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Catalysis of Angiotensin I Hydrolysis by Human Angiotensin-Converting Enzyme: Effect of Chloride and pH†

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ABSTRACT: The catalysis of the hydrolysis of angiotensin I, an important natural substrate, by human angiotensin-converting enzyme (ACE) was examined in detail as a function of chloride and hydrogen ion concentration. Chloride was found to be a nonessential activator over the pH range 5.0–10.0, with the chloride dependence increasing with increasing pH: the velocity enhancement at optimal [Cl^-] increased from 1.6- to 42-fold; the chloride optimum and K_a' increased from 20 to 520 mM and from 0.22 to 120 mM, respectively; and activity in the absence of chloride decreased from 60.9 to 2.4% (relative to maximal activation). Kinetic analyses at pH 6.0, 7.5, and 9.0 confirmed the nonessential activator mechanism. At all pH values tested chloride was found to be inhibitory (relative to maximal activation) at supraoptimal chloride levels. Depending on the [Cl^-] range, both apparent uncompetitive and competitive modes were demonstrated. From pH 6.0 to 9.0 K_is varied between 110 and 1140 mM (apparent). In all cases $K_i' \gg K_a'$. We suggest that at high [Cl^-] chloride binds to low-affinity inhibitory sites on the free enzyme and on the ES and EP complexes. The pH–rate profile demonstrated a chloride-dependent alkaline shift, with the pH optimum increasing from 7.1 at zero chloride to 7.6 at 400 mM NaCl. At [S] $\gg K_m$ a plot of log v vs pH revealed pKs of 5.9 and 9.4 in the ES complex in the absence of chloride, while at maximally activating [Cl^-] only one ionization at pK = 6.3 was observed. Thus, binding of chloride appeared to suppress the ionization of a basic group. A pK of 9.4 would be consistent with a lysyl residue in a positively charged, hydrophobic microenvironment, and a critical lysine has been implicated in chloride binding [Shapiro, R., & Riordan, J. F. (1983) *Biochemistry 22*, 5315–5321]. Our data indicate that human ACE is likely to be maximally activated for its natural substrate angiotensin I in most anatomic loci.

The striking activation of angiotensin-converting enzyme (ACE;[1] dipeptidyl carboxypeptidase, EC 3.4.15.1) by chloride and other monovalent anions is an unusual characteristic first noted by the enzyme's original discoverers (Skeggs et al., 1954) and since shown to be complex, dependent on substrate and pH, with both essential and nonessential activator mechanisms having been demonstrated (Cheung et al., 1980; Rohrbach et al., 1981; Bünning & Riordan, 1983; Shapiro et al., 1983). Furthermore, it is not unlikely that species differences in ACE may introduce an additional complicating factor in evaluating the effect of chloride on the hydrolysis of a particular substrate, since, for example, it has been shown that the activity for Hip-His-Leu differs for the rabbit and canine enzymes (Conroy et al., 1978).

Most of the kinetic work on the anion activation of ACE has been performed on the rabbit lung enzyme with synthetic N-blocked tripeptide substrates (Cheung et al., 1980; Bünning & Riordan, 1983; Shapiro et al., 1983). The information gained from these studies cannot necessarily be extrapolated to the human enzyme and its most important physiological substrate angiotensin I (AI). Work on human ACE has, until recently, been hampered by tedious purification procedures (Ehlers et al., 1986), while kinetic studies with AI were discouraged by its high cost and the lack of a continuous assay for this substrate. Thus, while the pathophysiological importance of the human enzyme in the conversion of AI to AII

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[1] Abbreviations: ACE, angiotensin-converting enzyme; AI, angiotensin I; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; His-Leu, L-histidyl-L-leucine; HSA, human serum albumin; ES, enzyme–substrate; EP, enzyme–product; EAS, enzyme–activator–substrate; EAP, enzyme–activator–product; Fa, 2-furanacryloyl; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; P_1' and P_2', penultimate and ultimate amino acid residues, respectively, of the substrate [terminology as described by Schechter and Berger (1967)].

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is being increasingly underscored by the clinical effectiveness of potent orally active ACE inhibitors (Robertson et al., 1987), no systematic kinetic evaluation of this reaction has been undertaken in vitro.

We have adapted a simple fluorometric assay of AI conversion (Friedland & Silverstein, 1977) for the kinetic investigation of the hydrolysis of this substrate by human ACE and here report on the pH and chloride dependence of this important process.

Materials and Methods

AI (human form), Hip-His-Leu, His-Leu, and *o*-phthaldialdehyde were from Sigma. HSA (>99% pure) was from Miles Laboratories. All other chemicals were of analytical grade. All buffers were made up in distilled water purified further by a Milli-Q water purifier.

Human kidney ACE was purified to electrophoretic homogeneity (Ehlers et al., 1986), with a specific activity of 90–100 units/mg (for 5 mM Hip-His-Leu, pH 8.3, 300 mM NaCl, 37 °C). Enzyme concentrations were determined by the method of Bradford (1976) and from the specific activity under standard conditions. The ACE and HSA were dialyzed exhaustively against 50 mM potassium phosphate (pH 8.0) over a period of 2–3 days before use. Prolonged dialysis was not found to affect the kinetic parameters of AI and Hip-His-Leu hydrolysis.

