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Irreversible Photolabeling of Active Site of Neutral Endopeptidase-24.11 "Enkephalinase" by Azidothiorphan and [^{14}C]-Azidothiorphan

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SUMMARY

Azidothiorphan and its [^{14}C]-labeled analogue have been developed as photoaffinity ligands for the active site of the neutral endopeptidase 24.11. In *in vitro* assays azidothiorphan inhibits the endopeptidase activity with a K_i of 0.75 nM. After ultraviolet irradiation the inhibitor binds irreversibly to the enzyme, and many factors suggest that the photolabeling occurs at the active site. The binding is accompanied by a loss of enzymatic activity, and the inclusion of the competitive inhibitor thiorphan protects the endopeptidase from this inactivation. In addition the binding of another competitive inhibitor [^{3}H]*N*-[(*R,S*)-3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl]-glycine to the active site of endopeptidase-24.11 is inhibited after irradiation with azidothiorphan. Experiments with [^{14}C]-azidothiorphan have shown that very little nonspecific binding of inhibitor to enzyme occurs and the labeled probe remains bound under denaturing conditions. Azidothiorphan has also been found to produce a long-lasting naloxone-reversible analgesia after intracerebroventricular administration. The results show that azidothiorphan should prove useful both for structural studies and for investigations on the synthesis and turnover of the neutral endopeptidase-24.11.

The neutral endopeptidase (EC 3.4.24.11) is a membrane bound Zn metallopeptidase that cleaves peptides at the amino side of hydrophobic residues. Although present in several mammalian tissues, notably the kidney (1), interest has been mainly centered on the enzyme in the central nervous system, where it has been shown to participate in the degradation of the opioid peptides, methionine and leucine enkephalin (2). This inactivation process, involving cleavage of the peptides at their Gly^{3}-Phe^{4} bonds, has led to the enzyme sometimes being referred to as "enkephalinase." A role for the peptidase in substance P degradation has also been proposed but remains to be definitively established (3, 4).

All the Zn metallopeptidases so far studied appear to share a similar mechanism of action and have, to a greater or lesser extent, certain features in common at their active sites. Two of these enzymes, carboxypeptidase A and the bacterial enzyme thermolysin, have been extensively studied by X-ray crystallography (5, 6) and simplified models of their active sites used as the basis for the synthesis of various endopeptidase-24.11 inhibitors (7). One of these, thiorphan, was the first of several to be shown to produce naloxone-reversible analgesia in mice (8). Comparisons of the *in vitro* potencies of these inhibitors have indicated that the active site of endopeptidase-24.11 is more closely related to that of thermolysin (7), an observation in agreement with chemical modification studies (9) and the similar specificities of these enzymes (7). Further confirmation of this has come from the recent cloning and sequence analysis of the rabbit kidney enzyme, which has shown that certain amino acids, known to be essential at the active site of thermolysin, are conserved in endopeptidase-24.11 (10).

Nevertheless the polypeptide chain of endopeptidase-24.11 consists of 750 amino acids while that of thermolysin is only 316, and initial analysis of the available data shows that there is probably little overall homology between the active sites of the enzymes. In the absence of crystallographic data a detailed picture of the active site of endopeptidase-24.11 can only be obtained by computer graphics and requires more specific knowledge of the amino acids present.

For this purpose a derivative of thiorphan, azidothiorphan (*N*-[(*R,S*)-3-mercapto-2-*p*-azidobenzyl-1-oxopropyl]glycine) has now been developed and radioactively labeled by use of [1-^{14}C]glycine. We present here the syntheses and biochemical and pharmacological properties of azidothiorphan and its radioactive analogue (*N*-[(*R,S*)-3-mercapto-2-*p*-azido-benzyl propanoyl] [1-^{14}C]glycine), which behave as the first reported irreversible inhibitors of endopeptidase-24.11.

