Metabolism of angiotensins by head membranes of the leech *Theromyzon tessulatum*

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Abstract Angiotensins (angiotensin I, angiotensin II, angiotensin II-amide) have been isolated in leeches and such peptides are involved in diuresis in these animals. To explore possible inactivation mechanisms of these peptides, angiotensins were incubated with head membranes of the leech *T. tessulatum*. Membranes derived from head parts of this leech are very rich in peptidases. They contain endopeptidase-24.11-like enzyme (NEP-like) associated with a battery of exopeptidase. The way that angiotensins are degraded by the combined attack of these membrane peptidases has been investigated. The contribution of individual peptidases was assessed by adding inhibitors (phosphoramidon, captopril and amastatin) to the membrane fractions, when they were incubated with the peptides. In the case of angiotensin I, the primary attack was performed by a combined action of the NEP-like and the ACE-like enzymes, followed by aminopeptidase attacks. Angiotensin II and III were hydrolyzed by NEP-like enzyme at the same Tyr-Ile bond, whereas the N-terminal arginine residue of angiotensin III was removed by an arginyl aminopeptidase. These results show that angiotensins are efficiently degraded by membranes and that NEP-like enzyme plays a key role in this process.

Key words: Angiotensin; Leech; Peptidase; Metabolism; Membrane

1. Introduction

Since immunocytochemical studies with polyclonal antisera raised against angiotensin II (a-AII) at the level of the leech *Theromyzon tessulatum* brain, evidence was given of neurons containing angiotensin-like material [1]. This material was isolated from *T. tessulatum* and corresponded to the vertebrate AII and AIII, as well as fragments (3–8) and (6–8) of AII [2], suggesting the existence of peptidases involved in the metabolism of AII. Moreover, in *Erpobdella octoculata*, an angiotensin II-amide, differing from the vertebrate AII by a carboxy-terminal amidation, has been sequenced [3]. Although the AII-like peptide is very similar to the vertebrate AII, its precursor seems to be different from the vertebrates one, the angiotensinogen protein of ca. 60 kDa. Western blot analyses performed with *T. tessulatum* central nervous systems (CNS) homogenate have revealed the existence of a multiple hormone AII-like precursor of ca. 19 kDa [2]. This protein possesses at least four epitopes recognized by three different polyclonal antisera (an anti-γ-MSH, an anti-angiotensin I and an anti-angiotensin II) and by a monoclonal antibody (Tt159) specific for epitopes contained in *T. tessulatum* supraesophageal ganglia [2]. Three of the four epitopes recognized have been identified. The N-terminal part of a fragment of this precursor has recently been determined and the first 14 amino acid residues (DRVYIHPFHLLXWG) reveal the existence of both angiotensin I and angiotensin II [4]. The third epitope is a peptide related to the vertebrate γ_1-MSH [5]. The fragment of the AII-like precursor presents in its sequence the processing sites of the metabolic enzymes: renin and angiotensin-converting enzyme (ACE) [4]. These two enzymes have been isolated and our findings demonstrate that these ectopeptidases are close to the renin and the ACE found in vertebrates, in activity, mass and N-terminal sequence [6,7], and are localized at the level of the dorsal commissure and glial cells (unpublished data). A renin-angiotensin-like system (RAS) similar to vertebrate RAS exists in leeches. Parallel to this RAS, a non-RAS would also be present in leeches. A neuropeptide endopeptidase of ca. 45 kDa (NEP-like enzyme) with characteristics close to those of endopeptidase-24.11 has been isolated from *T. tessulatum* head membranes [8].

Considering the existence of these two systems (RAS and non-RAS) in leeches, the aim of this work was to identify the different peptidases involved in angiotensins metabolism and to locate their sites of attack on these peptides.

2. Material and methods

2.1. Animals

Mature specimens of the rhynchobdellid leech *T. tessulatum*, reared under laboratory conditions as described by Malecha et al. [9], were used in this study.

2.2. Materials

Angiotensin I (DRVYIHPFHL: AI), angiotensin II (DRVYIHPF: AII), angiotensin III (RVYIHPF: AIII), DRVY, IHPF, HL, DRVYIHP, RVYIHP, VYIHPF, RVY, FHL peptides, the phenylalanine (F) residue, amastatin, captopril and phosphoramidon were obtained from Sigma.

