Stubbe, J. A., Ator, M., & Krenitsky, T. (1983b) *J. Biol. Chem. 258*, 1625–1630.
Thelander, L., & Reichard, P. (1979) *Annu. Rev. Biochem. 48*, 143–158.
Thelander, L., Larsson, A., Hobbs, J., & Eckstein, F. (1976) *J. Biol. Chem. 251*, 1398–1405.
Walling, C., & Johnson, R. A. (1975) *J. Am. Chem. Soc. 97*, 2405–2407.
Yoshikawa, M., Kato, T., & Takenishi, T. (1967) *Tetrahedron Lett. 50*, 5065–5068.

Inhibition of Angiotensin Converting Enzyme: Mechanism and Substrate Dependence†

Robert Shapiro and James F. Riordan*

ABSTRACT: The interaction of angiotensin converting enzyme with six metal-coordinating [(D-3-mercapto-2-methylpropanoyl)-L-Pro (captopril), *N*-[1(*S*)-carboxy-3-phenylpropyl]-L-Ala-L-Pro (MK-422), *N*-(phenylphosphoryl)-L-Phe-L-Phe, N^{α}-(3-mercaptopropanoyl)-L-Arg, N^{α}-[1(*S*)-carboxy-3-phenylpropyl]-Ala-L-Lys, and *N*-[1(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly] and three dipeptide inhibitors (Gly-L-Trp, L-Phe-L-Arg, and L-Ala-L-Pro) was examined at pH 7.5 in the presence of 300 mM NaCl. Inhibition modes, apparent K_i [K_i(app)] values, and shapes of $1/v$ vs. [I] plots were found to vary with the substrate employed. All inhibitors except Phe-Arg were competitive with the substrate furanacryloyl (Fa)-Phe-Gly-Gly, while five of seven tested with Fa-Phe-Phe-Arg as substrate produced mixed patterns. K_i(app) values for *N*-[1(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly, *N*-(phenylphosphoryl)-L-Phe-L-Phe, Gly-Trp, and MK-422 were 8.3-, 5.5-, 4.7-, and 2.6-fold lower, respectively, when Fa-Phe-Gly-Gly was substrate, compared with values measured with Fa-Phe-Phe-Arg. In contrast, K_i(app) values for Phe-Arg and (3-mercaptopropanoyl)-Arg were lower (2.8- and 2.2-fold, respectively) when Fa-Phe-Phe-Arg was the substrate. Plots of $1/v$ vs. [I] for most of the inhibitors were nonlinear, to an extent which was also substrate dependent. By curve fitting, these plots were shown to be consistent with a rate equation of the form $v/[E_0] = (1 + d[I])/(a + b[I] + c[I]^2)$, suggesting that inhibitor can bind to more than one enzyme form and that there are alternative pathways to product. Two inhibition mechanisms are described which incorporate these features and may account for the observed substrate dependence. These mechanisms attribute the unusual kinetics to (i) inhibitor binding to an enzyme–product complex or (ii) interaction of the enzyme with activating anions. A third mechanism, consistent with the kinetic observations, involves multiple inhibitor binding and appears unlikely on the basis of equilibrium dialysis measurements.

During the past several years, considerable effort has been devoted to the development of tight-binding, specific inhibitors of angiotensin converting enzyme (peptidyl dipeptidase, EC 3.4.15.1) (ACE)[1] for use as antihypertensive drugs. The effectiveness of these compounds in the management of hypertension, which has been demonstrated in numerous clinical studies (Gavras et al., 1978; Cushman & Ondetti, 1980), is presumably a consequence of the known physiological actions of ACE: it catalyzes both the generation of the potent, vasoconstricting octapeptide angiotensin II from the decapeptide angiotensin I and the inactivation of the vasodilating nonapeptide bradykinin (Soffer, 1976; Erdös, 1976; Peach, 1977).

The first orally active ACE inhibitor employed for control of blood pressure, (D-3-mercapto-2-methylpropanoyl)-L-proline (captopril), was designed on the basis of the active-site structure of the analogous zinc exopeptidase, carboxypeptidase A (Ondetti et al., 1977; Cushman et al., 1977). Its strength of binding to ACE (K_i = 1.7 nM) is thought to derive largely from the interaction of its sulfhydryl group with the zinc atom at the active site of the enzyme. Subsequently, Patchett et al. (1980) synthesized a series of *N*-(carboxyalkyl)dipeptide inhibitors containing a carboxylate group which presumably coordinates to the zinc atom. At least two of these, the *N*-[1(*S*)-carboxy-3-phenylpropyl] derivatives of Ala-Pro (MK-422) and Lys-Pro, have nanomolar IC_{50} values and are orally effective antihypertensive agents (Patchett et al., 1980; Gavras et al., 1981). Inhibitors incorporating a $-PO^-$ anion as the metal ligand have also been synthesized (Holmquist & Vallee, 1979; Galardy, 1980; Thorsett et al., 1982; Galardy et al., 1983), and some of these exhibit nanomolar K_i values.

Hundreds of potential ACE inhibitors have now been synthesized, resulting in the discovery of at least six classes of compounds effective at nanomolar concentrations. Despite this intense effort, relatively few reports have examined the mode of action of these inhibitors. Most of the nonclinical ACE inhibition literature concerns structure–activity relationships; inhibition mechanisms have received only cursory attention. Such work has been limited, in part, by the almost universal use of a discontinuous assay system, with reaction

† From the Center for Biochemical and Biophysical Sciences and Medicine and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115. *Received February 9, 1984.* This work was supported in part by National Institutes of Health Grant HL-22387. R.S. was supported by National Institutes of Health Predoctoral Training Grant GM 07306.

[1] Abbreviations: ACE, angiotensin converting enzyme; captopril, (D-3-mercapto-2-methylpropanoyl)-L-proline; MK-422, *N*-[1(*S*)-carboxy-3-phenylpropyl]-L-Ala-L-Pro; IC_{50}, the concentration of inhibitor producing 50% inhibition at the particular substrate and enzyme concentrations employed; Tris, tris(hydroxymethyl)aminomethane; Bz, *N*-benzoyl; SDS, sodium dodecyl sulfate; CA-Phe-Gly, *N*-[1(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly; PPPP, *N*-(phenylphosphoryl)-L-Phe-L-Phe; MP-Arg, N^{α}-(3-mercaptopropanoyl)-L-Arg; CP-Ala-Lys, N^{α}-[1(*S*)-carboxy-3-phenylpropyl]-L-Ala-L-Lys; Fa, 2-furanacryloyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; *t*-BOC, *tert*-butyloxycarbonyl.

0006-2960/84/0423-5225$01.50/0

velocities often based on a single time point, and buffers such as Tris or phosphate that have been shown to be inhibitory (Dorer et al., 1976; Bünning et al., 1983). In addition, many of these studies have employed enzyme of low purity [see, for example, Almquist et al. (1980)]. There has also been insufficient appreciation of the fact that standard kinetic analysis is not applicable to many of these inhibitors, which are both slow binding and tight binding. Finally, almost all of these studies have been carried out with only an angiotensin I analogue (usually Bz-Gly-His-Leu) or angiotensin I itself as substrate, even though ACE has broad specificity.

The present work examines in detail the kinetics of the interaction of ACE with three dipeptide and six metal-coordinating inhibitors, including captopril and MK-422. It was of particular interest to determine whether the effects of these inhibitors would vary with the substrate employed since the mechanisms by which anions activate ACE-catalyzed hydrolysis are markedly substrate dependent (Shapiro et al., 1983). We report here that inhibition of ACE, like anion activation, indeed varies with substrate. Thus, modes of inhibition and apparent K_i values for several of the inhibitors differ for different substrates. In addition, plots of $1/v$ vs. [I] are often nonlinear, to an extent that is also substrate dependent. These findings suggest a complex mechanism of inhibition involving multiple modes of interaction with enzyme.

