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Inhibition of Angiotensin Converting Enzyme by Aldehyde and Ketone Substrate Analogues†

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ABSTRACT: Three classes of carbonyl-containing substrate analogues and partial substrate analogues have been tested for their ability to inhibit angiotensin converting enzyme. (4-Oxobutanoyl)-L-proline is proposed to occupy the S_1' and S_2' subsites on the enzyme, thus locating its aldehyde carbonyl group at the position of the active site zinc atom. This aldehyde is 70% hydrated in aqueous solution and could mimic a tetrahedral intermediate occurring during enzyme-catalyzed substrate hydrolysis, but its K_i is only 760 μM. Carbobenzoxy-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalaninal is proposed to occupy the S_1 through S_4 subsites on the other side of the zinc atom. Its weak K_i of 60 μM is nearly equipotent to its parent peptide terminating in phenylalanine. However, ketoace, (5*RS*)-(5-benzamido-4-oxo-6-phenylhexanoyl)-L-proline [Almquist, R. G., Chao, W. R., Ellis, M. E., & Johnson, H. L. (1980) *J. Med. Chem. 23*, 1392–1398], one of the third class of inhibitors proposed to occupy subsites S_1 through S_2' on both sides of the zinc atom, has a K_i of 0.0006 μM under our assay conditions, orders of magnitude more potent than its parent peptide. The carbonyl carbon of ketoace is less than 3% hydrated in aqueous solution as determined by carbon-13 nuclear magnetic resonance spectroscopy. If the hydrate is the species bound to converting enzyme, its K_i must be less than 18 pM. Ketoace is a slow-binding inhibitor of converting enzyme, but its overall K_i is dependent on its concentration and therefore prevents calculation of kinetic constants for slow binding. The K_i of (5-benzamido-4-hydroxy-6-phenylhexanoyl)-L-proline, the alcohol analogue of ketoace, is 3 μM. Lengthening ketoace to give (5*RS*)-[5-(carbobenzoxyprolinamido)-4-oxo-6-phenylhexanoyl]-L-proline presumably allows occupation of the additional subsite S_2. However, this lengthened inhibitor has a K_i 10 000-fold weaker than that of ketoace.

Angiotensin converting enzyme is a dipeptidyl carboxypeptidase (EC 3.4.15.1) that catalyzes the hydrolysis of the carboxy-terminal dipeptide histidylleucine from the decapeptide angiotensin I to produce the pressor octapeptide angiotensin II. Several potent inhibitors of the enzyme have been shown to be orally active antihypertensive agents in animals and man (Cushman & Ondetti, 1980; Sweet et al., 1981).

Aldehydes and ketones have been proposed to be transition-state analogues for proteases due to their ability to add either an enzyme-bound nucleophile or a water molecule to form a tetrahedral adduct that mimics a tetrahedral intermediate known to occur in amide hydrolysis [Lewis & Wolfenden (1977a,b) and references cited therein]. Strong inhibition by aldehyde and ketone substrate analogues has been reported for 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) and references cited therein]. α-Fluoro ketones are also powerful inhibitors of converting enzyme and carboxypeptidase A (Gelb et al., 1985). Evidence for formation of a tetrahedral enzyme–inhibitor

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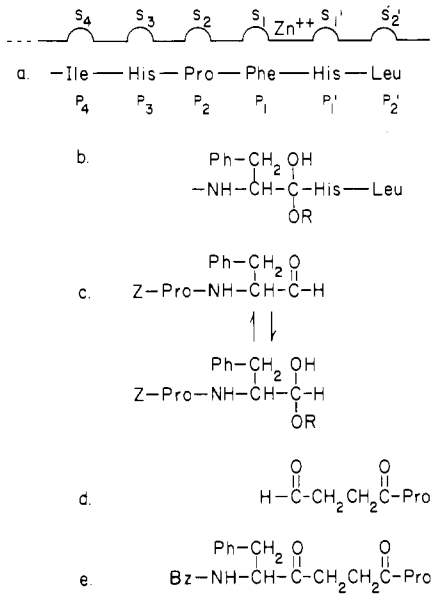

FIGURE 1: A model for the active site of angiotensin converting enzyme adapted from the model of Cushman et al. (1977). S_1 and S_2 are the binding subsites for the amino acid residues P_1 and P_2 to the left of the scissile amide bond and S_1' and S_2' are the binding subsites for the amino acid resiudes P_1' and P_2' to the right according to the nomenclature of Schechter and Berger (1968). (a) The peptide substrate angiotensin I; (b) the tetrahedral intermediate occurring during substrate hydrolysis; (c) an aldehyde inhibitor occupying the S_1 and S_2 subsites; (d) an aldehyde inhibitor occupying the S_1' and S_2' subsites; (e) the inhibitor ketoace.

complex has been acquired by carbon-13 NMR spectroscopy for aldehyde and ketone inhibitors of several classes of proteases but not for zinc metalloproteases [see review by Mackenzie et al. (1984)], thus confirming the transition-state analogy in these cases.

