Denaturation studies of active-site labeled papain using electron paramagnetic resonance and fluorescence spectroscopy

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ABSTRACT A spin-labeled *p*-chloromercuribenzoate (SL-PMB) and a fluorescence probe, 6-acryloyl-2-dimethylaminonaphthalene (Acrylodan), both of which bind to the single SH group located in the active site of papain, were used to investigate the interaction of papain (EC 3.4.22.2) with two protein denaturants. It was found that the active site of papain was highly stable in urea solution, but underwent a large conformational change in guanidine hydrochloride solution. Electron paramagnetic resonance and fluorescence results were in agreement and both paralleled enzymatic activity of papain with respect to both the variation in pH and denaturation. These results strongly suggest that SL-PMB and Acrylodan labels can be used to characterize the physical state of the active site of the enzyme.

INTRODUCTION

Papain (EC 3.4.22.2) is a protease isolated from the latex of *Carica papaya* (1–3). The enzyme consists of a single polypeptide chain with 212 residues with a thiol group in the active site. The complete primary sequence and the three-dimensional structure of this 23,000 dalton enzyme are known (1, 2). The structural conformation of papain is stabilized by three disulphide bonds, and the main chain of the enzyme is folded to form two domains of roughly equal size with a deep cleft between them. The catalytic site cysteine residue (Cys-25) is near the bottom of one wall of this cleft, and the catalytic site histidine residue (His-159) and asparatic acid residue (Asp-158) are on the other wall.

In the view of many investigators, a new generation of synthetic membranes involving membrane-bound enzymes (bioreactors) is needed to advance technological applications of membranes. However, to develop these systems, greater understanding of the structure, function, and stability of enzymes is required. The electron paramagnetic resonance (EPR) techniques of spin labeling have provided important advances in understanding the physical state of biological systems (for reviews and applications see references 4–12), including proteins (13, 14). In this paper we report the results of a study employing EPR spectroscopy of papain labeled exclusively at the active site thiol group of cysteine-25 with a spin-labeled *p*-chloromercuribenzoate (SL-PMB). Differential effects on the EPR spectra in urea- and guanidine hydrochloride-mediated denaturation have been investigated. In addition, fluorescence measurements of papain labeled with a sulfhydryl-specific fluorophore and enzymatic analysis were used to corroborate the EPR data.

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MATERIALS AND METHODS

The preparation of the spin-labeled *p*-chloromercuribenzoate (SL-PMB) used in this study was described by Zantema et al. (15). Briefly the procedure was as follows: 0.5 g sodium *p*-chlorimercuribenzoate (PMB) (Aldrich Chemical Co., Milwaukee, WI) was converted to the acid chloride by refluxing with 40 mL thionyl chloride for 4 h. The hot solution was filtered over glasswool to remove the residual PMB. After 2 d *p*-chloromercuribenzoyl chloride crystals were filtered by vacuum filtration under N_2 and washed with diethyl ether. 0.22 g of crude acid chloride was dissolved in 6.0 mL pyridine. 0.11 g 4-amino-2,2,6,6-tetramethyl piperidin-1-oxyl (Tempamine) (Aldrich Chemical Co.) was dissolved three times in freshly distilled pyridine and evaporated to dryness, and then dissolved in 2.5 mL pyridine. The solution was then added to the acid chloride solution and the mixture was stirred at room temperature for 1 h and pyridine removed by rotary evaporation. The residue was then four times suspended in chloroform and evaporated. Crude SL-PMB was suspended again in 4 mL chloroform, the white precipitate (PMB) was removed by centrifugation, and the orange solution was applied on a 20 g silicalgel column in chloroform. The column was eluted with increasing concentration of methanol. The yellow band eluted at 4.5% methanol was evaporated to dryness and dissolved in 1.5 mL hot ethanol. SL-PMB was crystallized from this solution. The resulting orange crystal was found to melt at 212–214 °C. The mass spectrum showed the characteristic HgCl isotope pattern at the correct mass.

