Inhibition of Angiotensin Converting Enzyme: Dependence on Chloride†

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ABSTRACT: In a previous report [Shapiro, R., Holmquist, B., & Riordan, J. F. (1983) *Biochemistry 22*, 3850], it was demonstrated that activation of angiotensin converting enzyme (ACE) by chloride is strongly dependent on substrate structure, and three substrate classes were identified on the basis of activation behavior. The present study examines the chloride dependence of the inhibition of ACE by nine inhibitors [(D-3-mercapto-2-methylpropanoyl)-L-Pro (captopril), *N*-[1(*S*)-carboxy-3-phenylpropyl]-L-Ala-L-Pro (MK-422), L-Ala-L-Pro, *N*-(phenylphosphoryl)-L-Phe-L-Phe, Gly-L-Trp, *N*-[1(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly, L-Phe-L-Arg, N^{α}-(3-mercaptopropanoyl)-L-Arg, and N^{α}-[1(*S*)-carboxy-3-phenylpropyl]-L-Ala-L-Lys] containing structural features characteristic of the three classes of substrates. Apparent K_i values for all inhibitors are markedly (70–250-fold) decreased by 300 mM chloride. However, the enhancement of inhibition is achieved at significantly lower chloride concentrations with those inhibitors having an ultimate arginine or lysine than with the remainder. This variability parallels that previously found for activation of substrate hydrolysis. The effect of chloride on the individual steps in the formation and dissociation of the steady-state enzyme–inhibitor complexes was determined with the slow-binding inhibitor MK-422. Pre-steady-state analysis indicates that binding of both MK-422 and captopril follows a (minimally) two-step mechanism:

$$E + I \overset{K_i}{\rightleftharpoons} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^*$$

in which rapid formation of an enzyme–inhibitor complex is followed by a slow isomerization. In the presence of 300 mM chloride, the calculated values for k_3, k_4, K_i, and the overall inhibition constant (K_i^*) with MK-422 are 1.9×10^{-2} s^{-1}, 1.1×10^{-4} s^{-1}, 9.2×10^{-9} M, and 5.0×10^{-11} M, respectively. In the presence of 20 mM chloride, k_3, k_4, K_i, and K_i^* values are 8.7×10^{-3} s^{-1}, 5.4×10^{-4} s^{-1}, 6.3×10^{-9} M, and 3.7×10^{-10} M, respectively. Thus, chloride influences isomerization, not initial complex formation, and serves largely to stabilize EI* and thereby slow the conversion of EI* to EI.

An unusual mechanistic feature of angiotensin converting enzyme (peptidyl dipeptidase, EC 3.4.15.1) (ACE)[1] is its strong activation by chloride and other monovalent anions (Skeggs et al., 1954). Since activation is largely accounted for by a decrease in K_m values (Bünning & Riordan, 1983; Shapiro et al., 1983), it would be reasonable to expect that inhibitor binding to the enzyme would also be dependent on the activator. Despite recent intense interest in ACE inhibitors [cf. Petrillo & Ondetti (1982)], this question does not appear to have been studied in detail previously. In the present report, it is shown that binding of both metal-coordinating and dipeptide inhibitors is indeed increased markedly by chloride.

We recently demonstrated that the effects of chloride on substrate hydrolysis are a function of the structure of the substrate (Shapiro et al., 1983). Hydrolysis of all substrates studied was enhanced at least 24-fold by chloride, but for different substrates, there were striking differences in the kinetic mechanism of activation, the amount of chloride required for optimal activity, and the effects of anion substitution and pH on activation. On the basis of these findings, substrates were divided into three classes. At pH 7.5, "class I" substrates (e.g., Fa-Phe-Gly-Gly) appear to be hydrolyzed by an ordered bireactant mechanism in which anion must bind before substrate (Bünning & Riordan, 1983), while hydrolysis of "class II" (e.g., Fa-Phe-Phe-Arg) and "class III" (e.g., Bz-Gly-Ala-Pro) substrates follows a nonessential activator mechanism. Apparent activation constants, representing the chloride concentrations required for half-maximal k_{cat}/K_m values, are 4, 25, and 100 mM with class II, III, and I substrates, respectively. In structural terms, class II substrates are distinguished by the presence of a positively charged side chain at the ultimate or penultimate position. The features distinguishing class I and III substrates are less clear, but all of the latter thus far identified have a penultimate alanine.

In view of this dependence of activation behavior on substrate structure, it was of interest to determine whether similar effects would be seen with inhibitors. Thus, the inhibitors employed in the present study were chosen to contain features characteristic of all three substrate classes: Gly-L-Trp, *N*-(phenylphosphoryl)-L-Phe-L-Phe (PPPP), and *N*-[1(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly (CA-Phe-Gly) are analogous to class I substrates, N^{α}-[1(*S*)-carboxy-3-phenylpropyl]-L-Ala-L-Lys (CP-Ala-Lys) and N^{α}-(3-mercaptopropanoyl)-L-Arg (MP-Arg) are analogous to class II, and L-Ala-L-Pro, (D-3-mercapto-2-methylpropanoyl)-L-Pro (captopril), *N*-[1(*S*)-carboxy-3-phenylpropyl]-L-Ala-L-Pro (MK-422) are analogous to class III. We find that the effect of chloride on ACE inhibition is indeed dependent on inhibitor structure.

Two of the inhibitors in the present study, captopril and MK-422, are slow binding and tight binding. Analysis of the pre-steady-state time interval suggests that binding proceeds by a two-step mechanism in which an enzyme–inhibitor complex forms rapidly and then undergoes a slow isomerization. With MK-422, it was possible to examine the effect of chloride on the individual steps in this process, and it was found that the anion influences isomerization and not initial complex formation.

