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Kinetics of Slow, Tight-Binding Inhibitors of Angiotensin Converting Enzyme†

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ABSTRACT: Five phosphorus-containing inhibitors of angiotensin converting enzyme were found to exhibit slow, tight-binding kinetics by using furanacryloyl-L-phenylalanylglycylglycine as substrate at pH 7.50 and $T = 25$ °C. Two of the inhibitors, (*O*-ethylphospho)-Ala-Pro (**2**) and (*O*-isopropylphospho)-Ala-Pro (**3**), are found to follow at minimum a two-step mechanism of binding (mechanism B) to the enzyme. This mechanism consists of an initial fast formation of a weaker enzyme–inhibitor complex (K_i = 130 nM for **2** and 180 nM for **3**) followed by a slow reversible isomerization to a tighter complex with measurable forward (k_3) and reverse (k_4) rate constants ($k_3 = 4.5 \times 10^{-2}$ s^{-1} for **2** and 5.4×10^{-2} s^{-1} for **3**; $k_4 = 9.2 \times 10^{-3}$ s^{-1} for **2** and 3.5×10^{-3} s^{-1} for **3**). For the remaining three inhibitors, phospho-Ala-Pro (**1**), (*O*-benzylphospho)-Ala-Pro (**4**), and (*P*-phenethylphosphono)-Ala-Pro (**5**), a one-step binding mechanism (mechanism A) is observed under the conditions of the experiment. The second-order rate constants k_1 (M^{-1} s^{-1}) for the binding of these inhibitors to converting enzyme are found to have values more than 3 orders of magnitude lower than the diffusion-controlled limit for a bimolecular reaction involving the enzyme, viz., 3.9×10^5 for **1**, 2.2×10^5 for **4**, and 4.8×10^5 for **5**. The rate constants, k_2, for the dissociation of the enzyme–inhibitor complexes following this mechanism are 5.1×10^{-3} s^{-1} for **1**, 2.0×10^{-3} s^{-1} for **4**, and 5.3×10^{-5} s^{-1} for **5**. The overall dissociation constants for these inhibitors as determined from Henderson plots are 13 nM for **1**, 22 nM for **2**, 11 nM for **3**, 9.2 nM for **4**, and 0.11 nM for **5**.

The reversible, slow, tight-binding inhibition of enzymes has been reviewed recently (Williams & Morrison, 1979; Morrison, 1982). A reversible, tight-binding inhibitor is one that reversibly inhibits the enzyme-catalyzed reaction at concentrations comparable to that of the enzyme either due to low K_i value or due to high concentrations of enzyme necessary for low turnover number enzyme–substrate systems or due to both. The classical Michaelis–Menten equation cannot be used to determine the potency of such inhibitors because an assumption is made in the derivation of this equation that the depletion

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of the inhibitor due to its binding with the enzyme is negligible, and this is generally not true for tight-binding inhibitors under the usual experimental conditions. Alternate equations that take into account the depletion of the inhibitor were developed by using steady-state theory (Morrison, 1969). The kinetic theory for tight-binding inhibitors due to Morrison was extended to slow, tight-binding inhibitors, i.e., for situations where the rate of establishment of equilibrium between the free enzyme, tight-binding inhibitor, and enzyme–inhibitor complexes is slow relative to the rate of catalytic reaction (Cha, 1975, 1976a,b).

Tight-binding inhibitors very often appear slow-binding solely due to the necessarily low concentrations employed for such inhibitors (Williams & Morrison, 1979; Cha, 1975). However, any inhibitors including tight-binding ones may also be slow-binding due to a low value for the overall rate constant for dissociation of enzyme–inhibitor complex(es), a low value for the overall second-order constant for the formation of such complex(es), or to a combination of both. In such a case it is instructive to try to find a detailed molecular basis for the occurrence of such low values for the rate constants.

Angiotensin converting enzyme is a dipeptidyl carboxypeptidase (EC 3.4.15.1) that catalyzes the hydrolysis of the carboxy-terminal dipeptide histidylleucine from the decapeptide angiotensin I to produce the pressor octapeptide angiotensin II. Two potent inhibitors of this enzyme, captopril and enalaprilat (MK-422), are orally active antihypertensive agents in man (Gavras et al., 1981; Petrillo & Ondetti, 1982) and have been shown to be slow-binding inhibitors (Shapiro & Riordan, 1984; Bull et al., 1984). These two inhibitors are slow-binding not simply because of the very low values of their dissociation constants but due to the small values for apparent rate constants for binding and dissociation of the inhibitors with the enzyme. In fact, instead of simple bimolecular formation of enzyme–inhibitor complex(es), the binding reaction was found to follow at minimum a two-step mechanism.

In the work presented in this paper five more inhibitors of angiotensin converting enzyme, which are about as potent as the two mentioned above, are also found to be slow-binding. Two different mechanisms of binding of these inhibitors to the enzyme are discussed in the context of processes that may be involved in the binding of tight-binding inhibitors.

