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Thiohemiacetal Formation by Inhibitory Aldehydes at the Active Site of Papain†

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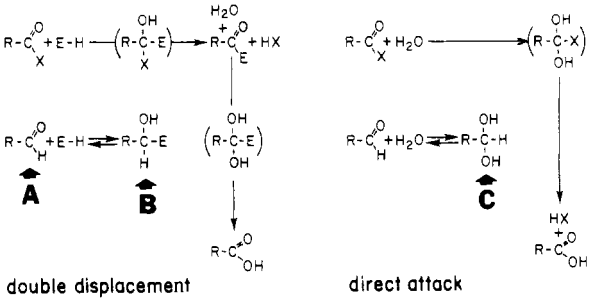
ABSTRACT: Papain is strongly inhibited by aldehydes resembling carboxylic acids released by hydrolysis of specific substrates (Westerik, J. O'C., and Wolfenden, R. (1972), *J. Biol. Chem. 247,* 8195-8197). Inhibitory complexes might involve binding of the aldehyde intact or as a covalent hydrate, or the aldehyde might undergo covalent addition of an active site sulfhydryl group to form a thiohemiacetal derivative. In an attempt to distinguish between these possibilities, benzamidoacetaldehyde-*1-d* has been synthesized, and its properties compared with those of the undeuterated inhibitor. After correction for differences in hydration, the observed effect on inhibition is found to be compatible with formation of a thiohemiacetal. In keeping with this conclusion, benzamidoethanol (a partial analogue of the covalent hydrate) and benzamide, *N*-methylbenzamide and *N*-ethylbenzamide (somewhat similar to the free aldehyde in size and hydrophobic character) are found to exhibit negligible affinity for the active site.

Aldehydes, designed to be capable of forming tetrahedral adducts with nucleophilic residues at the active sites of proteases, are unusually effective reversible inhibitors (Westerik and Wolfenden, 1972; Thompson, 1973, 1974). In the accompanying paper it was shown that peptide-related aldehydes are strongly hydrated, and secondary deuterium isotope effects on equilibrium addition of nucleophiles to aldehydes were compared (Lewis and Wolfender, 1977).

It is of interest to inquire whether aldehydes are indeed bound by enzymes as covalent adducts (Scheme I, case B). Among alternative possibilities, inhibition by an aldehyde hydrate (Scheme I, case C) would accord with a mechanism involving direct attack by water on the peptide bond (Findlater and Orsi, 1973). Aldehydes might also be bound intact, exhibiting unusual affinity in comparison with substrates because of their meager space-filling requirements. This has been suggested as a reasonable basis for the fairly strong inhibition of papain by nitriles (Lowe and Yuthavong, 1971). Recent evidence indicates that several derivatives of cinnamaldehyde are bound without chemical alteration by chymotrypsin (Breaux and Bender, 1975; Gorenstein et al., 1976).

This paper describes experiments designed to discriminate between these possibilities for peptide-related inhibitors of papain, making use of the effect of deuterium substitution on equilibrium addition of nucleophiles to aldehydes, and of analogues with properties resembling other forms in which these inhibitors might be bound.

SCHEME I: Alternative Forms of Bound Aldehyde.[a]



[a] (A) The intact carbonyl compound; (B) a covalent adduct (hemiacetal or thiohemiacetal) formed by addition of a nucleophilic residue at the enzyme's active site; (C) *gem*-diol formed by covalent hydration of the aldehyde. Hydroxyl groups have arbitrarily been drawn in the uncharged state.

