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Synthesis and Evaluation of an Inhibitor of Carboxypeptidase A with a K_i Value in the Femtomolar Range†

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ABSTRACT: Comparative studies among a series of tripeptide phosphonate inhibitors of the zinc peptidase carboxypeptidase A indicate that incorporation of the phosphonic acid analogue of valine at the P_1 position results in significantly higher affinity than the glycine, alanine, or phenylalanine analogues. When applied to the tripeptide analogue Cbz-Phe-Val^P-(O)Phe [ZFV^P(O)F], determination of the inhibition constant K_i was complicated by the very slow rate of dissociation. The rate of exchange of [^{3}H]ZFV^P(O)F with enzyme-bound [^{14}C]ZFV^P(O)F was followed for periods of 3–4 months to measure dissociation rate constants in the range of (1.7–4.4) × 10^{-9} s^{-1}, corresponding to half-lives of 5–13 years. Although the on- and off-rate constants differ for different carboxypeptidase isozymes, their ratios, corresponding to the inhibition constants K_i, are consistently in the range of 10–27 fM. Both the inhibition constants and the dissociation rate constants appear to be the lowest values yet determined for an enzyme–small inhibitor interaction.

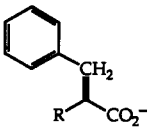
Considerable attention has been devoted to the synthesis of enzyme inhibitors for better understanding of enzyme mechanism, to probe the forces that bind small molecules to proteins, and for the purpose of drug design. The effectiveness of a molecule as a reversible enzyme inhibitor is gauged quantitatively, usually by the inhibition constant K_i but also by the rate constant for dissociation, k_{off} (Segel, 1975; Morrison & Walsh, 1987; Schloss, 1988a):

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{\cdot}I \qquad K_i = k_{off}/k_{on} = [E{\cdot}I]/[E][I] \quad (1)$$

As the strategies for inhibitor design are refined, the operational definitions of "potent inhibition" and "slow dissociation" change. In this respect, the evolution of carboxypeptidase A (CPA)[1] inhibitors is representative, as the partial list in Table I indicates. Although the progression of designs has not led monotonically to enhanced affinity, it is interesting to note that virtually every inhibitor in Table I was characterized as "potent" at the time it was reported.

As one of the prototypic zinc proteases, CPA occupies a prominent position in enzymology. It has been the subject of considerable scrutiny and ample debate in attempts to correlate structural information with mechanistic insights [e.g., Christianson and Lipscomb (1989)], and it has served as a model in the development of inhibition strategies that can be

Table I: Advancement in Inhibitor Design: Carboxypeptidase A



inhibitor (R)	K_i (nM)	reference
H	6200	Elkins-Kaufman and Neurath (1949)
$^-O_2CH_2$	450	Byers and Wolfenden (1973)
$^{2-}O_3PNH$	5000	Kam et al. (1979)
$HSCH_2$	11	Ondetti et al. (1979)
$^{2-}O_3PO$	140	Hofmann and Rottenberg (1980)
Cbz-Gly(PO_2^--NH)[a]	90	Jacobsen and Bartlett (1981)
O=$CHCH_2$	<480	Galardy and Kortylewicz (1984)
$^{2-}O_3PCH_2$	220	Grobelny et al. (1985)
$CF_3C(OH)_2CH_2$	200	Gelb et al. (1985)
Cbz-Phe-Ala-(PO_2^--O)[b]	0.001	Hanson et al. (1989)

[a] = ZG^{P}F. [b] = ZFA^P(O)F

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[1] Abbreviations: CPA, carboxypeptidase A; ZFV^P(O)F, the phosphonate ester analogue of Cbz-Phe-Val-Phe in which the Val-Phe peptide linkage has been replaced with PO_2^--O (related inhibitors are abbreviated similarly by using the single-letter amino acid code); dpm, decompositions per minute; SA, specific activity; LSC; liquid scintillation counting; fuaFF, furanacryloyl-L-phenylalanyl-L-phenylalanine.

