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SPIN-LABELING OF PORCINE PEPSIN AND *RHIZOPUS CHINENSIS* ACID PROTEASE BY DIAZOKETONE REAGENTS

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Summary: The active site of porcine pepsin and that of *rhizopus chinensis* acid protease were labeled with diazoketone type spin labels, 4-(3-diazo-2-oxopropylidene)-2,2,6,6-tetramethylpiperidine-1-oxyl (I) and 3-(4-diazo-3-oxo-*cis*-1-butenyl)-2,2,5,5-tetramethylpyrroline-1-oxyl (II), respectively. The values of τ_c showed that the nitroxide motion was only slightly restricted in the I bound enzymes. The *trans* isomer of II bound to another site of the enzymes. Addition of pepstatin reduced the nitroxide motion in all the labeled enzymes.

Considerable progress has been made in the elucidation of the structure of acid proteases. Thus, the amino acid sequence was determined completely for pepsin (1) and partially for *rhizopus chinensis* acid protease (RAP)(2). Recent X-ray diffraction study has revealed that RAP consists of a small and a large lobe and its active center is located in a cleft between the two lobes (3). As to pepsin an analogous structure of a large cleft was presented (4). The ESR spin-labeling technique seemed like the method of choice to investigate the conformation of such clefts, since a nitroxide radical, introduced through use of a particular attaching group, acts as a reporter group reflecting its motional freedom in clefts. In the present investiga-

Abbreviations used: RAP, *Rhizopus chinensis* acid protease; DAN, diazoacetyl norleucine methyl ester; BPB, *p*-bromophenacyl bromide.

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tion a few nitroxide spin-labels with a diazoketone attaching group (Figure 1) were synthesized and employed to probe the conformation of the clefts.

Materials and Methods

Materials: Porcine pepsin (2 x crystallized) was purchased from Sigma Chemical Co. and RAP (3 x crystallized) was from Seikagaku Kogyo Co., Tokyo. Pepstatin was presented by Drs. H. Umezawa and T. Aoyagi of Institute of Microbial Chemistry, Tokyo. The spin labels I - IV were prepared by the reaction of the corresponding carboxylic acids (5) with thionyl chloride followed by treatment with diazomethane.

Spin-Labeling of Acid Proteases: In a typical experiment, 5.0×10^{-5} mol of copper(II) acetate in 2.5 ml of 0.1 M sodium acetate buffer (pH 5.5) was added to 1 ml of 0.1 M sodium acetate buffer containing 15 mg of the enzyme, and the mixture was kept for 10 min at 14 °C. Then a solution of 25 mmol of the spin label in 50 µl methanol was added to the above mixture. Extent of inactivation of the enzyme was followed by the assay using hemoglobin as substrate. After most of the enzyme was inactivated, diazoacetylnorleucine methyl ester (DAN) was further added to complete the inactivation of the enzyme (6). The solution was dialyzed once against 0.1 M and then exhaustively against 1 mM sodium acetate buffer (pH 5.5, 2 l x 10). Pepstatin, the inhibitor of the enzymes (7), was added to the enzyme solution in equimolar amounts to the labeled enzymes when necessary.

ESR Measurement and Analysis: ESR spectra of the labeled enzymes were recorded by a JEOL JES-FE1X spectrometer with 100 kHz modulation at 22 °C. The rotational correlation time, τ_c, was calculated directly from the relative line heights and the width of the center line for slightly immobilized spin labels ($\tau_c = 10^{-11} - 10^{-9}$ sec) (8). For highly immobilized labels ($\tau_c \geq 10^{-8}$ sec), τ_c was characterized from the high and low field peak positions according to the method of McConnell et al. (9). The experimental parameter $A_{z'z'}$ was measured from the separation between the outer lines of the ESR spectrum.