Papain was isolated and purified from dried papaya latex (Sigma Chemical Co., St. Louis, MO) according to the method described by Baines and Brocklehurst (16). The protein was shown to be homogeneous by 7.5% polyacrylamide gel electrophoresis. The free thiol groups of papain was analyzed by means of Ellman's reagent, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) (17). The proteolytic activity of the enzyme was assayed using casein as substrate in 50 mM Tris-HCl

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buffer at 37°C in the presence of activators, which were comprised of 5 mM cysteine and 2 mM EDTA (18). The protein concentration was determined by the method of Lowry et al. (19).

The labeling of papain with SL-PMB was carried out at room temperature by activating the papain solution (5 mg/mL) in 50 mM phosphate buffer at pH 7.0, containing 5 mM NaCN and 2 mM disodium EDTA for 30 min with the activations noted above. This procedure was later followed by addition of a five-fold excess of SL-PMB in 50 μL ethanol to the papain solution, which was kept at room temperature for 1 h. Excess spin label was removed from the solution mixture by gel filtration on a Sephadex G-25 column using 10 mM NH_4HCO_3 buffer as eluent. The protein fractions detected at 280 nm were freeze-dried and redissolved in a minimum volume of phosphate buffer.

The EPR spectra of papain labeled with SL-PMB were measured in 50 mM phosphate buffer, pH 7.0 except where noted. The EPR resonance spectra were recorded on a Bruker ESP300 spectrometer with a rectangular cavity at room temperature. Typical EPR parameters for observation of the spin-label signals were the following: microwave frequency 9.7 GHz; microwave power 20 mW; modulation frequency 100 kHz; and modulation amplitude 0.32 G. The enzyme concentration was about 10^{-5} M. The labeled thiol group concentration of papain was directly determined by comparison of the double integration of standard spectra of a spin label in solution to the double integration of the EPR spectra of spin-labeled papain and that resulting from release of SL-PMB from papain by reaction with either dithiothreitol (DTT) or 2-mercaptoethanol (20).

The fluorescence probe, 6-acryloyl-2-dimethylaminonaphthalene (Acrylodan), was purchased from Molecular Probes (Eugene, OR). Papain was labeled with Acrylodan by the procedure described by Prendergast et al. (21) followed by elution through a Sephadex G-25 column as described above. Fluorescence emission spectra were obtained at room temperature on a Perkin-Elmer LS-50 A Spectrometer using an excitation wavelength of 393 nm. A UV quartz sample cell (NSG precision) with a 3-mm square path length was used for all samples.

Because of the pH dependence of papain, all EPR and fluorescence measurements for the denaturation study were carefully made at pH 7.0. The pH was adjusted by addition of 1.0 M HCl or 1.0 M NaOH.

RESULTS

The single SH group of papain located at the active site of this protease was covalently labeled by SL-PMB (Fig. 1 *A*) as evidenced by the absence of a spectrum in spin-labeled papain which had been previously treated with the SH reagent PMB (Fig. 1 *B*). These spectra also show that there is no nonspecific labeling of papain by SL-PMB. The binding ratio of SL-PMB to the enzyme was calculated to be 0.80:1 by double integration of EPR spectra and comparison with the spectra of the free spin-label standard. This finding is consistent with the results of DTNB titration of freshly prepared, nonspin labeled papain (Table 1) which also showed ~80% of papain molecules in the sample had a single free SH group. This finding confirms the DTNB titration data of Baines and Brocklehurst (16), who also reported ~80–85% of papain molecules could be activated to form a single SH group at the active site of the enzyme. And as