Materials and Methods

Enzyme. ACE was purified from frozen rabbit lungs (Pel-Freez Biologicals, Inc., Rogers, AR) by two procedures. For many of the experiments, the method of Bünning et al. (1983) was employed, except that the tissue extract was treated with 18% (w/v) ammonium sulfate to precipitate contaminants prior to hydroxylapatite chromatography. Most of the ACE remained in solution after this step, and up to 3-fold purification was achieved. For the remainder of the work, including equilibrium dialysis experiments, enzyme was isolated by affinity chromatography (Pantoliano et al., 1984). Kinetic results were independent of the isolation procedure employed. In all cases, the enzyme was >95% pure as judged by both specific activity and SDS–polyacrylamide gel electrophoresis. Enzyme concentrations were determined from the absorbance at 280 nm by using a molar absorptivity of 225 000 M^{-1} cm^{-1} (R. Shapiro and M. W. Pantoliano, unpublished experiments).

Inhibitors. All amino acids were of the L configuration. Gly-Trp·$2H_2O$ and Ala-Pro were obtained from Cyclo Chemical Corp. (Los Angeles, CA) and Vega Biochemicals (Tucson, AZ), respectively, and Phe-Arg was from either Vega Biochemicals or Sigma Chemical Corp. (St. Louis, MO). Thin-layer chromatography [1-butanol/acetic acid/water (4:1:1)] on silica gel (EM Reagents) revealed a single ninhydrin-positive spot for each of these dipeptides.

N-[1(*S*)-Carboxy-5-aminopentyl]-Phe-Gly (CA-Phe-Gly) and *N*-(phenylphosphoryl)-Phe-Phe (PPPP) were kindly provided by M. W. Pantoliano and B. Holmquist, respectively. Captopril and N^{α}-(3-mercaptopropanoyl)-Arg (MP-Arg) were gifts from the Squibb Institute and *N*-[1(*S*)-carboxy-3-phenylpropyl]-Ala-Pro (MK-422) and N^{α}-(1-carboxy-3-phenylpropyl)-Ala-Lys from Merck Sharp & Dohme Research Laboratories. The last compound was a mixture of diastereomers and was further purified by cation-exchange chromatography as described for CA-Phe-Gly (Pantoliano et al., 1984). Thin-layer chromatography of the initial material revealed two close but distinct ninhydrin-positive (weakly quenching) spots of equal intensity. The chromatographic fractions having the strongest inhibitory activity produced only the higher R_f spot, while less inhibitory fractions eluting later produced the lower R_f spot. Fractions containing only the higher R_f material were pooled and lyophilized. This compound is assumed, by analogy with results reported by Patchett et al. (1980), to be N^{α}-[1(*S*)-carboxy-3-phenylpropyl]-L-Ala-L-Lys (CP-Ala-Lys). Structures of the six metal-coordinating inhibitors are shown in Figure 1.

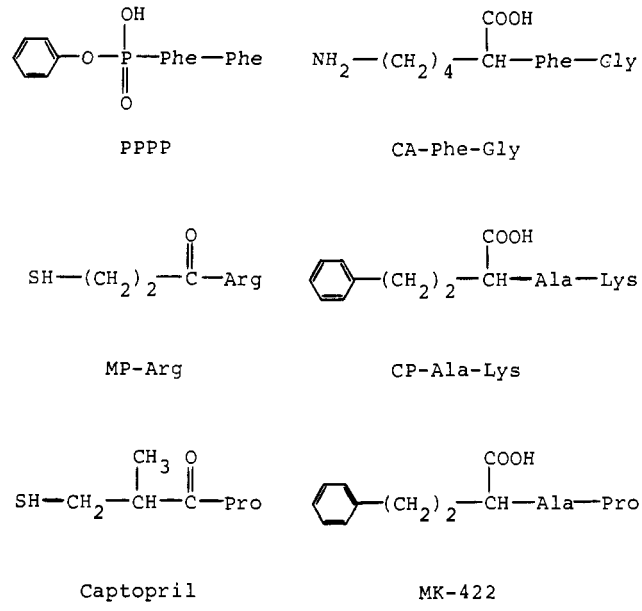


FIGURE 1: Structures of the six metal-coordinating inhibitors.

Assays. Enzyme activities were measured by using Fa-blocked tripeptides as described previously (Holmquist et al., 1979). An appropriate wavelength between 328 and 353 nm was chosen, depending on the substrate concentration, and the absorbance decrease accompanying hydrolysis was continuously monitored with a Varian Model 219 spectrophotometer equipped with a thermostated cuvette holder. A 2-, 10-, or 50-mm cuvette was employed as required to give initial absorbance readings between 0.15 and 2.0. The standard assay conditions were 25 °C and 50 mM Hepes/300 mM NaCl, pH 7.5. Initial velocities were measured during the first 10% of hydrolysis. All substrates were from previous studies, and their syntheses are described elsewhere (Holmquist et al., 1979; Bünning et al., 1983; Shapiro et al., 1983). For all experiments using captopril and MP-Arg, 2-mercaptoethanol (50–100 μM) was present in the assay mixture.

Kinetic Analysis. K_m and k_{cat} values were derived from Lineweaver–Burk plots. Methods used for determination of apparent K_i values are described elsewhere in the text. For inhibitors other than captopril and MK-422, the mode of inhibition was assessed from Lineweaver–Burk plots. The mode was termed "competitive" if there was no significant change in k_{cat} at inhibitor concentrations resulting in at least a 6-fold (usually >10-fold) increase in K_m. For "mixed" inhibition modes, an "α" value is provided which represents the 1/[S] intercept value in the absence of inhibitor divided by the 1/[S] value at the intersection between double-reciprocal lines obtained in the absence and presence of inhibitor. These α values are presented purely for descriptive purposes.

Nonlinear $1/v$ vs. [I] plots were fitted to

$$1/v = (a + b[I] + c[I]^2)/(1 + d[I]) \qquad (1)$$

by using a PASCAL program written for the Apple II Plus computer by Dr. Craig Luehr, where a, b, c, and d are constants that may contain various rate constants as well as the substrate concentration. This program performs a weighted linear regression on a form of the equation

$$1/v = a + b[I] + c[I]^2 - d(1/v)[I]$$

which is now linearized with respect to the constants a, b, c, and d (Miller, 1981). A v^2 or v^4 weighting was used in all cases.

Inhibition modes for the slow- and tight-binding inhibitors captopril and MK-422 were determined by an adaptation of the method of Cha (1976) utilizing the dependence of k_{obsd} on substrate concentration as detailed in the text, where k_{obsd} is the exponential decay constant associated with the decrease in reaction velocity as inhibitor binds to enzyme. Measurement of k_{obsd} is described in the following paper (Shapiro & Riordan, 1984).

Stoichiometry of Inhibitor Binding. Equilibrium dialysis was performed by using 1-mL TechniLab cells with Spectropor 2 dialysis tubing. Inhibitor solution (0.8–1.0 mL) was placed in one compartment and an equal amount of either enzyme or buffer in the other. Samples were equilibrated by rotation at a rate of ~25 turns/min for at least 4 h at 21–23 °C. The concentration of inhibitor remaining in the inhibitor compartment was determined from the effect of at least three different aliquots of this solution on the ACE-catalyzed hydrolysis of Fa-Phe-Gly-Gly. Quantitation was made by comparison with a standard curve: for CA-Phe-Gly, this was a plot of $1/v$ vs. [I] fitted to the ratio of polynomials given above, and for MK-422, it was a Henderson plot (see below). The CA-Phe-Gly concentration in the stock solution was determined by amino acid analysis.