Concentrations of AI were determined by absorption at 275 nm, in 0.104 M potassium phosphate (pH 7.5), 31.25 mM NaCl, and using $\epsilon = 1.31 \times 10^3$ M^{-1} cm^{-1} (Friedland & Silverstein, 1977). The AI from Sigma is supplied as the diacetate salt. ACE activity was measured over a range of [AI] both in the absence of added anion and in the presence of 2 mol equiv of acetate. At all [AI] no differences in activity were detected. Up to 2.0 mM acetate was without effect on ACE activity.

Assay Procedure. This was essentially the fluorometric method of Friedland and Silverstein (1977), adapted to obtain initial velocities under a wide range of experimental conditions. To 0.12 mL of substrate–buffer solution was added 0.005 mL of enzyme, the solution was incubated for 2–30 min at 37 °C, and the reaction was terminated by the addition of 0.725 mL of NaOH. HSA (0.12 mg/mL) was routinely added to all assay mixtures, since failure to do so often resulted in erratic, poorly reproducible results. $ZnSO_4$ (10^{-4} and 10^{-5} M) was added to assay solutions at pH 5.0 and 5.5, respectively. All buffers[2] were 100 mM. The amount of His-Leu formed was determined fluorometrically with *o*-phthaldialdehyde (0.05 mL of a 2% solution). Fluorescence was measured on Perkin-Elmer spectrophotometer 204-A fitted with a Perkin-Elmer 150 xenon power supply (λ_{ex} = 405 nm, λ_{em} = 500 nm). Results were quantitated by comparison with standard curves obtained with His-Leu standards. Initial rate conditions were established by assessing progress curves at pH 5.0, 6.0, 7.5, and 9.0 over a range of substrate (14–214 μM), chloride (0.075–20 mM), and ACE concentrations (0.16–1.15 μg/mL) at fixed time points of 0, 2, 4, 6, 10, and 15 min. Initial rates were then determined from two fixed-time readings, at zero time and at 4 or 6 min. Under these conditions generally between 1.5 and 8% substrate hydrolysis had occurred. Assays were performed in duplicate or triplicate, and differences in initial rate measurements were <15%, generally between 5 and 10%. At very low [S] and [Cl^-] and at extremes of pH (<6 and >9), product formation was minimal (<0.1 nmol) and fluorescence values were occasionally only 60% greater than those of the matched zero-time blanks. In such cases samples and blanks were performed in quadruplicate to obtain reliable initial rates. A best straight line was determined for all double-reciprocal plots by linear regression analysis. Correlation coefficients (r) varied between 0.955 and >0.999. In some cases kinetic constants were determined from more than one set of initial rate values; variability in the estimation of K_m was found to be 7–29% and 8–36% for V_{max}.

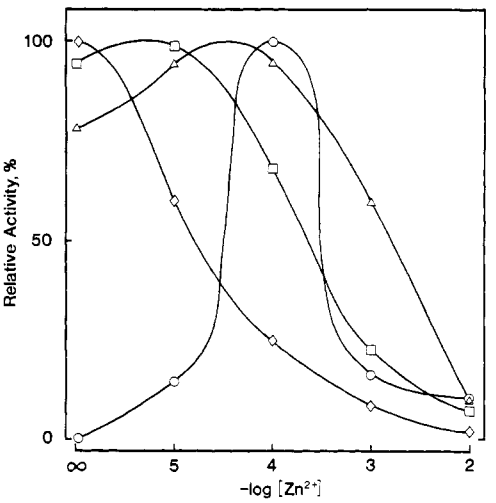


FIGURE 1: Effect of added Zn^{2+} on ACE activity at low pH. ACE activity was measured in the presence of added zinc (as $ZnSO_4$) at pH 5.0 (○), 5.5 (△), 6.0 (□), and 6.5 and 7.0 (◇) with 0.1 mM angiotensin I in 1 mM NaCl and 100 mM buffer (pH 5.0–6.0, sodium succinate; pH 6.5–7.0, MOPS).

Results

Zn^{2+} Addition at pH 5.0–7.0. Optimal [Zn^{2+}] at various pH values were found to be 10^{-4}–10^{-5} M at pH 5.5, <10^{-5} M at pH 6.0, and 0 M at pH 6.5 and 7.0. Marked inhibition occurred at all pH values at supraoptimal zinc concentrations (Figure 1).

Extent of Chloride Activation: Dependence on pH. The chloride dependence of ACE-catalyzed AI hydrolysis was determined at a number of pH values over a wide chloride concentration range at a single, fixed substrate concentration (80 μM, <K_m) (Table I). At all pH values chloride acts as a nonessential activator. Inhibition was observed at supraoptimal chloride concentrations. The extent of chloride activation was markedly pH dependent, increasing with increasing pH. This is illustrated in a plot of relative activity (i.e., percent of maximum) vs the logarithm of the chloride concentration at each pH value (Figure 2). At pH 5.0 the curve is flat, indicating a minimal dependence on Cl^- activation, a broad Cl^- optimum, and minimal inhibition at supraoptimal [Cl^-]. With increasing pH the curves become increasingly bell-shaped with stronger chloride activation, sharper chloride optima, and more marked inhibition at supraoptimal chloride levels. Of interest is the observation that while the chloride dependence increased strongly with pH, the chloride optimum remained relatively unchanged up to approximately pH 7.5 and only began to change significantly at pH values >7.5 (Table I).

