1830 *Biochemistry* **1982**, *21*, 1830–1835

Spin-Labeled Pepstatin Binding to Pepsin. A Study by Electron Spin Resonance and Nuclear Magnetic Resonance[†]

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ABSTRACT: Spin-labeled analogues of pepstatin were used to probe the active site of porcine pepsin. Pepstatin is Iva-Val-Val-Sta-Ala-Sta [where Sta is (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid], a potent inhibitor of acid proteases. Analogue **1** (pepstatin–Tempo-amide) (where Tempo is 2,2,6,6-tetramethylpiperidinyl-1-oxy) had spin-label 4-amino-Tempo condensed to the carboxyl-terminal Sta group of pepstatin, while **2** (Tempo-Val-Sta-Ala-Iaa-thiourea) was prepared by reaction of the synthetic peptide Val-Sta-Ala-isoamylamide with 4-isothiocyanato-Tempo to give the N terminus spin-labeled as a thiourea. Both **1** and **2** are strong inhibitors of pepsin with K_i's of 1.9×10^{-9} and 1.4×10^{-7} M, respectively, and both spin-labeled peptides are displaced from the active site by pepstatin. Electron spin resonance spectra of **2** bound to pepsin indicate almost complete immobilization of the label, while the spectrum of **1** shows partial restriction of motion relative to the enzyme. Resonances in proton nuclear magnetic resonance spectra of pepsin are selectively broadened by bound spin-labeled analogues. Paramagnetic difference spectra reveal that about 30 aromatic protons are within 12–14 Å of the nitroxide of bound analogue **2**. The single His-53 C-ϵ_1 ring proton and two methionine CH_3 groups are within 16 Å. His-53 is not broadened by the spin-label of bound analogue **1**, which, in general, affects a different set of protein resonances than **2**. On the basis of these data and in light of preliminary X-ray diffraction data on the pepstatin complex with the acid protease of *Rhizopus chinensis* (R. Bott and D. Davies, private communication), the spin-label of **2** is tentatively positioned within the pepsin active site, pointing toward the interior of the protein near Tyr-75 and Phe-111. The carboxyl end of pepstatin, on the other hand, is placed near the surface at one end of the active site cleft.

Pepsin is one of a group enzymes termed carboxyl, or acid, proteases. These molecules have active sites containing two catalytic aspartyl side chains and show specificity for cleaving peptide bonds between hydrophobic groups (Tang, 1979). Renin and cathepsin D belong to the class as do a number of mold enzymes. Acid proteases also share the property of being strongly inhibited by pepstatin, a hydrophobic pentapeptide first isolated by Umezawa and co-workers from cultures of *Streptomyces testaceus* (Umezawa et al., 1970). Pepstatin has the structure Iva-Val-Val-Sta-Ala-Sta[1] (Morishima et al., 1972). It binds exceptionally tightly to porcine pepsin with a K_{diss} of 4.5×10^{-11} M (Workman & Burkitt, 1979).

The present study is one of a series of steady-state kinetic (Rich et al., 1977; Rich & Sun, 1980) and NMR (P. G. Schmidt, M. Bernatowicz, and D. H. Rich, unpublished results) efforts designed to determine why pepstatin is such a potent inhibitor. We have shown that pepstatin binding proceeds through a mechanism containing very slow steps, which are the key events leading to its much tighter binding than some close analogues (Rich & Sun, 1980). NMR difference spectroscopy demonstrates that pepstatin and its analogues of sufficient length and correct stereochemistry induce significant conformational changes in the enzyme (P. G. Schmidt, M. Bernatowicz, and D. H. Rich, unpublished results). Such changes may play a role in catalysis, since it is known that addition of amino acids to dipeptide and tripeptide substrates can greatly accelerate catalysis by increasing k_{cat} (Fruton, 1976), possibly by facilitating the formation of the transition state.

In the work reported here we have sought to define the orientation of pepstatin in the pepsin active site and to gauge the freedom of motion of parts of the bound peptide. Pepstatin was spin-labeled at the free carboxyl of the terminal Sta (statine) residue to provide a paramagnetic locus at one end, and the analogue Val-Sta-Ala-isoamylamide was labeled at the N terminus to give a stable free radical at the other end of the chain. Both labeled peptides are strong inhibitors of pepstatin, both induce the same enzyme conformational change that pepstatin does, and they are both displaced from the enzyme by stoichiometric concentrations of pepstatin. ESR spectra yield parameters of internal motion of the labels for active site mapping (Hsia & Piette, 1969), and NMR paramagnetic difference spectra (Dwek et al., 1975) reveal specific amino acids within about 12–16 Å of the radicals. The results unequivocally define the overall orientation of pepstatin in the active site of porcine pepsin.