Results and Discussion

A striking feature of angiotensin converting enzyme is its strong activation by chloride and other monovalent anions (Skeggs et al., 1954). We recently reported (Shapiro et al., 1983) that the kinetic mechanism of activation, the amount of chloride required for optimal activity, and the effect of pH on activation are all markedly dependent on the substrate employed. Accordingly, we proposed an empirical division of the synthetic substrates examined into three classes. Peptides containing C-terminal or penultimate positively charged residues, such as the bradykinin analogue Fa-Phe-Phe-Arg, are "class II" substrates, while peptides lacking this feature are either "class I" (e.g., Fa-Phe-Gly-Gly) or "class III" (e.g., Fa-Gly-Ala-Pro). At pH 7.5, the angiotensin I analogue Fa-Phe-His-Leu displays characteristics intermediate between those of classes I and II, likely due to a partial charge on the penultimate histidine.

In light of this variability of kinetic properties with substrate structure, it seemed important from both a mechanistic and a pharmacologic viewpoint to examine the action of ACE inhibitors by using different substrates. Thus, the present study has been performed with both class I and class II substrates. In addition, it was of interest to determine whether inhibitors would fall into "classes" on the same structural basis as substrates. The nine inhibitors employed in this study were selected for this purpose. Phe-Arg, N^{α}-(3-mercaptopropanoyl)-Arg (MP-Arg), and N^{α}-[1(*S*)-carboxy-3-phenylpropyl]-Ala-Lys (CP-Ala-Lys) all contain positively charged side chains on the C-terminal amino acid, a feature which imparts class II properties to substrates. Similarly, Ala-Pro, captopril, and MK-422 are structurally analogous to class III substrates while the remaining three inhibitors, Gly-Trp, *N*-(phenylphosphoryl)-Phe-Phe (PPPP), and *N*-[1(*S*)-carboxy-5-aminopentyl]-Phe-Gly (CA-Phe-Gly), share structural features of class I substrates.

Most of these inhibitors can be studied by using standard kinetic approaches, but in two cases, this is not possible.

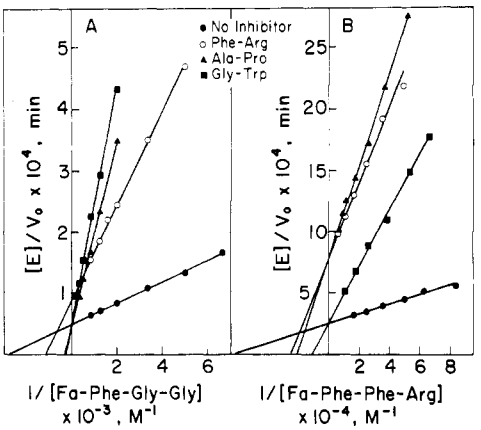


FIGURE 2: Lineweaver–Burk plots for ACE-catalyzed hydrolysis of (A) Fa-Phe-Gly-Gly in the absence (●) or presence of 300 μM Ala-Pro (▲), 300 μM Gly-Trp (■), or 800 μM Phe-Arg (○) and (B) Fa-Phe-Phe-Arg in the absence (●) or presence of 300 μM Ala-Pro (▲), 500 μM Gly-Trp (■), or 800 μM Phe-Arg (○). All assays were in 50 mM Hepes, 300 mM NaCl, and 1 μM $ZnCl_2$, pH 7.5 at 25 °C.

Captopril and MK-422 are slow-binding, tight-binding inhibitors, and as such, they require different analytical and experimental methodology (Williams & Morrison, 1979). The effects of these inhibitors will be presented separately following discussion of the other inhibitors.

Inhibition Modes. Phe-Arg appears to be a "mixed" inhibitor of the hydrolysis of the class I substrate Fa-Phe-Gly-Gly (Figure 2A), with $\alpha = 4$ at the highest inhibitor concentration employed (see Materials and Methods). The remaining non-tight-binding inhibitors produce competitive patterns, two examples of which (Ala-Pro and Gly-Trp) are shown in Figure 2A. In contrast, only Gly-Trp (Figure 2B) and possibly CA-Phe-Gly produce completely competitive plots with the class II substrate Fa-Phe-Phe-Arg. Inhibition by Phe-Arg and Ala-Pro (Figure 2B), and by MP-Arg, CP-Ala-Lys, and PPPP, is mixed, with α values of 3.5, 3.9, 4.0, 15, and 20, respectively.

Minimally, these mixed patterns indicate that the inhibitors can combine with more than one form of the enzyme. They do not necessarily imply that inhibitor can bind to the ES complex, as is frequently thought. The variation of inhibition mode with substrate is consistent with our previous finding that the class I substrate Bz-Gly-Gly-Phe is a mixed inhibitor of Fa-Phe-Phe-Arg hydrolysis (Shapiro et al., 1983). It may be a consequence of differences in the binding modes of the two substrates or their products, or in the kinetic mechanisms for hydrolysis of the two peptides. These points will be amplified below.

Inhibitor Effectiveness. It can be determined from Figure 2A,B that Gly-Trp is a more effective inhibitor of Fa-Phe-Gly-Gly hydrolysis than of Fa-Phe-Phe-Arg hydrolysis, while for Phe-Arg the reverse is true. The data in Table I demonstrate that the inhibitory effects of CA-Phe-Gly and PPPP are also substrate dependent. The remaining three inhibitors appear to be equally potent against both substrates, although some substrate dependence in their effects will be demonstrated below and in the following paper (Shapiro & Riordan, 1984).

The effectiveness of an inhibitor is generally expressed by its K_i value, which represents the dissociation constant for inhibitor and free enzyme, and which can be determined by several standard methods. When the inhibition mechanism is simple, plots of $1/v$ vs. [I] at various substrate concentrations yield straight lines which intersect at $[I] = -K_i$ (Dixon, 1953). In addition, plots of K_m/k_{cat} or K_m (for competitive inhibition) vs. [I] will also be linear, with an intercept at $[I] = -K_i$.

Table I: Effects of ACE Inhibitors on Hydrolysis of Fa-Phe-Gly-Gly and Fa-Phe-Phe-Arg[a]

inhibitor	[I] (μM)	inhibition mode[b] with Fa-Phe-Gly-Gly	inhibition mode[b] with Fa-Phe-Phe-Arg	x-fold increase[c] in K_m/k_{cat} with Fa-Phe-Gly-Gly	x-fold increase[c] in K_m/k_{cat} with Fa-Phe-Phe-Arg	ratio[d]
CA-Phe-Gly	2	competitive	competitive	11.3	2.4	4.7
PPPP	10	competitive	mixed	9.1	2.4	3.8
Gly-Trp	500	competitive	competitive	12.0	3.2	3.8
Ala-Pro	300	competitive	mixed	10.2	9.0	1.1
MP-Arg	0.12	competitive	mixed	13.0	12.0	1.1
CP-Ala-Lys	0.20	competitive	mixed	8.9	8.9	1.0
Phe-Arg	800	mixed	mixed	4.5	8.4	0.5

[a] Assay conditions: 50 mM Hepes and 300 mM NaCl, pH 7.5, 25 °C. [b] Determined from Lineweaver–Burk plots. [c] K_m/k_{cat} measured from the Lineweaver–Burk plot in the presence of the indicated amount of inhibitor divided by K_m/k_{cat} in the absence of inhibitor. [d] Increase in K_m/k_{cat} for Fa-Phe-Gly-Gly divided by increase for Fa-Phe-Phe-Arg.

