Observation of a Chloride-Dependent Intermediate during Catalysis by Angiotensin Converting Enzyme Using Radiationless Energy Transfer†

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ABSTRACT: Stopped-flow radiationless energy-transfer kinetics have been used to examine the effects of chloride on the hydrolysis of Dns-Lys-Phe-Ala-Arg by angiotensin converting enzyme. The kinetic constants for hydrolysis at pH 7.5 and 22 °C in the presence of 300 mM sodium chloride were K_M = 28 μM and k_{cat} = 110 s^{-1}, and in its absence, K_M = 240 μM and k_{cat} = 68 s^{-1}. The apparent binding constant for chloride was 4 mM, and the extent of chloride activation in terms of k_{cat}/K_M was 14-fold. The effects of chloride on the pre-steady-state were examined at 2 °C. In the presence of chloride, two distinct enzyme–substrate complexes were observed, suggesting multiple steps in substrate binding. The initial complex was formed during the mixing period (k_{obsd} > 200 s^{-1}) while the second complex was formed much more slowly (k_{obsd} = 40 s^{-1} when [S] = 5 μM and [NaCl] = 150 mM). Strikingly, in the absence of chloride, only a single, rapidly formed enzyme–substrate complex was observed. These results are consistent with a nonessential activator kinetic mechanism in which the slow step reflects conversion of an initially formed complex, $(E{\cdot}Cl^-{\cdot}S)_1$, to a more tightly bound complex, $(E{\cdot}Cl^-{\cdot}S)_2$.

Angiotensin converting enzyme (dipeptidyl carboxypeptidase, EC 3.4.15.1) (ACE)[1] is a zinc metallopeptidase that cleaves dipeptides from the C-terminus of a broad range of oligopeptides (Soffer, 1976). A striking characteristic of ACE is its marked activation by chloride and other monovalent anions. While this activation was first noted in 1954 (Skeggs et al., 1954), its kinetic and physical basis has only recently been examined in detail (Bünning & Riordan, 1981, 1983; Shapiro et al., 1983; Shapiro & Riordan, 1983). These studies revealed that activation is due largely to a lowering of K_M values and is independent of the active-site zinc atom. Furthermore, it was found that many features of activation are a function of the substrate being hydrolyzed. They include the extent of, and the anion concentration required for, activation as well as the kinetic mechanism of activation.

Most of the mechanistic studies cited have utilized initial rate assays based on conversion of substrate to product. In the present study, an alternative approach (Auld, 1977; Lobb & Auld, 1979, 1980, 1984) was chosen: measurement of radiationless energy transfer (RET) between tryptophan residues in ACE and a dansyl chromophore attached to peptide substrates. In combination with rapid-mixing techniques, this method allows the direct observation of enzyme–substrate complex formation and breakdown. Thus, both the steady-state and pre-steady-state portions of substrate hydrolysis can be examined. In addition, the effects of activators or inhibitors on substrate binding and turnover can be assessed (Auld, 1977; Williams & Auld, 1986).

In this study, the effect of chloride on the ACE-catalyzed hydrolysis of dansyl tri- and tetrapeptides has been investigated by using stopped-flow RET kinetics. The steady-state activation behavior observed is consistent with that found in the earlier studies. Examination of the pre-steady-state time interval reveals the existence of a slow step during hydrolysis in the presence of chloride. In the absence of chloride, no such step is observed.

Materials and Methods

ACE was isolated from frozen rabbit lungs (Pel-Freez Biologicals Inc., Rogers, AR) by using either a modification of the method of Bünning et al. (1983) or the affinity chromatographic procedure described by Pantoliano et al. (1984). The final preparation was at least 95% pure as judged by SDS–PAGE (Laemmli, 1970) and >93% pure based on specific activity toward Fa-Phe-Gly-Gly (Holmquist et al., 1979). Enzyme concentration was determined from the absorbance at 280 nm by using a molar absorptivity of 225 000 M^{-1} cm^{-1}.

Synthesis. Dipeptides and N- and C-blocked amino acids were purchased from Sigma Chemical Co. (St. Louis, MO) or Vega Biochemicals (Tucson, AZ). Dansyl tripeptides and peptide intermediates were synthesized by standard procedures (Auld & Holmquist, 1974). Thin-layer chromatography (TLC) was performed in 1-butanol/acetic acid/water (4:1:1) using silica gel plates (E. Merck).

