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Activation of Angiotensin Converting Enzyme by Monovalent Anions†

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ABSTRACT: The angiotensin converting enzyme catalyzed hydrolysis of furanacryloyl-Phe-Gly-Gly is activated by monovalent anions in the order $Cl^- > Br^- > F^- > NO_3^- > CH_3COO^-$. In the alkaline pH region, increasing anion concentrations decrease the K_M but do not change the k_{cat}. This behavior is characteristic of an ordered bireactant mechanism in which the anion binds to the enzyme prior to the substrate. At acidic pH values, however, the anion activation is a result of both a decrease in K_M and an increase in k_{cat}, implying a bireactant mechanism in which anion and substrate bind randomly. For both the ordered and the bireactant mechanisms the anion serves as an essential activator. The effect of chloride on enzyme activity was studied over the pH range 5-10 under k_{cat}/K_M conditions and demonstrates that the apparent chloride binding constant increases from 3.3 mM at pH 6.0 to 190 mM at pH 9.0. The k_{cat} vs. pH profile exhibits two pK values of 5.6 and 9.6, while the variation of K_M with pH is characterized by a pK of 8.9 and a 2-fold increase between pH 6.5 and 7.5. The chloride activation of the hydrolysis of furanacryloyl-Phe-Gly-Gly is compared with that of the physiological substrates angiotensin I and bradykinin.

Angiotensin converting enzyme (dipeptidyl carboxypeptidase, EC 3.4.15.1) (ACE)[1] catalyzes the hydrolytic release of dipeptides from the carboxyl terminus of oligopeptide substrates. Although the enzyme has broad specificity, its best known physiological functions are the conversion of the decapeptide angiotensin I into the vasoactive octapeptide angiotensin II (Skeggs et al., 1954, 1956a,b) and the inactivation of bradykinin (Yang et al., 1970).

One of the earliest properties of ACE to be recognized was its requirement for monovalent anions, notably chloride, for catalytic activity toward angiotensin I (Skeggs et al., 1954). Later it was demonstrated that the activity toward bradykinin is also enhanced by chloride but the presence of the anion did not seem essential for the hydrolysis of this substrate (Dorer et al., 1974). Moreover, chloride activation has been demonstrated for the hydrolysis of a number of synthetic oligopeptide substrates (Piquilloud et al., 1970; Cushman & Cheung, 1971; Dorer et al., 1976). Although the anion activation of ACE has been known for a long time, knowledge about the kinetic and structural basis of this phenomenon is still rather fragmentary. In part, this has been due to the lack of a rapid spectrophotometric assay of the enzyme. We have recently introduced FA-tripeptides as chromophoric substrates for ACE which allow continuous spectrophotometric monitoring of their hydrolysis (Holmquist et al., 1979). Utilizing this assay procedure, we have now investigated the effects of anions on the hydrogen ion and substrate concentration dependence of FA-Phe-Gly-Gly hydrolysis and have compared them to the hydrolysis of the physiological substrates angiotensin I and bradykinin. A preliminary account of these studies has been reported (Riordan et al., 1980).

Materials and Methods

Angiotensin I (human) and bradykinin were purchased from Peninsula Laboratories Inc. (San Carlos, CA). Solutions of Zn^{2+} were prepared from the chloride salt (ultrapure grade; Ventron Corp., Danvers, MA). All other chemicals were reagent grade or of the highest purity available.

ACE was purified to homogeneity from rabbit lung acetone powder (Pel-Freez Biologicals Inc., Rogers, AR) as previously described (Bünning et al., 1983). Concentrations of ACE were determined by measuring the absorbance at 280 nm and expressed in molar concentrations by using a molar absorptivity of 204 000 M^{-1} cm^{-1}. Adventitious metal ions were removed from all buffers and substrate solutions by extraction with

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[1] Abbreviations: ACE, angiotensin converting enzyme; Bz, benzoyl; FA, 2-furanacryloyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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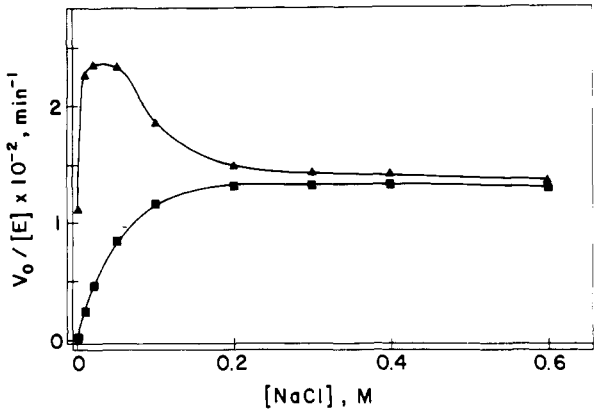


FIGURE 1: Effect of sodium chloride concentration on the hydrolysis of 2×10^{-5} M angiotensin I (■) and bradykinin (▲) in 50 mM Hepes, pH 7.5. The abscissa indicates the concentration of chloride added to the assay mixture and has not been corrected for any that might be present in substrate preparations.