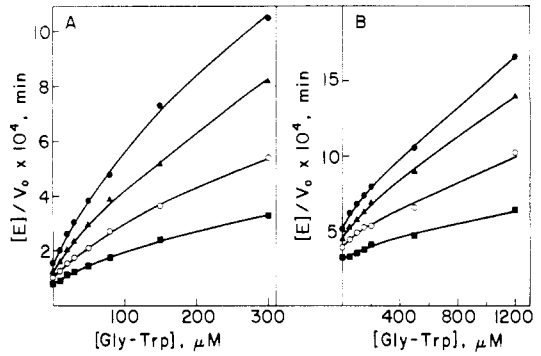


FIGURE 3: (A) Dixon plot for inhibition of Fa-Phe-Gly-Gly hydrolysis by Gly-Trp. Substrate concentrations are 150 (●), 200 (▲), 300 (○), and 500 (■) μM. (B) Dixon plot for inhibition of Fa-Phe-Phe-Arg hydrolysis by Gly-Trp. Substrate concentrations are 16 (●), 20 (▲), 28 (○), and 60 (■) μM. Assay conditions were as in Figure 2. Most of the lines drawn represent computer fits of the data to eq 1, as described under Materials and Methods. Additional data points at higher inhibitor concentrations were included when performing these fits. With 28 and 60 μM Fa-Phe-Phe-Arg, the range of velocities measured was insufficient to allow curve fitting.

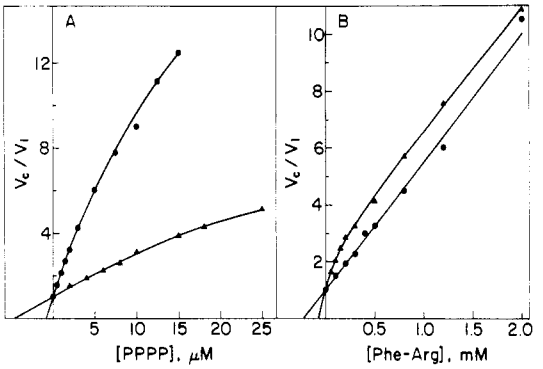


FIGURE 4: Plots of v_c/v_i vs. PPPP (A) or Phe-Arg (B) concentration with 50 μM Fa-Phe-Gly-Gly (●) or 3.2 μM Fa-Phe-Phe-Arg (▲) as substrate. These substrate concentrations are well below K_m in both cases. v_c and v_i are velocities in the absence and presence, respectively, of inhibitor. For the nonlinear plots of PPPP inhibition with Fa-Phe-Gly-Gly and Phe-Arg inhibition with Fa-Phe-Phe-Arg, the curves drawn represent computer fits of the data to eq 1. Assay conditions were as in Figure 2.

Application of these methods to the present inhibitors, however, yields nonlinear plots in most cases (e.g., Dixon plots for Gly-Trp are shown in Figure 3), indicating complex inhibition mechanisms.

The precise shape of the $1/v$ vs. [I] plots is highly variable, depending on both inhibitor and substrate. With Fa-Phe-Gly-Gly (at [S] $\ll K_m$), plots for Gly-Trp and CA-Phe-Gly deviate strongly from linearity beginning at concentrations producing 60–70% inhibition. With PPPP and MP-Arg, curvature does not become apparent until at least 80% inhibition, while with Ala-Pro, Phe-Arg, and CP-Ala-Lys, there is no curvature even up to inhibitor concentrations producing 90% inhibition. In contrast, all plots with Fa-Phe-Phe-Arg (again at [S] $\ll K_m$) are markedly nonlinear, with curvature in most cases (CA-Phe-Gly, Gly-Trp, Phe-Arg, and MP-Arg) already evident at concentrations producing 50% inhibition. Examples of these types of plots are shown in Figures 4 and 5. Additional ones will be found in the following paper (Shapiro & Riordan, 1984).

The curvature of these plots precludes determination of K_i values by standard kinetic methods. Inhibitor strength will therefore be expressed as "apparent" K_i [K_i(app)] values, obtained from $1/v$ vs. [I] plots at [S] $\ll K_m$ where v is proportional to k_{cat}/K_m. These plots approximate linearity at very low inhibitor concentrations, and K_i(app) is defined as the extrapolated −[I] intercept of this line. K_i(app) values for the seven inhibitors with each substrate are listed in Table II. All three of the inhibitors having a class I type structure produce severalfold lower K_i(app) values with the class I substrate than with the class II substrate. It should be noted that this difference is observed even with PPPP, an inhibitor that produces

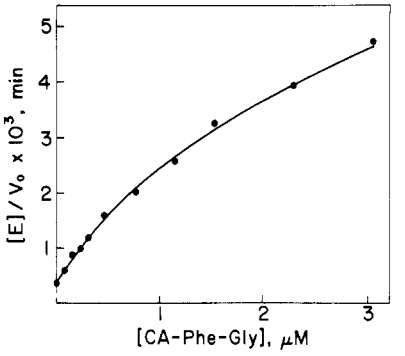


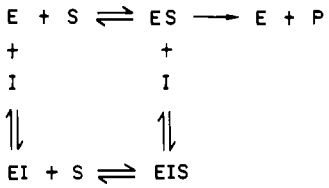
FIGURE 5: Standard curve of ACE inhibition by CA-Phe-Gly used to quantitate CA-Phe-Gly for determinations of binding stoichiometry. Assay conditions were as in Figure 2, using 50 μM Fa-Phe-Gly-Gly. The line drawn is a computer fit of the data to eq 1.

Table II: Apparent K_i Values for ACE Inhibitors[a]

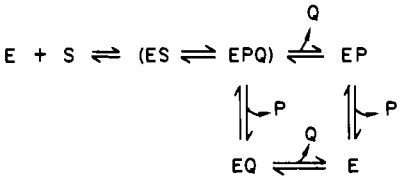
inhibitor	K_i(app) (μM) with Fa-Phe-Gly-Gly	K_i(app) (μM) with Fa-Phe-Phe-Arg
CA-Phe-Gly	0.12	1.0
PPPP	0.90	5.0
Gly-Trp	17	80
Ala-Pro	22	23
MP-Arg	0.022	0.010
CP-Ala-Lys	0.010	0.012
Phe-Arg	220	78

[a] Determined from plots of $1/v$ vs. [I] at [S] $\ll K_m$. The relatively linear portion at low [I] extrapolates to the [I] axis at $-K_i$(app). Substrate concentrations used were 50 μM for Fa-Phe-Gly-Gly and 3.2 μM for Fa-Phe-Phe-Arg, well below K_m in both cases [K_m values are 300 μM for Fa-Phe-Gly-Gly (Holmquist et al., 1979) and 15 μM for Fa-Phe-Phe-Arg (Shapiro et al., 1983)]. Assay conditions: 50 mM Hepes, 300 mM NaCl, pH 7.5, 25 °C.

Scheme I



Scheme II



$1/v$ vs. [I] plots that appear to be linear to fairly high [I] values. In constrast, two of the three inhibitors with a class II type structure give somewhat lower K_i(app) values with the class II substrate than with the class I substrate. The remaining two inhibitors show similar K_i(app) values with both substrates.

When two additional class I substrates, Fa-(N^{ϵ}-*t*-BOC)-Lys-Gly-Gly and Fa-Leu-Ala-Gly, are employed, the K_i(app) values and the shapes of the $1/v$ vs. [I] plots are almost identical with those obtained with Fa-Phe-Gly-Gly. Similarly, the kinetic plots with an additional class II substrate, Fa-Phe-Lys-Ala, are indistinguishable from those observed with Fa-Phe-Phe-Arg.

Curve Fitting. The nonlinearity of $1/v$ vs. [I] plots and the substrate dependence of K_i(app) values are clear indications that the inhibition mechanism is complex and that the K_i(app) values are not true dissociation constants. In considering possible mechanisms to account for the observed kinetics, it is useful to first determine the mathematical form of the rate equation. Downward curvatuve of $1/v$ vs. [I] plots is a frequent indication that $1/v$ bears a hyperbolic relation to [I], reaching a limiting value at high [I]. In this case, the compound being tested would be only a partial inhibitor. However, when measurements are made with high concentrations of the present inhibitors, it is evident that $1/v$ continues to increase appreciably, in fact almost linearly, with [I]. Cleland (1963) has described a function, referred to as a "2/1" function, that can produce such a shape. It is a nonrectangular hyperbola, with a curved line at low [I] that asymptotically approaches a straight line at high [I], having the form[2] of eq 1:

$$1/v = (a + b[I] + c[I]^2)/(1 + d[I])$$

Nonlinear $1/v$ vs. [I] plots for inhibitors in this study (Figures 3–5 and others not shown) were computer fitted to this equation as described under Materials and Methods. The resulting curves in virtually all cases closely follow the data points, even over an extremely wide range of [I] values.

