J. Enzyme Inhibition, 1989, Vol. 2, pp. 269–277
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INTERACTION OF ANGIOTENSIN I-CONVERTING ENZYME WITH TWO POTENT TRICYCLIC INHIBITORS

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(Received 5 April 1988)

Inhibition of rabbit lung angiotensin I-converting enzyme was studied with two inhibitors that combined tricyclic mimics of a substrate C-terminal dipeptide recognition unit with a 4-phenylbutanoic acid fragment. The overall inhibition constant for [4S-[4α,7α(R*),12bβ]]-7-[S-(1-carboxy-3-phenylpropyl)amino]-1,2,3,4,6,7,8,12b-octahydro-6-oxopyrido[2,1-a][2]benzazepine-4-carboxylic acid (MDL 27,088) was approximately 4 pM, whereas that for [4R-[4α,7α(S*),12bβ]]-7-[S-(1-carboxy-3-phenylpropyl)amino]-3,4,6,7,8,12b-hexahydro-6-oxo-1H-[1,4]thiazino[3,4-a][2]benzazepine-4-carboxylic acid (MDL 27,788) was estimated to be 46 pM. The formation of an initial complex of target enzyme and MDL 27,088 and its slower isomerization to a second complex were characterized kinetically. Both compounds appear to be among the most potent inhibitors known for this enzyme.

KEY WORDS: Angiotensin I-converting enzyme, ACE, MDL 27,088, MDL 27,788, slow, tight binding type, inhibition.

INTRODUCTION

Highly potent inhibitors of angiotensin I-converting enzyme (peptidyl dipeptidase, EC 3.4.15.1; ACE†) have been developed in recent years. Captopril[2] is the premier example of the class of mercaptoalkanoyl inhibitors, in which a substrate-mimicking fragment is coupled to a thiol fragment oriented to coordinate the ACE active site zinc ion. The potency of the sulfhydryl inhibitors has been surpassed by several compounds in which a carboxylate function serves as metal ligand. The lower affinity of this ligand for zinc ion, relative to a mercaptan, is compensated for by the incorporation of additional lipophilic bulk in that portion of the inhibitor which binds at the S1 subsite.‡ The carboxylate also may simulate to the enzyme a late transition state of substrate hydrolysis.[3] MK 422 is the prototype of the phenylbutanoic acid inhibitors.[4] Structure-activity relationships of myriad examples of both classes of in-

* Correspondence.

†Abbreviations: ACE, angiotensin I-converting enzyme; Captopril, D-3-mercapto-2-methylpropanoyl-L-Pro; HOE 498-diacid, 2-N-[1(S)-carboxy-3-phenylpropyl]-L-Ala-]-(1S,3S,5S)-2-azabicyclo[3.3.0]octane-3-carboxylic acid; MDL 27,088, [4S-[4α,7α(R*),12bβ]]-7-[S-(1-carboxy-3-phenylpropyl)amino]-1,2,3,4,6,7,8, 12b- octahydro-6-oxopyrido[2,1-a][2]benzazepine-4-carboxylic acid; MDL 27,788, [4R-[4α,7α(S*),12bβ]]-7-[S-(1-carboxy-3-phenylpropyl)amino]-3,4,6,7,8,12b-hexahydro-6-oxo-1H-[1,4]thiazino[3,4-a][2]benzazepine-4-carboxylic acid; MK 422, N-[1(S)-carboxy-3-phenylpropyl]-L-Ala-L-Pro.

‡The terminology of Schecter & Berger[1] for describing amino acid residues relative to a substrate scissile bond was employed.

hibitors have been reviewed[5] and modelling of the active site of ACE which best accommodates the variety of these inhibitors has been attempted.[6] The most effective members of each group inhibit ACE at subnanomolar concentration; several of them have been utilized in therapy of hypertension and congestive heart failure with outstanding benefit.

Rationalization of the ACE inhibitors which we prepared began with the carboxy terminal tripeptide fragment of angiotensin I. The -Phe-His-LeuOH portion of this physiological substrate is cleaved by ACE to His-LeuOH and the octapeptide angiotensin II. We designed rigid, aromatic, lipophilic tricyclic dipeptide surrogates and coupled them with the zinc liganding 4-phenylbutanoic acid side chain. Two inhibitors were synthesized which differed from each other by replacment of a methylene carbon in the tricyclic dipeptide fragment (MDL 27,088) with a sulfur atom (MDL 27,788) (Figure 1). One might expect differences in bond angles and distances in the tricyclic fragments would lead to variation in orientation of key functional groups of the two inhibitors.