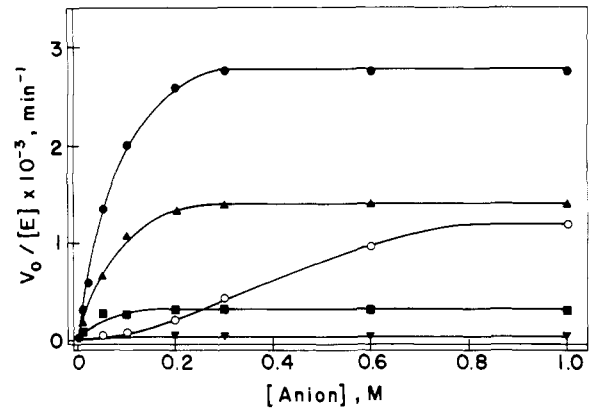


FIGURE 2: Effect of fluoride (○), chloride (●), bromide (▲), nitrate (■), and acetate (▼) concentrations on the activity of ACE measured with 5×10^{-5} M FA-Phe-Gly-Gly in 50 mM Hepes, pH 7.5. Anions were added as the respective sodium salts.

dithizone (0.01%) in carbon tetrachloride or by passage through a Chelex 100 column (Bio-Rad Laboratories, Richmond, CA).

Furanacryloyl-Tripeptide Hydrolysis. Standard conditions for enzyme assays were 5×10^{-5} M FA-Phe-Gly-Gly in 50 mM Hepes buffer and 300 mM NaCl, pH 7.5, 25 °C (Holmquist et al., 1979). Under these conditions, hydrolysis is first order in substrate ($[S] < K_M$). Activities, expressed as $V_0/[E]$ in units of reciprocal minutes, were obtained either from initial velocities or from half-lives after complete hydrolysis according to

$$V_0/[E] = 0.693[S]/(t_{1/2}[E])$$

where V_0 = the initial velocity, [E] = the total enzyme concentration in the reaction mixture, [S] = the initial substrate concentration, and $t_{1/2}$ = the half-life of the reaction.

Kinetic parameters for the hydrolysis of FA-tripeptides were obtained from Lineweaver–Burk plots. Initial velocities were measured during the first 10% of hydrolysis. Since the FA-tripeptides are chromophores themselves, two different cuvette path lengths were selected to achieve an adequate substrate concentration range without exceeding an initial absorbance of 2 while maintaining a constant wavelength of 343 nm. Thus, over the substrate concentration range from 0.05 to 1 mM, a 1-cm path length was employed, and over the range from 1 to 6 mM, a 0.2-cm path length was chosen. Control assays at 1 mM substrate concentration were carried out in 0.2- and 1-cm path-length cuvettes to assure that identical results were obtained.

Buffers used in the determination of the pH–rate profiles include Mes, Hepes, and borate (50 mM), which have been shown to have little effect on enzyme activity under the standard conditions of assay (Bünning et al., 1982). Below pH 7, ACE activity drops off markedly due to spontaneous loss of zinc from the enzyme. Therefore, 10^{-5} M Zn^{2+} between pH 7.5 and 7.0, 10^{-4} M Zn^{2+} between pH 7.0 and 6.0, 10^{-3} M Zn^{2+} between pH 6.0 and 5.5, and 10^{-2} M Zn^{2+} below pH 5.5 were added to assay mixtures to obtain full enzyme activity (Bünning et al., 1983).

Angiotensin I and Bradykinin Assays. The effects of chloride on the hydrolysis of angiotensin I and bradykinin were studied at 0.02 mM substrate concentration in 50 mM Hepes buffer, pH 7.5, containing different sodium chloride concentrations. Details of the assay procedure have been described (Bünning et al., 1983). Enzyme activities are expressed as $V_0/[E]$ in units of reciprocal minutes.

Table I: Effects of Various Anions on the Kinetic Parameters of the ACE-Catalyzed Hydrolysis of FA-Phe-Gly-Gly [a]

anion	k_{cat} (min^{-1})	K_M (M)
fluoride	9 000	6×10^{-4}
chloride	19 000	3×10^{-4}
bromide	15 600	5×10^{-4}
nitrate	18 000	1.8×10^{-3}
acetate	15 500	1.3×10^{-2}

[a] Assay conditions: 50 mM Hepes buffer, pH 7.5, containing 300 mM of the respective sodium salt, 25 °C.

Results

Activation of ACE by Monovalent Anions. The effect of chloride on the hydrolysis of the physiological substrates, angiotensin I and bradykinin, was studied at 2×10^{-5} M substrate concentration. For angiotensin I, this reflects k_{cat}/K_M conditions ($[S] < K_M$) while for bradykinin the hydrolysis kinetics are almost zero order due to a much lower K_M value (Dorer et al., 1974). In both cases, hydrolysis is activated by chloride. With angiotensin I, maximal turnover is observed at 200 mM chloride (Figure 1). A significantly different activation profile is observed for bradykinin. About 45% of the maximal activity is already observed in the absence of added chloride, and the maximal hydrolysis rate requires only 20 mM chloride. At higher chloride concentrations, activity toward bradykinin decreases to about 60% of the maximal activity.

In order to obtain detailed information on the mechanism of chloride activation of ACE, we carried out studies with the chromophoric substrate FA-Phe-Gly-Gly. The activating effects of fluoride, chloride, bromide, nitrate, and acetate were studied with this substrate, 5×10^{-5} M, in 0.05 M Hepes buffer, pH 7.5 (Figure 2). Under these conditions, the reaction is first order in substrate, i.e., $[S] < K_M$. In 0.05 M Hepes buffer in the absence of added anions, there is no detectable hydrolysis of substrate. With increasing concentrations of each of the anions, activity increases to reach a maximum at about 300 mM except in the case of fluoride which requires a concentration of 1 M. However, the maximal activities obtained with the various anions differ significantly. Chloride is the most effective activator whereas nitrate and acetate exhibit less than 10% of the activity observed for chloride. The effects of these anions on the kinetic parameters of the FA-Phe-Gly-Gly hydrolysis were studied with 0.3 M anion (Table I). Under these conditions, the k_{cat} values obtained with chloride, bromide, nitrate, and acetate are sim-

Table II: Effect of Chloride vs. Bromide on ACE Activity [a]

[NaCl] (M)	[NaBr] (M)	V_0/[E] (min^{-1})
0.3	0	2750
0	0.3	1400
0.3	0.3	2120
1.5	0.3	2730
0.3	1.5	1400

[a] Assay conditions: 5×10^{-5} M FA-Phe-Gly-Gly, 50 mM Hepes buffer, pH 7.5, 25 °C.