Materials and Methods

Hydroxybenzotriazole, diethylamine, and thiolactic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI),

ABBREVIATIONS: HPLC, high performance liquid chromatography; [^{3}H]HACBO-Gly, [^{3}H]*N*-[(*R,S*)-3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl]-glycine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

palladium-charcoal (10%) from Merck (Darmstadt, FRG), and glycine methyl ester hydrochloride from Bachem Fine Chemicals (Bubendorf, Switzerland). [^{3}H]HACBO-Gly (45 Ci/mmol) and thiorphan were synthesized as previously described (8, 11). [^{3}H]-Leu-enkephalin, [^{3}H]-D-Ala^2-Leu-enkephalin, and [I-^{14}C] glycine (52.4 mCi/mmol) were from CEA (Saclay, France), aminopeptidase N (EC 3.4.11.2) was from Boehringer Mannheim (FRG), and all other chemicals were purchased from Sigma (St. Louis, MO). Angiotensin converting enzyme (EC 3.4.15.1) was a generous gift from Professor P. Corvol (INSERM, Paris, France).

Syntheses of [*N*-[(*R,S*)-3-Mercapto-2-*p*-azidobenzyl propanoyl]glycine (Azidothiorphan) and *N*-[(*R,S*)-3-Mercapto-2-*p*-azidobenzyl propanoyl]-[1-^{14}C]glycine ([^{14}C]-Azidothiorphan)

Purity of intermediate products was checked by thin layer chromatography (Merck, 60F254 plates) with the following solvent systems (v/v): A, $CHCl_2$/ether (9:1); B, $CHCl_3$/MeOH (9:1); C, $CHCl_3$/MeOH/AcOH (9:1:9.1); D, butanol/AcOH/H_2O (4:1:1); E, $CHCl_3$/MeOHAcOH (7:3:0.5). Final products were run on HPLC (Waters, reverse phase μ-bondapak C_{18}, 3.9 × 30 cm) in CH_3CN/NH_4OAc (24:76), pH 4.2, at 1.2 ml/min and monitored at 210 nM. ^{1}H NMR spectra were taken with a Bruker WP 270 MHz in ($^2H_6$)Me_2SO using hexamethyldisiloxane as internal reference. Melting points of crystallized compounds are reported uncorrected. Microanalysis (C,H,N) of the synthesized products were in accordance with the theoretical values (±2%). A schematized representation of the syntheses is shown in Fig. 1.

Azidothiorphan

Step 1. 451 mg thiloacetic acid (5.9 mmol) were added to 767 mg *4* (3.7 mmol) of *p*-nitrobenzylacrylic acid (12) at 20°C. After stirring (30 min at 20°C, 24 hr at 70–80°C), excess thiolacetic acid was removed *in vacuo* and the crude product purified on silica gel by "Flash" chromatography (solvent $CHCl_3$:MeOH; 95:5) to give (*R,S*)-3-acetylthio-2-*p*-nitrobenzyl propanoic acid *1* as an oil (850 mg; 81%) R_F(B) 0.42.

Step 2. 5 ml of a 10% (w/v) suspension of Pd on charcoal in MeOH was saturated with H_2, and 400 mg of *1* (1.4 mmol) in 5 ml MeOH were added. After stirring under H_2 (3 hr, 20°C), filtration and evaporation *in vacuo*, (*R,S*)-3-acetylthio-2-*p*-amino benzyl propanoic acid *2* was obtained as a white solid (300 mg; 84%) m.p. 95°C, R_F(B) 0.33.

Step 3. 106 mg $NaNO_2$ (1.53 mmol) in 2 ml cold H_2O were slowly added to 243 mg of *2* (0.96 mmol) in 9.6 ml 2 M HCl at −10°C. After stirring (10 min, −10°C), excess $NaNO_2$ was destroyed with 8 M urea. Nitrous vapors were removed *in vacuo*, and 100 mg NaN_3 (1.53 mmol) in 2 ml H_2O were added at 0°C in the dark. After stirring (0°C, 1 hr) the pH was adjusted to 2 with 2 M NaOH and the solution extracted with ether; the organic phase was dried (Na_2SO_4) and evaporated *in vacuo* to give (*R,S*)-3-acetylthio-2-*p*-azidobenzyl propanoic acid *3* as an oil (2.13 mg; 80%) R_F(C) 0.52. Infrared absorption band was 2120 cm^{-1} (N_3).

