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Inhibition of Carboxypeptidase A by Ketones and Alcohols That Are Isosteric with Peptide Substrates

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ABSTRACT: The K_i's of three peptide ketone and three peptide alcohol inhibitors of carboxypeptidase A are compared with K_i's of their respective isosteric peptide substrates, N^{α}-benzoyl-L-phenylalanine, N^{α}-benzoylglycyl-L-phenylalanine, and N^{α}-carbobenzoxyglycylglycyl-L-phenylalanine. For the isosteric ketone analogues of these substrates, the respective K_i's are as follows: (2*RS*)-2-benzyl-4-(3-methoxyphenyl)-4-oxobutanoic acid, 180 ± 40 μM; (2*RS*)-5-benzamido-2-benzyl-4-oxopentanoic acid (V), 48 ± 7 μM; (2*RS*)-2-benzyl-5-(carbobenzoxyglycinamido)-4-oxopentanoic acid (IX), 9 ± 0.1 μM. For the alcohols derived by reduction of each of these ketones, K_i's are as follows: (2*RS*,4*RS*)-2-benzyl-4-(3-methoxyphenyl)-4-hydroxybutanoic acid, 190 ± 10 μM; (2*RS*,4*RS*)-5-benzamido-2-benzyl-4-hydroxybutanoic acid (IV), 160 ± 62 μM; (2*RS*,4*RS*)-2-benzyl-5-(carbobenzoxyglycinamido)-4-hydroxypentanoic acid (XI), 600 ± 100 μM. K_i values for the competitive peptide ketone inhibitors decrease with increasing peptide chain length. This is consistent with the possibility of increased binding interaction between inhibitor and enzyme by simple occupation of additional binding subsites by adding more amino acid residues to the inhibitor. In contrast, the K_i values of the alcohols (competitive or mixed inhibition) increased or remain essentially unchanged with increasing chain length. Increasing the chain length of ketone inhibitor V to give IX decreases K_i by one-fifth. The K_i of ketone IX is also less than $^1/_{30}$th the K_i of its isosteric peptide and almost $^1/_{70}$th that of its isosteric alcohol, XI. Taken together, these results suggest that binding of ketones V and IX to the enzyme may not be via a simple Michaelis-type complex but requires an additional interaction with the enzyme.

Carboxypeptidase A (EC 3.4.12.2) is a zinc metalloprotease that cleaves a single amino acid residue from the carboxy terminus of peptides. Aldehyde and ketone substrate analogues and isosteres strongly inhibit several zinc metalloproteases: angiotensin-converting enzyme (Almquist et al., 1980, 1982; Meyer et al., 1981; Gordon et al., 1984; Natarajan et al., 1984), leucine aminopeptidase (Andersson et al., 1982), and carboxypeptidase A [Galardy & Kortylewicz (1984, 1985) and references cited therein]. Carbonyl compounds have been proposed to be transition-state analogues for proteases due to their ability to add a nucleophile to form a tetrahedral species

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that mimics a tetrahedral intermediate occurring during amide hydrolysis (Lewis & Wolfenden, 1977a,b). Carbon-13 NMR spectroscopy has demonstrated tetrahedral enzyme–inhibitor complexes for aldehyde and ketone inhibitors of several classes of proteases but not for metalloproteases, thus confirming the transition-state analogy for the former enzymes [see review by MacKenzie et al. (1984)]. The strong inhibition of the metalloprotease angiotensin-converting enzyme by the ketone ketoace [5-benzamido-4-oxo-3-(6-phenylhexanoyl)-L-proline] suggests that it too is a transition-intermediate analogue (Almquist et al., 1984). Since inhibitors of the metalloprotease angiotensin-converting enzyme have been shown to be antihypertensive drugs (Petrillo & Ondetti, 1982) and other metalloproteases such as collagenase are also potential therapeutic targets in disease, it is important to know the mechanism of inhibition of this class of enzymes by ketones.

Peptide alcohols have been isolated from microorganisms (Umezawa, 1982) that are powerful slow binding inhibitors of the acid protease pepsin (Rich & Sun, 1980) and of aminopeptidases of the metalloenzyme class (Rich et al., 1984). However, these peptide alcohols (such as pepstatin, amastatin, and bestatin) are not isosteric with peptide substrates but contain a hydroxyethylene group inserted between the α-carbon atom and the carboxylic carbon atom of an α-amino acid (this β-hydroxy-γ-amino acid is called statine). In general, peptide alcohols that are isosteric with peptide substrates or isosteric with powerful aldehyde or ketone inhibitors are themselves found not to be strong inhibitors (Galardy & Kortylewicz, 1984; D. Grobelny and R. E. Galardy, unpublished results; Thompson, 1973; Lewis & Wolfenden, 1977b). The reasons for strong interaction of the statine family of inhibitors with their target proteases and weak interaction of the isosteric peptide alcohols are not known.