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[1] Abbreviations: ACE, angiotensin converting enzyme; Fa, 2-furanacryloyl; Bz, *N*-benzoyl; PPPP, *N*-(phenylphosphoryl)-L-Phe-L-Phe; CA-Phe-Gly, *N*-[1(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly; CP-Ala-Lys, N^{α}-[1(*S*)-carboxy-3-phenylpropyl]-L-Ala-L-Lys; MP-Arg, N^{α}-(3-mercaptopropanoyl)-L-Arg; captopril, (D-3-mercapto-2-methylpropanoyl)-L-Pro; MK-422, *N*-[1(*S*)-carboxy-3-phenylpropyl]-L-Ala-L-Pro; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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Materials and Methods

Methods for purification and assay of ACE are described in the preceding paper (Shapiro & Riordan, 1984), as are sources of substrates and inhibitors.

Kinetic Analysis. Inhibitor potency was characterized by an apparent K_i value [K_i(app)], derived from $1/v$ vs. [I] plots at [S] $\ll K_m$ for non-tight-binding inhibitors as described in the text. With Fa-Phe-Gly-Gly, the substrate concentration was 50 μM [K_m = 300 μM at pH 7.5 in the presence of 300 mM NaCl (Holmquist et al., 1979)], and with Fa-Phe-Phe-Arg, it was 3.2 μM [K_m = 15 μM (Shapiro et al., 1983)]. With the tight-binding inhibitors captopril and MK-422, K_i values were obtained from Henderson plots (Henderson, 1972) of $[I_T]/(1 - v_i/v_c]$ vs. v_c/v_i at [S] $\ll K_m$, where $[I_T]$ is the total inhibitor concentration and v_i and v_c are initial velocities in the presence and absence, respectively, of inhibitor. Assay mixtures containing enzyme and inhibitor in 50 mM Hepes, 1 μM $ZnCl_2$, and NaCl as specified (plus 50 μM 2-mercaptoethanol for captopril) were preincubated for at least 2 (captopril) or 6 h (MK-422) before the reaction was started by addition of substrate (1% of total volume). These lengthy preincubations were necessary because of the extremely slow binding of inhibitor to enzyme. At [S] $\ll K_m$, the initial velocity following preincubation closely approximates the steady-state velocity, and the slope of a Henderson plot equals K_i.

Methods for analysis of the slow binding of captopril and MK-422 were adapted from Cha (1975, 1976a,b), Strickland et al. (1975), and Williams & Morrison (1979). A cuvette containing a mixture of inhibitor and substrate in 50 mM Hepes, pH 7.5, plus the specified amount of NaCl, was equilibrated at 25 °C for at least 20 min inside the spectrophotometer. The assay was then begun by addition of enzyme. A pseudo-first-order rate constant (k_{obsd}) for the decrease in reaction velocity as inhibitor binds to enzyme was calculated by using eq 1 in the text. The decrease in absorbance at a convenient wavelength between 342 and 353 nm was continuously monitored until a steady-state velocity (i.e., a linear decrease in absorbance over at least a 10-min time interval) was reached. No more than 10% of the substrate was hydrolyzed during the assay. Changes in absorbance during successive equal time intervals were measured as approximations of the reaction velocity (in arbitrary units) at that time. Time intervals were sufficiently small (10–50 s) so that curves were essentially linear within each. A value for k_{obsd} was then determined from the slope of a plot of ln $(v - v_s)$ vs. time, where v and v_s are velocities at time t and at steady state, respectively. Between 6 and 10 points were used for each plot. Individual rate and dissociation constants were calculated from k_{obsd} values as detailed below.

Results

Effect of [Cl^-] on Inhibitor Binding. In cases where the mechanism of inhibition is simple, it should be possible to determine the effect of chloride concentration on inhibitor binding by measuring K_i, the dissociation constant for inhibitor and free enzyme, as a function of [Cl^-]. K_i can be determined kinetically from plots of K_m/k_{cat} vs. [I], or from Henderson plots if inhibitor binding is tight (i.e., if there is significant depletion of inhibitor by enzyme). However, it was demonstrated in the preceding paper (Shapiro & Riordan, 1984) that with most of the inhibitors under study, such plots are nonlinear and/or substrate dependent, precluding unambiguous determination of K_i. Therefore, in evaluating the effect of chloride on the interaction of ACE with inhibitors, we have to employ "apparent" K_i values [K_i(app)]. When K_m/k_{cat} vs. [I] plots or Henderson plots are linear, K_i(app) is defined as the −[I] intercept or slope, respectively. When K_m/k_{cat} vs. [I] plots are curved, linearity is approximated at very low inhibitor concentrations, and K_i(app) then represents the extrapolated −[I] intercept of this line.

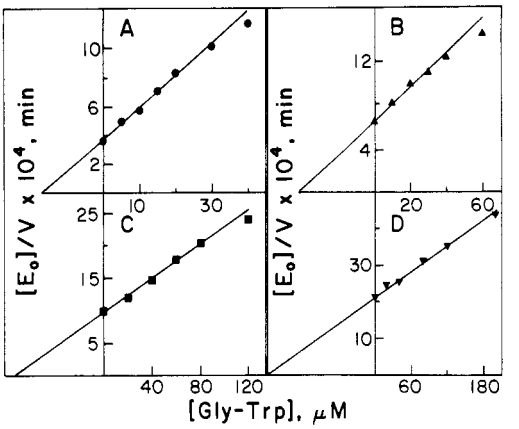

FIGURE 1: Plots of $[E_0]/v$ vs. [Gly-Trp] with 50 μM Fa-Phe-Gly-Gly as substrate. Assays were at 25 °C in 50 mM Hepes, pH 7.5, with 300 (A), 100 (B), 50 (C), or 20 mM (D) NaCl.

