Inhibition of Carboxypeptidase A by Aldehyde and Ketone Substrate Analogues†

Richard E. Galardy* and Zbigniew P. Kortylewicz

ABSTRACT: DL-2-Benzyl-3-formylpropanoic acid (XIVb) is a competitive inhibitor of carboxypeptidase A with an apparent K_i of 0.48 μM at pH 7.5 in 50 mM Tris buffer–0.5 M in sodium chloride with *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate as substrate. At pH 7.5 in deuterium oxide, DL-2-benzyl-3-formylpropanoic acid exists as an equilibrium mixture of 75% free aldehyde and 25% hydrated aldehyde. The species that binds to the enzyme may be either the free aldehyde or the hydrate. Therefore, the K_i of the species bound is significantly less than the observed K_i of 0.48 μM. The alcohol and dioxolane analogues of this aldehyde, DL-2-benzyl-4-hydroxybutanoic acid (XI) and 2-benzyl-4,4-(ethylenedioxy)butanoic acid (XXVII), are only weak inhibitors with K_i's of 0.54 mM and 2 mM, respectively. The ketone, (±)-3-(*p*-methoxybenzoyl)-2-benzylpropanoic acid [(±)-I; Sugimoto, T., & Kaiser, E. T. (1978) *J. Am. Chem. Soc. 100*, 7750–7751], was found to have a K_i of 180 μM, experimentally indistinguishable from that of the diastereomeric mixture of its alcohol analogue 2-benzyl-4-hydroxy-4-(*p*-methoxyphenyl)butanoic acid (III), K_i = 190 μM. The ketone (I) is not detectably hydrated (<2%) at pH 7.5 in deuterium oxide. These results suggest that the hydratable aldehyde DL-2-benzyl-3-formylpropanoic acid may mimic an intermediate resembling the transition state for amide hydrolysis by carboxypeptidase A while the nonhydratable ketone does not do so.

Aldehydes and halomethyl ketones are frequently strong reversible inhibitors of proteases known to employ an acyl enzyme intermediate such as elastase (Thompson, 1973) and papain (Lewis & Wolfenden, 1977a) and of certain aminopeptidases (Birch et al., 1972; Kettner et al., 1974; Anderson et al., 1982). The mechanism of inhibition probably involves the addition of a nucleophile to the carbonyl carbon atom, converting it to a tetrahedral configuration which resembles intermediates related to the transition state for amide substrate hydrolysis (Lewis & Wolfenden, 1977b). Tetrahedral addition complexes between trypsin and the irreversible inhibitor benzyloxycarbonyl[2-^{13}C]lysine chloromethyl ketone (Malthouse et al., 1983) and between pepsin and a carbon-13 labeled ketone (Rich et al., 1982) have been observed by carbon-13 NMR.[1] In the case of pepsin, the identity of the nucleophile involved in the tetrahedral complex was not established. It could be a water molecule or an enzyme-bound nucleophile.

The zinc metalloprotease carboxypeptidase A is irreversibly inhibited by the halomethyl ketone *N*-(bromoacetyl)-*N*-methylphenylalanine (Hass & Neurath, 1971a) accompanied by alkylation of glutamic acid-270 (Hass & Neurath, 1971b). The ketomethylene analogue of the substrate benzoyl-L-phenylalanine, (−)-3-(*p*-methoxybenzoyl)-2-benzylpropanoic acid [(−)-I], was reported to reversibly inhibit carboxypeptidase A with a K_i of 110 μM (Sugimoto and Kaiser, 1978). The reported studies with this ketone do not suggest whether the mechanism of inhibition involves reversible addition of a nucleophile to the ketone carbonyl carbon to produce a tetrahedral adduct. However, a base at the active site of carboxypeptidase was found to catalyze stereospecific exchange of one of the α protons of the ketone, and this base was proposed to be glutamic acid-270 or an intervening water molecule (Sugimoto & Kaiser, 1978). A ketomethylene analogue of a tripeptide substrate of angiotensin-converting enzyme has been shown to be a strong reversible inhibitor (K_i = 0.1 μM) of this carboxypeptidase-like zinc protease which is a target for antihypertensive therapy (Almquist et al., 1980). Thus, the interaction of aldehydes and ketones with carboxypeptidase A may serve as a model for the inhibition of converting enzyme by carbonyl compounds. This interaction could involve addition of a nucleophile to the carbonyl carbon to form a tetrahedral adduct resembling an intermediate in the enzyme-catalyzed reaction. In this case, the nucleophile could be an amino acid side chain of carboxypeptidase A such as glutamic acid-270 or a water molecule. Alternatively the carbonyl compound could simply act as a structural analogue of the substrate or product and bind with the carbonyl carbon in its trigonal planar configuration.

