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Biochemistry

Complete differentiation between enkephalinase and angiotensin-converting enzyme inhibition by *retro*-thiorphan

(enkephalinase inhibitors/*retro* peptides/analgesia)

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ABSTRACT **Thiorphan, *N*-[(*R,S*)-3-mercapto-2-benzylpropanoyl]glycine is a highly potent inhibitor (K_i = 3.5 nM) of "enkephalinase," a metalloendopeptidase cleaving the Gly-Phe bond (positions 3 and 4) of enkephalins in brain tissue. In accordance with this property, thiorphan displays antinociceptive activity after systemic administration. However, thiorphan also inhibits to a lesser extent (K_i = 140 nM) the widely distributed angiotensin-converting enzyme, a carboxydipeptidase implicated in blood pressure regulation. Therefore, in view of an eventual clinical use of enkephalinase inhibitors, it was very important to develop fully specific compounds. Such derivatives were obtained taking into account that *N*-methylation of the ultimate amide bond of dipeptides strongly decreases enkephalinase affinity without affecting angiotension-converting enzyme recognition, whereas *retro-inversion* of the amide bond leads to the inverse effect. Thus, the *retro-inverso* dipeptide (*R*)-H_2N-CH($CH_2\phi$)-*NHCO*-CH_2-CO_2H exhibits an inhibitory potency on enkephalinase ($IC_{50} \approx 12$ μM) close to that of the natural dipeptide L-Phe-Gly ($IC_{50} \approx 3$ μM). This result shows the topological analogy between the crucial components involved in enkephalinase recognition both in active dipeptides and structurally related *retro-inverso* isomers. Taking into account these observations, *retro*-thiorphan, (*R,S*)-HS-CH_2-CH-($CH_2\phi$)-*NHCO*-CH_2-COOH, was prepared. As compared to thiorphan, the *retro* isomer is 50% as potent (K_i = 6 nM) on enkephalinase but displays a drastic loss of potency on angiotension-converting enzyme (IC_{50} > 10,000 nM). This specificity was interpreted as a consequence of differences in the stereochemical constraints involving enzyme–inhibitor hydrogen bonding. This hypothesis is supported by reported crystallographic studies on related enzymes such as thermolysin and carboxypeptidase A. As expected, *retro*-thiorphan exhibits about the same analgesic potency as thiorphan on the hot plate and writhing tests in mice. Therefore, the topological concept of *retro-inverso* isomers could be extended to other enkephalinase inhibitors, allowing the design of potent and highly selective compounds occurring as new classes of analgesic and psychoactive agents.**

An important approach in the search for antinociceptive agents is to prevent degradation of the endogenous morphine-like peptides, enkephalins (1, 2), at the synaptic level. The characterization of a membrane-bound metalloendopeptidase called enkephalinase (3–5), which seems to be specifically involved in cleavage of the Gly-Phe (positions 3 and 4) bond of [Met]enkephalin (Tyr-Gly-Gly-Phe-Met) or [Leu]enkephalin (Tyr-Gly-Gly-Phe-Leu), made this approach possible. A highly potent enkephalinase inhibitor, thiorphan, *N*-[(*R,S*)-3-mercapto-2-benzylpropanoyl]glycine [K_i = 3.5 nM (6, 7)], was rationally designed taking into account the occurrence in this peptidase of (*i*) a Zn atom in its catalytic site able to coordinate a thiol group with a strong affinity (7, 8); (*ii*) a S_1' subsite exhibiting a specificity for aromatic or hydrophobic moiety (8, 9); (*iii*) a moderate but significant preference of the S_2' subsite for short side chains (9); and (*iv*) an enhanced affinity for substrates bearing a free COOH-terminal carboxyl group (10). As expected, thiorphan displays a naloxone-reversible analgesia after systemic administration in mice (7). The antinociceptive effect of thiorphan and its ability to prevent *in vivo* degradation of exogenously administered enkephalins has been widely confirmed recently (11–14).