Materials and Methods

Spin-Labeled Pepstatin Analogues. Pepstatin–Tempo-amide (**1**) was prepared from the condensation of 2,2,6,6-tetramethyl-4-aminopiperidinyl-1-oxy (Aldrich Chemical Co., Milwaukee, WI) with the free carboxyl of pepstatin A (Peninsula Laboratories, San Carlos, CA) by using DCC and HOBT in DMF. The purity of the product was checked by TLC and by NMR of the reduced (diamagnetic) compound in MeOH-d_4.

Tempo-Val-Sta-Ala-Iaa (**2**) was synthesized from 2,2,6,6-tetramethyl-4-isothiocyanatopiperidinyl-1-oxy (SYNVAR Corp., Palo Alto, CA) and Boc-Val-Sta-Ala-Iaa, whose syn-

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[1] Abbreviations: Iva, isovaleryl; Iaa, isoamylamide; Sta, statine [i.e., (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid]; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; DCC, dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; DMF, dimethylformamide; EtOAc, ethyl acetate; NMR, nuclear magnetic resonance; ESR, electron spin resonance; TLC, thin-layer chromatography. All amino acids are of the L configuration. Standard abbreviations for amino acids and protecting groups (i.e., Boc) as recommended by the IUPAC–IUB Commission on Biochemical Nomenclature are used.

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thesis has been described (Rich et al., 1980). The deprotected peptide in EtOAc was neutralized with triethylamine before addition of the Tempo-isothiocyanate. Purity was checked by TLC and by NMR in MeOH-d_4.

Enzyme Preparation. For ESR spectra commercial porcine pepsin from Worthington Biochemicals (Freehold, NJ) was used without further purification. For NMR the enzyme was prepared from pepsinogen that had been preexchanged in ^2H_2O at pH 9 to eliminate most amide proton resonances (P. G. Schmidt, M. Bernatowicz, and D. H. Rich, unpublished results). Protein concentrations were determined from the absorbance at 280 nm with $A_{280}^{1\%} = 14.3$. The pH values in ^2H_2O solutions are uncorrected readings taken from meters that had been standardized with H_2O buffers.

ESR Spectra. Spin-label stock solutions were made up in CH_3OH and added by microsyringe to solutions of pepsin in H_2O, pH 4.5. Sealed glass transfer pipets served as sample tubes. Spectra were obtained on a Varian E-9 spectrometer operating at 9.5 GHz. Power levels were below saturation, and the modulation level was 200 mG. Spectra were swept at the rate of 25 G/min, with a filter time constant of 0.3 s. The sample temperature was 30 °C.

NMR Measurements. Spectra were obtained at 270 MHz with a Fourier transform quadrature phase detection spectrometer with a Bruker Instruments magnet and probe and Nicolet 1180 computer. Sample temperatures were 28 °C. A solution of 0.5 mM pepsin, pH* 4.5, in "100 atom %" ^2H_2O (Aldrich Chemical Co., Milwaukee, WI) was prepared, and then 0.5 mM spin-labeled peptide was added (25 μL of a concentrated stock in CH_3OH-d_4). A spectrum of the sample was first collected and stored in the computer; then a freshly made, concentrated stock solution of ascorbic acid in ^2H_2O, pH* 4.5, was added to give a final concentration of 20 mM. After mixture and a 5-min incubation period to reduce the spin-label, the sample was run again under identical instrumental settings. The two spectra were subtracted (reduced minus oxidized) with an amplitude correction for lower concentration due to the added ascorbate. Chemical shifts are reported relative to DSS but were measured with respect to the H^2HO signal.

Kinetic Studies. Kinetic constants were determined with a heptapeptide substrate Phe-Gly-His-Phe(NO_2)-Phe-Ala-Phe-OMe as previously described (Rich & Sun, 1980). Hydrolysis of the substrate was measured at 25 °C in pH 4.0, 0.04 M formate buffer. Porcine pepsin (2 times crystallized and lyophilized, lot 83C-8080) used for kinetic studies was purchased from Sigma Chemical Co., St. Louis, MO. The 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.