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Experimental Section

Aminoacetaldehyde diethyl acetal was synthesized by a modification of the procedures for halogenation of acetaldehyde reported by Shchukina (1948) and the high pressure ammonolysis of chloroacetaldehyde described by Woodward and Doering (1955). Acetaldehyde (2.0 g) was cooled in an ice bath in a flask fitted with an efficient reflux condenser cooled with ice water. Bromine (7.1 g) was added dropwise through a long Pasteur pipet running through the coils of the condenser. A violent exothermic reaction initiated the bromination. Further additions of bromine were decolorized with concurrent evolution of HBr gas until nearly all the material had reacted. The slightly orange solution was allowed to stir in the cold for 15 min, during which the color disappeared. The acetal was formed by dropwise addition of ethanol (15–20 mL) and the mixture was allowed to stir for an additional 45 min. The acetal solution was diluted with 50 mL of deionized water, the pH adjusted to 7 with 1 M NaOH, and the acetal was extracted with several portions of chloroform which were combined and dried over $MgSO_4$. Rotary evaporation at 15 °C yielded 8.86 g of bromoacetaldehyde diethyl acetal (99% theoretical). NMR[1] (90 MHz, $CDCl_3$), 1.3 ppm (t, J = 7 Hz, 6 H), 3.42 ppm (s, 2 H), 3.68 ppm (dp, J = 7 Hz and 2.5 Hz, 4 H). Ammonolysis of the bromoacetal was accomplished in a high pressure hydrogenation bomb, containing a rimless glass liner of 50 mL capacity (Aminco No. 41-12580 or No. 41-19250). The liner and bomb were cooled in a dry ice bath and 5.4 g of acetal, dissolved in 5 mL of methanol, was added to the liner and ammonia gas was collected at −80 °C until a liquid volume of 40 mL had accumulated. This represents an approximately 100-fold excess of ammonia as recommended by Woodward and Doering (1955) to minimize formation of by-products. The cold ammoniacal solution was sealed in the bomb and incubated at 85 °C for 17 h. After the bomb had been cooled in dry ice for several hours, its contents was transferred to a round-bottomed flask. As the solvent was removed, crystalline NH_4Br was forced out of solution by addition of chloroform. Rotary evaporation of the filtrate yielded a dark viscous liquid, aminoacetaldehyde diethyl acetal hydrobromide, in a yield of about 51%: NMR (90 MHz, $CDCl_3$), 1.25 ppm (t, J = 7 Hz, 6 H), 3.06 ppm (s, 2 H), 3.7 ppm (dp, J = 7 Hz and 2.5 Hz, 4 H), 5.05 ppm (broad, 3 H).

Benzamidoacetaldehyde was prepared by a modification of the procedures published by Fischer (1893) and Westerik and Wolfenden (1972). To aminoacetaldehyde diethyl acetal (5.0 g) in 20 mL of chloroform, stirred in an ice bath, was added one-quarter of an aqueous solution of KOH (5 g in 10 mL). The remainder of the KOH was added alternately with 2.1 mL of benzoyl chloride, maintaining the solution basic to pH indicating paper, and the solution was allowed to stir for 3 h. The reaction mixture was extracted with chloroform, the chloroform layer was washed repeatedly with saturated NaCl solution and dried over $MgSO_4$, and solvent was removed by evaporation, yielding 7.4 g of crude benzamidoacetaldehyde diethyl acetal (69% of theory). Molecular distillation of this crude material was performed in a large sublimation apparatus with cold water cooling to yield a clear glass at 115 °C/0.1 mm, 3.4 g of spectroscopically pure benzamidoacetaldehyde diethyl acetal (50% recovery): NMR (90 MHz, $CDCl_3$), 1.25 ppm (t, J = 7 Hz, 6 H), 2.65 ppm (dp, J = 7 Hz and 2.5 Hz, 6 H), 6.35 ppm (broad, 1 H), 7.45 ppm (m, 3 H), 7.83 ppm (m, 2 H). This material was further purified by chromatography on silicic acid (Merck 70–230 mesh). Acetal (250 mg) was added to a column of 28 mL bed volume in carbon tetrachloride. After washing with 200 mL of pure solvent, elution was performed with 2% ethanol in carbon tetrachloride; the acetal emerged after approximately 70 mL.

