Pepstatin Binding to Pepsin. Enzyme Conformation Changes Monitored by Nuclear Magnetic Resonance†

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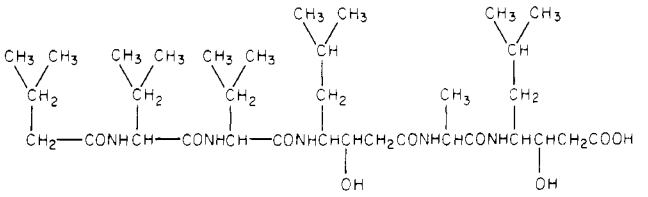
ABSTRACT: The ^1H nuclear magnetic resonance spectrum of porcine pepsin in ^2H_2O reveals a compact, globular molecule, consistent with the crystal structure. When pepstatin, a potent peptide inhibitor of acid proteases, including pepsin (K_i = 4.5 × 10^{-11} M), is added to the enzyme, changes appear in the NMR spectrum. Analysis of difference spectra shows that enzyme resonances are shifted largely due to changes in protein conformation and not directly by the diamagnetic anisotropy of loci on the inhibitor. Pepstatin is isovaleryl (Iva)-Val-Val-4-amino-3-hydroxy-6-methylheptanoic acid (Sta)-Ala-Sta (analogue **1**). Synthetic analogues of pepstatin also induce a pepsin NMR difference spectra. Iva-Val-Val-Sta-Ala-isoamylamide (Iaa) (**2**), *tert*-butoxycarbonyl-Val-Val-Sta-Ala-Iaa (**3**), and Boc-Val-Sta-Ala-Iaa (**4**) produce spectral effects very similar to pepstatin. When the Sta stereochemistry is changed from the natural 3*S*,4*S* to 3*R*,4*S* (analogue **5**), the NMR difference spectrum amplitude is attenuated about 5-fold, and the Boc methyl signal is in a different position compared to that of **4**. Shortening the chain to Boc-(3*S*,4*S*)-Sta-Ala-Iaa (**6**) produced new chemical shifts in the protein spectrum, while a change in stereochemistry to 3*R* (analogue **7**) left a difference spectrum containing essentially only the bound inhibitor resonances. Boc-(3*S*,4*S*)-Sta (**8**) also produced no enzyme conformational changes when bound. The NMR data, combined with steady-state kinetic inhibition results [Rich, D. H., & Sun, E. T. O. (1980) *Biochem. Pharmacol. 129*, 2205–2212], suggest that a general mechanism for pepstatin analogue binding involves formation of an initial enzyme–inhibitor complex EI, followed by an enzyme conformational change to EI*. Analogues **8** and **7** occupy only the first complex, **5** is partitioned 20% in the second complex, and **1**–**4** have complete access to EI*.

First isolated from cultures of actinomycetes by Umezawa et al. (1970), a compound they named pepstatin was found



pepstatin A

to be a potent inhibitor of carboxyl (acid) proteases (McKown et al., 1974). The hydrophobic pentapeptide bristles with branched aliphatic side chains and includes two copies of the unusual amino acid, statine (Sta),[1] at positions 3 and 5 in the sequence. Since its discovery, pepstatin has been shown to inhibit renin (McKown et al., 1974), cathepsin D (Aoyagi et al., 1971), and several acid proteases from microorganisms. Binding of the peptide to pig pepsin is exceptionally strong with a K_{diss} of 4.5 × 10^{-11} M (Workman & Burkitt, 1979). X-ray crystallographic data show that pepstatin binds to the active site of the acid protease from *Rhizopus chinensis* (Subramanian et al., 1977). Although X-ray evidence is presently lacking, the peptide is most likely a competitive inhibitor of pig pepsin as well (Rich & Sun, 1980). Exceedingly tight binding and its specificity for acid proteases are factors which led to the proposal that pepstatin is a transition-state analogue for pepsin (Marciniszyn et al., 1976).

Recent work from one of our laboratories has focused on the kinetics of inhibition of pepsin by pepstatin and synthetic analogues of the peptide (Rich & Sun, 1980). Inhibition is 1000–4000-fold weaker when the hydroxyl groups of the statine (Sta) residues are eliminated (Rich et al., 1977) or when chirality is changed from 3*S*,4*S* to 3*R*,4*S* on the interior statine, confirming a crucial role for the hydroxyl group (Marciniszyn et al., 1976). But the full effect of the Sta OH is seen only in sufficiently long peptides. Increasing chain length by addition of hydrophobic residues to the N terminus increased binding strength up to 10000 fold [for the change Ac-(3*S*,4*S*)-Sta-Ala-NH-*i*-C_5H_{11} to Iva-Val-(3*S*,4*S*)-Sta-Ala-NH-*i*-C_5H_{11}]. The latter example suggests key interactions for hydrophobic side chains near the N terminus.

Using a heptapeptide substrate, Phe-Gly-His-Phe(NO_2)-Phe-Ala-Phe-OMe, we discovered a remarkable characteristic of pepstatin inhibition and that of a select few analogues. When the reaction was initiated by addition of enzyme, these compounds developed maximal inhibition only after a lag period which, for pepstatin, was on the order of minutes when the pepstatin concentration was as high as 6 times the enzyme concentration. No pepsin activity was observed after 5-min preincubation of pepstatin with enzyme when substrate was added to initiate the reaction and enzyme and inhibitor were at a 1:1 molar ratio (Rich & Sun, 1980). On the other hand, Kitagishi et al. (1980) have observed a relatively fast first-order process (k = 600 s^{-1} at pH 5.0) for the binding of SPI to pepsin by stopped-flow kinetic studies. (SPI has the same structure as pepstatin except that the Iva group of pepstatin is replaced by an acetyl group.) Thus, it appears that both fast and slow first-order processes are involved in the binding of pepstatin-like molecules to pepsin.