The interaction of MDL 27,088 and MDL 27,788 with ACE is reported here. Their potency is compared to that of MK 422 and HOE 498-diacid, the latter of which is among the most potent of the phenylbutanoic acid derivatives described to date.[7] The strong inhibition of ACE observed for both MDL 27,088 and MDL 27,788 extends understanding of the topography of the active site of this enzyme.

MATERIALS AND METHODS

The procedure for synthesis of MDL 27,088 has been described.[8] MDL 27,788 was prepared in similar fashion, however, details have not as yet been published. The monoester of MDL 27,088, tritiated in the aryl portion of the 4-phenylbutanoic acid side chain, was converted to radioactive MDL 27,088 by hydrolysis in 0.9 M LiOH for ten minutes at 37°C. The radioactive monoester had been prepared at Merrell Dow Research Institute. MK 422 and HOE 498-diacid were courteously provided by Merck, Sharp & Dohme Research Laboratories and by Hoechst-Roussel Pharmaceuticals, respectively.

Angiotensin I-converting enzyme was partially purified from rabbit lung acetone powder (Pel-Freeze Biologicals, Rogers, AR) by chromatography on hydroxylapatite and DEAE-cellulose.[9] By comparison to the enhancement in specific enzyme activity in the report on which the procedure was based and based on the amounts of protein and inhibitor used in the studies reported here, it was estimated that ACE was of the order of 10% of the total protein in this preparation. Stored at −25°C, ACE activity was stable for years.

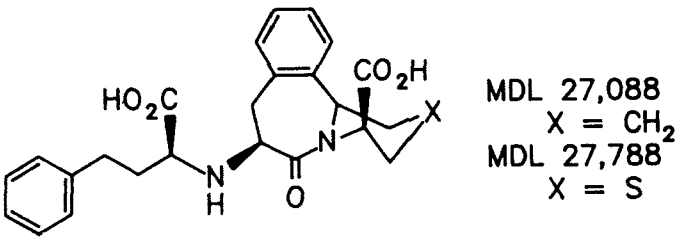

FIGURE 1 Structures of ACE inhibitors MDL 27,088 and MDL 27,788.

A radioassay[10] was used to assess the enzymatic activity of ACE in the presence of tight-binding inhibitors. ACE and inhibitor were incubated for 4 h at 37°C in 188 μL of buffer comprised of 0.6 M Na_2SO_4, 0.1 M NaCl, 0.05 M HEPES, pH 8.0, containing 1% bovine serum albumin. A 12 μL volume of tritiated substrate, 0.2 μCi of benzoylGly-His-Leu (product #01-1001, Ventrex Laboratories, Portland, Me), was added and incubation of the complete assay mixture was continued at 37°C for 1 to 4 h longer. The extent of hydrolysis of carrier-free substrate, 40 nM,[10] was kept to 15% or less. The Michaelis constant for the enzymatic reaction under these conditions has been reported[11] to be 0.08 mM. The assay was quenched by addition of an equal volume of 0.5 M HCl and 3 mL of Ventrex scintillation cocktail was added. Organic and aqueous phases were not separated,[10] as only that tritiated product extracted into the organic phase was measured in a beta counter (Packard Instrument Co.). The radioactivity of each assay mixture was plotted as a function of inhibitor concentration.

Characterization of the plot was carried out using the computer program ALLFIT.[12] Two inhibitors were tested in each trial and the fitting process was constrained so that for the two sets of results sigmoidal curves were generated which were parallel and had asymptotes equal to the average cpm of enzyme-free blanks and to uninhibited enzyme control assay mixtures; thus, the curves differed only in midpoint parameters, which were taken as IC_{50} values for each inhibitor at the specific ACE concentration of the trial. These assays were carried out using 0.04, 0.08 or 0.12 μg of the enzyme. A straight line was fit to multiple IC_{50} values determined at the three enzyme concentrations by nonparameteric regression;[13] 95% confidence intervals were obtained with an ordering algorithm.[14]