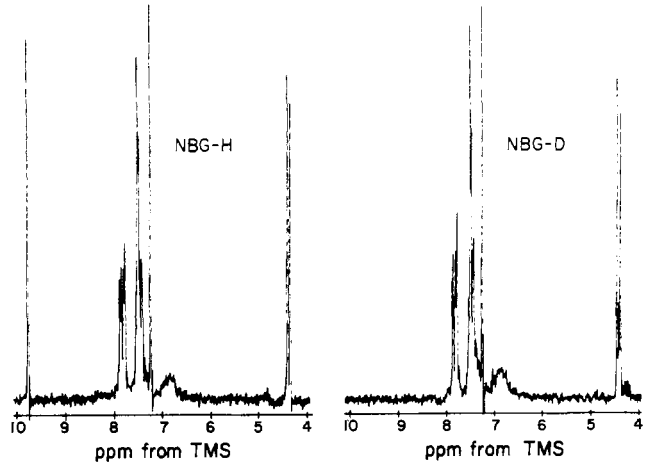

FIGURE 1: NMR spectra (90 MHz, $CDCl_3$) of benzamidoacetaldehyde (NBG-H) and benzamidoacetaldehyde-*1-d* (NBG-D) over the region shifted 4 to 10 ppm downfield from Me_4Si. No other peaks appear at higher field. Assignments: glycyl CH_2 4.3 ppm; amide H 6.8 ppm; $CHCl_3$ 7.3 ppm; aromatic H (meta and para) 7.5 ppm; aromatic H (ortho) 7.8 ppm; aldehyde H 9.8 ppm.

The free aldehyde was generated by stirring the acetal (0.5 g) in a mixture of acetone (50 mL) and 0.02 M aqueous HCl (50 mL) for 25 h at room temperature. After filtration and lyophilization the white crystalline product showed a melting point range of 66–72 °C, dependent upon the amount of water in the crystals; a large proportion of the product was found to be the crystalline *gem*-diol hydrate. Storage in vacuo over P_2O_5 slowly removed the water. NMR (100 MHz, Me_2SO-d_6), 4.06 ppm (dd, J = 5.5 Hz and 0.8 Hz, 2 H), 7.50 ppm (m, 3 H), 7.88 ppm (m, 2 H), 8.95 ppm (t, J = 5.5 Hz, 1 H), 9.55 ppm (t, J = 0.8 Hz, 1 H). Figure 1 shows spectra of the normal and deuterated products.

Papain (250 μL of Worthington suspension) was activated in the presence of 5×10^{-2} M cysteine in 2×10^{-2} M KOAc buffer, pH 5.0, containing 10^{-2} M EDTA (total volume 5 mL), under nitrogen for 45–60 min. The papain solution was then layered onto a Bio-Gel P-2 column (15 mL) previously equilibrated with the acetate buffer. Elution of active, cysteine-free papain from the column began after 2 mL of the eluting buffer had passed through the column. The concentration of papain was estimated from the ultraviolet absorption at 278 nm where a 1% solution of papain has an extinction of 25 (12). Papain activity was assayed at pH 5.5 (4×10^{-2} M KOac, 10^{-3} M EDTA, 0.3 M KCl) using the appearance of ultraviolet absorbance at 340 nm from the hydrolysis of *p*-nitrophenylhippurate ($K_m = 2.5 \times 10^{-5}$ M) (Westerik, 1974). The stock substrate solution (10^{-2} M) was prepared daily in spectroquality acetonitrile and was diluted at least 100-fold into the assay mixture so that the concentration of acetonitrile was always 1% or less. Assays were performed at 25 °C using a Perkin-Elmer 124 spectrophotometer. All rates were corrected for nonenzymatic hydrolysis of the substrate, which typically proceeded at 2% of the observed enzymatic reaction rate. Five replicates were run per set of experimental conditions of substrate and inhibitor concentrations, with the resulting standard deviations in measured initial velocities less than 5%.

Inhibition of papain was studied using standard assay conditions and adding known amounts of stock solutions of the

[1] Abbreviations used: Me_4Si (or TMS), tetramethylsilane; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetracetic acid.

TABLE I: Anticipated Effects of Deuterium Substitution on Inhibition of Papain by Benzamidoacetaldehyde.

		Normal	Deuterated[b]	App $K_i(D)/K_i(H)$
(A) Binding of the intact aldehyde	Fraction[a]	0.078	0.058	
	App K_i	2.5×10^{-5}	3.35×10^{-5}	1.341 ± 0.05
	True K_i	1.95×10^{-6}	1.95×10^{-6}	
(B) Binding as a thiohemiacetal	Fraction[a]	0.078	0.058	
	App K_i	2.5×10^{-5}	2.68×10^{-5}	1.073 ± 0.04
	True K_i	1.95×10^{-6}	1.56×10^{-6}	
(C) Binding as a covalent hydrate	Fraction[a]	0.922	0.942	
	App K_i	2.5×10^{-5}	2.45×10^{-5}	0.979 ± 0.04
	True K_i	2.3×10^{-5}	2.3×10^{-5}	