Results and Discussion

Mobility of Spin-Labels Bound to Pepsin. ESR spectra for spin-labels **1** and **2** bound to pepsin are shown in Figure 1 along with a spectrum of **1** free in solution. Spectra of bound spin-labeled peptides are broadened and spread out, reflecting restriction of motion of the radicals (Freed, 1976), while the free peptide spectrum is consistent with high mobility. A small concentration of mobile label is seen as narrow peaks on top of the main spectra for both bound spin-labels. With lower concentrations of peptide, at fixed protein concentration, the ratio of free to bound amplitudes remains constant. We assume that the free spectrum arises from a small contamination with spin-label starting materials or other paramagnetic trace impurities, which may have arisen from these during the synthetic reactions.

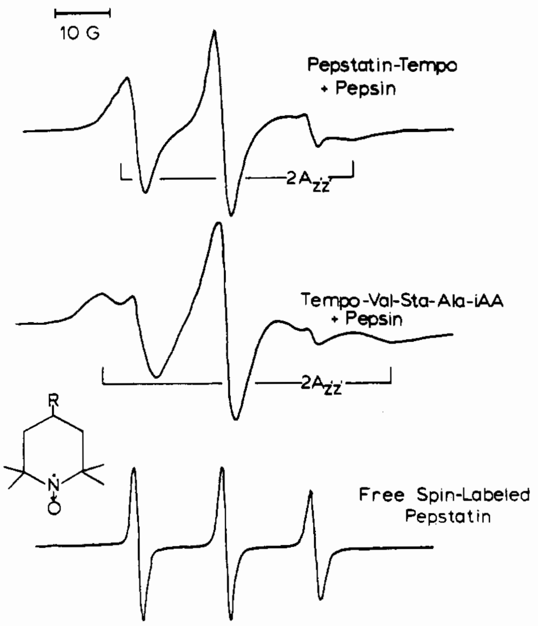

FIGURE 1: ESR spectra of spin-labeled pepstatin plus pepsin. The analogues **1** (pepstatin–Tempo) and **2** were added as stocks in CH_3OH to H_2O solutions of 0.5 mM pepsin, pH 4.5 at 30 °C. Spectral parameters: 25 G/min sweep, 0.2-G modulation amplitude, 0.3-s filter time constant. The lower spectrum is of analogue **1** in H_2O with the same spectral conditions as the upper two spectra but lower readout gain.

Values for $2A_{z'z'}$, the spacing between the outer extrema, are 44 G for **1** and 55 G for **2**. Pepsin has a mass of 34 000 daltons (Tang et al., 1973). For the protein in water at 30 °C we calculate a value of about 1×10^{-8} s for the rotational correlation time, τ_r, from the Stokes–Einstein relation (Dwek, 1973). Measurements of τ_r for other globular proteins by fluorescence (Haugland & Stryer, 1967; Yguerabide, 1972), ^{13}C NMR (Dill & Allerhand, 1979; Bauer et al., 1975), depolarized light scattering (Bauer et al., 1975), and spin-label methods (Shimshick & McConnell, 1972; McCalley et al., 1972) yield larger values than predicted by the Stokes–Einstein equation, possibly due to water of hydration. On the basis of these findings the appropriate value for pepsin may be closer to $(1.5 \pm 0.5) \times 10^{-8}$ s. If the labels were rigidly held with respect to the protein, the value of $2A_{z'z'}$ would be 57–59 G (McCalley et al., 1972), assuming a no motion, rigid glass limit for the label of 64 G (Sutton et al., 1977).

Differences between the observed values and the "rigidly bound to protein" limit most likely reflect internal motion of the label (Sutton et al., 1977). Several methods have been forwarded for extracting motional parameters from spin-label data. A study by Sutton et al. (1977) specifically deals with modes of anisotropic motion available to a particular label when the parent molecule is firmly attached to its binding site. Such is the case in this study, particularly for label **2**. We will reserve a detailed analysis for a future publication, but for the present, the value of $2A_{z'z'} = 55$ G for label **2** indicates that the six-membered ring nitroxide has available to it only limited amplitude excursions, possibly only ring flection (Sutton et al., 1977). Label **1** has a smaller splitting of 44 G consistent with substantial freedom, but the motion is still anisotropic, since isotropic tumbling of a label produces a value of $2A_{z'z'} \simeq 32$ G. We conclude that with analogue **1**, having the nitroxide on the carboxyl terminus of pepstatin, the label probably has sufficient room to rotate freely about one bond.