2.3. Antisera

Two polyclonal antisera directed either against AII (a-AII) or against AI (a-AI) were generated in rabbits using synthetic human AII or AI coupled to human serum albumin via glutaraldehyde. Their specificity has been described elsewhere [1,4]. In brief, for a-AII, 100% cross-reaction with AII and 0.46% for AI were obtained. For a-AI, 100% cross-reaction with AI, 10% for AII and 0% for the synthetic porcine angiotensinogen tetradecapeptide (DRVYIHPFHLLVYS) were observed.

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Abbreviations: AI, angiotensin I; AII, angiotensin II; AII-amide, angiotensin II-amide; a-AI, anti-angiotensin I; ACE, angiotensin-converting enzyme; AP, aminopeptidase; DIA, dot immunobinding assay; HPLC, high performance liquid chromatography; NEP, endopeptidase-24.11; RAS, renin-angiotensin system; TBS, Tris/HCl buffer saline; NAP, neutral aminopeptidase; DPAP, dipeptidyl aminopeptidase; Arg-AP, arginyl-aminopeptidase; Asp-AP, asparagyl-aminopeptidase.

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2.4. Immunoassays

Dot immunobinding assay (DIA) and enzyme linked immunosorbent assays (ELISAs) were based on the protocols of Salzet et al. [10,11].

2.5. Membrane preparation

After anesthesia of the animals in 0.01% chlorobutanol, head parts of *T. tessulatum* were excised, immediately frozen in liquid nitrogen and stored at −70°C. 500 head parts (5 g) were placed in 25 ml TBS (20 mM Tris/HCl, pH 7.4, containing 200 mM NaCl) and homogenized at 4°C with a polytron (5×15 s bursts on setting 9). After 30 min centrifugation at 10000 rpm at 4°C, the pellet was reextracted six times with the same amount of saline solution. The combined supernatants were centrifuged at 20000 rpm for 1 h at 4°C to yield a membrane pellet. These membranes were washed by resuspension in TBS, followed by sedimentation at 20000 rpm. The washing procedure was repeated until a clear supernatant was obtained. The washed membranes were resuspended in TBS and stored at −20°C. Protein concentration was determined with the Bradford procedure using γ-globulin as standard [12].

2.6. Identification of cleavage sites

Assays of peptidase activities were carried out with 15 μg of membranes incubated or not with 10 μM peptides and 10 μM inhibitors in TBS with a total volume of 100 μl. Reactions were terminated by addition of 30% trifluoroacetic acid (v/v). Samples were centrifuged at 12000 rpm for 15 min. Supernatants were diluted by adding 100 μl of 0.1% (v/v) trifluoroacetic acid and degraded fragments were analyzed on a narrow-bore C_{18} (250 mm×2 mm, Beckman) HPLC column, with a linear gradient of acetonitrile in acidified water (0.1% trifluoroacetic acid) from 0 to 80% in 30 min at a flow rate of 300 μl/min. Emerging peaks were detected by absorbance at 215 nm and collected manually before being dried in vacuo.

2.7. Analysis of peptide products

Identification of the metabolized products of the angiotensins was performed (a) by coelution with marker peptides in reversed-phase HPLC [13], (b) by reaction with specific antisera in immunoassays (DIA and/or ELISA) and (c) by Edman degradation on a pulse-liquid automatic sequencer (Applied Biosystems, model 473A). Molar ratios of the products were calculated from data given by Stephenson and Kenny [14] on the quantification of peptides by HPLC.

2.8. Inhibitor experiments

Phosphoramidon, captopril and amastatin were used at a final concentration of 10 μM and were preincubated for 30 min at 37°C with membranes before addition of 10 μl of the peptide solution. These inhibitors are respectively specific for endopeptidase-24.11 [15], ACE [16] and aminopeptidases [17].

2.9. Kinetics of degradation

Kinetic parameters were determined from the regression line fitted to the data plotted as 1/V vs. 1/[S]. Correlation coefficients were greater than 0.99 [18]. K_{cat} values were calculated assuming a molecular weight of 45 kDa for the purified NEP-like enzyme [8].

3. Results and discussion

Results obtained with head membrane preparations are identical to those obtained with membrane preparations of CNS. However, in order to have sufficient material for these studies, head parts including the whole of the CNS were used.