Here we report the relationship between the K_i's[1] for three peptide ketone and three peptide alcohol inhibitors of carboxypeptidase A and the K_i's for their respective isosteric peptide substrates. The three ketone and three alcohol inhibitors are designed to occupy respectively the S_1', the S_1' and S_1, and the S_1', S_1, and S_2 binding subsites on the enzyme (see Figure 1). Indeed, the respective K_i's of the ketones decrease with increasing chain length as if more interactions with additional subsites are occurring. In contrast the K_i's of the substrates and alcohols are approximately constant (or increase) with increasing chain length. For the most potent inhibitor, 2-benzyl-5-(carbobenzoxyglycinamido)-4-oxobutanoic acid (IX), a K_i of 9 μM was obtained. This K_i is less than $^1/_{30}$th that of its isosteric peptide substrate and about $^1/_{70}$th that of its isosteric alcohol, XI. However, this K_i is more than 1 order of magnitude higher than those reported for inhibitors proposed to be transition-state analogues for carboxypeptidase A (Galardy & Kortylewicz, 1984; Jacobsen & Bartlett, 1981). On the basis of these and previous results, a general scheme for the binding of carbonyl-containing inhibitors to carboxypeptidase A is proposed.

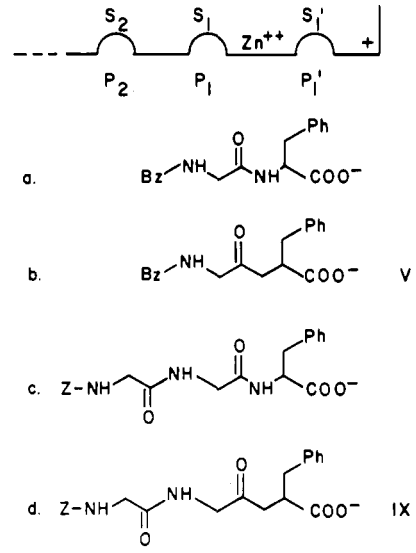

FIGURE 1: Model for the active site of carboxypeptidase A (Ondetti et al., 1979) showing proposed modes of binding for (a) benzoylglycylphenylalanine, (b) 5-benzamido-2-benzyl-4-oxopentanoic acid (V), (c) carbobenzoxyglycylglycyl-L-phenylalanine, and (d) 2-benzyl-5-(carbobenzoxyglycinamido)-4-oxopentanoic acid (IX). S_1', S_1, and S_2 are binding subsites on the enzyme for amino acid residues in the P_1', P_1, and P_2 positions in substrates and inhibitors (Schechter & Berger, 1968).

Experimental Procedures

Hippuryl-L-phenylalanine, N^{α}-carbobenzoxyglycylglycyl-L-phenylalanine, other protected amino acids, and carboxypeptidase A (Sigma type II) were from Sigma Chemical Co. Dicyclohexylcarbodiimide, *N*-(2,3-epoxypropyl)phthalimide, diethyl malonate, benzyl bromide, and other chemicals were obtained from Aldrich Chemical Co. Melting points, proton nuclear magnetic resonance spectroscopy (NMR), mass spectroscopy (MS), and thin-layer chromatography were as described (Galardy & Kortylewicz, 1984). The compositions of thin-layer solvent systems were, by volume, as follows: (A) chloroform/acetonitrile/hexane, 3:1:1; (B) chloroform/methanol, 9:1; (C) 2-propanol/concentrated ammonium hydroxide, 84:37. *O*-(*trans*-*p*-Chlorocinnamoyl)-L-β-phenyllactate was prepared according to Suh & Kaiser (1976), mp 125 °C (lit. mp 125–126.5 °C). 5-(Benzoylamino)-4-oxo-6-phenylhexanoic acid (X) was prepared according to Meyer et al. (1981), mp 179–182 °C (lit. mp 182–186 °C).