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translated to zinc proteases of greater medicinal importance (see, for example, the references in Table I). CPA has served as a useful target for inhibitor design, because effects on binding affinity can be understood on a structural level as a result of the crystallographic studies from the Lipscomb group [Kim & Lipscomb, 1990; Christianson and Lipscomb (1989) and references therein].

In this report, we describe further steps in the investigation of phosphorus-containing peptide analogues as zinc protease inhibitors. We had earlier observed the influence of methyl and benzyl substitution, in comparison to hydrogen, at the P_1 position in a limited series of tripeptide phosphonates. For example, we found a significant increase in affinity from ZAG^P(O)F (K_i = 710 pM) to ZAF^P(O)F (K_i = 4 pM) or ZAA^P(O)F (K_i = 3 pM) (Hanson et al., 1989), consistent with the conformational constriction induced by the substituted amino acids but revealing no generalized steric or hydrophobic effect. However, a comparison of the mode of binding to CPA of the phosphonate transition-state analogue ZAA^P(O)F (Kim & Lipscomb, 1990) with that of the naturally occurring potato inhibitor (Rees & Lipscomb, 1982) drew our attention to the structural basis for selectivity at the S_1 binding site on the enzyme. The presence of a valine residue at the P_1 position of the potato inhibitor and consideration of the geometric characteristics of the S_1 site suggested that further discrimination in this region was possible. We therefore studied the effect of incorporating the phosphonic acid analogue of valine at the P_1 position in the phosphonate inhibitors. This investigation led to the determination of what we believe to be the lowest inhibition constant and slowest dissociation rate constant measured for a low molecular weight enzyme inhibitor.

Experimental Procedures

Synthesis of Inhibitors. The phosphonate tripeptide analogues were synthesized by methods described previously (Hanson et al., 1989). The radiolabeled forms of ZFV^P(O)F were prepared from L-[2,3,4,5,6-^{3}H]phenylalanine (Amersham or New England Nuclear, diluted to a specific activity of ca. 50000 dpm/nmol) and L-[U-^{14}C]phenylalanine (ICN, diluted to a specific activity of 400 dpm/nmol). Full experimental details and characterization are provided in the supplementary material.

Stock Solutions. All solutions were prepared with doubly distilled water and filtered (0.45-μM pore size) before use.

Buffer. The buffer for all experiments and stock solutions was 25 mM Tris and 0.5 M NaCl, pH = 7.5; for long-term incubations, 0.1 mg/mL bovine serum albumin was included in the buffer to stabilize CPA at low concentrations.

CPA. CPA-δ (Allan et al., 1964) was purchased from Sigma (catalog no. C-0386), CPA-γ (Anson, 1937) was purchased from Boehringer-Mannheim (catalog no. 103225), and a sample of CPA-α (Cox et al., 1964) was obtained as a gift from Dr. David S. Auld. Stock solutions were prepared as described previously (Hanson et al., 1989).

Substrate. A ca. 125 μM stock solution of furanacryloyl-L-phenylalanyl-L-phenylalanine (fuaFF, Sigma) was prepared by dissolving the solid material in buffer with stirring at 80 °C overnight. The solution was brought to pH = 7.5 by the addition of HCl and then stored in a brown glass container (loss of substrate, presumably through adherence to the wall, has been observed with Nalgene containers). Substrate activity was checked before each assay session by observing any decrease in absorbance at λ = 305.5 nm.

Inhibitors. Inhibitor amounts were determined gravimetrically and corrected for purity on the basis of phosphorus analysis. Stock solutions were prepared by dissolving the inhibitor in water and diluting into Tris/NaCl buffer to a concentration of 3–25 μM.