Questions about the structural origin of these processes in pepstatin binding and possible conformational changes in the

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[1] Abbreviations: Iva, isovaleryl; Sta, 4-amino-3-hydroxy-6-methylheptanoic acid; Iaa, isoamylamide; SPI, *Streptomyces* pepsin inhibitor; TNS, 6-*p*-toluidinylnaphthalene-2-sulfonate. All amino acids are of the L configuration. Standard abbreviations for amino acids, protecting groups, and peptides as recommended by the IUPAC–IUB Commission on Biochemical Nomenclature are used.

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Table I: Structures and Kinetic Constants for Pepstatin Analogue Inhibitors of Pepsin

no.	compd	K_i (M)	time-dependent inhibition	diff spectrum type[a]
1	Iva-Val-Val-(3*S*,4*S*)-Sta-Ala-Sta (pepstatin A)	4.57×10^{-11} [b]	yes	P
2	Iva-Val-Val-(3*S*,4*S*)-Sta-Ala-Iaa	$\sim 10^{-10}$ [c]	yes	P
3	Boc-Val-Val-(3*S*,4*S*)-Sta-Ala-Iaa	$< 6.7 \times 10^{-9}$ [d]	yes	P
4	Boc-Val-(3*S*,4*S*)-Sta-Ala-Iaa	1.7×10^{-7}	no	P
4a	Iva-Val-(3*S*,4*S*)-Sta-Ala-Iaa[e]	1.1×10^{-9}	yes	
5	Boc-Val-(3*R*,4*S*)-Sta-Ala-Iaa	ND[f]		F
5a	Iva-Val-(3*R*,4*S*)-Sta-Ala-Iaa[e]	2.4×10^{-6}	no	
6	Boc-(3*S*,4*S*)-Sta-Ala-Iaa	4.5×10^{-6}	no	B
6a	Iva-(3*S*,4*S*)-Sta-Ala-Iaa[e]	3.4×10^{-7}	no	
7	Boc-(3*R*,4*S*)-Sta-Ala-Iaa	$> 3 \times 10^{-5}$ [g]	no	N
8	Boc-(3*S*,4*S*)-Sta	ND		N
8a	Ac-Sta[e]	1.2×10^{-4}	no	

[a] Characteristics of difference spectrum produced by a 1:1 complex of pepsin and inhibitor: P = pepstatin-like; F = fraction of P; B = unique to 6; N = no change other than peptide peaks. [b] K_{diss} (Workman & Burkitt, 1979). [c] Kinetic behavior is essentially identical with that of pepstatin in our assay system. [d] Solubility of inhibitor in the assay system leads us to believe this is a maximum value. Apparently inhibitor slowly precipitates from a 1% MeOH–buffer solution even at concentration as low as 10^{-8} M. [e] From Rich & Sun (1980). [f] Not determined. [g] At this concentration, no inhibition was observable. Solubility of inhibitor prevented use of high concentrations.

enzyme accompanying inhibitor interactions led to the present ^1H NMR study. Only a few unambiguous assignments of peaks can be made in the pepsin NMR spectrum since the enzyme is relatively large (34000 daltons) and thus contains an abundance of residues leading to broad, overlapping peaks. When pepstatin is added on a 1:1 molar basis, changes in the pepsin spectrum are barely discernible. However, NMR difference spectroscopy (Dwek et al., 1975) clearly reveals alterations in the trace. We show that these differences arise mostly from changes in the protein conformation, along with contributions from protons of pepstatin itself. The eight pepstatin analogues used in this study provoke four distinct classes of difference spectra, allowing for a detailed comparison of inhibition constants and other kinetic data with structural parameters of the inhibitor–enzyme complex.

Materials and Methods

Enzyme. Pig pepsin for NMR studies was prepared from pepsinogen (Sigma Chemical Co., St. Louis, MO 53178) by the method of Rajagopalan et al. (1966). Prior to activation, pepsinogen amide protons were exchanged for ^2H by incubation of the enzyme at pH* 9.3 and 40 °C for 2 h in a buffer of 50 mM borate in ^2H_2O. The mixture was then neutralized with 0.8 M acetate buffer, pH 4.5. Pepsinogen undergoes a reversible, partial denaturation at alkaline pH (Perlmann, 1963), a situation highly favorable to exchange of interior peptide amide protons in globular proteins (Markley & Porubcan, 1976). So that pepsin could be obtained, deuterium-exchanged pepsinogen was incubated at pH 2.0 in 0.55 M citrate buffer (^2H_2O) at 14 °C for 10 min. The resulting mixture of pepsin and small peptides was brought to pH 4.4 with 4 M acetate buffer, pH 5.2, in ^2H_2O, applied to a column of Sephadex C-25, and eluted at 4 °C with 0.4 M acetate buffer (H_2O), pH 4.4 (Rajagopalan et al., 1966). The pooled pepsin fractions were dialyzed against H_2O in the cold and then lyophilized. Enzyme activity was measured in the hemoglobin assay (Anson & Mirsky, 1932).

Peptides. Pepstatin A was from Sigma Chemical Co. It appeared >95% pure based on NMR spectra in CH_3OH-d_4 and $(CH_3)_2CO$-d_6. Synthetic analogues of pepstatin were prepared from optically pure synthetic (*tert*-butyloxycarbonyl)statine (Rich et al., 1978) by using a stepwise solution strategy. Their structures are listed in Table I. Each analogue was fully characterized and judged to be pure by amino acid analysis, microanalysis, mass spectrometry, TLC, and NMR. Details of the syntheses have been reported (Rich et al., 1980; Rich & Bernatowicz, 1982).