Kinetic Mechanism. From the preceding data, three criteria can be established for determining the adequacy of any kinetic mechanism proposed for the inhibitors under study. Such a mechanism should produce (i) a rate equation of the form

$$v = (1 + d[I])/(a + b[I] + c[I]^2) \qquad (2)$$

(ii) both competitive and mixed inhibition patterns, and (iii) substrate-dependent apparent K_i values. If steady-state assumptions are made, eq 2 would imply the occurrence of certain physical steps in the mechanism. It can readily be seen, for example, by application of the King–Altman method (King & Altman, 1956) for derivation of rate equations, that the denominator will only contain an $[I]^2$ term if the inhibitor can combine with two different forms of the enzyme. Similarly, an [I] term in the numerator implies that one of the enzyme–inhibitor complexes can break down to form products by a path other than simple reversal of the step by which the complex was formed. One mechanism containing these two features is conventional mixed inhibition (Scheme I). Steady-state analysis of this system yields a rate equation of the same form as eq 2 [see p 338 of Dixon & Webb (1979)]:

$$v/[E_0] = (C_1[S] + C_2[S]^2 + C_3[S][I])/(C_4 + C_5[S] + C_6[I] + C_7[I]^2 + C_8[I][S] + C_9[S]^2 + C_{10}[S][I]^2 + C_{11}[S]^2[I]) \qquad (3)$$

where C_1–C_{11} represent composites of the individual rate constants. The $[I]^2$ terms in the denominator reflect the fact that both E and ES can bind I, while the [I] term in the numerator derives from the pathway EI → EIS → ES → E + P. Scheme I is inadequate, however, because it predicts that k_{cat} will vary with inhibitor concentration, approaching zero at high [I]. This cannot be reconciled with the observation that most of the inhibition patterns for Fa-Phe-Gly-Gly are competitive, even with inhibitors such as Gly-Trp and CA-Phe-Gly which produce markedly nonlinear $1/v$ vs. [I] plots.

A 2/1 function can also result from product inhibition in a random Uni-Bi reaction (Cleland, 1963; Segel, 1975) (see Scheme II).

The steady-state rate equation in the absence of one of the products (Q) [adapted from Segel (1975)] is

$$v/[E_0] = \frac{C_1[S] + C_2[P][S]}{C_3 + C_4[S] + C_5[P][S] + C_6[P] + C_7[P]^2} \qquad (4)$$

In this case, the denominator contains a $[P]^2$ term because P can combine with both E and EQ. The [P] term in the numerator reflects the ability of EPQ (formed from EQ plus P) to break down to products by an alternative route, EPQ → EP → E.

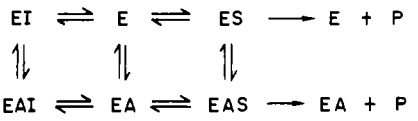
Thus, if ACE follows Scheme II, inhibition by dipeptide products such as Gly-Trp and Phe-Arg should follow eq 2. The metal-coordinating inhibitors could produce similar effects if they bind to both E and EP (or EQ) as long as P (or Q) can still be released at a reasonable rate. In fact, even if product dissociation is ordered, any inhibitor that can bind to both E and EQ (where Q is released second) without preventing release of Q can yield a 2/1 function (Cleland, 1963).

Scheme II (and the variations described) can also account for the substrate dependence of the K_i(app) values. These values will approximate $a/b - ad$) as [S] → 0 since the curve defined by eq 1 has a slope of $b - ad$ at [I] = 0 and an intercept at $1/v = a$. This expression contains rate constants that vary with substrate.

The single requirement that would appear not to be satisfied by this scheme is that the inhibition mode, in most cases, be competitive. In eq 4, k_{cat}, which is equal to $(C_1 + C_2[P])/(C_4 + C_5[P])$, varies with inhibitor concentration. However, the predicted dependence of $1/k_{cat}$ on [I] is hyperbolic, with $1/k_{cat}$ increasing from C_4/C_1 (at [I] = 0) to a limiting value of C_5/C_2 (at infinite [I]). Both the extent of the change in k_{cat} and the inhibitor concentration range over which this change will occur

[2] In general, the shape observed can be produced if $1/v$ equals the ratio of two polynomials in [I] with the numerator polynomial of at least second order and the denominator polynomial one order lower. The simplest and most prevalent example of this is the 2/1 function, where 2 and 1 refer to the highest power of [I] occurring in the numerator and denominator, respectively.

Scheme III



are dependent on the relative magnitudes of the various rate constants. Thus, the inhibition mode could in fact appear to be either competitive or mixed.

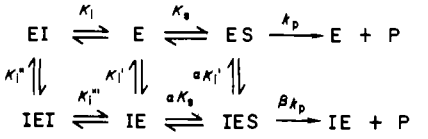
A third scheme to account for the observed kinetics of ACE inhibition is related to the anion activation properties of ACE. It is well-known that the enzyme is strongly activated by chloride which we have used in all assays at its optimal concentration of 300 mM. (Omitting chloride reduces k_{cat}/K_m by >1000-fold with Fa-Phe-Gly-Gly and by about 45-fold with Fa-Phe-Phe-Arg.) While the anion activation properties of ACE have been investigated in some detail (Bünning et al., 1983; Shapiro et al., 1983), the order of binding of activator and substrate is not known with certainty. The simplest interpretation of the available data is that class II and III substrates can bind to both the free enzyme and the enzyme–anion complex, while class I substrates can only bind to the latter. If inhibitor binds to both E and EA, as in Scheme III (the most general mechanism incorporating the desired feature), the steady-state rate expression will again have the same form as eq 2. An [I] term in the numerator will be needed to account for the breakdown of EAI (formed by E → EI → EAI) to products via the pathway EAI → EA → EAS → EA + P. Combination of the inhibitor with two forms of the enzyme, E and EA, will produce an $[I]^2$ term in the denominator. In the following paper (Shapiro & Riordan, 1984), we demonstrate that the inhibitors under study do bind, albeit weakly, to activator-free enzyme.

The rate equation for Scheme III is extremely complex. However, it is not necessary to examine this expression in full detail in order to assess the basic adequacy of the kinetic scheme. A consideration of the appropriate King–Altman patterns should suffice. For example, it is clear that K_i(app) $[=a/(b - ad)]$ will contain rate constants for steps involving substrate and, hence, be substrate dependent. On the other hand, the King–Altman patterns reveal that both numerator and denominator will contain $[S]^2$ terms, apparently conflicting with the linear Lineweaver–Burk plots observed for all of the inhibitors being studied. However, the relative importance of the $[S]^2$ terms is dependent on the precise rate constants, and there are many conditions under which they become negligible [see p 90 of Dixon & Webb (1979)].

The King–Altman patterns also indicate that the inhibition mode produced by this mechanism can be either competitive or mixed. If substrate binds only to E or only to EA, there will be no variation of k_{cat} with [I], nor will there be any $[S]^2$ terms. If substrate binds to both E and EA, the inhibition will be competitive if the $[S]^2$ terms are significant and mixed if they are not. In the latter case, $1/k_{cat}$ would be a hyperbolic function of [I] although it is possible that it would not differ significantly from its value in the absence of inhibitor at the inhibitor concentrations employed.

Finally, mechanisms involving binding of more than one molecule of inhibitor can be devised that would also be consistent with the observed kinetics. Interactions fairly distant from the catalytic site of ACE have been demonstrated to contribute significantly to the binding of oligopeptide substrates and inhibitors (Cushman et al., 1973). Thus, multiple binding of small inhibitors is physically plausible. In addition, such effects have been demonstrated spectroscopically for the similar zinc exopeptidase, carboxypeptidase A (Alter & Vallee, 1978).