Dns-Lys-Phe-Ala-Arg. Dns-(N^{ϵ}-*t*-Boc)Lys-ONSu (5.9 mmol) in 30 mL of DME was added to 2 mmol of Phe-Ala-OMe in 20 mL of 50% DME–water, pH 8.6. After being stirred overnight at room temperature, the pH was brought to 6.5. The solvent was then partially evaporated, resulting in crystallization. Product was recrystallized in ethanol–water. The ester was then hydrolyzed with 0.33 N NaOH in 67% methanol–water for 15 min. After neutralization with 1 N HCl, the methanol was removed by evaporation, and the mixture was extracted with ethyl acetate. The organic phase was washed with water, then dried with anhydrous $MgSO_4$, and evaporated to give Dns-(N^{ϵ}-*t*-Boc)Lys-Phe-Ala. The blocked tripeptide *N*-hydroxysuccinimide ester (1.47 mmol,

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[1] Abbreviations: ACE, angiotensin converting enzyme; Fa, 2-furanacryloyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Dns or dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; RET, radiationless energy transfer; HPLC, high-performance liquid chromatography; DCC, *N*,*N*′-dicyclohexylcarbodiimide; TLC, thin-layer chromatography; *t*-Boc, *tert*-butyloxycarbonyl; ONSu, succinimidooxy; DME, 1,2-dimethoxyethane; TFA, trifluoroacetic acid; C18, octadecylsilane; IC_{50}, concentration of inhibitor producing 50% inhibition at the particular substrate concentration employed; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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formed by standard procedures using DCC and HONSu) in dioxane containing 10% DME was added to 3 mmol of arginine free base and 7.5 mmol of $NaHCO_3$ in 50% DME–water. After 3 h at room temperature, the solution was concentrated by evaporation. Most of the Dns-labeled material became insoluble, and the liquid was decanted. The insoluble material, assumed to be largely Dns-(N^{ϵ}-*t*-Boc)Lys-Phe-Ala-Arg, was dissolved in TFA and allowed to stand for 30 min at room temperature. The TFA was removed by evaporation, and the residue was redissolved in ethanol and dried down 3 times. This material was reconstituted in 0.2 M pyridine formate, pH 2.9, and applied to a column of AG50W-X2 cation-exchange resin. A linear gradient of 0.2 M pyridine formate (pH 2.9) to 2 M pyridine formate (pH 5.75) was then applied. A broad peak of 330-nm-absorbing material eluted with finishing buffer and was lyophilized. TLC revealed one major fluorescent spot and a minor spot of about 10 times weaker intensity. This material was applied to a Radial Pak C18 column (Waters Associates, Milford, MA) and eluted with a 25-min linear gradient from 0% to 100% solvent B, where solvent A was 0.1% (v/v) TFA in water and solvent B was 0.08% TFA and 60% acetonitrile in water. The flow rate was 0.8 mL/min, and 1-min fractions were collected. A Waters Associates liquid chromatography system was employed. Eluent absorbance was monitored at 340 nm. Fractions containing the dansyl tetrapeptide (retention time = 16 min) were pooled and lyophilized to give a pale yellow powder. Amino acid analysis (Pico-Tag, Waters Associates; Bidlingmeyer et al., 1984) gave Phe (1.00):Ala (0.97):Arg (1.08). Dns-lysine is not hydrolyzed appreciably to free lysine under the conditions used (5.7 N HCl, 110 °C, 20–22 h).

Preparation of Dns-Lys-Phe. Dns-Lys-Phe-Ala-Arg (1.1 mg) was dissolved in 1 mL of 45 mM Hepes, pH 7.5, containing 300 mM NaCl. After addition of ACE (10 nM final concentration), the mixture was allowed to stand at room temperature overnight. The product Dns-Lys-Phe was then purified by using C18 reversed-phase HPLC as above except that a 30-min gradient was used.

Kinetic Measurements. Activities toward Fa tripeptides were measured spectrophotometrically as described previously (Holmquist et al., 1979). Assays were performed in 50 mM Hepes, pH 7.5, containing 300 mM NaCl at 25 °C. Stopped-flow RET measurements using dansyl peptides were performed in 50 mM Hepes, pH 7.5, with enzyme concentrations of 0.1–1.0 μM. Dansyl peptide concentrations were determined by using $\epsilon_{328} = 4500\ M^{-1}\ cm^{-1}$ (pH 7.5). RET kinetic measurements were carried out on either a Durrum-Gibson instrument or the low-temperature instrument of Hanahan and Auld (1980) as described previously (Lobb & Auld, 1980; Galdes et al., 1983). Enzyme tryptophan residues were excited with 285-nm light. Tryptophan quench signals were measured by using a band-pass filter centered at 360 nm while dansyl emission signals were measured by using a 430-nm cut-on filter.