3.1. Metabolism of angiotensins

After 1 h incubation of AI, AII and AIII peptides with membranes, the three angiotensins yielded multiple products which were collected, then dried in vacuo. The peptides or amino acids identified using ELISA, coelution with marker peptides and microsequencing are shown in Table 1.

3.2. Effect of inhibitors on the pattern of angiotensin hydrolysis (Table 1)

In trying to understand the way in which angiotensins are

Table 1
Effect of inhibitors on the hydrolysis of angiotensins by *T. tessulatum* head membranes

Peptide	Retention time (min)	Product formed in the absence of inhibitor (nmol)	Peptide remaining (%)		
			Phosphoramidon (10 μM)	Captopril (10 μM)	Amastatin (10 μM)
Angiotensin I					
HL	3.3	1.66	70	52	48
F	3.9	1.3	8	93	20
IHP	4.8	0.8	12	100	100
DRVY	6.4	1.5	12	100	100
DRVYIHP	10.3	1.2	13	100	100
VYIHP	11.5	0.3	32	95	100
FHL	12.7	1.75	15	88	100
DRVYIHPF	15.3	2.53	60	25	100
Angiotensin II					
HP	2.7	1.68	80	95	30
F	3.6	1.45	86	100	80
DRVY	6.2	2.6	3	100	100
HPF	7.5	1.2	2	100	100
DRVYIHP	10.7	0.78	100	100	100
RVYIHPF	11.3	0.5	100	100	30
IHPF	11.7	2.3	4	100	100
Angiotensin III					
F	3.8	1.8	80	100	100
RVY	4.5	2.8	5	100	100
RVYIHP	9.9	0.7	100	100	100
IHPF	11	3.2	12	100	100
VYIHPF	11.8	0.8	100	100	34

See section 2 for details. The amount of each product formed in 60 min in the absence of inhibitors is given in nmol h^{-1} mg $protein^{-1}$. The effect of each inhibitor on the product yield is expressed as a percentage of this value. The data are based on four experiments.

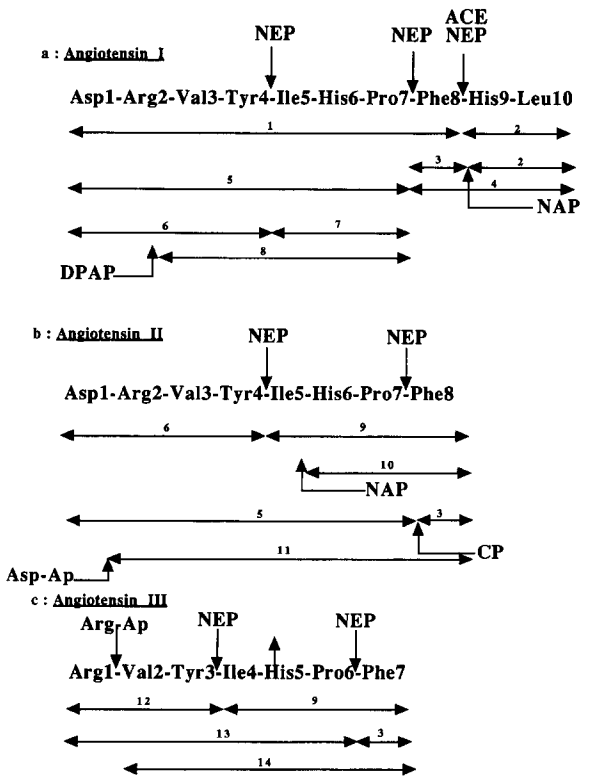

Fig. 1. Identities of the products formed by incubation of angiotensins with *T. tessulatum* membranes. The numbered peptides correspond to the HPLC peaks and were identified by coelution with peptide markers in HPLC, antisera recognition and spectral scanning comparison. (1) DRVYIHPF; (2) HL; (3) F; (4) FHL; (5) DRVYIHP; (6) DRVY; (7) IHP; (8) VYIHP; (9) IHPF; (10) HPF; (11) RVYIHPF; (12) RVY; (13) RVYIHP; (14) VYIHPF. The enzymes acting on the angiotensin metabolism are the following: ACE: angiotensin-converting enzyme; AP: aminopeptidase; Asp-AP: aspartyl aminopeptidase; Arg-Ap: arginyl aminopeptidase; CP: carboxypeptidase; DPAP: dipeptidyl aminopeptidase; NAP: neutral aminopeptidase; NEP: neuropeptide-degrading endopeptidase.