Materials and Methods

Chemicals. Furanacryloyl-L-phenylalanylglycylglycine (FAPGG)[1] and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co. Phospho-Ala-Pro (**1**), (*O*-ethylphospho)-Ala-Pro (**2**), (*O*-isopropylphospho)-Ala-Pro (**3**) (Galardy & Grobelny, 1985), (*O*-benzylphospho)-Ala-Pro (**4**) (Galardy, 1982), and (*P*-phenethylphosphono)-Ala-Pro (**5**) (Galardy et al., 1983) were available from previous studies. All amino acids in the inhibitors are the L stereoisomers.

Enzyme. Angiotensin converting enzyme was purified from rabbit lungs by affinity chromatography exactly as described previously (Pantoliano et al., 1984). The pure enzyme had a specific activity of 78 units/mg compared to the reported 82 units/mg. Since large amounts of enzyme were required in many of the kinetic experiments, partially purified enzyme was frequently used. This enzyme was from the ammonium sulfate fractionation step of Pantoliano et al. (1984) and had a specific activity of 0.4 unit/mg (compared to the reported 0.56 unit/mg). This enzyme exhibited slow-binding kinetics identical with those observed with pure enzyme with (*O*-benzylphospho)-Ala-Pro.

[1] Abbreviations: FAPGG, furanacryloyl-L-phenylalanylglycylglycine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; K_m, Michaelis constant for FAPGG hydrolysis by angiotensin converting enzyme (303 μM).

Table I: Range of Inhibitor Concentrations Used to Obtain k_{obsd} Values for Various Inhibitors[a]

inhibitor	concn of inhibitor (nM)
$-O_2P(O)$AlaPro (**1**)	43–220
$-O(C_2H_5O)P(O)$AlaPro (**2**)	230–1100
$-O(i\text{-}C_3H_7O)P(O)$AlaPro (**3**)	460–3000
$-O(PhCH_2O)P(O)$AlaPro (**4**)	90–470
$-O(PhCH_2CH_2)P(O)$AlaPro (**5**)	40–320

[a] The concentration of substrate (FAPGG, K_m = 303 μM) was 1 mM in all cases; the concentration of enzyme was less than 10% of the concentration of both inhibitor and the substrate (see text for more details).

Kinetic Analysis. The slow-binding kinetics was studied by the method employed by Shapiro and Riordan (1984). In a typical experiment a 2-mL cuvette (1 cm path length) containing FAPGG (1 mM) and various amounts of inhibitor in HEPES buffer (50 mM, pH 7.50, 300 mM in NaCl) was incubated at 25 °C for about 15–20 min inside the spectrophotometer. The reaction was initiated by the addition of the enzyme, and the rate of hydrolysis of the substrate was monitored by observing the decrease in absorbance at 343 nm. The initiation of recording of the absorbance change always took place in less than 15 s after addition of the enzyme to the mixture of substrate and the inhibitor. Progress curves were recorded until steady-state rates were obtained. The pseudo-first-order rate constants for the decrease in rate of hydrolysis of the substrate to the steady-state velocity were determined by plotting ln $(v - v_s)$ vs. time, where v and v_s are reaction rates at time t and at the steady state, respectively.

The concentration of the enzyme was adjusted such that the steady state was reached before 10% of the substrate was hydrolyzed so that the depletion of the latter during the pre-steady state was not significant, maximizing at the same time the *difference* between v_0 and v_s. Increasing the enzyme concentration does not change the ratio of v_0 and v_s for a reversible, competitive inhibitor but it does increase the value of $(v_0 - v_s)$. The concentration of enzyme was at least 10-fold lower than that of either the substrate or the inhibitor in every kinetic run. Only under these conditions may a pseudo-first-order decrease in velocity to the steady state be assumed (Cha, 1976b; Morrison, 1982). The ranges of inhibitor concentration used are given in Table I. The lower limit to usable inhibitor concentration was set to ensure that the steady state is achieved before 10% of the substrate is hydrolyzed (the concentration of substrate used in all runs being 1 mM) at conveniently high enzyme concentrations. The highest usable concentration is limited by the usual problem associated with the conventional mixing of reagents; i.e., only those first-order decays with half-lives of more than 15–20 s can be studied conveniently. As the concentration of inhibitor was raised without varying the substrate concentration, the amount of enzyme added was also increased so that a sufficient amount of substrate (but not more than 10% of the original amount) was hydrolyzed before the steady-state rates were obtained.