Steady-state kinetic parameters for hydrolysis of Dns-Lys-Phe-Ala-Arg were determined from the dansyl fluorescence signal at a single initial substrate concentration as described in detail by Lobb and Auld (1980, 1984). A series of fluorescence intensities, F_t, and associated areas, A_t (where $A_t = \int_t^{\infty} F_t\, dt$), were evaluated at every tenth time interval (a total of 1000 data points were recorded per experiment) and used to generate a set of instantaneous velocities, v_t, according to the expression $v_t = F_t[S]_t/A_t$. The substrate concentration at time t, $[S]_t$, was calculated from the initial substrate concentration, $[S]_0$, and A_t by using the expression $[S]_t = [S]_0[A]_t/[A]_0$. Kinetic constants were determined from plots of $1/v_t$ vs. $1/[S]_t$ using linear regression analysis. All k_{cat} and K_M values are the average of four or more determinations. The k_{cat}/K_M values for all dansyl tripeptides were calculated from the first-order decrease in fluorescence decay when $[S]_t << K_M$ (Lobb & Auld, 1980). In some cases, the k_{cat}/K_M values for hydrolysis of Dns-Lys-Phe-Ala-Arg were determined by using this method. Pre-steady-state rates were determined from plots of $-\log (F_{max} - F_t)$ vs. time, and a 100–300 point data base was used for the calculation.

Results and Discussion

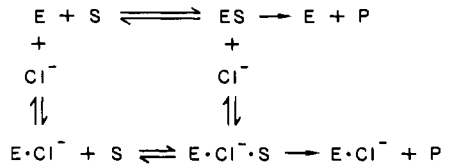
Initial studies from this laboratory on anion activation of angiotensin converting enzyme utilized Fa-Phe-Gly-Gly as substrate (Riordan et al., 1980; Bünning & Riordan, 1983). The kinetics of peptide hydrolysis were found to be consistent with an essential activator mechanism in which chloride must bind to the enzyme prior to substrate (Scheme I). Thus, the effect of the activator is to lower K_M without influencing k_{cat}. The apparent chloride binding constant (K'_A) measured with this substrate—i.e., the chloride concentration at which k_{cat}/K_M is half-maximal—is 80 mM at pH 7.5.

Scheme I

$$E + Cl^- \rightleftharpoons E{\cdot}Cl^- + S \rightleftharpoons E{\cdot}Cl^-{\cdot}S \rightarrow E{\cdot}Cl^- + P$$

A subsequent study (Shapiro et al., 1983) employed numerous additional Fa- and Bz-blocked tripeptide substrates of widely varying structures. Three different patterns of activation were observed, depending on the particular substrate being hydrolyzed. With "class I" substrates, the activation kinetics closely resembled that found with Fa-Phe-Gly-Gly. Many other substrates, however, were found to be hydrolyzed by a nonessential activator mechanism (Scheme II) although the degree of activation by chloride was still large (24–160-fold). With these peptides, the anion influenced both K_M and k_{cat}, but the primary effect was still on K_M. These substrates could be further subdivided, based largely on the K'_A values for chloride: one group (termed "class II") yielded K'_A values of 3–5 mM at pH 7.5, and the other (termed "class III") produced K'_A values of 18–30 mM. Structurally, class II substrates were distinguished by the presence of a positively charged side chain at either the P_1' or the P_2' position.[2] All class III substrates identified contained an alanine residue at P_1', although this was not by itself sufficient to impart kinetic properties typical of class III substrates.

Scheme II



In order to extend these studies, we have used radiationless energy-transfer (RET) kinetics under stopped-flow conditions. This method has proved to be extremely powerful in examining the mechanisms of other proteolytic enzymes (Auld, 1977; Lobb & Auld, 1979; Galdes et al., 1983). For example, with carboxypeptidase A, pre-steady-state measurements at subzero temperatures allowed the identification and characterization of two distinct enzyme–substrate complexes which are formed during hydrolysis of peptide and ester substrates (Galdes et

[2] The nomenclature for the individual amino acid residues (P_2, P_1, P_1', P_2') of a substrate is that of Schechter and Berger (1967).