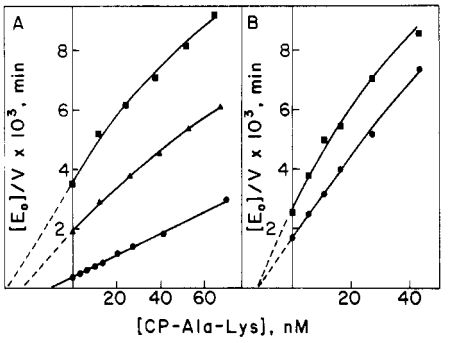

FIGURE 2: Plots of $[E_0]/v$ vs. [CP-Ala-Lys] with 50 μM Fa-Phe-Gly-Gly (A) or 3.2 μM Fa-Phe-Phe-Arg (B) as substrate. Assays were at 25 °C in 50 mM Hepes, pH 7.5, with 300 (●), 20 (▲), or 10 mM (■) NaCl.

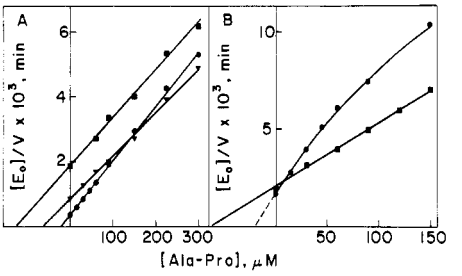

FIGURE 3: Plots of $[E_0]/v$ vs. [Ala-Pro] with 50 μM Fa-Phe-Gly-Gly (A) or 3.2 μM Fa-Phe-Phe-Arg (B) as substrate. Assays were at 25 °C in 50 mM Hepes, pH 7.5, plus 300 (●), 50 (▼), or 20 (■) mM NaCl.

With the seven, non-tight-binding inhibitors, plots of $1/v$ vs. [I] were obtained at several chloride concentrations by using the class I substrate Fa-Phe-Gly-Gly at [S] $\ll K_m$ where v is directly proportional to k_{cat}/K_m. In some cases, the class II substrate Fa-Phe-Phe-Arg was also employed. These plots, examples of which are shown in Figures 1–3, reveal variable effects of chloride concentration on K_i(app). Thus, with all three inhibitors having a class II type structure (i.e., Phe-Arg, CP-Ala-Lys, and MP-Arg), the ratio of K_i(app) values measured at 20 mM NaCl to those found at 300 mM NaCl with Fa-Phe-Gly-Gly as substrate is ~2, while with inhibitors having a class I or class III type structure the ratio is >5 (Table I). The k_{cat}/K_m value for Fa-Phe-Gly-Gly itself

Table I: Effect of [Cl⁻] on K_i(app) with Various Inhibitors

inhibitor	K_i(app)[a] (M) at 300 mM NaCl	K_i(app)[a] (M) at 20 mM NaCl	ratio[b]
Gly-Trp	1.7×10^{-5}	1.7×10^{-4}	10
MK-422	5.0×10^{-11}	3.7×10^{-10}	7.4
Ala-Pro	2.2×10^{-5}	1.4×10^{-4}	6.3
CA-Phe-Gly	1.2×10^{-7}	6.6×10^{-7}	5.5
captopril	3.3×10^{-10}	1.8×10^{-9}	5.5
PPPP	9.0×10^{-7}	4.7×10^{-6}	5.2
MP-Arg	2.2×10^{-8}	5.5×10^{-8}	2.5
CP-Ala-Lys	1.0×10^{-8}	2.3×10^{-8}	2.3
Phe-Arg	2.2×10^{-4}	4.0×10^{-4}	1.8

[a] K_i(app) values were obtained from plots of $1/v$ vs. [I] or from Henderson plots, as described in the text. All assays were performed in 50 mM Hepes, pH 7.5 at 25 °C, with 50 μM Fa-Phe-Gly-Gly as substrate. [b] The K_i(app) value at 20 mM NaCl divided by that at 300 mM NaCl.

changes about 5-fold over this range of chloride concentrations.

With the class II substrate Fa-Phe-Phe-Arg, the effect of chloride on K_i(app) is smaller than that observed with Fa-Phe-Gly-Gly (Figures 2 and 3). There is no significant change in K_i(app) when [Cl⁻] is lowered from 300 to 10 mM with the class II type inhibitors. With Ala-Pro, K_i(app) increases only 2.8-fold as [Cl⁻] is decreased from 300 to 20 mM, compared with a 6.3-fold change when Fa-Phe-Gly-Gly is the substrate.

The shapes of the $1/v$ vs. [I] plots are also chloride concentration dependent. With Fa-Phe-Gly-Gly as substrate, plots for the class II type inhibitors are nearly linear at 300 mM NaCl but become curved as [Cl⁻] is lowered (Figure 2). In contrast, plots that display marked curvature at 300 mM NaCl (i.e., those with the class I type inhibitors Gly-Trp and CA-Phe-Gly) become less curved as the chloride concentration is decreased. With Fa-Phe-Phe-Arg as substrate, a similar approach to linearity with lower [Cl⁻] is observed with Ala-Pro (Figure 3).

With the two tight-binding inhibitors captopril and MK-422, Henderson plots were obtained at different chloride concentrations by using the substrate Fa-Phe-Gly-Gly (at $[S] \ll K_m$). The plots are linear, and their slopes provide apparent K_i values (Figure 4 for MK-422). These K_i values increase 5.5- and 7.4-fold with captopril and MK-422, respectively, as the chloride concentration is lowered from 300 to 20 mM (Table I).

In order to assess the overall dependence of inhibitor binding on chloride, we also determined K_i values in its absence.[2] In this case, Fa-Phe-Phe-Arg was employed as the substrate, since there is no measurable hydrolysis of Fa-Phe-Gly-Gly under these conditions. K_i(app) values, obtained from plots of $1/v$ vs. [I] at $[S] \ll K_m$, are 5.8 mM, 170 μM, 1.2 μM, 0.84 μM, and 14 nM for Ala-Pro, CA-Phe-Gly, MP-Arg, CP-Ala-Lys, and MK-422, respectively. Compared with the values measured by using a class II substrate at 300 mM NaCl (Shapiro & Riordan, 1984), these represent increases of 250-, 170-, 120-, 70-, and 110-fold, respectively.