Scheme IV



Multiple binding most commonly results in parabolic $1/v$ vs. [I] plots rather than the shape observed in Figures 3–5. However, if binding of one of the inhibitor molecules is only partially inhibitory while binding of the other or the two together is completely inhibitory, as in Scheme IV, a rate equation of the same form as eq 2 would be generated (in this case by using rapid equilibrium assumptions):[3]

$$v/[E_0] = \{k_p[S][1 + \beta[I]/(\alpha K_i')]\}/\{K_s[1 + (1/K_i + 1/K_i')[I] + [I]^2/(K_iK_i'')] + [S][1 + [I]/(\alpha K_i')]\} \quad (5)$$

A similar rate equation will be obtained if the form EI is omitted. Lineweaver–Burk plots with this mechanism will be linear. Since the expression for K_i(app) contains both α and β, K_i(app) can be substrate dependent. The inhibition mode can be either competitive or mixed and can also vary with substrate. Since

$$k_{cat} = \frac{k_p[1 + \beta[I]/(\alpha K_i')]}{1 + [I]/(\alpha K_i')}$$

competitive inhibition will be observed if β is close to 1 or if the inhibitor concentrations employed are much smaller than $\alpha K_i'/\beta$ and $\alpha K_i'$.

Stoichiometry of Inhibitor Binding. Scheme IV can be tested by physical determination of the stoichiometry of inhibitor binding. For this purpose, equilibrium dialysis was performed using CA-Phe-Gly. The $1/v$ vs. [I] plots observed with this inhibitor are especially curved, and it has a conveniently low K_i(app) of 120 nM. Control experiments using several CA-Phe-Gly levels in the absence of ACE demonstrate that equilibrium is indeed reached under the dialysis conditions employed (see Materials and Methods) and that the inhibitor does not adhere to the apparatus. Stoichiometry of binding was determined from the concentration of CA-Phe-Gly present in the inhibitor compartment at equilibrium. This concentration was calculated from the inhibition of Fa-Phe-Gly-Gly hydrolysis caused by several aliquots of this solution, by comparison with a standard curve (Figure 5). (Additional details are under Materials and Methods.) This method allows use of inhibitor concentrations that are spectrophotometrically undetectable, circumvents the need to synthesize and use inhibitors of high specific radioactivity, and provides additional flexibility since any inhibitor can be employed as long as its K_i is sufficiently low (ACE concentrations significantly above 100 μM are difficult to achieve).

The results in Table III indicate that two molecules of this inhibitor can indeed bind to the enzyme, thus lending qualitative plausibility to Scheme IV. However, while a stoichiometry of 1 would be inconsistent with Scheme IV, a stoichiometry of 2 cannot be regarded as definitive evidence in its favor. In order to judge whether this multiple binding can account for the precise kinetics observed, it would be necessary to quantitate K_i'' and K_i'''. Unfortunately, obtaining values for a, b, c, and d from curve fitting (using eq 1) for Figure 5 is not sufficient to allow quantitation of all the

[3] Steady-state treatment will yield a rate expression considerably more complex, containing $[I]^3$ terms in the numerator and $[I]^4$ in the denominator. Thus, $1/v$ would be a "4/3" rather than a "2/1" function of [I].

Table III: Stoichiometry of CA-Phe-Gly Binding to ACE

[ACE][a] (μM)	[CA-Phe-Gly]$_0$[b] (μM)	[CA-Phe-Gly]$_{eq}$[c] (μM)	stoichiometry[d]
1.2	2.7	0.77	1.0
6.0	13.5	3.07	1.2
16.2	45	10.4	1.5
16.2	54	13.6	1.7
16.2	72	20.5	1.9

[a] Concentration of ACE added to compartment A of a 1-mL equilibrium dialysis cell, as determined by activity, using a k_{cat}/K_m value of 7.7×10^7 M^{-1} min^{-1} with Fa-Phe-Gly-Gly. [b] Concentration of CA-Phe-Gly added to compartment B of an equilibrium dialysis cell, based on amino acid analysis of a stock solution. [c] Concentration of CA-Phe-Gly remaining in compartment B at equilibrium, i.e., after 4–8 h. This concentration was determined from the effects of several different aliquots of this solution on the ACE-catalyzed hydrolysis of Fa-Phe-Gly-Gly (50 μM substrate, 50 mM Hepes, and 300 mM NaCl, pH 7.5), by comparison with a standard inhibition curve (Figure 5). (Additional details are under Materials and Methods.) [d] Number of molecules of CA-Phe-Gly bound per ACE molecule. In all cases, both enzyme and inhibitor solutions were in 50 mM Hepes and 300 mM NaCl, pH 7.5, and dialyses were performed at room temperature (21–23 °C).

equilibrium constants in this mechanism. Nonetheless, these values do allow limits to be set. Since [S] $\ll K_m$ and $\beta \simeq 1$ (i.e., competitive inhibition is observed)

$$a = K_s/(k_p[S])$$

$$b = \frac{K_s}{k_p[S]}\left(\frac{1}{K_i} + \frac{1}{K_i'}\right)$$

$$c = \frac{K_s}{k_p[S]}\left(\frac{1}{K_iK_i''}\right) = \frac{K_s}{k_p[S]}\left(\frac{1}{K_i'K_i'''}\right)$$

and

$$d = 1/(\alpha K_i')$$

The calculated values for these constants are $a = 3 \times 10^{-4}$ min, $b = 2.54 \times 10^{-3}$ min/μM, $c = 2.74 \times 10^{-4}$ min/μM^2, and $d = 0.642$ μM^{-1}. Substituting these values into the appropriate expressions, we obtain $1/K_i + 1/K_i' \simeq 8.47$ μM^{-1}, $K_iK_i'' = K_i'K_i''' = 1.09$ μM^2, and $\alpha K_i' = 1.56$ μM. Thus, if $K_i = K_i'$, the value of these constants will be 0.24 μM, and K_i'' ($=K_i'''$) will be 4.6 μM. In the more likely event that K_i and K_i' differ, then either K_i'' or K_i''' will be less than 4.6 μM. If, for example, $K_i' = 5K_i$, then $K_i = 0.14$ μM and $K_i''' = 1.5$ μM. Since a stoichiometry of 1.5 does not occur until the concentration of unbound inhibitor is $\sim$10 μM (Table III), multiple binding as in Scheme IV is unlikely to be the sole source of the unusual kinetics observed.

Slow-, Tight-Binding Inhibitors: (*A*) K_i *Values*. Analysis of the inhibition kinetics of captopril and MK-422 requires a different approach from that applied with the non-tight-binding inhibitors discussed thus far. Apparent K_i values for both of these inhibitors are subnanomolar at the pH and chloride concentrations employed. Since the enzyme concentration in the assays (>0.5 nM) is not well below K_i, significant depletion of free inhibitor by enzyme can occur. Thus, it is inappropriate to use the equations normally employed for kinetic analysis of inhibition (where the free and total inhibitor concentrations are assumed to be equal). Fortunately, methods for determining K_i values and inhibition modes under such conditions have been developed. In one of these methods (Henderson, 1972), plots of $[I_T]/(1 - v_i/v_c)$ vs. v_c/v_i, henceforth called "Henderson plots", are obtained at several substrate concentrations, where $[I_T]$ is the total inhibitor concentration and v_c and v_i are steady-state velocities in the absence and presence, respectively, of inhibitor. The slope of each line represents an apparent K_i, and a plot of slope vs. [S] indicates the inhibition mode and yields K_i (the apparent K_i extrapolated to [S] = 0).