Stock solutions of 10 mM peptides were made up in CH_3OH-d_4 (Aldrich Chemical Co., Milwaukee, WI 53233). Aliquots were added to pepsin solutions in the NMR tube with a long needle microsyringe. The NMR sample was gently mixed after each addition by streaming it along the length of the tube several times.

NMR Measurements. Proton NMR spectra were run at 270 MHz with a Bruker Instruments magnet and probe (5 mm), Nicolet 1180 data system, and home-built, quadrature rf transmitter–receiver system. Ninety-degree pulses were generally used with wait times between scans sufficient to allow at least 90% recovery of all protein peaks. Difference spectra were produced through use of the standard Nicolet software. Appropriate corrections were made for concentration changes due to addition of reagents.

Chemical shifts were referenced to the omnipresent H^2HO peak and are expressed relative to DSS. A separate calibration established the H^2HO–DSS chemical shift as a function of temperature. Most spectra reported here were run at the ambient probe temperature of 28 °C where H^2HO is at 4.77 ppm from DSS.

Protein solutions for NMR were generally 0.5 mM. Higher concentrations of pepsin showed evidence of aggregation, particularly at pH* values of 4 or less. Much lower concentrations required extended time averaging, and since pepsin is a proteolytic enzyme, there was the problem of autolysis. Ample signal-to-noise ratios were obtained in 10 min or less of time averaging, a short enough period to avoid detectable proteolysis.

Buffers. Pepsin spectra were taken in the pH range 3.5–6.5. We used 50 mM oxalate in the range 3.5–4.5 and 50 mM pyrophosphate at the higher end of the range. These buffers served well and contributed no nonexchangeable hydrogen resonances. In several cases no buffer was used; there was no detectable difference in NMR spectra for pepsin in comparing a sample at the same pH with or without buffer. Addition of 2 mM EDTA did not change the protein spectra, suggesting that paramagnetic metal ions are not a problem.

Values of pH in ^2H_2O solutions were measured on a Radiometer Model 26 by using a thin Ingold combination electrode standardized in H_2O buffers. Reported values are uncorrected for the deuterium glass electrode isotope effect and are denoted pH*.

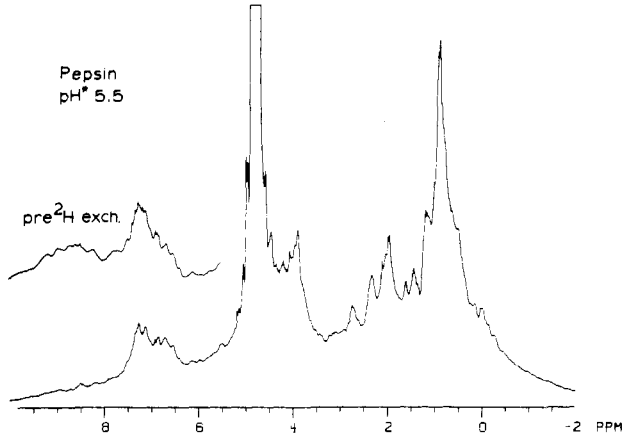

FIGURE 1: ^1H NMR spectrum of porcine pepsin. Pepsin, 0.5 mM in ^2H_2O, was from ^2H-exchanged pepsinogen, pH* 5.5, 23 °C. NMR parameters were 256 scans, 3-s pulse repetition period, and 90° pulses. The free induction decay was weighted by an exponential corresponding a 1-Hz line broadening. The inset shows the aromatic region of pepsin without ^2H exchange of buried amide protons.

Kinetic Studies. Kinetic constants were determined by using a heptapeptide substrate, Phe-Gly-His-Phe(NO_2)-Phe-Ala-Phe-OMe, as previously described (Rich & Sun, 1980; Rich et al., 1977). Hydrolysis of the substrate was measured at 25 °C in pH 4.0, 0.04 M formate buffer. Porcine pepsin (2 × crystallized and lyophilized, lot 83C-8080) used for kinetic studies was purchased from Sigma Chemical Co. Enzyme concentration was determined by its UV absorbance at 278 nm with $\epsilon = 5.17 \times 10^4$ M^{-1} cm^{-1}. Enzyme concentrations used ranged from 18 to 25 nM. Inhibition constants (K_i) were determined from IC_{50} values taken from plots of v_i/v_0 vs. inhibitor concentration where v_i is inhibited velocity and v_0 is the velocity in the absence of inhibitor. The IC_{50} values were converted to K_i by the following equation for competitive inhibition (Cha et al., 1975)

$$K_i = (IC_{50} - E_t/2)(1 + S/K_m)^{-1}$$

where E_t = total enzyme, S = substrate concentration, and K_m = Michaelis constant of the substrate.

Results

^1H NMR Spectra of Pepsin in ^2H_2O. A spectrum of pepsin at pH* 4.3 in ^2H_2O is shown in Figure 1. Like other proteases (Markley & Porubcan, 1976) and many proteins with extensive β structure, a large percentage of pepsin peptide NH protons do not readily exchange for ^2H in ^2H_2O under moderate conditions of pH and temperature. The result is a broad background of resonances in the aromatic region which persists for days and which, for this study, was an interference (Figure 1, inset). The best way to eliminate these peaks is to denature a protein in ^2H_2O (Markley & Porubcan, 1976), but pepsin does not readily denature reversibly. However, its *zymogen* does denature reversibly above pH 9 (Perlmann, 1963) so we took advantage of this fact to introduce ^2H into exchangeable sites on the interior before activating pepsinogen (whereupon 44 amino acids are cleaved from the N terminus) to yield pepsin. Enzyme from the activation of pepsinogen did not contain the partial proteolysis peptide fragments found in commercial pepsin preparations and had the highest possible specific activity.

