ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 310, No. 1, April, pp. 167–171, 1994

# Active Site Structure and Stability of the Thiol Protease Papain Studied by Electron Paramagnetic Resonance Employing a Methanethiosulfonate Spin Label

D. Allan Butterfield*,†,[1] and Jinbo Lee*

*Department of Chemistry and †Center of Membrane Sciences, University of Kentucky, Lexington, Kentucky 40506-0055

Received August 30, 1993, and in revised form December 8, 1993

**The electron paramagnetic resonance (EPR) spin labeling technique has been employed to study the properties and conformation of the thiol protease papain in solution, using (1-oxyl-2,2,5,5-tetramethyl-$\Delta^3$-pyrroline-3-methyl) methanethiosulfonate (MTS) as the spin label. The measurements of papain's amidase activity corroborate the EPR results. The major findings are: (i) the motion of the MTS spin label is very sensitive to the active site conformation of papain, which may reflect the location of the pyrroline ring of the spin label near the narrow portion of the active site cleft of papain, and thus there may be intimate interactions between the spin label and its environment; (ii) the active site cleft of papain may have a more open structure at intermediate pH (pH 4.2 to 8.0) than at higher (pH > 8.0) or lower (pH < 4.2) pH, which is consistent with the bell-shape pH curve of the enzyme's amidase activity with the optimum pH at pH 7.00; and (iii) the motion of spin label at the active site of free papain in solution becomes slower upon addition of a denaturant (urea or guanidine hydrochloride), suggesting that the denatured enzyme may have a more closed active site cleft. Urea is more effective than guanidine hydrochloride in denaturing papain at low concentration. However, both urea and guanidine hydrochloride can completely inactivate papain at high concentrations. When an appropriate spin label is selected to label the active site of papain (such as MTS spin label), the EPR spin labeling technique may offer additional insight into the conformation of papain over that obtained by optical methods. These results are discussed in terms of possible studies of biofunctional membranes, opaque assemblies in which a biological molecule is attached to a polymeric membrane.** © 1994 Academic Press, Inc.

Spin labeling of the active site of enzymes and other proteins has been used for a number of years to gain structural information about peptides (1). Insight into the conformation, polarity, and motion of the local microenvironment and the depth of the active site cleft can often be obtained using spin-labeled enzymes (1, 2). This electron paramagnetic resonance (EPR) method is applicable to opaque samples in which optical methods are not feasible, e.g., in biofunctional membranes, those entities in which an enzyme is covalently attached to a polymeric matrix cast in the form of porous membranes (3–5). Zhuang and Butterfield first employed EPR spin labeling methods to examine the conformation of the cysteine protease papain in biofunctional membranes using a lengthy nitroxide derivative of *p*-chloromercuribenzoate (Fig. 1) (3–5). The difficulty with this SH-group-specific spin label was that the piperidine ring containing the nitroxide moiety protruded out of the active site cleft; consequently, structural information about the active site cleft under a variety of perturbations could only be indirectly inferred. Further, the benzoic acid moiety of the *p*-chloromercuribenzoate nitroxide spin label was held firmly in the active site cleft, leading to anisotropic rotation of the piperidine ring with consequent atypical EPR spectra (e.g., the low-field resonance line was of larger amplitude than the central line, in contrast to normal nitroxide EPR spectra) (3, 6). Attempts to circumvent these difficulties in order to gain further insight into the structure of immobilized enzymes in biofunctional membranes were made with other SH-binding spin labels such as the maleimide derivative MAL-6 (Fig. 1). However, MAL-6 was found to bind to lysine $NH_2$ groups in addition to the single SH group of papain, located at the active site (7). Multiple binding sites on a single protein complicates the resulting EPR spectrum.

[1] To whom correspondence and reprint requests should be addressed.