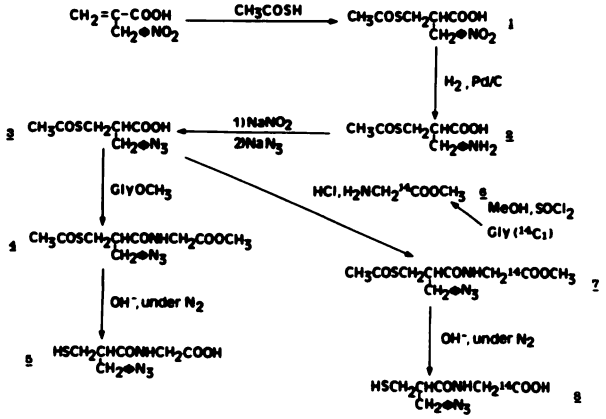

Fig. 1. Schematic representation of the syntheses of azidothiorphan and [^{14}C]azidothiorphan.

Step 4. Care was taken to avoid exposure to light during all the following steps. 68 mg glycine methyl ester hydrochloride (0.54 mmol), 55 mg triethylamine (0.54 mmol) in 3 ml AcOEt, 82 mg 1-hydroxybenzotriazole monohydrate (0.54 mmol) in 2 ml tetrahydrofuran, and 121 mg dicyclohexylcarbodiimide (0.6 mmol) in 1.5 ml AcOEt were successively added to 150 mg of *3* (0.54 mmol) in 1 ml AcOEt at 0°C. After stirring (hr at 0°C, 24 hr at 20°C) the dicyclohexylurea was removed by filtration, the solvents evaporated, and the residue dissolved in AcOEt. After further filtration the organic phase was washed (H_2O, 10% citric acid, H_2O, 10% $NaHCO_3$, H_2O, saturated NaCl) and evaporated *in vacuo*. *N*-[(*R,S*)-3-acetylthio-2-*p*-azidobenzyl 1-oxopropyl]-glycine methyl ester *4* was obtained as an oil (164 mg; 87%) R_F(B) = 0.72. Infrared absorption band was 2120 cm^{-1} (N_3).

Step 5. For this stage all solvents were degassed, and reactions were performed under a stream of N_2.

1 ml 1 M NaOH was slowly added (45 min) to 160 mg *8* (0.46 mmol) in 4 ml acetone/H_2O (2:1) at 0°C. After stirring (3 h, 20°C) the solvents were removed *in vacuo* and 1 ml H_2O added. The solution was washed (2 + 1 ml CH_2Cl_3), acidified (1 M HCl), extracted (3 × 3 ml $CHCl_3$) and the organic phase washed (saturated NaCl), dried (Na_2SO_4), and evaporated *in vacuo*. *N*[(*R,S*)-3-mercapto-2-*p*-azidobenzyl propanoyl]glycine *9* was obtained as beige solid (110 mg; 82%) m.p. 106–108°C; R_F(D) 0.74. Infrared absorption band at 2120 cm^{-1} (N_3). A single peak was obtained on HPLC, retention time (t_R) 10 min 36 sec.

[^{14}C]-Azidothiorphan

Step 1. 10.4 mg glycine (133 μmol) and 2.57 mg [1-^{14}C] glycine were suspended in 1 ml MeOH. 22 μl $SOCl_2$ (300 μmol) were added at 0°C and the mixture stirred (5 min at 0°C, 2 hr at 20°C) and refluxed (2.5 hr). The solvents were removed *in vacuo* and the residue dried in a dessicator overnight, then washed with ether. [1-^{14}C]glycine methyl ester hydrochloride *6* was obtained as a white solid (21 mg; 100%). R_F(E) 0.36, m.p. 175°C.

Step 2. *N*-[(*R,S*)-3-acetylthio-2-*p*-azidobenzyl propanoyl] [I-^{14}C]glycine methyl ester *11* was synthesized following the procedure described for *4*, using 21 mg [1-^{14}C] glycine methyl ester hydrochloride (167 μmol). An oil was obtained (42 mg; 70%) R_F(B) 0.7. Infrared absorption band at 2120 cm^{-1} (N_3).

Step 3. *N*[(*R,S*)-3-mercapto-2-*p*-azidobenzyl 1-oxopropyl] [1-^{14}C] glycine *8* was synthesized by the procedure described for *5* using 40 mg *7* (120 μmol). A beige solid was obtained (22.7 mg; 65%) m.p. 106–108°C, R_F(D) 0.72. Infrared absorption band at 2120 cm^{-1} (N_3). A single peak was obtained on HPLC (t_R 10 min 36 sec); specific activity was 10.41 mCi/mmol. The product was conserved in the dark in 10% ethanol solution containing 10 μM β-mercaptoethanol.