Table II: Inhibition of CPA by Tripeptide Phosphonates[a]

entry	inhibitor	k_{on} (10^5 M^{-1} s^{-1})	k_{off} (10^{-9} s^{-1})	K_i (pM)
1	BocAA^P(O)A[b]			646000
2	ZAA^P(O)A[b]			76000
3	ZFA^P(O)A[b]			56000
4	BocYA^P(O)A			24500
5	ZYA^P(O)A	5	(6000000)	12000
6	ZAG^P(O)F[b]	6.6	(470000)	710
7	BocYV^P(O)A	0.276	(11000)	370
8	ZAF^P(O)F[b]	2.0	800	4
9	ZAA^P(O)F[b]	2.1	750	3
10	ZFA^P(O)F[b]	2.2	220	1
11	ZFV^P(O)F-CPAδ	1.54 ● 0.06	1.6[c]	(0.011)
12	ZFV^P(O)F-CPAγ	1.60 ± 0.1	3.7[c]	(0.023)
13	ZFV^P(O)F-CPAα	2.12 ± 0.07	2.9	(0.014)

[a] Measured values, except values calculated from the relationship $K_i = k_{off}/k_{on}$ given in parentheses. [b] From Hanson et al. (1989). [c] Average of two experiments (Table III).

Enzyme Assays. Assays were performed at 25 °C with fuaFF as substrate. Product formation was monitored at λ = 330 nm ($\Delta\epsilon$ = 2100 M^{-1} cm^{-1}) and the data were evaluated by using HYPER+ (Cleland, 1979) or ENZFITTER (Leatherbarrow, 1987). Inhibition by Boc-YA^P(O)A and ZYA^P(O)A was measured by incubation of inhibitor and enzyme at 25 °C for 5 min prior to initiation of the reaction by addition of substrate. K_i values were determined from simple plots of v_o/v_i versus [I] at a substrate concentration equal to K_m.

Modifications in this procedure were made for BocYV^P-(O)A, because of its slower and tighter binding behavior. Enzyme and inhibitor were preincubated for 18 h to allow complex formation to proceed prior to addition of substrate; in turn, because of the slow off-rate ($t_{1/2}$ > 6 h), compensation for substrate binding could be neglected. Moreover, the K_i value was determined by the method of Henderson (1972), in order to correct for inhibitor depletion under the conditions of the assay ([E] = 0.15 nM, [I] = 0.36–1.0 nM). Complete details are provided in the supplementary material.

The inhibition constants of phosphonate inhibitors measured under steady state or equilibrium conditions are collected in Table II (entries 1–10).

Determination of Association Rate Constant (k_{on}) for ZFV^P(O)F Binding to CPA. k_{on}(app) was determined by observing the first-order decrease in enzyme activity at varying concentrations of enzyme and inhibitor: [fuaFF] = 65.5 μM, [CPA] = 61.8–742 pM, and [I] = 11.8–235 nM. The length of the assays depended on the concentration of enzyme and ranged from 8–120 min. k_{on} is calculated from a plot of k(app) vs [I]. Representative figures are presented in the supplementary material. In Table II are given the values measured for k_{on} for each of the preparations of CPA.

Assays of Radiolabeled Inhibitors. Liquid scintillation counting (LSC) was performed with an LKB 1209 Rackbeta Liquid Scintillation Counter interfaced with a Bios Model G terminal with UltroTerm II software using LKB Optiphase HI-SAFE II as cocktail. The ^{3}H and ^{14}C windows were set to minimize detection of ^{14}C in the ^{3}H channels; counting overlap was corrected by the quench curves internal to the instrument. Use of ^{14}C-labeled inhibitor of comparatively low specific activity also ensured a low degree of ^{3}H/^{14}C overlap: typical samples contained 1000 dpm of ^{14}C and 400–4000 dpm of ^{3}H.

Formation of the E·[^{14}C]I Complex. Samples of 1.2–2.4 mg of [^{14}C]I were dissolved in 500–550 μL of Tris/NaCl buffer. The concentrations of these solutions were determined

Table III: Biphasic Dissociation of ZFV^P(O)F from CPA Preparations

CPA sample[a]	expt	$k_{off}(1)$ (10^{-6} s^{-1})	$k_{off}(2)$ (10^{-9} s^{-1})	burst size (%)	$k_{off}(2)/k_{on} = K_i$ (fM)
CPS-δ	1	4.11 ± 0.47	1.67 ± 0.06	1.3	10.8
	2	2.62 ● 0.44	1.59 ± 0.10	1.3	10.3
CPA-γ	3	2.08 ± 0.28	4.36 ● 0.21	3.7	27.3
	4	0.94 ± 0.17	2.94 ± 0.42	4.5	17.8
CPA-α	5	2.92 ● 0.56	2.92 ± 0.21	1.7	13.8