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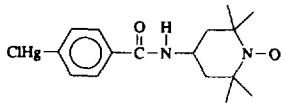
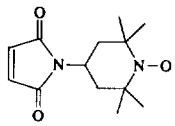
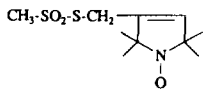


PMB-SL



MAL-6



MTS

**FIG. 1.** Structures of the SH-binding spin labels mentioned in this work. PMB-SL, *p*-chloromercuribenzoate amide derivative of 4-amino-2,2,6,6-tetramethylpiperidine-*N*-1-oxyl; MAL-6, 2,2,6,6-tetramethyl-4-maleimido-piperidine-*N*-1-oxyl; MTS, (1-oxyl-2,2,5,5-tetramethyl-$\Delta^3$-pyrroline-3-methyl) methanethiosulfonate.

Berliner and co-workers (8) introduced a SH-specific spin label of short length, the methanethiosulfonate (MTS)[2] spin label (Fig. 1). These workers showed that MTS is thiol-specific, and this label provided a potentially new approach to studying enzymes. However, relatively little was reported on the applicability of MTS to traditional studies of enzymes. For example, Berliner and co-workers (8) showed that MTS could be displaced from the thiol group of papain by DTT and that the bound spin label completely inhibited the activity of the protease; however, these workers did not report the pH dependence of the spectra and the effects of denaturants on the conformation of the enzyme. Consequently, in order to determine if MTS would be a useful spin label for investigating the structure of immobilized enzymes in biofunctional membranes, we thought it necessary and important to ascertain first whether this SH-specific spin label would be sensitive to subtle conformational changes in an isolated enzyme. Accordingly, this paper reports the results of EPR studies of MTS-labeled papain. The results are consistent with an active site structure in solution that is similar to that reported for papain in the solid state. These studies also suggest that MTS has great promise as a spin label for the investigation of the active site structure of enzymes immobilized in biofunctional membranes.

[2] Abbreviations used: MTS, methanethiosulfonate; DTT, dithiothreitol; BAPNA, *N*-benzoyl-DL-arginine-4-nitroanilide; DMSO, dimethyl sulfoxide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

## MATERIALS AND METHODS

*Materials.* Dried papaya latex, *N*-benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA), DL-cysteine, and guanidine hydrochloride were obtained from Sigma Chemical Co. Urea was obtained from BDH Chemicals Ltd., while disodium EDTA and dimethyl sulfoxide (DMSO) were obtained from Aldrich. The spin label, (1-oxyl-2,2,5,5-tetramethyl-$\Delta^3$-pyrroline-3-methyl) methanethiolsulfonate, was purchased from Reanal (Budapest, Hungary). The ultrafiltration membranes were obtained from Millipore Co. All other chemicals and solvents were reagent grade.

*Isolation and purification of papain.* Papain was isolated and purified from dried papaya latex according to the methods described by Baines and Brocklehurst (9). The purified papain was stored in deionized water at 4°C. The purity of papain was tested by SDS–PAGE, and a single band appeared at approximate molecular weight 23,000.

*Spin labeling papain active site using MTS spin label.* The concentration of the papain stock solution in deionized water was estimated by the method of Lowry *et al.* (10), and the protein content was adjusted to 8.0 mg/ml with deionized water. Four milliliters of this papain solution was activated in 50 mM phosphate buffer, pH 7.0, containing 2 mM sodium cyanide and 0.8 mM disodium EDTA at room temperature with stirring for 2 h. The MTS spin label (1.2–1.8 mg) was dissolved in 0.1 ml of acetone and added to the papain solution. The reaction was then carried out at room temperature for 10 min. Unreacted spin label was removed by gel filtration on a Sephadex G-25 (medium) column, using 5 mM $NH_4HCO_3$ buffer containing 0.5 mM disodium EDTA as the eluting buffer. The protein fractions detected at 280 nm were pooled, and the labeled papain was concentrated by ultrafiltration using an ultrafiltration membrane of 10,000 molecular weight cutoff.

*EPR spectra acquisition.* All the EPR spectra of MTS-labeled papain were recorded on a Bruker ESP-300 spectrometer with a TM rectangular cavity at room temperature. The enzyme concentration was about $10^{-5}$ M for all the EPR studies. Typical parameters for the acquisition of the EPR signals were the following: microwave frequency, 9.78 GHz; microwave power, 24.2 mW; modulation frequency, 100 kHz; modulation amplitude, 0.32 G; and scan width, 130 G.