[a] Fraction of total aldehyde (including both free aldehyde and hydrate) present in the form that would be expected to combine with enzyme. These are based on equilibria of hydration from the accompanying paper (Lewis and Wolfenden, 1977). [b] Values for the deuterated compound are calculated from values for the normal compound, after correcting for their differing fractional hydration. These calculations assume that deuterium substitution would not change the affinity of the enzyme for the intact aldehyde (case A) or for the covalent hydrate (case C), but that binding as a hemiacetal (case B) would result in a true isotope effect of 1.25, corresponding to that observed for thiohemiacetal formation from acetaldehyde, as described in the accompanying paper (Lewis and Wolfenden, 1977). It is assumed that equilibrium isotope effects on oxygen and sulfur addition to benzamidoacetaldehyde are the same as those for acetaldehyde. Small differences would not affect the principal conclusions suggested by these findings. That equilibrium isotope effects are characteristic of the bonds formed, but insensitive to substituents, is indicated by the observed agreement between isotope effects on the addition of water and methanol to pentanal (Hill and Milosevich, 1976).

potential inhibitors to the assay buffer prior to addition of enzyme and substrate. Determination of the secondary deuterium isotope effect on peptidyl aldehyde inhibition of papain required special attention to the relative concentrations of inhibitor stock solutions. Routinely, 1×10^{-4} mol of each of the two acetals were weighed into separate flasks and dissolved in 5 mL of spectroquality acetonitrile. Ultraviolet spectra of 300- and 600-fold dilutions of these solutions were recorded and then 5 mL of 0.02 to 0.1 M HCl was added so that the final HCl concentration ranged from 0.01 to 0.05 M. After reacting 24 h (over 10 half-times), ultraviolet spectra were again recorded at several dilutions of the hydrolysis mixture into acetonitrile, and extinctions at 225 nm were compared to determine the relative concentrations of the two inhibitor solutions. Initial velocities (five replicates) were measured at substrate concentrations in the vicinity of K_m, in the absence and presence of the aldehydes.

Results

To obtain an accurate value for the isotope effect on K_i, it was necessary to obtain very pure preparations of inhibitors. Benzamidoacetaldehyde was chosen because its inhibition constant lies in a range in which conventional kinetic procedures can be used. The synthetic precursor acetaldehyde-*1*-*d* is available commercially, and there was reason to suppose that a distinctive isotope effect would be observed if thiohemiacetal formation accompanied binding, as indicated in Table I.

Benzamidoacetaldehyde diethyl acetal, the precursor of the inhibitor, was prepared by bromination of acetaldehyde, conversion to the diethyl acetal, high temperature ammonolysis, high temperature ammonolysis, and benzoylation by the Schotten–Bauman procedure. After molecular distillation, the product was free of impurities detectable by ^{1}H NMR spectroscopy, and was further subjected to partition chromatography on silicic acid (2% EtOH in CCl_4) in order to remove trace impurities that might have passed undetected.

Identical procedures were used for the synthesis of the normal and the deuterated inhibitor. Quantitation of the inhibitor was performed by determining the ultraviolet absorbance of a standard solution of the diethyl acetal, immediately before initiating its hydrolysis by addition of a very small volume of concentrated HCl. The extinction coefficient of benzamidoacetaldehyde diethyl acetal-*1*-*d* was assumed without proof to be precisely the same as that of the undeuterated compound. This assumption seems reasonable in view of the separation of the benzene chromophore from the position of isotopic substitution and was consistent with the results of gravimetric analysis. In the last step, the free aldehyde was generated by acid hydrolysis of the acetal for 24 h at 20 °C in dilute HCl (0.01 M). Under these conditions the half-time for hydrolysis of the acetal was 150 min. The rate constant for hydrolysis of the deuterated acetal was virtually the same (see also Hill and Milosevich, 1976). Spectra of the aldehyde products are shown in Figure 1. Aliquots of the hydrolysis mixtures were diluted directly into enzyme assay mixtures in kinetic studies of inhibition.