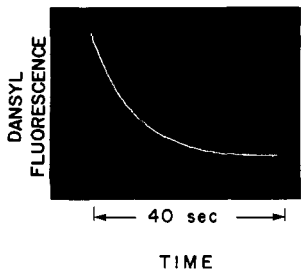


FIGURE 1: Oscilloscope trace of dansyl fluorescence signal for the ACE-catalyzed hydrolysis of 100 μM Dns-Ala-Phe-Arg. Conditions were pH 7.5, 50 mM Hepes, 300 mM NaCl, and 1 μM ACE at 25 °C. Instrument parameters were adjusted such that the initial fluorescence increase was near full scale.

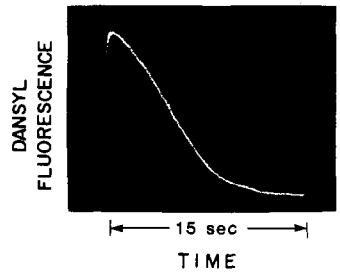


FIGURE 2: Oscilloscope trace of dansyl fluorescence signal for the ACE-catalyzed hydrolysis of 38 μM Dns-Lys-Phe-Ala-Arg. Conditions were pH 7.5, 50 mM Hepes, 300 mM NaCl, and 0.1 μM ACE at 22 °C. Instrument parameters were adjusted such that the initial fluorescence increase was near full scale.

Table I: Hydrolysis of Dns Tripeptides by ACE[a]

substrate	k_{cat}/K_M ($\times 10^{-4}$ M^{-1} s^{-1})
Dns-Ala-Phe-Arg	13.0
Dns-Phe-Ala-Lys	6.3
Dns-Phe-Lys-Gly	3.5
Dns-Phe-Ala-(N^{ϵ}-*t*-Boc)Lys	2.1

[a] pH 7.5, 50 mM Hepes, 300 mM NaCl, at 25 °C. Values of k_{cat}/K_M were determined from the rate of substrate hydrolysis under first-order conditions as described under Materials and Methods.

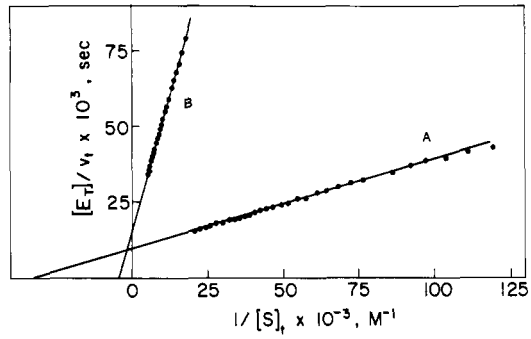


FIGURE 3: Plots of $[E_T]/v_i$ vs. $1/[S]_t$ for the ACE (0.1 μM) catalyzed hydrolysis of Dns-Lys-Phe-Ala-Arg. Assay conditions were (A) 50 μM initial substrate, pH 7.5, 50 mM Hepes, and 300 mM NaCl at 22 °C or (B) 200 μM initial substrate, pH 7.5, and 50 mM Hepes at 22 °C.

al., 1983). In addition, RET kinetics have been used to examine the mechanism of inhibition of carboxypeptidase A by chloride and other anions (Williams & Auld, 1986). In the present case, it was hoped that RET measurements would provide an independent means of assessing the accuracy of the kinetic picture of ACE delineated above and, more importantly, provide further insight into the mechanism of anion activation by allowing the effect of chloride on pre-steady-state enzyme–substrate complexes to be examined.