Ketoace, (5*RS*)-(5-benzamido-4-oxo-6-phenylhexanoyl)-L-proline (XXV) (Almquist et al, 1980), is an analogue of benzoyl-L-phenylalanylglycyl-L-proline, which presumably occupys the S_1 through S_2' subsites at the active site of converting enzyme (see Figure 1). The extraordinarily tight binding of ketoace to converting enzyme is confirmed here by the fluorometric single time point assay of the release of histidylleucine from hippurylhistidylleucine as used in Galardy et al. (1983), giving a K_i of 0.0006 μM. We also show here that ketoace is less than 3% hydrated in aqueous solution as determined by carbon-13 nuclear magnetic resonance spectroscopy. Therefore, if the hydrate is the species bound, its K_i must be less than 18 pM. In the continuous spectrophotometric assay using furanacryloyl-L-phenylalanylglycylglycine (Holmquist et al., 1979), ketoace is a slow-binding inhibitor. Two classes of peptide aldehydes designed to occupy either the S_1 through S_4 subsites or the S_1' and S_2' subsites inhibit converting enzyme with weak K_i's not significantly different from their parent peptide analogues.

Experimental Procedures

The synthesis of all of the inhibitors is described in the supplementary material for this paper (see paragraph at end of paper regarding supplementary material). A proton-decoupled carbon-13 nuclear magnetic resonance spectrum of 80 mg of diastereomeric ketoace (XXV) in 1 mL of D_2O at pD 7 and ambient temperature was acquired on the Varian XL-300 instrument in the Department of Chemistry at the University of Louisville.

Angiotensin Converting Enzyme. Converting enzyme was partially purified (Galardy, 1982) from frozen rabbit lungs, the last step of lectin affinity chromatography (Das & Soffer, 1985) being omitted. Yields were lower than those achieved by Das and Soffer. The specific activity of converting enzyme averaged 19 units/mg of protein. One unit of converting enzyme hydrolyzes 1 μmol of hippurylhistidylleucine/min at 37 °C in 100 mM potassium phosphate buffer, pH 8.3, 300 mM in sodium chloride.

Kinetic Studies. All inhibitors were assayed with hippurylhistidylleucine as substrate in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) adjusted to pH 7.5 with sodium hydroxide, 300 mM in sodium chloride (Cheung & Cushman, 1973; Galardy, 1980), by the fluorometric assay of the release of histidylleucine and a single 30-min time point. K_m was 0.5 mM. The reaction was initiated by the addition of enzyme to a final concentration of 0.05 nM. At this enzyme concentration, depletion of inhibitor by enzyme is insignificant (Webb, 1961). Hydrolysis was linear with time to well beyond 30 min with less than 5% total substrate hydrolyzed for all substrate concentrations. K_i's were determined by averaging the K_i's found from a Lineweaver–Burk plot and a Dixon plot. Every K_i was determined at least twice. The standard deviations of the mean K_i's averaged about 30% of the value. These K_i's are compared to K_i's and IC_{50}'s from the literature determined under different conditions of buffer and pH. K_i's determined under different conditions are not strictly comparable, nor are IC_{50}'s and K_i's.

The mode of inhibition was reported as competitive if the Lineweaver–Burk plots intersected on the ordinate and mixed if off the ordinate but not on the abscissa. If multiple determinations of K_i did not clearly discriminate between mixed and competitive inhibition, then the inhibition was reported to be mixed.

Kinetics of Inhibition of Converting Enzyme by Ketoace. The kinetics of the time-dependent inhibition of converting enzyme by ketoace were investigated with the substrate furanacryloyl-L-phenylalanylglycylglycine and the spectrophotometric assay of Holmquist et al. (1979) at 25 °C in pH 75.5 50 mM 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, 0.3 M in NaCl.

K_i*, the overall inhibition constant after slow binding had reached equilibrium, was determined by a Henderson plot as described by Shapiro et al. (1984b) with 2 nM converting enzyme, 50 μM substrate, and 0.5–60 nM ketoace. Velocities at each inhibitor concentration were determined in triplicate. The pre-steady-state kinetics of the slow-binding reaction were also determined as described by Shapiro and Riordan (1984b). The progress curves for slow binding were recorded at various enzyme, substrate, and inhibitor concentrations until conditions for observing slow-binding kinetics were optimized at concentrations of around 3 nM enzyme, 2 mM substrate, and 50–4000 nM ketoace. Approximately 15 s elapsed between starting the reaction by addition of enzyme and starting the recording of absorbance vs. time.

Results

K_i's for Inhibition of Converting Enzyme by Peptide Aldehydes and Peptide Ketones. The K_i's for all of the inhibitors tested against converting enzyme with hippurylhistidylleucine as substrate are reported in Table I. The peptide aldehydes that have amino acid residues presumed to occupy the S_1 through S_n subsites on the enzyme, such as N^{α}-carbobenzoxy-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalaninal (XVI), were not significantly more potent than their peptide analogues with carboxylate in place of the aldehyde group. The qualitative similarity of their K_i's with the K_i's of their parent carboxylic acids and the weak binding observed for all of these