In studies of the inhibition of papain, it was initially confirmed that benzamidoacetaldehyde appears, within experimental error, to be a strictly competitive inhibitor as reported by Westerik and Wolfenden (1972); the same was true for the deuterated inhibitor. Provided with these assurances, it seemed desirable to maximize precision in determining the relative degree of inhibition by the normal and deuterated inhibitor, by comparing their effects under identical conditions permitting most accurate determination of the inhibited and uninhibited reaction rates. In 17 complete experiments on separate days, the activity of papain was first measured in the presence of substrate at a concentration in the neighborhood of K_m. Five replicate measurements were performed. Inhibitor was then added at a concentration sufficient to produce substantial (40–80%) inhibition, and five replicate measurements of the rate were performed. The same procedure was followed with the deuterated inhibitor. Within each set of measurements, the standard deviation in rate did not exceed 3%, and was less than 2.5% in most sets.

In each of 17 experiments, benzamidoacetaldehyde was found to produce slightly more powerful inhibition than its deuterated analogue. K_i values were determined from eq 1

$$\frac{v_i}{v_0} = \frac{1 + (K_m/S)}{1 + \dfrac{K_m(1 + I/K_i)}{S}} \qquad (1)$$

where v_i is the rate of reaction observed in the presence of the

TABLE II: Corrected Dissociation Constants of Inhibitor Complexes of Papain.

	K_i (M)
$C_6H_5CONHCH_2CH_2OH$[a]	≥1.0
$C_6H_5CONHCH_3$	≥5.0×10^{-1}
$C_6H_5CONH_2$	≥2.5×10^{-1}
$C_6H_5CONHCH_2CONH_2$[b]	2.0×10^{-1} (K_s)
$C_6H_5CONHCH_2COOH$[c]	1.7×10^{-2}
$C_6H_5CONHCH_2CH_3$	1.0×10^{-2}
$C_6H_5CONHCH_2COOCH(CH_3)_2$[b]	1.0×10^{-2} (K_s)
$C_6H_5CONHCH_2C{\equiv}N$[a]	3.8×10^{-4}
$C_6H_5CONHCH_2CHO$ (7.8 %)[d]	2.0×10^{-6}
$C_6H_5CONHCH_2CDO$ (5.8 %)[d]	1.6×10^{-6}
Ac-L-Phe-$NHCH_2C{\equiv}N$[e]	7.3×10^{-7}
Ac-L-Phe-$NHCH_2CHO$ (11.2)[d]	5.2×10^{-9}

[a] Data of Lucas and Williams (1969). [b] Data of Lowe and Williams (1965) and Smith et al. (1958). [c] Sluyterman (1964). [d] Values for the free aldehyde, corrected for hydration, calculated from the apparent values of Westerik and Wolfenden (1972). Free aldehyde is indicated here as % of total aldehyde. [e] Data of Westerik (1974).

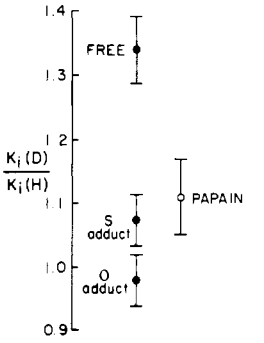

FIGURE 2: Standard deviation of isotope effects expected for various modes of binding of aldehydes, as compared with values actually observed for papain.

inhibitor, v_0 is the rate observed in its absence, and I, S, K_i, and K_m are the concentration of the inhibitor, the concentration of the substrate, the inhibition constant, and the Michaelis constants, respectively.

Average values observed for the inhibition constant were 2.50×10^{-5} M for benzamidoacetaldehyde, and 2.78×10^{-5} M for its deuterated analogue. When ratios were computed separately for each experiment, the mean ratio from the collected values for 17 experiments was $K_i(D)/K_i(H) = 1.11$, with a standard deviation of 0.06.

Benzamide, *N*-methylbenzamide, *N*-ethylbenzamide, and benzamidoacetone were also tested as potential inhibitors. None of them gave detectable inhibition at a concentration of 10^{-2} M, in the presence of substrates at concentrations near K_m, and in several cases these compounds failed to give detectable inhibition even at much higher concentrations (Table II).