[a] Enzyme sources described under Experimental Procedures.

from their specific activity: [I] = 5.18–7.61 mM. The E·[^{14}C]I complex was formed by incubation of a solution of inhibitor ([I] = 1.57–2.54 mM) and CPA ([E] = 291–333 μM) at 25 °C for 18 hours. In one experiment, incubation of CPA-δ (420 μM) and [^{14}C]I (22.8 mM) was allowed to proceed for 6 days. In another experiment, the CPA-δ preparation was incubated in the presence of 66 μM $ZnCl_2$ (ca. 0.1 mol equiv, based on total CPA) for 18 h prior to incubation with [^{14}C]-I. Each complex was separated from excess inhibitor by elution through a Bio-Rad Econopac 10DG size-exclusion gel column. Fractions were taken at 10-drop intervals (approximately 0.5 mL); fractions were monitored by LSC and A_{278} to identify those containing the E·I complex. The yield from the column was typically 86–95%, based on protein absorbance, and the stoichiometry of inhibitor binding, based on LSC, corresponded within 6–18% to the expected 1:1 complex.

Exchange of [^{3}H]I into the E·[^{14}C]I Complex. Solutions of tritiated inhibitor and of the E·[^{14}C]I complex were combined with buffer to a volume of 10 mL at the following initial concentrations: E·[^{14}C]I, 36.2–38.1 μM, and [^{3}H]I, 331–473 μM. At various time intervals, aliquots of 125–500 μL were removed from the incubation mixture, and the E·I complex was separated from free inhibitor by size-exclusion gel filtration with a Bio-Rad Econopac 10DG column. The eluant from these columns was collected in 10-drop fractions (approximately 0.5 mL), and each fraction was submitted in its entirety for LSC. Two scintillation vials containing the buffer were also submitted for LSC to measure the background radiation level. The counts from the E·I-containing fractions were added and the percent exchange was determined from

$$\% \text{ exchange} = \frac{\dfrac{\text{dpm of } ^{14}\text{C}}{\text{SA of } ^{14}\text{C}}}{\dfrac{\text{dpm of } ^{14}\text{C}}{\text{SA of } ^{14}\text{C}} + \dfrac{\text{dpm of } ^{3}\text{H}}{\text{SA of } ^{3}\text{H}}} \quad (2)$$

The percent exchange as a function of time was fit to a double-exponential equation by the least-squares method (Leatherbarrow, 1987) to determine the dissociation rate constants corresponding to each step of the biphasic exchange process. Representative curves are shown in Figure 1.

Dissociation of this inhibitor from the CPA complexes was biphasic, with between 1.3% and 4.5% of the ^{14}C-containing material dissociating with a first-order rate constant 3 orders of magnitude higher than the rest of the complex. The values of k_{off} for each preparation of CPA are presented in Table III, along with the calculated values of K_i. In comparison experiments in which ^{14}C-labeled inhibitor and CPA-δ were incubated for 18 h and 6 days prior to exchange with ^{3}H-labeled material, the initial phase of exchange comprised 2.2% and 1.6% of the initially bound [^{14}C]-labeled inhibitor, respectively. Incubation of a sample of CPA-δ with $ZnCl_2$ prior to formation of the E·[^{14}C]I complex had no effect on the biphasic exchange process.

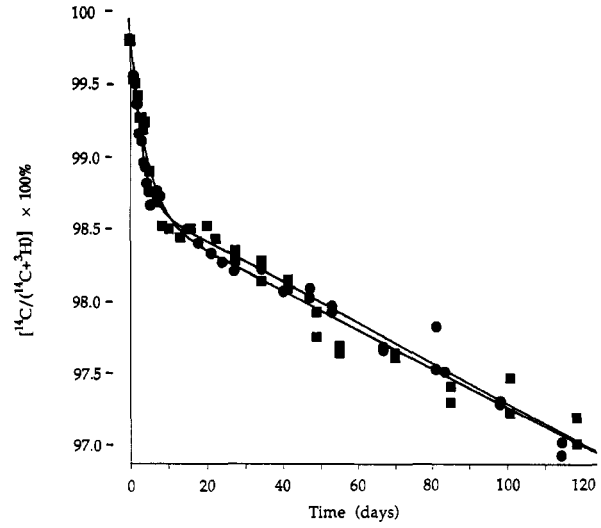

FIGURE 1: Exchange of labelled ZFV^P(O)F from CPA-δ complexes. Experiment 1 (■) and 2 (●); theoretical curves derived from fit of each data set separately to a double exponential.

