2 nm) was continuously recorded at 37° C (a). The first order plot of substrate degradation (b) was obtained using measurements every 2 min, on the graph shown.

nm the emission spectrum of dansyl-Gly-Trp-Gly showed a maximum at 540 nm, with no fluorescence detectable at 350 nm. In contrast, under the same conditions, the emission spectrum of the peptides dansyl-Gly and Trp-Gly in equimolar concentration showed a maximum at 350 nm, and a complete disappearance of fluorescence at 540 nm (Figure 2a). In a similar way, upon excitation at 277 nm, the emission spectrum of peptides dansyl-Gly-Tyr-Gly and dansyl-Gly-Tyr-Gly-NH2 showed a maximum at 540 nm, while the maximum was at 315 nm for a mixture of the peptides dansyl-Gly and Tyr-Gly or, dansyl-Gly and Tyr-Gly-NH2 (Figure 2b). This change in emission spectrum is indicative of a fluorescence transfer between the tryptophan residue and the dansyl group in the peptide dansyl-Gly-Trp-Gly, or between the tyrosine residue and the dansyl group in the peptides dansyl-Gly-Tyr-Gly and dansyl-Gly-Tyr-Gly-NH2.

When dansyl-Gly-Trp-Gly was incubated with purified rat kidney enkephalinase and under excitation at 280 nm, the hydrolysis of the Gly-Trp amide bond of the substrate induced an increase in fluorescence intensity at 350 nm, which could be continously recorded (Figure 3). Similarly, the

Table 1

Kinetic parameters for the hydrolysis of various substrates by purified rat kidney enkephalinase

	Substrate	K_m(uM)	kcat (min^{-1})	kcat/K_m (uM^{-1} min^{-1})
	P_2 P_1 P'_1 P'_2			
I	Gly-Trp-Gly		53	
II	N-acetyl-Gly-Trp-Gly		218	
III	dansyl-Gly-Trp-Gly	30	902	30
IV	dansyl-Gly-Tyr-Gly	41	1143	28
V	dansyl-Gly-Tyr-Gly-NH_2	90	248	3

K_m and kcat values for substrates III, and IV and V were obtained by measuring the increase in tryptophan or tyrosine fluorescence after 1 h incubations of increasing concentrations of substrates (from 1 uM to 1 mM) with 1 ng purified enzyme at 37° C. The kcat values for these substrates and peptides I and II were also obtained by HPLC analysis of 1 h incubations of 1 mM peptides with 200 ng (substrate I), 50 ng (substrates II and V), or 10 ng (substrates III and IV) enzyme. The kcat values obtained by both methods for substrates III, IV and V, were in close greement.

hydrolysis of peptides dansyl-Gly-Tyr-Gly and dansyl-Gly-Tyr-Gly-NH2 could be continuously recorded by following the increase in fluorescence intensity at 315 nm under excitation at 277 nm (not shown).

The kinetic parameters for the hydrolysis of dansyl-Gly-Trp-Gly, dansyl-Gly-Tyr-Gly and dansyl-Gly-Tyr-Gly-NH2 by purified rat kidney enkephalinase were measured by incubating increasing concentrations of the substrates with the enzyme and stopping the reaction by the addition of EDTA before fluorescence measurements were taken (Table 1). Because tryptophan fluorescence increases with pH, the use of EDTA to stop the reactions resulted in an enhanced sensitivity in the assay when dansyl-Gly-Trp-Gly was used as the substrate.

The specificity constant (kcat/K_m) of dansyl-Gly-Tyr-Gly-NH2 was much lower than that of the corresponding free carboxylic acid substrate dansyl-Gly-Tyr-Gly. This decrease in specificity constant was due both to an increased Km value and a decrease in kcat (Table 1).

DISCUSSION

Enkephalinase activity was initially characterized using tritiated (Leu^5)enkephalin as the substrate (1). Because all of the peptide bonds in

the enkephalin molecule are sensitive to hydrolysis by various peptidases (3), in particular aminopeptidases, other more specific substrates were developed. One of these substrates was an aminopeptidase-resistant analog of (Leu^5)enkephalin, tritiated ($DAla^2$, Leu^5)enkephalin (6). The assays for these substrates were based on measuring the release of a characteristic tritiated metabolite, ^{3}H-Tyr-Gly-Gly or ^{3}H-Tyr-DAla-Gly, after its separation from the intact substrate by polystyrene beads chromatography (26).

More recently, indirect fluorescent assays using substrates unrelated to the enkephalins have been developed. The first step in these assays is the hydrolysis of an internal peptide bond in the substrates by enkephalinase. The second step is the release of a fluorescent group by the action of a purified commercial aminopeptidase added to the reaction mixture (20-22). Because these two-step assays depend on two enzyme activities they are obviously not suitable for a thorough enzymological study of enkephalinase; for example, any decrease in measured activity induced by an inhibitor could be due to inhibition of either enkephalinase or the aminopeptidase. Also, these substrates cannot be used for continuous recording of enkephalinase activity. Recently, Florentin et al (23) developed the first direct fluorometric assay for enkephalinase, which allows continous recording of its activity. The assay is based on an increase in dansyl fluorescence induced by the hydrolysis of the Gly-Phe(pNO_2) amide bond in the substrate dansyl-DAla-Gly-Phe(pNO_2)-Gly. The increase in fluorescence is due to the disappearance of intramolecular quenching of the dansyl fluorescence by the nitrophenyl residue.