Table III: Effect of Cations on the Activity of ACE [a]

	V_0/[E] (min^{-1})				
cation	F^-	Cl^-	Br^-	NO_3^-	CH_3COO^-
Na^+	430	2800	1400	330	35
K^+	450	2700	1500	360	35
Mg^{2+}		2100	1400	350	28
Ca^{2+}		2700	1800	470	37

[a] Assay conditions: 5×10^{-5} M FA-Phe-Gly-Gly and 50 mM Hepes buffer, pH 7.5, containing 300 mM each of the respective anion, 25 °C.

ilar whereas that for fluoride is significantly lower. Moreover, there is a marked variation of K_M with the anion, ranging from 3×10^{-4} M for chloride to 1.3×10^{-2} M for acetate.

Chloride and bromide were studied in combination to determine if they are mutually competitive (Table II). In the presence of equimolar concentrations of chloride and bromide, the observed activity is intermediate between that found with either anion alone. Increasing the bromide concentration at a constant chloride concentration diminishes enzyme activity to that observed for bromide alone, and the converse is true when chloride displaces bromide from the enzyme. Thus, the two anions compete with each other with apparently similar binding constants. Moreover, nitrate, acetate, and, less effectively, fluoride also exhibit competition with chloride.

Cations have been tested for their effect on ACE activity at a constant anion concentratioin of 300 mM (Table III). Virtually identical activities are obtained for the sodium and potassium salts whereas magnesium salts exhibit somewhat lower activities throughout. The activities observed for calcium chloride and acetate are the same as those for the respective sodium or potassium salts whereas those for calcium bromide and nitrate are slightly higher. It appears that the nature of the cation has little effect on ACE activity and that enzyme activation is primarily a specific anion effect.[2]

Effect of Anions on the Kinetic Parameters for ACE Catalysis. The effect of the substrate concentration on the anion activation of FA-Phe-Gly-Gly hydrolysis was investigated in 0.05 M Hepes buffer, pH 7.5. At all anion concentrations studied, Lineweaver–Burk plots are linear over a concentration range from at least 5×10^{-5} to 6×10^{-3} M. The sections that reflect high substrate concentrations are shown in Figure 3. Increasing the chloride concentration decreases K_M for FA-Phe-Gly-Gly hydrolysis from 11 mM at 2 mM chloride to 0.3 mM to 300 mM chloride whereas k_{cat}, 19 000 min^{-1}, is constant over this chloride concentration range (Figure 3A). For bromide concentrations from 2 to 300 mM, a slightly lower k_{cat} value of 15 600 min^{-1} is found, and the K_M value decreases from 14 to 0.5 mM (Figure 3B). Thus, the effects of bromide and chloride on FA-Phe-Gly-Gly hydrolysis are closely similar. The same behavior is also found for the chloride activation

[2] One exception to this is ammonium ion which inhibits the enzyme (R. Shapiro, unpublished experiments).

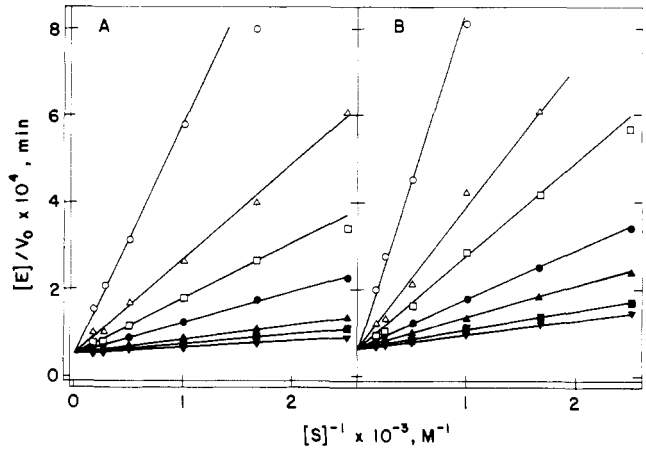


FIGURE 3: Lineweaver–Burk plots for the ACE-catalyzed hydrolysis of FA-Phe-Gly-Gly in 2 (○), 5 (△), 10 (□), 20 (●), 50 (▲), 100 (■), and 300 (▼) mM sodium chloride (A) or sodium bromide (B). Assays were carried out in 50 mM Hepes, pH 7.5.

