Solid-State ^{13}C NMR Study of a Transglutaminase–Inhibitor Adduct†,‡

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ABSTRACT: We have used solid-state ^{13}C NMR to study the structure of the adduct resulting from the inactivation of the enzyme transglutaminase by 3-halo-4,5-dihydroisoxazoles. These inhibitors were conceived on the assumption that they would inhibit transglutaminase by attack of an enzyme active site cysteine thiol on the imine carbon of the dihydroisoxazole ring. The tetrahedral intermediate formed could then break down with the loss of the halide group and the subsequent formation of a stable imino thioether adduct. We have compared the ^{13}C CPMAS spectra of the chloro-, bromo-, and (ethylthio)dihydroisoxazole inhibitors, and the results indicate that the chemical shift of the C-3 carbon is sensitive to the nature of the heteroatom. Subtraction of the natural-abundance ^{13}C solid-state NMR spectrum of the enzyme from that of the enzyme inactivated by C-3-labeled chlorodihydroisoxazole reveals a broad peak at 156 ppm. The chemical shift of this peak is very close to that observed for a model 3-ethylthio compound and suggests the formation of a stable imino thioether enzyme adduct. Similar results were obtained for lyophilized enzyme adducts and for frozen solutions of the enzyme adduct in the absence and presence of Ca^{2+}. We have also compared these results with those obtained by solution NMR on an aqueous solution of the enzyme–inhibitor complex. The ^{13}C-labeled C-3 resonance was not observed in this case.

Transglutaminases are a class of calcium-dependent enzymes that catalyze the covalent coupling of the γ-carboxamide group of peptide-bound glutamine residues with an ε-amino group of peptide-bound lysine residues. The critical intermediate in the catalytic sequence is a thioester acyl-enzyme formed between a glutaminyl-peptide acyl donor and the active site cysteine residue, as illustrated in Figure 1A. A variety of important physiological events are catalyzed by transglutaminases, including the cross-linking of fibrin in the final step of hemostasis (Lorand et al., 1980) and epidermal cell envelope formation (Rice & Green, 1977), as well as a number of immunologic phenomena (Fesus, 1982). Moreover, transglutaminases have been implicated in a variety of disease states, including acne (DeYoung et al., 1984), psoriasis (Bernard et al., 1986), cataracts (Azari et al., 1981), and immunologic diseases (Fesus, 1982).

Highly specific inactivators are required to clarify the role of transglutaminases in cellular phenomena. A plausible scheme for transglutaminase inactivation by isoxazole inhibitors based on the formation of a tetrahedral intermediate can be formulated, analogous to the normal mechanism of action of the enzyme. This proposed mechanism is shown in Figure 1B. A putative proton donor activates the ring imine to attack by the active site cysteine thiol. Breakdown of the tetrahedral intermediate with the loss of the halide is accompanied by the formation of a stable adduct, an imino thioether, in which the cysteine sulfur is linked to the imine carbon of the ring (Castelhano et al., 1988).

The molecular weight of guinea pig liver transglutaminase (a monomer of M_r 76 620) (Ikura et al., 1988) is quite large by the standards of high-resolution NMR.[1] In particular, the size of the protein leads to a long correlation time and, therefore, to broad NMR lines. In addition, a variety of other problems are often encountered in systems of this size, including aggregation and/or precipitation at the concentrations necessary for NMR measurements. Solid-state NMR provides a unique method for structural studies of enzymes and soluble proteins that complements and extends structural methods such as X-ray diffraction and solution NMR (Smith & Griffin, 1988). There are now several examples of the successful application of solid-state NMR to the study of enzyme–inhibitor complexes. Such examples include investigation of the mechanism of inhibition of the enzymes alanine racemase (Copié et al., 1988) and thermolysin (Copié et al., 1990) and the study of the adduct formed from the reaction of the enzyme D-alanyl-D-alanine ligase, ATP, and a dipeptide analogue (McDermott et al., 1990). Moreover, solid-state NMR spectra of protein crystals of α-lytic protease have been utilized to resolve conflicting results between X-ray diffraction and solution NMR studies (Smith et al., 1989).

In the present study, we used solid-state ^{13}C NMR to study the structure of the adduct formed from the inhibition of the enzyme transglutaminase by 3-halo-4,5-dihydroisoxazoles. The results present evidence for the nature of the inactive transglutaminase–isoxazole complex. Specifically, we observed a chemical shift consistent with the formation of a

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[1] Abbreviations: NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; CPMAS, cross polarization and magic angle spinning; MAS, magic angle spinning; TMS, tetramethylsilane; DMSO, dimethyl sulfoxide.