Slow-Binding Inhibitors: Mechanism at 300 mM NaCl. When the hydrolytic reaction in the presence of captopril or MK-422 is initiated by addition of enzyme, the onset of inhibition is slow, and in some cases, a true steady-state velocity is not reached for several hours. Examination of the pre-steady-state time interval can, in theory, provide information concerning the inhibition mechanism and individual rate constants for formation and dissociation of the EI complex.

[2] The chloride concentration in the buffer was estimated to be ~10 μM on the basis of measurements with a chloride-selective electrode (Graphic Controls, Buffalo, NY).

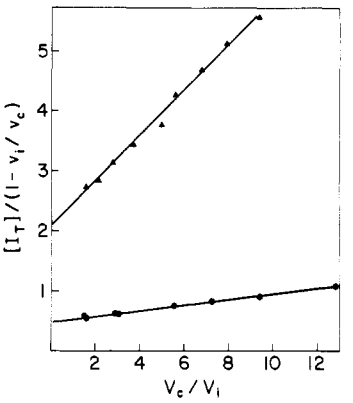

FIGURE 4: Henderson plots for inhibition of Fa-Phe-Gly-Gly hydrolysis by MK-422. Assays were in 50 mM Hepes, pH 7.5, with 300 (●) or 20 (▲) mM NaCl at 25 °C. Assay mixtures were preincubated for at least 6 h at 25 °C before the reaction was begun by addition of substrate (1% of total volume). The substrate concentration was well below K_m.

Analytical methods for this purpose have been developed by Cha (1975, 1976a,b), Stickland et al. (1975), Williams & Morrison (1979), and Williams et al. (1979), and the following treatment has been adapted from these sources. It is generally assumed that the enzyme–substrate complex reaches a steady-state concentration instantaneously. If no further assumptions are made, determination of rate constants and the inhibition mechanism is extremely complex. However, the analysis can be greatly simplified if (i) the amount of substrate hydrolyzed during the assay is small, (ii) the concentration of enzyme ($[E_0]$) is much smaller than that of substrate, and (iii) there is no significant depletion of inhibitor by enzyme. In the present experiments, less than 10% of the substrate was hydrolyzed during the course of each assay, and [S] was always at least 10^4 times higher than $[E_0]$. Further, the inhibitor concentration was 6–10 times greater than $[E_0]$ (with exceptions as discussed below), and any error due to inhibitor depletion should thus be relatively small.

An additional condition which must be met in order for the analysis to be straightforward is that the decrease in velocity during the slow onset of inhibition follow the equation

$$v = v_s + (v_0 - v_s) \exp(-k_{obsd} t) \quad (1)$$

where v, v_s, and v_0 are velocities measured at time t, steady state, and zero time, respectively, and k_{obsd} is an apparent first-order rate constant (Cha, 1976b). Equation 1 predicts that a plot of ln $(v-v_s)$ vs. t will be linear, with a slope of $-k_{obsd}$. With both MK-422 and captopril, these plots do appear to be linear (Figure 5), although in some cases there is a somewhat more rapid decrease in velocity during the first ~1 min of the reaction.

If the mode of inhibition is competitive [as demonstrated for captopril and MK-422 in the preceding paper (Shapiro & Riordan, 1984)], there are three simple mechanisms which can account for a slow onset of inhibition:

mechanism A

$$E \underset{k_2}{\overset{k_1[I]\ (slow)}{\rightleftharpoons}} EI$$

mechanism B

$$E \underset{k_2}{\overset{k_1[I]}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3\ (slow)}{\rightleftharpoons}} EI^*$$

mechanism C

$$E \underset{k_2}{\overset{k_1\ (slow)}{\rightleftharpoons}} E^* \underset{k_4}{\overset{k_3[I]}{\rightleftharpoons}} E^*I$$

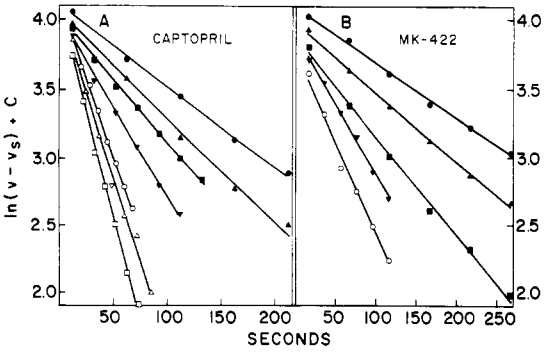

FIGURE 5: Determinations of k_{obsd} for the slow onset of inhibition of Fa-Phe-Gly-Gly hydrolysis by captopril (A) and MK-422 (B), according to eq 1. Assays were initiated by addition of ACE to mixtures of 1 mM substrate in 50 mM Hepes, pH 7.5, containing inhibitor and 300 mM NaCl. Inhibitor concentrations were 20 (●), 30 (▲), 40 (■), 60 (▼), 100 (○), 150 (▲), or 250 (□) nM for captopril and 10 (●), 15 (▲), 25 (■), 40 (▼), or 80 (○) nM for MK-422. Enzyme concentration ([E_0]) was varied along with inhibitor concentration. With captopril, [I]/[E_0] was 6–10, and with MK-422, it was 6. Velocities at different times were determined as described under Materials and Methods. A factor C was added to ln ($v - v_s$) for each plot and was chosen for graphic purposes only. It is of no consequence since the desired parameter, k_{obsd}, is obtained solely from the slopes of these lines.

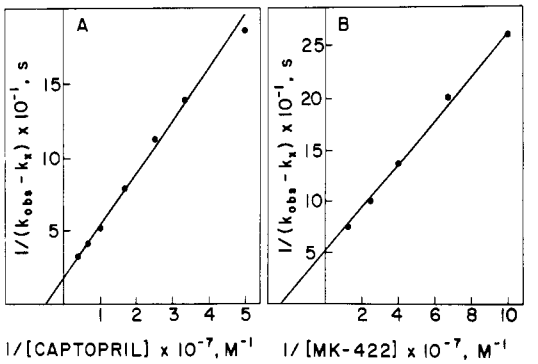

FIGURE 6: Dependence of $1/(k_{obsd} - k_x)$ on 1/[I] for captopril (A) and MK-422 (B). Values for k_{obsd} are from Figure 5. k_x represents k_2 if mechanism A applies and k_4 if mechanism B is followed. The k_x values employed are 4.2×10^{-4} s^{-1} with captopril and 1.0×10^{-4} s^{-1} with MK-422 and were obtained by using eq 5.