We show here that (±)-3-(*p*-methoxybenzoyl)-2-benzylpropanoic acid (I), K_i = 180 μM, is not appreciably hydrated in water and is not a better inhibitor of carboxypeptidase A than either its parent amide, benzoyl-L-phenylalanine, K_i = 88 μM, or its alcohol analogue (±), (±)-2-benzyl-4-hydroxy-4-(*p*-methoxyphenyl)butanoic acid (III), K_i = 190 μM. Therefore, addition of a nucleophile to the ketone (I) is probably not involved in its modest inhibition of carboxypeptidase. DL-2-Benzyl-3-formylpropanoic acid (XIVb) is a potent inhibitor with a K_i of 0.48 μM, over 1000-fold more potent than its nonhydratable alcohol analogue DL-2-benzyl-4-hydroxybutanoic acid (XI), K_i = 0.54 mM. This aldehyde is 25% hydrated in water. Therefore, the mechanism of inhibition could involve addition of water or perhaps an enzyme-bound nucleophile to the aldehyde carbonyl carbon. Such a mechanism would be consistent with the tight binding of hydratable aldehyde XIVb compared to unhydratable ketone (±)-I based on the resemblance of the tetrahedral aldehyde adduct to an intermediate related to the transition state for amide hydrolysis. The design of the potent aldehyde inhibitor DL-2-benzyl-3-formylpropanoic acid (XIVb) was sug-

† From the Department of Biochemistry and the Sanders-Brown Research Center on Aging, University of Kentucky, Lexington, Kentucky 40536. *Received June 8, 1983; revised manuscript received November 9, 1983.* This project was supported by National Institutes of Health Grants HL 27368 and RR 05374.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; PPTS, pyridinium *p*-toluenesulfonate; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; DMF, dimethylformamide; THF, tetrahydrofuran; MHz, megahertz; SD, standard deviation.

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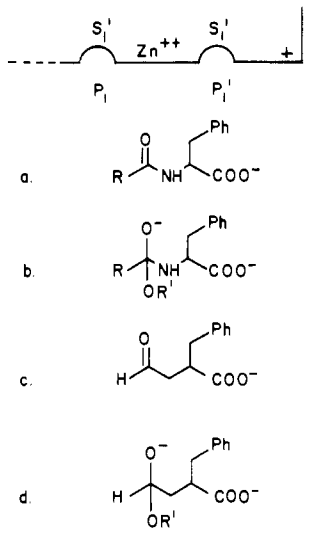

FIGURE 1: Model for the active site of carboxypeptidase A (Ondetti et al., 1979) showing proposed modes of binding for (a) substrate, (b) an intermediate resembling the transition state for substrate hydrolysis, where R′ is –H or an amino acid side chain from the enzyme such as that of glutamic acid-270, (c) the aldehyde (XIVb), and (d) a tetrahedral adduct of a nucleophile with the aldehyde (XIVb).

gested by its dicarboxylic analogue, the byproduct carboxypeptidase A inhibitor, DL-2-benzylsuccinate (Byers & Wolfenden, 1973).

A simple model of the active site of carboxypeptidase A (Ondetti et al., 1970) and the proposed interaction of substrates and the aldehyde inhibitor (XIVb) is shown in Figure 1.

Experimental Procedures

Benzoyl-L-phenylalanine and carboxypeptidase A (Sigma type II) were from Sigma Chemical Co., St. Louis, Mo. *trans-p*-Chlorocinnamic acid from Aldrich Chemical Co., Milwaukee, WI, was recrystallized until its NMR spectrum was free of reesonances from the cis isomer. L-β-Phenyllactic acid was from Aldrich. *O*-(*trans-p*-Chlorocinnamoyl)-L-β-phenyllactate was prepared according to Suh & Kaiser (1976); mp 125 °C (lit. mp 125–126.5 °C).