5-Benzamido-5-benzyl-4-hydroxypentanoic Acid Potassium Salt (IV). A solution of 9 g (28.4 mmol) of δ-phthalimido-α-carbethoxy-γ-valerolactone (Dey, 1937) in 20 mL of anhydrous tetrahydrofuran was added slowly to a suspension of 0.68 g (28.4 mmol) of sodium hydride in 2 mL of anhydrous tetrahydrofuran at 0 °C under a nitrogen atmosphere. The mixture was stirred for 1 h (until the evolution of hydrogen had stopped), and 4.85 g (28.3 mmol) of benzyl bromide was added. The resulting mixture was stirred at room temperature for 1 h and then refluxed for an additional 1 h. The organic solvent was evaporated, and the residue was diluted to 150 mL with methylene chloride, washed with water and saturated sodium chloride solution, and dried over anhydrous magnesium sulfate. Evaporation of the solvent gave an oily residue, which was crystallized from wet ethanol to give 9.2 g (83% yield) of δ-phthalimido-α-benzyl-α-carbethoxy-γ-valerolactone (I): mp 109–110.5 °C; R_{fA} 0.75; NMR ($CDCl_3$) δ 1.25 (t, 3 H, OCH_2CH_3), 2.50 (m, 2 H, lactone CH_2), 3.25 (m, 2 H, $PhCH_2$), 3.50–4.40 (m, 5 H, OCH_2CH_3, NCH_2CHO), 7.20 (m, 5 H, $PhCH_2$), and 7.75 (m, 4 H, Pht).

A mixture of I (5 g, 12.3 mmol) and 15 mL of 6 N hydrochloric acid in 15 mL of ethanol was refluxed for 30 h. After evaporation of the organic solvent, the residue was diluted to 150 mL with water and washed with ether (3 × 50

[1] Abbreviations: Bz, benzoyl; Bzl, benzyl; SD, standard deviation; IC_{50}, inhibitor concentration causing 50% inhibition of a given enzyme concentration at a given substrate concentration; k_{cat}, first-order rate constant for the formation of products from the Michaelis complex; K_i, kinetically determined enzyme–inhibitor dissociation constant; K_m, Michaelis constant; Tris, tris(hydroxymethyl)aminomethane; Z, carbobenzoxy; MS, mass spectrum.

mL). The aqueous phase was evaporated to dryness, and the residue was recrystallized from absolute ethanol to give 2.73 g (92% yield) of the hydrochloride salt of δ-amino-α-benzyl-γ-valerolactone (II): mp 209–211 °C; NMR ([$^2H_6$]-Me_2SO) δ 1.60–2.40 (m, 2 H, CH_2), 2.45–3.60 (m, α-CH, δ-CH_2, *Ph*CH_2), 4.60 (m, 1 H, γ-CH), 7.20 (s, 5 H, *Ph*CH_2), and 8.54 (br s, 3 H, NH_3). A 1.05-g (7.5 mmol) aliquot of triethylamine was added to a vigorously stirred suspension of 0.91 g (3.7 mmol) of II and 0.53 g (3.7 mmol) of benzoyl chloride in 15 mL of methylene chloride at 0 °C. After being stirred for 10 h at room temperature, the mixture was diluted to 150 mL with methylene chloride, was washed successively with water, 0.1 N hydrochloric acid, 5% sodium bicarbonate, and saturated sodium chloride solution, and was dried over anhydrous magnesium sulfate. After evaporation of the solvent, the residue was recrystallized from a mixture of methylene chloride/hexane to give 1.12 g (98% yield) of δ-benzamido-α-benzyl-γ-valerolactone (III): mp 110–115 °C; R_{fA} 0.62; NMR ($CDCl_3$) δ 1.35–2.40 (m, 2 H, β-CH_2), 2.50–4.10 (m, 5 H, δ-CH_2, α-CH, PhC*H*$_2$), 4.40 (m, 1 H, γ-CH), 6.72 (m, 1 H, NH), 7.10 (m, 5 H *Ph*CH_2), and 7.20–7.70 (m, 5 H, *Ph*CO); MS (chemical ionization) *m/e* 310 (M + 1), 338 (M + 29), 350 (M + 41). A mixture of III (0.187 g, 0.62 mmol) and 0.62 mL of 1 N potassium hydroxide in 5 mL of tetrahydrofuran was refluxed for 4 h. After evaporation of the organic solvent, the residue was diluted to 10 mL with water and frozen and lyophilized to give 0.21 g (100%) of IV: mp 122–132 °C; NMR (D_2O) δ 1.50 (m, 2 H, 3-CH_2), 2.66 (m, 3 H, PhC*H*$_2$, 2-CH), 3.20 (m, 2 H, 5-CH_2), 3.60 (m, 1 H, 4-CH), 7.00 (m, 5 H, *Ph*CH_2), and 7.10–7.70 (m, 5 H, *Ph*CO).