In mechanism A, the binding of inhibitor to enzyme is slow. In mechanism B, an EI complex is formed rapidly and then undergoes a slow tightening or isomerization to EI*. (It should be noted that if EI formation is also slow, eq 1 will not be followed.) In mechanism C, the enzyme itself must slowly isomerize before it can bind inhibitor. These three mechanisms can be distinguished by measuring k_{obsd} as a function of inhibitor concentration, since for mechanism A k_{obsd} equals

$$k_2 + k_1[I]/(1 + [S]/K_m) \quad (2)$$

for mechanism B k_{obsd} equals

$$k_4 + k_3[I]/([I] + K_i') \quad (3)$$

and for mechanism C k_{obsd} equals

$$k_1/(1 + [S]/K_m) + k_2K_i/([I] + K_i) \quad (4)$$

where $K_i' = K_i(1 + [S]/K_m)$ and $K_i = k_2/k_1$ (mechanism B) or k_4/k_3 (mechanism C).

In the present case, mechanism C is immediately eliminated by the observation that k_{obsd} increases, rather than decreases, as [I] increases. Mechanisms A and B can be distinguished by several criteria. First, both yield k_{obsd} values that increase with inhibitor concentration, but with mechanism A, k_{obsd} is a linear function of [I], while with mechanism B the dependence is sigmoidal, with k_{obsd} reaching a limiting value of $k_3 + k_4$ at high [I]. In practice, however, these two mechanisms may be differentiated most readily by plotting $1/(k_{obsd} - k_2)$ or $1/(k_{obsd} - k_4)$ vs. 1/[I]. In both cases, the plots will be linear, but with mechanism A, the line will pass through the origin, while with mechanism B it will have a $1/k_{obsd}$ intercept at $1/k_3$ and a 1/[I] intercept at $-1/K_i'$. Determination of k_{obsd} at various inhibitor concentrations are shown in Figure 5 for captopril and MK-422. The concentration of the substrate Fa-Phe-Gly-Gly was held constant at 1 mM ($3.3K_m$). Values for k_2 or k_4 can be obtained from each assay by using the relationship (Morrison, 1982)

$$k_2 \text{ or } k_4 = k_{obsd}v_s/v_0 \quad (5)$$

These values are only approximate since it is difficult to accurately measure v_0 (see below) and, in some instances, v_s. However, in all cases, the ratio v_s/v_0 is small (<0.07 with captopril and <0.03 with MK-422), so that k_{obsd} is much larger than k_2 or k_4 and the uncertainty in the latter values will not strongly influence the result. The plots in Figure 6 do not pass through the origin and, hence, are consistent with mechanism B for both captopril and MK-422. The values for k_3 and K_i obtained are shown in Table II. A more accurate calculation of k_4 can then be made employing the relationship

$$K_i^* = K_ik_4/(k_3 + k_4) \quad (6)$$

where K_i^* is the overall inhibition constant for E + I ⇌ EI* which is measured by Henderson plots. These k_4 values do not differ greatly from those derived by using eq 5.

Table II: Rate and Equilibrium Constants for Inhibition of ACE by MK-422 and Captopril[a]

inhibitor	[Cl⁻] (mM)	K_i^* (pM)	K_i (nM)	k_3 (s^{-1})	k_4 (s^{-1})
captopril	300	330	47	5.6×10^{-2}	4.0×10^{-4}
MK-422	300	50	9.2	1.9×10^{-2}	1.1×10^{-4}
MK-422	20	370	6.3	8.7×10^{-3}	5.4×10^{-4}

[a] All assays were performed in 50 mM Hepes, pH 7.5 at 25 °C, with Fa-Phe-Gly-Gly as substrate. Values for K_i^* were obtained from Henderson plots (Figure 4 for MK-422). K_i, k_3, and k_4 were derived from Figures 6 and 8 as described in the text and in the legends to those figures.

Mechanisms A and B can also be distinguished by two additional criteria. If mechanism A is followed, the initial reaction velocity (v_0) will be independent of inhibitor concentration. On the other hand, mechanism B predicts that v_0 will decrease as [I] increases, due to rapid formation of EI. With MK-422, the spectrophotometer tracings at constant [S] and [E_0] (not shown) indicate the latter relationship.

Finally, a plot of $1/(k_{obsd} - k_2)$ (mechanism A) or $1/(k_{obsd} - k_4)$ (mechanism B) vs. [S] at constant [I] will produce different [S] intercepts for the two mechanisms: $[S] = -K_m$ with mechanism A, and $[S] = [-K_m([I] + K_i)]/K_i$ for mechanism B. The intercept determined with MK-422 (Figure 7) is 1.2 mM, well above the K_m of 300 μM for Fa-Phe-Gly-Gly. The value of K_i calculated from this intercept is 7 nM, in good agreement with the value derived from Figure 6.