All the starting materials and solvents used in this work were reagent grade in 98% or higher purity and used without further purification or prepared by standard literature procedures. Anhydrous THF was obtained by distillation from $LiAlH_4$. Acetonitrile and DMF were purified by the usual methods and stored over molecular sieves. Thionyl chloride was distilled from boiled linseed oil. Flasks and stirring bars used for generation and reactions of lithio derivatives were dried at 120 °C and allowed to cool in a desiccator over P_4O_{10}. Thin-layer chromatography (TLC) was on silica gel 60F-254 (EM-Reagents), and compounds were visualized by ultraviolet light and iodine. Purification by column chromatography was performed on silica gel 100–200-mesh size (EM-Reagents). Infrared (IR) measurements were done on a Perkin-Elmer 257 grating spectrophotometer. Liquid samples were measured neat on NaCl plates and solid samples as KBr pellets. Proton nuclear magnetic resonance (NMR) spectra were recorded on a Varian EM-390 or XL-200. Chemical shifts (δ) are parts per millon (ppm) downfield from tetramethylsilane. Mass spectra were recorded on a Hitachi Perkin-Elmer RMV-6E mass spectrometer. Pyridinium *p*-toluenesulfonate (PPTS) was prepared by treating *p*-toluenesulfonic acid monohydrate with 3.5 equiv of pyridine at 0 °C, and crude product was recrystallized from acetone (Miyashita, 1977); mp 160 °C; 96% yield. 2-(β-Chloroethoxy)tetrahydropyran was prepared by treating 2-chloroethanol with an excess of dihydropyran and stirring at room temeprature for 3 h; bp 85–87 °C (12 mm); 76% yield. Melting points were taken on a hot stage and are corrected. Boiling points which are given for short-path evaporative distillation correspond to bath temperatures and are uncorrected. The yields given were based on isolated products after purification.

The preparation of the following compounds is described in the supplementary material for this paper (see paragraph at end of paper regarding supplementary material): 3-(*p*-methoxybenzoyl)-2-benzylpropanoic acid, both (+) and (–) isomers [(+)-I, (–)-I]; 2-benzyl-4-hydroxy-4-(*p*-methoxyphenyl)butanoic acid sodium salt (III); DL-2-benzylbutanoic acid (V); DL-2-benzyl-4,4-(ethylenedioxy)butanoic acid (XXVII). The preparation of the aldehyde DL-2-benzyl-3-formylpropanoic acid potassium salt (XIVa) by oxidation of the alcohol DL-2-benzyl-4-hydroxybutanoic acid (V) is described here.

Diethyl [2-(Tetrahydropyranyloxy)ethyl]malonate (VI). To the solution of (0.146 mol) of MeONa in 50 mL of ethanol (freshly prepared from 3.40 g of sodium and 25 mL of methanol) were added 24.05 g (0.146 mol) of 2-(β-chloroethoxy)tetrahydropyran, 23.37 g (0.146 mol) of diethyl malonate, and 0.1 g of powdered NaI. This mixture was refluxed 20 h. After this time, the solution was cooled and the solvent evaporated under reduced pressure. The residue was poured into a mixture of 100 mL of 2 N H_2SO_4 and 150 g of ice. The resulting solution was extracted with ether (5 × 20 mL). After the solvent was dried and evaporated, the residue was distilled under vacuum, and 24.5 g (60% yield) of pure product was obtained bp 94–97 °C (0.025 mm); NMR ($CDCl_3$) δ 1.25 (t, 6 H, 2 × CH_3), 1.38–2.00 (m, 6 H, $CH_2CH_2CH_2$ from pyran ring), 3.31 (m, 2 H, CH_2O pyran ring), 3.46–3.98 (m, 5 H, OCH_2CH_2CH), 4.16 (q, 4 H, 2 × CH_2O), and 4.70 [m, 1 H, C(O–)(O–)H from pyran ring].