Nevertheless, the inhibitory potency of thiorphan (K_i = 3.5 nM) is only 40 times stronger against enkephalinase than against angiotensin-converting enzyme [ACE; K_i = 140 nM (7)], a carboxydipeptidase that also displays some ability to split the Gly-Phe bond (positions 3 and 4) of enkephalins (15, 16). Therefore, given the wide distribution of ACE and its involvement in blood pressure regulation, it is of great importance to develop enkephalinase inhibitors exhibiting both a strong potency and a selectivity as high as possible, in preview of their eventual clinical use.

Several groups purified enkephalinase from various tissues (5, 17–20). It was suggested that the mouse brain enkephalinase was identical (19) to an endopeptidase from bovine pituitary (18) and to a neutral endopeptidase from rabbit kidney (21). Although enkephalinase and ACE belong to different groups of peptidases it seems difficult to obtain highly potent and selective inhibitors by systematic chemical changes of the residues implicated in S_1' or S_2' active sites recognition. On the contrary, amide bond *N*-methylation of selected dipeptides strongly decreases their inhibitory potency on enkephalinase without significant changes on ACE (8, 9). This suggests that amide bond modification is a definite means of differentiation between enkephalinase and ACE binding sites. Synthesis and biological activities of *retro*-thiorphan (*R,S*)-HS-CH_2-CH($CH_2\phi$)-*NHCO*-CH_2-COOH reported in this paper demonstrate the validity of this heuristic approach in the design of highly potent and fully selective enkephalinase inhibitors (6).

MATERIALS AND METHODS

Chemistry

(*R,S*)-1-Acetylthio-2-(*N*-Benzyloxycarbonyl)-Amino-3-Phenylpropane. To a solution of (*R,S*)-3-acetylthio-2-benzylpropa-

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Abbreviations: ACE, angiotension-converting enzyme; i.c.v., intracerebroventricular(ly); i.p., intraperitoneal(ly).

noic acid (22) (2.6 g; 11 mmol) in toluene 2.5 ml (11.5 mmol) of diphenylphosphorylazide and 1.6 ml of triethylamine were added. The mixture was stirred at 80°C for 1 hr, brought at room temperature for the addition of 1.4 ml of benzylic alcohol, and heated again at 80°C for 18 hr.

The organic layer was evaporated to dryness and the residue was dissolved in ethyl acetate. The solution was washed, dried (Na_2SO_4), and evaporated to dryness. A solid, recrystallized from Et_2O, was obtained (3.45 g; 93%); melting point = 97°C; R_f ($CHCl_3/CH_3OH/H_2O$, 9:1:saturated) = 0.93.

Analysis Calculated for $C_{19}H_{21}NO_3S$: C, 66.46; H, 6.16; N, 4.08; S, 9.32. Found: C, 66.12; H, 6.28; N, 4.19; S, 9.21.

1,1′-Dithiobis- [(*R,S*)-2-(*N*-Benzyloxycarbonyl)- Amino-3-Phenylpropane]. To a solution of 2.2 g (6.4 mmol) in methanol of the preceding compound, cooled at 0°C, were added 7.7 ml of 1 M NaOH and the mixture was stirred for 2 hr at 0°C. A solution of I_2 in ethanol was added dropwise until a yellow persistent color was obtained.

The white precipitate, 1.92 g (77%), was recrystallized from methanol; melting point = 134°C; R_f ($CHCl_3/CH_3OH$, 8:2) = 0.73.

Analysis Calculated for $C_{34}H_{36}N_2O_4S_2$: C, 67.98; H, 6.04; N, 4.66; S, 10.66. Found: C, 68.30; H, 6.13; N, 4.54; S, 10.82.