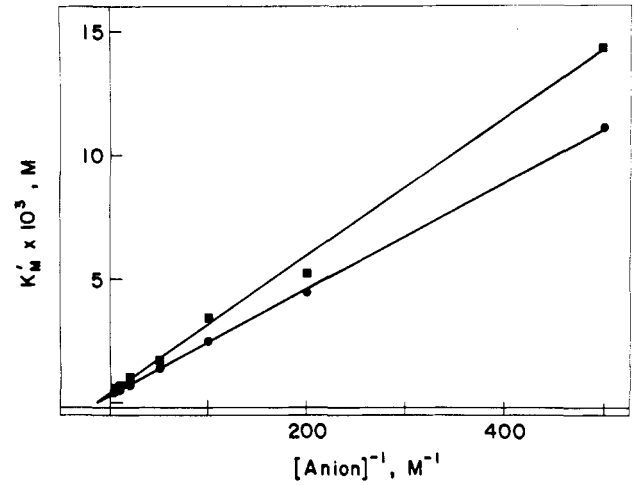


FIGURE 4: Plot of K_M' values for the hydrolysis of FA-Phe-Gly-Gly at various sodium chloride (●) or sodium bromide (■) concentrations vs. [anion]$^{-1}$. Data are taken from Figure 3A,B.

of FA-Phe-Leu-Gly hydrolysis. The k_{cat} value is constant at 3800 min^{-1} whereas K_M decreases from 1.1×10^{-3} to 2.5×10^{-4} M when the chloride concentration is increased from 20 to 300 mM (not shown).

Replots of the data in Figure 3 according to Segel (1975) yield a linear relationship between the apparent K_M, i.e., K_M' which is defined as

$$K_M' = K_M(1 + K_A'/[A])$$

and the reciprocal of the anion concentration (Figure 4). For both chloride and bromide activation of the ACE-catalyzed hydrolysis of FA-Phe-Gly-Gly, the apparent anion binding constants, K_A', obtained from the intercepts with the abscissa, are identical, 80 mM at pH 7.5. The K_M values obtained from the ordinate intercepts are 2.3×10^{-4} M for chloride and 3.8×10^{-4} M for bromide.

pH Dependence of the Anion Activation of ACE. The effect of chloride on the hydrolysis of FA-Phe-Gly-Gly, 5×10^{-5} M, was examined at pH 6.0 and 9.0. At pH 6.0, enzyme assays were carried out in the presence of 10^{-4} M zinc chloride to prevent dissociation of the metal from the enzyme. As a consequence, it was not possible to carry out activity measurements in the complete absence of added chloride. The use of nitrate or other salts of zinc would not have obviated this problem since these anions would themselves interfere with the activity measurements. The activity observed at pH 6.0

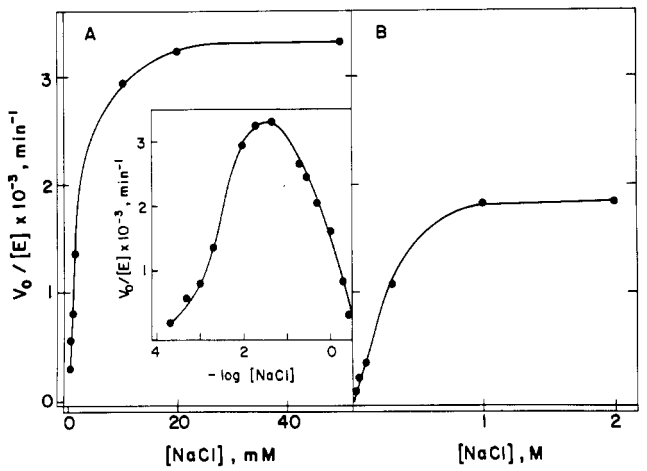


FIGURE 5: Effect of sodium chloride concentration on the activity of ACE measured with 5 × 10^{-5} M FA-Phe-Gly-Gly in either 50 mM Mes, pH 6.0, containing 10^{-4} M zinc chloride (A) or in 50 mM borate, pH 9.0 (B).

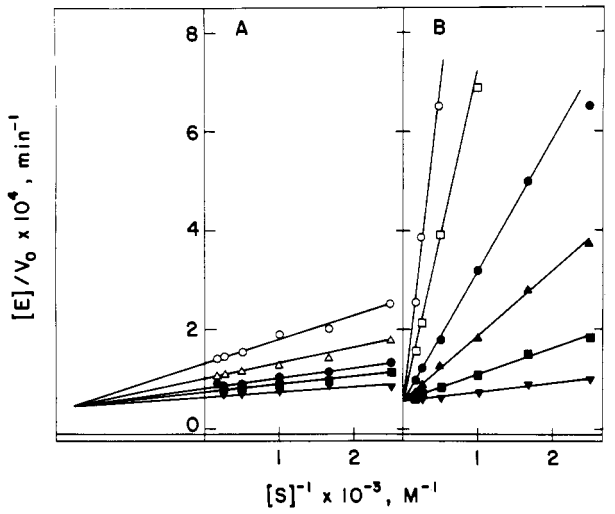


FIGURE 6: (A) Lineweaver–Burk plots for the ACE-catalyzed hydrolysis of FA-Phe-Gly-Gly in 0.5 (○), 1 (△), 2 (●), 10 (■), and 50 (▼) mM sodium chloride in 50 mM Mes, pH 6.0, containing 10^{-4} M zinc chloride. (B) Lineweaver–Burk plots for the ACE-catalyzed hydrolysis of FA-Phe-Gly-Gly in 10 (○), 20 (□), 50 (●), 100 (▲), 300 (■), and 1000 (▼) mM sodium chloride in 50 mM borate, pH 9.0.

in the presence of 0.1 mM $ZnCl_2$ was 300 min^{-1}. On addition of chloride, enzyme activity increases over 11-fold and reaches a maximum of 3400 min^{-1} at 50 mM (Figure 5A). If the chloride concentration is increased above 50 mM, enzyme inhibition is observed (Figure 5A, inset). At pH 9.0, a chloride concentration of 1 M is required to achieve maximal activation of ACE, but no inhibition is observed when the chloride concentration is raised to 2 M or higher (Figure 5B). The apparent chloride binding constants obtained from double-reciprocal plots of activity vs. anion concentration are 3.3 mM at pH 6.0 and 190 mM at pH 9.0, respectively.