Diethyl (2-Hydroxyethyl)benzylmalonate (VIII). In 40 mL of absolute ethanol, 1.12 g (0.049 mol) of sodium metal was dissolved, and 14.0 g (0.049 mol) of VI was added. Then 8.35 g (0.05 mol) of benzyl bromide was added dropwise, and the immediate precipitate of KBr was collected. The mixture was refluxed at 80 °C for 5 h, and after cooling the solvent was evaporated. The residue was treated with 60 mL of 2 N H_2SO_4 and 100 g of ice. The mixture was extracted with ethyl ether (3 × 60 mL), and the ether extracts were washed with saturated $NaHCO_3$, 5% $Na_2S_2O_3$, and water, dried over $MgSO_4$, and evaporated. A total of 15.5 g (83% yield) of crude (2-(tetrahydropyranyloxy)ethyl)benzylmalonate (VII) was obtained. Complete deprotection of VII was achieved by using PPTS in absolute ethanol at 45 °C for 3 h. After reaction, PPTS was removed by filtration after the ethanol was evaporated, and the residue was treated with 100 mL of ethyl ether. The crude product was recrystallized from Et_2O/*n*-hexane (1:2 v/v) giving 8.5 g (79% yield) of VIII: mp 57–60 °C; NMR ($CDCl_3$) δ 1.18 (t, 6 H, 2 × CH_3), 2.05 (m, 2 H, CH_2C), 3.23 (s, 2 H, CCH_2), 3.65 (m, 2 H, OCH_2), 4.23 (q, 4 H, 2 × OCH_2) and, 7.26 (m, 5 H, Ph).

3-Benzyloxacylopentan-2-one (α-Benzyl-γ-butyrolactone) (X). The hydrolysis of 8.0 g (0.03 mol) of VIII was accomplished by heating for 4 h with alcoholic KOH (7.5 g in 35 mL of ethanol). When the TLC plates showed that the reaction was completed, 200 mL of ether was added, and the mixture was kept at 5 °C overnight. The sodium salt of the diacid of VIII was collected by filtration under a nitrogen atmosphere: mp 140–143 °C (6.2 g, 83% yield); NMR (D_2O) δ 1.82 (t, 2 H, CH_2C), 3.16 (t, 2 H, CCH_2), 3.64 (t, 2 H,

OCH_2), and 7.34 (m, 5 H, Ph). The pure potassium salt of the diacid of VIII (6.0 g, 0.024 mol) was then dissolved in 20 mL of water, and this solution was acidified with 6 N H_2SO_4 to pH 2.0 with cooling. After stirring for 30 min, the mixture was extracted with warm ether (5 × 50 mL) and dried ($MgSO_4$), and the solvent was evaporated. The residue consisted of two products: α-carboxyl-α-benzyl-γ-butyrolactone (IX) and α-benzyl-γ-butyrolactone (X) in a 2:3 ratio (calculated from the NMR spectrum). This mixture was then decarboxylated at 170 °C to give crude lactone (X) which was purified by silica gel column chromatography ($CHCl_3$) to give pure X, a colorless liquid (2.85 g, 83% yield): IR (film) 1030, 1510, 1600, 1760, 2900, 3020 cm^{-1}; NMR ($CDCl_3$) δ 1.62–3.29 (m, 2 H, CH_2 from lactone ring), 2.41–3.34 (m, 3 H, Ph CH_2 and CH group from lactone ring), 4.05 (m, 2 H, OCH_2 from lactone ring), and 7.22 (m, 5 H, Ph). Anal. Calcd for $C_{11}H_{12}O_2$: C, 75.00; H, 6.82. Found: C, 74.76; H, 6.93.

DL-*2-Benzyl-4-hydroxybutanoic Acid Potassium Salt (XI).* Pure lactone X (0.89 g, 0.005 mol) was suspended in 5.1 mL of 1.0 N KOH, and the mixture was heated at 60 °C with stirring for 3 h. The organic layer disappeared, and the clear solution was lyophilized, giving 1.13 g of the potassium salt of acid XI: mp 202–203 °C; NMR (D_2O) δ 1.20–1.72 (m, 2 H, CH_2) 2.19–2.94 (m, 3 H, $CHCH_2$), 3.26 (t, 2 H, OCH_2), 7.14 (m, 5 H, Ph); mass spectrum, *m/z* (70 eV) 176 (M), 148, 131, 104, 91, 65.