Table I: Inhibition of Converting Enzyme by Peptide Aldehydes and Peptide Ketones with Hippurylhistidylleucine as Substrate

inhibitor	$K_i \pm$ SD (μM), mode
Z-Pro-Phe-OH (III)	2200 ± 1000, competitive
Z-Pro-Phe-H (VIII)	260 ± 50, mixed
Z-Pro-Gly-H (X)	>4000
Z-Pro-Phe-OH (IX)	5500 ± 1000, mixed
Z-Ile-His-Pro-Phe-OH (XII)	20 ± 3, mixed
Z-Ile-His-Pro-Phe-H (XVI)	60 ± 30, mixed
chymostatin	240 ± 100, mixed
CH_3CO-Gly-Pro (XXXV)	800 ± 100, mixed[a]
$HCOCH_2CH_2CO$-Pro (XXII)	760 ± 300, competitive
$HOOCCH_2CH_2CO$-Pro (XXXVI)	6 ± 3[b]
BzNHCH(Bzl)$COCH_2CH_2CO$-Pro (XXV)	0.0006 ± 0.0002, competitive or mixed[c]
$BzNHCH_2COCH_2CH_2CO$-Pro (XXXVII)	2600[d]
CH_2(Bzl)$COCH_2CH_2CO$-Pro (XXXVIII)	2600[d]
BzNHCH(Bzl)$CH(OH)CH_2CH_2CO$-Pro (XXVI)	3.4 ± 0.6, mixed
BzNHCH(Bzl)$COCH_2CH_2CO$-Pro-Ala (XXX)	26 ± 7, mixed
BzNHCH(Bzl)$COCH_2CH_2COOH$ (XXIII)	>5000
Z-Pro-NHCH(Bzl)$COCH_2CH_2CO$-Pro (XXXIV)	3 ± 2, mixed

[a] IC_{50} = 27 000 μM, Petrillo & Ondetti (1982). [b] IC_{50} = 330 μM, Petrillo & Ondetti (1982). [c] Ketoace, literature values for the 5*S* diastereomer: IC_{50} = 0.11 μM, Almquist et al. (1980); IC_{50} = 3.2 nM, Meyer et al. (1981); IC_{50} = 1 nM, Almquist et al. (1982). [d] IC_{50}, Petrillo & Ondetti (1982).

aldehydes suggest that they are binding as structural analogues of their parent peptides and not as transition-state analogues. The best of these aldehydes XVI has a K_i only 4-fold lower than the peptide aldehyde chymostatin, whose sequence is unrelated to angiotensin I. (4-Oxobutanoyl)-L-proline (XXII), designed to occupy the S_1' and S_2' positions with a K_i of 760 μM, is bound only as tightly as its peptide analogue acetylglycyl-L-proline (XXXV) and over 100-fold less tightly than its carboxylic acid analogue succinyl-L-proline. The high K_i of (4-oxobutanoyl)-L-proline suggests that it is not a transition-state analogue. [4-Oxo-4-(trifluoromethyl)butanoyl]-L-proline has a K_i of 15 μM for converting enzyme, possibly due to the lower pK_a of its hydrate compared to that of the aldehyde (Gelb et al., 1985).

Figure 2 shows Lineweaver–Burk and Dixon plots for the inhibition of converting enzyme by ketoace (XXV) and [5-(carbobenzoxy-L-prolinamido)-4-oxo-6-phenylhexanoyl]-L-proline (XXIV). The intersection of the Lineweaver–Burk plots for ketoace suggests nearly competitive inhibition while longer ketone XXXIV is clearly a mixed inhibitor. The K_i observed for ketoace varied with the concentration range of ketoace employed with hippurylhistidylleucine as substrate. At higher ranges of ketoace concentrations, the K_i appeared to be up to 5-fold higher than the K_i that we report here. Dixon plots over wide ranges of inhibitor concentrations therefore exhibited curvature similar to the type found by Shapiro and Riodan (1984a) for other inhibitors of converting enzyme. The complex kinetics of binding of substrates and inhibitors to converting enzyme are described in detail by these authors (Shapiro & Riodan, 1984a,b). Ketoace binds more than 10 000-fold more tightly than its peptide analogue ben-

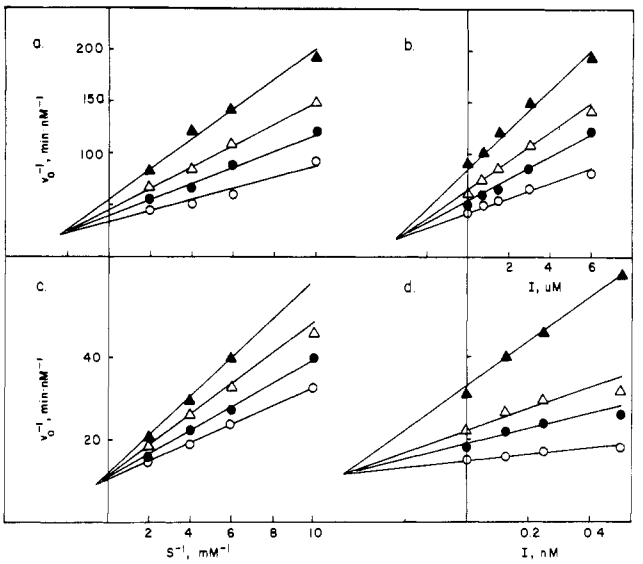

FIGURE 2: Lineweaver–Burk (a and c) and Dixon (b and d) plots of the inhibition of angiotensin converting enzyme by [5-(carbobenzoxy-L-prolinamido)-4-oxo-6-phenylhexanoyl]-L-proline (XXXIV) (a and b) and ketoace (XXV) (c and d). (a) Inhibitor concentrations are (○) 0.0, (●) 0.75, (△) 3.0, and (▲) 6.0 μM. (b) Substrate (hippurylhistidylleucine) concentrations are (○) 0.5, (●) 0.25, (△) 0.17, and (▲) 0.10 mM. (c) Inhibitor concentrations are (○) 0.0, (●) 0.125, (△) 0.50, and (▲) 1.0 nM. (d) Substrate (hippurylhistidylleucine concentrations are (○) 0.5, (●) 0.25, (△) 0.17, and (▲) 0.10 mM.