The C-ϵ_1 proton of the single His-53 is visible in Figure 1 near 8.5 ppm, a value appropriate for the protonated form (Wuthrich, 1976). This peak is well resolved in spectra of pepsin produced from ^2H-exchanged pepsinogen. It is the only peak that persists below 7.5 ppm in partial transverse relaxation spin-echo spectra after a delay time of 20 ms, consistent with the His C-ϵ_1 assignment (Campbell et al., 1975). Over the pH* range of 3.5–6.5 the peak has no change in chemical shift (within ±0.02 ppm). Below pH* 3.5, pepsin is not soluble to NMR and above pH* 6.5 the protein rapidly denatures irreversibly (Bovey & Yanari, 1960). At pH* 7 the C-ϵ_1 peak appears at 8.26 ppm, reflecting a more normal His pK_a (Markley & Porubcan, 1976) for denatured pepsin. Line widths throughout the spectrum increase progressively from pH 4 to 3.5, indicating aggregation at the lower pH values.

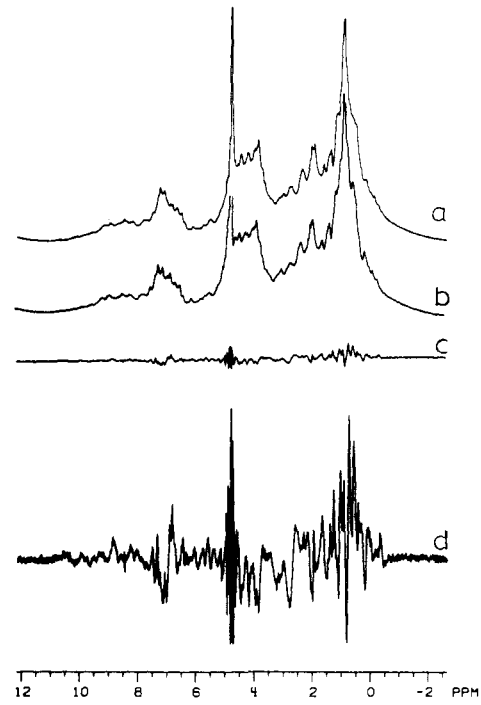

FIGURE 2: Pepstatin-induced difference spectrum. (a) Pepsin in ^2H_2O, 0.5 mM, pH* 4.3; 400 scans, 2-Hz line broadening. (b) 0.5 mM pepsin plus 0.5 mM pepstatin. The peptide (25 μL) was added as a stock solution of 10 mM in CH_3OH-d_4, then the sample was lyophilized, and 500 μL of ^2H_2O was readded. NMR parameters as in (a). (c) (a) – (b) at same vertical readout scale as in (a) and (b). (d) Spectrum c with vertical readout gain increased 10×.

Pepstatin Binding to Pepsin. When pepstatin (10 mM in CH_3OH-d_4) is added in aliquots to a 0.5 mM pepsin solution at pH* 4.5 the inhibitor binds and produces changes in the NMR spectrum. Parts a and b of Figure 2 show the pepsin spectrum with and without bound pepstatin. The differences are subtle but become apparent in the *difference spectrum* (Figure 2c). When the vertical gain is increased (Figure 2d) the difference spectrum can be seen more readily. There are changes in chemical shifts of pepsin methyl (or possibly methylene) groups as shown by the dispersion peaks between 0 and 1 ppm. Near 2 ppm it appears that a singlet is shifted by pepstatin binding. We assign this to a methionine methyl. Pepstatin protons contribute substantially to the spectrum too. The region between 0.5 and 1.5 probably contains most of the 11 pepstatin side chain methyl group resonances.

The aromatic region shows evidence of a number of protons having been displaced relative to other anisotropic groups. The region near 7.1 ppm forms a distinctive "W" shape that is characteristic of the pepstatin–pepsin interaction. Since pepstatin has no aromatic groups of its own, the changes in the region 5–9 ppm might be expected to arise largely from changes in the pepsin conformation and not from a direct effect of pepstatin residues. Data on the binding of other peptides (vide infra) support this conclusion. The histidine C-ϵ_1 proton at 8.48 ppm in pepsin is shifted only slightly downfield to 8.5,

but it shows up clearly in a difference spectrum because of the very narrow line width.

Comparing the area above and below the baseline in a difference spectrum, with the area of a defined region in a normal spectrum, leads to an estimate of the number of nuclei contributing. Approximately 160 protons make up the aromatic region of a pepsin spectrum between 6 and 8 ppm when amide protons are exchanged for ^2H. From this we find 10 ± 2 protons in the aromatic region difference spectrum of pepsin plus pepstatin (i.e., area equivalent to 10 protons both above and below the base line). Possible errors in this value arise mostly from uncertainties in defining a proper base line under the carbon-bound aromatic proton spectrum.

In the aliphatic region from −1 to 1.8 ppm the analysis yields 14 ± 6 protons below the base line and 47 ± 5 above. The extra area above comes from resonances of the inhibitor itself. The greater estimated uncertainty in these values is in large part due to the difficulty of drawing a resonable base line since the difference spectra are highly susceptible to small variations in phase, offset, and relative amplitude in the methyl region.

In general, a peak appears in the difference spectrum if its shift due to pepstatin is on the order of, or exceeds, its line width. Because of cancellations, fewer peaks will be found than are actually shifted, so exact quantitation of the number of peaks which move is probably impossible at this stage. Instead, we have used difference spectra mostly as a *qualitative* measure of the peptide–protein interaction.