(2RS)-5-Benzamido-5-benzyl-4-oxopentanoic Acid (V). V was prepared from IV by the method of Parikh & Doering (1967). Crystallization from a mixture of chloroform/hexane gave pure II in 35% yield: mp 102–104 °C; R_{fB} 0.43; NMR ($CDCl_3$) δ 2.20–3.40 (m, 5 H, 2-CH, 3-CH_2, PhC*H*$_2$), 4.20 (d, 2 H, 5-CH_2), 6.80 (br m, 1 H, NH), 7.10 (m, 5 H, *Ph*CH_2), 7.20–7.80 (m, 5 H, *Ph*CO), and 9.75 (br s, 1 H, OH); MS (chemical ionization) *m/e* 326 (M + 1), 354 (M + 29), 366 (M + 41), 308 (M − H_2O + 1).

(2RS)-5-Amino-2-benzyl-4-oxopentanoic Acid Hydrochloride Salt (VI). A solution of V (0.15 g, 0.461 mmol) in acetic acid (2.3 mL), concentrated hydrochloric acid (2.3 mL), and water (1.15 mL) was refluxed for 12 h. After evaporation of the solvent, crude VI was crystallized from acetonitrile to give 0.076 g (64%) of VI: mp 172–175 °C; R_{fC} 0.51; NMR (CD_3OD) δ 2.35–3.40 (m, 5 H, PhCH_2C*H*CH_2), 3.95 (s, 2 H, NCH_2CO), and 7.20 (s, 5 H, *Ph*CH_2).

(2RS)-4-(N$^\alpha$-Carbobenzoxyglycinamido)-2-benzyl-4-oxopentanoic Acid (IX). Coupling of N$^\alpha$-carbobenzoxyglycine to II with dicyclohexylcarbodiimide gave 5-(N$^\alpha$-carbobenzoxyglycinamido)-2-benzyl-γ-valerolactone (VII) in 77% yield, after crystallization from ethanol: mp 98–103 °C; R_{fA} 0.23; NMR ($CDCl_3$) δ 1.40–2.40 (m, 2 H, β-CH_2), 2.50–3.60 (m, 5 H, δ-CH_2, α-CH, PhCH_2CH), 3.75 (c, 2 H, Gly CH_2), 4.35 (m, 1 H, γ-CH), 5.50 (s, 2 H, *Ph*CH_2O), 5.68 (m, 1 H, Gly NH), 6.80 (m, 1 H, lactone NH), 7.15 (m, 5 H, *Ph*CH_2CH), and 7.28 (s, 5 H, *Ph*CH_2O); MS (chemical ionization) *m/e* 3.97 (M + 1), 4.25 (M + 29), 437 (M + 41). A mixture of VII (0.72 g, 1.81 mmol) and 1.8 mL of 1 N potassium hydroxide was stirred overnight at room temperature. After evaporation of the organic solvent, the residue was diluted to 10 mL with water, frozen, and lyophilized to give crude 5-(N$^\alpha$-carbobenzoxyglycinamido)-2-benzyl-4-hydroxypentanoic acid (VIII).

The Parikh–Doering oxidation of VIII, performed as described for IV, gave 0.1 g (13% yield) of IX after purification by column chromatography (silica gel; chloroform/methanol, 95:5): mp 135–137 °C (crystallization from chloroform/hexane); R_{fB} 0.22; NMR ([$^2H_6$]Me_2SO, 56 °C) δ 2.40–3.40 (m, 5 H, PhCH_2C*H*CH_2CO), 3.90 (m, 4 H, Gly CH_2, NHCH_2COCH_2), 5.10 (s, 2 H, PhCH_2O), 6.50 (m, 1 H, Gly NH), and 7.10–8.30 (m, 12 H, Ph, NH, OH); MS (chemical ionization) *m/e* 413 (M + 1).

5-Benzamido-4-oxo-6-phenylhexanoic acid (X) was prepared as described (Meyer et al., 1981).

2-Benzyl-5-(carbobenzoxyglycinamido)-4-hydroxypentanoic Acid (XI). Ketone IX (63 mg, 0.153 mmol) was dissolved in 7 mL of 80% ethanol, and a 2 molar excess of $NaBH_4$ in 0.5 mL of ethanol was added in one portion. The reaction mixture was stirred at room temperature overnight. Ten milliliters of water was added, and the pH was adjusted to 3.5 with concentrated HCl. The solvent was partially evaporated at room temperature, and the residue was extracted with diethyl ether (3 × 15 mL). The combined extracts were dried (Na_2SO_4), and the solvent was evaporated. The oily residue was purified. After purification on a silica gel column ($CHCl_3$/MeOH, 3:1 (v/v), 18 mg of clear oil was collected (R_{fB} 0.70) and treated with an equimolar amount of 0.2 M NaOH until dissolution was completed. The solution was freeze-dried, giving 21 mg (14% yield) of white solid, mp 109–112 °C.