Discussion

Papain offers an unusually favorable opportunity to examine the binding of inhibitory aldehydes by proteases, since the presence of sulfur as a potential nucleophile should lead to a distinctive isotope effect if covalent addition occurs. Quite a different effect would be expected if the aldehyde were bound intact. Still another effect would be expected for an oxygen adduct, and it would not be possible, in the case of a serine protease, to distinguish between inhibitor binding as a covalent hydrate or a hemiacetal using the present method. Precursors of aldehydes suited to the specificity of papain are readily available in deuterated form. Other methods might be used to study the structure of the inhibitory complex of papain with inhibitory aldehydes, and such work is in progress. However, papain has proven unusually refractory to the preparation of active site derivatives that are suitable for crystallography (with the exception of the chloromethyl ketone derivative recently described by Drenth et al. (1976)), and the enzyme is not sufficiently soluble to be easily studied by nuclear magnetic resonance spectrometers currently availabe to us.

The method used here relies on several assumptions. First, we assume that the inhibition constant K_i is a true dissociation constant for a competitive inhibitor that undergoes no irreversible chemical modification as a result of its interaction with the enzyme. Second, we assume that inhibitory aldehydes are bound in only one form. This appears reasonable for peptide-related aldehydes that exhibit strong and specific activity for papain (Westerik and Wolfenden, 1972). Third, we assume that substitution of hydrogen by slightly bulkier deuterium does not result in steric hindrance to aldehyde binding. The effect of such hindrance, if it occurred to some small extent, would presumably be to increase K_i for the deuterated inhibitor (relative to the value that would be observed in its absence). So far as we are aware, there is little experimental evidence that might bear on this possibility. However, a major aim of the present study was to discriminate between binding of the aldehyde intact, or alternatively as a covalent adduct. Steric hindrance would be expected to bias the results in such a way as to make it appear that noncovalent binding had occurred, and, since the results in fact appear to argue strongly against this possibility (Figure 2), they are unlikely to have been misleading in this respect.

If the inhibitor were bound as the intact aldehyde, unaltered by covalent addition, then the observed inhibition would be expected to reflect, almost in full, the favorable effect of deuterium substitution on equilibrium hydration of the aldehyde. As a result, the deuterated (and more strongly hydrated) inhibitor would be expected to exhibit a larger K_i (expressed in terms of the total concentration of aldehyde plus hydrate in solution) than the normal inhibitor, with $K_i(D)/K_i(H) = 1.34$ (Table I, case A).

If binding involved formation of a thiohemiacetal at the active site of papain, then deuterium substitution might be expected to disfavor inhibitor binding, because the favorable effect of deuterium on equilibrium addition of sulfur (25%) is somewhat less than its favorable effect on equilibrium addition of oxygen (37%). The expected effect of deuteration on observed K_i values, uncorrected for hydration, would be $K_i(D)/K_i(H) = 1.07$ (Table I, case B).

Noncovalent binding of the aldehyde hydrate (or *gem*-diol) at the active site would be expected to result in a very small difference in K_i between the normal and deuterated inhibitor, corresponding to the slightly greater abundance of hydrate in the deuterated aldehyde when these compounds are compared at the same total concentration in solution, with $K_i(D)/K_i(H) = 0.98$ (Table I, case C). The same result would be expected if the aldehyde were bound as a hemiacetal of a serine protease such as elastase, because an oxygen adduct is present in both cases.

It is evident from these comparisons that secondary isotope effects on K_i are likely to be most useful for distinguishing between covalent and noncovalent binding, whereas the distinction between oxygen and sulfur addition would require reduction of the experimental error in the isotope effect to a much lower level. In the present experiments, the K_i value of benzamidoacetaldehyde-*1*-*d* was found to be slightly larger

than that of its normal analogue, with $K_i(D)/K_i(H) = 1.11$. This result appears to exclude appreciable noncovalent binding of the intact aldehyde and suggests that a thiohemiacetal is formed at the active site, as shown in Figure 2.