DL-*2-Benzyl-3-formylpropanoic Acid Potassium Salt (XIVa). Method A.* To 5 mL of dry pyridine was added 1.76 g (0.01 mol) of lactone X, and the mixture was stirred at room temperature for 2 h. After this time the excess of pyridine was evaporated under vacuum. The residue was dissolved in 50 mL of water and was cooled to 0 °C. Chromium trioxide (1.25 g, 0.0125 mol) was dissolved in 3 mL of water, and this solution was added to 13 mL of pyridine at 0 °C. The cooled solution of pyridinium salt of X was added in one portion, and the yellow mixture was stirred at room temperature for 1 h. The solution was diluted with water to 125 mL and acidified with 2 N H_2SO_4 to pH 4.0, extracted with ethyl ether (5 × 25 mL), and the organic extracts were washed twice with water, and solid K_2CO_3 (15 g) was added. After filtration, solvent was evaporated, and the crude hydroxylactone (XIII) was obtained as a yellow residue. After short-path distillation, 0.85 g (27% yield) of 3-benzyl-5-hydroxyoxacyclopentan-2-one (XIII) was obtained: bp 97–101 °C (0.015 mm); NMR ($CDCl_3$) δ 1.63–3.34 (m, 4 H, $PhCH_2$ and CH_2 from lactone ring), 3.61 (t, 1 H, CHCO from lactone ring), 4.21 (m, 1 H, CH from lactone ring), 5.73 (m, 1 H, COH, exchangeable with D_2O), and 7.26 (m, 5 H, Ph); mass spectrum, *m/z* (70 eV) 175 (M^+ – OH), 166, 163, 148, 146 (M^+ – HCOOH), 91, 46. Anal. Calcd for $C_{11}H_{12}O_3$: C, 68.75; H, 5.73. Found: C, 68.49; H, 6.16.

To 4.0 mL of 1.0 N KOH was added 0.80 g (0.004 mol) of lactone XIII, and this mixture was stirred at room temperature until the organic layer disappeared. lyophilization gave 0.90 g (98% yield) of 2-benzyl-3-formylpropanoic acid potassium salt (XIVa): mp 152–155 °C dec; NMR ($CDCl_3$) δ 1.77–3.62 (m, 5 H, CH_2CHCH_2), 7.34 (m, 5 H, Ph), and 9.31 (t, 1 H, CHO).

Two other methods for preparing XIV are described in the supplementary material.

Kinetic Studies. The hydrolysis of *O*-(*trans-p*-chlorocinnamoyl)-L-β-phenyllactate by carboxypeptidase A was measured spectrophotometrically at 25 °C as described by Suh & Kaiser (1976) in 50 mM Tris base–0.5 M in sodium chloride adjusted to pH 7.5 with hydrochloric acid. The

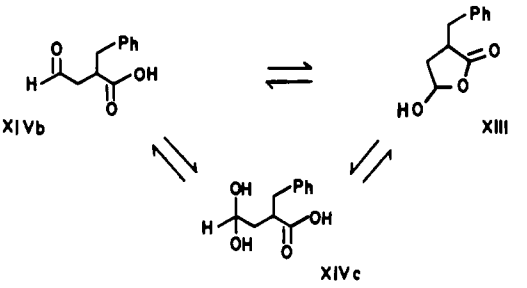

FIGURE 2: Proposed equilibrium between DL-2-benzyl-3-formylpropanoic acid (XIVb), its hydrate (XIVc), and its lactone hemiacetal (XIII) in water.

suspension of enzyme as received was diluted to give a stock solution with a concentration of 7 nM in the assay buffer. The enzyme concentration in the kinetic assay was 0.36 nM. Substrate concentrations were varied from 80 to 400 μM. K_m was found to be 170 ± 30 μM. Initial velocities were calculated from the linear initial slopes of the change in absorbance vs. reaction time curves where the amount of substrate consumed was always less than 10%. For each substrate and inhibitor at least four K_i's were determined, one from a Lineweaver–Burk plot and one from a Dixon plot from each of two independent experiments done on different days. The four K_i's were averaged, and a standard deviation was calculated. K_i's were determined from four independent experiments for the ketones (±)-I, (+)-I, and (−)-I with ketones prepared in two totally independent syntheses.

The reversibility of inhibition by DL-2-benzyl-3-formylpropanoic acid (XIVa) was demonstrated by a 50-fold dilution of 32 nM enzyme inhibited 67% by 7.5 μM XIVa and redetermining the amount of inhibition. After dilution the inhibited enzyme regained 91% of the activity of uninhibited enzyme. The lack of progressive inhibition of the enzyme by XIVa was demonstrated by determining the degree of inhibition as a function of incubation of enzyme and inhibitor for a period of 4 h. Enzyme at 0.74 nM and XIVa at 0.4 μM were incubated at 25 °C. At 0, 1, 2, 3, and 4 h, the solution was diluted by 2-fold into 200 μM substrate and assayed. The decrease in initial velocity was about 2% h^{-1}, identical with that found for a control incubation without inhibitor.