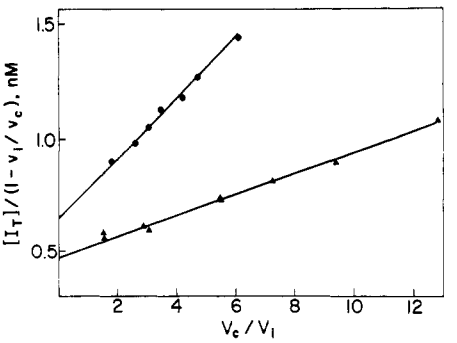


FIGURE 6: Henderson plots for inhibition of ACE-catalyzed hydrolysis of 50 μM Fa-Phe-Gly-Gly (▲) or 21 μM Fa-Phe-Lys-Ala (●) by MK-422. $[I_T]$ represents the total concentration of inhibitor, and v_c and v_i are velocities in the absence and presence, respectively, of inhibitor. With each substrate, the enzyme concentration in all assays was kept constant. A 20% larger aliquot of enzyme solution was employed with Fa-Phe-Lys-Ala than with Fa-Phe-Gly-Gly. Assay mixtures (enzyme and inhibitor in 50 mM Hepes, 300 mM NaCl, and 1 μM $ZnCl_2$, pH 7.5) were preincubated for at least 6 h at 25 °C before the reaction was begun by addition of substrate (1% of total volume). The substrate concentrations employed are well below K_m in both cases.

Captopril and MK-422 present a further complication: their onset of inhibition is extremely slow, in some cases requiring several hours before steady-state velocities are reached. However, a reasonable assessment of K_i can still be obtained from a Henderson plot using substrate concentrations well below K_m, where the slope approximates K_i. Enzyme and inhibitor are preequilibrated, and the assay is started by addition of substrate. With captopril and MK-422, at least 2 and 6 h of preincubation, respectively, are required to ensure full equilibration at all inhibitor concentrations. [These times were calculated from the rate constants provided in the following paper (Shapiro & Riordan, 1984).] Activities in the absence of inhibitor did not change over 16 h, indicating that the enzyme is stable under these preincubation conditions. It was also determined that neither captopril nor MK-422 loses potency during the preincubation period (captopril solutions contain 50 μM 2-mercaptoethanol).

Henderson plots yield K_i values of 330 pM for captopril and 50 pM for MK-422 (Figure 6) with Fa-Phe-Gly-Gly as substrate.[4] This K_i for captopril is about 5-fold lower than the value (1.7 nM) determined by Cushman et al. (1977) and much less than values reported by others [e.g., 72 nM by Almquist et al. (1980)]. The difference between our value and that of Cushman et al. is probably largely attributable to the difference in assay pH (7.5 vs. 8.3), since the K_i values of metal-binding ACE inhibitors appear to increase markedly with pH (Pantoliano et al., 1984). Reports of significantly higher K_i values may well have overlooked the lability of this sulfhydryl compound.

Henderson plots were also obtained by using Fa-Phe-Lys-Ala, a class II substrate (Fa-Phe-Phe-Arg could not be employed for technical reasons). With captopril, a K_i value of 370 pM was measured, virtually identical with that found with Fa-Phe-Gly-Gly. However, with MK-422 (Figure 6), the K_i value (130 pM) is about 2.5-fold higher with the class II

[4] The enzyme concentrations employed in these assays were 1.0 nM with captopril and 0.56 nM with MK-422, on the basis of the absorbance at 280 nm.

Scheme V

substrate, similar to the pattern seen with several of the inhibitors discussed previously.

The Henderson plots appear to be linear up to v_c/v_i values of at least 5 for captopril and >10 for MK-422. Any mechanism that would yield curved $1/v$ vs. [I] plots (Figures 3–5) would produce nonlinear Henderson plots as well (in this case, with upward curvature). In addition, Henderson plots provide an assessment of the enzyme concentration ($[E_0]$ = intercept with ordinate). With all four inhibitor–substrate combinations, the $[E_0]$ value is within 15% of that determined from the absorption at 280 nm.

The variation of the apparent K_i value for MK-422 with different substrates is not as large as that observed with some inhibitors (Table II), but it is reproducible. In order to determine whether this discrepancy may be due to multiple binding of the inhibitor, equilibrium dialysis was performed as described above. Enzyme and inhibitor concentrations were 1.0×10^{-7} and 2.5×10^{-7} M, respectively. In contrast with results for CA-Phe-Gly, a stoichiometry of 0.95 was obtained. This finding supports the idea that the kinetic anomalies observed are not due to multiple binding of inhibitor.

(B) Inhibition Mode. Because of the extremely slow approach to steady-state, Henderson's method cannot be used to assess the mode of inhibition. However, Cha (1976) has noted that the inhibition mode can be determined from the dependence of k_{obsd} on substrate concentration, where k_{obsd} is the pseudo-first-order rate constant for the approach to steady state, determined from

$$v = v_s + (v_0 - v_s) \exp(-k_{obsd}t) \quad (6)$$

where v_s, v_0, and v represent velocities at steady-state, zero time, and time t. In the following paper (Shapiro & Riordan, 1984), evidence is presented that captopril and MK-422 bind by a (minimally) two-step mechanism, with fairly rapid initial binding followed by a slow tightening or isomerization. If this is fully competitive (see Scheme V), then

$$k_{obsd} = k_4 + k_3[I]/([I] + K_i')$$

where $K_i' = K_i(1 + [S]/K_m)$ and $K_i = k_2/k_1$. Thus, if $1/(k_{obsd} - k_4)$ is plotted as a function of [S], a straight line with a positive slope will be obtained. In contrast, if the inhibition is mixed, $1/(k_{obsd} - k_4)$ will bear a hyperbolic relation to [S], becoming independent of [S] for pure noncompetitive inhibition. Such a plot reveals a competitive inhibition mode for both MK-422 and captopril (shown in Figure 7) [in agreement with Cushman et al. (1977) but not with Mendelsohn et al. (1981) or Schwab et al. (1983)].[5] In these experiments, k_{obsd} was determined from eq 6 and k_4 from

$$K_i^* = K_i k_4/(k_3 + k_4)$$

as outlined in the following paper [see Shapiro & Riordan (1984)] where K_i^* represents the overall inhibition constant

[5] These experiments were performed with the class I substrate Fa-Phe-Gly-Gly. It was not possible to determine the inhibition mode with a class II substrate for technical reasons.

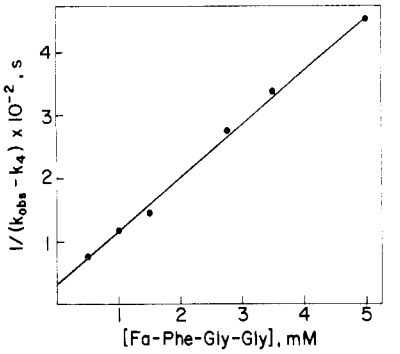


FIGURE 7: Dependence of $1/(k_{obsd} - k_4)$ on Fa-Phe-Gly-Gly concentration for the slow onset of inhibition by captopril. k_{obsd} represents the pseudo-first-order rate constant for the velocity decrease observed as the inhibitor (40 nM) binds to ACE (5.0 nM). k_4 is the rate constant for conversion of EI* to EI, as defined in Scheme V. Assays were performed as described for Figure 2, except that 50 μM 2-mercaptoethanol was also present.

measured in the Henderson plots. An additional assessment of k_4 was obtained from the relationship $k_4 = k_{obsd}v_s/v_0$ for each assay (Morrison, 1982). Values of k_4 obtained by the two methods differ by up to 30% and were averaged. This uncertainty is of little consequence since k_{obsd} is at least severalfold larger than k_4 in all cases.