Purification and Assay of Endopeptidase-24.11

Endopeptidase-24.11 was purified from rabbit kidney as previously described (13), concentrated in an Amicon ultrafiltration unit using a YM10 membrane, and equilibrated against 50 mM Tris-HCl (pH 7.4 at 4°C) containing 1% wt/vol *n*-octyl-β-D-glucopyranoside. Enzyme activity was determined by measuring the initial rate of [^{3}H]-Tyr-Gly-Gly formation from [^{3}H]-leucine enkephalin, as previously described (14). For reversible inhibition studies with azidothiorphan all incubations were performed in the dark. The K_i was calculated from the Cheng-Prusoff equation assuming competitive inhibition (15). The inhibitory potency of azidothiorphan was also tested against aminopeptidase N, a dipeptidylaminopeptidase activity partially purified from rat brain (14) and angiotensin converting enzyme, using assays previously described (14).

Irreversible Binding of Azidothiorphan Ultraviolet Irradiation

Preincubation and irradiation were performed in 5-ml cylindrical polycarbonate tubes (1.1 cm diameter). Enzyme, with or without inhibitor, was first incubated in the dark for 30 min at 25°C in 50 mM Tris-HCl (pH 7.0) containing 1% *n*-octylglucosamine in a total volume of 100 μl. Irradiation, at 4°C, was performed at 254 nm using a 150-watt lamp (Prolabo, Paris, France). The flux was varied by changing the distance between the lamp and the surface of the liquid, and the intensity of the flux was measured with a VLX-254 radiometer (Vilber Lourmat, France). After irradiation the contents of the tubes were pooled, if required, or diluted to a volume of 1 ml and dialyzed against several changes of Tris buffer at 4°C. Nonirradiated samples, with and without inhibitor, were treated in the same manner. Enzyme without inhibitor was taken as control, and the dialysis was stopped when the activity of a nonirradiated sample, preincubated with azidothiorphan, reached control levels.

[^{3}H]-HACBO-Gly Binding

Enzyme was incubated with 10 nM [^{3}H]-HACBO-Gly and with or without 1 μM thiorphan at 25°C in 50 mM Tris-HCl, pH 7.0, in a volume of 100 μl. After 40 min bound and free ligand were separated over a column of G-25 (PD10, Pharmacia, Uppsala, Sweden) equilibrated in the same buffer. The column was eluted at a flow rate of 0.5 ml/min, and 0.25-ml fractions were collected for determination of radioactivity.

Irreversible Binding of [^{14}C]-Azidothiorphan to Endopeptidase-24.11

When [^{14}C]-azidothiorphan was used for irradiation, aliquots were taken after dialysis, mixed with an equal volume of 10% SDS, 0.1 mM dithiothreitol, and heated at 100°C for 10 min. The samples were then either subjected to SDS-PAGE followed by autoradiography, as described below, or passed over a column of PD10 equilibrated in 50 mM Tris-HCl (pH 7.0) containing 0.1% SDS. Fractions were collected for determination of radioactivity.

One dimensional SDS-PAGE was performed as described (16) using 8% polyacrylamide slab gels. The gels were dried under vacuum at 55°C, exposed to LKB (Les Ulis, France) Ultrofilm for 2 wk, and the film developed with D19 (Kodak).

Radioactivity was determined using a Betamatic (Kontron) liquid scintillation counter and biofluor (New England Nuclear) as scintillant, with efficiencies of 46 and 70% for [^{3}H] and [^{14}C], respectively

Protein was determined by the method of Bradford (1976) (17).

Analgesic Studies

Male Swiss mice (25 g, Charles River, Paris, France) received an intraventricular injection of either azidothiorphan or thiorphan (50 μg in 10 μl) 30 min before the start of the tests. Controls received an equivalent volume of physiological saline, and some animals were also injected intraperitoneally with 25 μg naloxone 15 min before the start of the tests. Analgesic potency was assessed, as previously described, using the hot plate jump latency test (18) and vocalization after electrical stimulation of the tail (19).