*Assay for the amidase activity of papain in solution.* The amidase activity of papain was determined by reaction with BAPNA, as described by Arnon (11). A BAPNA stock solution (2 mM) in 50 mM Tris buffer, pH 7.5, was prepared by dissolving 43.5 mg BAPNA in 1 ml DMSO and then dispersed into 49 ml of Tris buffer. Thus, the final DMSO content in the substrate stock solution was 1% (v/v). The papain stock solution was adjusted to 2 mg/ml with deionized water. An activating solution, 20 mM cysteine and 4 mM EDTA in 50 mM Tris buffer, pH 7.5, was also prepared.

In order to determine the appropriate activation time, 0.1 mg of papain was activated in 0.5 ml of 0.1 M Tris buffer, pH 7.5, containing 10 mM cysteine and 2 mM EDTA for different time periods at 37°C. Then 0.25 ml of Tris buffer and 0.2 ml of substrate (final substrate concentration, 0.5 mM) were added and reacted for 15 min in a 37°C water bath with agitation. The amount of 4-nitroaniline released was estimated spectrophotometrically at 410 nm ($\epsilon$ = 8800 $M^{-1}$ $cm^{-1}$).

In the enzymatic assay procedure, all the solutions used were prewarmed to 37°C in the water bath. Papain (0.1 mg) was activated in the activating solution containing 10 mM cysteine and 2 mM EDTA for 2 min at 37°C with agitation. Different amounts of substrate (final substrate concentration, 0.01–1 mM) were added and the final volume of the reaction mixture was adjusted to 1 ml with 50 mM Tris buffer, pH 7.5. The reaction mixtures were incubated for 16 min at 37°C with agitation. The amount of 4-nitroaniline released was estimated spectrophotometrically as noted above. Two controls were used in the assay. One contained all the reagents except the substrate (Tris buffer as the

substitute). The other one contained all the reagents except papain. The kinetic parameters, $K_m$ and $V_{max}$, were obtained from Lineweaver–Burk plots of the experimental data.

*pH studies.* The buffers (0.2 M) used in the pH study were acetate buffer for pH 3–5, phosphate buffer for pH 5.8, and Tris buffer for pH 9–10. In the study of the effect of pH on the papain active site conformation, papain was incubated in buffers of different pH (pH 3.03–9.81) for 15 min at room temperature before the EPR spectrum was acquired. The distance between the high-field and the low-field line, $2A'_{zz}$, was used as a parameter for the motion of the spin label at the papain active site.

When measuring the papain activity under different pH, the activating solution and the substrate solution were prepared as described above but in deionized water. A solution of 0.1 mg of papain in deionized water was added to the tube, and the total volume was adjusted to 0.25 ml with the buffer of appropriate pH. The solution was incubated at 37°C for about 2 min. Then 0.25 ml of activating solution was added and papain was activated in 10 mM cysteine and 2 mM EDTA for 2–4 min. Deionized water (0.25 ml) and 0.25 ml substrate solution were then added and reacted at 37°C for 15 min with agitation (final substrate concentration, 0.5 mM BAPNA). The amount of 4-nitroaniline released was estimated spectrophotometrically as noted above. The solutions containing all the reagents but papain (deionized water as substitute) were used as control in each pH sample.

*Denaturant studies.* The effects of the denaturants urea and guanidine hydrochloride on the papain active site conformation and papain amidase activity were studied. Ten molar urea and 8 M guanidine–HCl stock solution in deionized water were prepared, respectively. In the conformation studies, MTS-labeled papain was incubated in urea (0–8 M) or guanidine–HCl (0–6 M) solutions at different concentrations for a half hour at room temperature before the EPR spectrum was acquired. When measuring the papain activity under different concentrations of urea or guanidine hydrochloride, the activating solution, the substrate solution, and the enzymatic assay were prepared or performed as above.