Difference spectra generated by less than 1:1 ratios of pepstatin:pepsin are simply lower amplitude versions of the 1:1 complex. Difference peaks do not shift as peptide is added, and no peaks are exchange broadened. This implies that the peptide is in slow exchange on the NMR time scale; a lower limit of $\tau_{ex} \gg 0.1$ s can be set for the exchange lifetime, based on the His C-ϵ_1 proton shift of only 8 Hz. In fact, the lifetime from the earlier kinetic analysis (Rich & Sun, 1980) is $\tau \cong 2 \times 10^4$ s. As aliquots of pepstatin are added to pepsin, the difference spectrum increases in amplitude up to a 1:1 molar ratio (data not shown). Further addition of pepstatin results in precipitation of the very hydrophobic peptide and no further change in the difference spectrum, implying a 1:1 stoichiometry.

Pepstatin and most of the analogues used are virtually insoluble in H_2O. They were added to pepsin solutions as 10 mM stocks in CH_3OH-d_4 to a final concentration of 5% methanol (v/v) or 1.2 M. But methanol is a known inhibitor of pepsin (Tang, 1965) with a K_i of about 0.6 M. All the analogues will displace the alcohol from the active site; however, a general effect of methanol on protein structure needs to be considered (Tang, 1965). Addition of 5% CH_3OH-d_4 alone to pepsin produced an NMR difference spectrum whose amplitude was only 10% of that produced by pepstatin. The features of the methanol difference spectrum did not match the pepstatin-induced changes, except for the residual protonated methyl peak of CH_3OH-d_4. Finally, lyophilization, followed by readdition of ^2H_2O only, produced a spectrum very similar to that before drying, lacking only the methanol peak and possibly having a somewhat different pattern near 1 ppm, but that is a difficult region to deal with since the protein has its highest amplitude there. Methanol by itself does have detectable effects on pepsin structure, but these effects are small and do not appear to influence structural changes produced by pepstatin.

Pepstatin Analogues. The synthetic analogues used are shown in Table I. They were added in CH_3OH-d_4 solution to pepsin solutions in the same manner as pepstatin. Compound **2** lacks only a 3-hydroxypropionic acid group bonded to the C-terminal Sta of pepstatin but is otherwise identical. It induces a difference spectrum almost superimposible on that of pepstatin (Figure 3b). In general, isoamylamide (Iaa) substitution for Sta at the carboxyl terminus of pepstatin analogues has a minimal effect on K_i (Rich & Sun, 1980; Rich & Bernatowicz, 1982) and likewise on NMR spectra.

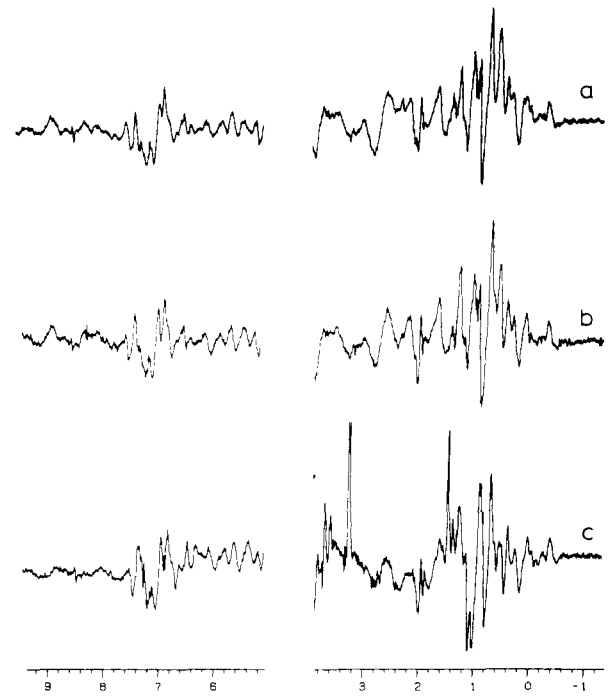

FIGURE 3: Effects of pepstatin and analogues on pepsin: difference spectra for (a) 0.5 mM pepstatin plus 0.5 mM pepsin. Experimental conditions as in Figure 2. (b) As in (a) but with Iva-Val-Val-Sta-Ala-Iaa. (c) As in (a) but with Boc-Val-Val-Sta-Ala-Iaa. In this case the sample was not lyophilized before the NMR run, accounting for the residual methyl peak at 3.25 ppm from CH_3OH-d_4. Some of the differences seen near 1 ppm may be due to direct effects of 5% CH_3OH in the sample. The peak at 1.46 ppm is from the Boc methyl protons.

tert-Butyloxycarbonyl (Boc) substituted for isovaleryl in **2** gives a difference spectrum in the aromatic region again like pepstatin (Figure 3c) except for the methanol peak at 3.25 ppm which remains in this particular spectrum because the sample was not lyophilized. The aromatic region difference spectrum for **3** is virtually identical with that for **2**. Unlike most peptide resonances, the Boc methyl peak with its nine equivalent protons is easily picked out as a narrow singlet in the aliphatic region at 1.46 ppm, a downfield shift of 0.06 ppm from the free solution position. Some features near 1 ppm do not match the pepstatin-induced spectrum. They may reflect a real difference in binding or possible effects of CH_3OH, present in this sample but not in the other two.

