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THE PEROXIDASE-CATALYZED OXIDATION OF ENKEPHALINS

M. A. ROSEI, C. BLARZINO, C. FOPPOLI°, R. COCCIA and C. DE MARCO°

Dipartimento di Scienze Biochimiche, Università "La Sapienza"
° Centro di Biologia Molecolare del C.N.R., Rome, Italy

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SUMMARY: In vitro experiments are reported showing that Leu-enkephalin and Met-enkephalin, in the presence of hydrogen peroxide, can be oxidized by horseradish peroxidase. The products formed are strongly fluorescent and characterized by absorption peaks with maxima at 290 nm and 315 nm. The effects of substrate and enzyme concentrations on the oxidation rate of enkephalins are described. Amino acid analysis of the hydrolysates from peroxidase-treated enkephalins provides evidence for the presence of dityrosine. The data suggest that the oxidation leads to the production of enkephalin dimers with a linkage between the N-terminal tyrosine residues. Data are also obtained indicating that enkephalins function as hydrogen donors for mammalian peroxidases.

The pentapeptides Leu-enkephalin (Leu-enk) and Met-enkephalin (Met-enk) are of great interest because of their opiate-like activity (1) and their role as neurotransmitters or neuromodulators is well established (2,3). Many studies (4,5) have been devoted to the action of specific peptidases which are able to produce the rapid cleavage of enkephalins but little work has been performed on alternative pathways. Recently we have demonstrated that enkephalins, esorphins and some of their derivatives are good substrates for mushroom tyrosinase showing an affinity for the enzyme higher than tyrosine itself (6,7). In order to gain further information on alternative routes of enkephalins conversion, we have tested these peptides as substrates for another oxidative enzyme, the horseradish peroxidase. It is known that peroxidases catalyze the oxidation of a large variety of substrates (8,9) and, among the possible hydrogen donors, aromatic amines, catechols and tyrosine could be ascribed (10,11).

In this paper we present evidence for the role of enkephalins as hydrogen donors for horseradish peroxidase; the reaction leads to the formation of dityrosine, which links peptide molecules into dimers.

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MATERIALS AND METHODS

Materials: Leu-enk and Met-enk were obtained from Serva. Tyrosine, horseradish peroxidase (HRP), myeloperoxidase (MPO) and lactoperoxidase (LPO) were purchased from Sigma. Peroxidase concentrations were determined spectrophotometrically using the following millimolar extinction coefficients: for HRP 91 at 403 nm (11), for MPO 178 at 430 nm and for LPO 114 at 412 nm (12).
Hydrogen peroxide solutions were freshly prepared by diluting a 30% stock solution (Merck); the concentration was measured using a molar extinction of 72.4 at 230 nm (13). Dityrosine was synthetized according to the enzymatic procedure described by Amadò et al.(14). All other chemicals were analytical grade.
Optical measurements: Spectral data were recorded using thermostated cuvettes at 25°C. The rate of dityrosine formation was estimated measuring the optical density at 315 nm as a function of time (9). Except where indicated the assay mixture for kinetic measurements contained: 0.1 mM enkephalin, 0.1 mM H_2O_2, 33 nM horseradish peroxidase, 0.1 M potassium phosphate buffer (pH 8.2) in a volume of 1 ml. The reaction was started by the addition of the enzyme whereas an identical mixture without the enzyme was used as reference. Suitable blanks of enkephalins in presence of H_2O_2 ruled out a non-enzymatic oxidation of the substrates.
Synthesis of enkephalin dimers: The experiments were performed according to Amadò et al. (14). The mixture contained 2 mM enkephalins or tyrosine, 4 mM H_2O_2, and 1.2 μM HRP in a final volume of 1 ml. After addition of the enzyme, the mixture was incubated at 37°C with shaking; at various time the reaction was stopped by adding sodium metabisulfite to destroy H_2O_2 and samples were subjected to chromatographic analysis.
High performance liquid chromatography: Samples were applied on a reverse-phase column (Aquapore RP 300, 7 x 250 mm, Brownlee Laboratories) using the following chromatographic conditions: eluent A was 0.2 % trifluoracetic acid and eluent B was 70 % CH_3CN in 0.2 % trifluoracetic acid. The gradient applied was 0-50 % B in 30 minutes. Flow rate was 2 ml/min and chromatography was carried out at 25°C with a thermostated apparatus. The absorbance of the effluents was monitored at 220 and 280 nm.
Ion-exchange chromatography: In order to ascertain dityrosine formation in peroxidase-treated enkephalins, the samples (1 ml) were added with an equal volume of HCl 12 M and hydrolyzed for 20 h at 120°C. Amino acids were quantitatively determined by amino acid analyzer, in the condition already described (15).