Hydrolysis of Dansyl Tripeptides. We first investigated the action of ACE on six dansyl tripeptides having structures similar to those of Fa tripeptides from all three substrate classes. TLC analysis revealed that one of these peptides, Dns-Phe-Gly-Gly, is cleaved extremely slowly: the half-life for hydrolysis of 1 mM peptide by 86 nM ACE at 25 °C is greater than 1 h. Thus, this substrate was not investigated further. Much faster rates were observed by TLC for the remaining five dansyl tripeptides, and hydrolysis of these substrates (100 μM) by ACE (1 μM) in the presence of 300 mM NaCl was studied by using the Durrum–Gibson stopped-flow instrument at 25 °C. With Dns-Phe-(N^{ϵ}-*t*-Boc)-Lys-Gly, there was no detectable increase in the dansyl fluorescence signal after mixing, indicating extremely poor binding. With the remaining four peptides, Dns-Phe-Ala-Lys, Dns-Ala-Phe-Arg, Dns-Phe-Lys-Gly, and Dns-Phe-Ala-(N^{ϵ}-*t*-Boc)Lys, significant fluorescence increases were observed. Figure 1 shows a typical trace with Dns-Ala-Phe-Arg. Dansyl fluorescence reaches its maximum value within the instrument dead time, reflecting attainment of the steady-state concentration of ES, while the subsequent fluorescence decrease reflects substrate hydrolysis. With all four substrates, the ES fluorescence signal undergoes a first-order decay over the entire reaction time course, suggesting that the initial substrate concentration is well below K_M. The k_{cat}/K_M values calculated from the first-order decay rates are listed in Table I.[3] Lowering the chloride concentration to 20 mM with Dns-Ala-Phe-Arg, Dns-Phe-Ala-Lys, and Dns-Phe-Lys-Gly had no significant effect on k_{cat}/K_M, consistent with results obtained for Fa and Bz tripeptides of similar (i.e., class II) structure, where 20 mM chloride is sufficient for optimum activation (Shapiro et al., 1983).

The dansyl tripeptides examined are poor substrates compared to Fa tripeptides with similar sequences. The rate of Dns-Phe-Gly-Gly hydrolysis observed by TLC is at least 200-fold slower than that for Fa-Phe-Gly-Gly. The k_{cat}/K_m values for Dns-Phe-Ala-Lys and Dns-Phe-Ala-(N^{ϵ}-*t*-Boc)Lys are both ~70-fold lower than those for the corresponding Fa tripeptides (Shapiro et al., 1983). Much of this difference is clearly due to decreased substrate binding, as evidenced by the weak fluorescence signals and first-order kinetics obtained at a substrate concentration (100 μM) that is near or above K_M for the Fa peptides. Thus, it appears that a dansyl group at the P_2 position is unfavorable for substrate binding.

Hydrolysis of Dns-Lys-Phe-Ala-Arg. The poor binding of the dansyl tripeptides obviates their use in detailed RET kinetic studies. We therefore prepared the tetrapeptide Dns-Lys-Phe-Ala-Arg as a potential ACE substrate to see if the dansyl moiety could be better accommodated at the P_3 position. The design of this substrate was based largely on substrate sequences reported previously. The P_1, P_1', and P_2' amino acids chosen are those of the best binding Fa tripeptide substrates [K_M = 6.5 μM (Shapiro et al., 1983)]. The selection of the P_2 residue was based on structure–function studies of the potent inhibitor/substrate <Glu-Lys-Trp-Ala-Pro (Cushman et al., 1973). These studies demonstrated that a lysine residue at the P_2 position contributes significantly to binding, since its replacement by norleucine, glutamine, threonine, or glutamic acid markedly attentuates inhibition.

Dns-Lys-Phe-Ala-Arg proved to be an excellent ACE substrate. A strong dansyl fluorescence signal is observed upon

[3] The absorbance properties of the dansyl chromophore make it difficult to accurately monitor substrate hydrolysis at concentrations significantly above 0.2 mM. Therefore, the individual kinetic parameters, k_{cat} and K_M, could not be determined for these substrates.

Table II: Kinetic Parameters for the ACE-Catalyzed Hydrolysis of Dns-Lys-Phe-Ala-Arg[a]

[NaCl] (mM)	k_{cat}[b] (s^{-1})	K_M (μM)	k_{cat}/K_M[b] ($\times 10^{-6}$ M^{-1} s^{-1})	k_{cat}/K_M[c] ($\times 10^{-6}$ M^{-1} s^{-1})
300	110	28	3.9	3.3
0	68	240	0.29	0.31

[a] pH 7.5, 50 mM Hepes at 22 °C. [b] Determined from the dependence of F_t/A_t on substrate concentrations as described under Materials and Methods. [c] Determined from the rate of substrate hydrolysis under first-order conditions as described under Materials and Methods.