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 sodium chloride adjusted to pH 7.5 with hydrochloric acid. The suspension of enzyme as received was diluted into the assay buffer to give a stock solution with a calculated concentration of 7 nM on the basis of enzyme weight in the suspension. The k_{cat} values found for the enzyme (see below) confirmed the calculated enzyme concentration. 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 and k_{cat} to be 155 s^{-1} (K_m = 136 μM, k_{cat} = 144 s^{-1}; Suh & Kaiser, 1976). 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. For the inhibitors 2-benzyl-4-(*p*-methoxyphenyl)-4-oxobutanoic acid and IX, the percentage of the inhibition that was of a competitive nature was calculated from the ratio of the V^{-1} coordinate of the intersection point on the Lineweaver–Burk plot [$V^{-1} = V_m^{-1}(1 - \alpha^{-1})$] to the V^{-1} intercept of the line with inhibitor concentration equal to zero (V_m), where α was found to be 3.3 for the former inhibitor and 5 for the latter (Webb, 1963). K_i's were also determined spectrophotometrically with hippuryl-L-phenylalanine as described by Folk & Schirmer (1963) in single experiments. The K_m was 620 μM and k_{cat} was 110 s^{-1}. Since the peptides of Table I are known substrates, they are assumed to be competitive inhibitors with hippuryl-L-phenylalanine as substrate. The K_i of 2-benzyl-4-(*p*-methoxyphenyl)-4-oxobutanoic could not be determined against this substrate due to its strong absorbance at 254 nm.

In determining K_i's for the three peptide substrates, significant hydrolysis of the peptide during the assay would introduce a large error in K_i. The expected amount of hydrolysis

Table I: Dissociation Constants for the Interaction of Three Peptide Substrates and Their Isosteric Ketone and Alcohol Analogues with Carboxypeptidase A Using *O*-(*trans*-*p*-Chlorocinnamoyl)-L-β-phenyllactate as Substrate[a]

	K_i (mean ± SD), mode
PhCONHCH(Bzl)COOH	88 ± 13,[b] competitive
MeOPhCOCH$_2$CH(Bzl)COOH	180 ± 40,[b] 70–100% competitive
MeOPhCHOHCH$_2$CH(Bzl)COOH	190 ± 10,[b] mixed (270, competitive)
PhCONHCH$_2$CONHCH(Bzl)COOH	280 ± 100,[c] competitive
PhCONHCH$_2$COCH$_2$CH(Bzl)COOH (V)	48 ± 7, competitive (80, competitive)
PhCONHCH$_2$CHOHCH$_2$CH(Bzl)COOH (IV)	160 ± 62, competitive (270, competitive)
PhCONHCH(Bzl)COCH$_2$CH$_2$COOH (X)	86 ± 9, competitive
NH$_2$CH$_2$COCH$_2$CH(Bzl)COOH (VI)	1000 ± 500, mixed
Z-NHCH$_2$CONHCH$_2$CONHCH(Bzl)COOH	290 ± 10, competitive
Z-NHCH$_2$CONHCH$_2$COCH$_2$CH(Bzl)COOH (IX)	9 ± 0.1, 82–100% competitive (10, competitive)
Z-NHCH$_2$CONHCH$_2$CHOHCH$_2$CH(Bzl)COOH (XI)	600 ± 100, competitive or mixed

[a] The chiral atoms in the ketones and alcohols are racemic. Peptides all contain L-phenylalanine. The K_i's in parentheses are with hippuryl-L-phenylalanine as substrate. [b] Galardy & Kortylewicz, 1984. [c] Quiocho & Lipscomb, 1971.

during the assays can be calculated from K_m and k_{cat} values for these substrates. For benzoylglycyl-L-phenylalanine, K_m = 1750 μM and k_{cat} = 131 s^{-1} (Folk & Schirmer, 1963). At the highest concentration used (2 mM), less than 0.05% hydrolysis occurred during the first 10 min of the assay, the time period where the initial velocity was measured. For carbobenzoxyglycylglycyl-L-phenylalanine, K_m = 250 μM and k_{cat} = 8000 min^{-1} (Auld & Vallee, 1970). At 2 mM, less than 5% hydrolysis occurred during the first 10 min. Thus, the determination of K_i for these peptides is not significantly affected by their hydrolysis. For benzoyl-L-phenylalanine, $k_{cat}K_m^{-1}$ is so low [<0.1 min^{-1} μM^{-1}, estimated from Hofmann & Bergman (1940)] that hydrolysis must be negligible.