Kinetic Inhibition Constants. 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 the inhibited velocity and

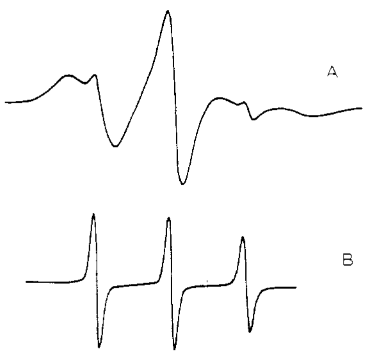

FIGURE 2: Competition of analogues with pepstatin. (A) 0.5 mM pepsin plus 0.4 mM analogue **2**. Instrumental conditions as in Figure 1. (B) 0.5 mM pepsin plus 0.6 mM analogue **2** plus 0.7 mM pepstatin. Vertical readout gain less than in (A).

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, 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.

Pepstatin and some of its analogues display an unusual time dependence of inhibition whereby the apparent K_i decreases markedly after initial mixing of enzyme and inhibitor, over a period of up to several minutes (Rich et al., 1977; Rich & Sun, 1980). Analogue **1** shows this time dependence, while **2** does not have the lag; both are competitive inhibitors with K_i values of 1.9×10^{-9} M for label **1** (pepstatin–Tempo-amide) and 1.4×10^{-7} M for label **2**. By way of comparison, pepstatin itself has a K_i of 4.5×10^{-11} M, and Iva-Val-Sta-Ala-Iaa, with isovaleryl in place of the Tempo-thiourea of analogue **2**, has a K_i of 1.1×10^{-9} M (Rich & Sun, 1980). Both of these non-spin-labeled peptides have a time lag before maximum inhibition and are stronger inhibitors than the spin-labels by factors of 40–100, probably reflecting steric hindrance by the nitroxide groups. Yet the spin-labeled peptides are still very strong inhibitors of pepsin.

Competition of Spin-Labels with Pepstatin for Binding at the Active Site. When pepstatin is added to solutions containing analogue **1** or **2** and pepsin, the bound label spectrum disappears and is replaced by the spectrum of free label. This would be expected from the K_i's of **1** and **2**, which are more than 40 times greater than for pepstatin. Stepwise titration of pepstatin into solutions of pepsin containing an excess of **2** progressively increased the free spin-label signal up to a 1:1 ratio of pepsin to pepstatin. Further pepstatin addition did not change the ESR spectrum. Figure 2 shows that a solution of 0.5 mM pepsin, 0.6 mM **2**, and 0.7 mM pepstatin displays only the free label spectrum. Similar data are found for label **1**. With these data and the structural similarity between pepstatin and the labeled peptides, the latter most likely bind at the same site as does pepstatin and have no other sites on pepsin, at least none where the label is restricted in motion.

Effects of Bound Spin-Labels on ^1H NMR Spectra of Pepsin. The pepsin NMR spectrum in ^2H_2O contains approximately 1800 proton signals (P. G. Schmidt, M. Bernatowicz, and D. H. Rich, unpublished results), making direct interpretation rather difficult. When analogues **1** and **2** bind to pepsin, the paramagnetic centers broaden resonances of nearby protein protons due to the strong, fluctuating magnetic field generated by a free electron spin (Krugh, 1976). The broadened peaks are revealed in paramagnetic difference spectra (Dwek et al., 1975) whereby the spectrum for the protein, selectively broadened by bound spin-label, is subtracted from the spectrum without label. But analogues **1** and **2** by themselves cause substantial changes in enzyme conformation, leading to extensive chemical shifts of protein peaks (P. G. Schmidt, M. Bernatowicz, and D. H. Rich, unpublished results). We circumvented this problem by obtaining difference spectra between spectra of pepsin plus bound label before and after addition of ascorbate, which reduces the paramagnetic nitroxide to the diamagnetic hydroxylamine but leaves the complex essentially unaltered. Separate ESR experiments established that indeed ascorbate eliminated the spin-label spectrum. The reaction rate was dependent on the ascorbate level, so a concentration of 20 mM was chosen to assure rapid reduction in the NMR experiments. Separate controls showed that ascorbate had no effect on the unliganded pepsin NMR spectrum beyond the appearance of ascorbate proton signals at 4.03, 4.5, and 3.72 ppm.

Figure 3a shows a spectrum of pepsin plus **2** after reduction by ascorbate. The spectrum of the aromatic region is essentially identical with a spectrum of pepsin plus pepstatin or pepsin plus Boc-Val-Sta-Ala-Iaa, the closest analogue to **2** that we have tested (P. G. Schmidt, M. Bernatowicz, and D. H. Rich, unpublished results). It is also the same as that produced by label **1** after reduction.