Influence of Stereochemistry. The pepstatin analogues used above contained statine residues with 3*S*,4*S* configuration. We tested the importance of stereochemistry at the 3 carbon, with its hydroxyl group, using analogues **4** and **5**, which are shorter by one Val unit than analogue **3** and differ in their statine configuration. Figure 4 shows the dramatic contrast in spectral change induced by these two analogues. The 3*S* configuration yields a difference spectrum very close to that of **1**, **2**, and **3** while the changes accompanying binding of **5** (3*R*) are almost 1 order of magnitude smaller and are, in some ways, qualitatively different. The *tert*-butyl group of pepsin-bound **4** is at 1.65 ppm while in **5** it appears as a broad resonance near 1.5 ppm. Other features of the **5** difference spectrum (e.g.,

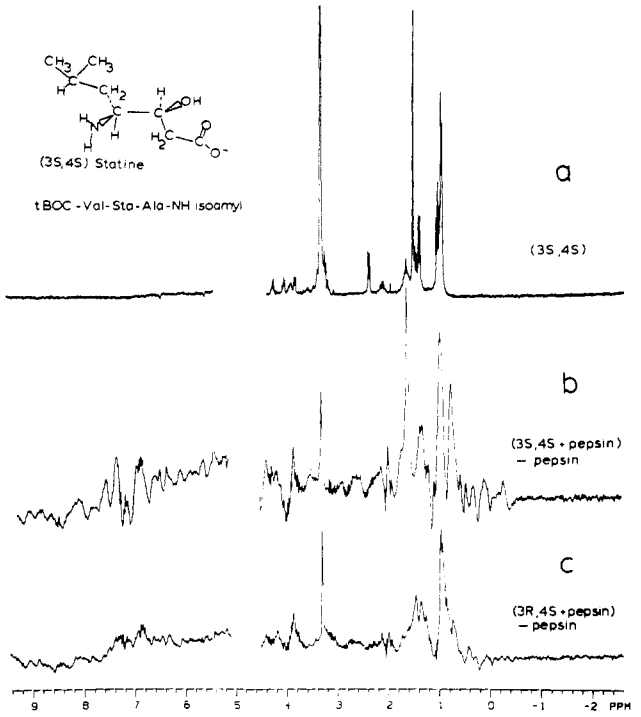

FIGURE 4: Effects of stereochemistry of statine in Boc-Val-Sta-Ala-Iaa. (a) Boc-Val-(3*S*,4*S*)-Sta-Ala-Iaa alone in CH_3OH-d_4 solution. (b) Difference spectrum induced in pepsin by binding of the peptide having the (3*S*,4*S*)-statine configuration. Experimental conditions similar to those of Figure 3. (c) Pepsin difference spectrum generated upon binding of the peptide with (3*R*,4*S*)-Sta. Conditions and vertical gain as in (b).

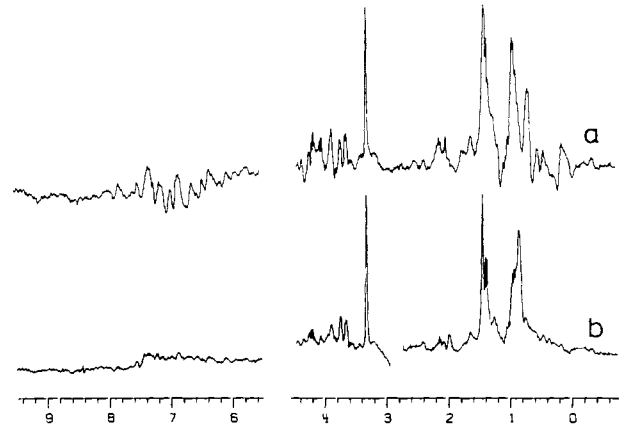

FIGURE 5: Binding of Boc-Sta-Ala-Iaa and effect of Sta stereochemistry. (a) Pepsin difference spectrum generated by Boc-(3*S*,4*S*)-Sta-Ala-Iaa. Experimental conditions as in Figure 3. (b) As in (a) but with the peptide Boc-(3*R*,4*S*)-Sta-Ala-Iaa.

the "W" structure near 7.1 ppm) appear to be smaller (about 20%) amplitude versions of the **4** spectrum, possibly indicating that a conformational equilibrium exists for both peptide–pepsin complexes for which **4** favors a more "tightened" complex (more amplitude in perturbed difference spectrum) than analogue **5**.

Analogues **6** and **7** are also stereoisomers. The peptide Boc-Sta-Ala-Iaa lacks the Val residue of the previous inhibitors. This time the difference spectrum (Figure 5) of the 3*S*,4*S* isomer does not so closely resemble that produced by pepstatin. But significant changes are found throughout the aromatic region and in the 0–1-ppm methyl region. The Boc methyls are not shifted, in contrast to Boc-Val-Sta-Ala-Iaa. In this case, the difference spectrum generated by the 3*S*,4*S* stereoisomer is about 1 order of magnitude greater than that

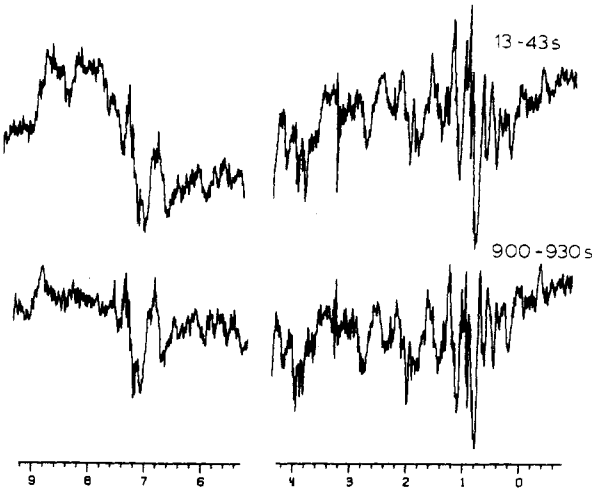

FIGURE 6: Time dependence of difference spectrum appearance. (a) 30-s accumulation after addition of a total of 1 mM pepstatin to 1 mM pepsin in ^2H_2O. 13 s was required to add the sample, mix, and begin data acquisition. Another sample of pepsin with the same amount of CH_3OH-d_4 added, without peptide, was accumulated for the same time period and subtracted to give the difference spectrum shown. (b) Similar to (a) except the sample with pepstatin added stood for 15 min before a 30-s run was made.

with 3*R*,4*S*. By and large the difference spectrum for **7**, with the nonbiological stereochemistry, contains only the new resonances due to the peptide.