The time course of the inhibition of carboxypeptidase A by 5-benzamido-2-benzyl-4-oxopentanoic acid (V) and 5-(carbobenzoxyglycinamido)-2-benzyl-4-oxopentanoic acid (IX) was determined at single enzyme, substrate, and inhibitor concentrations of, respectively, 60 pM, 36 μM, and 64 μM V or 9.4 μM IX. The velocity of hydrolysis was invariant up to 40 min for both V (at a velocity of 36% of the uninhibited reaction) and IX (at a velocity of 50% of the uninhibited reaction). Recording of initial velocity started within 1 min of addition of enzyme to the reaction. Changes in velocity that might have occurred within this 1-min interval thus were not observed.

Results

Table I gives the dissociation constants for the interaction of the three peptide substrates and their isosteric ketones (competitive inhibition) and alcohols (competitive or mixed inhibition) with carboxypeptidase A. Figure 2 shows a Lineweaver–Burk and a Dixon plot of the inhibition of carboxypeptidase by 2-benzyl-5-(carbobenzoxyglycinamido)-4-oxopentanoic acid (IX), from which the K_i of this inhibitor was determined. For the peptide substrates, k_{cat} increases and K_m decreases as the peptide chain length increases, i.e., hydrolysis becomes more efficient. In contrast, the K_i's of these peptide substrates determined under our assay conditions are similar.

K_i's of both the peptide substrates and their isosteric alcohols remain nearly constant (or increase slightly for the alcohols) with increasing length of the inhibitor. In contrast, the K_i's of the ketones decrease dramatically as the length of the inhibitor increases. This suggests some fundamental differences in the interaction of the substrates and their alcohol isosteres with the enzyme compared to the interaction of the ketone isosteres. For the ketone inhibitor (V), moving the benzyl side chain from the 2-position to the 5-position give 5-benzamido-5-benzyl-4-oxopentanoic acid (X), the ketone isostere of benzoyl-L-phenylalanylglycine. This inhibitor has a K_i approximately equal to that of ketone V. This is surprising

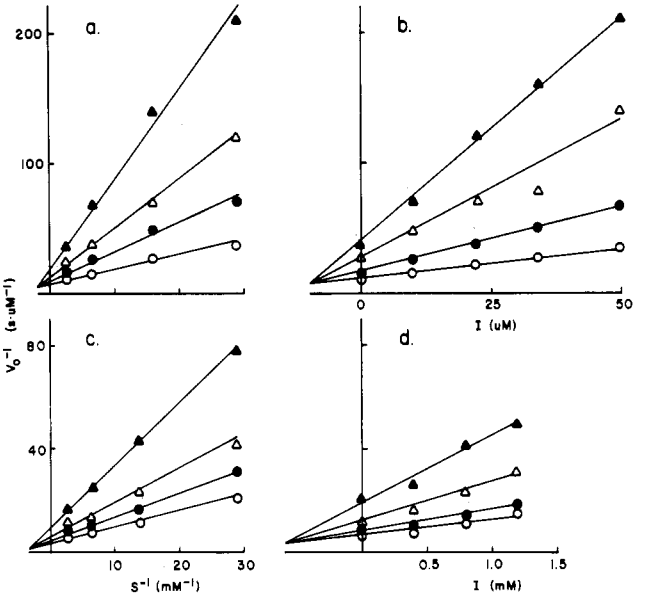

FIGURE 2: Lineweaver–Burk (a and c) and Dixon (b and d) plots of the inhibition of carboxypeptidase A catalyzed hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate by 2-benzyl-5-(carbobenzoxyglycinamido)-4-oxopentanoic acid (IX) (a and b) and 2-benzyl-5-(carbobenzoxyglycinamido)-4-hydroxypentanoic acid (XI) (c and d). Inhibitor concentrations are as follows: (a) (○) 0, (●) 10, (△) 22, and (▲) 49 μM; (c) (○) 0, (●) 0.4, (△) 0.8, and (▲) 1.8 μM. Substrate concentrations are as follows: (b) (○) 380, (●) 150, (△) 64, and (▲) 34 μM; (d) (○) 360, (●) 150, (△) 71, and (▲) 35 μM.

considering the preference of carboxypeptidase A for a terminal phenylalanine compared to glycine (Hofmann & Bergmann, 1940). Since ketone V is a relatively weak inhibitor to begin with, interchanging the side chains to give ketone X simply must not make much difference in its modest interaction with the enzyme. Removal of the benzoyl group from V gives 5-amino-2-benzyl-4-oxopentanoic acid (VI), whose K_i is 20-fold higher than that of V.