The initial interaction of enzyme and inhibitor was followed by continuous spectrophotometric assay.[15] Substrate, 1 mM 2-furan acryloyl-L-Phe-Gly-Gly (Sigma Chemical Co., St. Louis, MO), and inhibitor in a 2 mL volume of buffer (the buffer used for the radioassay, but without bovine serum albumin) were thermally equilibrated to 25°C in the sample compartment of a Cary 210 spectrophotometer. ACE was added to complete the assay mixture. Absorbance values monitored at 345 nm were transferred through a digital interface port (Varian Associates) to a VIC 20 microcomputer which recorded up to 100 time and absorbance observations per assay. A change in absorbance of 0.62 for hydrolysis of 1 mM substrate was used to calculate product amounts and was determined using assay mixtures carried to complete hydrolysis. Michaelis-Menten parameters for the enzyme preparation under these assay conditions were K_m = 0.18 mM and V_{max} = 23 μmol per minute per mg protein.

Inhibition data were obtained by this procedure at MDL 27,088 concentrations of 2, 5, 10, 20 and 40 nM and a 16-fold range of enzyme concentrations (0.4–6.4 μg). The constraints necessary for simplification of data analysis, namely, limited product formation, considerable excess of substrate over enzyme concentration and no significant depletion of inhibitor by enzyme[16] were recognized. Progress curves were smoothed by polynomial regression and normalized to a single concentration of ACE. Seven assays carried out with identical inhibitor concentration were combined, principally in order to provide error estimates for assays done under each set of conditions. There were no obvious differences in comparable progress curves normalized to a single concentration of enzyme. This is expected when inhibitor concentration exceeds by several fold total enzyme concentration.[17] From the pooled data, seven product amounts, with associated error, were calculated at roughly equally spaced

time intervals out to 2–4 half-lives for inhibition. These product amounts were fit to an equation describing inhibition in which a rapidly-formed enzyme-inhibitor complex is more slowly converted to a second enzyme-inhibitor complex.[18] NONLIN84 (Statistical Consultants, Inc., Lexington, KY) was used for weighted fitting to the integrated expression (product amount as the dependent variable).

Dissociation of MDL 27,088 from the more stable enzyme-inhibitor complex was measured using radioactive inhibitor, an approach[3] also used in characterizing interaction of MK 422 with ACE. Tritiated MDL 27,088, 0.2 μCi/mL, was equilibrated for about 4 h at 37°C with approximately an equimolar concentration (*ca* 15 nM) of enzyme. The buffer employed in the radioassay described above was used. The mixture was cooled to 25°C and 50 μM unlabeled MDL 27,088 was added. Aliquots (0.2 mL) of the mixture were removed periodically and enzyme-bound inhibitor was precipitated with ethanol.[19] The fluid phase was concentrated under vacuum, then mixed with scintillation fluid (Aqualyte, J.T. Baker Chemical Co.). Samples taken at the time of adding unlabeled inhibitor and samples made 0.5 M in HCl before the addition of ethanol provided values for fully bound and fully unbound radioactivity, respectively. A semi-logarithmic plot of percent enzyme-bound inhibitor vs time was fitted by least squares regression.

RESULTS

Both MDL 27,788 and MDL 27,088 were potent inhibitors of rabbit lung angiotensin I-converting enzyme, effective in subnanomolar concentrations. The carbocyclic compound was more potent than the compound containing sulfur in the heterocyclic fragment of the molecule (Figure 2). Concentrations producing 50% inhibition of enzymatic activity were determined several times at each of three concentrations of ACE. A line fitting these IC_{50} data was extrapolated to the ordinate[10] to provide an estimate of the overall inhibition constant,[18] K^*_i. The extrapolation to infinitely dilute enzyme concentration, under the assay condition that $[S] \ll K_m$, yields a thermodynamically valid descriptor for the two inhibitor complex model. Figure 3 illustrates the fit for MDL 27,088.

Inhibition of ACE was determined similarly for MK 422 and for HOE 498-diacid. K^*_i values estimated from these trials are presented for the four inhibitors in Table I. The K^*_i estimate for MDL 27,008 was determined from a total of 21 IC_{50} values, whereas K^*_i estimates for MDL 27,788, HOE 498-diacid and MK 422 were based on 13, 19 and 13 values, respectively. The precision with which these estimates were made was not great enough to permit assigning statistical significance to all the observed differences in K^*_i values; confidence intervals determined by the fitting routine pointed to a difference significant at the 5% level between the K^*_i of MDL 27,088 and MK 422, but not between MDL 27,088 and the two other inhibitors; the K^*_i estimate for MDL 27,788 was not significantly different at the 5% level from that of any of the other inhibitors.