## RESULTS

The MTS spin label has been shown to specifically label the Cys-25 residue at the active site of the papain molecule, yielding a mixed disulfide bond between them (8). A typical EPR spectrum of MTS-labeled papain is shown in Fig. 2. The mobility of the spin label is slow, but isotropic, reflective of a nitroxide group which interacts rather strongly with its environment (active site of papain). The rotational correlation time of the spin label under slow isotropic motion can be estimated by the change in splitting of the outer extrema, $2A'_{zz}$ (12–16). The larger the value of $2A'_{zz}$, the larger the rotational correlation time and thus the more immobilized the spin label. This separation between the outer peaks of an EPR spectrum, $2A'_{zz}$, has been used to detect minute changes of the molecular motion of spin labels in several enzyme active site studies (17–19).

Papain is a multifunctional enzyme which has the activities of protease, esterase, and amidase (11). The papain amidase activity was studied in this research in order to determine if there were a correlation with the EPR studies. A synthetic amide, BAPNA, was used as the substrate and the unit of the activity is micromoles of 4-nitroaniline released per milligram of enzyme per minute (M/mg · min).

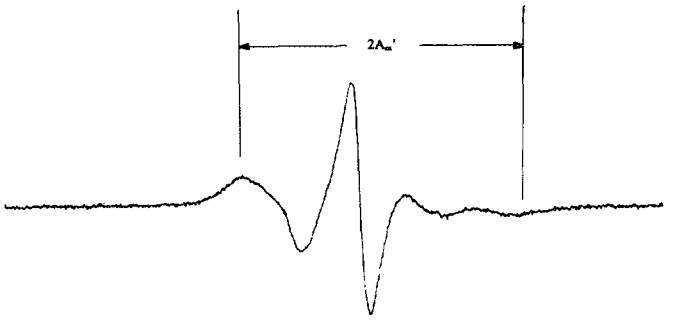



**FIG. 2.** A typical EPR spectrum of MTS-labeled papain in 0.1 M phosphate buffer, pH 7.0. The $2A'_{zz}$ motional parameter determined by the computer in the EPR spectrometer from the maximum of the $M_I = +1$ low-field line and the minimum of the $M_I = -1$ high-field line is indicated. The latter was measured from spectra whose amplitude was greatly expanded compared to that of the spectrum shown.

The reaction conditions in the activity assay were first optimized. The appropriate time period for activating papain was found by activating the papain at 37°C for different time periods prior to measuring the activity. Two minutes was sufficient to fully activate the enzyme and the activities for longer activation time periods remained constant. Therefore, a 2-min activation time was chosen for all the activity measurements of free papain in solution. There was no absorption observed for the two controls used in the assay and thus no correction needed to be made for the absorption of the samples.

The amidase activity follows Michaelis–Menten kinetics. The kinetic parameters, $V_{max}$ and $K_m$, were obtained from the Lineweaver–Burk plot of the experimental data. In our studies, free papain in 50 mM Tris buffer, pH 7.5, has an average maximum reaction velocity of 68.6 M/mg · min and an average $K_m$ of 0.377 mM.

Changes in pH may change the ionization state of the amino acid residues at the enzyme active site and thus may change the conformation of the active site. The spin label motion parameter, $2A'_{zz}$, of MTS in papain is pH dependent. $2A'_{zz}$, is smaller at intermediate pH (pH 4.2–8.0) and larger at both high (pH > 8.0) and low (pH < 4.2) ends of the pH curve (Fig. 3). Therefore, the spin label at the active site of papain has faster motion at intermediate pH and slower motion at high or low pH, which suggests that the active site cleft of papain is more open at the intermediate pH than at high or low pH. The enzymatic activity is almost constant at intermediate pH from 5.1 to 7.0, and the activity drops at both high and low pH, in general agreement with the EPR results (data not shown).