Effects of Weak Inhibitors. If the Ala-Iaa function is eliminated from **6**, the resulting Boc-Sta **8** is still an inhibitor, albeit a relatively weak one (Ac-Sta has a K_i of 1.2×10^{-4} M). Saturation of the pepsin active site with Boc-Sta produced no detectable change of enzyme conformation as evidence by the appearance of only the added amino acid peaks in the difference spectrum (data not shown).

Kinetics of Conformation Changes. The observation of a time lag in attaining full inhibition by pepstatin (Rich & Sun, 1980) prompted us to do a crude "stop-flow NMR" experiment. This simply involved collecting NMR spectra in short intervals immediately after addition of pepstatin to a pepsin solution. The shortest time-averaging interval that still gave usable signal-to-noise ratios was 30 s, and there was a 13-s delay between addition of peptide and start of the first acquisition. The results are shown in Figure 6. The difference spectrum from the first interval (13–43 s) is essentially identical with that from 30-s intervals taken 3 and 15 min (Figure 6b) after addition of peptide. Exchangeable protons were present in these protein samples so a broad band of peaks between 6 and 10 ppm shows up, indicating exchange of NH resonances for solvent ^2H over the course of the experiment. All the major features of pepstatin-induced difference spectra are found in the earliest scan including the 7.1-ppm "W" and other aromatic proton peaks, the Met CH_3 shift at 2 ppm, and the pattern from 0.5 to −0.5 ppm. On the basis of the signal-to-noise ratio we can put a limit of about 5% on the "non-pepstatin-like" conformation in the first spectrum. Assuming that the "pepstatin-like" difference spectrum develops in a first-order rate process, the time constant is less than 4–5 s. This is definitely an upper limit, and the real value may be orders of magnitude less.

Discussion

Relation of Difference Spectra to Pepsin Tertiary Structure. Since some pepstatin analogues, while binding, do *not* cause significant changes in the enzyme spectrum, we conclude that most of the protein chemical shifts resulting from pepstatin

binding are caused by reorientation of protein anisotropic groups. Recent high-resolution X-ray crystallographic data for acid proteases from *Penicillium janthinelum* (M. James et al., 1982, private communication) and *Rhizopus chinensis* (R. Bott et al., 1982, private communication) show that the only significant movement in the pepstatin (or analogue)-bound form occurs for a portion of the structure called the "β flap", including residues 70–80. Tyr^{75} is on this flap in pepsin and its ring appears to be part of a hydrophobic pocket for the aliphatic side chain of the Sta^3 residue in pepstatin (Bott et al., 1981). Tyr^{75} lies very close to the Phe^{111} and to Trp^{39} (the ring centers are 5–7 Å apart). These three residues are conserved in at least four acid protease sequences including pepsin and penicillopepsin (Tang et al., 1973; Foltmann & Pedersen, 1977). Aromatic rings in such close proximity will mutually affect each other's chemical shifts through the ring current anisotropy effect. With 14 protons between them, movement of the Tyr^{75} side chain upon pepstatin binding could provide a major share of the observed aromatic region difference spectrum wherein about 10 net protons are shifted.

Pepstatin Structural Features for Enzyme Conformational Changes. Eight analogues produced four major classes of difference spectra (Table I). Analogues **1–4** generated pepstatin-like changes in pepsin, **5** produced a fraction (about 20%) of a pepstatin effect, **6** created a unique pattern, and **7** and **8** provoked no protein difference spectrum beyond their own proton signals. These spectral classes can be analyzed in light of the pepstatin structure, containing loci P_4–$P_{2'}$ (Schecter & Berger, 1967):

sequence:	Iva-Val-Val-Sta-Ala-Sta
locus:	P_4 P_3 P_2 P_1 $P_{1'}$ $P_{2'}$

Prerequisites for the full pepstatin-like difference spectrum are a branched side chain at locus P_3 and a configuration of 3*S*,4*S* for Sta in P_1. The P_4 locus need not be present (**4** works as well as **2**) nor is the 3-hydroxylpropionic acid function needed in $P_{2'}$ (**2** vs. **1**). Lack of both P_4 and P_3 loci substantially alters the pattern (analogue **6**). The difference spectrum does not closely resemble that for **1–4**, implying that a different enzyme conformation is adopted when the N terminus is short by two residues. Finally, loss of the carboxyl-terminal loci, $P_{1'}$ and $P_{2'}$, along with the loss of P_4 and P_3, eliminates any detectable enzyme conformational changes.

Inhibition Constants and Conformation Changes. Comparison of structural effects of inhibitors to their inhibition constants from steady-state kinetics is instructive. While analogues **1–4** produce essentially the same difference spectra, their K_i values vary from 4.5×10^{-11} M to 1.7×10^{-7} M; obviously all are very strong inhibitors. Shortening the chain to lose the P_3 locus increases the inhibition constant more than 300-fold when comparing their respective Iva derivatives. At the same time loss of P_3 produces a new enzyme–inhibitor conformation. The P_3 locus clearly plays a key role in the pepsin–pepstatin interaction. A change in Sta configuration from 3*S* to 3*R* increases K_i by a factor of 2000 for the Iva derivatives of analogues **4** to **5** and a factor of at least 7 times (as a minimum limit due to solubility of the 3*R*,4*S* isomer) for the change of **6** to **7**. Again stronger binding correlates with a larger conformational change in these examples.

