Ionization states of the complex formed between 2-benzyl-3-phosphonopropionic acid and carboxypeptidase A

Umesh B. GOLI, Damian GROBELNY and Richard E. GALARDY

Department of Biochemistry and Sanders-Brown Research Center on Aging, University of Kentucky, Lexington, KY 40536, U.S.A.

The binding to carboxypeptidase A of two phosphonic acid analogues of 2-benzylsuccinate, 2-DL-2-benzyl-3-phosphonopropionic acid (inhibitor I) and 2-DL-2-benzyl-3-(-O-ethylphosphono)propionic acid (inhibitor II) was studied by observing their ^{31}P resonances when free and bound to the enzyme in the range of pH from 5 to 10. The binding of I by co-ordination to the active-site Zn(II) lowered the highest pK_a of I from a value of $7.66(\pm 0.10)$ to a value of $6.71(\pm 0.17)$. No titration of any protons on II occurred over the pH range studied. The enzyme-bound inhibitor II also did not titrate over the pH range 6.17–7.60. The pH-dependencies of the apparent inhibition constants for I and II were also investigated by using *N*-(2-furanacryloyl)-L-phenylalanyl-L-phenylalanine as substrate. Two enzymic functional groups with pK_a values of $5.90(\pm 0.06)$ and $9.79(\pm 0.14)$ must be protonated for binding of inhibitor I, and two groups with pK_a values of $6.29(\pm 0.10)$ and $9.19(\pm 0.15)$ for binding of inhibitor II. Over the pH range from 6.71 to 7.66, inhibitor I binds to the enzyme in a complex of the enzyme in a more protonated form, and the inhibitor in a less protonated form than the predominant unligated forms at this pH. Mock & Tsay [(1986) *Biochemistry* 25, 2920–2927] made a similar finding for the binding of L-2-(1-carboxy-2-phenylethyl)-4-phenylazophenol over a pH range of nearly 4 units. The true inhibition constant for the dianionic form of inhibitor I (racemic) was calculated to be $54.0(\pm 5.9)\text{ nM}$ and that of the trianionic form to be $5.92(\pm 0.65)\text{ nM}$. The true inhibition constant of the fully ionized II (racemic) was calculated to be $79.8(\pm 6.4)\text{ nM}$.

INTRODUCTION

The zinc-metalloproteinase carboxypeptidase A (EC 3.4.12.2) has been used as a model for the design of inhibitors of human angiotensin-converting enzyme (Cushman *et al.*, 1977). The use of this model system has resulted in converting-enzyme inhibitors which are widely marketed as effective antihypertensive agents in man (Kostis & DeFelice, 1987). The specific states of ionization of inhibitors and the enzyme in the complex determine the binding affinity of the inhibitor at physiological pH. For converting enzyme, little is known about the optimum ionization states of enzyme and inhibitor in their complexes. The results reported here, together with previous studies by others, define these optimum ionization states for the model enzyme carboxypeptidase A. For example, L-benzylsuccinic acid, one of the most powerful inhibitors of carboxypeptidase A, was thought on the basis of the pH profile of its apparent binding affinity (Byers & Wolfenden, 1973) to bind to the enzyme as a monoanion. A later ^{13}C n.m.r. study of the enzyme-inhibitor complex at different pH values indicated that the inhibitor binds as a dianion (Palmer *et al.*, 1982), which was reconciled with the results of the previous pH profile of the inhibition constant by assuming that the protonation of a group on the enzyme close to the inhibitor binding site is necessary for stabilizing the enzyme-inhibitor complex. The side-chain carboxylic acid of Glu-270 was proposed to be the group responsible for this stabilization.

More recently, an analogue of L-benzylsuccinic acid, namely L-2-(1-carboxy-2-phenylethyl)-4-phenylazophenol (III) (Fig. 1), has been shown (Mock & Tsay, 1986) to be a reversible competitive inhibitor, albeit with a value for minimum $K_{i,\text{app}}$, two orders of magnitude larger than for the $K_{i,\text{app}}$ at the pH optimum for L-benzylsuccinic acid. Those authors concluded, by studying how the apparent dissociation constant of this inhibitor varied with pH over the range 5–10, and by spectrophotometric titration of the enzyme-inhibitor complex, that an enzyme group with a pK_a of 6.2 must be protonated for strongest inhibitor binding. They suggest that this group was the zinc-bound water molecule, and that the fully ionized inhibitor is the most potent over the pH range studied.