As expected for a nonessential activator, double-reciprocal plots of $1/v$ vs $1/[Cl^-]$ curved downward (not shown), but double-reciprocal plots in which the velocity in the absence

[2] A number of buffers were used over the pH range 5.0–10.4, including potassium phosphate. Although phosphate has been shown to be inhibitory for the hydrolysis of Fa-Phe-Gly-Gly by rabbit ACE at acidic pH (Bünning et al., 1983), this was not noted for the substrate Hip-His-Leu (Cushman & Cheung, 1971). Similarly, we did not find a significant difference between the effect of phosphate and other buffers, such as succinate, Tris, Hepes, and borate, on the rate of AI hydrolysis.

Table I: Chloride Dependence of the ACE-Catalyzed Hydrolysis of Angiotensin I: Importance of pH[a]

pH	activity at 0 $[Cl^-]$ (%)	optimal $[Cl^-]$ (mM)	K_a' (mM)	velocity enhancement at optimal $[Cl^-]$ (x-fold)
5.00	60.9	20	0.22	1.6
5.40	39.6	25	0.71	2.5
6.00	25.0	20	1.15	4.0
6.80	18.3	25	1.47	5.5
7.50	11.5	32	1.92	8.7
7.90	10.2	55	2.32	9.8
8.30	6.6	70	3.85	15.2
8.65	5.4	105	5.05	18.5
9.00	3.5	320	20.6	28.6
9.50	2.7	430	45.3	37.0
10.00	2.4	520	120	41.7
10.40	ND[b]	>1600	885	ND[b]

[a] Initial velocities were determined at each pH value in 80 μM angiotensin I over a $[Cl^-]$ range of 0–800 mM (up to 1600 mM for pH 9.5–10.4) in 100 mM buffer: pH 5.0 and 5.4, sodium succinate; pH 6.0–8.3, potassium phosphate; pH 8.65, Tris-$PO(OH)_3$; pH 9.0–10.0, sodium borate; pH 10.4, sodium carbonate/bicarbonate. Values for percent activity at 0 $[Cl^-]$, optimal $[Cl^-]$, and velocity enhancement at optimal $[Cl^-]$ were determined from v vs $[Cl^-]$ plots. K_a' values were obtained from $1/v - v_0$ vs $1/[Cl^-]$ plots (see Figure 3). [b] Not determined.

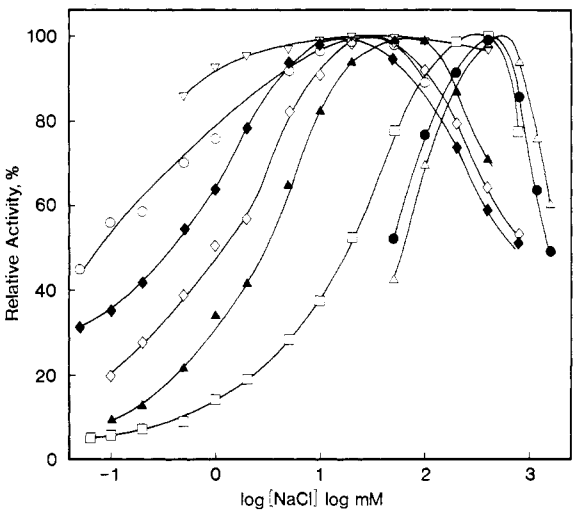


FIGURE 2: Chloride dependence of angiotensin I hydrolysis as a function of pH. Initial rates were determined at pH 5.0 (▽), 5.4 (○), 6.0 (◆), 7.5 (◇), 8.3 (▲), 9.0 (□), 9.5 (●), and 10.0 (△) at 0–1600 mM NaCl with 80 μM angiotensin I in 100 mM buffer (pH 5.0 and 5.4, sodium succinate; pH 6.0–8.3, potassium phosphate; pH 9.0–10.0, sodium borate). Activities are expressed as percent of maximum at optimal $[Cl^-]$ (see Table I). Plots at pH 6.8 and 8.65 were not included as they were very similar to those at pH 7.5 and 8.3, respectively. No plot was possible at pH 10.4 as the optimal $[Cl^-]$ was not determined (>1600 mM).

of chloride was subtracted from the velocities determined at different chloride concentrations ($1/v - v_0$) were linear over a 40- to 100-fold chloride concentration range (Figure 3). This behavior is predicted (Dixon & Webb, 1979a) for a nonessential activator mechanism in which substrate and activator bind randomly to the enzyme and product can be formed from both the ES and EAS complexes (Scheme I). Additional data supporting this mechanism are presented below.