In the present work, we have used a different property of fluorescence, namely energy transfer. Enkephalin-degrading aminopeptidase substrates have already been developed (27) using fluorescent energy transfer between the N-terminal tyrosine of the enkephalins and a dansyl group attached to their C-terminus.

A comparison of the emission spectra of Dansyl-Gly-Trp-gly and a mixture of Dansyl-Gly plus Trp-Gly under excitation at 280 nm demonstrates that the energy transfer from the donor (tryptophan) to the acceptor (dansyl) is essentially complete, since the fluorescence intensity of the tryptophan (at 350 nm) in Dansyl-Gly-Trp-Gly is decreased to background levels (Figure 2a). Hydrolysis of the glycyl-tryptophanyl bond in Dansyl-Gly-Trp-Gly thus results in the appearance of tryptophan fluorescence which can be continuously monitored (Figure 3). Similarly, there was an almost complete energy transfer between tyrosine (donor) and dansyl (acceptor) in the peptides Dansly-Gly-Tyr-Gly and Dansyl-Gly-Tyr-Gly-NH_2 (Figure 2b). However, because the relative fluorescence intensity of a

tyrosine is much lower than that of a tryptophan, the signal-to-noise ratios were lower than with the substrate Dansyl-Gly-Trp-Gly.

We had previously reported (16) that tetrapeptides are the smallest peptides that can be hydrolyzed by enkephalinase, and this conclusion had recently been confirmed by others (28). However, in this study, we have found that the tripeptide Gly-Trp-Gly can be hydrolyzed by the purified enzyme, and that this hydrolysis is blocked by thiorphan, a potent enhephalinase inhibitor (29) (Figure 1), but it should be noted that the rate of hydrolysis of the tripeptide was very low. Furthermore, N-acetylation of the peptide which mimics a peptide bond, induced a greater than 4-fold increase in sensitivity to hydrolysis. This finding strongly suggests that the P_2-P_1 amide bond in substrates binds to some component in the active site of the enzyme, and helps to maintain the P_1-P'_1 amide bond in proper position for hydrolysis. This could explain the observation that the N-terminally substituted chemotactic tripeptide formylMet-Leu-Phe can be efficiently hydrolysed by enkephalinase (30).

Because enkephalinase preferentially hydrolyses peptide bonds on the amino-side of hydrophobic residues, it was initially suggested that it is a thermolysin-like enzyme (10,13). We had previously presented evidence showing that, although having endopeptidase activity, enkephalinase preferably functions as a dipeptidyl-carboxypeptidase (9). We also showed in a direct comparison of enkephalinase and thermolysin that these two enzymes have clearly different substrate specificities. This conclusion was recently confirmed by two other groups (31,32). Our present results point to another difference between the two enyzmes; Morihara and Tsuzuki (33) had shown that the hydrophobic pocket in thermolysin cannot accomodate a tryptophan residue in position P'_1. In agreement with this observation, we also have found that Dansyl-Gly-Trp-Gly is not hydrolysed by thermolysin (not shown). In contrast, the hydrophobic pocket in S'_1 in enkephalinase easily accommodates a tryphophan residue, and there is no major difference in either K_m or kcat values between the substrates Dansyl-Gly-Trp-Gly and Dansyl-Gly-Tyr-Gly (Table 1). This further demonstrates that enkephalinase is not a thermolysin-like enzyme.

Several researchers have reported that amidation of substrates in the position P'_2 decreases their specificity constant by a factor of approximately 10 (9,11,16,31). We had suggested that this behavior is due to the interaction of the carboxyl end of substrates in position P'_2 with an arginine residue at the active site of the enzyme (9,19,34). This conclusion was recently confirmed (35,36). Our present results again show the importance of a free carboxyl group in the P'_2 position, since

Dansyl-Gly-Tyr-Gly-NH_2 has a specificity constant approximately 10 fold less than Dansyl-Gly-Tyr-Gly.

The specificity constant of the fluorescent substrates we developed is significantly higher than that of many other fluorescent enkephalinase substrates. Particularly, Dansyl-Gly-Trp-Gly seems to be a better substrate (by 20 fold) than Dansyl-DAla-Gly-Phe (pNo_2)-Gly, the only other fluorescent substrate which can be used in a one-step assay (23). It is hoped that these new substrates will lead to a better understanding of the mechanisms underlying the substrate specificity of enkephalinase.

ACKNOWLEDGMENTS: We thank Dr. Bryan Finkle for his continous support, and Dan Burdick and Martin Struble for excellent technical assistance.

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