Paramagnetic difference spectra for **2** and **1** are shown in parts b and c of Figure 3. These spectra graphically illustrate the simplification achievable by selecting only a subset of protons. The aromatic regions contain only protein resonances, while the aliphatic region is complicated by the presence of spin-label peaks from the nitroxide ring and from the valine and statine side chains of the analogue. However, one feature near 2 ppm deserves attention. Two narrow singlets are found here when **2** is bound; their line widths are about 8 Hz, and the peaks contain approximately 2.5 protons each. The downfield peak is also seen in the spectrum with **1**, along with a smaller amplitude peak upfield. These peaks are present, barely resolved, in spectra of pepsin alone and pepsin plus non-spin-labeled pepstatin analogues, so they do not come from the nitroxide ring. We conclude that they are pepsin resonances and, to be narrow singlets in this region of the spectrum, they most probably are methionine methyl protons.

In general, paramagnetic difference spectra for **1** and **2** do not resemble each other, implying that their nitroxide groups are probably separated by at least 10 Å. This in turn suggests that pepstatin binds in a relatively extended conformation, a conclusion supported by the preliminary X-ray diffraction analysis of the pepstatin complex with the acid protease of *Rhizopus chinensis* (Subramanian et al., 1979).

In the aromatic region, analogue **2** produces a strong effect on a narrow peak at 8.5 ppm, while **1** has no effect on this group at all. Pepsin has the one good NMR characteristic of containing a single histidine in its sequence at position 53. We earlier made the assignment of the 8.5-ppm peak to the C ϵ_1 of His-53 (Schmidt, 1980). A broad group of peaks near 6.9 ppm appears in both spectra, and a series of peaks between 7.2 and 7.5 ppm in Figure 3c may have counterparts in Figure 3b, but most of the peaks in the two spectra do not match.

A closer look at the aromatic difference spectrum for **2** is shown in Figure 4. Higher protein concentration in this spectrum makes for a better signal-to-noise ratio, so that we can do quantitative analysis with some confidence. On the assumption that the histidine peak at 8.5 ppm and the resonance at 7.5 ppm contain one proton each and the peaks at 6.37, 6.10, and 5.67 ppm have two protons each, then the total area corresponds to 29 protons with an uncertainty of about ±5. One way to produce this spectrum involves two Tyr, three

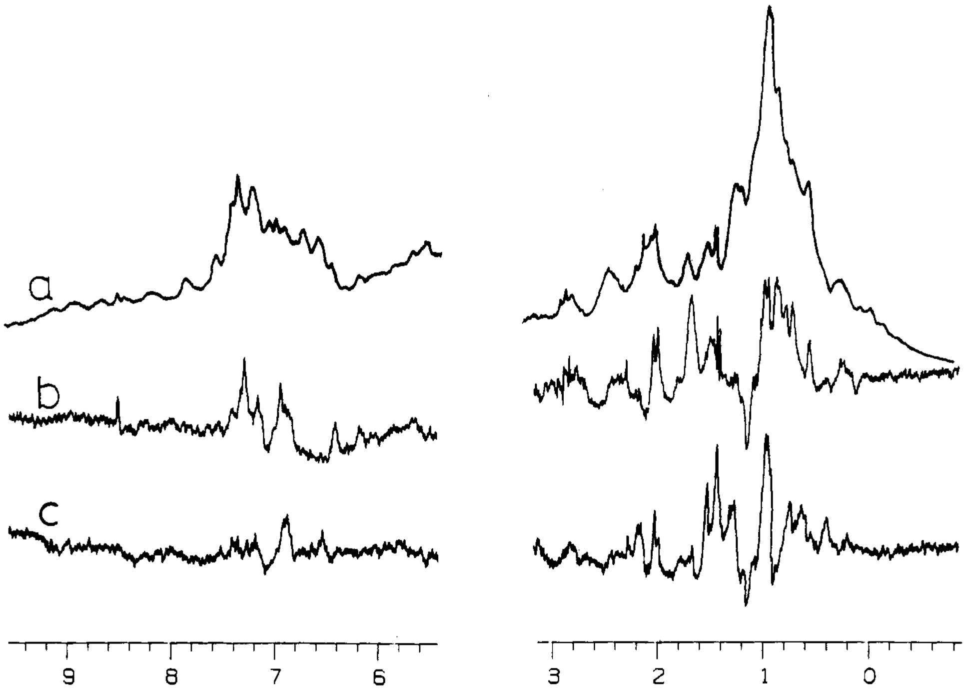

FIGURE 3: Paramagnetic difference spectra. (a) ^{1}H NMR spectrum of 0.5 mM pepsin plus 0.5 mM **2**, reduced with 20 mM ascorbate, pH 4.5. The vertical gain of the aromatic region (left side) is 2 times that of the aliphatic region; 400 scans; 90° pulses; 3-s pulse period. (b) From the spectrum in (a) was subtracted one taken under identical conditions before reduction of the nitroxide by ascorbate. The vertical gain has been increased for this display. (c) Same as (b) except with the analogue **1**.