metabolized by leech membranes, we can be hampered by some degree of ignorance concerning enzymes that may be involved. However, three enzymes have previously been well characterized in *T. tessulatum* head membranes: the renin-like, the ACE-like, and the endopeptidase-24.11-like (NEP-like) enzymes [6–8]. This background knowledge gives us some confidence in attributing the attack to one or more of the peptidases known to be present in *T. tessulatum* head membranes. Such conclusions clearly depend on the specificity of the inhibitors employed. By employing 10 μM phosphoramidon, the leech NEP activity completely disappears. By contrast, the ACE-like enzyme remains active. 10 μM captopril are sufficient to stop the leech ACE activity but have no effect on the NEP-like enzyme. In the case of the aminopeptidases (AP), amastatin, a potent inhibitor [8], was used and blocked at least the neutral aminopeptidase (NAP) (unpublished data).

3.2.1. Angiotensin I (Fig. 1a). The main product (DRVYIHPF: AII) resulting from hydrolysis of the Phe^8-His^9 bond was suppressed for 75% by captopril, 40% by phosphoramidon and 95% by a cocktail of phosphoramidon and captopril, reflecting attack by both ACE-like and NEP-like enzymes. Hydrolysis at the Pro^7-Phe^8 bond yielding the DRVYIHP peptide was suppressed for 87% by phosphoramidon and unaffected by other inhibitors. The second fragment (FHL) yielded by this attack was transitorily detected and was rapidly metabolized to HL peptide and Phe residue. The production of the peptide HL was suppressed for 58% by captopril and only 30% by phosphoramidon. This peptide is generated by both the ACE-like and the NEP-like enzymes followed by an AP attack inhibited by amastatin, liberating the Phe residue. Peptides DRVY and IHP from attacks at Tyr^4-Ile^5 and Pro^7-Phe^8 bonds by a NEP-like enzyme were about 88% suppressed by phosphoramidon. The N-terminal dipeptide DR was not detected, even in the presence of amastatin. However, peptide VYIHP has been isolated, reflecting an attack at the Arg^2-Val^3 bond by a dipeptidyl aminopeptidase (DPAP).

3.2.2. Angiotensin II (Fig. 1b). Conversion of AII (DRVYIHPF) in AIII (RVYIHPF) by cleavage at the Asp^1-Arg^2 bond is about 70% suppressed by amastatin reflecting an attack by an aspartyl aminopeptidase (Asp-AP). However, the main attack on AII occurs at the Tyr^4-Ile^5 bond generating DRVY and IHPF peptides. This attack is wholly suppressed by phosphoramidon. The IHPF peptide was rapidly degraded to either IHP and F or HPF. Appearance of these peptides is inhibited by phosphoramidon and amastatin, reflecting a NEP-like enzyme and an AP action, successively. None of the inhibitors used suppressed the formation of DRVYIHP. As compared to what is found in vertebrates, these results could be consistent with a degradation by a carboxypeptidase P which hydrolyses Pro-Xaa bonds [14,19].

3.2.3. Angiotensin III (Fig. 1c). Three points of primary attack were revealed. The N-terminal arginine residue was removed to yield the VYIHPF peptide, partially suppressed by amastatin. Phenylalanine was released from the C-terminus, and this cleavage was inhibited 20% by phosphoramidon. The main attack was at the Tyr^3-Ile^4 bond, yielding the RVY and the IHPF peptides and this was strongly inhibited (88%) by phosphoramidon.

3.3. Site of attack (Table 2)

3.3.1. Angiotensin I. In addition to the conversion of AI to AII by the combined action of ACE-like and NEP-like enzymes at the Phe^8-His^9 bond, during the first 15 min, AI is degraded by the NEP-like enzyme at the Tyr^4-Ile^5 and Pro^7-Phe^8 bonds. Then, between 15 and 30 min, this last event becomes the most important and peptide products are further degraded by a DPAP which attacks at the Arg^2 level and by a neutral aminopeptidase (NAP) (recently characterized, unpublished data) which cleaves at the Phe^8-His^9 bond the metabolized product (FHL) coming from the previous NEP-like enzyme attack.