The time course of inhibition of carboxypeptidase A by ketones V and IX was determined at the enzyme and substrate concentrations given under Experimental Procedures. No change in initial velocity was observed during 40 min of substrate hydrolysis in the presence of V or IX at concentrations above their K_i's. Therefore, the K_i's reported for these ketones are based on velocity measurements at steady state, which is achieved within 1 min of adding enzyme to a solution of substrate and inhibitor. These ketones do not appear to be "slow-binding" inhibitors of carboxypeptidase A. [For an example of slow-binding inhibitors of the zinc metalloprotease angiotensin-converting enzyme, see Shapiro & Riordan (1984).]

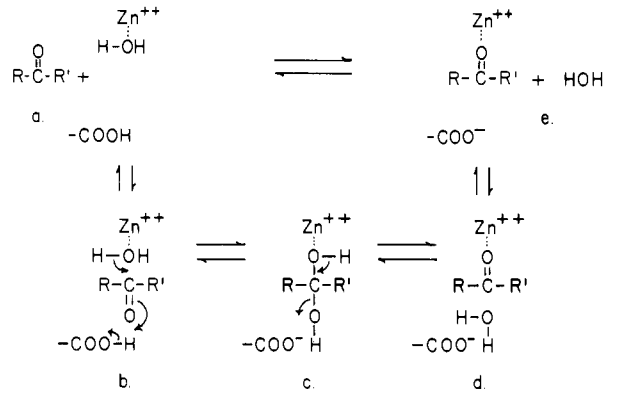

FIGURE 3: Possible modes of binding of ketones to carboxypeptidase A. The carboxyl group is that of glutamic acid-270. The ketone could bind without displacing water from the active site to give b or d. The ketone could displace water from enzyme a to give e directly or could abstract water from the active site proceeding through b and the tetrahedral intermediate c to give e. e was the structure proposed for binding of the ketone (−)-2-benzyl-4-(*p*-methoxyphenyl)-4-oxobutanoic acid to carboxypeptidase A (Rees et al., 1980). c is the structure observed for the binding of 2-benzyl-4-oxobutanoic acid (Galardy & Kortylewicz, 1984) to carboxypeptidase A (Christianson and Lipscomb, personal communication). c could be achieved either via the path a, b, c or the path a, e, d, c. This figure is intended to be only a schematic representation of the binding of carbonyl compounds to carboxypeptidase A. The state of ionization of the tetrahedral adduct in c, for example, could be anionic [the pK_a of $CH_2(OH)_2$ is about 13; Bell, 1966] or binding could proceed from a directly to d. The positions of protons, the position of water molecules, and the participation of other enzyme groups are also not meant to be strictly specified in this scheme.

Discussion

In this study the K_i's of three peptide ketones and the peptide alcohols derived from them were compared with the K_i's of their isosteric peptide substrates. Starting with the poor substrate benzoyl-L-phenylalanine, K_i remained relatively constant with increasing peptide chain length by the addition of one or two glycine residues to the left of the phenylalanine. For the peptide alcohols isosteric with these substrates, K_i remained constant or increased slightly with increasing chain length. K_i for the ketone isosteres decreased significantly with increasing chain length.

The weak binding of the alcohol isosteres was expected from our previous demonstration that 2-benzyl-4-hydroxybutanoic acid binds more than 1000-fold more weakly to carboxypeptidase A than 2-benzyl-4-oxobutanoic acid (Galardy & Kortylewicz, 1984). We attributed this weak binding to the inability of the alcohol to mimic a tightly bound transition intermediate occurring during normal substrate hydrolysis, while the aldehyde can mimic this intermediate by adding an oxygen nucleophile. However, the peptide alcohol pepstatin is an extraordinarily powerful inhibitor of the acid protease pepsin (K_2 = 0.05 nM; Rich & Sun, 1980), and other peptide alcohols have been shown to be potent inhibitors of the zinc metalloprotease angiotensin-converting enzyme with IC_{50}'s as low as 28 nM (Gordon et al., 1985). None of these inhibitors are isosteric with peptide substrates, and the mechanism of interaction with the target enzymes is unknown [see Discussion in Hofman & Fink (1984) and Gordon et al. (1985)]. The present series of peptide alcohols is clearly a different class of inhibitor with K_i's about equal to the K_i's of their substrate isosteres.