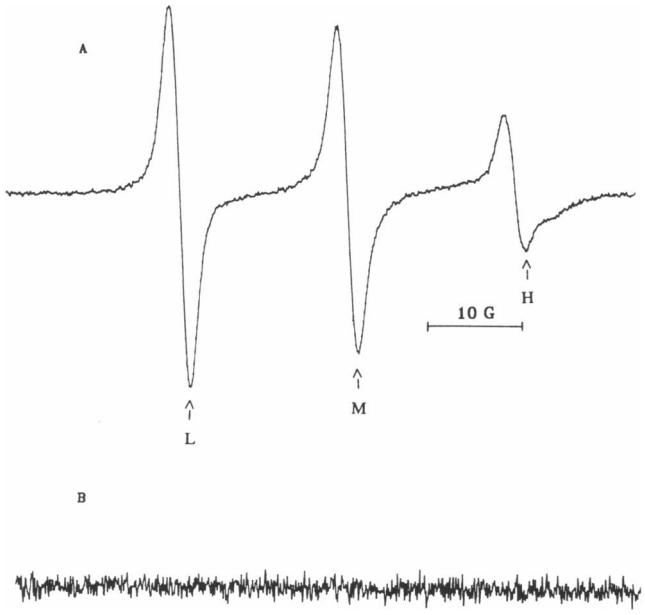

FIGURE 1 EPR spectra of SL-PMB labeled papain in 50 mM phosphate buffer, pH 7.0, (*A*) untreated with PMB, and (*B*) after prior PMB treatment. (L, M, and H refer to low, middle, and high field EPR lines, respectively. The peak-to-peak amplitudes of M and H are used to assess motion and denaturation. See text.)

seen in Fig. 1, it is only free SH groups that can bind the spin label used. Therefore, a 1:1 ratio of spin label incorporation to SH availability at the active site was found.

Berliner et al. (20) showed that for SH-group bound spin labels, exposure to DTT or 2-mercaptoethanol would displace these bound labels resulting in a sharp, three-line spectrum of the free tumbling nitroxide. We performed a similar study with SL-PMB labeled papain. Comparison of the double integration of the spectrum before exposure to DTT or 2-mercaptoethanol and after exposure to these reagents indicated that all the bound spin label was released (data not shown). This result is consistent with Fig. 1 which indicates that only the single SH group of the active site of papain was labeled by SL-PMB. In addition, comparison of the double integration of the spectrum of the DTT or 2-mercaptoethanol-induced release of the spin label to that of the solution

TABLE 1 Mole ratio of free SH group of papain to the enzyme as determined by DTNB titration

Enzyme added	Free SH group found	Average free SH group
0.10	0.0812	80.1
0.10	0.0791	

spectra of a spin label of known concentration also suggested that ~80% of the total papain molecules present were spin labeled by SL-PMB, consistent with the DTNB titration results in Table 1.

It should also be noted that both SL-PMB and Acrylodan inactivate the protein equally well. Therefore, it is highly likely that both the spin label and the fluorophore separately bind to the same, active-site sulfhydryl group.

Fig. 2 shows a comparison of the EPR signals obtained for the SL-PMB spin label attached to the protein (Fig. 2 *B*) and for the spin-label free in buffer solution (Fig. 2 *A*). The three-line pattern is due to the hyperfine interaction between the nitroxyl electron magnetic moment and the magnetic moment of the ^{14}N nucleus in the nitroxyl group. The EPR signal from the label covalently bound to papain shows a characteristic asymmetric broadening of the high-field line with a loss in amplitude due to the influence of the protein structure on the rotational motion of the attached spin-label. The signal from the bound label also shows that the peak-to-peak amplitude of the low-field line is higher than that of the middle-field line. Such an unusual EPR spectrum was also reported by other groups (22–24). Based on the results of these workers, the unusual spectrum is probably due to the rapid anisotropic motion of the piperidine moiety of the spin label molecule with respect to the enzyme and the benzoic acid ring. According to the three-dimensional structure of papain (1, 2), the active site Cys-25 of this protein is situated close to the bottom of the cleft between two domains. Spectroscopic studies of the active site of papain in solution (25) have suggested that the depth of the papain active site cleft from the surface of the protein was ~10 A. This is schematically illustrated in Fig. 3. In the present work, the distance between the two ends of the spin label, i.e., the nitroxide radical and the S-Hg bond, was estimated to be ~14 A (15). It is likely that the aromatic ring component of the SL-PMB label is fairly tightly bound to the cleft and the nitroxide head has some freedom to rotate relative to the enzyme. This idea is consistent with the results of elegant experiments by Brocklehurst et al. (26) who showed that His-159 in the active site of papain can be hydrogen bonded by appropriate substrates.