3.3.2. Angiotensin II. AII, one of the biologically active peptides in leeches, is only degraded at 50% when 30 min have elapsed. AII is firstly cleaved by an asparagyl aminopeptidase (Asp-AP) which carries off the Asp^1 amino acid in AIII, then by the NEP-like action on the same bonds as in the second attack of the AI. The latter enzyme seems to cleave at the Ile^5 level. Early in the course of the reaction, a carboxypeptidase begins to take off the Phe^8 residue, which inactivates AII.

3.3.3. Angiotensin III. The Asp-AP already mentioned at-

Table 2
Time course of formation of peptides upon treatment with *T. tessulatum* head membranes

Peptide	Incubation time (min)						
	0	5	10	15	30	45	60
Angiotensin I	8.8	8	6	5	2.6	2	
HL				0.3	0.8	1.2	1.4
F					0.9	1.1	1.1
IHP		0.05	0.1	0.1	0.3	0.4	0.5
DRVY		0.2	0.5	0.8	1.1	1.5	1.5
DRVYIHP			0.3	0.6	0.9	1.2	1.1
VYIHP						0.1	0.2
FHL		0.1	0.4	0.7	0.9	0.9	1.4
DRVYIHPF (AII)		0.5	1.2	1.4	1.4	1.7	2.1
Angiotensin II	9.5	9.3	8	6.8	5	2.2	
HP					0.2	1.2	1.7
F		0.05	0.1	0.2	0.7	1	1.5
DRVY				0.5	1.3	1.6	2.6
HPF				0.6	0.8	1.2	1.2
DRVYIHP		0.1	0.1	0.2	0.4	0.5	0.8
RVYIHPF		0.3	0.5	0.8	0.6	0.5	0.5
IHPF			0.5	0.8	1.3	1.7	2.3
Angiotensin III	10.5	10	9.2	7	5.1	2.4	
F				0.3	0.8	1.2	1.9
RVY				0.8	1.5	2.2	3.3
RVYIHP			0.1	0.4	0.5	0.7	0.9
IHPF		0.1	0.6	1.3	1.6	2.5	3.2
VYIHPF		0.5	0.6	0.6	0.7	0.8	0.9

See section 2 for details. Samples were incubated at 37°C for 0, 5, 10, 15, 30, 45, 60 min. The amount of each product formed is given in nmol.

tacks the Asp^1-Arg^2 bond of AII to produce AIII, the other active angiotensin in leeches [1]. As soon as AIII is yielded, the first enzymes to begin its degradation are an arginyl aminopeptidase (Arg-AP) and then the NEP-like enzyme. Indeed, VYIHPF (AIV) is the major product obtained after 5 min. By contrast, after 10 min, the amount of VYIHPF is constant while IHPF has greatly increased.

3.3.4. Summary of the kinetic scheme (Fig. 1). Finally, it appears to us that the main attack is done by the NEP-like enzyme. The metabolized products are then degraded by AP in a secondary attack. The AP attacks, at the Phe-Xaa, the Arg-Xaa and the Asp-Xaa bonds, would not be performed by the same AP. According to the background knowledge in vertebrates, two types of AP could be implicated in such cleavages, i.e. a NAP and acidic aminopeptidases [19]. In leech membrane, an enkephalin-degrading aminopeptidase close to the NAP has been isolated and characterized (unpublished data). This enzyme cleaves peptides at the Tyr-Xaa, Phe-Xaa bonds and could also be implicated in degradation of angiotensins at the Phe-Xaa bond. The other cleavages at the Arg-Xaa and Asp-Xaa could be due to acid aminopeptidases as in vertebrates.