The k_{obsd}'s were determined three times for each inhibitor concentration for all inhibitors, and the average of these three k_{obsd}'s was used for further analysis. In general, the values of k_{obsd} and the overall dissociation constant for any given inhibitor were reproducible to within 10% of the average value. The calculated values of other rate and equilibrium constants are averages of at least three independent experiments, the

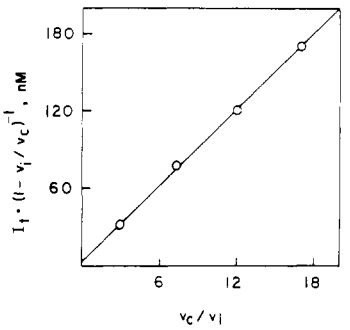

FIGURE 1: Determination of the overall dissociation constant, K_i, for (*O*-benzylphospho)-Ala-Pro (**4**) using a Henderson plot. Assays were initiated by the addition of 20 μL of 5 mM FAPGG in the HEPES buffer (50 mM, pH 7.50) containing 300 mM NaCl to a preincubated (at least 20 min at 25 °C) mixture (1.98 mL) of inhibitor and ACE in the same buffer. Enzyme concentration was equal to 3 nM. I_t is the total inhibitor concentration in nM; v_c is the steady-state velocity in the absence of the inhibitor; v_i is the steady-state velocity in the presence of inhibitor at concentration I_t. The slope of the straight-line plot yields the value of K_i under the conditions of this experiment, i.e., when [S] is very much smaller than the K_m value.

results of which were distributed within a range of 15–20% about the average.

The stock solution of phospho-Ala-Pro (**1**) was prepared in a small quantity of water so that the pH was greater than 10, and the solution was kept in ice and used within 2 h. The serial dilutions of the stock solution were made in buffer just before each kinetic run. This phosphopeptide is easily dephosphorylated at neutral pH at room temperature with a half-life of around 1–2 h (Poncz et al., 1984).

All of the peptides used are slow, tight-binding inhibitors. Traditional steady-state analysis to determine the overall dissociation constant (K_i for mechanism A and K_i^* for mechanism B) for the enzyme–inhibitor complexes cannot be employed since the concentration of unbound inhibitor is significantly different from the total inhibitor concentration. The Henderson plot (Figure 1) was therefore used to determine the overall dissociation constant (Henderson, 1972). For a typical Henderson plot, inhibitor was incubated with the enzyme in a 2-mL cell (1 cm path length) in the spectrophotometer cell compartment at 25 °C for about 20–60 min. The concentrations of inhibitor used were such that equilibrium between enzyme, inhibitor, and enzyme–inhibitor complexes was achieved within this incubation time. This time can be easily calculated from approximate values for kinetic and equilibrium constants involved in the binding of enzyme to the inhibitor as obtained from preliminary slow-binding experiments. To the incubated solution containing both the enzyme and the inhibitor was added a small quantity (1% of the final volume of the reaction mixture) of a concentrated solution of substrate such that its final concentration after dilution in the reaction mixture was 50 μM. The hydrolysis of the substrate was monitored at 328 nm. In every case the steady-state velocity was obtained almost instantaneously as expected since the already established equilibrium between the enzyme, inhibitor, and all forms of enzyme–inhibitor complexes is not expected to be shifted significantly because of substrate binding. The fraction of enzyme bound to the substrate is very small compared to that bound to the inhibitor; i.e., the [I]/(overall dissociation constant) value is much larger than the $[S]/K_m$ value. These steady-state velocities were used to obtain the Henderson plot. The slope of the straight-line plot gives the value of the overall dissociation constant when the value of [S] is smaller than the K_m value, and the intercept on the ordinate is that of enzyme concentration.

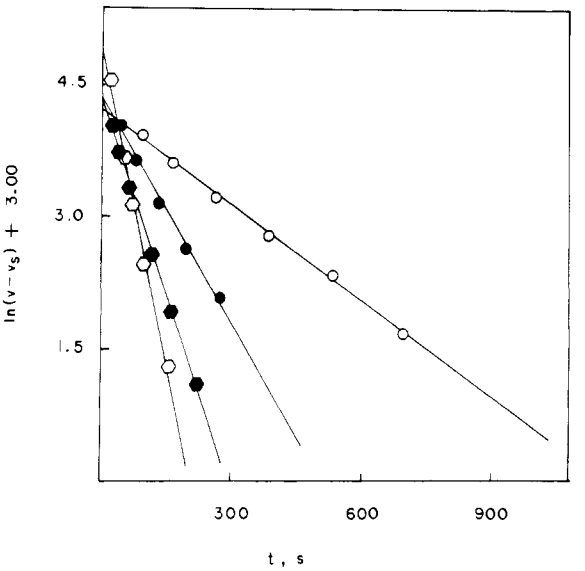

FIGURE 2: Determination of pseudo-first-order rate constants, k_{obsd}'s, at 25 °C for the approach to equilibrium between converting enzyme, the inhibitor (*P*-phenethylphosphono)-Ala-Pro (**5**) and enzyme–inhibitor complex(es) in the presence of substrate (FAPGG) at various inhibitor concentrations. Concentrations of inhibitor were 40 (○), 110 (●), 180 (●), and 320 (○) nM. The constant, 3.00, used in the ordinate is arbitrary and is used for graphical purposes only. Slopes of the straight-line plots yield the rate constants (k_{obsd}) at various inhibitor concentrations.