Chloride Activation: Kinetic Analyses at pH 6.0, 7.5, and 9.0. Initial velocities were measured at six fixed chloride concentrations while substrate was varied over at least a 10-fold concentration range. Lineweaver–Burk plots at each pH value were linear, intersecting at a point above the $1/[S]$ axis, as predicted by the general scheme for nonessential activation

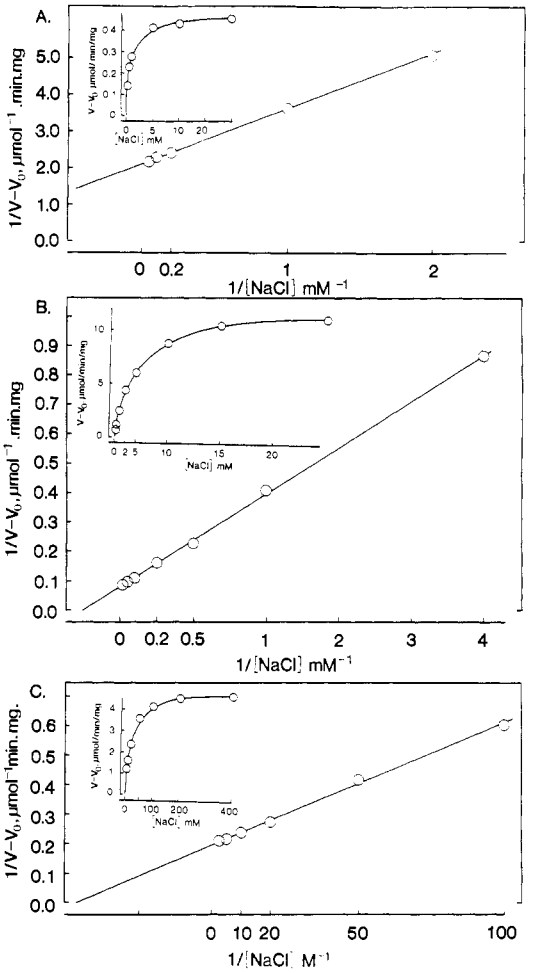
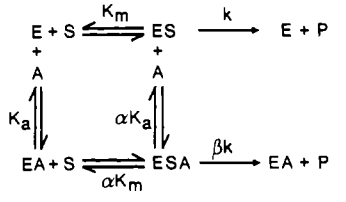


FIGURE 3: Chloride activation of the ACE-catalyzed hydrolysis of angiotensin I. Activities were determined at pH 5.4 (A), 7.5 (B), and 9.0 (C) as described in the legend to Figure 2, and expressed as reciprocals of $v - v_0$, where v is the activity in the presence of various chloride concentrations and v_0 is the activity in the absence of chloride. In all cases intersection of the plots with the abscissa gives $-1/K_a'$. Insets: Source plots of $v - v_0$ vs $[Cl^-]$ at each pH.

Scheme I



(Segel, 1975; Dixon & Webb, 1979a). Furthermore, secondary plots of the reciprocals of the change in slope ($1/\Delta$slope, where Δslope = slope in the absence of chloride − slope at each chloride concentration) and change in intercepts ($1/\Delta$intercept, where Δintercept = y-intercept$_{Cl^-}$ − y-intercept$_0$) vs the reciprocal of the chloride concentration (Segel, 1975) were linear at all three pH values tested and intersected on the abscissa. These plots allowed the determination of K_a' α, and β (eq 1–3) since for the $1/\Delta$slope vs $1/[Cl^-]$ plot the y intercept is given as

$$y = \beta V_{max}^0/[K_m^0(\beta - \alpha)] \quad (1)$$

for the $1/\Delta$intercept vs $1/[Cl^-]$ plot

$$y = \beta V_{max}^0/(\beta - 1) \quad (2)$$

and the common x intercept is

$$x = -\beta/\alpha K_a \quad (3)$$

Table II: Kinetic Parameters for the ACE-Catalyzed Hydrolysis of Angiotensin I as a Function of pH

[NaCl] (mM)	(A) Data from Lineweaver–Burk Plots[a] K_m (mM) pH 6.0	pH 7.5	pH 9.0	V_{max} (μmol min^{-1} mg^{-1}) pH 6.0	pH 7.5	pH 9.0
0	0.54			3.49	4.07	0.86
0.075	0.44			4.95		
0.15	0.36			6.16		
0.2		0.21			7.04	
0.3	0.29			6.79		
0.5	0.21	0.18	0.42	7.74	10.6	1.90
1.0	0.16	0.16	0.33	10.2	13.9	2.92
2.0	0.13	0.12	0.29	12.1	17.7	3.79
10.0		0.075	0.18		24.4	6.29
50.0			0.10			7.38

pH	(B) Data from Secondary Plots[b] α	β	K_a (mM)
6.0	0.052,[c] 0.054[d]	3.6,[c] 3.4[d]	7.23,[c] 5.75[d]
7.5	0.197,[c] 0.22[d]	6.84,[c] 6.46[d]	6.91,[c] 6.18[d]
9.0	0.086,[c] 0.15[d]	10.1,[c] 9.55[d]	37.3,[c] 33.2[d]

[a] Data are derived from linear regression analysis of Lineweaver–Burk plots. Initial rates were determined over a 10-fold [AI] range in 100 mM buffer: pH 6.0 and 7.5, potassium phosphate; pH 9.0, sodium borate. [b] Values for the constants α, β, and K_a, defined in eq 1–3, were derived from secondary plots as described in the text. [c] From 1/Δintercept and 1/Δslope vs 1/[Cl$^-$] plots. [d] From $K_m^0/\Delta K_m$ and $V_{max}^0/\Delta V_{max}$ vs 1/[Cl$^-$] plots (Rohrbach et al., 1981).