Conclusions

The present studies demonstrate that with several of the ACE inhibitors examined the inhibition modes, apparent K_i values, and shapes of $1/v$ vs. [I] plots are markedly dependent on the substrate employed. A number of patterns have emerged. (1) A more complex inhibition mechanism is evident with class II than with class I substrates; i.e., the inhibition mode is more frequently mixed, and $1/v$ vs. [I] plots show a much greater degree of curvature. (2) All three inhibitors with a class I type structure (Gly-Trp, CA-Phe-Gly, and PPPP) produce severalfold lower apparent K_i values with class I than with class II substrates (Table II). (3) Two of the three inhibitors with a class II type structure (Phe-Arg and MP-Arg) produce somewhat lower K_i(app) values with class II substrates. (4) Inhibitors with a class III type structure (Ala-Pro, MK-422, and captopril) display the most "normal" kinetics, as judged by the relative linearity of $1/v$ vs. [I] and Henderson plots and the greater substrate independence of K_i values.

These results indicate that many, if not all, of the measured K_i values do not represent true dissociation constants. By curve fitting, we have determined that the rate expression in eq 2 is consistent with the unusual $1/v$ vs. [I] plots obtained. Four mechanisms capable of generating such an expression have been examined, and two of them cannot be eliminated by the data. These mechanisms attribute the abnormal kinetics to (i) inhibitor binding to an enzyme–product complex or (ii) interaction of the enzyme with activating anions. Another mechanism (Scheme IV) considered in detail involves multiple inhibitor binding. It was demonstrated by equilibrium dialysis that two molecules of CA-Phe-Gly can bind to ACE. However, binding of the second molecule occurs at higher inhibitor concentrations than predicted by Scheme IV. In addition, only one molecule of MK-422 binds to ACE, even at $[I] > 1000K_i$, although substrate-dependent "K_i" values are also found with this inhibitor. Clearly, multiple binding cannot be the sole explanation for the unusual kinetics. It may, however, contribute along with the other two mechanisms in some cases. It should be noted that these three schemes are by no means mutually exclusive. Additional mechanisms, not considered here, may also be consistent with the observed kinetics.

Nonetheless, they would have to share two features of these schemes: (i) the inhibitor must be able to combine with more than one form of the enzyme, and (ii) there must exist alternative pathways to products.

The present results were obtained by using synthetic N-blocked tripeptides as substrates. If similar phenomena are observed with the natural ACE substrates angiotensin I and bradykinin, this would suggest ways in which inhibitors might be targeted preferentially against each of these. Thus, a tight-binding class I type inhibitor [e.g., CA- or CP-Gly-Trp should have a K_i(app) value below 10 nM] might inhibit angiotensin II production severalfold more effectively than bradykinin inactivation. In contrast, a class II type inhibitor (e.g., MP-Arg) might affect bradykinin more than angiotensin I hydrolysis. Use of such inhibitors in vivo could then provide information concerning the individual roles of the angiotensin and bradykinin systems. In addition, it may be desirable therapeutically to inhibit one of these two physiological systems more than the other.

Acknowledgments

We thank Dr. A. A. Patchett for generously providing the inhibitors MK-422 and N^{α}-(1-carboxy-3-phenylpropyl)-L-Ala-L-Lys, Dr. Z. P. Horovitz for the captopril and MP-Arg, Dr. B. Holmquist for PPPP, and Dr. M. W. Pantoliano for CA-Phe-Gly. We also thank Drs. M. W. Pantoliano, B. Holmquist, and A. Galdes for many valuable discussions, C. Luehr for writing the computer program employed for curve fitting, and B. L. Vallee for his continued advice and support.

References

Almquist, R. G., Chao, W.-R., Ellis, M. E., & Johnson, H. L. (1980) *J. Med. Chem. 23*, 1392.

Alter, G. M., & Vallee, B. L. (1978) *Biochemistry 17*, 2212.

Bünning, P., & Riordan, J. F. (1983) *Biochemistry 22*, 110.

Bünning, P., Holmquist, B., & Riordan, J. F. (1983) *Biochemistry 22*, 103.

Cha, S. (1976) *Biochem. Pharmacol. 25*, 2695.

Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 173.

Cushman, D. W., & Ondetti, M. A. (1980) *Prog. Med. Chem. 17*, 41.

Cushman, D. W., Pluscec, J., Williams, N. J., Weaver, E. R., Cheung, H. S., & Ondetti, M. A. (1973) *Experientia 29*, 1032.

Cushman, D. W., Cheung, H. S., Sabo, E. F., & Ondetti, M. A. (1977) *Biochemistry 16*, 5484.

Dixon, M. (1953) *Biochem. J. 55*, 170.

Dixon, M., & Webb, E. C. (1979) *Enzymes*, 3rd ed., Academic Press, New York.

Dorer, F. E., Kahn, J. R., Levene, M., & Skeggs, L. T. (1976) *Biochim. Biophys. Acta 429*, 220.

Erdös, E. G. (1976) *Am. J. Med. 60*, 749.

Galardy, R. E. (1980) *Biochem. Biophys. Res. Commun. 97*, 94.

Galardy, R. E., Kontoyiannidou-Ostrem, U., & Kortylewicz, Z. P. (1983) *Biochemistry 22*, 1990.

Gavras, H., Brunner, H. R., Turini, G. A., Kershaw, C. P., Tifft, C. P., Cuttelod, S., Gavras, I., Vukovich, R. A., & McKinstry, D. N. (1978) *N. Engl. J. Med. 298*, 991.

Gavras, H., Bernard, W., Gavras, I., Biollaz, J., Brunner, H. R., & Davies, R. (1981) *Lancet 22*, 543.

Henderson, P. J. F. (1972) *Biochem. J. 127*, 321.

Holmquist, B., & Vallee, B. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6216.

Holmquist, B., Bünning, P., & Riordan, J. F. (1979) *Anal. Biochem. 95*, 540.

King, E. L., & Altman, C. (1956) *J. Phys. Chem. 69*, 1375.

Mendelsohn, F. A. O., Csicsmann, J., & Hutchinson, J. S. (1981) *Clin. Sci. 61*, 277s.

Miller, A. R. (1981) PASCAL *Programs for Scientists and Engineers*, Chapter 10, Sybex, Berkeley, CA.

Morrison, J. F. (1982) *Trends Biochem. Sci. (Pers. Ed.) 7*, 102.

Ondetti, M. A., Rubin, B., & Cushman, D. W. (1977) *Science (Washington, D.C.) 196*, 441.

Pantoliano, M. W., Holmquist, B., & Riordan, J. F. (1984) *Biochemistry 23*, 1037.

Patchett, A. A., Harris, E., Tristram, E. W., Wyvratt, M. J., Wu, M. T., Taub, D., Peterson, E. R., Ikeler, T. J., ten Broeke, J., Payne, L. G., Ondeyka, D. L., Thorsett, E. D., Greenlee, W. J., Lohr, N. S., Hoffsommer, R. D., Joshua, H., Ruyle, W. V., Rothrock, J. W., Aster, S. D., Maycock, A. L., Robinson, F. M., Hirschmann, R., Sweet, C. S., Ulm, E. H., Gross, D. M., Vassil, T. C., & Stone, C. A. (1980) *Nature (London) 288*, 280.

Peach, M. J. (1977) *Physiol. Rev. 57*, 313.

Schwab, A., Weinryb, I., Macerata, R., Rogers, W., Suh, J., & Khandwala, A. (1983) *Biochem. Pharmacol. 32*, 1957.

Segel, I. H. (1975) *Enzyme Kinetics*, pp 646–649, Wiley-Interscience, New York.

Shapiro, R., & Riordan, J. F. (1984) *Biochemistry* (following paper in this issue).

Shapiro, R., Holmquist, B., & Riordan, J. F. (1983) *Biochemistry 22*, 3850.

Skeggs, L. T., Marsh, W. H., Kahn, J. R., & Shumway, N. P. (1954) *J. Exp. Med. 99*, 275.

Soffer, R. L. (1976) *Annu. Rev. Biochem. 45*, 73.

Thorsett, E. D., Harris, E. E., Peterson, E. F., Greenlee, W. J., Patchett, A. A., Ulm, E. H., & Vassil, T. C. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2176.

Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol. 63*, 437.