RESULTS

The kinetic analysis of data was carried out by the method of Shapiro and Riordan (1984), which is an adaptation of methods developed by Cha (1975, 1976a,b), Strickland et al. (1975), Williams and Morrison (1979), and Williams et al. (1979). All the equations used are from these sources. The analytical method is briefly described here. The approximation made in these methods is that the steady state can be assumed for a reaction between substrate and enzyme but the binding reaction of inhibitor to the enzyme is in the presteady state for a time usually in the range of minutes depending upon the concentrations of the substrate, inhibitor, and enzyme. It is assumed that the difference between the presteady-state rate and the steady-state decreases (when the reaction is initiated by the addition of the enzyme to the mixture of substrate and inhibitor) exponentially with an observed first-order rate constant, k_{obsd}

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

where v = the pre-steady-state rate at time t, v_0 = the initial pre-steady-state rate at $t = 0$, and v_s = the steady-state rate.

The k_{obsd}'s are the slopes of straight lines obtained by plotting ln $(v - v_s)$ vs. time (Figure 2). The values of k_{obsd} were found to increase with concentrations of the inhibitor. Two types of slow-binding inhibition mechanism (mechanism A and mechanism B) are consistent with this observed dependence of k_{obsd} values on inhibitor concentration (Shapiro & Riordan, 1984).

mechanism A

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \quad (2)$$

mechanism B

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^* \quad (3)$$

The approximate expressions for the k_{obsd}'s are

$$k_{obsd} = k_2 + k_1[I]/(1 + [S]/K_m) \quad \text{for mechanism A} \quad (4)$$

$$k_{obsd} = k_4 + k_3[I]/([I] + K'_i) \quad \text{for mechanism B} \quad (5)$$

where

$$K'_i = K_i (1 + [S]/K_m) \quad (6)$$

and

$$K_i = k_2/k_1 \text{ (mechanism B)} \quad (7)$$

In mechanism A, the equilibrium between E, I, and EI is reached slowly. In mechanism B the equilibrium between E, I, and an initial EI complex is attained rapidly but this EI complex subsequently isomerizes to a tighter EI* complex. The rate at which the EI* complex comes to equilibrium with EI, or with E and I, is, however, slow, and this is responsible for the slow onset of inhibition by mechanism B. It is to be noted that in practice the two steps in mechanism B may appear as just one step as in mechanism A if the initial complex, EI, is much weaker than the slowly formed but tigher EI* complex in mechanism B and the concentration of the inhibitor is varied in the region lower than the K_i value (Morrison, 1982). This can be verified by neglecting [I] in comparison with the K'_i value in eq 5 (mechanism B). Equation 5 will now have the same form as eq 4, which is the expression for k_{obsd} in mechanism A.

The approximate values for the first-order rate constants, k_x, for the reverse reaction of the slow step (k_x is k_2 for mechanism A and k_4 for mechanism B) can be calculated by using the relationship

$$k_x = k_{obsd} \cdot v_s/v_0 \quad (8)$$

where v_s is the steady-state velocity and v_0 is the initial, pre-steady-state velocity.

In many cases it is difficult to determine the value of v_0 directly. v_0 can be approximated by extrapolating the plot of ln $(v - v_s)$ vs. t back to time $t = 0$. As also noted by Shapiro and Riordan (1984), the rate for the first minute or less was a little faster than expected on the basis of a nearly first-order decay of $(v - v_s)$ as evidenced by an otherwise linear plot of ln $(v - v_s)$ vs. t (not shown in Figure 2). This departure from first-order decay seems to be especially apparent at low inhibitor concentrations.

The average values of $(k_{obsd} - k_x)$ for experiments run in triplicate at each inhibitor concentration were calculated. Then the plot of $1/(k_{obsd} - k_x)$ vs. $1/[I]$ yielded straight lines with definite intercepts for (*O*-ethylphospho)-Ala-Pro (**2**) and (*O*-isopropylphospho)-Ala-Pro (**3**) (Figure 3b). For the remaining inhibitors the lines pass through the origin (Figure 3a). For **2** and **3**, which clearly follow mechanism B, k_3 and K_i can be calculated from the $1/(k_{obsd} - k_x)$ and $1/[I]$ intercepts, respectively (see eq 5–7), where k_3 is the first-order rate constant for isomerization of the weak EI complex to the tighter EI* complex and K_i is the dissociation constant of the weak EI complex.

More accurate values of k_4 can be obtained from the relationship

$$K_i^* = K_i k_4/(k_3 + k_4) \quad (9)$$

where K_i^* is the overall inhibition constant obtained from a Henderson plot, which actually is a measure of the degree of dissociation of all forms of enzyme–inhibitor complexes into E and I, i.e.

$$K_i^* = \frac{[E][I]}{[EI + EI^* + ...]} \quad (10)$$

For phospho-Ala-Pro (**1**), *O*-benzylphospho-Ala-Pro (**4**), and (*P*-phenethylphosphono)-Ala-Pro (**5**), linear plots of $1/(k_{obsd} - k_x)$ vs. $1/[I]$ passed too close to the origin to be justifiably classified as following mechanism B. For these three inhibitors, a plot of $(k_{obsd} - k_x)$ vs. [I] yields a straight line passing through the origin, the slope of the straight line being equal to $k_1/(1 + [S]/K_m)$ from which the values of k_1 can be obtained with [S] = 1 mM and K_m = 300 μM. (See Figure 4 and eq 4 and 8 for mechanism A).