At pH 6.0, the activation of ACE by chloride is the composite effect of a decrease in K_M' from 0.37 to 0.15 mM and an increase in k_{cat} from 7700 to 15 000 min^{-1} as the chloride concentration is raised from 0.5 to 50 mM (Figure 6A). Double-reciprocal plots of activity vs. anion concentration (not shown) are also linear. This kinetic behavior is characteristic of a random bireactant mechanism in which either anion or substrate can bind to the enzyme first. Chloride still appears to be an essential activator, but, as indicated above, it is not possible to measure activity in the absence of any added anion.

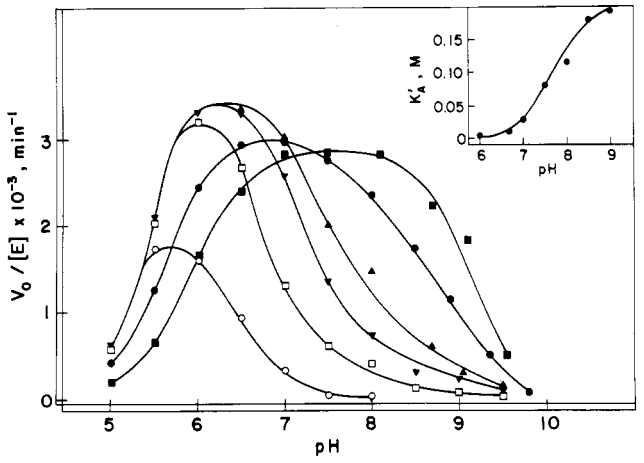


FIGURE 7: pH dependence of the ACE-catalyzed hydrolysis of 5 × 10^{-5} M FA-Phe-Gly-Gly in 2 (○), 20 (□), 50 (▼), 100 (▲), 300 (●), and 1000 (■) mM sodium chloride. Conditions of the assay are described under Materials and Methods. Inset: Variation of the apparent chloride binding constant, K_A', with pH.

At pH 9.0, however, the activation of ACE is solely due to a decrease in K_M' while k_{cat} is constant at 17 000 min^{-1} as the chloride concentration increases from 10 mM to 1 M (Figure 6B). This activation is similar to that observed at pH 7.5. Thus, the anion activation follows an ordered bireactant mechanism at alkaline pH values whereas in the acidic pH region activation is mediated by a random bireactant mechanism.

The pH profile of ACE activity was examined at various chloride concentrations (Figure 7). With 5 × 10^{-5} M FA-Phe-Gly-Gly, the reaction is first order in substrate and reflects k_{cat}/K_M' conditions at all chloride concentrations studied. The pH–rate profile is characterized by a decrease in activity below pH 6 at the low chloride concentrations and above pH 8 at the highest chloride concentration used. Furthermore, there is a decrease in the maximal activity from 3400 to 2800 min^{-1} as the pH is raised from 6.0 to 7.5. As the chloride concentration is raised from 2 mM to 1 M, there is a progressive shift in the alkaline limb of the pH profile; it is not shifted further by chloride concentrations higher than 1 M. This is a reflection of a pH-dependent change in the apparent chloride binding constant, K_A', obtained from double-reciprocal plots of activity vs. chloride concentration at each pH value studied, which increases from 3.3 mM at pH 6.0 to 190 mM at pH 9.0 (Figure 7, inset).

The pH–activity profile of the ACE-catalyzed hydrolysis of FA-Phe-Gly-Gly has been resolved into the pH dependences of the kinetic parameters k_{cat} and K_M' (Figure 8). These data were derived from Lineweaver–Burk plots which were linear over at least a 10-fold substrate concentration range spanning the respective K_M' values. The variation of log k_{cat} as a function of pH (Figure 8A) was studied at chloride concentrations ranging from 20 mM at acidic pH values to 1 M in the alkaline pH region. Chloride concentrations were employed which fully activate the enzyme under k_{cat}/K_M' conditions at the respective pH values (Figure 7). The increase of log k_{cat} between pH 5 and 7 could likely reflect ionization of a group in the enzyme–substrate complex with a pK of 5.6. Thereafter, the rate remains constant up to pH 8.5 and then decreases, suggesting the ionization of a second group of the enzyme–substrate complex with a pK of 9.6. The pH dependence of pK_M' was studied at both 20 mM and 1 M chloride (Figure 8B). At 20 mM chloride, pK_M' exhibits its maximal value at pH 5.2 and decreases as the apparent chloride binding constant increases. At pH 5.5 in 1 M chlo-