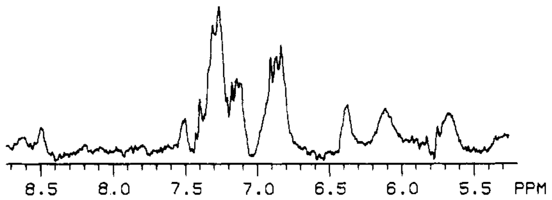

FIGURE 4: Aromatic region of analogue **2** paramagnetic difference spectrum. Conditions as in Figure 3b except the sample was 1.0 mM in protein for better signal-to-noise ratio.

Phe, one Trp, and one His, for a total of 30 protons. The His is certain, but there could easily be other combinations of Phe, Trp, and Tyr producing the spectrum. The two peaks at 5.67 and 6.10 ppm have the same line widths and areas and may come from a tyrosine ring. The possibility also exists that these peaks are from tryptophan or are α protons shifted far downfield of their usual positions, but that seems unlikely in view of the area of the histidine peak that is half of these.

The paramagnetic broadening effect of a free electron is exceedingly large at close range but falls off as r^{-6}, where r is the proton–electron distance (Dwek, 1973). Dwek and co-workers have discussed in detail paramagnetic difference spectra in a situation very similar to the present one (Dwek et al., 1975). For a label that is close to being rigidly held on the protein, the correlation time for the magnetic interaction is approximately the rotational correlation time of the protein, in their case 0.7×10^{-8} s and in ours about $(1-2) \times 10^{-8}$ s.

In the present case protons less than 16–18 Å will have line widths broadened by more than 9 Hz and for those less than 12–13 Å, 50 Hz. Peaks whose natural width is on the order of 10 Hz (e.g., methionine and histidine) will appear in a difference spectrum (Dwek et al., 1975) if they are within about 18 Å of the radical and those whose widths are 20–30 Hz (many aromatics) when they are within about 13–14 Å.

We used the X-ray diffraction coordinates of the acid protease from *Penicillium janthinelum*, solved to 2.4-Å resolution by James and co-workers (Hsu et al., 1977), in an attempt to locate the spin-label of **2** in the pepsin active site. Using a Kendrew model (Cambridge Repetition Engineers) of penicillopepsin, we fit a model of **2** into the site such that the hydroxyl group of the statine residue pointed between the side chain carboxyls of Asp-32 and Asp-215 (Subramanian et al., 1979). The backbone of the inhibitor was then positioned to generate comfortable contacts with protein side chains, in the approximate position where it appears in preliminary studies of the rhizopuspepsin (R. Bott and D. Davies, private communication). Coordinates were taken from the model for the nitroxide nitrogen and atoms connecting the ring to the peptide. It was not obvious from the model what orientation the nitroxide ring would take, although the ESR data suggested that only limited motion should be possible. We therefore made a computer search, at 20° intervals, over conformations involving rotation around the thiourea nitrogen–piperidine ring bond. The program involved determining distances from the nitroxide nitrogen to γ carbons of the penicillopepsin molecule or aromatic carbons when the amino acid was identical. Close tertiary structure homology exists between penicillopepsin and porcine pepsin (Andreeva & Gustchina, 1979), but the sequence homology is only about 30% (Tang et al., 1973), His-53 being one that is identical.

The program found only a limited region (±40° of arc) in the pepsin–penicillopepsin hybrid structure where the nitroxide was within 16 Å of at least two pepsin methonines (80 and either 290 or 246) and the histidine C-ϵ_1 carbon. In this region there was approximately the right number of aromatic protons as well. Table I lists the aromatic amino acids of pepsin whose γ carbons (or oxygen) were within 12 Å of the nitroxide nitrogen, a distance chosen to be sure they would appear in the paramagnetic difference spectrum. One Trp, four Tyr, and two Phe rings are in range, along with the His for a total of 33 aromatic protons. Given the approximations involved, it must be regarded as coincidence that close to the same number of protons appear in the paramagnetic difference spectrum.