Effect of [Cl⁻] on Slow Binding of MK-422. The data reported in the preceding section were obtained in the presence of 300 mM NaCl. A similar analysis was carried out with MK-422 at 20 mM NaCl (Figure 8) in order to determine which steps in mechanism B are affected by chloride. As noted earlier, K_i^* is increased 7.4-fold as [Cl⁻] is lowered from 300 to 20 mM. Calculation of k_4 from eq 5 reveals that this reverse isomerization constant is severalfold faster at the lower chloride

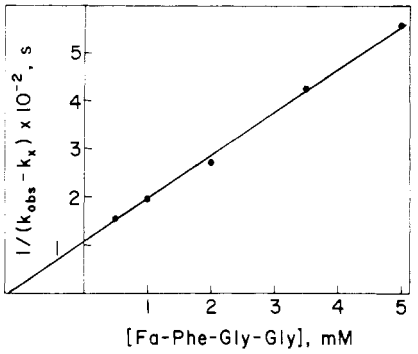

FIGURE 7: Dependence of $1/(k_{obsd} - k_x)$ on Fa-Phe-Gly-Gly concentration for the slow onset of inhibition by MK-422. k_{obsd} represents the pseudo-first-order rate constant for the velocity decrease observed as the inhibitor (20 nM) binds to ACE (3 nM). k_x is k_2 if mechanism A is followed and k_4 if mechanism B is followed. It is determined from eq 5. Assays were performed at 25 °C in 50 mM Hepes, pH 7.5, containing 300 mM NaCl.

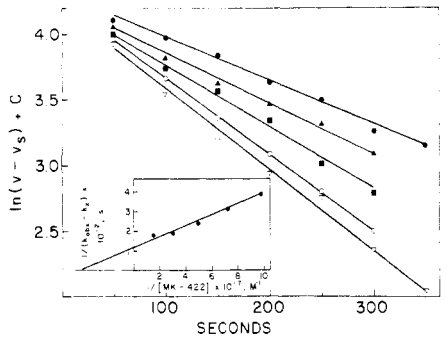

FIGURE 8: Determination of k_{obsd} for the slow onset of inhibition of Fa-Phe-Gly-Gly hydrolysis by MK-422 in the presence of 20 mM NaCl as in Figure 5. Assays were at 25 °C in 50 mM Hepes, pH 7.5. The substrate concentration was 4.5 mM. Initial inhibitor concentrations were 15 (●), 20 (▲), 30 (■), 50 (○), or 100 (▽) nM. $[I]/[E_0]$ was kept constant at 2.5. Inset: Dependence of $1/(k_{obsd} - k_x)$ on $1/[I]$ as in Figure 6.

concentration. Values of K_i, k_3, and k_4 derived from a plot of $1/(k_{obsd} - k_4)$ vs. $1/[I]$ (Figure 8) and eq 6 confirm the change in k_4 and indicate that K_i is not significantly altered, while there is a 2-fold decrease in k_3 (Table II). Thus, chloride appears to influence primarily the isomerization of the EI complex rather than the initial binding of the inhibitor. It must be noted, however, that the data plotted in Figure 8 were not obtained in precisely the same manner as those at 300 mM NaCl. With 20 mM chloride, it was necessary for technical reasons to use a higher $[E_0]/[I]$ ratio (~0.4) in order to obtain absorbance changes sufficient to allow determination of k_{obsd}. Thus, the depletion of inhibitor by enzyme is no longer insignificant, and k_{obsd} will vary somewhat during the course of the assay as the concentration of free inhibitor decreases. The resulting error introduced by this can be minimized by calculating k_{obsd} (Figure 8) from velocities reached after 50% inhibition so that [I] remains relatively constant during the time interval examined. The inhibitor concentrations used to derive K_i, k_3, and k_4 values (Figure 8 inset) then represent values ($[I_f]$) which have been corrected for depletion [$[I_f] = [I_T] - [E_0](v_0 - v_a)/v_0$ where v_a is the average velocity measured in each plot in Figure 8]. We estimate that the resultant uncertainty in [I] is below 10% and should not drastically influence the calculated rate and equilibrium constants.

The interaction of MK-422 with ACE was also examined in the absence of added chloride by using the substrate Fa-Phe-Phe-Arg. No slow onset of inhibition was observed. As stated earlier, an apparent K_i value of 14 nM was obtained. This is in reasonable agreement with the K_i values for E + I ⇌ EI calculated at 300 and 20 mM NaCl (Table II).

Discussion

The present results demonstrate that binding of inhibitors to converting enzyme, like that of substrates, is greatly enhanced by chloride. The inhibitors employed incorporate a variety of structural characteristics. Three are dipeptides, and six are amino acid or dipeptide derivatives containing a metal-liganding group (carboxylate, sulfhydryl, or $-PO^-$). In two instances the amino acid at the ultimate position is aromatic, in another three it is positively charged, and in three more it is proline. The latter is particularly noteworthy since it has been claimed that both the binding of BPP_{5a} (<Glu-Lys-Trp-Ala-Pro) and the hydrolysis of substrates containing Ala-Pro at the C-terminus are inhibited rather than activated by chloride (Cheung & Cushman, 1973; Cheung et al., 1980). In constrast, we find that the apparent K_i values for Ala-Pro and MK-422 (an N-substituted Ala-Pro) are decreased over 100-fold by addition of 300 mM chloride. A similar enhancement of MK-422 binding by chloride, measured by using physical rather than kinetic methods, has been found by Bull and co-workers [cited in Patchett & Cordes (1984)].

Inhibitors, like substrates, can be assigned to different classes on the basis of the effects of chloride on their interaction with ACE. The change in K_i(app) as the chloride concentration is lowered varies markedly with inhibitor (Table I). Inhibition by compounds having a structure analogous to that of class II substrates (Phe-Arg, MP-Arg, and CP-Ala-Lys) is clearly not decreased as greatly as is inhibition by the remaining compounds. However, the effects of chloride on binding of inhibitors with structures analogous to those of class I (Gly-Trp, PPPP, and CA-Phe-Gly) and class III (Ala-Pro, captopril, and MK-422) substrates appear to be similar. This suggests that there are two rather than three classes of inhibitors, with respect to anion activation. This conclusion must be considered tentative, however, since the number of inhibitors examined is relatively small and the K_i(app) values upon which the divisions are based derive from nonlinear plots and bear an unknown relationship to true dissociation constants.