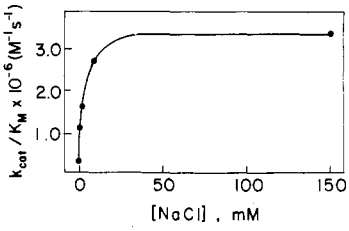


FIGURE 4: Effect of sodium chloride concentration on the hydrolysis of Dns-Lys-Phe-Ala-Arg by ACE. Assay conditions were pH 7.5, 50 mM Hepes at 22 °C. Values of k_{cat}/K_M were calculated from the first-order portion of the fluorescence signals, where $[S]_t << K_M$ as described under Materials and Methods.

mixing a 38 μM solution of the peptide with ACE (0.1 μM) at 22 °C in the presence of 300 mM chloride (Figure 2). A complementary signal is obtained when quenching of enzyme tryptophan fluorescence is monitored (not shown). In contrast to the pattern observed with dansyl tripeptides, the fluorescence decrease is not first order. A plot of $1/v_t$ vs. $1/[S]_t$ generated from such a trace is shown in Figure 3. The k_{cat} and K_M values obtained are 110 s^{-1} and 28 μM, respectively (Table II).

When chloride is omitted from the reaction mixture, the fluorescence increase is only one-third of that observed with 300 mM chloride, indicating that chloride significantly enhances substrate binding. In addition, the decrease in dansyl fluorescence accompanying substrate hydrolysis is first order over the entire time course, indicating that this substrate concentration is now well below K_M. It is therefore necessary to use a higher initial substrate concentration (200 μM) to determine k_{cat} and K_M. The K_M and k_{cat} values obtained from a plot of $1/v_t$ vs. $1/[S]_t$ (Figure 3, Table II) are 8.6-fold higher and 1.6-fold lower, respectively, than those at 300 mM chloride. Thus, the total extent of chloride activation in terms of k_{cat}/K_M is 14-fold. Additional measurements were performed by using intermediate chloride concentrations, and the resultant k_{cat}/K_M values are plotted in Figure 4. The concentration of chloride required for half-maximal activation is about 4 mM. These findings—detectable binding and turnover in the absence of chloride and a K'_A value for chloride of ~4 mM—identify Dns-Lys-Phe-Ala-Arg as a class II substrate, as expected on the basis of its P_2' arginine residue.

Values for k_{cat}/K_M were also calculated independently by using the rate constant for the first-order decrease in dansyl fluorescence which occurs during the latter stages of the reaction, i.e., where $[S] << K_M$. While the k_{cat}/K_M values obtained by the two methods in the absence of chloride are in close agreement, the value determined under first-order conditions in the presence of 300 mM chloride is 15% lower than that calculated from the individual parameters (Table II). In order to determine whether this is due to product inhibition, the effects of both hydrolysis products on ACE activity were investigated. Dns-Lys-Phe (50 μM) did not inhibit the ACE-catalyzed hydrolysis of Fa-Phe-Gly-Gly (50

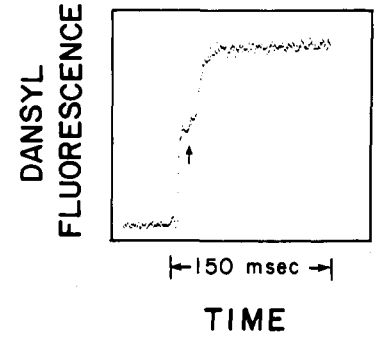


FIGURE 5: Dansyl fluorescence signal for the binding of 20 μM Dns-Lys-Phe-Ala-Arg by ACE. Assay conditions were pH 7.5, 50 mM Hepes, 10 mM NaCl, and 0.4 μM ACE at 25 °C. The arrow indicates the end of the mixing period. A Durrum–Gibson instrument was used.

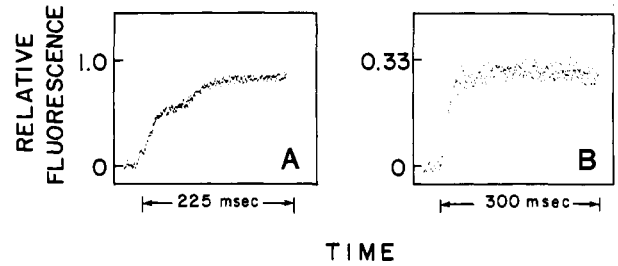


FIGURE 6: Dansyl fluorescence signal for the binding of 5 μM Dns-Lys-Phe-Ala-Arg to 0.1 μM ACE at 2 °C. Conditions were pH 7.5 and 50 mM Hepes (A) in the presence of 150 mM NaCl and (B) in the absence of added chloride. Measurements were performed on the low-temperature instrument of Hanahan and Auld (1980). The initial apparent plateau at about 0.5 relative fluorescence in (A) is an artifact of mixing/flow time.