A spin-label attached at the *carboxyl* end of pepstatin (analogue **1**), oriented in the active site as above, is 22–27 Å

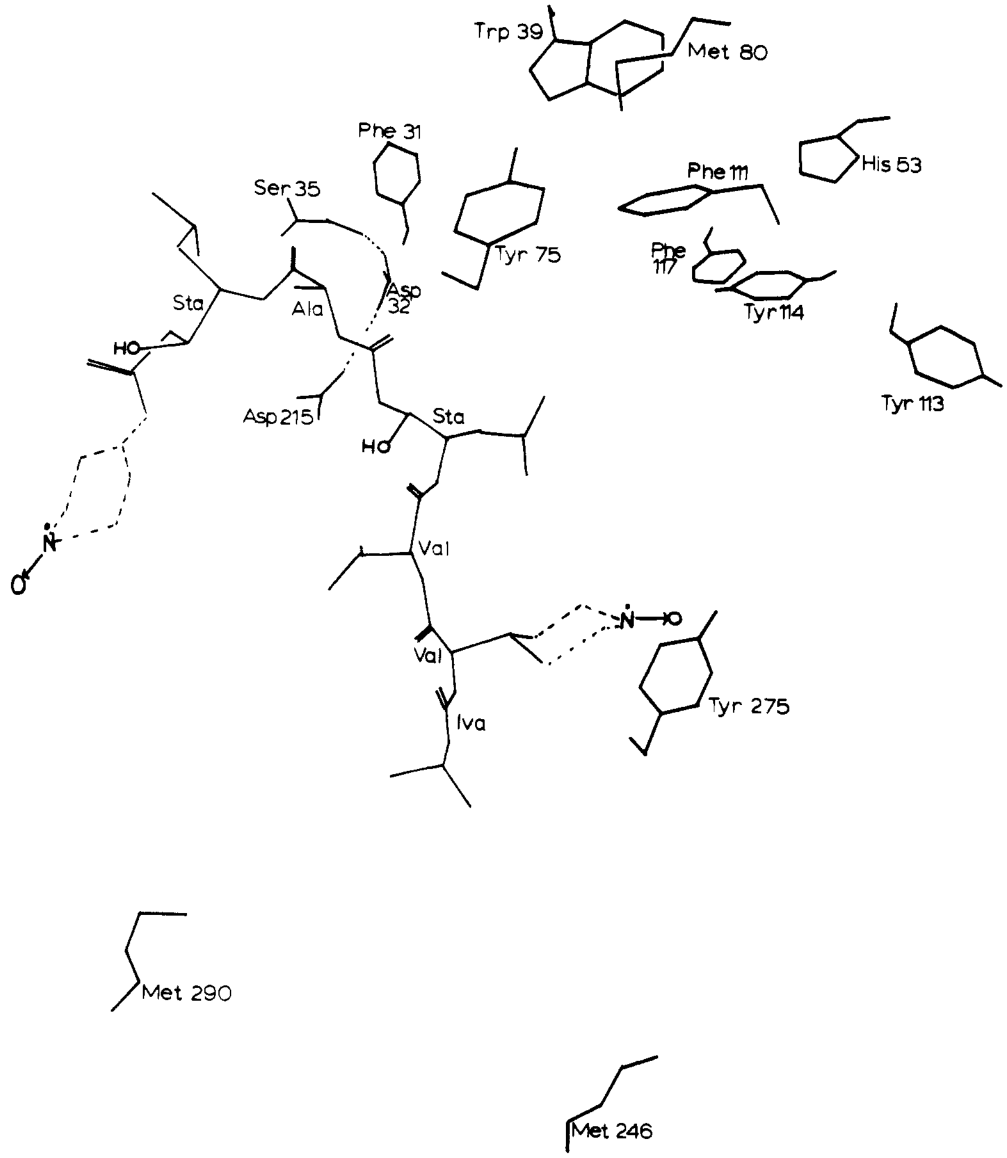

FIGURE 5: Schematic drawing of selected side chains near the active site of penicillopepsin. Pepstatin (Iva-Val-Val-Sta-Ala-Sta) is drawn in approximately the position found in the X-ray structure of the pepstatin–rhizopuspepsin complex. Side chains are those of porcine pepsin when different from penicillopepsin. Tempo rings are included in the approximate positions they would occupy in the two pepstatin analogues tested.

from His-53, well out of range of paramagnetic broadening. Tyr-75 ring protons are approximately 14–18 Å from the nitroxide nitrogen of **1**, possibly close enough to experience detectable broadening by the free electron spin.