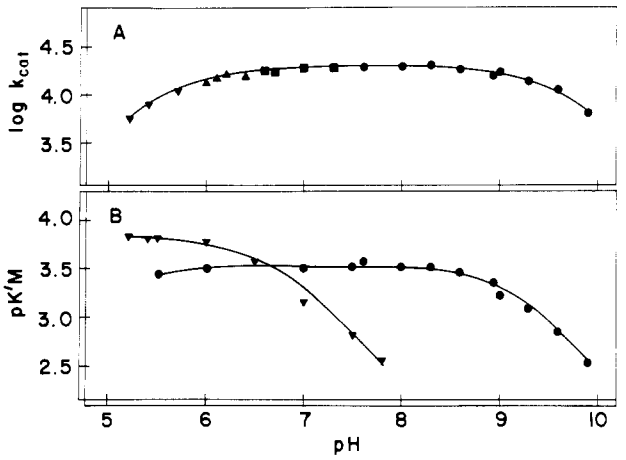


FIGURE 8: pH dependence of log k_{cat} (A) and pK_M' (B) for FA-Phe-Gly-Gly hydrolysis in 20 (▼), 50 (▲), 300 (■), and 1000 (●) mM sodium chloride. Conditions of the assay are described under Materials and Methods.

ride, the pK_M' value is somewhat lower than that in 20 mM chloride due to anion inhibition. It remains constant between pH 5.5 and 8, and above pH 8, it decreases in a manner that suggests ionization of a group on the enzyme with a pK of 8.9.

Discussion

Angiotensin converting enyzme is a central component of the renin–angiotensin–kinin system. In the last few years, it has been demonstrated that substances which are potent inhibitors of the enzyme in vitro can act as effective antihypertensive agents in vivo. It is not clear, however, if such substances serve mainly to prevent the activation of angiotensin I, inhibit the inactivation of bradykinin, or, since the enzyme can act on a wide range of peptides, have additional but as yet unknown effects. Nevertheless, it seems clear that the changes in blood pressure that have been observed both in humans as well as in laboratory animals are due to the inhibition of ACE. Thus, control of ACE activity is physiologically quite important.

Chloride activation is a characteristic feature of ACE and is the only process thus far known to control the activity of the enzyme that could operate naturally and potentially have important physiological significance. It was one of the earliest properties of the enzyme to be recognized (Skeggs et al., 1954) and has served as a means to differentiate ACE from other peptidyldipeptide hydrolases (Gorenstein & Snyder, 1979). In spite of its obvious significance, chloride activation of ACE has not been the subject of extensive investigation. Under the conditions used in these studies, activity toward angiotensin I reaches a maximum in the presence of about 200 mM chloride and remains constant up to 600 mM chloride. It is noteworthy that activity toward this substrate cannot be detected in the absence of added chloride (Figure 1). In contrast, hydrolysis of bradykinin occurs in the absence of added chloride, and the maximal turnover rate is observed with only 20 mM chloride, i.e., tenfold lower than with angiotensin I. In this case, increasing the chloride concentration above 20 mM actually leads to a partial decrease in activity. Other investigators have also found that bradykinin hydrolysis requires less chloride than angiotensin I, but attempts to deduce mechanisms of anion activation either have been very limited or have been carried out under circumstances that were suboptimal for measuring ACE activity. Furanacryloyl peptides and in particular FA-Phe-Gly-Gly have proved to be suitable substrates for examining this problem. At pH 7.5, ACE is virtually inactive toward FA-Phe-Gly-Gly in the absence of monovalent anions. The most potent activators are halides, notably chloride, whereas nitrate and acetate have rather small effects. Maximal ACE activity is reached at about 300 mM anion, except for fluoride which is required in a concentration of 1 M. Competition experiments demonstrate that the anions studied bind to the same site on the enzyme (Table II). A comparison of the kinetic parameters obtained in the presence of 300 mM anion shows that the k_{cat} values for chloride, bromide, nitrate, and acetate are similar whereas that for fluoride is only half of these. On the other hand, the apparent K_M values differ markedly, ranging from 3×10^{-4} M for chloride to 1.3×10^{-2} M for acetate.

The unusual activation behavior of fluoride is an expression of the unique properties of this anion. Fluoride is poorly polarizable and possesses an intense electrostatic field. Therefore, it may retain its dipolar-oriented hydration sphere even in the vicinity of a cationic site on the enzyme. This could prevent close approach of fluoride to that site. In contrast, larger monovalent anions do not have sufficiently intense external fields to hold hydration spheres tenaciously and may therefore make a close approach to a cationic binding site.

The activation of ACE by anions appears to be a specific effect rather than one due to ionic strength. As shown in Table II, activity in the presence of 0.3 M NaBr (line 2) is not increased further by the presence of 0.3 M NaCl plus 1.5 M NaBr (line 5) even though the ionic strength is increased almost 6-fold. Similarly, in Table III, activity in the presence of 0.3 M NaCl or KCl (ionic strength = 0.3) is virtually identical with that seen with 0.15 M $CaCl_2$ (ionic strength = 0.45). Further, as shown in Figure 2, increasing the acetate concentration up to 1.0 M has virtually no effect on activity. In all cases examined, mixtures of anions activate ACE to the extent predicted on the basis of their individual K_A values rather than total ionic concentration or ionic strength. Perhaps most significantly, the degree of activation obtained with a given concentration of chloride varies widely, depending on the particular substrate used for the assay (Shapiro et al., 1982), an effect which cannot be attributable to ionic strength. Dorer et al. (1976) interpreted the activating effect of sulfate as being due to ionic strength, but we have found that sulfate specifically lowers the K_A for chloride activation (P. Bünning and J. F. Riordan, unpublished experiments).