(Segel, 1975). The data derived from these primary and secondary plots are summarized in Table II.

Chloride Inhibition at High [Cl$^-$]: Dependence on pH. Under conditions similar to those described in the previous section, initial rates were determined at fixed high [Cl$^-$]. At pH 7.5, Lineweaver–Burk plots at 300, 600, and 800 mM chloride were linear and parallel in an apparent uncompetitive pattern (Figure 4A). However, a replot of $1/V_{max}$ vs [Cl$^-$] (Figure 4A, inset) gave a line that curves upward, suggesting a more complex mechanism. A straight line fitted to this curve gave an apparent K_i of 260 mM. At even higher chloride concentrations, 800, 1000, and 1400 mM, Lineweaver–Burk plots were found to intersect on the y axis in a competitive pattern (Figure 4B), although again the replot of slope vs [Cl$^-$] was nonlinear (Figure 4B, inset). At pH 6.0, Lineweaver–Burk plots at 150, 300, and 600 mM NaCl intersected on the y axis in a competitive pattern similar to that shown for pH 7.5 between 800 and 1400 mM (Figure 4B), although in this case the replot of slope vs [Cl$^-$] was linear, with K_i = 110 mM. At pH 9.0, Lineweaver–Burk plots at 800, 1000, and 1400 mM NaCl were linear and parallel in a pattern similar to that seen at pH 7.5 over the 300–800 mM range (Figure 4A), and once again the replot of y intercept vs [Cl$^-$] gave an upward sloping curve. A tangent drawn to the lower part of this curve intersected the x axis to give an apparent K_i of 1140 mM.

pH Dependence of Angiotensin I Hydrolysis. At low substrate concentration (0.03 mM, [S] < K_m) initial velocities were determined over the pH range 5.0–10.0 at five chloride concentrations: 0, 0.2, 2.0, 20, and 400 mM. The results indicate a modest increase in the pH optimum, from 7.1 at zero chloride to 7.6 at 400 mM NaCl, and a progressive and noticeable increase in the alkaline limb of the pH–rate profile with increasing chloride concentration (not shown). Although at [S] < K_m v is proportional to V_{max}/K_m, and therefore a plot of log v vs pH should give some information about the pKs of the free enzyme (Dixon & Webb, 1979b; Tipton & Dixon, 1979), such a plot was not useful in this case, as the straight-line portions of the graphs did not have integer slopes (not shown). It seems likely that this anomalous behavior is due to the fact that these graphs reflect ionizations in both

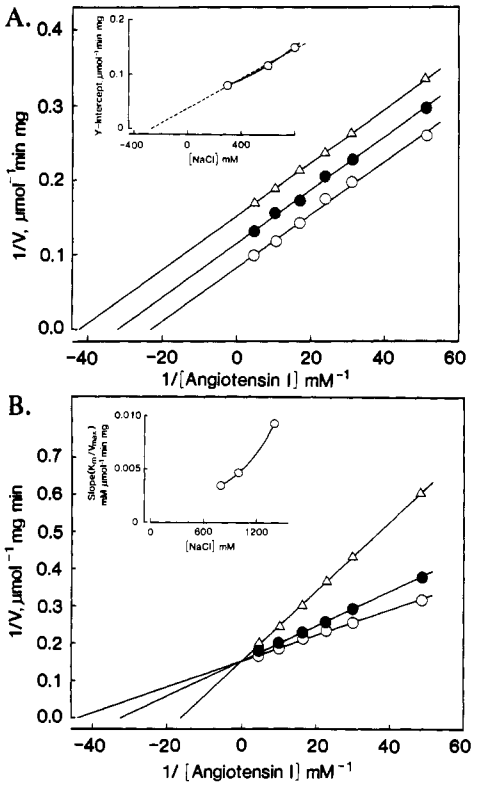


FIGURE 4: Lineweaver–Burk plots for the hydrolysis of angiotensin I at high [NaCl]. (A) 300 (○), 600 (●), and 800 (△) mM NaCl in 100 mM potassium phosphate, pH 7.5. Inset: Secondary plot of y intercept ($1/V_{max}$) vs [Cl$^-$]. (B) 800 (○), 1000 (●), and 1400 (△) mM NaCl in 100 mM potassium phosphate, pH 7.5. Inset: Secondary plot of slope (K_m/V_{max}) vs [Cl$^-$].