μM) at 300 mM NaCl, pH 7.5. On the other hand, the dipeptide Ala-Arg inhibited ACE significantly, yielding an IC_{50} value of 40 μM with the class II substrate Fa-Phe-Phe-Arg ([S] = 3 μM, well below K_M).[4] Thus, accumulation of Ala-Arg could be responsible for the 15% lower k_{cat}/K_M value calculated from the first-order rate for Dns-Lys-Phe-Ala-Arg hydrolysis.

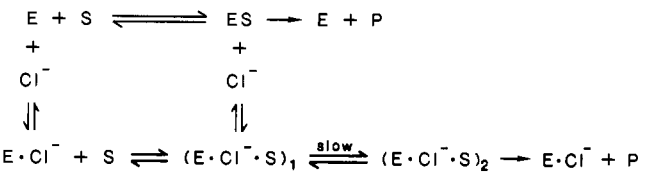
Pre-Steady-State Kinetics. Figure 5 shows the dansyl fluorescence signal observed during the first 150 ms of the reaction of 20 μM Dns-Lys-Phe-Ala-Arg with 0.4 μM ACE in the presence of 10 mM NaCl. The initial rapid fluorescence increase that occurs during the mixing period is followed by a somewhat slower increase to a maximum reflecting attainment of the steady-state within 50 ms. This apparent diphasic pattern suggests that there may be one or more intermediates in substrate hydrolysis (see below). However, under these conditions, this process is too rapid to allow detailed study. Thus, the temperature was decreased to 2 °C in subsequent experiments.[5] At 300 mM chloride, this temperature change results in a 4.6-fold decrease in k_{cat} but has no effect on K_M. The effect of chloride concentration on k_{cat}/K_M is similar to that measured at 22 °C.

The fluorescence signal observed upon mixing 0.1 μM ACE with 5 μM Dns-Lys-Phe-Ala-Arg at 2 °C in the presence of 150 mM chloride is shown in Figure 6A. Attainment of the steady-state now requires ~200 ms and is clearly a biphasic

[4] As with several other ACE inhibitors (Shapiro & Riordan, 1984a) the $1/v$ vs. [I] plots are markedly nonlinear and vary with the substrate employed. We therefore report an IC_{50} rather than a K_i value.

[5] Subzero temperatures could not be employed since these would require the use of organic solvents, which alter the anion activation properties of ACE considerably (unpublished results).

Scheme III



process. Such a biphasic pattern can be interpreted in terms of a reaction mechanism involving two (or more) distinct ES complexes (Lobb & Auld, 1979; Galdes et al., 1983). In this case, the initial rapid rise in fluorescence during mixing represents formation of the first complex, ES_1. The subsequent slower fluorescence increase then reflects formation of the second complex, ES_2, and the consequent greater degree of enzyme saturation by substrate as the steady-state concentrations of ES_1 and ES_2 are approached. At 5 μM substrate (Figure 6A), the observed rate constant k_{obsd} for the slow process is 40 s^{-1}. Values for k_{obsd} increase with substrate concentration and at 50 μM peptide approach the limit for rates resolvable by this method, i.e., $\sim$150 s^{-1}.

The slow process is markedly affected by chloride concentration. Using 20 μM Dns-Lys-Phe-Ala-Arg, k_{obsd} decreases from 104 s^{-1} at 150 mM chloride to 75 s^{-1} at 10 mM chloride and 50 s^{-1} at 2.5 mM chloride. Simultaneously, the extent of the fluorescence increase associated with the formation of ES_2 diminishes. Strikingly, when chloride is omitted altogether from the reaction mixture (Figure 6B), the full steady-state ES concentration is reached during mixing and no second step is observed. Thus, it appears that the formation of ES_2 is associated with chloride activation.