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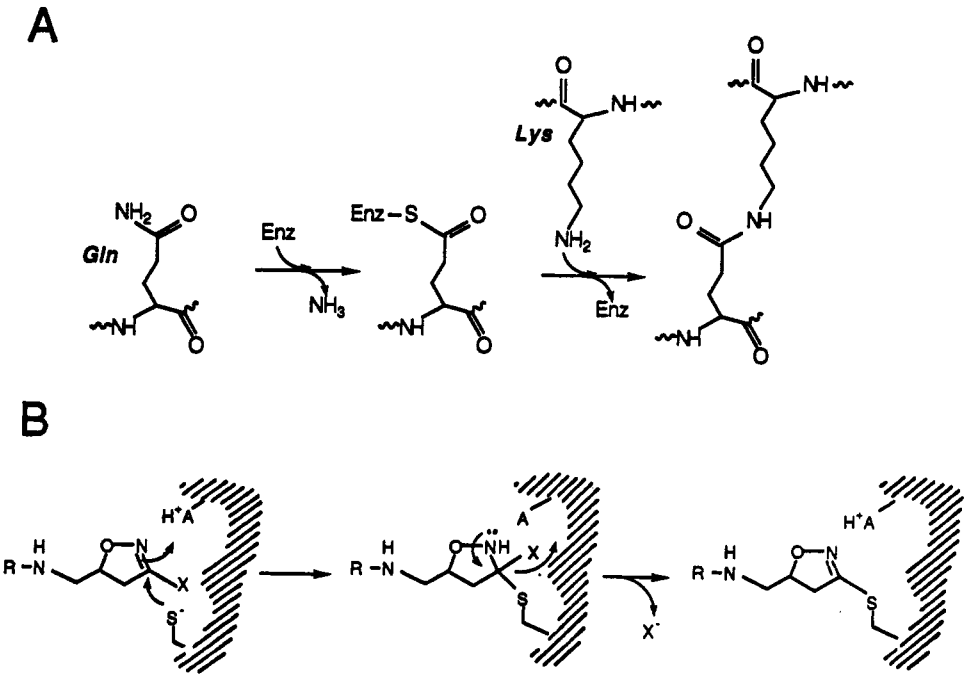


FIGURE 1: (A) Acyl transfer catalyzed by transglutaminases. (B) Proposed mechanism for the inactivation of transglutaminase by 3-halo-4,5-dihydroisoxazole.

stable thioimine-like enzyme adduct. Similar results were obtained for both the lyophilized form and frozen solutions of the inactivated enzyme in the absence and presence of Ca^{2+}. We have also compared the results with those obtained by solution NMR on an aqueous solution of the enzyme–inhibitor complex.

MATERIALS AND METHODS

Synthesis. Preparation of the unlabeled dihydroisoxazole inhibitors has been described previously (Castelhano et al., 1990). The isotopically labeled analogues were prepared similarly by using [^{13}C]dibromoformaldoxime (prepared from [^{13}C]glyoxal and hydroxylamine) in the [2 + 3] cycloaddition reaction with *N*-(carbobenzyloxy)phenylalanine allyl amide. Conversion of the 3-bromo to the 3-chloro inhibitor analogue was effected with diphosgene (as a source of HCl). Mass spectra (FAB): ^{13}C-labeled C-3 Br, 461 $(M + H)^+$; ^{13}C-labeled C-3 Cl, 417 $(M + H)^+$, 419 $(M + H)^{2+}$.

Purification of Transglutaminase. Guinea pig liver transglutaminase was obtained by using the procedure of Folk and Chung (1985). Very active enzyme was obtained in high yield by using female livers exclusively. Enzyme activity was monitored by measurement of the rate of incorporation of hydroxylamine into Cbz-Gln–Gly-OH, again following the procedure of Folk and Chung (1985). The enzyme used for NMR experiments was stored frozen at –80 °C in 10 mM Tris–acetate buffer, pH 6.0, containing 1 mM EDTA and 0.16 M KCl.