zoyl-L-phenylalanylglycyl-L-proline (IC_{50} = 9.4 μM; Almquist et al., 1980) and more than 5000-fold more tightly than its alcohol analogue (5-benzamido-4-hydroxy-6-phenylhexanoyl)-L-proline (XXVI). This suggests that ketoace is a transition-state analogue for converting enzyme. The weak binding of alcohol XXVI, in spite of the fact that the alcoholic carbon is tetrahedral, must be due in part to the lack of the second heteroatom (presumably the oxygen of hydroxide) present in the tetrahedral transition state and present in the tetrahedral adduct of ketoace proposed to occur at the active site of the enzyme (see Discussion).

Extension of the peptide chain of ketoace to the right by the addition of alanine in the P_3' position to give XXX raises the K_i by nearly 50 000-fold as expected from the known substrate specificity of this enzyme (Soffer, 1975). That is, the distance between the C-terminal carboxyl and the ketone group in XXX is too great to allow correct alignment of both groups in the active site, and XXX is no longer a transition-intermediate analogue. Likewise, ketone XXIII lacking the C-terminal proline (K_i > 5000 μM) is too short to allow correct alignment of both the carboxyl and the ketone groups. Extension of ketoace by one amino acid to the left to occupy the P_3 position in the inhibitor gives [5-(carbobenzoxy-L-prolinamido)-4-oxo-6-phenylhexanoyl]-L-proline (XXXIV), which has a K_i over 5000-fold greater than that of ketoace.

Ketoace Is Not Significantly Hydrated in D_2O at pD 7. The proton-decoupled carbon-13 NMR spectrum of diasteromeric ketoace [(5*RS*)-(5-benzamido-4-oxo-6-phenylhexanoyl)-L-proline] in D_2O is as follows: δ [ppm downfield from Me_4Si (peak height in mm, assignment)] 23.0 and 24.5 (21 and 28, Pro γ-CH_2), 27.7, 30.3, and 32.0 (18, 26, and 22, Pro β-CH_2 and PhCH_2), 35.0 and 36.1 (10 and 34, $COCH_2CH_2CON$), 47.5 and 48.2 (29 and 31, Pro δ-CH_2), 61.8, 62.0, 61.6, and 62.6 (30, 32, 31, and 28 Pro α-CH, $CHCOCH_2$) 127.3, 127.8, 128.0, 128.7, 132.7, 133.0, and 137.5 (73, 197, 210, 157, 64, 24, and 47, two Ph), 170.2 (18, Pro COO^-), 172.5, 172.8, 180.0, and 180.3 (16, 13, 19, and 20, two $CONH$), and 210.9

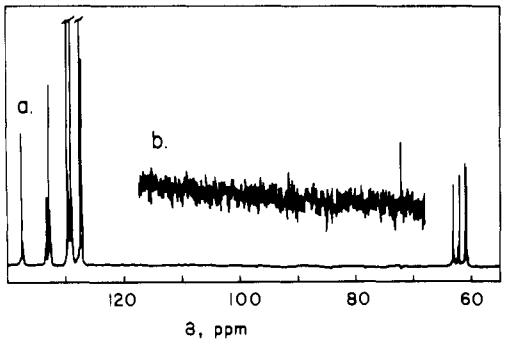

FIGURE 3: (a) Carbon-13 NMR spectrum of diastereomeric ketoace (XXV), (5*RS*)-(5-benzamido-4-oxo-6-phenylhexanoyl)-L-proline, in D_2O at pD 7 from 55 to 114 ppm. (b) No hydrate resonance [$-C(OD)_2$] is visible in the region from 78 to 117 ppm expanded to 10-fold the amplitude of (a). We assign the resonance at 73.0 ppm to chloroform or an artifact. The assignments of 11 resonances are given under Results.

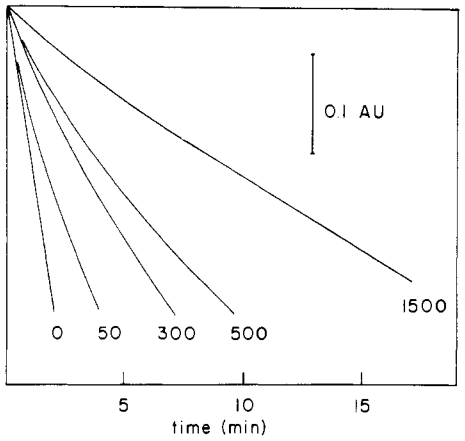

FIGURE 4: Time course of inhibition of converting enzyme by ketoace (XXV): 2 mM furanacryloyl-L-phenylalanylglycylglycine as substrate, 3 nM angiotensin converting enzyme, and 0–1500 nM ketoace. Recording of the hydrolysis of substrate was at 346 nm. Since the absolute absorbance varied enormously with substrate concentration, the bar indicates 0.1 AU on the ordinate scale.