Two potent inhibitors of carboxypeptidase A, 2-DL-2-benzyl-3-phosphonopropionic acid (I) and 2-DL-2-benzyl-3-(-O-ethylphosphono)propionic acid (II), which are analogous to benzylsuccinic acid (Fig. 1), have been synthesized and their inhibition kinetics at pH 7.5 previously reported (Grobelny *et al.*, 1985). The phosphono group is an analogue of the tetrahedral transition state for amide and ester hydrolysis (Biryukov *et al.*, 1978). Thus inhibitors I and II are transition-state-analogue inhibitors of carboxypeptidase A (Grobelny *et al.*, 1985). Proposed modes of binding of these inhibitors to the active site of carboxypeptidase A are shown in Fig. 1. Inhibitor I has three ionizable protons. The present study reports the pH titration of ^{31}P n.m.r. resonance of inhibitors I and II, both free and bound to

Abbreviations used: (inhibitor) I, 2-DL-2-benzyl-3-phosphonopropionic acid; II, 2-DL-2-benzyl-3-(-O-ethylphosphono)propionic acid; III, L-2-(1-carboxy-2-phenylethyl)-4-phenylazophenol; FAPP, *N*-(2-furanacryloyl)-L-phenylalanyl-L-phenylalanine; IV, L-2-benzylsuccinic acid.

Fig. 1. Model for the active site of carboxypeptidase A (based on that described by Ondetti *et al.*, 1979), showing proposed modes of binding for inhibitors I, II, III and IV

The proposed ionization states of the bound inhibitors are not indicated in this Figure. S_1 is the binding site for the amino acid residue P_1 to the left of the scissile amide bond according to the nomenclature of Schechter & Berger (1968) for papain. Abbreviations: Ph, C_6H_5 -; Et, C_2H_5 -.

the enzyme. The third acid dissociation constant of I when bound to carboxypeptidase A is found to have a pK_a value that is about 1 unit lower than that of free inhibitor. The positions of the ^{31}P resonances associated with the phosphono group on inhibitor II are found not to change with pH over the range 5–10, as expected. Similarly, the enzyme-bound inhibitor II peak positions are found not to change with pH over the range studied, that is, from pH 6.17 to 7.60. We also report here our investigation of the pH-dependence of kinetically determined apparent-inhibition-constant values for these two inhibitors in the pH range 5–10. The analysis indicates that the protonation of the enzyme group with pK_a value around 6 is necessary for binding of both inhibitor I (both dianionic and trianionic forms) and inhibitor II, as found by Mock & Tsay (1986) for the azophenol inhibitor (III). There also is another group with a pK_a value above 9 that needs to be protonated for the binding of both I and II. These pK_a values have been repeatedly observed before by others [Makinen *et al.*, 1979; Vallee & Galdes 1984 and references therein; Mock & Tsay, 1986].

EXPERIMENTAL

Materials

Inhibitors I and II (both racemic) were available from previous studies (Grobelny *et al.*, 1985). The carboxypeptidase A (Allan form; catalogue no. C0386, an

aqueous suspension) and DL-benzylsuccinic acid were obtained from Sigma Chemical Co. Four different buffers (50 mM each) were used to cover the range of pH from 5 to 10: 2,6-bis(hydroxymethyl)pyridine for pH 4.2–5.3, Mes for pH 5.3–7.25, Tris for pH 7.25–8.75, and 2-amino-2-methylpropane-1,3-diol (ammediol) for pH 8.75–10. Each of these buffers is known to be non-inhibitory at these concentrations (Mock & Tsay, 1986). The ionic strength was maintained nearly constant by making buffers 1 M with respect to NaCl. Both Mes and 2,6-bis(hydroxymethyl)pyridine buffers were adjusted to a $ZnCl_2$ concentration of approx. $100 \mu M$ to suppress the metal-ion dissociation from the active site; Mock & Tsay (1986) used 50 – $100 \mu M$ $ZnCl_2$ at pH 6.4 and below. The substrate employed in the inhibition kinetic assays of I and II was FAPP [*N*-(2-furanacryloyl)-L-phenylalanyl-L-phenylalanine] (Riordan & Holmquist, 1984) and was obtained from Sigma. The excess zinc ions are a competitive inhibitor for carboxypeptidase A (Hirose *et al.*, 1987). Indeed in our experiments it was found that, at $100 \mu M$ substrate at pH 7, the zinc ions at concentrations of $100 \mu M$ inhibited the rate of hydrolysis by about 40%. However, the apparent-inhibition-constant value for inhibitor II at the same pH was found to change little when zinc ions were added to this concentration ($K_i = 0.8 \mu M$ with $100 \mu M$ $ZnCl_2$, $K_i = 0.6 \mu M$ in the absence of zinc, as determined from Dixon plots only).