RESULTS

Spectral changes observed when Leu-enk is used as substrate of horseradish peroxidase are shown in Fig. 1. When the enzyme acts upon the enkephalin in the presence of hydrogen peroxide two characteristic absorption peaks with maxima at about 290 nm and 315 nm soon appear. Such spectral features are exactly alike to those reported in literature for dityrosine absorption (14, 16,17). The spectra obtained when Met-enk is utilized as substrate are very similar. For both enkephalins, equilibrium is reached after twenty minutes (inset Fig. 1).
Chromatographic detection of reaction products and dityrosine quantitation: In order to identify the reaction products, a large-scale incubation of enkepha-

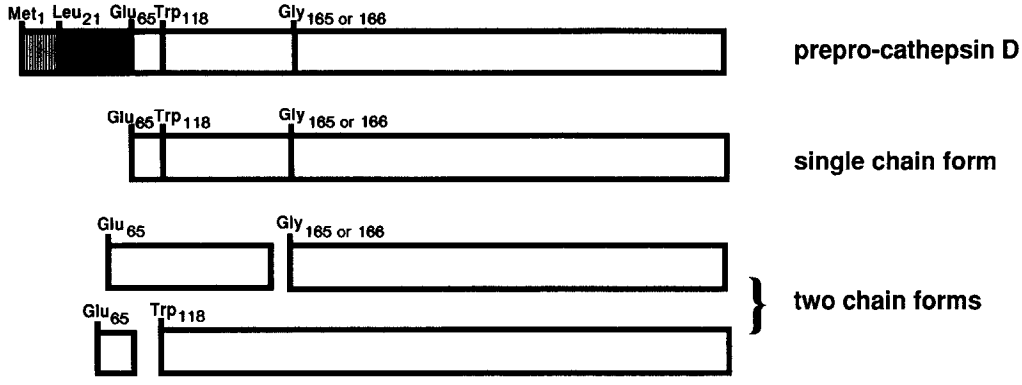

Fig. 4. Schematic representation of proteolytic processing of rat cathepsin D. Vertical straight lined area shows a signal peptide and hatched area the propeptide.

sequence contained 2 potential *N*-glycosylation sites at positions 134 and 258 and the active site aspartyl residues are also well conserved, as is the case with other aspartyl proteases.

Comparison of rat, human, and porcine cathepsin D

Porcine spleen cathepsin D and rat liver cathepsin B, H, L, are mostly composed of heavy and light chains (5, 22-24), the so-called two-chain form. However, cathepsin D purified from the rat spleen contains only a single chain form (6) and as can be seen in Fig. 1, cathepsin D purified from rat liver contains an abundant single chain form and small amounts of two two-chain forms. One two-chain form was composed of a 34 kDa heavy chain and a 9 kDa light chain while the other one has 30 kDa and 14 kDa heavy and light chains, respectively. The deduced amino acid sequence of the mature cathepsin D has an 82% homology to those of human and porcine cathepsin D. The propeptides from rat and human cathepsin D have a 72% homology. Despite such a high homology, rat cathepsin D polypeptide is shorter than that in human's by 5 amino acid residues. Five amino acids from 162 to 166 residues of the human enzyme are deleted in the rat one, and the deletion is near the splitting position which generates the two-chain form in the rat enzyme (Fig. 3). Faust et al.(9) found that porcine cathepsin D is 7 amino acid residues shorter than that in human's and suggested that these 7 residues are lost during proteolytic conversion from the single to the two-chain form. In this case, 7 amino acids from 160 to 166 residues of the human enzyme lacked in the porcine one (Fig. 3). Since the splitting sites which generate the two-chain forms in rat and porcine cathepsin D, respectively, are located in close positions in each sequence, the lack of 7 amino acid residues of the porcine enzyme may be a result of deletion in a genomic level, as in the case of rat one. Since Faust et al. (9) suggested that the similarity in amino acid sequences between cathepsin D and renin, pepsin, and chymosin was 46,49 and 47%, respectively, we examined the similarity between the whole sequence of rat cathepsin D and human cathepsin E (25). We revealed that there is a 46% similarity and aspartic acid in active sites and 6 cysteines are well conserved, hence the three-dimensional structure in this family of aspartyl proteases is conserved. The proteolytic split sites of rat cathepsin D are shown in Fig. 4.