Discussion

As more potent inhibitors are discovered, more sophisticated techniques must be utilized to determine their K_i values. These constants are typically determined under steady-state conditions in the presence of substrate, using the reduction in enzymatic activity as a measure of the position of the enzyme–inhibitor equilibrium. Such an approach cannot be used for inhibitors with inhibition constants in the subnanomolar range, since the requirements that a significant fraction of enzyme activity be present at equilibrium and that this equilibrium be attained prior to significant consumption of substrate cannot be satisifed simultaneously (Cha, 1975; Williams & Morrison, 1979). In these cases, it may still be possible to measure the equilibrium between enzyme and inhibitor directly, assaying for residual activity as this equilibrium is approached, since the degree of dissociation of the enzyme·inhibitor complex is not significant during the course of an individual assay. However, this approach is in turn complicated by the requirement for long-term stability on the part of the enzyme and the need for a very sensitive assay; this method also becomes impractical when the time required for equilibration, which depends on k_{off}, exceeds several weeks.

The remaining approaches to the determination of very low inhibition constants rely on the independent determination of the association and dissociation rate constants, k_{on} and k_{off}, respectively. If inhibition is reversible, the ratio of k_{off}/k_{on} can be taken as K_i. While k_{on} is most readily determined by measuring the rate of inactivation of the enzyme as a function of inhibitor concentration, methods for determining k_{off} vary. Dissociation or exchange of radiolabeled ligands has been used for some tight-binding ligands [e.g., Green (1963) and Schloss (1988b)]; monitoring the return of enzymatic activity on high dilution of the enzyme·inhibitor complex has even been used to determine a half-life for dissociation of 180 days (Bartlett & Marlowe, 1987).

For practical reasons, the effects of incorporating the P_2-tyrosine and the P_1-valine residues of the potato inhibitor in phosphonate analogues were first assessed with derivatives containing an alanine mimic at the C-terminal (P_1') position rather than the preferred phenylalanine. Comparisons of related inhibitors with alanine, phenylalanine, and tyrosine at P_2 indicated that relatively modest alterations in binding affinity are obtained on incorporating the tyrosine moiety

(compare entries 1 and 2 with 4 and 5, Table II). As analyzed in more detail in the accompanying paper (Kim & Lipscomb, 1991), this modest discrimination appears to result from the relatively exposed position of the P_2 side chain in the S_2 pocket.

In contrast, comparison of Boc-YV^P(O)A (entry 7) with Boc-YA^P(O)A (entry 4) reveals that valine at the P_1 position increases the affinity by nearly 2 orders of magnitude. Extrapolation of this effect[2] to analogues with phenylalanine at P_1' suggested that inhibition constants on the order of 15 fM would be observed, on the basis of the K_i value of 1 pM for ZFA^P(O)F (Hanson et al., 1989). It was clear that any approach to determination of such an inhibition constant could not depend on equilibrium techniques. Nor could recovery of enzymatic activity be monitored in order to determine the off-rate constant.

The magnitude of the challenge of determining the inhibition constant for ZFV^P(O)F was underscored by the finding that k_{on} is 1.54×10^5 M^{-1} s^{-1} for CPA-δ (experiment 1, Table III). While this rate constant represents slower binding to the enzyme than expected for a normal peptide substrate, it is not unusual for related phoshonate inhibitors of CPA or other zinc peptidases (Barlett & Marlowe, 1987; Hanson et al., 1989). This value was significant, however, since with an anticipated K_i value in the 15 fM range, a half-life for dissociation of more than 8 years could be predicted.