### *Denaturant Studies*

*Urea.* A common protein denaturant, urea, was applied to assess the effect of a denaturant on the active site conformation of papain in solution. The dependence of spin label motion parameter, $2A'_{zz}$, on the urea concen-

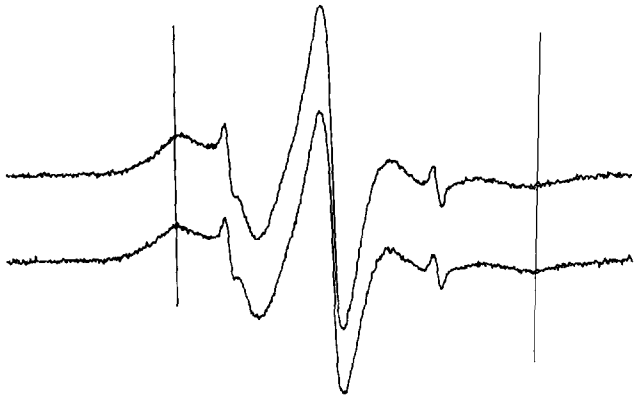


FIG. 3. The effect of pH on MTS-labeled papain. Top spectrum, pH 7.0; bottom spectrum, pH 9.8. The $2A'_{zz}$ parameter is indicated from the bottom spectrum and is clearly larger than that for the top spectrum. A small amount of unbound spin label is shown by the more narrow lines of each spectrum.

tration is given in Fig. 4. Urea is effective in changing the active site conformation of papain. As the urea concentration increases, the value of $2A'_{zz}$ increases correspondingly. Even at 1 M urea, the change in conformation is dramatic, judging from the nearly 6 G increase of $2A'_{zz}$ compared with the sample without urea (Fig. 4). A change in $2A'_{zz}$ of as much as 12 G was observed when the urea concentration increased from 0 to 8 M (Fig. 4), implying that the motion of the spin label at the active site of papain decreases substantially as a function of urea concentration. Thus, the active site cleft, at least the portion near Cys-25 that the spin label detected, may have a more closed-structure upon urea-induced denaturation.

Corresponding to the decrease in motion of MTS at the active site, the activity of papain drops significantly upon addition of urea (Fig. 4), and the results of the activity measurements are highly correlated to the results of conformational studies by EPR spin labeling methods. As observed in the pH studies, the more open the active site cleft (at least the portion that the spin label detected) the higher the papain amidase activity.

*Guanidine hydrochloride.* Another protein denaturant, guanidine hydrochloride, was also used to denature papain. The results of the conformation studies using EPR and the activity measurements also are shown in Fig. 4. Guanidine hydrochloride is not as effective as urea in denaturing papain at low concentrations. When the concentration changes from 0 to 1 M, the increase of $2A'_{zz}$ is only about 2 G for guanidine hydrochloride but nearly 6 G for urea. However, the motion of MTS spin label at the active site of papain decreases ($2A'_{zz}$ increases) continuously as the concentration of guanidine hydrochloride increases. At a concentration as high as 6 M, guanidine hydrochloride is as effective as urea at 8 M in totally denaturing papain, and the increase in $2A'_{zz}$ is 11.2 G compared to 12.2 G for urea.

The enzymatic activity measurements are consistent with the EPR results. Guanidine hydrochloride is not as effective as urea in denaturing the enzyme at low concentrations. Upon addition of 1 M guanidine hydrochloride, the amidase activity of papain almost did not change, with just a 1.3% decrease compared to that of the control (Fig. 4). However, guanidine hydrochloride becomes more effective in denaturing papain as the concentration increases. Comparison of the EPR results and activity measurements suggests the same inference as that in the previous studies (pH, urea); namely, the more closed the active site cleft of papain (the less the motion of the spin label at the active site), the lower the activity.

## DISCUSSION

Previous X-ray studies of papain suggest that the active site cleft of papain in the solid state appeared to become wider at around 8.4 Å and then narrower at 9.6 Å from the bottom, and the depth of the cleft was estimated to be 10 Å (17, 18). Considering the length of the MTS spin label and the formation of the mixed disulfide bond between the Cys-25 residue and the spin label, the pyrroline ring of the spin label may locate near the narrow portion of the active site cleft of papain. Thus, there may be intimate interactions between the spin label and its environment. Furthermore, the motion of the spin label may be very sensitive to the active site conformation of papain. The results of the current study showed that the motional parameter, the splitting between the high-field and the low-field lines of the EPR spectrum ($2A'_{zz}$), is very sensitive to the active site conformation of papain under different conditions (pH, urea, and guanidine hydrochloride).