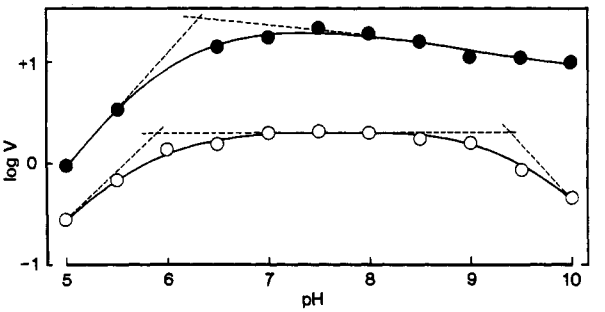


FIGURE 5: pH dependence of log v at saturating angiotensin I concentration (1.5 mM) at zero (○) and maximally activating (●) chloride concentrations: pH 5.0–7.5, 20 mM; pH 8.0, 40 mM; pH 8.5, 100 mM; pH 9.0, 320 mM; pH 9.5, 430 mM; pH 10.0, 520 mM. Buffers were 100 mM: pH 5.0 and 5.5, sodium succinate; pH 6.0–7.5, potassium phosphate; pH 8.0 and 8.5, Tris-PO(OH)$_3$; pH 9.0–10.0, sodium borate.

the free enzyme and the free substrate, the latter being a decapeptide with seven interfering ionizing groups. Under conditions of substrate saturation[3] ([S] = 1.5 mM), i.e., [S] $\gg$ K_m, initial velocities were measured over the pH range 5.0–10.0 at zero and at maximally activating chloride concentrations (these values are pH dependent and were determined earlier, see Table I). The pH–rate profiles in the presence and absence of chloride showed, as expected, a common pH optimum, pH 7.5, but differed in form, with the

[3] Saturation with substrate was assessed at pH 5.4, 7.5, and 9.0, at both zero and maximally activating chloride concentrations, over the substrate range 0.5–2.0 mM. In all cases a plateau was reached on the velocity vs [S] curve at or before 1.5 mM with no evidence of substrate inhibition, indicating that there were reasonable grounds for assuming that the enzyme was saturated with substrate under all conditions tested.

alkaline limb becoming once again more prominent in the presence of chloride. In substrate excess the velocity of the reaction is simply the rate of breakdown of the ES complex (effects on affinity having been eliminated), and thus a plot of log v vs pH is expected to yield information about the state of ionization of the ES complex (Dixon & Webb, 1979b). Such a plot, shown in Figure 5, revealed that at zero chloride the ES complex appeared to have two ionization constants with pKs of 5.9 and 9.4, while at maximally activating chloride the pK of the first group appeared to have shifted from 5.9 to 6.3, while no second ionization was detectable.

Discussion

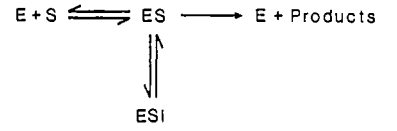
The kinetic data collected in this study reaffirm the complexity of the interacting effects of Cl^- and pH on ACE activity previously noted by others (Cheung et al., 1980; Bünning & Riordan, 1983; Shapiro et al., 1983) and provide a basis for understanding these effects on the ACE-catalyzed hydrolysis of AI. Comparison with data in the literature indicate that there may be species-dependent differences in the chloride activation/inhibition of ACE for this substrate.

Zinc addition at low pH (Figure 1) indicates that the levels of zinc required by the human enzyme for optimal activity were at least 2 orders of magnitude lower than those reported by Bünning et al. (1983) for rabbit ACE, and in fact marked inhibition was found at all pH values at zinc levels greater than optimal $[Zn^{2+}]$.

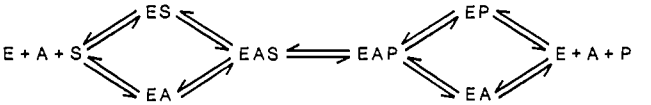
Studies on the extent of chloride activation of the human enzyme at various pHs led to a number of observations. First, chloride acts as a nonessential activator of human ACE for the catalysis of AI hydrolysis at all pH values tested, contrasting with equine (Fernley, 1977) and rabbit ACE (Bünning et al., 1983) that were found to have an absolute chloride requirement for this substrate. Second, the level of chloride required for maximal activity was lower than reported for other species. At pH 7.5 we found maximal activation with 32 mM NaCl, which is considerably lower than the 200 and 100 mM reported for rabbit (Bünning et al., 1983) and porcine (Inokuchi & Nagamatsu, 1981) ACE, respectively. Third, activity was inhibited at supraoptimal chloride levels over the entire pH range tested. This inhibition was relative to the activity of maximally activated ACE, and in no case was activity diminished to less than that observed in the absence of chloride. This behavior was previously reported by Dorer et al. (1972) for porcine ACE but has not been noted by other workers for this substrate. Indeed, Bünning and Riordan (1983) found that with AI rabbit ACE was maximally activated by >0.2 M NaCl, with no change occurring at least up to 0.6 M.