The slow rate of dissociation of the avidin–biotin complex ($k_{off} = 9 \times 10^{-8}$ s^{-1}) was determined by measuring the displacement of radiolabeled ligand by unlabeled material (Green, 1963). Recently, Schloss used this method in a double-labeled version to determine a dissociation rate constant of 1.5×10^{-8} s^{-1} and a K_i value of 190 fM for 4-carboxy-D-arabinitol 1,5-bisphosphate inhibition of ribulose-bisphosphate carboxylase (Schloss, 1988b). By following the displacement of one label from the enzyme·inhibitor complex by inhibitor containing another label of higher specific activity, very small amounts of exchange can be measured accurately. This method has the additional advantage that measurement of protein-bound radiolabels compensates for any nonspecific loss of inhibitor from the active site as a result of enzymatic degradation or denaturation or for variable recovery of protein-bound label in different aliquots (Schloss, 1988b).

Figure 1 depicts the progress of two such experiments with CPA-δ and ZFV^P(O)F monitored for a period of 4 months, during which time some 3% of the initial complex underwent exchange in each case. It reveals a biphasic process, with an inital "burst" corresponding to ca. 1.3% exchange ($t_{1/2}$ = 2–3 days) followed by a slower step with rate constants of $(1.67 \pm 0.06) \times 10^{-9}$ s^{-1} and $(1.59 \pm 0.10) \times 10^{-9}$ s^{-1} ($t_{1/2}$ =13 years). The slower phase, which is attributed to exchange of ZFV^P(O)F out of the majority of the enzyme and therefore represents the relevant value of k_{off}, leads to a calculated value for K_i of 11 fM, quite close to that predicted above.

Two explanations for the rapid exchange process were considered.[3] The CPA sample may contain a protein contaminant from which the inhibitor dissociates more rapidly. It would not be surprising for the enzyme to contain 1.3% of material that has lost the zinc atom or suffered partial proteolysis. Such material would not be observed during the on-rate experiments, and although attempts to discern such minor components by analytical chromatography or by native gel electrophoresis of an enzyme·inhibitor complex were not successful, these experiments do not preclude the possibility that different but closely related forms are present. To determine if demetallated apoenzyme is responsible for the burst, a sample of CPA was incubated with zinc chloride at a concentration corresponding to 0.1 mol equiv, i.e., in 5–10-fold excess to the "minor" component. No diminution of the biphasic exchange behavior was observed, indicating that the rapid exchange component does not involve the apoenzyme.

An alternative explanation would attribute the burst phase of dissociation to a contaminating form of the inhibitor, perhaps a truncated or epimerized compound that is bound in kinetic competition with ZFV^P(O)F during formation of the complex. Equilibration among inhibitor forms with off-rate constants less than 10^{-5} s^{-1} would not occur on overnight incubation, with the result that the distribution of bound species would reflect the product of their individual k_{on} values and their mole fraction in the mixture. A burst phase that results from inhibitor contamination should therefore be abolished if equilibration of the ^{14}C-labeled inhibitor with the enzyme is allowed to take place prior to separation of unbound inhibitor and initiation of the exchange with tritiated material. When CPA-δ and ^{14}C-labeled inhibitor were incubated for 6 days before exchange was initiated, corresponding to 2–3 half-lives of the observed burst phase, a reduction in the magnitude of the burst from 2.2% to 1.6% was observed. This reduction is considerably less than predicted, indicating that the more rapidly exchanged enzyme·inhibitor complex cannot be attributed to a contaminant in the inhibitor.

Similar initial bursts were also observed in the previously described exchange experiments. Both ligand contamination and partial protein denaturation were considered to explain the initial phase of rapid dissociation observed with the biotin–avidin system (Green, 1963); for ribulose-bisphosphate carboxylase inhibition, this behavior was ascribed to contamination of the tight-binding inhibitor with a weaker epimer (Schloss, 1988b).