1-1′-Dithiobis-[(*R,S*)-2-Amino-3-Phenylpropane],Bis Bromhydrate. To a solution of 1.5 g (2.5 mmol) of the preceding compound in 15 ml of acetic acid were added 0.80 ml of 30% HBr in acetic acid and 15 ml of methanol. The mixture was stirred for 5 hr at room temperature; then it was evaporated to dryness and the residue was washed with anhydrous ether. A white solid was obtained (1.20 g; 97%); melting point = 260°C; R_f (butanol/acetic acid/H_2O, 4:1:1) = 0.50.

1-1′-Dithiobis-[(*R,S*)-2-(*N*-Benzyloxymalonyl)-Amino-3-Phenylpropane]. To a solution of 0.76 g (1.54 mmol) of the preceding disulfur and 0.44 ml of triethylamine in $CHCl_3$ were successively added 0.6 g (3.08 mmol) of benzyl hydrogen malonate and 0.47 g (3.08 mmol) of 1-hydroxybenzotriazole in tetrahydrofuran; then 0.70 g (3.4 mmol) of dicyclohexylcarbodiimide in $CHCl_3$ was added. After stirring overnight at room temperature and filtration of the precipitate, the solvents were evaporated to dryness. The residue was dissolved in ethyl acetate and the solution was washed successively with 10% citric acid, H_2O, and 10% $NaHCO_3$. The organic solvent was evaporated to dryness and the residue was chromatographed over silica gel ($CHCl_3$/ether, 5:5). A white solid (0.61 g; 58%) was obtained and recrystallized from methanol; melting point = 102°C; R_f ($CHCl_3/CH_3OH/H_2O$, 9:1:saturated) = 0.81.

Analysis Calculated for $C_{38}H_{40}N_2O_6S_2$: C, 66.65; H, 5.89; N, 4.09; S, 9.35. Found: C, 66.23; H, 5.80; N, 4.28; S, 9.60.

(*R,S*)-1-Mercapto-2- (*N*-Malonyl)- Amino-3-Phenylpropane: *Retro*-Thiorphan. The preceding compound (0.50 g; 0.73 mmol) was dissolved in 10 ml of acetone and 1.5 ml of 1 M NaOH was added dropwise at 0°C. The mixture was stirred for 1 hr at 0°C and for 3 hr at room temperature. The solvent was evaporated and the residue was dissolved in water. The solution was washed with ethyl acetate, acidified to pH 2, and extracted with ethyl acetate. The organic layer was dried (Na_2SO_4) and evaporated to dryness. The yellow oily product (0.30 g; 82%) obtained was dissolved in methanol and 5.2 ml of 1 M HCl and 0.15 g of Zn powder were added. The mixture was stirred for 4 hr at 20°C. After filtration and evaporation of the solvent, the residue was purified by chromatography over silica gel [$(CH_3)_2CO/H_2O$/AcOH, 20:0.1:0.1]. A white solid was obtained (0.18 g; 60%); R_f (butanol/acetic acid/H_2O, 4:1:1) = 0.73; melting point = 84–86°C.

Analysis Calculated for $C_{12}H_{15}NO_3S$: C, 56.92; H, 5.97; N, 5.53; S, 12.64. Found: C, 56.35; H, 6.03; N, 5.38; S, 12.48.

NMR (deuterated dimethylsulfoxide): *HS* = 2.20 ppm; CH_2SH = 2.42 ppm; $CH_2\beta$ = 2.62 and 2.74 ppm; $COCH_2$ = 2.99 ppm; CH_α = 2.84 ppm; ϕ = 7.15 ppm; NH = 8 ppm; COOH = 12.39 ppm.

Synthesis of *Retro-Inverso* Dipeptides. *Retro-inverso* dipeptides were synthesized according to previously described methods (23, 24) by condensation of a monoprotected *gem*diamine obtained through Curtius rearrangement and a monoester of malonic or substituted malonic acid. Saponification of the ester group followed by removal of the protecting residue (*t*-butyloxycarbonyl) by trifluoroacetic acid led to the free *retro-inverso* dipeptides. Curtius rearrangement proceeding with retention of configuration, the *gem*diamino part of *retro-inverso* peptides was obtained with a full optical purity. The ethyl hydrogen methylmalonate being used as a racemic mixture, the *retro-inverso* Phe-Ala was obtained as a 50:50 diastereoisomeric mixture of *RR* and *RS* isomers. Structures of the different compounds were checked by ^1H NMR (270 MHz).