Two compounds were tested contemporaneously in each of the trials and additional information was obtained from rank comparisons. In 4 trials in which the two inhibitors were compared side-by-side (one trial is illustrated in Figure 2), the IC_{50} for MDL 27,088 was less than that of MDL 27,788. In each of 8 trials, the IC_{50} for MDL 27,088 was less than that of MK 422, whereas in each of 9 comparsions, the IC_{50} for MDL 27,088 was less than that of HOE 498-diacid. In 4 of 6 comparisons, the IC_{50}

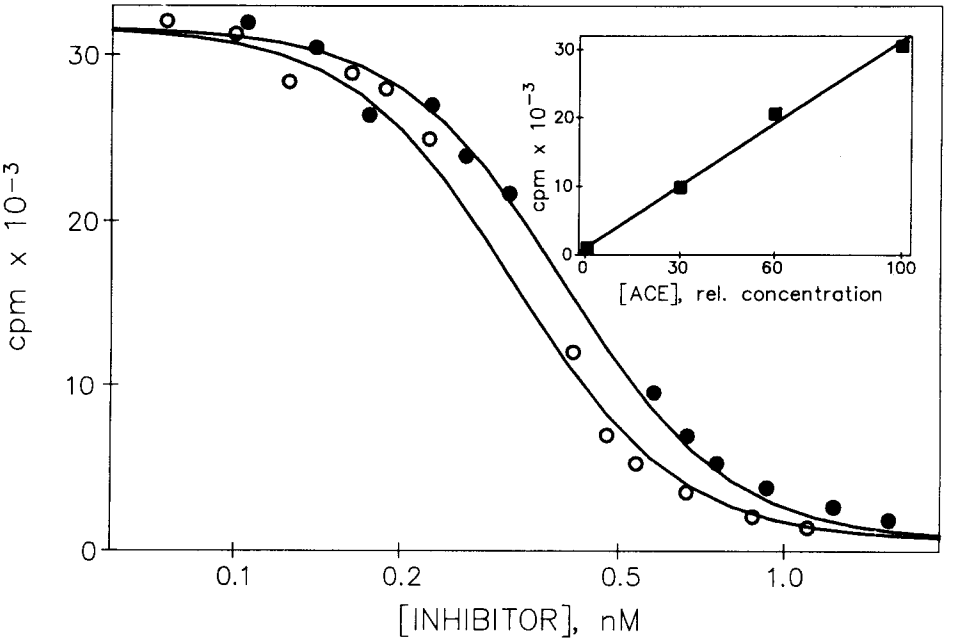

FIGURE 2 Effect of tricyclic inhibitors on ACE activity. Inhibition by MDL 27,088 (open circle) was greater at a given inhibitor concentration than that by MDL 27,788 (filled circle). The midpoints of the two curves were taken as the IC_{50} values. In the inset is illustrated the linear assay response to several concentrations of (inhibitor-free) enzyme; 0.12 μg of enzyme was used in the inhibitor assays.