The dependence of substrate k_{cat}/K_m values on $[Cl^-]$ provides an apparent activation constant ($^sK_A'$) for chloride (Bünning & Riordan, 1983; Shapiro et al., 1983), representing the chloride concentration at which k_{cat}/K_m is half-maximal. (The physical meaning of this constant will be discussed below.) An apparent activation constant ($^iK_A'$) can also be derived from the dependence of K_i(app) on $[Cl^-]$ for the inhibitors; i.e., a plot of K_i(app) vs. $1/[Cl^-]$ will have a $1/[Cl^-]$ intercept at $-1/^iK_A'$. With the class I type inhibitors (replots not shown), $^iK_A'$ is about the same (130 mM for PPPP and 140 mM for CA-Phe-Gly) or higher (500 mM for Gly-Trp) than $^sK_A'$ measured with class I substrates (75–150 mM). With all three class II type inhibitors, $^iK_A'$ values (20–30 mM) are severalfold greater than class II $^sK_A'$ values (3–5 mM). An even larger difference is observed with class III type inhibitors vs. substrates: $^iK_A'$ is 140–250 mM while $^sK_A'$ is 18–30 mM.

In the preceding paper (Shapiro & Riordan, 1984), several aspects of ACE inhibition were shown to be substrate dependent, including inhibitor effectiveness [K_i(app)], inhibition mode, and shapes of $1/v$ vs. [I] plots. An additional substrate dependence is observed when the effects of anions on inhibition are examined: $^iK_A'$ values are significantly higher when inhibition is measured with a class I than with a class II substrate. Virtually no change in K_i(app) for Phe-Arg, MP-Arg, and CP-Ala-Lys (Figure 2B) is seen with Fa-Phe-Phe-Arg as substrate when the chloride concentration is lowered from 300

Scheme I

$$E + A \overset{K_A}{\rightleftharpoons} EA + S \overset{K_S}{\rightleftharpoons} EAS \xrightarrow{k_p} E + P$$

Scheme II

$$\begin{array}{ccccc} E + S & \overset{K_S}{\rightleftharpoons} & ES & \xrightarrow{k_p} & E + P \\ + & & + & & \\ A & & A & & \\ {\scriptstyle K_A}\downarrow\uparrow & & {\scriptstyle \alpha K_A}\downarrow\uparrow & & \\ EA + S & \overset{\alpha K_S}{\rightleftharpoons} & EAS & \xrightarrow{\beta k_p} & EA + P \end{array}$$

to 10 mM. The ${}^iK_A'$ obtained by using the inhibitor Ala-Pro with Fa-Phe-Phe-Arg as a substrate is 40 mM, compared to 180 mM with Fa-Phe-Gly-Gly.

An interesting consequence of the enhancement of inhibitor effectiveness by chloride is that the presence of relatively small amounts of inhibitor (e.g., as dipeptide impurities or products) can drastically alter the observed dependence of substrate hydrolysis on chloride concentration. If ${}^iK_A'$ is greater than ${}^sK_A'$, $1/v$ vs. [I] lines at different [Cl^-] will intersect at positive [I] values. Thus, with sufficient inhibitor present, the reaction velocity will first increase but then decrease as the chloride concentration is raised (Figure 3). This effect should be particularly severe when class III substrates such as Bz-Gly-Ala-Pro are employed, since ${}^iK_A'$ measured with the dipeptide product is about 10-fold higher than ${}^sK_A'$ obtained with the substrate itself. If ${}^iK_A'$ is less than or equal to ${}^sK_A'$, there will be no decrease in velocity at high [Cl^-], but the observed ${}^sK_A'$ in the presence of inhibitor will be lower than that found in its absence.

In earlier studies on the effects of chloride on ACE-catalyzed hydrolysis (Bünning & Riordan, 1983; Shapiro & Riordan, 1983), it was suggested that at alkaline pH class I substrates can only bind to the enzyme–anion complex (Scheme I) while class II and III substrates can bind to free enzyme as well (Scheme II). If rapid equilibrium assumptions are made for each mechanism, a dissociation constant K_A for chloride and *free enzyme* can be calculated from the dependence of K_m on [Cl^-]. Paradoxically, K_A values differing by 20-fold are obtained when class I and class II substrates are employed. Two possible explanations for this discrepancy have been offered. First, the kinetic results could reflect the existence of two chloride binding sites, one regulating binding of class I substrates and the other that of class II substrates. Second, the variation of the measured K_A with substrate could indicate that these values do not represent true dissociation constants. Steady-state treatment of Scheme II yields a rate equation considerably more complex than that obtained by assuming that the substrate and anion binding steps are rapid equilibrium processes. The measured K_A values can now deviate from the true K_A and be substrate dependent. Computer modeling (R. Shapiro, unpublished results) suggests that, as long as the pathway E → EA → EAS makes a significant contribution, it is not possible for the observed K_A values to differ by 20-fold. If this pathway is eliminated, however, as in Scheme III, a steady-state rate equation is obtained:

$$v/[E_0] = \{k_1[S][k_3k_4[A] + k_2(k_{-3} + k_4)]\}/\{(k_{-1} + k_2) \times (k_{-3} + k_4) + k_3k_4[A] + k_1(k_{-3} + k_4)[S] + k_1k_3[A][S]\}$$

in which the measured K_A [$=(k_{-3} + k_4)(k_{-1} + k_2)/(k_3k_4)$] can indeed vary with substrate over a wide range of values.

While Scheme III is consistent with the previously reported data for activation of substrate hydrolysis (Shapiro et al., 1983), it is less readily reconciled with the effects of chloride on ACE inhibition. If it is assumed that inhibition follows a

Scheme III

$$\begin{array}{ccccc} E + S & \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} & ES & \xrightarrow{k_2} & E + P \\ & & + & & \\ & & A & & \\ & & {\scriptstyle k_{-3}}\uparrow\downarrow{\scriptstyle k_3} & & \\ & & EAS & \xrightarrow{k_4} & E + A + P \end{array}$$

simple competitive mechanism and that inhibitor binds before anion (as with substrates in Scheme III):

$$E \overset{k_1[I]}{\underset{k_{-1}}{\rightleftharpoons}} EI \overset{k_2[A]}{\underset{k_{-2}}{\rightleftharpoons}} EIA$$

then the measured inhibition constant K_i will equal

$$\frac{k_{-1}k_{-2}}{k_1k_2[A] + k_1k_{-2}}$$

At high chloride concentrations where K_i is much less than k_{-1}/k_1 (i.e., K_i in the absence of anion), K_i will be directly proportional to $1/[Cl^-]$. This appears to be inconsistent with the observation that K_i(app) decreases only 2–10-fold when [Cl^-] is increased 15-fold (Table I).