Retro-inverso-*L-Phe(D,L)-Ala* (R,R *and* R,S *isomers*), *compound 2.* R_f (butanol/acetic acid/H_2O, 4:1:1) = 0.35.

NMR (deuterated dimethylsulfoxide): two series of signals for the methylmalonyl part of the spectrum: CH_3 = 0.92 and 1.07 ppm; $CH_2\beta$ = 2.84 ppm; *CH*-COOH = 3.11 and 3.14 ppm; CH_α = 4.82 ppm; ϕ = 7.20 ppm; NH = 8.67 and 8.72 ppm.

Analysis Calculated for $C_{12}H_{16}N_2O_3$: C, 61.00; H, 6.83; N, 11.86. Found: C, 60.82; H, 6.95; N, 11.99.

Retro-inverso-*L-Phe-Gly* (R *configuration*), *compound 4.* R_f (butanol/acetic acid/H_2O, 4:1:1) = 0.15; melting point = 151°C; $[\alpha]_D^{20} = +13.4 \pm 1.2°$.

NMR (deuterated dimethylsulfoxide): $CH_2\beta$ = 2.95 ppm; CH_2-COOH = 3.09 ppm; CH_α = 5.02 ppm; ϕ = 7.27 ppm; NH = 9 ppm.

Analysis Calculated for $C_{11}H_{14}N_2O_3$: C, 59.45; H, 6.35; N, 12.60. Found: C, 59.86; H, 6.52; N, 12.75.

Retro-inverso-*D-Phe-Gly* (S *configuration*), *compound 6.* R_f (butanol/acetic acid/H_2O, 4:1:1) = 0.15; melting point = 151°C; $[\alpha]_D^{20} = -12.9 \pm 1.2°$.

Analysis Found: C, 59.78; H, 6.22; N, 12.83.

Biological tests

Assays for Enkephalinase Activity. Enkephalinase activity was monitored by using the particulate P_2 fraction of mouse striatum (25) or partially purified enkephalinase obtained from mouse brain according to previously described methods (5, 20). The solubilized enzyme exhibits a specific activity of 1,400 pmol/min per mg, close to that reported for rat brain enkephalinase (5, 20). [*Tyrosyl*-3,5-^{3}H(N)][D-Ala^2,Leu]enkephalin was used as substrate and the formed metabolite, [^{3}H]Tyr-D-Ala-Gly, was measured by liquid scintillation spectrometry after chromatography separation on a polystyrene bead column as reported in detail elsewhere (7, 25).

Assays for ACE Activity. P_2 fraction of mouse striatum or purified ACE from porcine plasma (Calbiochem) was used as the enzyme source. Inhibitory potency of *retro*-thiorphan was determined by using hippuryl-His-Leu (Bachem, Bubendorf, Switzerland) as the substrate following described procedures (7).

Pharmacological assays

Male Swiss albino mice (CD 1, Charles River Breeding Laboratories, 24–26 g) were used. All drugs are injected intracerebroventricularly (i.c.v.), except naloxone, which was administered intraperitoneally (i.p.). The i.c.v. injections (10 μl) were performed according to the method of Haley and McCormick (26). Control animals were injected with saline.

Hot Plate Test. This test was derived from that of Eddy *et al.* (27). Ten minutes after the i.c.v. injection of the drug, the animals were set on a plate heated to 55 ± 0.5°C and the jump latency was measured up to 240 s (cut-off time).

Phenylbenzoquinone Test. This test was derived from that of Siegmund *et al.* (28). Ten minutes after i.c.v. injection of the drug, the animals were injected i.p. with a phenylbenzoquinone solution and 10 min later abdominal writhings were counted over a period of 10 min. Phenylbenzoquinone (Sigma) was dissolved in absolute ethanol (2 mg in 0.5 ml) and the solution was adjusted to 10 ml with distilled water. This latter solution was kept at 37°C during the experiments and was injected at this temperature.