Inhibition kinetics

Inhibition kinetic data were collected typically at two substrate (FAPP) concentrations ($40 \mu M$ and $100 \mu M$) and at least four different inhibitor concentrations covering a range 0–4 times the apparent inhibition constant. The progress of the reaction was monitored by observing the decrease in A_{328} . The initial rates were measured from slopes of the spectrophotometric traces representing less than 10% of the reaction. The initial estimates of $K_{i,app}$ (from the intercept), V_{max} , and K_m were obtained from Dixon plots. (The Lineweaver–Burk plots obtained at substrate concentrations from $0.25 K_m$ to K_m at some selected pH values demonstrated that inhibitor I exhibits classically competitive behaviour from pH 5.5 to 9.5, and that inhibitor II exhibits the same type of inhibition from pH 5.5 to 9. At pH 10, however, both inhibitors exhibited apparently mixed inhibition.) These initial estimates were then used to fit directly the velocity data to the competitive-inhibition equation by an iterative non-linear least-squares program to obtain more reliable values of apparent inhibition constants. These values were found to be close to their initial estimates obtained from Dixon plots, and the general pH profiles of apparent inhibition constants were same for both initial estimates and the least-squared-fitted values, thus validating the use of two substrate concentrations and a wide range of inhibitor concentrations to obtain apparent inhibition constants for these two classically competitive inhibitors. The average values for the inhibition constants determined in duplicate sets of experiments were used to fit the data to non-linear inhibition equations; each data point was weighted proportionally to the inverse square of the difference between $K_{i,app}$ values obtained in duplicate sets of experiments. The s.d. values for all the fitted constants reported are obtained from the least-square fitting routine. The pH of all solutions was measured with a

Corning pH-meter 125 using a Radiometer combination pH-electrode calibrated with standard buffers. These pH values are uncorrected for ionic-strength effects (expected to be insignificant).

pH titration of ^{31}P n.m.r. resonances

The ^{31}P n.m.r. studies were carried out on a Varian XL-200 (at the Department of Chemistry, University of Kentucky, Lexington, KY, U.S.A.), a Varian XL-300 (at the Magnetic Resonance Imaging and Spectroscopy Center, University of Kentucky) and a Bruker CXP-200 (at Yale University Medical School, New Haven, CT, U.S.A.) instrument using 10 mm sample tubes. The temperature of the probe was maintained at 22 °C. The same buffers used in the inhibition kinetic studies were used in the n.m.r. experiments. The pH values were measured both before and after the n.m.r. run to make sure the pH had not changed.

The ^{31}P n.m.r. resonances of free inhibitor I and II at various pH values were obtained at approx. 40 mM-inhibitor in appropriate buffers. The buffer pH values were changed within their respective ranges by adding 1 M-NaOH or 1 M-HCl.

The carboxypeptidase A for the n.m.r. study was prepared as follows: 10 ml of the enzyme in aqueous suspension was collected by centrifugation, washed three times with 3 ml portions of distilled water and dissolved in 2 ml of appropriate buffer containing 10 % (v/v) $^2\text{H}_2\text{O}$ (for n.m.r. lock) to yield protein solutions typically at 1.7 mM concentration. Protein concentrations were determined by measuring A_{278} ($\epsilon_{278} = 6.42 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$; Simpson *et al.*, 1963). The pH was varied by adding 1 M-NaOH. For lower pH values, 0.1 M-HCl was added, whereupon there was some precipitation. In such cases spectra were obtained by using slightly turbid solutions. In all but a few cases mentioned below inhibitor was at least three times in excess of enzyme so that both free-inhibitor and enzyme-bound-inhibitor peaks could be observed. In experiments involving identification of multiple enzyme-bound-inhibitor resonances, separate runs with a deficient quantity of inhibitor and excess inhibitor were used.

All of the chemical shifts reported are relative to 85 % (v/v) H_3PO_4 used as an external reference or 1 % H_3PO_4 in $^2\text{H}_2\text{O}$ used as an external reference in a concentric tube inside the sample tube.

RESULTS

The pH titration of free inhibitors I and II

Inhibitor I has two acidic protons on its *phosphonic* acid group. The more acidic of these is already ionized at the lower limit of pH range used, i.e. at pH 5.00. The second proton titrates with a $\text{p}K_a$ of $7.66(\pm 0.10)$. This value was obtained by *three-parameter* (F_{max} , F_{min} , and K_a) non-linear least-squares fitting of chemical-shift data from pH 5 to pH 11 to eqn. (1):

$$\delta_{\text{obs.}} = \frac{[\text{H}]}{([\text{H}] + K_a)} \left(F_{\text{max.}} + \frac{K_a}{[\text{H}]} F_{\text{min}} \right) \quad (1)$$

where $F_{\text{max.}}$ is the low-pH limit of chemical shift corresponding to the dianionic form of the inhibitor, $F_{\text{min.}}$ is the high-pH limit of chemical shift corresponding to the trianionic form of the inhibitor (Fig. 2, ○), $[\text{H}]$ is the hydrogen-ion concentration and $\delta_{\text{obs.}}$ is the chemical

Fig. 2. pH titration of ^{31}P resonances of free (○) and carboxypeptidase A-bound (●) inhibitor I