Figure 5 is a drawing of a portion of the penicillopepsin model showing the placement of the nitroxide groups for the two analogues relative to pepstatin and some of the protein side chains nearby. When they differ from penicillopepsin, the corresponding porcine pepsin groups have been drawn in with arbitrary side chain orientations. (Note that this two-dimensional representation distorts relative distances.)

Considering the pitfalls of combining data from two different crystal structures to analyze NMR results on yet another species (porcine pepsin), Figure 5 and Table I must be considered highly schematic. Nonetheless, the general picture emerges whereby the spin-label of **2**, occupying the side chain position of Val-1 in pepstatin, faces toward the interior of the protein where it is strongly immobilized, where it broadens a large number of aromatic side chain protons, and where it is within 16 Å of the His-53 imidazole ring. The nitroxide on the carboxyl end of pepstatin (**1**) has more mobility, broadens fewer aromatic resonances, and is out of paramagnetic reach of the histidine side chain. Especially interesting is the finding that Tyr-75 is predicted to be among those rings strongly affected by the spin-label of **2**. This group has been

Table I: Pepsin Amino Acids near Bound Spin-Label 2

group in porcine pepsin	distance to γ carbon (Å)[a]	comments[b]
Trp-39	>15	ring carbons <12 Å
His-53	12	C ϵ_1
Tyr-75	9.7	ring carbons <11 Å
Met-80	13	Ser[c]
Phe-111	8.4	ring carbons <8 Å
Tyr-113	7.7	Gln[c]
Tyr-114	6.9	Asp[c]
Phe-117	10.3	Asn[c]
Tyr-275	12.3	ring carbons <11 Å
Met-246	~15[d]	Tyr[c]
Met-290	~15[d]	Asn[c]

[a] Distances from nitroxide nitrogen to γ carbon of side chains for "best fit" positioning of spin-label. [b] Amino acids conserved between pepsin and penicillopepsin unless otherwise noted. [c] Group in penicillopepsin. [d] Distance to CH_3 of methionine side chain is poorly determined since these residues are not conserved in penicillopepsin.

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implicated in the catalytic mechanism (Hsu et al., 1977) since it lies so close to the active site carboxyl groups. Other spin-label studies are planned to probe possible conformational changes of Tyr-75 upon substrate and inhibitor binding.

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Direct Assignment of the Dihydrouridine-Helix Imino Proton Resonances in Transfer Ribonucleic Acid Nuclear Magnetic Resonance Spectra by Means of the Nuclear Overhauser Effect[†]

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Abstract: The NMR resonances from the hydrogen-bonded ring NH protons in the dihydrouridine stem of *Escherichia coli* $\mathrm{tRNA}_{\mathrm{I}}^{\mathrm{Val}}$ have been assigned by experiments involving the nuclear Overhauser effect (NOE) between adjacent base pairs. Irradiation of the 8–14 tertiary resonance produced a NOE to base pair 13. Irradiation of the CG13 ring NH produced NOEs to base pairs 12 and 14. Similarly, base pair 12 was shown to be dipolar coupled to 11 and 13, and base pair 11 was found to be coupled to 10 and 12. These sequential connectivities led to the assignment of CG13 at −13.05 ppm, UA12 at −13.84 ppm, CG11 at −12.23 ppm, and GC10 at −12.60 ppm. The results are compared with previous, less direct assignments for these four base pairs and with the expected proton positions from the crystal structure coordinates for this helix.

High-resolution NMR[1] is potentially the most informative spectroscopic tool for studying the molecular structure and dynamics of transfer RNA in solution (Crothers & Cole, 1978; Schimmel & Redfield, 1980). Although several regions of the ^{1}H NMR spectrum containing different types of protons have been studied in several laboratories (Kastrup & Schmidt, 1975, 1978; Kan et al., 1977; Robillard et al., 1977a,b; Schmidt & Edelheit, 1981), the most useful region of the NMR spectrum of tRNA is probably the extreme low-field spectrum between

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[1] Abbreviations: tRNA, transfer ribonucleic acid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; DSS, 4,4-dimethylsilapentane-1-sulfonate; D, dihydrouridine; s^{4}U, 4-thiouridine; Ψ, pseudouridine.

0006-2960/82/0421-1835$01.25/0