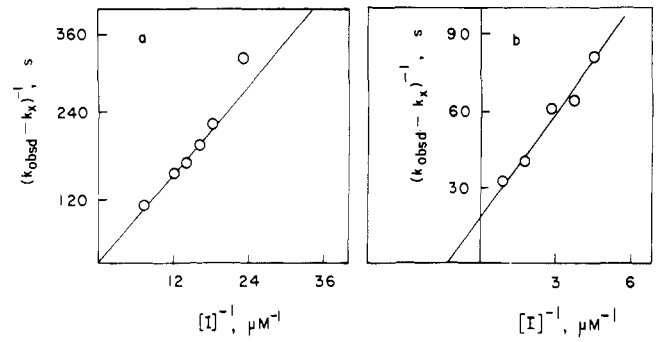

FIGURE 3: Reciprocal relationship of $(k_{obsd} - k_x)$ values and inhibitor concentration for phospho-Ala-Pro (**1**) and for (*O*-ethylphospho)-Ala-Pro (**2**) (parts a and b, respectively). The values of k_{obsd} were obtained from plots similar to those shown in Figure 2. Note that the straight-line plot passes through the origin in plot a but not in plot b.

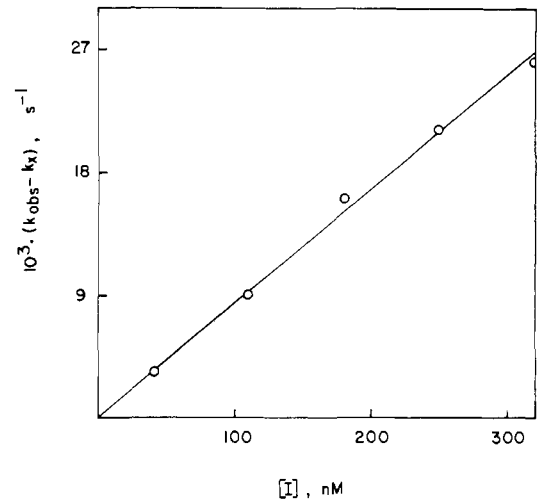

FIGURE 4: Direct relationship of $(k_{obsd} - k_x)$ and [I] observed for (*P*-phenethylphosphono)-Ala-Pro (**5**). The data are obtained from Figure 2 (the straight line in Figure 2 corresponding to the data point at 250 nM in the present figure has been omitted for a better presentation of the graph).

K_i for mechanism A is given by the expression

$$K_i = k_2/k_1 \quad (11)$$

Hence, an accurate value of k_2 was obtained from this k_1 and K_i determined from a Henderson plot. The values of K_i and k_1 and the value of k_2 obtained from these two constants are given in Table II. Included also in this table are the apparent values of k_1 and k_2 for the reaction

$$E + I \underset{k_2(app)}{\overset{k_1(app)}{\rightleftharpoons}} EI^* \quad (12)$$

where EI* is the enzyme–inhibitor complex slowly formed via a rapidly formed but weaker EI complex (mechanism B). Only inhibitors **2** and **3** clearly follow this two-step mechanism, and apparent values of k_1 and k_2 for these two inhibitors are therefore obtained from known values (from Table III) of k_3, k_4, and K_i (k_1(app) = k_3/K_i and k_2(app) = k_4).

All of the kinetic and equilibrium constants can be obtained from slow-binding studies alone or from slow-binding experiments together with dissociation constants determined from

Table II: Kinetic and Equilibrium Constants Associated with Formation of the Final Enzyme–Inhibitor Complex from Free Enzyme and Free Inhibitor by Mechanism A or Mechanism B

inhibitor	k_1 (M^{-1} s^{-1})	k_2 (s^{-1})	K_i or K_i^* (nM)[c]
1[a]	3.9×10^5	5.1×10^{-3}	13
2[b]	3.5×10^5	9.2×10^{-3}	22
3[b]	3.0×10^5	3.5×10^{-3}	11
4[a]	2.2×10^5	2.0×10^{-3}	9.2
5[a]	4.8×10^5	5.3×10^{-5}	0.11
captopril[b]	1.2×10^6	4.0×10^{-4}	0.33
enalaprilat[b]	2.1×10^6	1.1×10^{-4}	0.050

[a] These inhibitors appear to follow mechanism A under the conditions of our experiment (see eq 2 and 11). [b] For inhibitors **2**, **3**, captopril, and enalaprilat, clearly following the two-step mechanism B, k_1 is the apparent rate constant of formation from $k_1(\text{app}) = k_3/K_i$ and k_2 is the apparent rate constant of dissociation into free enzyme and inhibitor ($k_2(\text{app}) = k_4$) of slowly formed EI* complex (see eq 9 and 12). The k_3, k_4 and K_i, and K_i^* values were obtained from Table III. [c] K_i represents the overall dissociation constant for inhibitors following mechanism A and K_i^*, for those following mechanism B (see eq 9–11).