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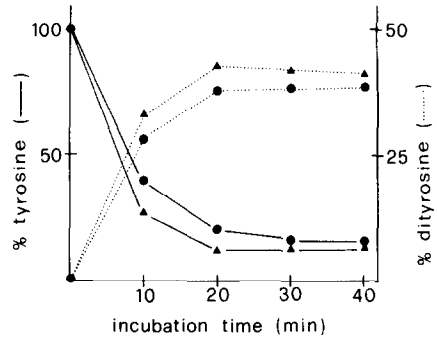

Fig. 3. Dityrosine production in function of the incubation time.
The values reported were calculated taking as 100 the initial tyrosine concentration. Each value is the average of three different experiments.
Leu-enk (●), Met-enk (▲); tyrosine (full line), dityrosine (dotted line).

Kinetic measurements: In table I the apparent K_m values for the oxidation of Leu-enk, Met-enk and tyrosine by HRP are reported. The affinity of the enzyme for the two opioid peptides is higher with respect to tyrosine; the apparent K_m value for the amino acid is in fact 3.3×10^{-3} M while Leu-enk and Met-enk show apparent K_m values respectively of 0.83×10^{-3} M and 0.55×10^{-3} M. The reaction appears to be linear until the Vmax is reached.

Action of some mammalian peroxidases on enkephalins: In order to establish if enkephalins could be substrates also of mammalian peroxidases, we have tested enkephalins with myeloperoxidase and lactoperoxidase. The opioid peptides actually function as hydrogen donors for these enzymes and the spectral changes observed are identical to those shown in Fig. 1.
In table II a comparison of the activity of the three peroxidases on enkephalins and tyrosine is reported.

Table I. Kinetic measurements of the oxidation of enkephalins by HRP

SUBSTRATE	K_m app ($M \times 10^{-3}$)	k_{cat} app (Vmax/mgE)	k_{cat}/K_m app
Leu-enk	0.83 ±0.02	66	79.5
Met-enk	0.55 ±0.02	56	102.0
Tyrosine	3.3 ± 0.2	13	3.9

Vmax is expressed as Δ of optical density at 315 nm/minute.
Each value ± S.D. is the average of three experiments.

Table II. Comparison of the activity of HRP, LPO and MPO on enkephalins and tyrosine

Enzyme	Leu-enk	Met-enk	Tyr
	(Δ D.O. 315 nm/min)		
HRP	4.22	4.70	0.13
LPO	3.94	5.00	3.15
MPO	0.64	0.85	0.52

The values are referred to the same enzyme concentration (1×10^{-6} M) and substrate concentration (1×10^{-3} M). Each value is the average of five experiments.

DISCUSSION

The results reported in this paper show that Leu-enk and Met-enk are good substrates for HRP; by the reaction, the corresponding enkephalin dimers are produced, as evidenced by spectroscopic and chromatographic data. The conversion of enkephalins into the dimers is very high (85-90 %): however, a small amount of pentapeptide is yet recovered after 24 h incubation. This conversion is very rapid, the reaction reaching the equilibrium within 20 minutes; for the formation of dityrosine from tyrosine the reaction proceeds more slowly (14).

Kinetic measurements indicate that the oxidation reactions by the HRP/H_2O_2 system seem to follow Michaelis-Menten kinetics. All kinetic parameters indicate that enkephalins are preferred as substrates with respect to the tyrosine.

This result suggests that the presence of a peptidic bond could be a favourable factor for the oxidation of the phenolic ring. The fact may be explained considering that peptidic bond could help electronic release to the ring, facilitating the formation of the radical whose presence is required for the mechanism of action of peroxidase (18). This behaviour is similar to that observed with tyrosinase that utilizes these peptides very easily as substrates (6) and even kyotorphin, a simple dipeptide (Tyr-Arg) with opioid-like activity, is oxidized by tyrosinase faster than tyrosine (7). The presence of naturally occurring dityrosine is well documented (19,20). It has been suggested that dityrosine formation is responsible for natural cross-linking in proteins and peptides and the biological significance of dityrosine occurrence has been outlined (21).

The oxidative coupling of tyrosine residues in enkephalins by the HRP/H_2O_2 system leads to the production of dimers with a linkage between the N-terminal

tyrosine residues. Previous studies performed on dimeric enkephalins, obtained by chemical coupling at the Leucine terminal, have shown a strong increase of opioid-like activity (22) and an enhanced affinity for the delta opiate receptor (23). Work is in progress to determine whether the dimerization at the tyrosine terminal could change the opioid activity of the compounds.

Next task would be to test whether these oxidation reactions could be effective in vivo and whether these substances are to be simply considered as catabolic products or may possess some metabolic functions.

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