Additional animals, treated as above with saline, azidothiorphan, or thiorphan, were killed 90, 180, and 480 min after injection. The brains were removed and homogenized in 1 ml 50 mM Tris-HCl (pH 7.4), and aliquots (50 μl) were taken for determination of endopeptidase-24.11 activity. Included in the final assay volume of 100 μl were the aminopeptidase inhibitor bestatin (10^{-5} M) and the angiotensin converting enzyme inhibitor captorpil (10^{-6} M). Control tubes also contained the endopeptidase inhibitor, thiorphan (10^{-6} M). Reactions were initiated by the addition of substrate [^{3}H]-D-Ala^2-Leu-enkephalin (20 nM final concentration) and terminated after 45 min at 25°C by the addition of 10 μl of 0.5 M HCl, followed by centrifugation at 10,000 × *g* for 5 min. The metabolite [^{3}H]-Tyr-D-Ala-Gly was separated from [^{3}H]-Leu-D-Ala^2-enkephalin using columns of Poropak Q as previously described (14). Aliquots of the homogenate were also taken for protein determination.

Results

The K_i of azidothiorphan for endopeptidase-24.11 was calculated to be 0.75 ± 0.05 nM, compared with that previously reported for thiorphan of 1.8 nM (8). The introduction of an azido group into the para position of the phenyl ring of thiorphan therefore causes a slight increase in the affinity of the inhibitor for the enzyme. Moreover azidothiorphan displayed a high selectivity for the enzyme as shown by its K_i values for angiotensin converting enzyme (0.5 μM), aminopeptidase N (>10 mM), and the dipeptidylaminopeptidase (>10 mM). The latter two enzymes have also been proposed to participate in enkephalin degradation *in vivo* (21, 22), and angiotensin converting enzyme can cleave enkephalins at their Gly^3-Phe^4 bonds, although this is probably without *in vivo* significance in the central nervous system (23).

Analgesic studies. As previously reported (8, 10), intracerebro ventricular injections of thiorphan into mice were found to produce analgesia in both the vocalization and hot plate jump latency tests (Fig. 2). Azidothiorphan was also found to elicit analgesia in these tests (Fig. 2), and the antinociceptive activity of both inhibitors was blocked by prior administration of the opiate antagonist naloxone (data not shown). However, although the same dose (50 μg) of each inhibitor was found to produce equivalent levels of analgesia at the start of the tests the antinociceptive effect of azidothiorphan had a much longer duration. In both cases thiorphan-induced analgesia had fallen to nonsignificant levels at around 1 hr, whereas for azidothiorphan nonsignificant levels were only reached between 4 and 8 hr after the start of the tests.

Endopeptidase-24.11 activity was measured in brain homog-

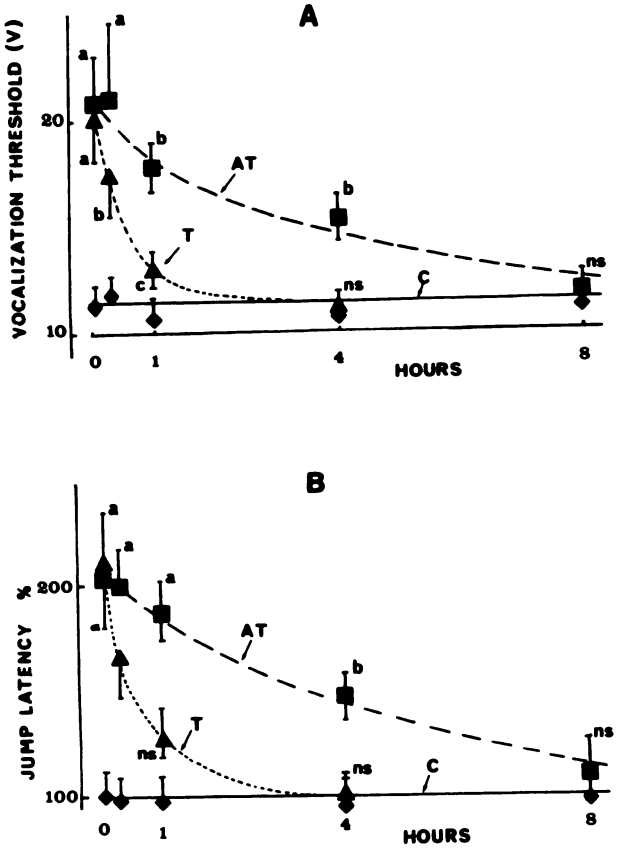

Fig. 2. Analgesic effect of intracerebroventricular administration of 50 μg thiorphan (T) and azidothiorphan (AT) as described in Methods. A: vocalization test; B: hot plate jump test. Values are means ± SD of between 15 and 20 separate determinations. Student's *t* test: a, $p < 0.001$; b, $p < 0.01$; c, $p < 0.05$ compared with control values (C); *ns*, nonsignificant.