(19, $CHCOCH_2$). This spectrum is in agreement with the partial spectrum reported by Almquist et al. (1980) in $CDCl_3/CD_3OD$. Two resonances are observed for most carbons due to either the presence of two diastereomers or the occurrence of both cis and trans isomers of the acylproline amide bond (Almquist et al., 1980). Figure 3a shows this spectrum from 55 to 114 ppm. No hydrate resonance [$-CH(OD)_2$] is visible in this region. In contrast, (4-oxobutanoyl)-L-proline is 70% hydrated in D_2O at pD 7 as determined by proton NMR at 90 MHz of the aldehyde proton: δ 5.00 [m, 0.70 H, $-CH(OD)_2$] and 9.55 (s, 0.30 H, $-CHO$). The signal at 73 ppm in Figure 3b is too far upfield to be the ketone hydrate. We attribute it to chloroform present in the ketoace (δ 77, neat; chloroform is the crystallization solvent) or to an artifact. The ketone hydrate resonance should appear in the range of 100 ppm on the basis of the following model compounds: acetone hydrate, 95 ppm; "ketopepstatin" adduct, 99 ppm (Rich et al., 1982); fructose hemiacetal, 99.8–107 ppm (Funcke & Klemer, 1976). The ratio of half of the total amplitude of the noise to the peak height of the resonance at 62.6 ppm is 1/60. Assuming that a hydrate resonance of double this intensity could be observed, the extent of hydration must be less than 1/30 or 3%.

Kinetics of Binding of Ketoace to Converting Enzyme. Figure 4 shows the progressive inhibition of converting enzyme by ketoace with furanacryloyl-L-phenylalanylglycylglycine as substrate at concentrations of enzyme, substrate, and inhibitor that tend to optimize the observation of slow-binding inhibition. The difference in velocity at the start of the progress curves (15 s after initiating the reaction by addition of enzyme) and the velocity after the steady state had been reached was only a factor of about 2 at best.

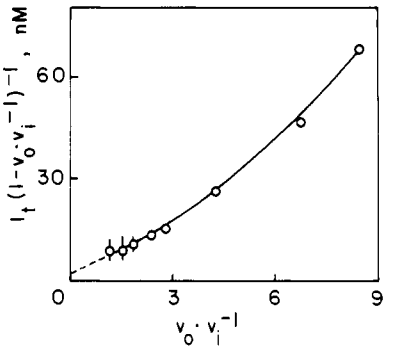

FIGURE 5: Henderson plot of inhibition of converting enzyme (2 nM) catalyzed hydrolysis of furanacryloyl-L-phenylalanylglycylglycine (50 μM) by ketoace (XXV) at 0–60 nM concentration. V_0 is the velocity of hydrolysis without inhibitor, V_i is the velocity of hydrolysis at steady-state conditions at inhibitor concentration i, and I_t is the total inhibitor concentration present. The vertical bars show the range of values observed in triplicate determinations of velocities. For experimental points without bars, the range fell within the size of the point on this graph. The dashed line drawn through the lower part of the curve gives a K_i^* of 6 nM.

Figure 5 shows a Henderson plot for the inhibition of converting enzyme by ketoace with furanacryloyl-L-phenylalanylglycylglycine as substrate from which the overall inhibition constant K_i^* was determined. The marked curvature of the plot demonstrates an increase in K_i^* with increasing inhibitor concentration, as was also observed in the assay using hippurylhistidylleucine as substrate. K_i^* was determined from the lower part of the curve to be 0.006 μM, 10-fold higher than the K_i determined in the fluorometric assay using hippurylhistidylleucine as substrate. The best fit of the points by a curve rather than a straight line was proven by a residual plot (Netter & Wasserman, 1979).

Discussion

Inhibition of Converting Enzyme by Peptide Aldehydes and Peptide Ketones. Peptide aldehyde inhibitors designed to occupy either the S_1–S_4 subsites or the S_1' and S_2' subsites on converting enzyme have weak K_i's near those of their parent peptides and thus do not seem to be transition-state analogues. This result was surprising since an aldehyde designed to occupy the S_1' subsite on carboxypeptidase A (Galardy & Kortylewicz, 1984) and an aldehyde designed to occupy the S_1 subsite on leucine aminopeptidase (Andersson et al., 1982) both exhibited low K_i's in the range expected for transition-state analogues. The high K_i found for (4-oxobutanoyl)-L-proline (760 μM) is especially disappointing considering the low K_i observed for phosphorylglycyl-L-proline (K_i = 0.05 μM; Galardy & Grobelny, 1983), whose structure is analogous to that of the hydrate of (4-oxobutanoyl)-L-proline, which is present as 70% of the total amount in solution at pH 7. Clearly, phosphoramidate and aldehyde analogues of the same substrate-like peptide acylglycyl-L-proline interact very differently with converting enzyme. For carboxypeptidase A, aldehyde and phosphoramidate inhibitors analogous to acyl-L-phenylalanine are nearly equipotent [2-benzyl-4-oxobutanoic acid, K_i = 0.48 μM (Galardy & Kortylewicz, 1984); phosphoryl-L-phenylalanine, K_i = 5 μM (Kam et al., 1979), K_i = 2.1 μM (Holmquist & Vallee, 1979), and K_i = 0.24 μM (D. Grobelny and

R. E. Galardy, unpublished result)]. These two enzymes, then, have very different interactions with aldehyde and phosphoramidate analogues of substrate-like peptides.