The experiments depicted in Figure 1 were performed with the δ ("Allan") form of CPA (Allan et al., 1964), corresponding to residues 8–307. To explore the potential influence of protein structure on the association and dissociation pro-

[2] The expectation that side-chain effects would be approximately additive for CPA was based on the assumption that there is no significant interaction between the side chains in solution or in the E·I complex. The latter case is clearly supported by the structural work of Kim and Lipscomb (1990). Moreover, while there are few direct comparisons available, those that have been reported do show effects to be additive within an order of magnitude (Hanson et al., 1989; Abramowitz-Kurn & Schechter, 1974).

[3] A third possibility has been suggested; namely, that the biphasic exchange process reflects two forms of the E·I complex in equilibrium, according to the equation

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E^*{\cdot}I$$

with the minor component (E·I) responsible for the rapid phase of exchange and the major component (E*·I) responsible for the slow phase. Precedents for this kinetic mechanism are well established for a number of slow-binding enzyme inhibitors (Morrison & Walsh, 1987). However, in the present instance it can be ruled out by consideration of the two limiting cases. Case 1: $k_2 < k_3$. Equilibration of E·I and E*·I would be faster than dissociation of E·I and no burst would be observed. Case 2: $k_3 < k_2$. In this situation, isomerization of E*·I to E·I is rate limiting for dissociation in the slow phase and the measured off-rate constant (ca. 2×10^{-9} s^{-1}) would correspond closely to k_4. The observation that $[E^*{\cdot}I]/[E{\cdot}I] < 100$ and the equilibrium relationship $k_3[E{\cdot}I] = k_4[E^*{\cdot}I]$ then require that k_3 be less than 2×10^{-7} s^{-1}. However, if this were the case, $t_{1/2}$ for the conversion of E·I to E*·I would be more than 40 days and E*·I would never have been formed under the conditions of our experiments.

cesses, the exchange experiments were repeated with additional samples of CPA: the γ ("Anson") form (Anson, 1937; also corresponding to residues 8–307 but prepared in a different fashion) and a highly purified sample of the α ("Cox") form (Cox et al., 1964; residues 1–307). In each case, the k_{on} and k_{off} values were determined, and the magnitude of the dissociative burst was noted (Table III). It is clear that the kinetic aspects of the binding process differ in detail between the various forms and even preparations of the enzyme. Nonetheless, the calculated inhibition constants for each enzyme agree within a factor of 2 or 3, as would be expected for complexes that differ only at positions remote from the active site.

Over the 4-month period of some of the exchange experiments, during which time only 3–4% of the initially bound [^{14}C]I dissociated (relative to the sum of the bound [^{14}C]I plus bound [^{3}H]I), there was no decrease in total amount of bound inhibitor isolated in succeeding aliquots. This result indicates that there was no degradation of the protein and reflects a remarkable degree of stabilization of the protein by bound inhibitor.

The assumption that ZFV^{P}(O)F is a noncovalent, competitive inhibitor of CPA, and therefore that the ratio k_{off}/k_{on} can be taken as the measure of K_i, rested on its structural similarity with the related inhibitors of Table II, many of which have been shown to be competitive inhibitors by conventional steady-state kinetic measurements (Hanson et al., 1989). The structure determinations reported by Kim and Lipscomb for the CPA complexes of ZAA^{P}(O)F, ZAG^{P}(O)F, and ZFV^{P}-(O)F itself (Kim & Lipscomb, 1990, 1991) provide unequivocal evidence of the noncovalent nature of the binding interactions and the location of these inhibitors in the active site. Of greater concern with respect to the validity of the K_i values calculated for ZFV^{P}(O)F is the fact that the values of k_{on} and k_{off} were determined at considerably different inhibitor and protein concentrations: for the on-rate experiments, [CPA] = 62–740 pM and [I] = 12–235 nM; for the exchange experiments, [CPA·I] = 36–38 μM and [I] = 300–500 μM. Although the traditional mechanisms for inhibitor–enzyme equilibration involve a single inhibitor and a single enzyme molecule in the rate-determining steps, it is conceivable that exchange of bound inhibitors could proceed through a ternary complex. However, within the limited range explored, we did not observe any dependence of exchange rate on inhibitor concentration. Moreover, if the observed exchange rate reflects the intrusion of a nontraditional, associative mechanism, it would imply that the actual unimolecular dissociation rate constant, and therefore the K_i value, are even lower.[4]