The chemical shifts are relative to 85 % H_3PO_4 . The continuous lines are theoretical lines obtained from three-parameter (F_{max} , F_{min} and K_a) non-linear least-squares fit of chemical-shift data to eqn. (1) (see the text). The crosses (+) give the chemical shifts of the minor resonance closest to the major free-inhibitor-I peak, which is a contaminant in the inhibitor. This peak was observed only in the higher-sensitivity and -resolution instrument (Varian XL-300) and not on the Varian XL-200.

shift. The least-squares-fitted values and their standard deviations for the ^{31}P chemical shift of the free trianionic form of the inhibitor is $20.9(\pm 0.5)$ and that of the free dianionic form is $24.5(\pm 0.0)$ p.p.m. relative to 85 % H_3PO_4 .

As expected, no pH variation of the position of the ^{31}P resonance was observed for inhibitor II over the pH range of 5–11. But a sharp upfield change in the chemical shift was observed at a pH just below 5. The chemical shift at pH 5.6 was 26.2, and that at pH 3.9 was 24.7 p.p.m. both relative to 1 % H_3PO_4 . The $\text{p}K_a$ of the group responsible for this was estimated to be approx. 4.5. The titration of ethylphosphonic acid yielded two $\text{p}K_a$ values, 7.3 and 2.3, with the ^{31}P resonances shifted downfield at lower pH. Therefore the group with the $\text{p}K_a$ of approx. 4.5 for inhibitor II is thought to be the carboxylic acid group.

pH titration of the enzyme-bound inhibitors I and II

Inhibitor II at pH 7.60 had a single peak at 26.3 p.p.m. relative to 1 % H_3PO_4 . On binding to the enzyme, however, it had two additional peaks at 28.9 and 26.6 p.p.m. The intensity ratio of the peaks at 28.9 and 26.6 p.p.m. was about 1:3 when the inhibitor was about 3.3 times in excess of the enzyme. The intensity ratio was about 1:1 when the relative concentration was about 1:1. This suggests that the peaks of 26.6 and 28.9 p.p.m. are due to two enzyme-bound enantiomers and that the more potent of the two is responsible for the peak at 26.6 p.p.m.

For free inhibitor I, at pH 8.3 and a very high signal-to-noise ratio, three peaks were observed: a major one

Fig. 3. ^{31}P n.m.r. resonances of free and carboxypeptidase A-bound inhibitor I at pH 8.3

The chemical shifts are relative to 85% H_3PO_4 as an external standard. (a) The spectrum when the ratio of the concentrations of the inhibitor and the enzyme was about 4.5. (b). The spectrum when the concentrations of the enzyme and the inhibitor were comparable. (c) The spectrum when the concentrations of the enzyme and the inhibitor were comparable, but in the presence of a large excess of the competitive inhibitor IV. The peaks at 24.1 and 26.2 p.p.m. are due to two enantiomers of inhibitor I bound to the enzyme. The remaining three peaks are free-inhibitor peaks, the minor two of which appear only under high-sensitivity and -resolution conditions.

at 21.8 and two very small peaks at 21.4 and 19.7 p.p.m. downfield from 85% H_3PO_4 . In the presence of a deficient amount of the enzyme at the same pH an additional peak appeared at 24.1 p.p.m. (Fig. 3a), which was assigned to the more potent of the enantiomers of inhibitor I bound to the enzyme, as for inhibitor II. When a nearly-equi-molar mixture of the enzyme and the inhibitor at the same pH was run overnight, one more enzyme-bound peak was observed at 26.2 p.p.m. (Fig. 3b), which was broader than all the other peaks. This peak was assigned to the less potent enantiomer of inhibitor I bound to the enzyme, as it was seen only when the enzyme concentration was comparable with that of the inhibitor. The peak at 21.7 p.p.m. (Fig. 3b) is the free-inhibitor-I peak. When the inhibitor-I concentration was

in excess over that of the enzyme and in the presence of a very large excess of the competitive inhibitor DL-benzylsuccinic acid (Byers & Wolfenden, 1973), only the free-inhibitor peak (21.8 p.p.m. at pH 8.3) was observed. This indicates that the enzyme-bound-inhibitor peaks at 24.1 and 26.2 p.p.m. arise from the inhibitor I binding to the *active site* of the enzyme. The peaks due to the impurities in inhibitor I (21.4 and 19.7 p.p.m.) do not appear in Fig. 3(c) owing to the low concentration of inhibitor I used in this experiment.