Chloride activation appears to follow a nonessential mechanism according to Scheme I. This was suggested by the finding that plots of $1/v - v_0$ vs $1/[Cl^-]$ were linear (Figure 3) and supported by the demonstration that a family of Lineweaver–Burk plots generated by a more detailed kinetic analysis intersected at a common point above the x axis with secondary plots of the reciprocals of the change in intercept and change in slope versus the reciprocal of the activator concentration being linear and intersecting on the x axis. Shapiro et al. (1983) identified a group of Fa tripeptides whose hydrolysis is activated by such a mechanism and designated these "class II". Class II substrates have K_a' values between 2.9 and 5.0 mM at pH 7.5, and all have positively charged side chains at P_1' or P_2'. On the basis of the activation mechanism and magnitude of K_a' (see Table I), AI appears to fall into class II in the pH range 5.0–8.65. Since histidine has a pK of 5.97 (imidazole) (Greenstein & Winitz, 1961), the P_1' histidyl may be considered charged in AI at pHs up to 7.0, but in alkaline conditions the residue would essentially be uncharged. Thus, AI should behave as a "class I" substrate (Shapiro et al., 1983), for this class chloride acts as an essential activator by an ordered bireactant mechanism with apparent K_as on the order of 75–100 mM. While there is no evidence for an essential activator mechanism at either pH 7.5 or 9.0, we did find that at pH >7.5 the $[Cl^-]$ required for maximal activation increased sharply, the relative activity at zero chloride decreased, and there was a parallel increase in K_a' (Table I), suggesting that as the pH was increased AI tended toward a class I substrate. Alternatively, the change in chloride dependence with pH may not be a result of the changes in the ionization states of substrate residues, but rather of such changes in catalytically important groups in the enzyme, including a putative chloride binding residue. This will be discussed further in light of the pH dependence below.

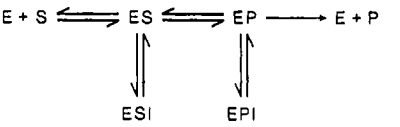
Scheme II



Scheme III



Scheme IV



While the *inhibition* of ACE by supraoptimal $[Cl^-]$ for various substrates has been observed by a number of investigators (Dorer et al., 1972, 1974; Cheung et al., 1980; Bünning & Riordan, 1983), its kinetic basis has not been determined. We found this phenomenon to be complex. At pH 7.5 an apparent uncompetitive pattern was observed between 300 and 800 mM NaCl (Figure 4A), suggesting that the anion is binding to the ES complex, retarding the breakdown to E and products, thereby decreasing V_{max}, as well as slowing the dissociation of ES to E and S, and hence also decreasing the apparent K_m (Dixon & Webb, 1979c) (Scheme II).

A second possibility may be binding of chloride to the EP complex, as suggested by Bünning and Riordan (1983) for the inhibition of Fa-PheGlyGly hydrolysis by chloride at pH 6.0 at >50 mM NaCl. These authors proposed the mechanism of Scheme III. Inhibition would be a consequence of chloride binding to EP to form EAP, thereby retarding product release and decreasing V_{max}. Furthermore, we suggest that since ES and EAS are in equilibrium with EAP, this mechanism could also account for a decrease in the rate of breakdown of ES to E and S and thereby decrease the apparent K_m, producing an apparent uncompetitive picture. A combination of the two mechanisms could be envisaged in which chloride binds to both ES and EP (Scheme IV). Such a mechanism may account for the finding that the secondary plot of $1/V_{max}$ vs $[Cl^-]$ is not linear but curves upward, possibly implying that with increasing $[Cl^-]$ more than one inhibitory site is occupied, resulting in an accelerated decrease in V_{max}. Support for the existence of more than one inhibitory binding site is given by the occurrence at even higher chloride levels, 800–1400 mM, of an apparent competitive inhibitory pattern (Figure 4B). Chloride may be an "allosteric" inhibitor binding to a different

site than the substrate, with binding of either causing a conformational change preventing the other form binding, thus generating apparent competitive kinetics (Monod et al., 1963). Again, the secondary plot of slope vs [Cl^-] was nonlinear, implying a more complex mechanism. As was the case with chloride activation, the inhibition was markedly pH dependent. Thus, at pH 6.0 the competitive pattern was observed at relatively low anion levels, 150–600 mM; at pH 9.0 the uncompetitive pattern was seen at relatively high anion levels, 800–1400 mM. In general, the pH dependence of the effect of chloride appears to be such that the degree of activation and relative dependence on anion increases with increasing pH, while the converse holds true for the occurrence of inhibition, and there is a parallel increase in both apparent K_a and K_i values with increasing pH. Furthermore, at all pH values $K_a' \ll K_i'$, and therefore the activating chloride binding site has a considerably higher affinity than the inhibitory site(s).