Statistical Comparisons. Means were compared to respective controls by Student's *t* test.

RESULTS AND DISCUSSION

According to the concept of topological analogy developed by Goodman and Chorev (23, 29), a strict structural similarity between a natural dipeptide and its *retro-inverso* isomer requires: (*i*) respective replacement of both the NH_2- and COOH-terminal amino acids by their corresponding *gem*diaminoalkyl and malonyl analogs and (*ii*) appropriate chirality of the new residues allowing similar spatial orientation of the side chains in both the natural and modified dipeptides. All of these manipulations, leading to the end-modified *retro-inverso* dipeptides 2, 4, and 6 (Table 1), were performed according to previously described methods (23, 24).

Synthesis of the *retro-inverso*-Phe-Ala, compound 2, results in a mixture of *R,S* and *R,R* diastereoisomers. The separation of this mixture failed despite several assays performed by use of HPLC with a reversed-phase μBondapak C_{18} column used under different conditions. The relatively good activity of the mixture of *R,S* and *R,R* isomers of *retro-inverso*-Phe-Ala, compound 2, was tentatively interpreted as a consequence of the structural analogy between the *R,R* isomer and the natural dipeptide L-Phe-L-Ala, compound 1 ($IC_{50} \approx 1$ μM). Indeed, the dipeptide L-Phe-D-Ala corresponding to the *R,S* isomer of compound 2 displays a much lower activity than L-Phe-L-Ala on enkephalinase ($IC_{50} \approx 100$ μM). This drastic loss of enkephalinase inhibition after a single modification of one of the two amino acids of a natural dipeptide agrees with previous studies (8, 9). Nevertheless, to confirm the assumed relationship between enkephalinase inhibition and spatial orientation of the amino acid side chains, pure *R* and *S* isomers of the *retro-inverso*-Phe-Gly were synthesized. As shown in Table 1, potency of the (*R*)-*retro-inverso*-Phe-Gly, compound 4 ($IC_{50} \approx 12$ μM), is close to that of its corresponding dipeptide L-Phe-Gly, compound 3 ($IC_{50} \approx 3$ μM). On the contrary, the (*S*)-*retro-inverso*-Phe-Gly, compound 6, displays an activity that is 1/8th as much ($IC_{50} \approx 100$ μM), corresponding to that of D-Phe-Gly, compound 5. These results show that the crucial components involved in enkephalinase active site recognition (P_1' benzyl residue, NH_3^+ and COO^- groups) can fit to their respective subsites in the active dipeptides as well as in their structurally related *retro-inverso* isomers. These results also show that despite the amide bond reversal, the oxygen and hydrogen atoms fill similar spatial positions in both *retro* and natural amide bonds (Fig. 1). Obviously, due to the differences in subsite selectivity between enkephalinase and ACE (8, 9), all of the compounds shown in Table 1 are inactive on the latter enzyme.

Table 1. Inhibitory potency of dipeptides and their *retro-inverso* analogs on enkephalinase activity from mouse striata*

	Compound	IC_{50}, μM†
1	H_2N-CH($CH_2\phi$)-CONH-CH(CH_3)-COOH (*S,S*)	1.0 ± 0.2
2	H_2N-CH($CH_2\phi$)-*NHCO*-CH(CH_3)-COOH (*R,R* and *R,S*)	10.0 ± 2.0
3	H_2N-CH($CH_2\phi$)-CONH-CH_2-COOH (*S*)	3.0 ± 0.5
4	H_2N-CH($CH_2\phi$)-*NHCO*-CH_2-COOH (*R*)	12.0 ± 2.0
5	H_2N-CH($CH_2\phi$)-CONH-CH_2-COOH (*R*)	≈100
6	H_2N-CH($CH_2\phi$)-*NHCO*-CH_2-COOH (*S*)	≈100

* Concentration inhibiting 50% of the activity of mouse striatal enkephalinase (P_2 fraction) in Tris·HCl (pH 7.4): 50 μM; 25°C with 40 nM [*tyrosyl*-3,5-^{3}H(N)][D-Ala^2,Leu]enkephalin as substrate (25). Similar values were obtained for compounds 1–4 by using partially purified enkephalinase from mouse brain.