In order to determine the pK_a of enzyme-bound inhibitor I, an approx. 5-fold excess of the inhibitor was added to carboxypeptidase A at approx. 1.7 mM, and chemical shifts of the free and enzyme-bound inhibitor at different pH values were studied from pH 5.6 to 9.3. Chemical shifts of free inhibitor changed with pH as expected for an acid with a pK_a of 7.66. The non-linear least-squares fitting to eqn. (1) of chemical-shift data for the enzyme-inhibitor I complex involving the more potent of the enantiomers yielded a pK_a of $6.71 (\pm 0.17)$ (see Fig. 2, ●), which is about 1 unit lower than that of free inhibitor I. The least-squares-fitted values for chemical shift of the enzyme-inhibitor I complex were more than 3 p.p.m. downfield at both the high- and the low-pH limits from the free-inhibitor chemical shift at the respective pH extremes. It was also observed that the position of the minor resonance closest to the major free-inhibitor-I peak shifted with pH in the same direction as the movement of the major enzyme-bound peak (Fig. 2, +). The enzyme-bound-inhibitor-II peak positions did not change with pH, as checked at three different pH values from 6.17 to 7.6.

pH-dependence of the apparent inhibition constant

Both inhibitors I and II exhibited a very strong dependence of the competitive inhibition constant $K_{i,\text{app}}$ on pH. For inhibitor I the $\text{pK}_{i,\text{app}}$ values were constant at pH values below 5.9 (Fig. 4), but decreased as the pH was increased with inflections at pH 6.71 and 7.66. The data fits very well (in the least-squares sense) to eqn. (2):

$$K_{i,\text{app}} = \frac{K_i \left(1 + \frac{K_1}{[\text{H}]}\right) \left(1 + \frac{K_3}{[\text{H}]}\right) \left(1 + \frac{K_4}{[\text{H}]}\right)}{\left(1 + \frac{K_2}{[\text{H}]}\right)} \quad (2)$$

K_3 is the acid dissociation constant for the free inhibitor I. K_1 and K_4 are the successive acid dissociation constants (molecular dissociation constants in the strictest sense; Tipton & Dixon, 1979) of the dibasic enzyme, which differ by a factor of more than three orders of magnitude (pK_1 5.9 and pK_2 9.79 as obtained by fitting the data to eqn. 2). Here K_2 is the acid dissociation constant of a group in the enzyme-inhibitor complex that is reported by ^{31}P n.m.r. titration of the resonance due to the enzyme-bound inhibitor. The pH-independent true inhibition constant, K_i in eqn. (2), represents the dissociation constant of dianionic inhibitor I from its complex with the fully protonated enzyme.

The iterative non-linear least-squares fit to eqn. (2) was made by varying K_i , K_1 and K_4 keeping K_3 and K_2 values constant at $10^{-7.66}$ and $10^{-6.71}$ respectively. Both K_3 and K_2 values were obtained from pH titration of n.m.r. resonances (see Fig. 2). The values of K_i , pK_1 , and pK_4 obtained by such a procedure are $54.0 (\pm 5.9)$ nM, $5.90 (\pm 0.06)$ and $9.79 (\pm 0.14)$ respectively.

Fig. 4. Variation with pH of apparent inhibition constant $K_{i,app}$ for inhibitor I (○) and inhibitor II (●)

The continuous lines are theoretical lines obtained by three-parameter (K_i , K_1 and K_2 for inhibitor I and K_i , K_1 and K_2 for inhibitor II) non-linear least-squares fit of inhibition-constant data to eqns. (2) and (3) respectively. The vertical bar gives the highest and the lowest values at all pH values. All apparent inhibition constants were determined twice, except those at pH 5.5, 8.5 and 10 for inhibitor I and at pH 5.5, 9.0 and 9.5 for inhibitor II, which were determined three times.

The inhibition constants for inhibitor II were fitted to eqn. (3):

$$K_{i,app} = K_i \left(1 + \frac{K_1}{[H]} \right) \left(1 + \frac{K_2}{[H]} \right) \quad (3)$$

where K_1 and K_2 represent the successive acid dissociation constants of the enzyme that differ by a factor of about three orders of magnitude. The K_i is the true dissociation constant of dianionic inhibitor II from its complex with the fully protonated enzyme. Each one of the variables [K_i (the pH-independent true inhibition constant), pK_1 and pK_2] were varied. When non-linear least-squares fits of inhibition data for inhibitor II to eqn. (3) were performed, the data points at pH values equal to 6.5 and 7.00 were ignored, because these two points deviate substantially from the rest of the profile (Fig. 4). The best values (in the least-squares sense) for K_i , pK_1 and pK_2 obtained are $79.8(\pm 6.4)$ nM, $6.29(\pm 0.10)$ and $9.19(\pm 0.15)$ respectively. In order to incorporate the inflection feature at about pH 7 into the fit, two more pK_a values would have to be invoked, about 5 and 7 for the free enzyme and the inhibitor-bound enzyme respectively. The higher of these pK_a values is not seen in the ^{31}P n.m.r. spectrum of inhibitor II complexed with the enzyme. Therefore, even by obtaining many more data points over this pH range, it may not be possible to improve the accuracy of determination of pK_1 , pK_2 and the true inhibition constant, K_i , by including this inflection, because two more unknown constants have to be determined now by curve-fitting. It is to be noted, however, that the phenomenon responsible for this