In the preceding paper (Shapiro & Riordan, 1984), however, we demonstrated that with many (perhaps all) of the compounds under study, the inhibition mechanism is complex, indicative of multiple modes of interaction between enzyme and inhibitor. The complete kinetic scheme must therefore involve more steps than simply E ⇌ EI ⇌ EIA, and it may no longer predict that K_i(app) be directly proportional to $1/[Cl^-]$. In addition, since the relationship of K_i(app) values to true inhibition constants is unknown, it is conceivable that the latter would in fact show the chloride dependence expected from Scheme III. In order to establish the actual activation and inhibition mechanisms, it is clearly crucial that the present kinetic work be supplemented by physical determinations of the dissociation constants for free enzyme and chloride and for enzyme and inhibitor as a function of [Cl^-]. Such methods are currently being explored in this laboratory.

With two of the inhibitors in the present study, captopril and MK-422, the onset of inhibition is slow. This is not surprising in light of the extremely low K_i values measured for these compounds (330 and 50 pM, respectively) (Shapiro & Riordan, 1984). Cha (1975) has pointed out that when K_i values are below 10^{-9} M, binding will frequently be slow because the diffusion limit for a second-order rate constant is $\sim 10^9$ M^{-1} s^{-1} and these constants are typically at least 1 or 2 orders of magnitude smaller. In the present case, however, the slow onset of inhibition does not seem to reflect slow binding of the inhibitor (mechanism A) but rather an isomerization of the enzyme–inhibitor complex, which is itself formed rapidly (mechanism B).

At least 20 instances of slow-binding inhibition have previously been described [reviewed by Morrison (1982)]. In several of these cases, a two-step mechanism is followed, e.g., inhibition of dihydrofolate reductase by methotrexate (Williams et al., 1979), ribulose-1,5-bisphosphate carboxylase by 2-carboxy-D-arabinitol 1,5-bisphosphate (Pierce et al., 1980), and pepsin by pepstatin (Rich & Sun, 1980). Three pieces of evidence favor this mechanism for ACE. First, when the reaction is started by addition of enzyme, the initial velocity appears to decrease as the inhibitor concentration is increased. It must be noted, however, that it is difficult to obtain true initial velocities in these assays: some decrease in velocity inevitably occurs during the mixing time before the spectrophotometer tracing begins. Since k_{obsd} increases with [I], the amount of the reaction that is missed also increases with [I].

If the mixing time is sufficiently long, a decrease in v_0 with [I] could be observed when there is in fact no change. While our mixing time (<15 s) should have been short enough to minimize such errors, additional evidence is clearly required in order to establish the mechanism.

Stronger support for mechanism B is provided by the observation that plots of $1/(k_{obsd} - k_4)$ vs. $1/[I]$ yield lines which do not pass through the origin (Figures 6 and 8). This implies that k_{obsd} reaches a limiting value at high [I] as expected for mechanism B but not for mechanism A. Finally, a plot of $1/(k_{obsd} - k_4)$ vs. [S] for MK-422 has an intercept at $|[S]|$ values much greater than K_m (Figure 7). (The linearity and positive slope of this plot indicate a competitive inhibition mode no matter which mechanism applies.) Mechanism A predicts that the intercept will be at $[S] = -K_m$, while with mechanism B the intercept is at $[S] = [-K_m([I] + K_i)]/K_i$. The value for K_i obtained from this plot is similar to that derived from Figure 6B.

During the preparation of this paper, work by Bull and co-workers [cited in Patchett & Cordes (1984)], examining the slow binding of captopril and MK-422 to ACE in the presence of 300 mM NaCl by somewhat different methodology, was communicated to us. Their data are also consistent with mechanism B. The values of k_4, K_i, and K_i^* suggested by these investigators are in reasonable agreement with those reported here.

The slow formation of EI* makes it possible to examine the effect of chloride on the individual steps in mechanism B. The lowering of K_i^* with increasing chloride concentration could be attributable to either a decrease in K_i or a shift in the equilibrium between EI and EI* toward the latter. Comparison of the rate and equilibrium constants obtained at 300 and 20 mM NaCl (Table II) indicates that it is the isomerization and not the initial binding which is chloride dependent. Within the isomerization step, it is primarily k_4 which is decreased by raising $[Cl^-]$, although there is also some increase in k_3. Consistent with this, the onset of inhibition in the absence of added chloride is rapid, and the measured K_i is roughly the same as for $E + I \rightleftharpoons EI$ in mechanism B. Patchett & Cordes (1984) have proposed that the initial rapid interaction involves the active-site zinc atom and the (nonterminal) carboxylate function of the inhibitor. In this case, it would be reasonable for this step to be chloride independent, since neither chloride nor substrate binding appear to involve the metal (Bünning & Riordan, 1981).

The present data on slow binding do not establish which form or forms of the enzyme bind chloride. Similar effects could be observed whether anion binds to E, EI, EI*, or some combination of the three. It is clear, however, that chloride binding is not the rate-limiting process in $EI \rightarrow EI^*$, since the magnitude of k_3 would then be directly proportional to chloride concentration.

An obvious question raised by the present results is whether binding of substrates and of non-slow-binding inhibitors also follows mechanism B, but with much faster k_3 values. Preliminary stopped-flow radiationless energy transfer measurements using *N*-dansyl-blocked peptides suggest that formation of the steady-state ES complex is indeed a (minimally) two-step process (R. Shapiro, unpublished experiments). Further work in this area should establish whether these two steps correspond to those seen with captopril and MK-422.

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