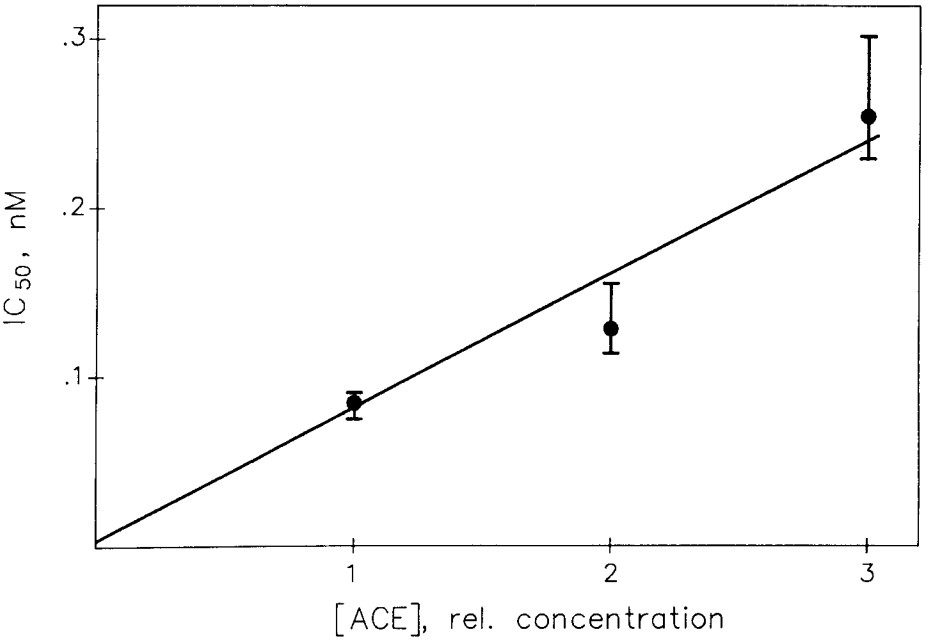

FIGURE 3 Extrapolation of IC_{50} values for inhibition of ACE by MDL 27,088 to infinitely dilute enzyme concentration. The intercept on the ordinate, 0.004 nM, was considered the overall inhibition constant, K_i^*. The 95% confidence interval for the intercept parameter ranged from 0 to 0.033 nM. Error bars represent 68% confidence intervals determined by an ordering routine, with at least 6 assays per point.

TABLE I
ACE inhibition constants

inhibitor	K^*_i (pM)
MDL 27,088	4
MDL 27,788	46
HOE 498-diacid	51
MK 422	76

Literature values (not determined under the same assay conditions) are 50 pM for MK 422[16] and 7 pM for HOE 498-diacid.[7] K^*_i is an overall inhibition constant comprised of terms for rapid and slow formation of enzyme-inhibitor complexes.[18]

estimate for MDL 27,788 was less than that of HOE 498-diacid, whereas in 3 trials, the IC_{50} for MDL 27,788 was less than that of MK 422. Thus, these paired comparisons of relative potency were consistent with the ordering of the inhibitors presented in Table I.

Inhibition of ACE by MDL 27,088 and MDL 27,788 was time dependent. The process was slow and could be monitored in a standard spectrophotometric assay. The initial rate of product formation in a mixture of substrate and inhibitor to which

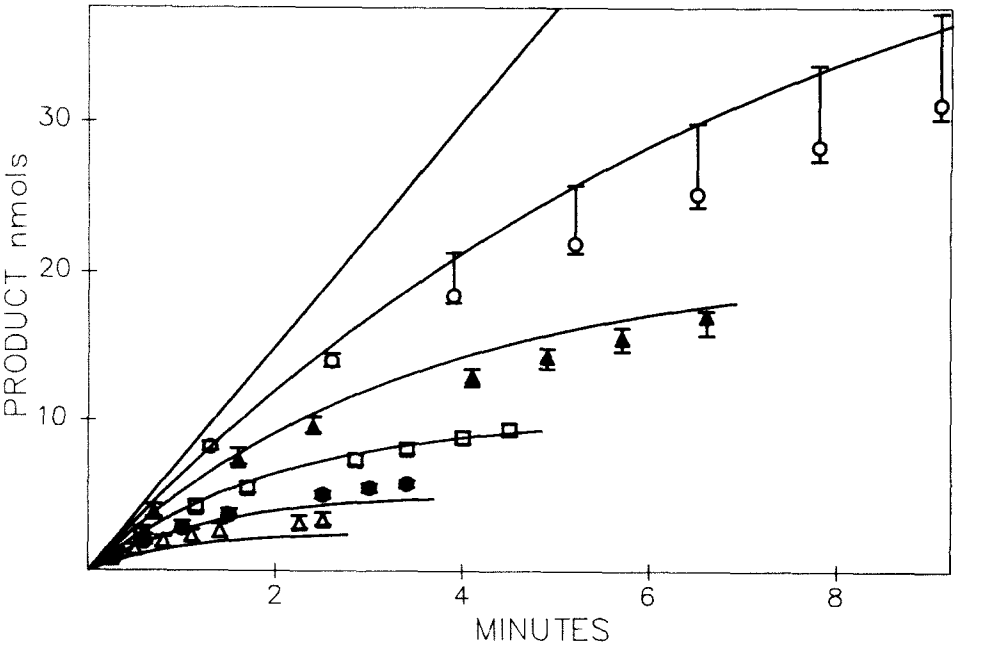

FIGURE 4 Progress curves for hydrolysis of 1 mM 2-furanacryloyl-L-Phe-Gly-Gly by ACE at 25°C in the presence of MDL 27,088. Inhibitor concentrations were 2 (open circle), 5 (filled triangle), 10 (open square), 20 (filled circle) and 40 (open triangle) nM. The line with no associated symbol is the progress curve obtained in the absence of inhibitor. Enzyme concentration was varied as well as inhibitor, but the curves are normalized to a single concentration. Seven progress curves were combined at each concentration of inhibitor. Error bars represent 68% confidence intervals for central values as determined by an ordering routine. The curves were drawn using rate and inhibition parameters obtained as described in the text; weights assigned to each observation in the fitting process were inversely proportional to the errors illustrated.

