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SPIN-LABELING STUDY ON THE DEPTH OF THE ACTIVE SITE OF PAPAIN

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Summary: Papain is alkylated with a series of haloacetamide spin labels with varying distance between the haloacetamide residue and the nitroxide portion. The dependence of τ_c on the distance and temperature revealed that the active site has a narrow neck and one auxiliary binding site around the neck. The depth of the active site is estimated about 10 Å.

Papain is a sulfhydryl protease which contains only one thiol group at the active site and its three dimensional structure was determined by X-ray crystallographic analysis (1). Recently, the fluorescence (2) and the ^{19}F labeling (3) techniques were applied to the native papain, and the microscopic dissociation constants of the active site histidine-159 and aspartic acid-158 were determined. We labeled the active site thiol group of cysteine-25 with a series of haloacetamide spin labels I - VI (Table I), in which the distance, d, between the haloacetamide residue and the nitroxide portion (2,2,6,6-tetramethylpiperidene-1-oxyl group) was gradually increased, and determined the depth of the active site hole in native papain.

Materials and Methods

Materials: Papain (2 x crystallized) was purchased from P-L Biochemical, Inc. and α-N-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride was from Nakarai Chem. Ltd. The spin label I was synthesized according to the known method (4) and the other spin labels II - VI were newly prepared by similar procedures (5).

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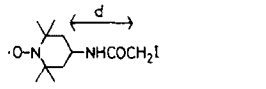
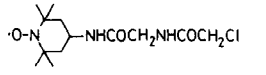
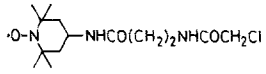
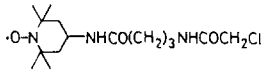
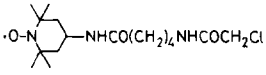
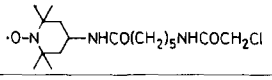
Table I. Haloacetamide Spin Labels I - VI

Spin Label	Structure	d(A)
I		3.7
II		7.1
III		8.4
IV		9.6
V		10.9
VI		12.1

Spin-Labeling of Papain: In a typical experiment, 2.2×10^{-7} mole of papain was dissolved in 1.5 ml of 0.05 M acetate buffer (pH 5.7), containing NaCN (5×10^{-3} M) as an activator of papain and disodium EDTA (3×10^{-3} M), and the mixture was kept for 10 min at room temperature. Then, 7.4×10^{-7} mole of the spin label reagent in 50 µl ethanol was added to the enzyme solution. Extent of inactivation of the papain was followed by the assay using α-N-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride as substrate as described by Arnon with a slight modification (6). After the papain was completely inactivated, the solution was exhaustively dialyzed against distilled water (2 l x 8) to remove the remaining spin label reagent.

ESR Measurement and Analysis: ESR spectra were recorded by a JEOL JES-FE1X spectrometer with 100 kHz modulation. 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) (7). For highly immobilized labels ($\tau_c > 10^{-8}$), τ_c was characterized from the high and low field peak positions according to the method of McConnell *et al.* (8).

Results and Discussion

The free thiol group of papain is specifically alkylated by a variety of haloacetic acids and their derivatives (6), and thus, is considered to be labeled by the spin labels I - VI.

The X-ray study showed that the active site thiol group is located at the bottom of a shallow hole in crystalline papain (1). The structure of this active site hole may not change seriously in native papain. Accordingly, as the distance, d, between the haloacetamide residue and the nitroxide

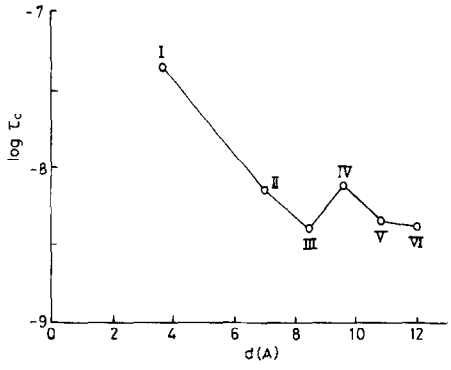


Figure 1. Dependence of τ_c on the distance, d.

portion increases, the nitroxide portion would go out of the hole and the mobility of the nitroxide portion would become increased. Indeed this expectation was found partially true.

The values of τ_c obtained for each labeled papain were plotted against the distance, d, as shown in Fig. 1. (We abbreviate hereafter, for example, the I-labeled papain as papain-I and so on.) It can be seen that, as d is increased, the mobility of the nitroxide portion of the labeled papains increased up to papain-III, becomes slightly decreased on going from papain-III to -IV, and then, increases again in papain-V and -VI. These findings suggest that the nitroxide motion is restricted in some ways in papain-IV, but not in papain-III, -V, and -VI. In papain-V, d may be long enough for the nitroxide portion to go out of the active site hole.

In order to gain more insight into the unexpected behavior of papain-IV the temperature dependence on τ_c was investigated for papain-III, -IV, and -V (Fig. 2). The curve for papain-IV is likewise different from those for papain-III and -V. The mobility of the nitroxide portion in papain-III and -V increases gradually on increasing temperature, but slight stepwise increments of the mobility were observed at about 37 °C, which may be ascribed to the structural transition of papain.

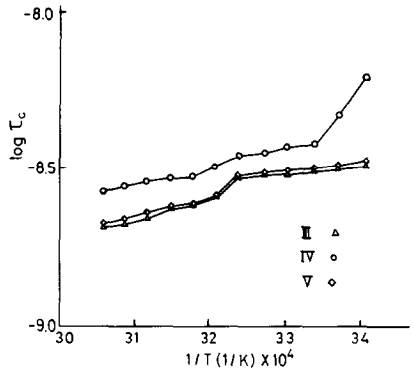


Figure 2. Temperature dependence of τ_c for papain-III, -IV, and -V.

In contrast to papain-III and -V, papain-IV exhibits a larger change in the mobility in the temperature range of 21 - 27 °C. This fact suggests that the nitroxide motion is restricted in papain-IV due to temperature dependent specific interaction between the nitroxide portion and auxiliary binding site(s) in the active site hole. Indeed the structural consideration of papain supports this view: The hydrogen bond could be formed between the carboxyl group of asparatic acid-158 and the N-H group attached to the 4 position of the piperidine moiety.

If the nitroxide motion in papain-IV is reduced only by this interaction, the curve for papain-IV should come near to ones for papain-III and -V in Fig. 2. However, above 30 °C the curve for papain-IV runs parallel to those for papain-III and -V, and the mobility of the nitroxide portion in papain-IV is always lower than those in papain-III and -V independent of temperature. Thus, the presence of other interaction such as steric hindrance is probable. The model consideration suggests the presence of the steric interaction between glycine-65 and the piperidine moiety in papain-IV.

Similar abnormal motion of the nitroxide portion against distance changes was also observed by Wee and his co-workers in the spin-labeling study of bovine carbonic anhydrase (9). Their findings might be explained in similar ways.

In conclusion the active site hole of papain is found to have a narrow neck and one auxiliary binding site around the neck. The depth of the hole is estimated to be about 10 A.

References

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