The K_i that we report for ketoace (0.0006 μM) is equivalent to the IC_{50} reported by Almquist et al. (1982) of 0.001 μM and in the range reported by Meyer et al. (1981) (IC_{50} = 0.003 μM). Since ketoace appears to be nearly in a steady-state binding equilibrium with converting enzyme under our assay conditions (a 30-min assay), this K_i appears to be close to an equilibrium K_i. The anomalies observed in Lineweaver–Burk and Dixon plots for ketoace over wide ranges of ketoace concentrations described under Results seem to be similar to the complex kinetics of binding of other inhibitors and substrates to converting enzyme (Shapiro & Riodan, 1984a,b). The fact that ketoace has a K_i over 10000-fold lower than the IC_{50} of its parent peptide benzoyl-L-phenylalanylglycyl-L-proline suggests that ketoace mimics a transition state for converting enzyme.

Extension of ketoace by adding proline in the P_2 position yields [5-(carbobenzoxy-L-prolinamido)-4-oxo-6-phenylhexanoyl]-L-proline with a K_i over 50000-fold higher than ketoace itself. This result is unexpected since occupation of one more subsite on the enzyme should decrease K_i by providing an additional binding interaction between enzyme and inhibitor. An analogous result was found for ketone peptide inhibitors of *Clostridium histolyticum* collagenase (Grobelny & Galardy, 1985). For this collagenase, extension of (5-benzamido-4-oxo-6-phenylhexanoyl)-L-prolyl-L-alanine to [5-(carbobenzoxy-L-prolinamido)-4-oxo-6-phenylhexanoyl]-L-prolyl-L-alanine increased the IC_{50} by a factor of 50 instead of causing the expected decrease (or no change). Compounds XXXVII and XXXVIII in Table I demonstrate that both the benzyl side chain and the benzamido group are crucial to the low K_i of ketoace. Replacement of either group with a hydrogen atom raises the K_i by over 10000-fold. Meyer et al. (1981) have shown that only furan-2-carboxamido will substitute for benzamido in ketoace and still preserve the low K_i. Even substitution of carbobenzoxamido for benzamido increases the K_i by nearly 2 orders of magnitude (Meyer et al., 1981). However, Almquist et al. (1985) have shown that substitution of cyclobutylcarbonyl-L-lysinamido for benzamido decreases the IC_{50} by a factor of 10. The widely different binding affinities of ketoace analogues containing various carboxamido groups in the P_2 position is difficult to rationalize.

Time Course of Inhibition of Converting Enzyme by Ketoace. The observed progressive inhibition of converting enzyme by ketoace is unusual in that the maximum pre-steady-state velocities were only about double the final steady-state velocities. In contrast, for captopril and MK-422 these velocities differed by 1–2 orders of magnitude (Shapiro & Riodan, 1984b). We find the same slow-binding kinetics for MK-422 (not shown) as reported by these authors. We also find that progressive inhibition by (phenethylphosphonyl)-L-alanyl-L-proline (Galardy et al., 1983) exhibits kinetics similar to those of MK-422, with a maximum of nearly 2 orders of magnitude difference in pre-steady-state and steady-state velocities (Umesh B. Goli and Richard E. Galardy, unpublished results). The progressive inhibition kinetics of ketoace are not pronounced compared to these two inhibitors and are detected only under carefully optimized conditions, as shown in Figure 3. A further peculiarity of ketoace is the curved Henderson plot shown in Figure 4 from which K_i* was calculated to be 0.006 μM at low inhibitor concentration. The increase in K_i* with increasing ketoace concentrations shown in Figure 4 was also observed in the determination of K_i from Lineweaver–Burk and Dixon plots by the fluorometric assay with a single 30-min time point. The discrepancy between K_i* from Figure 4 (6 nM, spectrophotometric assay) and K_i (0.6 nM, fluorometric assay) is probably due to the different buffers and substrates employed in the two assays. Even if enzyme and inhibitor are not in the steady-state in the fluorometric assay, the K_i determined in this assay is probably near the steady-state value since a maximum difference of only 2-fold was observed in initial and steady-state velocities in the spectrophotometric assay. The strong dependence of K_i* on inhibitor concentration and the small differences between pre-steady-state and steady-state velocities prevents the determination of the mechanism or the rate constants for the slow binding of ketoace.