We studied the effect of chloride on the pH–rate profile of the ACE-catalyzed hydrolysis of AI. Under V_{max}/K_m conditions, increasing [Cl^-] resulted in a modest alkaline shift of the pH optimum and a more marked increase in the alkaline limb of the pH–rate profile. A similar shift of even greater magnitude was previously reported for rabbit ACE with the substrate Fa-PheGlyGly (Bünning & Riordan, 1983) and appears to be a general phenomenon of the anion activation of enzymes (Dixon & Webb, 1979a; Bünning & Riordan, 1983). It has been proposed that the anion acts by combining with a basic group adjacent to either of the two ionizable groups (acidic or basic) in the active center that determine the pH curve: The suppression of an adjacent positive charge will increase its pK and shift its titration curve in an alkaline direction (Massey, 1953). The pKs we determined from log v vs pH plots at saturating [S] ([S] $\gg K_m$) support such a mechanism: In the absence of chloride evidence for two ionizing groups in the ES complex was found (Figure 5), with pKs of 5.9 and 9.4, while in the presence of maximally activating [Cl^-] the acidic pK shifted to 6.3, and the basic ionization was no longer apparent over the pH range tested (pH 5.0–10.0). This may mean that suppression of an adjacent positive charge by combination with chloride induced both a small shift in the acidic ionizing group and a larger shift in the basic group that now has a pK >10.0. Alternatively, the chloride may be binding directly to the basic group in the active center, suppressing its ionization. There is considerable evidence that lysine is a critical residue in the active site of ACE (Bünning et al., 1978; Weare, 1982) and that this lysine is a component of the chloride binding site (Shapiro & Riordan, 1983). A pK of 9.4 would be consistent with such a residue since the pK_a of a lysyl ϵ-amine can be reduced from 10.28 (Greentein & Winitz, 1961) in a positively charged microenvironment (Lifshitz & Levitzki, 1976), such as near the active-site Zn^{2+}, and/or in a hydrophobic environment that stabilizes the un-ionized forms of groups (Tipton & Dixon, 1979) [ACE is thought to be a hydrophobic protein (Erdös & Skidgel, 1985)]. Furthermore, the pK of a unique chloride binding lysyl residue was also found to be reduced to 9.1 in α-amylase (Lifshitz & Levitzki, 1976), an enzyme similarly activated by chloride.

The in vitro studies presented here indicate that the ACE known to occur in high concentrations in human vascular endothelium and renal proximal tubular epithelium (Defendini et al., 1983; Danilov et al., 1987) is probably maximally activated for catalyzing the hydrolysis of AI, since at these sites pH and [Cl^-] are optimal and do not vary significantly (Guyton, 1976), and thus a regulatory role for chloride is unlikely. On the other hand, in the intestinal microvilli, which also contain large amounts of ACE (Ward et al., 1980; Defendini et al., 1983), ion fluxes (Guyton, 1976) could conceivably regulate ACE activity. The significance of this remains uncertain, however, in the face of complicating effects such as the recently described potentiating effect of sulfate (Bünning & Riordan, 1987).

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Mechanism of Adenylate Kinase. Histidine-36 Is Not Directly Involved in Catalysis, but Protects Cysteine-25 and Stabilizes the Tertiary Structure†

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ABSTRACT: Several previous reports on muscle adenylate kinase (AK) have suggested that histidine-36 (His-36) is located in the binding site of adenosine 5′-triphosphate (ATP) and is involved in catalysis. We have tested the role of His-36 using site-specific mutagenesis on chicken muscle AK expressed in *Escherichia coli*. Three mutant proteins (H36Q, H36N, and H36G) were obtained by substituting His-36 with glutamine, asparagine, and glycine, respectively. Steady-state kinetic studies showed that the mutants have similar kinetic properties to those of the wild-type (WT) AK, which suggested that His-36 is not directly involved in catalysis. However, His-36 is likely to interact with or protect cysteine-25 (Cys-25) on the basis of the following evidence: The crystal structure of porcine muscle AK revealed a close proximity between His-36 and Cys-25; the mutants were unstable during purification (the order of stability was WT > H36Q > H36N > H36G); the H36G mutant readily dimerized; the sulfhydryl groups of mutants became more reactive (WT < H36Q < H36N) toward 5,5′-dithiobis(2-nitrobenzoic acid). Furthermore, His-36 was found to stabilize the tertiary structure of AK on the basis of guanidine hydrochloride induced denaturation studies, which showed that the conformational stability decreases in the order WT > H36Q > H36N. Three models are proposed to explain the structural roles of His-36: (i) His-36 forms a charge-transfer complex with Cys-25; (ii) His-36 forms a hydrogen bond (N3-H···S-H) with Cys-25; and (iii) His-36 protects Cys-25 by steric shielding and stabilizes the tertiary structure via H-bonding with Asp-93. ^{1}H NMR experiments were also performed as a function of pH, and the pK_a of His-36 was determined as 6.3. Two other histidines showed pK_a = 6.5, and the fourth histidine showed nontitrating behavior. The chemical shifts and pK_a of the histidines in H36Q and H36N are the same as those in the wild-type AK within experimental error.

Adenylate kinase (AK)[1] (EC 2.7.4.3) catalyzes the interconversion between AMP, ADP, and ATP: MgATP + AMP ⇌ MgADP + ADP (Noda, 1973). The enzyme from mammalian muscle (unless otherwise specified, our discussion will be confined to muscle AK) is one of the smallest ($M_r \approx 21\,700$) and most extensively studied kinases. The crystal structure

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; AK, adenylate kinase; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; dCTPαS, 2′-deoxycytidine 5′-*O*-(1-thiotriphosphate); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; Gdn-HCl, guanidine hydrochloride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PEP, phosphoenolpyruvate; SDS, sodium dodecyl sulfate; TNB, thionitrobenzoic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; TSP, 3-(trimethylsilyl)propionic acid; UV, ultraviolet.

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