Angiotensin-convertining enzyme was prepared and assayed as described (Galardy, 1982).

Results

The synthesis of the aldehyde (XIVb) was complicated by formation of the lactone hemiacetal (XIII) unless the carboxylic acid function was in the form of a salt which prevented lactone formation. The proposed equilibirium between the aldehyde (XIVb), its hydrate (XIVc) and the lactone hemiacetal (XIII) which occurs in the presence of water is shown in Figure 2. The free aldehyde (XIVb) could only be isolated in stable form as its potassium salt (XIVa) after mild base hydrolysis of the lactone hemiacetal (XIII). A 90-MHz proton NMR spectrum of the aldehyde (XIVb) in deuterated dimethyl sulfoxide containing 10% deuterium oxide showed 68% aldehyde (XIVb), 22% hydrate (XIVc), and 10% lactone hemiacetal (XIII).

Since the aldehyde hydrate (XIVc) was not isolated, its existence is inferred from the NMR spectrum and the known chemical shifts of the proton attached to the hydrated aldehydic carbon in model compounds (Lewis & Wolfenden, 1977b). Immediately after dissolution of XIVb in deuterium oxide at pH 7.5, the amounts of the three isomers XIVb, XIII, and XIVc and the chemical shifts of the relevant protons were

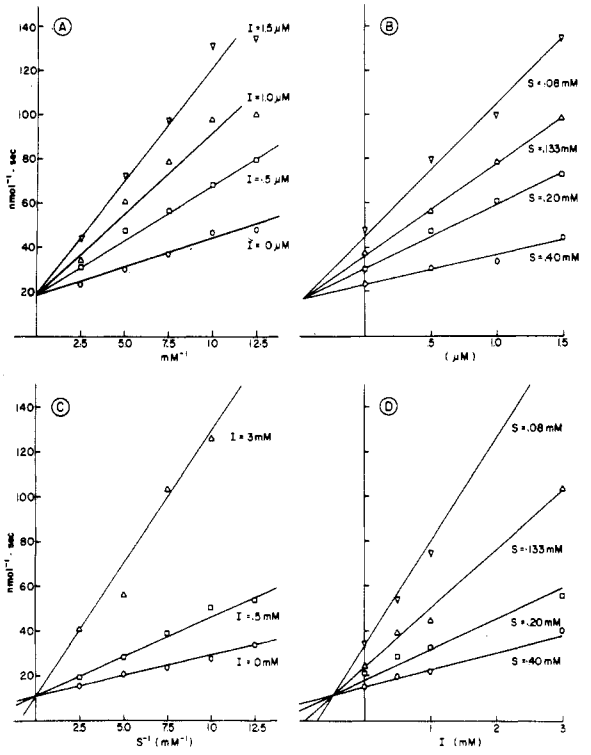

FIGURE 3: Lineweaver–Burk (A and C) and Dixon (B and D) plots for the inhibition of carboxypeptidase A catalyzed hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate by DL-2-benzyl-3-formylpropanoic acid potassium salt (XIVa) (A and B) and DL-2-benzyl-4-hydroxybutanoic acid (XI) (C and D).

as follows: XIVb, 68% and δ 7.33 (Ph), 8.57 (CHO); XIII, 10% and δ 7.10 (Ph), 6.30 (OC*H*OH); XIVc, 22% and δ 7.10 (Ph), δ 5.16 (HOC*H*OH). After 30 min at 35 °C, the lactone resonances disappeared to give a mixture of 75% aldehyde (XIVb) and 25% hydrate (XIVc).

Figure 3 shows Lineweaver-Burk and Dixon plots for the inhibition of carboxypeptidase A by the aldehyde DL-2-benzyl-3-formylpropanoic acid potassium salt (XIVa) and its alcohol analogue DL-2-benzyl-4-hydroxybutanoic acid (XI). The inhibition by the aldehyde is competitive. Both the potassium salt (XIVa) and the freshly prepared acid form (XIVb) gave the same K_i and competitive inhibition. Table I gives the K_i's and modes of inhibition for all of the compounds tested. Benzoyl-L-phenylalanine, K_i = 88 μM, is included as the amide analogue of ketone I. DL-Benzylsuccinate is the byproduct analogue inhibitor (Byers & Wolfenden, 1973) which is the dicarboxylic acid analogue of the aldehyde DL-2-benzyl-3-formylpropanoic acid (XIVb).