Pepstatin Analogue Binding Mechanisms. The minimum mechanism to account in a general way for the NMR data of all inhibitors except **6** is

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^* \qquad (1)$$

EI is the initial collision complex between enzyme and inhibitor, involving little or no accommodation of the enzyme conformation to the peptide. Analogues **7** and **8** bind only in the EI form, for example. EI* is the pepstatin-bound conformation; in this scheme inhibitors **1–4** occupy EI* exclusively. Analogue **5** shows the very interesting property of a difference spectrum that appears in many ways to be that of its stereoisomer **4** but has only 20% of the latter's amplitude. The simplest explanation for such behavior is that the EI → EI* equilibrium constant is only 0.2 for **5** while it is >10 for **1–4**. EI* is probably somewhat different in structure for **5** than for **1–4** because of the (3*R*,4*S*)-Sta, but the NMR suggests that the differences are minor, manifested most apparently in the different chemical shift for the Boc protons but not in the protein spectrum.

Analogue **6** presents a special case. Its effect on pepsin structure is qualitatively different from the others. In the scheme of eq 1, EI* could be simply a different enzyme–inhibitor conformation than for pepstatin, or there could be another intermediate between EI and EI*, at which **6** stops but the others do not. Our data do not discriminate between these possibilities.

In stop-flow experiments Kitagishi et al. (1980) measured an apparent rate constant for the binding reaction between pepsin and *Streptomyces* pepsin inhibitor (SPI), a close analogue of pepstatin. In these experiments a decrease in fluorescence of TNS was followed as the inhibitor displaced the dye from the enzyme. From the concentration dependence of the rate constant they deduced the mechanism of eq 1 with a very rapid initial binding step (diffusion controlled?) followed by a unimolecular isomerization process. In their case $k_3 \cong 600\ s^{-1}$ at 25 °C and pH 5. While the transient kinetic data do not directly measure a conformational change for the unimolecular step, the mechanism derived is entirely consistent with the NMR results where it is clear that an enzyme conformational change accompanies the EI → EI* step.

Origin of Lag Transient for Pepstatin Inhibition. In earlier steady-state kinetic studies (Rich & Sun, 1980) pepstatin and Iva-Val-Sta-Ala-Iaa **4a** showed a time lag before developing their full inhibitory power. Compounds **2** and **3** (Rich & Bernatowicz, 1982) also are slow-binding inhibitors of pepsin (Table I) and have the very long half life of 30 s. We looked for evidence that this slow step involved a further conformational change beyond EI* of eq 1 and concluded that changes beyond EI* are not detectable by NMR using the methods described here. Fast-binding inhibitors (e.g., **4**) give the same difference spectrum as the slow-binding inhibitors **1–3**; thus, the NMR difference spectrum develops within a much shorter time period than the lag time needed for maximum inhibition by **1–3**. One rationalization for these observations is that the tight-binding inhibitors bind rapidly to form complexes (with average bound lifetimes of about 30 s) in which several enzyme aromatic residues are reoriented to produce the observed difference spectra. On a longer time scale these complexes partition into more tightly bound complexes (with very long lifetimes) without measurable further rearrangement of the aromatic residues. It is reasonable to postulate that the difference NMR spectra in the aromatic region arise from reorientations of aromatic residues in the mobile "flap" and that changes in the intensity of the difference spectra reflect the distribution between open- and closed-flap forms of the enzyme–inhibitor complexes. The origin of the slow formation of very tightly bound complexes remains unknown.

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Covalently Activated Glycogen Phosphorylase: A Phosphorus-31 Nuclear Magnetic Resonance and Ultracentrifugation Analysis†

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ABSTRACT: Glycogen phosphorylase *b* reconstituted with pyridoxal pyrophosphate in place of the natural coenzyme, pyridoxal phosphate, is shown to exist in a more activated (R) conformation than does native phosphorylase. Addition of nucleotide activator to the reconstituted enzyme traps it totally in this activated conformation. These conclusions were arrived at on the basis of tertiary structural information obtained from ^{31}P nuclear magnetic resonance studies, which allowed measurement of the nucleotide binding constant, and on the basis of quaternary structural information obtained via ultracentrifugal analysis of the enzyme in the presence of various effectors. Control experiments were performed with another modified form of the enzyme, pyridoxal phosphorylase. It is suggested that the transition-state analogue pyridoxal pyrophosphate, bound at the active site, mimics the actual configuration of enzyme plus substrate achieved during the normal catalytic reaction and therefore traps the enzyme in an activated conformation. These findings agree well with recent results obtained with the alternate transition-state analogue pyridoxal pyrophosphate glucose [Withers, S. G., Madsen, N. B., Sykes, B. D., Takagi, M., Shimomura, S., & Fukui, T. (1981) *J. Biol. Chem. 256*, 10759] and therefore provide further evidence for the "interacting phosphates" hypothesis presented in the latter paper.

The focal point of most studies on the catalytic mechanism of glycogen phosphorylase in recent years has been the role of the coenzyme pyridoxal phosphate. The phosphate moiety has been shown to be the catalytically essential component of the coenzyme by a variety of studies involving analogue replacement (Kastenschmidt et al., 1968; Shaltiel et al., 1969; Pfeuffer et al., 1972; Feldmann et al., 1972, 1974; Vidgoff et al., 1974; Feldmann & Helmreich, 1976; Parrish et al., 1977; Shimomura & Fukui, 1978; Hoerl et al., 1979; Chang & Graves, 1982). The phosphate moiety has been studied quite

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