Table III: Kinetic and Equilibrium Constants, Calculated from both k_{obsd} Data and the Values of the Slopes of Henderson Plots, for the Inhibitors Clearly Following Mechanism B

inhibitor	K_i (nM)	k_3 (s^{-1})	k_4 (s^{-1})	K_i^* (nM)	k_3/k_4
2	130	4.5×10^{-2}	9.2×10^{-3}	22	4.9
3	180	5.4×10^{-2}	3.5×10^{-3}	11	15
captopril[a]	47	5.6×10^{-2}	4.0×10^{-4}	0.33	140
enalaprilat[a]	9.2	1.9×10^{-2}	1.1×10^{-4}	0.050	170

[a] Shapiro and Riordan (1984).

Table IV: Kinetic Constants Calculated from k_{obsd} Data Alone (the Slow-Binding Data) for the Interaction of Three of the Inhibitors with the Enzyme Appearing To Follow Mechanism A

inhibitor	k_1[a] (M^{-1} s^{-1})	k_2[b] (s^{-1})	K_i (nM)
1	3.9×10^5	8.6×10^{-4}	2.2
4	2.2×10^5	1.7×10^{-3}	7.7
5	3.6×10^5	1.4×10^{-4}	0.39

[a] Second-order rate constant for the formation of the EI complex from free enzyme E and free inhibitor I obtained from slope of plots like that shown in Figure 4. [b] First-order rate constant for the dissociation of the EI complex into free enzyme E and free inhibitor I. This is obtained from known values of k_1 (see footnote *a* above) and K_i (Henderson plot) by using the relationship $K_i = k_2/k_1$ for mechanism A.

Henderson plots. For example, for inhibitors **1**, **4**, and **5** the values of k_1 and k_2 (average of k_x) from the slow-binding studies alone and K_i obtained from these two rate constants are given in Table IV. The values of K_i and k_2 determined by these two procedures (compare Table II and Table IV) were in fair agreement with each other, confirming the validity of the assumption made in the analytical method used to determine the kinetic and equilibrium constants from the data.

Discussion

Among the five inhibitors of angiotensin converting enzyme employed in this study, (*O*-ethylphospho)-Ala-Pro (**2**) and (*O*-isopropylphospho)-Ala-Pro (**3**) were shown to follow at minimum a two-step mechanism of binding to the enzyme (mechanism B). The first step involves the rapid formation of an initial complex, which then slowly isomerizes in the second step to a tighter EI* complex with a forward rate constant of k_3 and a reverse isomerization rate constant of k_4. For these inhibitors the tighter complex, EI*, is about 5–15-fold more stable than the rapidly formed EI complex as indicated by k_3/k_4 values in Table III.

From the plots of $1/(k_{obsd} - k_x)$ vs. $1/[I]$ it was not possible to demonstrate that phospho-Ala-Pro (**1**), (*O*-benzylphospho)-Ala-Pro (**4**), and (*P*-phenethylphosphono)-Ala-Pro (**5**) follow mechanism B. These reciprocal plots pass through the origin (Figure 3a) even when quite high concentrations of inhibitors were used (Table I). The second-order rate constants, k_1, for the binding of these three inhibitors (**1**, **4**, and **5**) to converting enzyme are of the same order of magnitude within experimental error. The values are around 4×10^5 $M^{-1} \cdot s^{-1}$ (Table II), a value more than 3 orders of magnitude lower than the diffusion-controlled limit for a bimolecular reaction involving the enzyme (Fersht, 1984). While the unimolecular dissociation rate constants, k_2, of the enzyme–inhibitor complexes in mechanism A for (*O*-benzylphospho)-Ala-Pro (**4**) and phospho-Ala-Pro (**1**) are of the same order of magnitude (10^{-3} s^{-1}), the value for (*P*-phenethylphosphono)-Ala-Pro (**5**) is smaller by a factor of 100. This parallels the trend in K_i values of these inhibitors. The overall dissociation constant for **5** is much lower than those of the other inhibitors studied, and this lower value is mainly due to the very slow reverse-isomerization reaction.

It is possible that (*O*-benzylphospho)-Ala-Pro (**4**), (*P*-phenethylphosphono)-Ala-Pro (**5**), and phospho-Ala-Pro (**1**) may appear to follow a one-step mechanism due to a very high value for the dissociation constant of the initial EI complex in mechanism B relative to the overall inhibition constant. If this is so, the steady-state concentration of this initial EI complex is very small under the conditions of the experiment (a necessarily low concentration of inhibitor compared to the dissociation constant value for the initial complex), as compared to the free enzyme. Thus it may appear as if the final EI* complex is formed directly from free enzyme and free inhibitor instead of by the isomerization of an initial EI complex.