TABLE 1

Endopeptidase-24.11 activity in brain homogenates from animals previously injected intracerebroventricularly with 50 μg of thiorphan or azidothiorphan

Injections, tissue preparation, and endopeptidase-24.11 assay were as described in Methods. Activity is expressed as percentage of controls and has been adjusted to include the relative protein concentrations. Results are means of two experiments performed in triplicate.

	Time after injection		
	90 min	180 min	8 hr
	% control		
Thiorphan	24.90 ± 1.57	60.20 ± 7.3	75.51 ± 16.5
Azidothiorphan	5.19 ± 1.51	14.01 ± 2.4	27.62 ± 3.3

enates various times after the intracerebro ventricular injection of either azidothiorphan or thiorphan to give a relative indication of the levels of enzyme inhibition. As shown in Table 1 the long-lasting analgesic effect of azidothiorphan appears to be paralleled by the degree of inhibition of the peptidase. Enzymatic activity in thiorphan treated animals was 25, 60, and 75% of the values found in control animals 90 min, 180 min, and 8 hr after injection, respectively. For azidothiorphan the comparable figures were 5, 14, and 27%. As previously discussed (7, 24), the involvement of both aminopeptidase N and the neutral endopeptidase in enkephalin metabolism means that a high level of inhibition of the latter enzyme is required to give a significant analgesia.

Irradiation conditions. To determine the conditions necessary for the irreversible binding of azidothiorphan, initial irradiation experiments were performed with the enzyme at a concentration of 20 nM. The concentration of inhibitor was varied (0.1–10 μM) as well as the flux (0.5–1.5 mW/cm²) and time (0–15 min) of irradiation.

When enzyme and inhibitor were irradiated at a flux of 0.5 mW/cm², full enzyme activity was recovered after dialysis, whereas at 1.5 mW/cm² the enzyme alone was rapidly inactivated. Optimum apparent irreversible inactivation of the enzyme was achieved by irradiating at 1.00 mW/cm² (72 cm between the lamp and liquid surface) with 1 μM azidothiorphan (Fig. 3). Immediately after irradiation no enzyme activity could be detected, and only 24% of this activity was recovered after dialysis, compared with nonirradiated controls. Enzyme irradiated without inhibitor retained 98% of its activity. The recovery of activity after dialysis is probably due to one or a combination of two factors. Either not all of the azide groups in the active sites were activated or a certain percentage of the bonds formed between the activated azide and the enzyme were unstable.

Increasing the concentration of azidothiorphan did not result in any further inactivation, and at 10^{-7} M the level of inhibition was reduced to 50%. Inclusion of the competitive inhibitor thiorphan (10^{-4} M) in the incubation was found to completely protect the enzyme from apparent irreversible inactivation by azidothiorphan.

[^{14}C]-Azidothiorphan. For work with [^{14}C]-azidothiorphan the concentration of the enzyme was greatly increased (8 μM) to facilitate future structural studies. The irradiation parameters were as before using an irradiation time of 2 min, and parallel experiments were performed with varying concentrations of both azidothiorphan and [^{14}C]-azidothiorphan to find conditions that gave maximum irreversible inhibition with minimum nonspecific binding. Over 80% apparent irreversible inhibition was achieved by using 29 μM azidothiorphan and by repeating the incubation-irradiation step three times. Fresh