enzyme was added was decreased by increasing inhibitor concentration. Such a progress curve is consistent with a reversible, slow, tight-binding mechanism of inhibition in which an initial enzyme-inhibitor complex is formed, which, within the time frame of the assay, generates a second complex.[18] This is also the minimal mechanism used to characterize inhibition of ACE by MK 422[16] and by HOE 498-diacid[7]. Data from such assays with MDL 27,088 were fitted to a model with two enzyme-inhibitor complexes, which generated three parameters: K_i, the dissociation constant of the rapidly formed enzyme-inhibitor complex, 3.3 ± 1.1 nM; k_3, the forward formation rate to the slowly-generated complex, 1.6 ± 0.8 min^{-1}; and k_4, the reverse isomerization rate constant. The latter value was poorly determined by these data collected at relatively high inhibitor concentrations, as was explained by Bull *et al.*[3] and was disregarded. The curves in Figure 4 are those calculated by use of the parameters obtained in the fitting process. The designations of these parameters follow the notation used for MK 422[16] and HOE 498-diacid.[7] Since steady-state velocities were not determined in the spectrophotometric assays, data analysis by a technique such as the Henderson plot was not appropriate.

The reverse isomerization rate was measured by release of enzyme-bound tritiated inhibitor in the presence of a great excess of nonradioactive MDL 27,088 (Figure 5). k^4 was calculated from these observations to be 1.6 ± 0.1 × 10^{-3} min^{-1}. The parameters were used to evaluate the expression[18] $K^*_i = k_4 K_i/(k_3 + k_4)$. The result was K^*_i = 3 pM, which was comparable to the value of 4 pM determined from the radioassays.

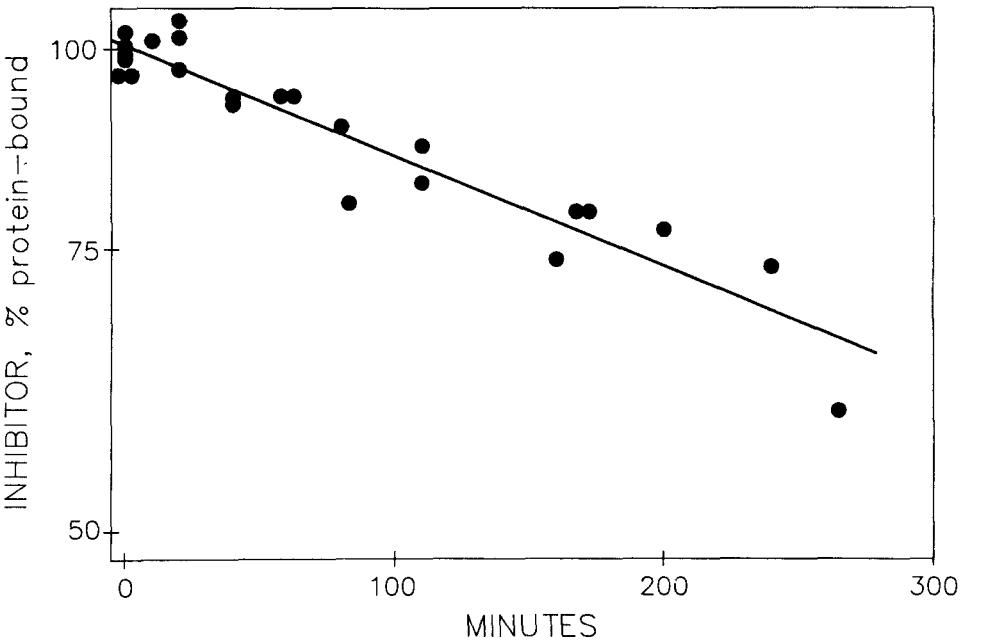

FIGURE 5 Dissociation of radioactive MDL 27,088 from ACE at 25°C. At zero time, > 1000-fold excess of unlabeled inhibitor was added to enzyme-inhibitor mixtures. At subsequent times the remaining ethanol-precipitable-radioactivity was plotted as a percentage of the zero time vlaue. The semi-logarithmic plot was best characterized by a first-order half-life of 436 minutes.