The lower value of k_2 for **5** relative to the values obtained for **1** and **4** is due to the much better binding interaction of **5** with the enzyme in the EI complex, probably because of favorable interaction of the phenyl group with the hydrophobic S_1 subsite on the enzyme in the EI complex. It is not clear however why the phenyl group in (*O*-benzylphospho)-Ala-Pro (**4**) is not as effective in the same mode. The only structural difference beween **4** and **5** is that a P to C bond in **5** is replaced by a P to O bond in **4**. The phosphorus atom in both is supposed to be mimicking the carbonyl carbon of the scissile peptide bond in the transition state for substrate hydrolysis. It is possible that **5** more nearly resembles the transition state for the hydrolysis, in terms of the bond lengths of affected bonds than does **4**, which does not have a long P–C bond that might be necessary to mimic the C–C bond adjacent to the scissile pepide bond in the transition state. On the other hand, the more strongly basic phosphinates are generally stronger inhibitors of converting enzyme than the more weakly basic phosphonates (Petrillo & Ondetti, 1982). Likewise phosphonamidates should be more strongly basic than phosphoramidates [pK_a's for methyl phosphate of 1.54 and 6.31 compared to those for phosphonomethane of 2.35 and 7.10 (Fasman, 1976)]. Thus the more basic phosphonamidate **5** is a better ligand for zinc and a better inhibitor than the phosphoramidate **4**.

It is well-known that monoanions, particularly chloride ions, strongly activate some converting enzyme catalyzed hydrolytic reactions (Skeggs et al., 1954; Cheung et al., 1980; Shapiro et al., 1983; Bunning & Riordan, 1983). Likewise inhibitor binding is also influenced by chloride ions as demonstrated by Shapiro & Riordan (1984) for a potent inhibitor of this enzyme, MK-422, which also follows at minimum a two-step binding reaction. By analyzing the effect of chloride ion concentration on rate and equilibrium constants of individual

steps in the reaction, these authors have concluded that chloride ion binding is not the rate-determining step in the formation of EI* from the EI complex though it may be involved in the stabilization of the former complex, resulting in slow reverse isomerization. An attempt was made to study the binding of (*P*-phenethylphosphono)-Ala-Pro (**5**) to converting enzyme with chloride ions at a concentration of about 2 μM. As a result the turnover number for FAPGG hydrolysis decreased dramatically, as expected, and the relative rate of binding of inhibitor was not slow any longer. For the same reason (low turnover) the overall dissociation constant, K_i, was determined from Henderson plots since the concentration of the enzyme used was comparable to that of inhibitor concentration. The value of K_i for **5** increased to 30 nM at this low chloride ion concentration as expected (Shapiro & Riordan, 1984).

The overall dissociation constant value of 110 pM as determined from Henderson plots for (*P*-phenethylphosphono)-Ala-Pro (**5**) is considerably less than the published value of 500 pM or more (Galardy et al., 1983, and references cited therein). The higher published value for the dissociation constant for **5** may be due to the fact that either the slow, tight-binding nature of this inhibitor or inhibitor depletion was not taken into consideration during these previous determinations. The inhibitor **5** therefore is a better inhibitor in vitro than the orally active hypertensive drug captopril (K_i^* = 330 pM) and only a slightly poorer inhibitor than MK-422 (K_i^* = 50 pM). Both captopril and MK-422 have been shown to follow mechanism B, and the dissociation constants of the weaker EI complexes for these two inhibitors were found to be less than 50 nM (Shapiro & Riordan, 1984). The values for k_3, the rate constant for isomerization of the EI to EI* complex in mechanism B for both captopril and MK-422, are of the same order of magnitude (10^{-2} s^{-1}) as for the two inhibitors **2** and **3**, which clearly followed mechanism B in our study. From the K_i, K_i^*, k_3, and k_4 data on captopril and MK-422 it is possible, as discussed before, to calculate the apparent values of k_1 and k_2 for formation and dissociation of the tighter EI* complex as in eq 12 for these two inhibitors. The values of k_1(app) are generally smaller for the inhibitors in our study compared to those of captopril and MK-422, which means that the ratio of K_i to k_3 (mechanism B) has a larger value for our inhibitors. The absolute values of K_i and k_3 are expected to be larger for (*P*-phenethylphosphono)-Ala-Pro (**5**) if is can be shown that it follows mechanism B too. This is one of the main reasons why mechanism B could be demonstrated for captopril and MK-422 and not **5**, which has a comparable overall dissociation constant. (The value of this ratio is proportional to the slope of the straight-line plot of $1/(k_{obsd} - k_4)$ vs. $1/[I]$.) The same kind of argument can be applied to inhibitors **1** and **4**.

Though the EI complex may be the usual Michaelis-type complex, the nature of the physical process that further stabilizes this complex for **2** and **3** is not very clear. It has been shown by a fluorescence quenching method that the slow binding of some transition-state-analogue inhibitors of adenosine deaminase is due to slow conformational change accompanying the formation of transition-state-like complexes (Kurz & Frieden, 1983; Kurz et al., 1985). The authors concluded that the same amount of conformational change occurred for all transition-state-analogue inhibitors studied although they differed by a factor of 10^6 in their overall dissociation constant values.