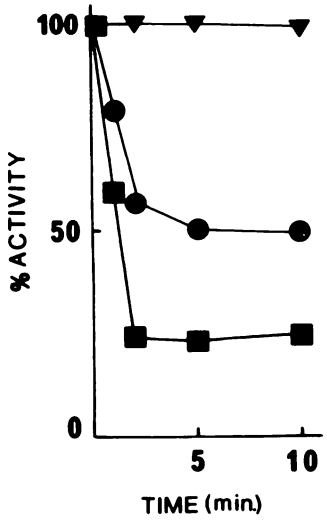

Fig. 3. Changes in endopeptidase-24.11 activity after irradiation with azidothiorphan. Enzyme (20 nM) was preincubated with the inhibitor before irradiation and dialysis, as described in Methods. Results are expressed as a percentage of nonirradiated controls. Irradiation conditions and inhibitor concentrations were: ▼, 0.5 mW/cm², 1 μM azidothiorphan; ●, 1.0 mW/cm², 0.1 μM azidothiorphan; ■, 1.0 mW/cm², 1 μM azidothiorphan.

inhibitor was added after each irradiation to bring the concentration back to original levels.

In addition to decreasing enzymatic activity, irradiating with azidothiorphan was also found to reduce the binding of the competitive inhibitor [^{3}H]-HACBO-Gly (K_i = 1.2 nM) to the active site of the enzyme. As shown in Fig. 4, when enzyme and inhibitor were preincubated as described in Methods and then passed over a column of PD10, two peaks of radioactivity were eluted, one at the void volume coeluting with enzyme and the second at the total column volume, representing free inhibitor. Including 1 μM thiorphan in the preincubation reduced the radioactivity eluted in the void volume by 98.5%, indicating that in the former case [^{3}H]-HACBO-Gly was bound to the active site of the enzyme. When aliquots of enzyme, irradiated with azidothiorphan as described above, were treated in a similar manner, binding of [^{3}H]-HACBO-Gly to endopeptidase-24.11 was found to be reduced to 18% of control levels.

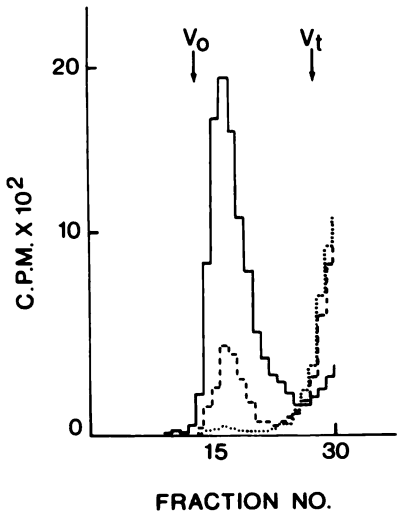

Fig. 4. Elution profile of [^{3}H]HACBO-Gly from a PD 10 column after incubation with endopeptidase-24.11 (0.1 μM) as described in Methods.—, unmodified enzyme; · · ·, in the presence of 1 μM thiorphan; – – –, after ultraviolet irradiation of enzyme and azidothiorphan. V_0, void volume; V_t, total volume.

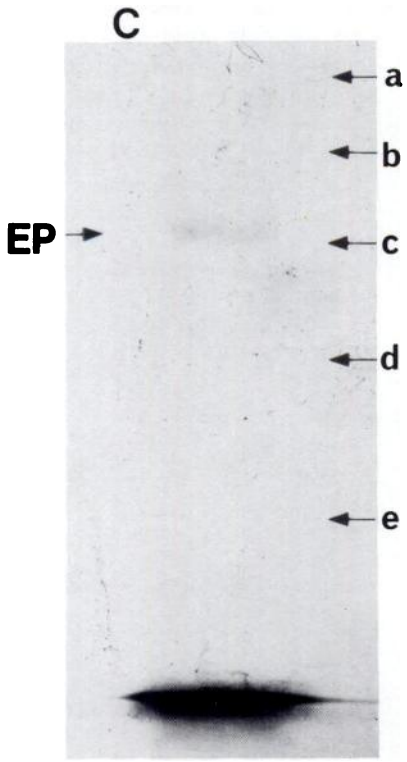

Fig. 5. Irreversible binding of [^{14}C]-azidothiorphan to endopeptidase-24.11 after ultraviolet irradiation. Enzyme and inhibitor were incubated and irradiated as described in Methods; 10 μg of enzyme were then run on SDS-PAGE, and after staining, the gel was subjected to autoradiography. Running positions of marker proteins are indicated: a, 200 K; b, 120 K; c, 92.5 K; d, 62.2 K; e, 45 K; EP, endopeptidase-24.11. C, control track containing nonirradiated enzyme and inhibitor.