The alcohol analogue (XI), the fully saturated analogue (V), and the dioxolane (XXVII) of the aldehyde (XIVb) were over 1000-fold, 10000-fold, and 4000-fold less potent, respectively, than XIVb. Insertion of one additional methylene group between the benzyl group in the P_1' position and the aldehyde group in XIVb to give DL-2-benzyl-4-formylbutanoic acid (XXII) reduced the potency by 100-fold. Since XXII was found to be hydrated in water to the same extent as XIVb, this reduced potency must be due to incorrect positioning of the benzyl or the aldehyde group at the active site. Propionaldehyde, possessing neither a benzyl side chain to occupy the S_1' subsite on the enzyme nor a free carboxyl group, is a very weak inhibitor with K_i > 25 mM. XIVb inhibits angiotensin-converting enzyme with a K_i of 7 mM and thus binds 1400-fold less tightly than to carboxypeptidase A. The relative K_i's of the two ketone enantiomers [(+)-I and (−)-I] are consistent with the reported estimated K_i's (Sugimoto & Kaiser, 1978). The alcohol analogue (III) of the racemate [(±)-I] occurs as two diastereomers. This mixture of all four isomers was equipotent to (±)-I within the error of the experiments.

Table I: Inhibition of Carboxypeptidase A Catalyzed Hydrolysis of *O*-(*trans*-*p*-Chlorocinnamoyl)-L-β-phenylacetate by Aldehydes, Ketones, and Their Analogues

inhibitor	K_i (μM)
benzoyl-L-phenylalanine	88 ± 13, competitive
DL-benzylsuccinate (IV)	0.2 ± 0.1, competitive[a]
DL-2-benzyl-3-formylpropanoic acid (potassium salt) (XIVa, XIVb)	0.48 ± 0.1, competitive
DL-2-benzyl-4-hydroxybutanoic acid (XI)	540 ± 80, competitive
DL-2-benzylbutanoic acid (V)	6300 ± 420, mixed
DL-2-benzyl-4,4-(ethylenedioxy)-butanoic acid (XXVII)	2000 ± 250, mixed
DL-2-benzyl-4-formylbutanoic acid (XXII)	23 ± 6, competitive
propionaldehyde	>20 000
(±)-3-(*p*-methoxybenzoyl)-2-benzylpropanoic acid [(±)-I]	180 ± 40, competitive or mixed
(+)-3-(*p*-methoxybenzoyl)-2-benzylpropanoic acid [(+)-I]	47 ± 15, competitive[b]
(−)-3-(*p*-methoxybenzoyl)-2-benzylpropanoic acid [(−)-I]	700 ± 140, mixed[c]
2-benzyl-3-hydroxy-3-(methoxyphenyl)propanoic acid (III)	190 ± 10, mixed

[a] Literature value, 1.1 μM (Byers & Wolfenden, 1973). [b] Literature value, 49 μM (Sugimoto & Kaiser, 1978). [c] Literature value, 110 μM (Sugimoto & Kaiser, 1978).

Discussion

The facile addition of nucleophiles to aliphatic aldehydes is illustrated by the spontaneous cyclization or hydration of the aldehyde (XIVb) to its lactone hemiacetal (XIII) or hydrate (XIVc). The equilibrium shown in Figure 2 between free aldehyde and its two addition compounds may be a model for the interaction of XIVb with the active site of carboxypeptidase A as shown in Figure 1d. The nucleophile in carboxypeptidase which is presumed to add to the aldehyde could be either a water molecule as in the hydrate (XIVc) or the γ-carboxylic acid group of glutamic acid-270 as in the lactone hemiacetal (XIII). It is not possible to distinguish between these two possibilities at this time. It is also not known whether the free aldehyde or its hydrate is the species which binds to the enzyme. Anderson et al. (1982) have suggested that L-leucinal binds to leucine aminopeptidase as the free aldehyde and then adds a nucleophile from the active site of the enzyme based on the drastically lower inhibition found for L-leucinol and the absence of an isotope effect for the binding of [1-^{1}H]- and [1-^{2}H]leucinal. An isotope effect of 1.37 was predicted if the hydrate were bound on the basis of the protio and deuterio aldehydes having a 1.37-fold difference in equilibrium degree of hydration.