† Values are the means ± SEM from five independent experiments computed by log probit analysis of five inhibitor concentrations.

Taking into account the good potency of *retro-inverso* dipeptides of appropriate chirality on enkephalinase, the amide bond modification was introduced into *retro*-thiorphan through the steps outlined in Fig. 2. As in the case of thiorphan (7), *retro*-thiorphan was obtained as a mixture of *R,S* enantiomers and this mixture was used without separation for biological studies. Fig. 3 shows that *retro*-thiorphan is a highly potent and competitive inhibitor of enkephalinase. Indeed, when the enzymatic assays were performed under exactly the same conditions, the K_i of *retro*-thiorphan (6 nM) was very close to that of thiorphan, K_i = 3.5 nM. No significant change in the K_i values of these inhibitors was observed by using P_2 fractions of mouse striatal membranes instead of partially purified enzyme. The same observation was reported for inhibition of membrane-bound or purified enkephalinase from rat kidney by thiorphan (19). Furthermore, *retro*-thiorphan exhibits a very weak inhibitory potency ($IC_{50} >$ 10 μM) on several metallopeptidases, such as the membrane-bound aminopeptidases cleaving the Tyr-Gly bond (positions 1 and 2) of enkephalins (30), the microbial endopeptidase thermolysin, and carboxypeptidase A. Nevertheless, the most exciting result is the high selectivity of *retro*-thiorphan for enkephalinase, clearly demonstrated by its IC_{50} value that is >10,000 nM on ACE activity. This feature is probably related to different structural requirements for hydrogen bond formation of the amide (or *retro*-amide) groups within active sites of both enzymes.

Recent structural determination by x-ray crystallography of the

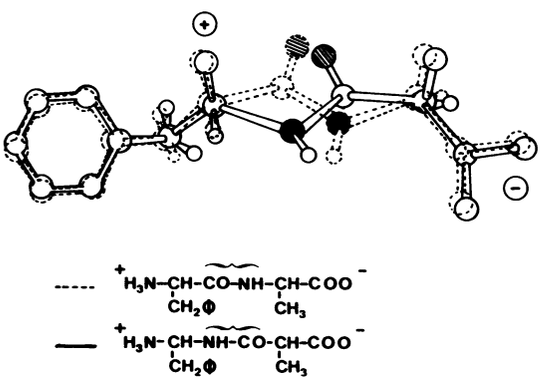

FIG. 1. Topological analogy between L-Phe-L-Ala and its (*R,R*)-*retro-inverso* analog.

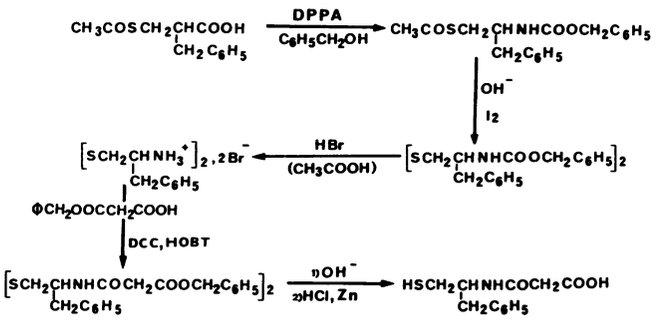

FIG. 2. Scheme for the synthesis of *retro*-thiorphan.