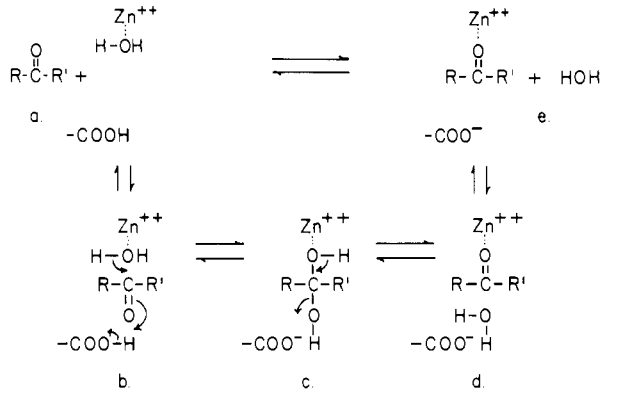

FIGURE 6: Possible modes of binding of ketones to zinc metalloproteases such as angiotensin converting enzyme. The ketone could displace water from the enzyme a to give e directly or could abstract water from the active site proceeding through the tetrahedral intermediate c to give e. e was the structure proposed for binding of the ketone (−)-2-benzyl-4-(3-methoxyphenyl)-4-oxobutanoic acid to carboxypeptidase A (Rees et al., 1980). b is the structure found for this ketone [D. W. Christianson and W. N. Lipscomb (Harvard University), personal communication]. c is the structure observed for the binding of 2-benzyl-4-oxobutanoic acid (Galardy & Kortylewicz, 1984) to carboxypeptidase A (D. W. Christianson and W. N. Lipscomb, personal communication).

Mechanism of Inhibition of Converting Enzyme by Ketoace. Figure 6 shows several possible modes of binding for carbonyl compounds to converting enzyme. The simple active site model of Figure 6 assumes that there is an active site carboxyl group as found by crystallography in the zinc metalloproteases carboxypeptidase A (Quiocho & Lipscomb, 1971) and thermolysin (Kester & Matthews, 1977) and demonstrated by chemical modification for converting enzyme (Harris & Wilson, 1983). The possible modes of binding of carbonyl compounds to zinc metalloproteases shown in Figure 6 are based on X-ray crystallography of the complex of (−)-4-(3-methoxyphenyl)-2-benzyl-4-oxobutanoic acid (Rees et al., 1980) and 2-benzyl-4-oxobutanoic acid [D. W. Christianson and W. N. Lipscomb (Harvard University), personal communication; Galardy & Kortylewicz, 1984] to carboxypeptidase A.

The binding of carbonyl compounds to the enzyme could simply displace water from the active site shown in Figure 6a to give the complex shown in Figure 6e. Although Figure 6e appears to be a Michaelis-type complex, it could be argued that the loss of water from the active site leaves an active site without the ability to hydrolyze (add water to) substrate, and thus incapable of proceeding to products. This kind of complex between glycyl-L-tyrosine and carboxypeptidase A that does not include a water molecule trapped at the active site has been called a nonproductive complex (Kester & Matthews, 1977). Figure 6e therefore could represent this type of non productive

complex. Rees et al. (1980) proposed that the ketone (−)-2-benzyl-(3-methoxyphenyl)-4-oxo-4-butanoic acid binds to carboxypeptidase A as indicated in Figure 6e, i.e., without locating a tightly bound water molecule. However, more recent results at high resolution show that this ketone binds as shown in Figure 6b, without displacing water from the active site [D. W. Christianson and W. N Lipscomb (Harvard University), personal communication].

An alternative pathway from a to e in Figure 6 goes through the intermediates b, c, and d. For the highly electrophilic aldehydes (Galardy & Kortylewicz, 1984) and haloketones (Galardy & Kortylewicz, 1985), the tetrahedral state 6c is populated. For less electrophilic ketones such as 2-benzyl-4-(3-methoxyphenyl)-4-oxobutanoic acid, the tetrahedral structure in Figure 6c can be less stable. The less electrophilic ketones therefore could pass through state 6c to 6d or 6e or bind directly in state b, d, or e without passing through tetrahedral state c.

On the basis of these results with carboxypeptidase A, we propose that ketoace binds to converting enzyme as shown in Figure 6c, i.e., as its hydrate. The evidence for this structure is the extraordinarily tight binding of ketoace (in the range expected for a transition-intermediate analogue, the structure 6c observed for 2-benzyl-4-oxobutanoic acid and carboxypeptidase A, and the tetrahedral complex observed by NMR for the ketone analogue of pepstatin and pepsin (Rich et al., 1982). In spite of the low electrophilicity of ketoace (less than 3% hydrated in water), we propose that it is the tetrahedrally hybridized ketone that is bound to the enzyme. If the tetrahedral hydrate of ketoace is the form that binds to the enzyme, the K_i of this species must be less than 0.03×0.0006 μM or 18 pM. The binding of the tetrahedral form of ketoace, a species not detectable in solution, would be analogous to the demonstrated binding of the tetrahedrally hybridized ketone analogue of pepstatin to pepsin, where only the trigonal planar ketone is observed in the absence of binding to enzyme (Rich et al., 1982). The longer ketone XXXIV may be bound as in b, d, or e of Figure 6 on the basis of its low electrophilicity *and weak* K_i. The aldehyde inhibitors could be bound as in Figure 6c due to their high electrophilicity since they are highly hydrated in aqueous solution. However, their weak K_i's suggest that they lack other elements required for the tight binding normally associated with transition-state analogues. Thus, the presence of the tetrahedral hydrate alone is not sufficient for mimicking a transition state.

In summary, the peptide aldehydes that do not span the binding subsites on both sides of the zinc atom of converting enzyme do not bind with the affinity expected for transition-state analogues. Ketoace, which does span these sites, appears to be a transition-state analogue. If the hydrate of ketoace is the species bound, its K_i must be less than 18 pM since it is present at less than 3% of the total amount of ketoace in solution as determined by carbon-13 NMR. The alcohol analogue of ketoace, although tetrahedrally hybridized, binds more than 5000 times less strongly than ketoace itself. Extension of ketoace to occupy one more binding subsite on converting enzyme by adding a proline residue in the P_2 position yields an inhibitor that is more than 5000-fold weaker than ketoace, contrary to expectations.