3.4. Comparison of kinetic constants for purified NEP-like enzyme

Purified NEP-like enzyme according to Laurent and Salzet [8] was used to follow the hydrolysis of angiotensins. With a K_m of 72 μM for AI, 110 μM for AII and 85 μM for AIII occurring at the Pro^7-Phe^8 and Tyr^4-Ile^5 bonds of angiotensins, these values are in line with those obtained on bradykinin (92 μM), lysylbradykinin (186 μM), and substance P (32 μM) with vertebrate NEP [14,18]. However, among the three angiotensins, the NEP-like enzyme seems to cleave preferentially AII, then AIII and AI. K_{cat} values (4480 min^{-1}; 5225 min^{-1}; 4259 min^{-1}) obtained for angiotensins AI, AII and AIII, respectively confirm the preceding hypothesis. NEP-like Kcat values are in line with those registered with vertebrate NEP on bradykinin (6360 min^{-1}) or substance P (5060 min^{-1}) [18]. The ratio K_{cat}/K_m (10^6/mol per min) corresponding to the catalytic efficiency of the enzyme are the following: 62.2, 47.5, 50.1 for AI, AII and AIII, respectively. Compared to K_m values, NEP-like K_{cat}/K_m reflects a better efficiency of this enzyme towards AI than towards AIII and AII. These results could be explained by the fact that NEP-like enzyme attacks AI at the level of three bonds instead of two bonds in the case of AII and AIII. In AI, cleavages mediated by NEP-like enzyme were on the amino side of F and are consistent with the known specificity of vertebrate NEP, for cleavages on the amino side of hydrophobic residues [18]. Moreover, as regards experiences conducted with vertebrate NEP on different substrates, the ratio K_{cat}/K_m changes with the substrate used, e.g. K_{cat}/K_m: 69 for bradykinin, 16.7 for lysylbradykinin, 158 for substance P [14,18]. As suggested by Rosenbaum et al. [18], residues in the P2 through P4 positions also contribute to substrate binding, whereas those in a more N-terminal position contribute little to this process. The kinetics of degradation of AII and AIII are equivalent, because they possess the same N-terminal sequence except for a lack of an Asp residue in AIII. In the case of AI, its N-terminal part is identical to the ones of AII and AIII, but its C-terminal side is different. Its C-terminal extension (FHL) allows the NEP-like enzyme to attack the Phe residue at both sides (N and C). These arguments could explain the degradation kinetics constants of AI compared to those of AII and AIII.

4. Conclusion

In the first 15 min, about 30% of AI, AII and AII are metabolized. However, among this 30% degradation, a high percentage, ca. 20%, is attributed to the conversion of AI into AII, of AII into AIII and of AIII into AIV, the three active peptides in leech osmoregulation [1]. These results are in line with those of Zerbst-Boroffka [20]. This author has demonstrated by physiological experiments conducted in *Hirudo medicinalis* an 8-fold increase of the urine volume excreted 15 min after a blood meal, which persisted for many hours. Moreover, we have established that the AII amount greatly increased just after a blood meal [1] and we speculate that AII, AIII and IV increase diuresis by acting on their different targets as tegument, stomach or nephridia [1,3]. These data allow us to present the kinetic scheme of the relations existing between a blood meal, effective diuresis and the central nervous system (CNS) in which the angiotensin metabolism takes place. According to Wenning [21], stimulation of abdominal stretch receptors during feeding would cause hormonal release from the CNS. One of the hormone systems implicated in such a phenomenon is the angiotensins in leeches [1–3]. If we try to connect this physiological event to the enzymes involved in metabolism of angiotensins in CNS, we can distinguish three implicated enzymes: the ACE-like, the NEP-like enzymes and the AP whose efficiencies are in line with those of vertebrates [19].

The main enzyme participating in the degradation of the

angiotensins is the NEP-like enzyme. This peptidase acts firstly to yield the AII and secondly to inactivate AI, AII, AIII, by the action at Tyr^4-Ile^5 and Pro^7-Phe^8 bonds. The metabolized products are then cleaved by the action of AP. These enzymes could constitute the degradative pattern of angiotensins. In addition, our findings have led us to discover the existence of a different AP, confirmed with the use of amastatin. First, a DPAP seems to play a role at the N-terminal side of AI and its existence in invertebrates is sustained by recent results obtained in Cnideria [22]. Moreover, existence of an Asp-AP is sustained by the presence of AIII and by its diuretic activity in *T. tessulatum* [1]. For the Arg-AP, we established its presence in head membranes by detection of the AIV peptide. These different enzymes would be implicated at a high level in degradation of AI, AII and AIII, 30 min after their release and could be activated by a feedback after AII action on its targets. However, only the localization of these enzymes in the CNS would allow us to confirm this hypothesis and further immunocytochemical experiences with antisera raised against these purified enzymes are needed.

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