To our knowledge, the K_i values of 10–20 fM for ZFV^{P}(O)F represent the lowest inhibition constants determined for any reversible, low molecular weight inhibitor, although even more tightly bound ones certainly exist. For example, a K_i value of ca. 10^{-19}–10^{-20} M has been projected for methionine sulfoximine phosphate as an inhibitor of glutamine synthase, on the basis of an extrapolated value for k_{off} (Maurizi & Ginsburg, 1982). A dissociation constant of 0.71 fM has been determined for the angiogenin·placental ribonuclease inhibitor complex, based on the ratio of dissociation and association rate constants (Lee et al., 1989). Although not an enzyme–inhibitor system, to date the tightest measured noncovalent dissociation constant for a protein–small molecule interaction is the 1 fM value of K_d referred to above for the avidin–biotin complex (Green, 1963).

The side-chain specificity at the P_1 position of CPA substrates has not been investigated extensively; among a series of (carbobenzoxy)dipeptides, substituted amino acids (Ala, Leu, Phe) are preferred to glycine, and the effect is manifested more strongly in k_{cat}/K_m (reflecting transition-state binding) than in K_m (indicative of ground-state binding) (Abramowitz-Kurn & Burger, 1974). However, anomalous binding has been observed for some glycine-containing dipeptide inhibitors (Christianson & Lipscomb, 1988; Christianson et al., 1987) and it is inferred for the corresponding substrates (Hanson et al., 1989). It is not surprising, therefore, that the modest incremental effect observed in a limited number of dipeptide substrates contrasts with the dramatic influence of alkyl substituents at the P_1 position of the tripeptide phosphonates (Table II). Comparison of ZAG^{P}(O)F with ZAA^{P}(O)F and of ZFA^{P}(O)F with ZFV^{P}(O)F reveals increases in binding energy of 3.3 and 2.8 kcal/mol, respectively. Incremental binding effects of this magnitude are significantly greater than expected from a general hydrophobic effect (Knowles, 1965; Fastrez & Fersht, 1973), and they approach those observed for highly specific systems such as the tRNA synthetases (Fersht et al., 1980). The observation that the phosphonate tripeptides are transition-state analogues (Hanson et al., 1989; A. P. Kaplan and P. A. Bartlett, unpublished results), i.e., that the binding affinity of phosphonate inhibitors and the rate of hydrolysis of the corresponding peptide substrates show the same dependence on side-chain variation, suggests that peptides containing valine at the P_1 position should be good CPA substrates.

In considering the possibility of carrying the quest for tight-binding inhibitors even further, we note that significant additional reductions in the K_i value of a CPA inhibitor can be predicted from the incorporation of Tyr in place of the P_2-Phe, and potentially by replacement of the phosphonate ester oxygen with a methylene group [cf. Morgan et al. (1991)]; these alterations could conceivably lead to inhibitors with *projected* K_i values in the attomolar (10^{-18} M) range. However, even with an exceedingly sensitive assay, it is not likely that the dissociation constant for such a phosphonate CPA inhibitor could be determined, in view of the anticipated off-rate. Indeed, we would suggest that the only practical way to devise a higher affinity CPA inhibitor whose K_i value can be determined under normal conditions is to find a way to accelerate the association process, as opposed to decelerating further the dissociative step.[5]

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Supplementary Material Available

Experimental details for the synthesis, characterization, and enzymatic assay of the inhibitors described in this report and seven representative figures showing determination of binding

[4] There appears to be no reason to suggest an atypical exchange process, especially in view of the observation that the binding enhancement due to valine substitution of alanine at P_1 is similar to that for Boc-YV^{P}(O)A, whose K_i value was determined under thermodynamic conditions.

[5] As a final comment, we note that the crystallographic structure determination of the complex between CPA and ZFV^{P}(O)F (Kim & Lipscomb, 1991) required less time to carry out than did the experiments (the results of which are depicted in Figure 1) to determine the dissociation rate constant.

constants (18 pages). Ordering information is given on any current masthead page.

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