The same experiment was repeated using [^{14}C]-azidothiorphan and aliquots taken and subjected either to SDS-PAGE and autoradiography or dialysis followed by PD10 chromatography under denaturing conditions. After SDS-PAGE and autoradiography a band of radioactivity was detected at 94 kD showing that the [^{14}C]-azidothiorphan was irreversibly bound to the enzyme (Fig. 5). In a control track containg nonirradiated enzyme, no evidence was found for binding of inhibitor to the enzyme without irradiation. The amount of radioactivity eluting in the void volume of a PD10 column, under denaturing conditions, was used to determine the degree of labeling of the enzyme. If inhibitor and enzyme were to bind with a 1:1 ratio, then 17,200 cpm/100 μg enzyme would be expected to be recovered. Under the conditions described above a ratio of total enzyme:inhibitor of 1:0.87 was found. Thus despite the fact that [^{14}C]-azidothiorphan was present in a large molar excess during irradiation very little nonspecific binding to the enzyme seemed to have occurred.

Discussion

It has recently been suggested that inhibitors of endopeptidase-24.11 represent "the next generation of analgesics" (25). However, for this to become a reality more detailed knowledge is required on the structure, function, synthesis, and turnover of the enzyme. The development of azidothiorphan and [^{14}C]-azidothiorphan should now facilitate both *in vitro* and *in vivo* studies.

Azidothiorphan acts as a strong competitive inhibitor of endopeptidase-24.11 and has been shown to bind irreversibly to the enzyme after ultraviolet irradiation. This is accompanied by a loss of enzyme activity and a concomitant reduction in the binding of the competitive inhibitor [^{3}H]HACBO-Gly to the active site, whereas another competitive inhibitor, thiorphan, protects the enzyme from loss of activity. In addition, studies using [^{14}C]-azidothiorphan showed that the degree of labeling of the enzyme is close to that expected on a 1:1 molar basis. It would therefore appear that during ultraviolet irradiation azidothiorphan binds irreversibly and primarily to the active site of the enzyme and that the covalent bond formed is stable under conditions such as prolonged dialysis and denaturing conditions (heating at 100°C and SDS-PAGE).

Azidothiorphan can thus be regarded as the first reported irreversible inhibitor of endopeptidase-24.11 and should prove useful not only for labeling large quantities of enzyme for structural studies but also for studying the synthesis and turnover of the enzyme in *in vitro* systems.

Originally designed for *in vitro* work on the active site of endopeptidase-24.11, the antinociceptive properties of azidothiorphan show that it should be additionally useful for *in vivo* studies. Its long-lasting analgesic activity, compared with thior-

phan, is unlikely to be related to inhibition of other enkephalin degrading enzymes, since azidothiorphan has a higher selectivity for endopeptidase-24.11 than its parent compound. Although obviously only an indirect assessment of the *in vivo* situation, the duration of analgesia appears to be reflected in endopeptidase-24.11 activity in brain homogenates from animals previously injected with the inhibitors. In thiorphan-treated animals analgesia had fallen to nonsignificant levels 90 min after injection, although there was still sufficient inhibitor remaining in the brain to inhibit 76% of the endopeptidase activity in the *in vitro* conditions employed. In contrast, with the azidothiorphan-treated animals, the level of *in vitro* inhibition was initially higher (95.5% after 90 min and 86% after 180 min) but had fallen to comparable levels 8 hr after injection, a time when its analgesic activity was also nonsignificant.

The differences in duration of action might therefore be partly due to differences in the K_I values and/or be due to differences in the metabolism of the two inhibitors, with the levels of azidothiorphan remaining above the threshold required for analgesic activity for a much longer time. Azidothiorphan may also irreversibly inhibit endopeptidase-24.11 *in vivo* even though no evidence was found for this to occur *in vitro* without ultraviolet irradiation. Nevertheless azides are known to form covalent bonds by direct σ-substitution of aromatic rings or through nucleophilic displacement of amino, hydroxyl, or thiol groups (26). One or more of these reactions might therefore occur with the appropriate amino acids of the enzyme in its *in vivo* environment without the necessity for ultraviolet irradiation. Further experiments, using tissues more enriched in endopeptidase-24.11 than the brain are now in progress to investigate this possibility.

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