Slow binding of pepstatin, a powerful inhibitor of the aspartic proteinase pepsin, is argued to be due to the displacement of water in the initial weaker pepsin–pepstatin complex by the statine 3(*S*)-hydroxyl group (Rich, 1985). The slow-binding inhibition in our study does not seem to originate from slow transfer of the phosphoryl group of the inhibitors from the outer coordination sphere to the inner coordination sphere of Zn(II) by displacing one or two molecules of zinc-bound water since the first-order rate constant, k_3, for the movement of a ligand L from an outer-sphere complex with an aquated Zn^{2+} to the inner sphere is on the order of more than 10^7 s^{-1}, the rate constant for the formation of the outer-sphere complex typically being diffusion-controlled (Basolo & Pearson, 1967).

$$Zn(H_2O)_6^{2+} + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{\text{outer-sphere complex}}{Zn(H_2O)_6^{2+},L} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \underset{\text{inner-sphere complex}}{Zn(H_2O)_5L^{2+}} + H_2O \quad (13)$$

This value of k_3 is larger by a factor of around 10^8 or more than the k_3 value of isomerization of EI to EI* in our experiments with inhibitors **2** and **3**. It is to be noted however that the ligand environment around Zn(II) in angiotensin converting enzyme is probably much different from that around aquated zinc ion, which may cause the dissociation of a zinc-bound water molecule or other coordinated ligand in the enzyme to be slower than anticipated on the basis of water displacement rates on ordinary aquated ion.

In summary, five different potent transition-state-analogue inhibitors of angiotensin converting enzyme have been studied for their kinetic mechanism of binding to the enzyme. Two of the inhibitors, (*O*-ethylphospho)-Ala-Pro (**2**) and (*O*-isopropylphospho)-Ala-Pro (**3**), were found to follow at minimum a two-step mechanism of binding to the enzyme: the initial fast formation of a weaker enzyme–inhibitor complex followed by a slow isomerization of this to a tighter complex. The same mechanism, if followed at all, could not be demonstrated for phospho-Ala-Pro (**1**), (*O*-benzylphospho)-Ala-Pro (**4**), and (*P*-phenethylphosphono)-Ala-Pro (**5**). For these three inhibitors mechanism B appears to have degenerated into the one-step mechanism A under the conditions of the experiment. The overall dissociation constant of (*P*-phenethylphosphono)-Ala-Pro (**5**) has been redetermined to be 110 pM, a value that is considerably smaller than the one previously reported (500 pM). Kinetic and thermodynamic constants obtained for these five inhibitors are also compared with the values reported earlier for captopril and MK-422.

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Reaction Energetics of a Mutant Triosephosphate Isomerase in Which the Active-Site Glutamate Has Been Changed to Aspartate†

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ABSTRACT: The essential catalytic base at the active site of the glycolytic enzyme triosephosphate isomerase is the carboxylate group of Glu-165, which directly abstracts either the 1-*pro-R* proton of dihydroxyacetone phosphate or the 2-proton of (*R*)-glyceraldehyde 3-phosphate to yield the *cis*-enediol intermediate. Using the methods of site-directed mutagenesis, we have replaced Glu-165 by Asp. The three enzymes chicken isomerase from chicken muscle, wild-type chicken isomerase expressed in *Escherichia coli*, and mutant (Glu-165 to Asp) chicken isomerase expressed in *E. coli* have each been purified to homogeneity. The specific catalytic activities of the two wild-type isomerases are identical, while the specific activity of the mutant enzyme is reduced by a factor of about 1000. The observed kinetic differences do not derive from a change in mechanism in which the aspartate of the mutant enzyme acts as a general base through an intervening water molecule, because the D_2O solvent isotope effects and the stoichiometries of inactivation with bromohydroxyacetone phosphate are identical for the wild-type and mutant enzymes. Using the range of isotopic experiments that were used to delineate the free-energy profile of the wild-type chicken enzyme, we here derive the complete energetics of the reaction catalyzed by the mutant protein. Comparison of the reaction energetics for the wild-type and mutant isomerases shows that only the free energies of the transition states for the two enolization steps have been seriously affected. Each of the proton abstraction steps is about 1000-fold slower in the mutant enzyme. Evidently, the excision of a methylene group from the side chain of the essential glutamate has little effect on the free energies of the intermediate states but dramatically reduces the stabilities of the transition states for the chemical steps in the catalyzed reaction.

The opportunity to tinker with the amino acid sequence of enzymes by using the methods of site-directed mutagenesis is proving to be irresistible. Yet to avoid the aimless and the uninterpretable, the enzymologist must be prepared to analyze both the structural and functional consequences of an amino acid alteration in detail. The most informative first steps will therefore be taken with enzymes that are already well characterized in structural, mechanistic, and energetic terms. We report here the effects on the reaction energetics of a single change at the active site of such an enzyme, triosephosphate isomerase.

The three-dimensional structure of native triosephosphate isomerase is known to atomic resolution. Additionally, since the isomerase catalyzes the interconversion of one substrate and one product, the structure of a productive complex of the

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