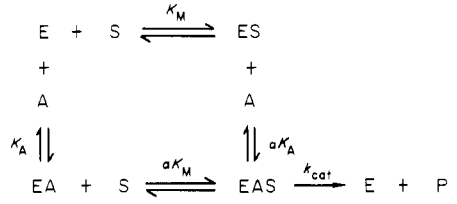
The investigation of ACE activation by chloride as a function of the substrate concentration demonstrates that, at pH 7.5, with increasing anion concentration K_M' decreases but k_{cat} does not change (Figure 3). This kinetic behavior is indicative of an ordered bireactant mechanism:

$$E + A \overset{K_A}{\rightleftharpoons} EA + S \overset{K_M'}{\rightleftharpoons} EAS \xrightarrow{k_{cat}} E + P$$

in which the anion binds to the enzyme first and then the substrate binds to the enzyme–anion complex followed by substrate hydrolysis (Segel, 1975). Replots of K_M' vs. the reciprocal of the anion concentration are linear, consistent with this mechanistic scheme, and yield values for K_A and K_M (Figure 4). Thus, chloride functions as an essential activator mediating substrate binding. Direct evidence in favor of the ordered bireactant mechanism of ACE has been obtained by stopped-flow studies of the enzyme with a fluorescent substrate. In the presence of chloride, there is both binding and hydrolysis of the substrate whereas in the absence of chloride peptide binding to the enzyme is not observed (Bünning & Riordan, 1981a)

The ordered bireactant mechanism for ACE activation does not pertain at all pH values, however. This is evident from

the marked effect of pH on the concentration of chloride that is necessary for complete activation. Thus, at least 10 times less chloride is required for maximal activation at pH 6.0 as at pH 7.5 while at pH 9.0 about 4 times more chloride is needed. Lineweaver–Burk plots of data obtained at pH 6.0 indicate that increasing chloride concentrations again decrease K_M', but now, in contrast to at pH 7.5, the apparent V_{max} increases as well (Figure 6A). This kinetic behavior is typical of a bireactant mechanism in which activator and substrate bind to the enzyme randomly followed by product formation from the resultant ternary complex.



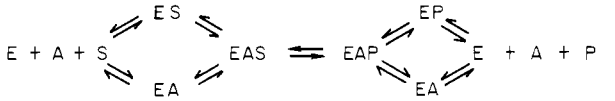
According to this scheme, chloride still appears to be an essential activator since product can only be detected as arising from EAS. Some breakdown of ES to form product may occur under these circumstances, but if so, it is at a rate which is very much less than that of EAS. On the other hand, Lineweaver–Burk plots of data obtained at pH 9.0 (Figure 6B) are similar to those at pH 7.5 where chloride only affects K_M'. The transition from a random to an ordered binding mechanism thus occurs between pH 6 and 7.5. Substrate apparently cannot bind to the free enzyme at the higher pH, but it can at the lower pH. Under all conditions, however, substrate binding is influenced by chloride. At pH 6.0, the initial velocity of substrate hydrolysis as a function of anion and substrate concentration is described by

$$\frac{V_0}{[E]} = \frac{k_{cat}[S]}{\alpha K_M(1 + K_A/[A]) + [S](1 + \alpha K_A/[A])}$$

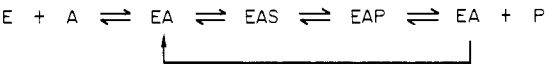
The parameters K_A, K_M, and k_{cat} are defined in the reaction scheme; V_0 is the initial velocity of substrate hydrolysis, [E] is the total enzyme concentration in the reaction mixture, [A] is the total anion concentration, and [S] is the initial substrate concentration. K_M can be obtained directly from the intersection of the Lineweaver–Burk plots (Figure 6A), and K_A is obtained from a replot of the reciprocal of the anion concentration vs. $1/\Delta K_M$ (Segel, 1975) where ΔK_M is the difference between K_M' and K_M. The effect of anion binding on the binding of substrate or vice versa is described by the factor α, which for activation must have a value less than unity. This value is also obtained from double-reciprocal plots of activity vs. substrate concentration (Figure 6A). The point of intersection of the family of lines is given by $(1/V_{max})(1 - \alpha)$ and $-1/K_M$ while the intercept on the $1/V$ axis at saturating concentrations of chloride gives $1/V_{max}$ (Segel, 1975).

As the pH shifts from acidic to alkaline values, the sequence of anion and substrate binding changes from a random to an ordered process. This phenomenon can also be described in terms of a pH-dependent change of α from 0.26 at pH 6.0 to values much less than this in the alkaline pH region (Segel, 1975). In other words, as α decreases in value, the point of intersection of the Lineweaver–Burk plot will approach the $1/V$ axis (Figure 6A,B). Thus, the ACE-catalyzed hydrolysis of FA-Phe-Gly-Gly, in general, follows a random bireactant mechanism in which chloride serves by and large as an essential activator. In the alkaline pH region, however, α becomes very small, and, thus, the kinetics of ACE catalysis resemble those of an ordered bireactant mechanism.

A random bireactant mechanism for substrate binding suggests that a similar scheme may also pertain for product dissociation.



Such a scheme could, in fact, account for the observation that at pH 6.0 high chloride concentrations (>50 mM) result in inhibition (Figure 5A, inset). Inhibition would be the consequence of chloride binding to EP to form EAP, thereby preventing product formation via this route. At pH 7.5 or 9.0, where the mechanism is ordered bireactant with chloride binding before substrate, product would be expected to dissociate before chloride, and hence, chloride inhibition by this specific process would be unlikely to occur. This is apparent from the following scheme:



It is not clear at this time if the chloride inhibition observed with bradykinin (Figure 1) is a manifestation of a random product dissociation mechanism or if it is related to some feature of substrate binding. Studies in progress are addressing this question.