While the detailed nature of the slow step cannot yet be defined, it seems unlikely that it corresponds to any of the chloride-dependent steps in Scheme II (see above), the simple mechanism suggested by steady-state kinetics. It cannot represent binding of chloride to free enzyme since identical fluorescence traces are obtained whether chloride is present in the enzyme solution or only in the substrate solution prior to mixing. It does not reflect slow binding of S to $E{\cdot}Cl^-$ since the extent of fast binding during mixing (in this case due to the step E + S $\rightarrow$ ES) does not decrease as the chloride concentration increases. Finally, the slow step does not represent binding of chloride to ES since this would produce a linear dependence of k_{obsd} on [Cl^-]. Our data show a markedly nonlinear relationship between these two variables. Further, this pathway would essentially be eliminated at low substrate and high chloride concentrations. In contrast, at [S] $<<$ K_M and with chloride saturating (with respect to k_{cat}/K_M), the slow process is clearly observed.

These considerations suggest that Scheme II must be expanded in order to account for the slow step. An extended scheme that is consistent with the data is shown in Scheme III. Here, the slow step reflects conversion of the initially formed $(E{\cdot}Cl^-{\cdot}S)_1$ to $(E{\cdot}Cl^-{\cdot}S)_2$ in which S is more tightly bound to the enzyme. Such a two-step substrate binding process is closely analogous to the two-step mechanism described previously for tight-binding ACE inhibitors, including enalaprilat and captopril (Shapiro & Riordan, 1984a,b; Bull et al., 1985), i.e., E + I $\rightleftharpoons$ EI $\rightleftharpoons$ EI*. In this mechanism, the rapid formation of one complex (EI) is followed by a slower "isomerization" to form the tighter complex EI*. Significantly, this two-step process is observed only in the presence of chloride (Shapiro et al., 1984b). When chloride is omitted, only the single complex EI can be detected. Thus, the mechanism by which chloride enhances the activity of ACE toward substrates may be closely related to that by which chloride modulates inhibitor binding.

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REFERENCES

Auld, D. S. (1977) in *Bioorganic Chemistry* (Van Tamelin, E. E., Ed.) Vol. 1, pp 1–17, Academic Press, New York.

Auld, D. S., & Holmquist, B. (1974) *Biochemistry 13*, 4355–4361.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. C. (1984) *J. Chromatogr. 336*, 93–104.

Bull, H. G., Thornberry, N. A., Cordes, M. H. J., Patchett, A. A., & Cordes, E. H. (1985) *J. Biol. Chem. 260*, 2952–2962.

Bünning, P., & Riordan, J. F. (1981) *Isr. J. Chem. 21*, 43–47.

Bünning, P., & Riordan, J. F. (1983) *Biochemistry 22*, 110–116.

Bünning, P., Holmquist, B., & Riordan, J. F. (1983) *Biochemistry 22*, 103–110.

Cushman, D. W., Pluscec, J., Williams, N. J., Weaver, E. R., Sabo, E. F., Kocy, O., Cheung, H. S., & Ondetti, M. A. (1973) *Experientia 29*, 1032–1035.

Galdes, A., Auld, D. S., & Vallee, B. L. (1983) *Biochemistry 22*, 1888–1898.

Hanahan, D., & Auld, D. S. (1980) *Anal. Biochem. 108*, 86–95.

Holmquist, B., Bünning, P., & Riordan, J. F. (1979) *Anal. Biochem. 95*, 540–548.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lobb, R. R., & Auld, D. S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2684–2688.

Lobb, R. R., & Auld, D. S. (1980) *Biochemistry 19*, 5297–5302.

Lobb, R. R., & Auld, D. S. (1984) *Experientia 40*, 1197–1206.

Pantoliano, M. W., Holmquist, B., & Riordan, J. F. (1984) *Biochemistry 23*, 1037–1042.

Riordan, J. F., Bünning, P., & Holmquist, B. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 23*, 2214.

Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.

Shapiro, R., & Riordan, J. F. (1983) *Biochemistry 22*, 5315–5321.

Shapiro, R., & Riordan, J. F. (1984a) *Biochemistry 23*, 5225–5233.

Shapiro, R., & Riordan, J. F. (1984b) *Biochemistry 23*, 5234–5240.

Shapiro, R., Holmquist, B., & Riordan, J. F. (1983) *Biochemistry 22*, 3850–3857.

Skeggs, I. T., Marsh, W. H., Kahn, J. R., & Shumway, N. P. (1954) *J. Exp. Med. 99*, 275–282.

Soffer, R. L. (1976) *Annu. Rev. Biochem. 45*, 73–94.

Williams, A. C., & Auld, D. S. (1986) *Biochemistry 25*, 94–100.