The peptide ketones exhibit a trend of inhibition that is quite different from that of their isosteric alcohols. With increasing peptide chain length, K_i drops from 180 to 9 μM. This trend suggests a stronger interaction between the ketones and the enzyme compared to that of the alcohols and substrates. Possible modes of binding of ketones to carboxypeptidase A are shown in Figure 3. Structure e with the carbonyl oxygen atom displacing water from zinc was proposed for the weak inhibitor 2-benzyl-4-(*p*-methoxyphenyl)-4-oxobutanoic acid (Rees et al., 1980), an analogue of the poor substrate benzoyl-L-phenylalanine (see Table I). However, a crystallographic structure at higher resolution now shows that this inhibitor does not bind by displacing water from the zinc (D. S. Christianson and W. N. Lipscomb, Harvard University, personal communication). Structure c has recently been found for the complex of carboxypeptidase A with the strong inhibitor 2-benzyl-4-oxobutanoic acid (Galardy & Kortylewicz, 1984; D. W. Christianson and W. N. Lipscomb, Harvard University, personal communication). The latter inhibitor thus seems to be a true transition-state analogue that binds as a tetrahedral adduct with the nucleophile water. Structures b and d in Figure 3 show two alternate modes of binding without tetrahedral adduct formation and without complete displacement of water from the active site. Substrates and substrate isosteres could bind as in b, d, or e with state c accessible only to transition-state analogues.

We propose that the alcohols and ketones of this study bind in one of the states b, d, or e. The stronger binding of the ketones compared to their alcohol and peptide isosteres is probably not due to formation of a tetrahedral adduct as in c since their electrophilicity is low compared to that of aldehydes (Lewis & Wolfenden, 1977a) and their K_i values are not in the range expected for transition-state analogues. The fact that ketone IX (K_i = 9 μM) develops a K_i 20-fold lower than that of its alcohol or amide isostere must be due to an interaction of its carbonyl group with the enzyme and the fact that a similar state is not accessible to its isosteres. This interaction could be the ability to add water from the active site of the enzyme and then eliminate it via the pathways a–e. Alternatively, the ketone group could interact more strongly simply due to the greater polarization of its carbon–oxygen bond compared to that of the alcohol or amide. Whatever interaction occurs between the ketones and the enzyme must be more favorable with ketone isosteres of better substrates since K_i for the ketones decreases with increasing chain length.

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Reaction of Both Active Site Thiols of Reduced Thioredoxin Reductase with *N*-Ethylmaleimide†

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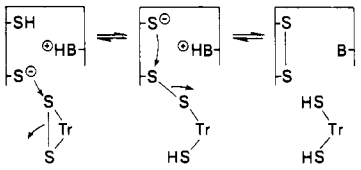
ABSTRACT: Thioredoxin reductase from *Escherichia coli*, only in its reduced state, reacts rapidly with 2 mol of *N*-ethylmaleimide, which specifically alkylates both active site cysteine residues. This dual modification supports previous studies indicating that a base lowers the p*K* of both active site cysteine residues. The dual modification also indicates that the region around the active site dithiol is more open than is the case with the related enzymes lipoamide dehydrogenase and glutathione reductase, both of which can be alkylated only on one nascent thiol. Enhanced nucleophilicity of the active site thiols is consistent with the proposed chemical mechanism of thioredoxin reductase. The sequence of the amino-terminal 16 residues is presented.

Thioredoxin reductase from *Escherichia coli* catalyzes the reversible transfer of electrons between NADPH[1] and the disulfide of thioredoxin, a small protein (Moore et al., 1964). The active center of thioredoxin reductase contains a FAD and an oxidation–reduction active disulfide (Zanetti & Williams, 1967; Thelander, 1968). It is thought that the electrons flow from NADPH to the FAD, from the FAD to the disulfide, and from the dithiol to the disulfide of thioredoxin. On the basis of model studies, electrons would be transferred from the active site dithiol moiety on thioredoxin reductase to the disulfide of thioredoxin in two steps (Scheme I): (1) nucleophilic attack by one enzyme thiol on the disulfide of thioredoxin to form a mixed disulfide bridge between enzyme and substrate and (2) nucleophilic attack by the other enzyme

Scheme I: Roles of the Active Site Thiols of Thioredoxin Reductase in the Reduction of Thioredoxin



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[1] Abbreviations: NEM, *N*-ethylmaleimide; APADP$^+$, oxidized 3-acetylpyridine adenine dinucleotide; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; DTE, 2,3-dihydroxy-1,4-dimercaptobutane; NADPH, reduced nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide; TNB, 5-thio-2-nitrobenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; dpm, disintegrations per minute.