complex between the Zn metalloendopeptidase thermolysin and the thiol inhibitor (2-benzyl-3-mercaptopropanoyl)-L-alanylglycinamide provided evidence for (*i*) the expected coordination of the sulfur atom with the Zn of the peptidase and (*ii*) the involvement of the carbonyl oxygen of the inhibitor Ala-Gly peptide bond in two strong hydrogen bonds with the guanidinium moiety of the Arg-205 residue of thermolysin (31). It is interesting to note that, even when a free carboxyl group is present in the vicinity of the ultimate amide bond of a complexed inhibitor, the arginine side chain of thermolysin remains hydrogen-bonded to the amide carbonyl group. This was shown in the complex between thermolysin and phosphoramidon *N*-(α-L-rhamnopyranosyl-oxyhydroxyphosphinyl)-L-leucyl-L-tryptophan (32). Owing to the endopeptidase activity of enkephalinase (17–20) and to the occurrence of an arginine residue in its active site (19), it can be inferred that the geometry of thiorphan binding to enkephalinase is similar to that of the (2-benzyl-3-mercaptopropanoyl)-L-alanylglycinamide inhibitor to thermolysin. Therefore, the formation of hydrogen bonds between the amide carbonyl group of *retro*-thiorphan and enkephalinase is still possible because the arginine side-chain flexibility probably allows overcoming the small change in the position of the oxygen atom in the *retro*-amide bond as compared to the natural amide bond.

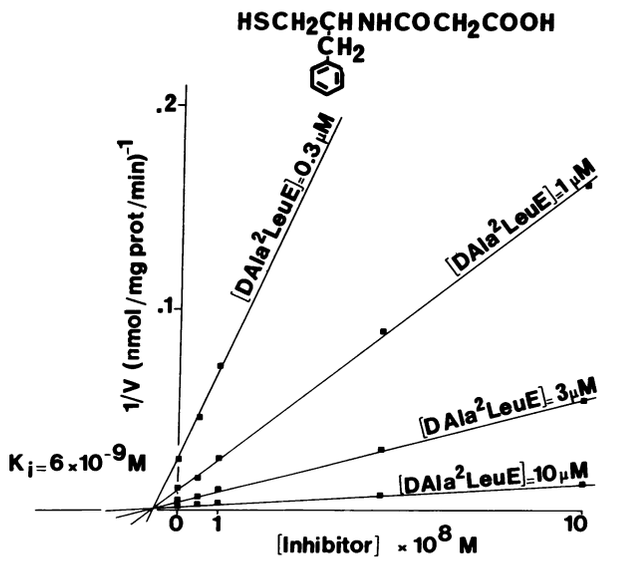

FIG. 3. Inhibition of partially purified enkephalinase from mouse brain by *retro*-thiorphan performed at 25°C in 50 mM Tris·HCl buffer (pH 7.4) by using 40 nM [*tyrosyl*-3,5-^{3}H(N)][D-Ala^2,Leu]enkephalin as substrate and unlabeled [D-Ala^2,Leu]enkephalin at the indicated concentrations (incubation time, 20 min); K_m = 20 μM for [D-Ala^2,Leu]enkephalin. Each point of the Dixon plots represents the mean of triplicate determinations. This experiment was repeated five times, leading to a mean K_i value (±SEM) of 6 ± 1.5 nM.

Because the molecular structure of carboxydipeptidases remains unknown, their active sites were often compared to that of carboxypeptidase A (33, 34). Crystallographic measurements performed on carboxypeptidase A showed that, unlike thermolysin, the guanidinium group of the arginine residue present in the active site strongly interacts with the free carboxyl group of substrates or inhibitors (35). Therefore, in a carboxydipeptidase such as ACE, it was assumed that the carbonyl group of the ultimate amide bond of substrates or inhibitors was hydrogen-bonded to an acceptor group of the enzyme backbone (33, 36). This type of interaction is much more stringent than that involving a flexible arginine side chain. Therefore, the specific loss of *retro*-thiorphan inhibitory potency on ACE suggests that this crucial hydrogen bond cannot be formed in ACE.