Indirect support for this conclusion is also provided by the striking failure of papain to bind analogues that resemble alternate forms of aldehyde. Thus 2-benzamidoethanol, a partial analogue of the aldehyde hydrate, exhibits a K_i value in excess of 1 M, as shown by Lucas and Williams (1969). In the present study it was found that benzamide, *N*-methylbenzamide, and *N*-ethylbenzamide are also very weakly bound. In comparison with substrates, these compounds have relatively minor space-filling requirements, and they are sufficiently hydrophobic (cf. Wolfenden, 1976) that their extraction from solvent should present no serious thermodynamic obstacle. They share these characteristics with aldehydes and nitriles. Table II shows apparent dissociation constants for various complexes of small molecules with papain, corrected for hydration in the case of aldehydes. In the hippuric acid series, the only compound exhibiting an affinity within three orders of magnitude of that of benzamidoacetaldehyde is benzamidoacetonitrile. Lowe and Yuthavong (1971) have suggested that the small size of the nitrile may account for its high affinity; however, the total failure of the benzamides to bind suggests that there may be other explanations. It appears possible that nitriles may also bind covalently at the active site of papain. In that event, it is conceivable that the enzyme might be sufficiently "broad-minded" as to serve as a catalyst for nitrile hydrolysis. Despite repeated efforts, we have been unable to detect such a reaction, and Sluyterman and Wijdenes (1973) report similar failure. It has, however, been observed that L-asparaginase, an enzyme that is also strongly inhibited by aldehyde analogues of the substrate (Westerik and Wolfenden, 1974), catalyzes hydrolysis of the corresponding nitrile (Jackson and Handschumacher, 1970). It seems not unlikely, in view of these findings, that papain undergoes the first stage of this reaction, adding to the nitrile, but that the barrier to further activation is too high for hydrolysis of the imidothioester to proceed at a detectable rate. C–N bond cleavage appears to be the rate-determining step in normal reaction catalyzed by papain (O'Leary and Kluetz, 1972; O'Leary et al., 1974), and even greater barriers may be present in this nonphysiological reaction.

In contrast to the present findings with peptide-related aldehyde inhibitors of papain, cinnamaldehyde derivatives are found to be bound intact by chymotrypsin (Breaux and Bender, 1975; Gorenstein et al., 1976). These inhibitors exhibit somewhat higher affinity than the corresponding substrates but (as indicated by these authors and earlier discussed by Rawn and Lienhard (1974)) these substrates are not among those for which chymotrypsin exhibits high activity as a catalyst. Possibly these rather hydrophobic aldehydes (and the corresponding substrates) are bound in largely nonproductive modes, and it will be of interest to determine whether more specific aldehyde inhibitors of chymotrypsin are also bound intact.

In another study, Findlater and Orsi (1973) have shown that acetaldehyde is a strong reversible inhibitor of a bacterial amidase. Acetaldehyde–ammonia was found to be even more effective, and it was concluded that the hydrate of acetaldehyde is probably the inhibitory species. This possibility is especially interesting because it would accord with a mechanism involving direct water attack on the substrate. Further information about the structure of this inhibitory complex would be desirable, in view of the possibility that acetaldehyde–ammonia, as an aldimine, undergoes covalent addition of an active site nucleophile to generate a tetrahedral adduct.

There is evidence that acyl-enzymes of papain are stabilized, relative to simple thioesters, to an extent comparable with the special stabilization of specific thiohemiacetals by papain (Hinkle and Kirsch, 1971; Westerik and Wolfenden, 1972). It seems not unlikely that the transition state for a partial reaction of papain may be located on the reaction coordinate between the acyl-enzyme and a tetrahedral intermediate, since the enzyme stabilizes complexes with both sp^2 and sp^3 geometry exceedingly well. This would accord with the occurrence of tetrahedral species as metastable intermediates rather than transition states, as postulated for chymotrypsin by Caplow (1969) and Fersht and Requena (1971), and demonstrated recently for elastase and α-lytic protease by Hunkapiller et al. (1976).

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Conformational Studies on Somatostatin and Analogues†