Results and Discussion

The diazoketone spin labels I and II inactivated pepsin in the presence of copper(II) ion by the formation of the corresponding 1:1 covalent complexes. (We abbreviate hereafter, for example, the covalent complex of I as pepsin-I and so on.) The chemical modification study showed that diazoketone reagents

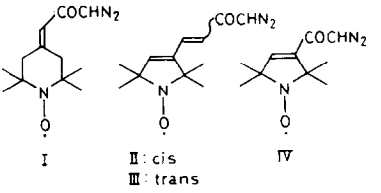

FIGURE 1. DIAZOKETONE SPIN LABELS I - IV.

Table I. Effect of Pepstatin on $2A_{z'z'}$

Labeled enzyme	$2A_{z'z'}$ (gauss)	
	Without pepstatin	With pepstatin
Pepsin-I	52.0	62.0
-II	55.6	61.1
-III	61.0	62.8
RAP-I	40.3	60.5
-II	59.4	62.5
-III	60.2	62.7

esterify pepsin specifically at the carboxylic group of aspartic acid-215 in the active center (10). In view of the similarity of the reactive part of I and II with that of diazoketone reagents we postulate that the same amino acid residue has been labeled in our cases. The value of τ_c for pepsin-I is calculated as 5×10^{-9} sec and indicates that the nitroxide motion is only slightly restricted and the cleft space, where the nitroxide resides, is rather wide. The value of $2A_{z'z'}$ can be used as a qualitative estimate of the degree of the nitroxide motion, because it becomes large on increasing immobilization of the nitroxides (9). As shown in Table I the value of $2A_{z'z'}$ for pepsin-II is larger than that for pepsin-I, suggesting that the nitroxide motion of pepsin-II is more restricted than that of pepsin-I, probably due to steric hindrance of the *cis* structure of II.

The spin label III bound similarly to pepsin in a 1:1 ratio, but not at the active site amino acid, because the labeling did not cause inactivation of pepsin. The reason for the unexpected result remains to be not explained. The III bound pepsin was further inactivated with DAN to protect autolysis during ESR measurement. The large value of $2A_{z'z'}$ for a DAN modified complex (pepsin-III) suggests that the space around the binding site of III is narrow from the first or becomes crowded on modification with DAN.

With the spin label IV no labeling of pepsin occurred at all. The reactive site of IV is sterically blocked and may be inaccessible to the active site residue of pepsin.

Pepstatin, the inhibitor of the enzyme, binds even to the diazoketone-modified pepsins in a non-covalent manner (7,11). When pepstatin was added to the labeled pepsins, the value of $2A_{z'z'}$ increased as shown in Table I. In the case of pepsin-I τ_c was calculated as 1×10^{-8} sec. Apparently pepstatin binds even to the labeled pepsins and reduces the mobility of the nitroxide. These observations indicate that the binding site of pepstatin is close to the active site aspartic acid-215. A similar conclusion was reported previously (11, 12).

p-Bromophenacyl bromide (BPB) reacts also with pepsin by the formation of a 1:1 covalent complex (13). Aoshima et al. modified pepsin with a bromoacetamide type spin label which has the same reacting group as BPB (14). The complex with BPB, however, can be further modified with DAN at the active site (13) and accordingly, the labeling with the bromoacetamide spin label at the active site is suspected.

As to RAP similar results were obtained. The spin labels I and II linked to RAP in a 1:1 ratio, probably at the active site aspartic acid, whereas III coupled to a different residue. The value of τ_c is 3×10^{-9} sec in RAP-I and is smaller than in pepsin-I, indicating that the cleft space of RAP is larger than that of pepsin. Survey of the $2A_{z'z'}$ values (Table I) shows that the nitroxide motion is restricted in RAP-II and -III. The addition of pepstatin reduced the nitroxide motion in all the labeled RAP. An increase of τ_c to 1×10^{-8} sec due to the addition of pepstatin to RAP-I supports that pepstatin binds to the site(s) close to the active center.

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