The marked effect of pH on chloride activation is reflected in the pH profiles obtained at various chloride concentrations (Figure 7). Under first-order reaction conditions, the pH profile shifts progressively into the alkaline region with increasing chloride concentrations. This behavior is brought about by the fact that the apparent chloride binding constant increases from 3.3 mM at pH 6.0 to 190 mM at pH 9.0. Thus, e.g., with 20 mM chloride, activity increases to an optimum at about pH 6.0 but then falls to less than 10% of this value at pH 8.0. This concentration of chloride is well in excess of K_A' at pH 6.0 but is well below K_A' at pH 8.0.

In addition to the effect of pH on chloride activation, ACE is inhibited by high chloride concentrations in the acidic pH region. Thus, at all pH values from 5.0 to 7.0, activity falls off at the higher chloride concentrations (Figure 7). Above pH 7.0, however, this inhibition is no longer apparent. The pH–rate profile of the ACE-catalyzed hydrolysis of FA-Phe-Gly-Gly is therefore obtained with 50–100 mM chloride below pH 7 and 1 M chloride above pH 7. This profile is characterized by a decrease in activity between pH 6 and 5 and again between pH 8.5 and 9.5. Furthermore, the maximal turnover rate decreases between pH 6.5 and 7.5 from 3400 to 2800 min^{-1}.

The pH–activity profile of ACE catalysis has been resolved into the pH dependences of the kinetic parameters k_{cat} and K_M'. The variation of k_{cat}, obtained at the optimal chloride concentration for each pH value, is characterized by two pKs, 5.6 and 9.6. The pK_M' vs. pH profile is affected by a pK of 8.9 in the presence of 1 M chloride. At 20 mM chloride, however, the apparent pK_M is 6.2, demonstrating that the pH dependence of K_A mainly affects the apparent pK_M. The decrease in ACE activity between pH 6.5 and 7.5, observed under k_{cat}/K_M conditions, is caused by an increase in K_M', and the chloride inhibition of ACE in the acidic pH region also reflects an increase in K_M'.

The mechanism of chloride activation varies with the reaction conditions and the nature of the substrate. For the hydrolysis of most substrates containing uncharged amino acid side chains, including FA-Phe-Gly-Gly, chloride serves as an essential activator. The activation of angiotensin I hydrolysis

also seems to follow an ordered bireactant mechanism with chloride as the essential activator (Fernley, 1977), whereas a nonessential activator mechanism for its analogue Bz-Gly-His-Leu has been proposed (Rohrbach et al., 1981). The hydrolysis of bradykinin seems to proceed in the absence of chloride according to a nonessential activator mechanism (Dorer et al., 1974). However, most of these studies have not been carried out under technically ideal conditions, and, therefore, definitive mechanistic conclusions cannot be drawn. The availability of FA-tripeptides as substrates has enabled us to overcome these problems and to apply optimal assay conditions (Holmquist et al., 1979). The investigation of the chloride activation of a wide variety of FA-tripeptides is now in progress and will contribute further to the understanding of the mechanism of ACE activation.[3]

The phenomenon of enzyme activation by anions has been known for a long time. The first enzyme demonstrated to be activated by monovalent anions was α-amylase (Cole, 1904). In this case, chloride mediates an increase in k_{cat}, but does not change K_M (Levitzki & Steer, 1974). It also shifts the alkaline side of the pH–activity profile to higher pH values (Myrbäck, 1926; Wakim et al., 1969). In addition, the α-amylase Ca^{2+} binding constant is increased by the presence of anions (Levitzki & Steer, 1974). The anion binding site is a lysyl residue which apparently has a counterpart in ACE (Lifschitz & Levitzki, 1976; Bünning et al., 1978; Bünning & Riordan, 1981b). Cathepsin C belongs to a second group of anion-activated enzymes (McDonald et al., 1966, 1969; Gorter & Gruber, 1970) where anions solely affect K_M. Whether anions activate by affecting k_{cat} or K_M, in all cases studied the activation is accompanied by a shift of the pH–activity profile toward the alkaline pH region.

The control of ACE activity through such a complex mechanism as anion activation raises the question of its physiological relevance. Both pH and chloride concentration are strictly controlled and show only little variation in blood plasma, to which ACE is exposed. Under normal physiological conditions, pH 7.4 and 100 mM chloride, ACE will exhibit only about 50% of its maximal activity toward angiotensin I but would be fully active toward bradykinin. Hence, one might speculate that anion activation serves as a means to regulate substrate specificity. In addition, the location of ACE in kidney, where chloride concentrations vary widely, or in brain may require a complex mechanism to adapt enzyme activity to local environmental conditions.

Acknowledgments

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[3] Preliminary studies have revealed three classes of substrates based on chloride activation of ACE. Class I substrates are typified by FA-Phe-Gly-Gly and have a K_A of 80 mM at pH 7.5. Class II substrates have Arg or Lys in the penultimate or N-terminal position and a K_A of 4 mM. Class III substrates have a K_A of about 25 mM. Mechanisms of activation for the three classes are different (Shapiro et al., 1982).

Registry No. ACE, 9015-82-1; FA-Phe-Gly-Gly, 64967-39-1; angiotensin I, 484-42-4; bradykinin, 58-82-2; fluoride, 16984-48-8; chloride, 16887-00-6; bromide, 24959-67-9; nitrate, 14797-55-8; acetic acid, 64-19-7.

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