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ABSTRACT: Somatostatin is a 38-membered cyclic tetradecapeptide with the following structure: NH_2-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-COOH. In an effort to increase our knowledge of the conformational aspects of somatostatin in aqueous solution, and to understand better the individual contributions of the four aromatic residues to circular dichroic (CD) spectra and overall conformation, comparative physicochemical studies have been performed on analogues with the following replacements: for phenylalanine, D-Phe^6, D-Phe^7, and D-Phe^{11}; for tryptophan, Gly^8, Ala^8, D-Ala^8, and D-Trp^8. Also, [Leu^{12}]-somatostatin (i.e., replacement of Thr^{12}) was investigated as a derivative unable to form a hydrogen bond via its side chain. Based on the ability of the analogues to inhibit the spontaneous secretion of somatotropin from pituitary cells in vitro, the analogues exhibited varying potencies relative to somatostatin. [D-Trp^8]-Somatostatin was eightfold more potent; [D-Phe^6]-somatostatin, [D-Phe^{11}]-somatostatin, and [Leu^{12}]-somatostatin were about 5 to 15% as potent; and the other analogues were less than 1% as potent as somatostatin. Conformational studies were performed using near- and far-ultraviolet CD spectroscopy in buffered aqueous solution and in 6 M guanidinium chloride (GdmCl), a strong denaturing solvent. Ultracentrifugation, involving sedimentation equilibrium to ascertain monodispersity and an approach-to-equilibrium technique to determine diffusion constants with an estimated error of ±5%, was also employed. Diffusion constants were of particular importance since they permitted calculation of the frictional ratio, f/f_0, i.e., a measure of hydrodynamic asymmetry. The diffusion constants of somatostatin and the analogues ranged between 1.83 and 2.95 $\times 10^{-6}$ cm^2/s, corresponding to f/f_0's of 1.51 to 0.96. On the basis of direct CD spectra, solvent-induced difference CD spectra (i.e., between compounds in aqueous solution and the same with 6 M GdmCl), and frictional ratios, somatostatin and the analogues were grouped into three major classes. Somatostatin, [D-Phe^6]-somatostatin, and [Leu^{12}]-somatostatin appeared to have similar conformations and comparable asymmetries ($f/f_0 \simeq 1.1$-1.2). [D-Ala^8]-Somatostatin, [D-Trp^8]-somatostatin, and [D-Phe^{11}]-somatostatin seemed to have a greater degree of asymmetry ($f/f_0 > 1.3$) and an altered β bend as monitored by difference CD measurements. [Gly^8]-Somatostatin, [Ala^8]-somatostatin, and [D-Phe^7]-somatostatin appeared to have quite different conformations from somatostatin and widely different asymmetries. Based on model construction, these results are consistent with a proposed β bend in somatostatin. The average contribution of a single phenylalanyl residue to the CD spectrum of somatostatin in 6 M GdmCl was estimated to be +18 000 deg cm^2 dmol^{-1} (molar ellipticity) at 219 nm, with a rotational strength of $+1.22 \times 10^{-39}$ (cgs units). Under the same conditions, the contribution of Trp^8 was estimated to be +27 000 deg cm^2 dmol^{-1} (molar ellipticity) at 226 nm; the rotational strength of this band was $+1.47 \times 10^{-39}$ (cgs units). From these results and from the CD spectrum of somatostatin, it is possible to estimate the total (i.e., combined) contribution at 225 nm of the single disulfide and the thirteen peptide chromophores to the ellipticity of somatostatin in the denaturing solvent to be approximately −43 400 deg cm^2 dmol^{-1}. This represents the first study in which estimates of average aromatic contributions to the CD spectrum of a peptide greater than several residues have been obtained in the important spectral region of the peptide chromophore.

Since the original reports on the purification and sequence of ovine hypothalamic somatostatin (Brazeau et al., 1973; Burgus et al., 1973), numerous studies have appeared on the chemistry (Rivier, 1974), conformation (Holladay and Puett, 1975, 1976a), distribution and physiology (cf. Guillemin and Gerich, 1976; Vale et al., 1977) of this cyclic tetradecapeptide. In addition to the inhibition of somatotropin (Breazeau et al., 1973), somatostatin also inhibits the release of other pituitary hormones, the pancreatic hormones, and gut hormones (Vale et al., 1977). These release-inhibitory properties of somatostatin have proven quite useful in clarifying the role of particular hormones in glucose homeostasis (Guillemin and Gerich, 1976; Vale et al., 1977).

Due to the availability of a large number of synthetic somatostatin analogues (Rivier et al., 1976b), it became possible to attempt to refine a recently proposed conformation of somatostatin (Holladay and Puett, 1976a) with new physicochemical evidence. This work is concerned with CD[1] and hydrodynamic studies on synthetic somatostatin and eight (cy-

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[1] Abbreviations used: CD, circular dichroic; GdmCl, guanidinium chloride.