DISCUSSION

MK 422 and HOE 498-diacid are highly potent competitive inhibitors of ACE; MDL 27,088 and MDL 27,788 appear to have similar efficacy. Indeed, MDL 27,088 was more potent than MDL 27,788, HOE-diacid or MK 422. These four inhibitors are of a slow, tight-binding type. For MK 422, a minimal mechanism involving rapid formation of an initial complex with slower generation of a second complex has been established;[16] the slow step is dependent on the concentration of chloride ion in the assay medium. The forward and reverse isomerization rates for the slowly-formed complex and the inhibition constant for the rapidly-formed complex were experimentally determined for MDL 27,088, according to this mechanism. The overall inhibition constant (K^*_i) obtained as a function of these parameters was 3 pM. A K^*_i value for the inhibition of ACE by MDL 27,088 also was obtained by extrapolation of IC_{50} values assessed at three enzyme concentrations to a value corresponding to infinite dilution of enzyme. This procedure copes with the difficulty that under practical assay conditions a meaningful fraction of total inhibitor is bound to enzyme for such potent inhibitors. The inhibition constant obtained in this way was estimated as 4 pM, although the statistically justified conclusion was that the likelihood was less than 5% that the value was greater than 33 pM.

The principal determinant of potency of the several slow-binding and tight-binding ACE inhibitors appears to be the reverse isomerization rate from the chloride-influenced complex to the rapidly-formed enzyme-inhibitor complex. This rate decreases from captopril ($4.0 \times 10^{-4}\,sec^{-1}$) to MK 422 ($1.1 \times 10^{-4}\,sec^{-1}$) to HOE 498-diacid ($1.8 \times 10^{-5}\,sec^{-1}$) which is also the order of potency of these three inhibitors.[7,16] The value of k_4 observed for MDL 27,088 ($2.6 \times 10^{-5}\,sec^{-1}$) is similar to that of HOE 498-diacid. However, the assay buffer employed in the present studies was one described for the radiometric ACE assay,[10] whereas detailed analysis of the kinetics of ACE inhibition by captopril, MK 422[16] and by HOE 498-diacid[7] used a buffer of different pH, higher chloride ion concentration and no added sodium sulfate; all these factors may be expected to influence kinetics both of substrate hydrolysis by ACE and of inhibition of ACE.[16,20,21] Thus, a comparison of rate and inhibition constants between the literature values and those observed in the present studies is not justified, despite numerical similarities.

HOE 498-diacid differs structurally from the prototype 4-phenylbutanoic acid inhibitor, MK 422, only in the alanylproline dipeptide fragment where a 5-membered carbocyclic ring is fused on the proline ring *cis* to the terminal carboxy group. This appears to lead to a favorable hydrophobic interaction within the ACE binding site, because the potency of HOE 498-diacid is several-fold greater than that of MK 422.[7] The MDL inhibitors differ from MK 422 more strikingly in this region. The tricyclic fragment of MDL 27,088 may be simplistically viewed as tying together a phenylalanyl-norleucine dipeptide so as to fix the conformation of the amino acid side chains with respect to the amide bond. The dipeptide mimic is then joined to the zinc-liganding fragment to complete the inhibitor. Computer modeling studies of these inhibitors are as yet incomplete. However, the predicted minimum energy conformation of the tricyclic fragment of MDL 27,088 has been confirmed by X-ray crystallography.[8] Mayer *et al.*[6] studied 28 active inhibitors, including several bicyclic lactams and benzolactams and found that all could be placed into the pharmacophore topography of ACE which was envisioned with common orientation of three important functionalities: the amide carbonyl, the C-terminal carboxylate and the zinc-

liganding moiety. Further description of the ACE active site may result from evaluation of the limited conformation possible for the amide and carboxylate functionalities and the apparently favorable lipophilic interaction with ACE of the tricyclic fragments of MDL 27,088 and MDL 27,788.

Administered intravenously or orally as the monoethyl ester pro-drug, MDL 27,088 demonstrated antihypertensive effects in laboratory animals.[8]

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