Two active site residues, Cys-25 and His-159, are thought to form an ion pair and three possible states exist as the pH changes. At intermediate pH (4–8.5), a thiol–

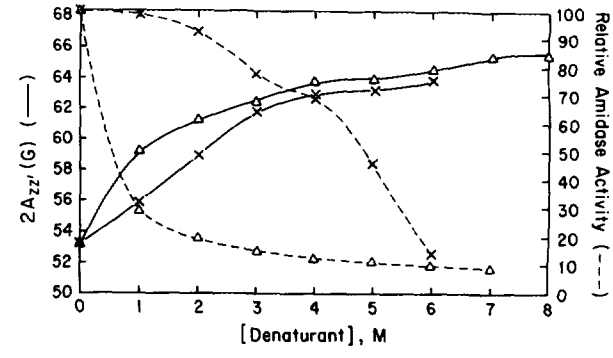



FIG. 4. Effect of urea and guanidine hydrochloride on the active site conformation and amidase activity of papain in solution. The splittings between the high- and the low-field lines are plotted against the concentration of urea (— △ —) or guanidine hydrochloride (— × —), as are the effects of urea (- - △ - -) or guanidine hydrochloride (- - × - -) on the respective relative amidase activity. Each point on each curve is the mean of three trials and the standard deviation is less than 5% of the respective mean value.

imidazole pair is assumed to exist predominantly as the ion pair $S^- \cdots {}^+HIm$, which should be the most active form of papain (20). The pH studies in this research show that the activity of papain remains almost constant between pH 5 and 7. The activities drop a little at both ends of this pH range (pH 4.2 and pH 8.0). The decrease in activity is more significant at low pH than at high pH; that is, the relative activity (relative to that at pH 7.0) decreases 67.6% at pH 3.03, while the decrease is only 37.5% at pH 9.81. This finding is again consistent with the active site model developed by Polgar and Halasz (20), as are the EPR results. At pH 4.2 to 8.0, the conformation of papain changes such that the interaction between the spin label with its environment at the active site of papain is decreased with subsequent faster motion of the spin label.

We previously used a 14-Å, mercuribenzoate spin label to study papain in solution (6). The reporter group of this label was outside the active site cleft of this enzyme and consequently was unable to detect some of the more subtle changes in active site conformation observed by the much shorter MTS spin label. With MTS as the spin label, EPR is a very sensitive technique for studying the effect of denaturants (urea and guanidine hydrochloride) on the active site conformation of papain (Fig. 4). Others have used MTS to study bacteriorhodopsin and colicin E1 (21, 22).

Although only slight changes in the conformation of papain could be detected by optical rotatory dispersion (ORD) and viscosity measurements even in 8 M urea (23), the change in conformation (at least the portion of the active site cleft that the spin label probed) detected by the EPR spin labeling technique in this study is significant upon addition of urea (Fig. 4). The amidase activity of the enzyme is almost totally lost at urea concentrations higher than 4 M (Fig. 4). The results of guanidine hydrochloride are fairly consistent with those of other workers (19); guanidine hydrochloride (6 M) completely denatures the enzyme and causes a significant increase in $2A'_{zz}$. Thus EPR spin labeling using MTS not only can detect the conformation change in the enzyme but also can distinguish how the conformation is changed, which is beyond the capability of activity measurements, and is apparently more informative than measurements using optical methods.

This research provides additional insight into the structure of papain employing MTS and, as such, extends the initial work of Berliner and co-workers (8). With greater insight into the structure and stability of papain in solution, it is now possible to compare these properties upon immobilization to opaque polymeric matrices. MTS shows promise as a spin label for examining these biofunctional membranes, and such studies are currently in progress (24).

## ACKNOWLEDGMENTS

This work was supported in part by grants from NSF (EHR9108764) and NIH (AG-10836).

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