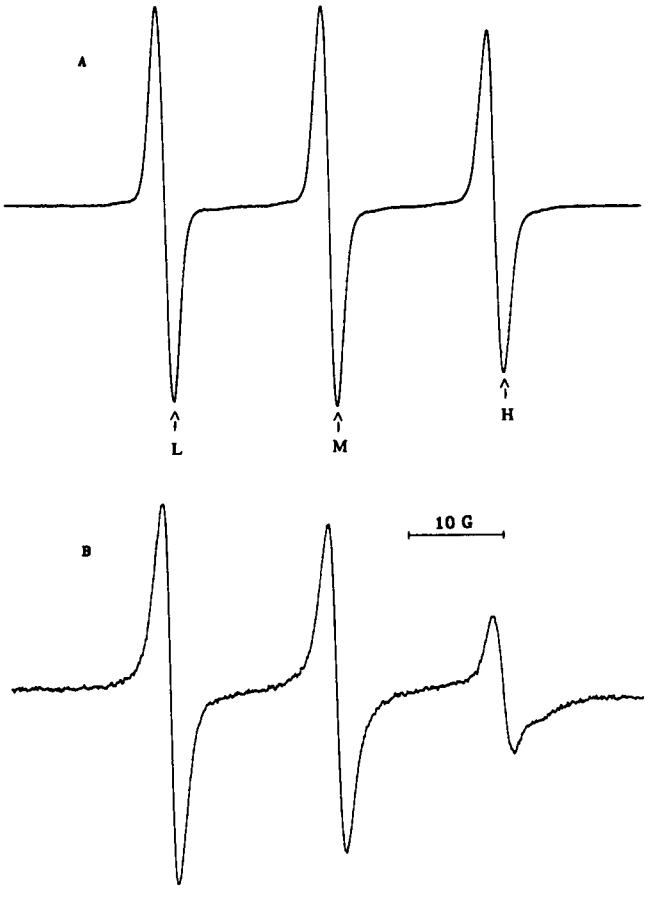

FIGURE 2 EPR spectra of SL-PMB spin label (*A*) in 50 mM phosphate buffer, pH 7.0 and (*B*) covalently attached to papain, dissolved in 50 mM phosphate buffer, pH 7.0. (L, M, and H are defined in the legend to Fig. 1.)

The hyperfine interaction constant characterizing the triplet of the spin-label EPR spectrum was found to be 17.0 ± 0.1 G. This result indicates that the environment of the label on the protein is accessible to the solvent. Spectra such as those in Fig. 1 *A* are further characterized as described previously (27, 28). Morrisett and Broomfield (27) have demonstrated that the change in the ratio of the EPR spectral amplitudes of the high field line (H) to the middle field line (M) can be used as a reliable measure to monitor the unfolding of the enzyme's tertiary structure. Berliner (28) used the inverse square root of this ratio, $(M/H)^{1/2}$, to indicate the change

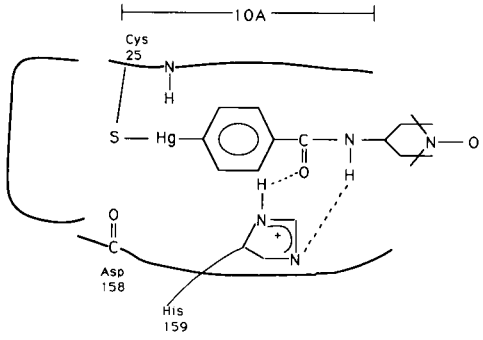

FIGURE 3 Schematic diagram showing the binding of SL-PMB to the SH group of Cys-25 in the active site of papain and the binding putatively stabilized by hydrogen bonding to His-159 (26).

in the molecular tumbling rate of the spin label. As spin label motion increases, $(M/H)^{1/2}$ decreases.