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Supplementary Material Available

Syntheses of inhibitors (12 pages). Ordering information is given on any current masthead page.

Registry No. II, 59830-60-3; III, 17350-17-3; IV, 59830-61-4; V, 55707-43-2; VI, 3304-59-4; VII, 99783-31-0; VIII, 88105-67-3; IX, 88084-14-4; X, 99783-32-1; XI, 99783-33-2; XII, 99783-34-3; XII (free acid), 99783-35-4; XIII, 99796-76-6; XIV, 99796-77-7; XV, 99783-36-5; XVI, 4220-65-9; XVIII, 4220-65-9; XIX, 99783-38-7; XX, 98992-63-3; XXI, 99783-39-8; XXIII, 83148-55-4; XXIV, 99828-07-6; XXV, 82336-10-5; XXVI, 97991-89-4; XXVII, 52616-95-2; XXVIII, 99783-41-2; XXIX, 98992-64-4; XXX, 98992-59-7; XXXI, 99783-42-3; XXXII, 98992-62-2; XXXIII, 99783-43-4; XXXIV, 99783-44-5; XXXV, 99783-45-6; XXXVI, 63250-32-8; chymostatin, 9076-44-2; N^{α}-carbobenzoxy-L-prolyl-L-phenylalanine ethyl ester, 18532-06-4; aminoacetaldehyde dimethyl acetal, 22483-09-6; N^{α}-carbobenzoxy-L-prolyl-L-glycinal dimethyl acetal, 98992-52-0; N^{α}-carbobenzoxy-L-proline, 1148-11-4; 4-amino-1,2-dihydroxy-4-phenylbutane hydrochloride, 99783-46-7; N^{α}-carbobenzoxy-L-isoleucyl-L-histidyl hydrazide, 76408-33-8; L-proline benzyl ester hydrochloride, 16652-71-4; 4-oxobutanoyl-L-proline lithium salt, 98992-47-3; 4-oxobutanoyl-L-proline, 99783-40-1; *tert*-butyloxy-L-proline, 15761-39-4; L-alanine benzyl ester hydrochloride, 5557-83-5; N^{α}-carbobenzoxy-L-prolyl-L-phenylalanine (azlactone), 69935-19-9; 3-carbomethoxypropionyl chloride, 1490-25-1; angiotensin converting enzyme, 9015-82-1; acetylglycine, 543-24-8.

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Development of a Radioimmunoassay for Quantitation of Calregulin in Bovine Tissues†

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ABSTRACT: Experimental conditions are described for a convenient and simple one-step method for radioimmunoassay (RIA) of the calcium binding protein calregulin [Waisman, D. M., Salimath, B. P., & Anderson, M. J. (1985) *J. Biol. Chem. 260*, 1652–1660]. The radioimmunoassay utilizes ^{125}I-labeled calregulin and pansorbin cells (*Staphylococcus aureus*) coated with rabbit anti-calregulin antibody. Binding equilibrium is attained in 30 min, and the total time of the assay is 1 h. The assay is sensitive to about 30 fmol of calregulin. Calregulin was quantitated in various bovine tissue extracts and was detected in all tissues except erythrocytes. It was present in particularly high amounts in pancreas (540 μg/g of tissue), liver (375 μg/g of tissue), and testis (256 μg/g of tissue). While about 80% of the total tissue calregulin is soluble, about 20% of this protein was found to be associated with particulate fractions. Unmasking of particulate calregulin required the presence of nonionic detergent. Gel permeation chromatography of bovine brain 100000*g* supernatant in the presence or absence of calcium has resolved a single peak of calregulin by RIA. In addition, the distribution of calregulin in the soluble or particulate fraction of bovine brain was unaffected by the presence or absence of calcium during homogenization, suggesting that calregulin does not form either calcium-dependent or calcium-independent association with soluble or particulate proteins. These results identify calregulin as a major tissue Ca^{2+} binding protein.

The development of techniques to allow quantitation of changes in cytosolic free Ca^{2+} concentrations with varied stimuli has catalyzed a resurgence of interest in the Ca^{2+} second messenger system. Information obtained from intracellular Ca^{2+} indicators such as aequorin (Shimomura et al., 1962) and quin 2 (Tsein, 1980) has provided solid evidence for a second messenger role of Ca^{2+} in many cellular processes. Critical to an understanding of the second messenger function of Ca^{2+} is the elucidation of changes in cytosolic free Ca^{2+} concentration into cellular activation. Considering the millimolar Mg^{2+} concentration of the cytosol (Rink et al., 1982), a prerequisite for the demonstration of a cytosolic calcium binding protein is the binding of calcium with a micromolar dissociation constant in the presence of millimolar Mg^{2+}.

We have begun a systematic analysis of the 100000*g* supernatant of several tissues for calcium binding proteins (Waisman, 1983; Waisman et al., 1983, 1985a,b). Our experimental approach has involved chromatography of tissue 100000*g* supernatant on DEAE-cellulose and analysis of resultant fractions for calcium binding activity by the chelex-100 competitive calcium binding activity assay (Waisman & Rasmussen, 1983). In bovine liver 100000*g* supernatant, we

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