inflection is not a factor in decreasing the apparent affinity of inhibitor II to the enzyme at pH values above 7, the approximate value of the pK_a causing the inflection around this pH. This phenomenon could be the greater stability of the protonated enzyme-inhibitor II complex, which could involve a hydrogen-bonded interaction of the phosphonyl moiety of inhibitor II with the side-chain carboxylic acid of Glu-270 compared with the complex at higher pH, where this proton is absent (see, for example, Palmer *et al.*, 1982; Christianson & Lipscomb, 1985).

DISCUSSION

Inhibitors analogous to the ones used in the present study and pseudo-substrates are known to bind directly to the metal centre of both the native and metal-ion-substituted carboxypeptidase A [Lipscomb *et al.*, 1968; Navon *et al.*, 1968, 1970; Hartsuck & Lipscomb, 1971; Rees & Lipscomb, 1981; Palmer *et al.*, 1982; Vallee & Galde, 1984 (and references therein)]. It is also observed that the hydrate of 2-benzyl-3-formylpropionic acid, an inhibitor analogous to inhibitor I, binds to the active-site Zn(II) by displacement of the metal-bound water molecule (Christianson & Lipscomb, 1985). Therefore it is reasonable to expect that inhibitors I and II also bind to Zn(II) through at least one oxygen atom of the phosphonic acid moiety, with the displacement of a water molecule. The total ^{31}P chemical-shift change with pH observed for enzyme bound inhibitor I is about the same as the similar change for the free inhibitor I (see Fig. 2 and its legend, which describes the way the constants F_{max} , F_{min} and K_a values were determined). Moreover the chemical-shift change with pH for the bound inhibitor I is in the same direction as for the free inhibitor. This makes it unlikely that movement of the chemical shift of the enzyme-bound inhibitor with changing pH is due to the titration of some remote enzyme functionality with a pK_a of 6.71 indirectly affecting the ^{31}P resonances. The possibility that this pK_a is that of the active-site water molecule bound to Zn(II) in the enzyme-inhibitor I complex is also ruled out, because similar enzyme-bound-inhibitor-II peaks do not titrate over the pH range 7.60–6.17, although the apparent-dissociation-constant-versus-pH profile of inhibitor II does show an inflection in this region. Moreover, the water molecule is very likely displaced from Zn(II) in the complex (Christianson & Lipscomb, 1985) and thus may be unlikely to affect the ^{31}P resonances of either inhibitor when bound to the enzyme. The only plausible explanation is that the pK_a of 6.71 does really refer to the ionization of the enzyme-bound inhibitor I, which means that, upon binding to the enzyme and on co-ordination to the active-site Zn(II), the pK_a of the inhibitor I phosphonic acid group is lowered from a value of 7.66 to 6.71. Therefore it is concluded that the trianionic form of inhibitor I binds about 9 times better than dianionic inhibitor I (the same as the ratio of acid dissociation constants of the enzyme-bound inhibitor and the free inhibitor).

The position of the hydrogen ion cannot be obtained by kinetic analysis. However, on the basis of the arguments above, the variation of apparent inhibition constants with pH can be interpreted to mean that inhibitor I binds exclusively to the enzyme whose acid groups exhibiting pK_a values of about 5.9 and 9.79 are

both protonated. Both dianionic and trianionic forms of the inhibitors bind, the latter better than the former by a factor of about 9. For inhibitor **II** a similar, and only marginally weaker, pH-dependence (at pH values less than 7.66 only; see below) is observed.

The K_i value for inhibitor **I** (54 nM) obtained by using eqn. (2) represents the true dissociation constant of the dianionic form of the inhibitor. The true inhibition constant that represents the dissociation constant of the fully ionized trianionic form of the inhibitor bound to the protonated form of the enzyme is therefore about 5.9 nM, which of course refers to a racemic mixture. Assuming that one enantiomer inhibits much more strongly than does the other, the inhibition constants are about 3 nM and 27 nM for one enantiomer of fully ionized **I** (pK_a 7.66) and dianionic **I** (pK_a 2.3) respectively. The modest increase in binding (about 9-fold) between dianionic **I** and trianionic inhibitor **I** occurs in spite of their affinities for a proton differing by more than 10^5 ($pK_a \sim 2.3$ as against pK_a 7.66). The analogous inhibitors L-azophenolate (**III**) (pK_a 8.76; Mock & Tsay, 1986) and L-2-benzylsuccinate (**IV**, Fig. 1; pK_a 4.6; Byers & Wolfenden, 1973; Palmer *et al.*, 1982) have corresponding true inhibition-constant values for fully ionized inhibitors of 1.9 nM and (at most) 6 nM respectively. In these inhibitors a large range of basicities of oxyanions with pK_a values of conjugate acids of 2.3–8.8 causes only a modest change in binding affinity to the enzyme.