EPR and fluorescence studies demonstrate that papain possesses properties that are markedly pH-dependent around a pH range of 3–6 (25, 29, 30, and this work). Fig. 4 shows the changes of EPR spectra, relative fluorescence intensity, and proteolytic activity of papain as a function of pH. A correspondence among all three methods is seen, with maximal activity and maximal spectroscopic characteristics occurring at around pH 7. The rapid rise in these parameters and the maximal effect as a function of pH appear to result from the ionization of two ionizable groups, Asp-158 and His-159, respectively, as suggested by Bendall et al. (29), and consistent with the results of Brocklehurst et al. (26).

The EPR spectra of papain labeled with SL-PMB and enzymatic activity were also used to investigate the interactions of two protein denaturants with the enzyme (Fig. 5). As the urea concentration increases the three spectral lines do not change very much, while, in the case of guanidine hydrochloride, the high field line narrows in width and increases in peak height, thereby decreasing $(M/H)^{1/2}$. The change in peak amplitude with guanidine hydrochloride reflects the increased motion of the spin label with respect to its environment, consistent with denaturation of the protease. It is apparent that a large concentration dependence of $(M/H)^{1/2}$ is noted in the case of guanidine hydrochloride, while urea seems to have very little effect on this ratio. A similar trend is observed in the activity measurement of papain (Fig. 5). Relative to control, the proteolytic activity of papain is still maintained at ~80% active even in 8 M urea solution, but the activity of papain has dropped to

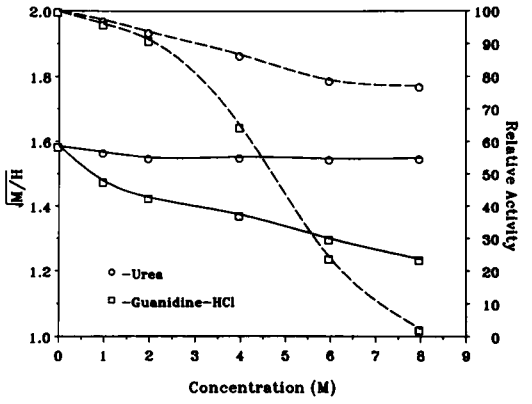

FIGURE 5 Changes of the EPR parameter $(M/H)^{1/2}$ of SL-PMB labeled papain (solid line) and proteolytic activity of papain (broken line) as a function of urea and guanidine hydrochloride concentrations.

almost zero in 8 M guanidine hydrochloride. Also, we observed that the activity loss in guanidine hydrochloride is not reversible.

To provide additional evidence for the differential effects of urea and guanidine hydrochloride on the motion of the active site of papain and its three-dimensional structure, fluorescence spectroscopy of papain labeled at active site with Acrylodan was used. Others have shown this label is specific for SH groups (21), consistent with our finding that the activity of papain is completely lost upon labeling of this fluorescence probe. Fig. 6 demonstrates that the relative fluorescence intensity of Acrylodan-labeled papain decreases with concentration of guanidine hydrochloride

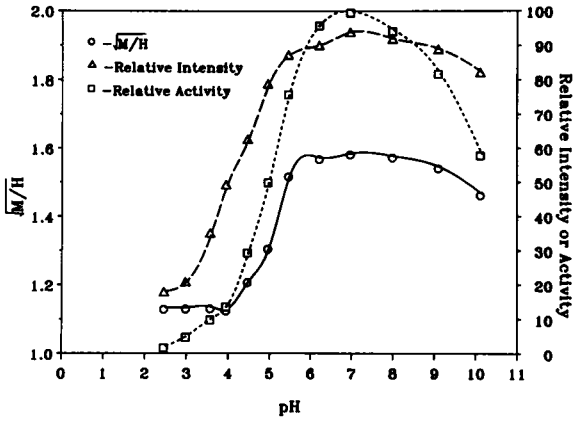

FIGURE 4 pH dependence of EPR spectra, relative fluorescence intensity and proteolytic activity of papain. In 50 mM phosphate buffer.