Retro-thiorphan and thiorphan, having similar inhibitory potencies on enkephalinase, also elicit similar biological activities. Indeed, Fig. 4 shows that i.c.v. injection of both thiol inhibitors leads to similar antinociceptive responses in mice when using the hot plate and writhing tests. The analgesic effect of thiorphan and *retro*-thiorphan arises from the protection of endogenous enkephalins. The involvement of these peptides in pain regulation through opiate receptor stimulation is clearly demonstrated by the antagonist effect of naloxone in the pharmacological assays (Fig. 4). Because of the somewhat large concentration required to obtain analgesic responses, thiorphan and

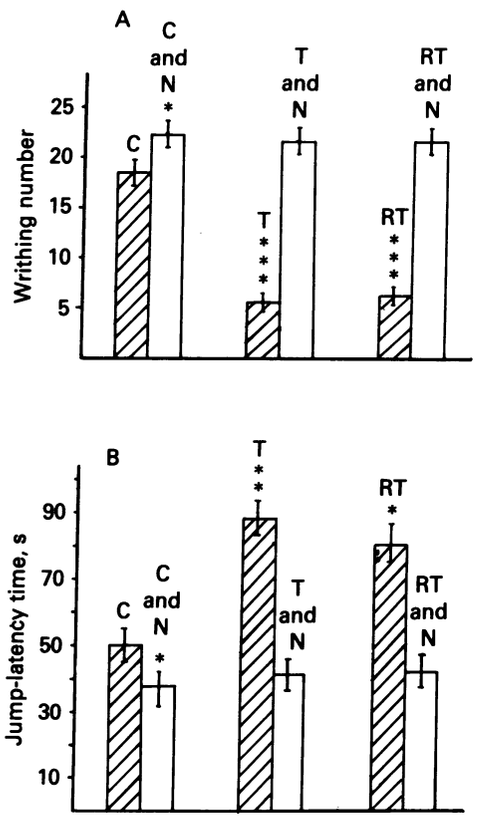

FIG. 4. Comparative analgesic effects of thiorphan and *retro*-thiorphan. (*A*) Writhing test. Thiorphan and *retro*-thiorphan were injected i.c.v. (50 μg/10 μl of saline per mouse; n = 12) 10 min after phenylbenzoquinone was administered i.p. and 10 min later the writhing number was determined over a period of 10 min. Naloxone (1 mg/kg) was injected i.p. 10 min before i.c.v. administration of thiorphan or *retro*-thiorphan. (*B*) Hot plate test. Temperature, 55 ± 0.5°C. Thiorphan and *retro*-thiorphan were injected i.c.v. (50 μg/10 μl of saline per mouse; n = 12). Naloxone (1 mg/kg) was injected i.p. 5 min before administration of inhibitors. T, thiorphan; RT, *retro*-thiorphan; N, naloxone; C, control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

retro-thiorphan are used at this time only as very useful probes to study the physiological role of the enkephalinergic system (7, 11–14).

As discussed in the first part of this work, the design of potent and highly selective enkephalinase inhibitors is of crucial importance for their eventual therapeutical use. Therefore, a very active and specific inhibitor, *retro*-thiorphan, was prepared taking into account both the active site model (33, 36) and the topological concept of *retro-inverso* isomers (23, 29). In addition, the *retro-inversion* of an amide bond in peptides or related compounds bestows on these molecules an enhanced stability to proteolytic enzymatic degradation. Obviously, this heuristic approach can be extended to other types of potent inhibitors, because most of them contain a terminal amide bond such as carboxyl-alkyl dipeptides (37) or phosphoryl dipeptides (38).

Finally, it is very interesting to observe that, from a molecular point of view, incorporation of a proline as last residue in inhibitors as well as *retro-inversion* or *N*-methylation of their ultimate amide bond play opposite roles in regard to enkephalinase or ACE inhibition. Therefore, systematic use of these changes in several kinds of inhibitors is necessary to clarify the differences between the active sites of the two enzymes.

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