The $K_{i,app.}$ values for inhibitor **II** fit to the scheme whereby it binds exclusively to the enzyme where two groups with $pK_a = 6.29$ and 9.19 are in the protonated form. There is no reason to suspect that these groups are not the same as the groups responsible for the binding of inhibitor **I** and the same as the groups with these pK_a values reported by many others, assumed to be zinc-bound water at pH 6 and either Tyr-248 or zinc-bound His-69 or -196 [Makinen *et al.*, 1979; Vallee & Galdes, 1984 (and references therein); Gardell *et al.*, 1985; Mock & Tsay, 1986; Rutter *et al.*, 1987]. The true inhibition-constant value for inhibitor **II** is $79.8(\pm 6.4)$ nM, which is only slightly higher than for the dianionic form of inhibitor **I** which is $54.0(\pm 5.9)$ nM. Unlike inhibitor **I**, inhibitor **II** is in its fully ionized state even at the lowest limit of the pH range studied. Moreover, in the case of inhibitor **I**, the fully ionized form is only about 9 times better an inhibitor (comparing the values of true inhibition constants) than the dianionic form. Therefore the $pK_{i,app.}$ -versus-pH profile of inhibitor **II** resembles that of inhibitor **I** with a roughly constant difference in $pK_{i,app.}$ of about 1 unit or so, especially after pH 7.66, where the last ionizable proton of inhibitor **I** begins to ionize.

For inhibitor **I**, Fig. 4 shows that $pK_{i,app.}$ rises as the pH falls, reaching a plateau below the enzyme pK_a of 5.9. An important feature of this curve is that the profile shows an inflection toward higher inhibition-constant values at pH 7.66 (which is the pK_a value for the inhibitor). The slope of the curve is very close to -1 (an enzyme group with a pK_a of 5.9 started ionizing at much lower pH) until the group with a pK_a of 9.79 begins to ionize, whereupon the slope starts to increase further in the negative direction. At just above pH 6.71 (which is the pK_a of enzyme-bound inhibitor **I**), the downward trend in the curve due to ionization of the group with pK_a of 5.9 is retarded until pH 7.66 is reached. Therefore, for effective binding of inhibitor **I**, both enzyme groups

with pK_a values of 5.9 and 9.79 need to be protonated, and it is the dianionic form of the inhibitor **I** that is a major binding species at pH values below 6.71. Above this pH it is the trianionic form of inhibitor **I** that is the major binding species. The trianionic form binds about 9 times more strongly than does the dianionic form. Remembering that the pK_a of free inhibitor **I** is 7.66 and that of enzyme-bound inhibitor is 6.71, it is clear that at pH values below 6.71 the major inhibitory species (the dianionic form) predominates in solution over this pH range. Between pH 6.71 and 7.66 the major inhibitory species is the trianionic form of inhibitor **I**, but this is present in only minor amounts over this pH range.

As pointed out above, the enzyme group with pK_a of 5.9 needs to be protonated for effective binding of inhibitor **I**, which means that, above pH 5.9, it is always the species in minor amount in solution that is active. Therefore over the narrow pH range of 6.71–7.66 the reverse-protonation-state (minor protonation state) inhibition mechanism is operative as found for the inhibitor azophenol (**III**) (Mock & Tsay, 1986). That is, enzyme and inhibitor forms that are major partners in the inhibition mechanism are only minor species in the solution.

Between pH 6.71 and 7.66 the increase in $K_{i,app.}$ due to the decreased amount of species with pK_a 5.90 is partly offset by ionization of dianionic inhibitor **I** bound to enzyme, which tends to decrease the apparent inhibition-constant value. This compensation mechanism ceases above pH 7.66, as trianionic inhibitor **I** now is already a major species. This is the reason why the $pK_{i,app.}$ -versus-pH profile rapidly decreases at pH values above 7.66, owing to the ever-decreasing concentration of the protonated form of the enzyme group with pK_a 5.90.