DL-2-Benzyl-3-formylpropanoic acid (XIVb), K_i = 0.48 μM, has high specificity for carboxypeptidase since its K_i for angiotensin-converting enzyme is 7 mM. This high specificity is similar to that found for substituted mercaptopropanoic acid inhibitors of carboxypeptidases compared to their inhibition of converting enzyme (Ondetti et al., 1979). The substituted mercaptopropanoic acid inhibitors have absolute K_i's for the carboxypeptidases up to 1000-fold lower than their analogous substituted succinic acid inhibitors and the aldehyde (XIVb) reported here (Ondetti et al., 1979). The inhibition of car-

boxypeptidase by XIVb is not due to irreversible chemical modification since the inhibition does not increase progressively with time and is reversible by dilution. Propionaldehyde, which is not a substrate analogue for carboxypeptidase, does not inhibit the enzyme. We propose that DL-2-benzyl-3-formylpropanoic acid (XIVb) is an analogue of an intermediate resembling the transition state for normal substrate hydrolysis due to addition of a nucleophile to the aldehydic carbon. Since the alcohol (XI) has only $^1/_{1000}$ the activity of its aldehyde analogue (XIVb), two oxygen atoms (or one oxygen and one other heteroatom) must be attached to the crucial tetrahedral carbon for strong binding. The weak inhibition by the dioxolane (XXVII) further demonstrates that both of these atoms cannot be alkylated.

Simple ketones are negligibly hydrated in water compared to aldehydes. For example, acetone is approximately 0.02% hydrated while acetaldehyde is more than 50% hydrated (Lewis & Wolfenden, 1977b). (−)-3-(*p*-Methoxybenzoyl)-2-benzylpropanoic acid (I) is not appreciably hydrated in water (<2%) and probably merely sterically mimics the amide analogue benzoyl-L-phenylalanine. This result is confirmed by the K_i's reported in Table I, 88 μM for the amide and 700 μM for the ketone [(−)-I]. The inability of this ketone to form a tetrahedral adduct with a nucleophile may be responsible for its lack of strong interaction with the enzyme. The experimentally indistinguishable K_i's of the ketone [(±)-I] and its alcohol analogue (III) further demonstrate that addition of a nucleophile is not involved in inhibition by the ketone since the alcohol, which cannot add a nucleophile, is equally potent. Sugimoto & Kaiser (1978) demonstrated stereospecific, carboxypeptidase A catalyzed exchange of one α-keto proton in (−)-I. Our results suggest that if this exchange is catalyzed by nucleophile addition to the ketone carbonyl carbon, the equilibrium for the formation of the adduct is very unfavorable.

In summary, we propose that the aldehyde (XIVb) strongly inhibits carboxypeptidase A by its ability to add an as yet unidentified nucleophile, either water on an enzyme-bound group, to form a tetrahedral adduct resembling a tetrahedral intermediate on the reaction pathway for substrates. If this is true, the mechanism for binding is distinctly different from the dicarboxylic acid byproduct analogue inhibitors of the carboxypeptidases described by Byers & Wolfenden (1973) and McKay & Plummer (1978) which cannot add a nucleophile. These latter inhibitors bind with one carboxylate in its trigonal planar configuration as a ligand of the active site zinc atom as shown by Bolognesi & Matthews (1979) for thermolysin. The ionized state of the carboxylate bound to the active site zinc in the benzylsuccinate–thermolysin complex (Bolognesi & Matthews, 1979), however, could be mimicked by the presumed tetrahedral aldehyde adduct if aldehyde hydrates have pK_a's in the appropriate pH range. However, the pK_a of acetaldehyde hydrate is 13.5 (Fasman, 1976) and seems to be too high for significant ionization to occur at pH 7.5. The tetrahedral aldehyde adduct could both mimic an intermediate resembling the transition state for substrate hydrolysis and act as an anionic byproduct analogue if this ionization could occur.

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Supplementary Material Available

Description of the preparations for (+)-I, (−)-I, III, V, and XXVII (7 pages). Ordering information is given on any current masthead page.

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