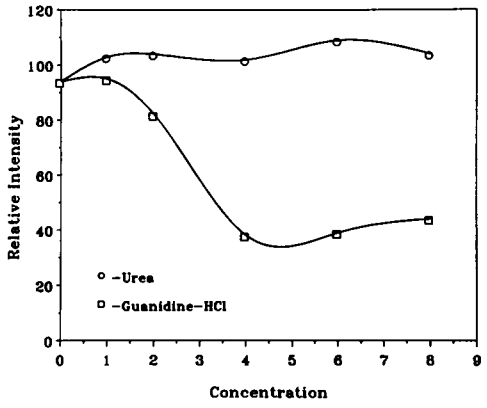

FIGURE 6 Maximum relative fluorescence intensity of Acrylodan-papain in 50 mM phosphate buffer, pH 7.0, as a function of urea and guanidine hydrochloride concentrations. All spectra were measured in 50 mM phosphate buffer, pH 7.0 with excitation wavelength of 393 nm. The slit width used was 5 mm.

and is unaffected by urea, consistent with the EPR results (Fig. 5). Also, we noted that the position of maximum emission intensity was shifted to higher wavelength upon denaturation by guanidine hydrochloride (data not shown). Prendergast et al. (21) have shown that Acrylodan fluorescence provided a good indication of the polarity of the environment. The red-shifted emission spectra suggest that the active site of papain undergoes a conformational change with increased exposure to the polar water environment. This change is accompanied by a decline in intensity. These results, together with the enzymatic activity study, corroborate the data found for spin-labeled papain.

DISCUSSION

The EPR spectrum of SL-PMB labeled papain does not present any detectable signal heterogeneity, consistent with only one type of binding site. The binding sites of both SL-PMB and Acrylodan were determined to be the active site cysteine residue of papain based on reaction with *p*-(chloromercuri) benzoic acid, sodium salt (PMB), a reagent which reacts specifically with free sulfhydryl groups of proteins to form mercaptides (31) and by the enzyme activity measurements. From these experiments it is apparent that both electron paramagnetic resonance and fluorescence spectroscopy reflect the changes in the active site region of the enzyme since the SL-PMB and Acrylodan are both attached to the active site cysteine. Therefore, we can assume that any structural changes detected by EPR and fluorescence spectroscopy are reflective of those changes at the active site of the native enzyme, consistent with the finding that the activity loss of papain follows so closely the changes of EPR and fluorescence (Figs. 5 and 6).

EPR, fluorescence, and kinetic studies of papain as a function of pH (Fig. 4) show that the active site conformation of this enzyme changes as the pH is varied. With all three methods, it is observed that there is a rapid increase around pH 4 in all parameters measured followed by maximal values around pH 7. The pH dependence of the conformation of the active site observed by the two different spectroscopic methods and by kinetic analysis are consistent with conformational changes in the active site SH group being influenced by Asp-158 and His-159 (26), which when in solution yield pK values of 4 and 7–8, respectively.

The EPR study shows that the motion of the spin label attached to the enzyme does not change very much in urea solutions, but this motion increases in guanidine solutions. This information is also complemented by the fluorescence study. From these results it is evident that papain is an enzyme of unusual stability in high concentration of urea, and shows inactivation in high concentration of guanidine hydrochloride (32–36). The changes in the mobility of the spin-label observed by EPR might be due to the conformational change induced by denaturation, and be related to the unfolding of one or more domains. Shapira and Arnon (35) reported that in 8 M urea, only slight changes in conformation could be detected by optical rotatory dispersion and viscosity measurements. The catalytic activity of papain was also preserved in the presence of 8 M urea. But more drastic denaturing conditions such as 6 M guanidine were found to cause complete and irreversible inactivation of papain (35). These workers also found that only one disulfide bond per molecule was opened in the 8 M urea, and all three disulfide bonds were disrupted in the presence of 6 M guanidine hydrochloride.

The agreement between conformational changes in the active site of papain and its enzymatic activity found in the present study demonstrates that selective active-site spin labeling of proteins can provide detailed molecular information on the physical state of enzymes.

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