In summary, the binding of two potent inhibitors of carboxypeptidase A, 2-DL-2-benzyl-3-phosphonopropionic acid (**I**) and 2-DL-2-benzyl-3-(*O*-ethylphosphono)propionic acid (**II**) has been investigated by using ^{31}P n.m.r. shifts of the free and enzyme-bound inhibitors as a function of pH. The value of the highest pK_a of inhibitor **I** has been found to be diminished to 6.71 on binding to the enzyme from its pK_a value in the free state of 7.66. In all cases enzyme-bound peaks were downfield from free-inhibitor peak at the pH values studied. The pH-dependence of inhibition constants in conjunction with the n.m.r. data led us to the conclusion that the enzyme that is active in binding inhibitors **I** and **II** is in the fully protonated state, two relevant acidic groups having pK_a values of about 5.9 and 9.79 as observed in inhibitor **I** kinetics, and 6.29 and 9.19 as observed in inhibitor **II** kinetics. The true inhibition constant for the dianionic form of inhibitor **I** (racemic) was calculated to be $54.0(\pm 5.9)$ nM and that of trianionic form to be $5.92(\pm 0.65)$ nM. The corresponding value for the fully ionized inhibitor **II** (racemic) was found to be $79.8(\pm 6.4)$ nM. Mock & Tsay (1986) and others have attributed the lower pK_a of the enzyme to the ionization of the water molecule bound to active-site zinc ion and the higher pK_a to the imidazole of one of the histidines co-ordinated to the metal centre. For inhibitor **I** over the narrow pH range 6.71–7.66 the reverse protonation mechanism of inhibition is demonstrated. These studies serve as a model for the binding of antihypertensive drugs containing similar functional groups (Kostis & DeFelice, 1987) to the zinc-metalloproteinase angiotensin-converting enzyme.

This study was supported by National Institutes of Health Grant HL 27368.

REFERENCES

- Biryukov, A. I., Ishmuratov, B. Kh. & Khomutov, R. M. (1978) *FEBS Lett.* **91**, 249–252
- Byers, L. D. & Wolfenden, R. (1973) *Biochemistry* **12**, 2070–2078
- Christianson, D. W. & Lipscomb, W. N. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6840–6844
- Cushman, D. W., Cheung, H. S., Sabo, E. F. & Ondetti, M. A. (1977) *Biochemistry* **16**, 5484–5490
- Gardell, S. J., Craik, C. S., Hilvert, D., Urdea, M. S. & Rutter, W. J. (1985) *Nature (London)* **317**, 551–555
- Gettins, P. (1986) *J. Biol. Chem.* **261**, 15513–15518
- Grobelny, D., Goli, U. B. & Galaray, R. E. (1985) *Biochem. J.* **232**, 15–19
- Hartsuck, J. A. & Lipscomb, W. N. (1971) *Enzymes* 3rd Ed. **3**, 1–56
- Hirose, J., Ando, S. & Kidani, Y. (1987) *Biochemistry* **26**, 6561–6565
- Kostis, J. B. & DeFelice, E. A. (1987) in *Angiotensin Converting Enzyme Inhibitors* (Kostis, J. B. & DeFelice, E. A., eds.), pp. 213–262, Alan R. Liss, New York
- Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N., Quiocho, F. A., Bethge, P. H., Ludwig, M. L., Steitz, T. A., Muirhead, H. & Coppola, J. C. (1968) *Brookhaven Symp. Biol.* **21**, 24–90
- Makinen, M. W., Kuo, L. C., Dymowski, J. J. & Jaffer, S. (1979) *J. Biol. Chem.* **254**, 356–366
- Mock, W. L. & Tsay, J.-T. (1986) *Biochemistry* **25**, 2920–2927
- Navon, G., Shulman, R. G., Wyluda, B. J. & Yamane, T. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **60**, 86
- Navon, G., Shulman, R. G., Wyluda, B. J. & Yamane, T. (1970) *J. Mol. Biol.* **51**, 15–30
- Ondetti, M. A., Condon, M. E., Reid, J. C., Sabo, E. F., Cheung, H. S. & Cushman, D. W. (1979) *Biochemistry* **18**, 1427–1430
- Palmer, A. R., Ellis, P. D. & Wolfenden, R. (1982) *Biochemistry* **21**, 5056–5059
- Rees, D. C. & Lipscomb, W. N. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5455–5459
- Riordan, J. F. & Holmquist, B. (1984) in *Methods of Enzymatic Analysis* (Bergmeyer, H. W., ed.), 3rd edn., vol. 5, pp. 44–55, Verlag Chemie, Deerfield Beach, FL
- Rutter, W. J., Gardell, S. J., Rocznik, S., Hilvert, D., Sprang, S., Fletterick, R. J. & Craik, C. S. (1987) in *Protein Engineering* (Oxender, D. L. & Cox, C. F., eds.), pp. 257–267, Alan R. Liss, New York
- Schechter, I., & Berger, A. (1968) *Biochem. Biophys. Res. Commun.* **32**, 898–902
- Simpson, R. T., Riordan, J. F. & Vallee, B. L. (1963) *Biochemistry* **2**, 616–622
- Tipton, K. F. & Dixon, H. B. F. (1979) *Methods Enzymol.* **63**, 183–234
- Vallee, B. L. & Galdes, A. (1984) *Adv. Enzymol. Relat. Areas Mol. Biol.* **56**, 283–430

Received 20 January 1988/6 April 1988; accepted 3 May 1988