Preparation of Samples for NMR Experiments. The frozen enzyme was thawed on ice and diluted 5-fold with 100 mM Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$ and 1 mM dithiothreitol. Ten equivalents of inhibitor was dissolved in dimethyl sulfoxide and added to the active enzyme such that the final concentration of organic solvent was 5%. The enzyme activity was monitored until none remained, approximately 20 min. The solution was then concentrated using Centricon centrifugal microconcentrators (Amicon Corp., Lexington, MA) equipped with 30 000 mol wt cutoff filters. The enzyme was washed three times with water, to remove all traces of small molecules, and concentrated to a final volume of 0.5 mL. Several drops of D_2O were added to serve as a lock in the solution-state experiments. For the solid-state experiments, the samples were either lyophilized or frozen. Final weights of 67, 35, and 48 mg for the ^{13}C-labeled C-3 chlorodihydroisoxazole, ^{13}C-labeled C-8 bromodihydroisoxazole, and unlabeled bromodihydroisoxazole enzyme adducts, respectively, were obtained.

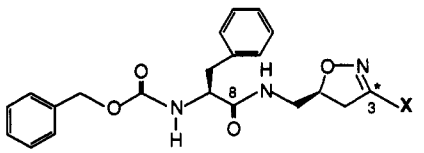
Solid-State NMR Experiments. The ^{13}C NMR CPMAS spectra were acquired on a home-built spectrometer operating at a ^{13}C frequency of 79.85 MHz and a proton frequency of 317 MHz. A home-built probe was used, with a stator and rotors from Doty Scientific Inc. (Columbia, SC). Typical 90° ^{13}C and ^{1}H pulse lengths were 4.0 and 3.0 μs, respectively. Cross polarization (Pines et al., 1973) from ^{1}H to ^{13}C was used to enhance the ^{13}C signal and shorten the effective spin–lattice relaxation time. Typically, cross polarization times were 2 ms, spinning speeds were 3–5 kHz, and recycle delays were 3 s. A 100-Hz line broadening was applied to all spectra. Isotropic chemical shifts in ppm are referenced relative to external TMS.

Solution NMR Experiments. The spectra were acquired on a Bruker AM 500 spectrometer equipped with a 5-mm dual frequency probe at a ^{13}C frequency of 125.759 MHz. Carbon spectra (32K data points) were generally acquired by using a 30° pulse angle and an acquisition time of 0.56 s, with the probe temperature maintained at 27 °C. Protons were decoupled by using composite pulses. The spectral width was 29 412 Hz, and the spectra were acquired with quadrature detection. A 10-Hz line broadening was applied. Chemical shift assignments were based on a solution of D_2O containing 5% DMSO-d_6 at 39.5 ppm.

RESULTS AND DISCUSSION

Solution ^{13}C NMR spectra obtained for several 3-halo-4,5-dihydroisoxazole inhibitors indicate that the isotropic chemical shift of the C-3 carbon is dependent upon the heteroatom attached to that carbon. In order to compare these chemical shifts with those obtained for the solid inhibitor,

Table I: Isotropic Chemical Shifts (σ_{iso}) Derived from Solid-State MAS Spectra of Labeled 3-Halo-4,5-dihydroisoxazole Inhibitors as a Function of the Heteroaom X



X	σ_{iso} (ppm)
Br	137
Cl	149
SCH_2CH_3	156

we measured the solid-state CPMAS ^{13}C spectra of 3-halo-4,5-dihydroisoxazole inhibitors as a function of the heteroatom at the C-3 position. In order to further confirm the assignment of the C-3 carbon resonance, we synthesized the chloro and bromo inhibitors, ^{13}C labeled at the C-3 position of the isoxazole ring, and measured their solid-state spectra. Table I presents the values of the C-3 carbon isotropic chemical shift as a function of different heteroatoms. Comparison of the results obtained for the chloro and bromo inhibitors indicates that the chemical shift of the C-3 carbon is sensitive to the chemical nature of the substituent, the chemical shift value for the chloro inhibitor (149 ppm) being about 12 ppm downfield from that obtained for the bromo inhibitor (137 ppm). These values, as well as the chemical shifts at the other positions, are in agreement with those obtained from the solution NMR spectra and suggest that the structure of the inhibitor in the solid state is similar to that of the solubilized form.

In order to determine if it would be possible to detect the formation of an imino thioether adduct in the enzyme–inhibitor complex on the basis of the chemical shift of the C-3 carbon in dihydroisoxazole inhibitors, we synthesized 3-(ethylthio)-4,5-dihydroisoxazole as a model for the putative adduct between the dihydroisoxazole inhibitor and a cysteine residue of the enzyme transglutaminase. The chemical shift value of the C-3 carbon obtained for that model compound, from both solution and solid-state spectra, is about 156 ppm (Table I). This chemical shift is 19 ppm downfield from that observed for the bromo derivative and 7 ppm downfield from that observed for the chloro inhibitor. These results clearly indicate that the chemical shift of the C-3 carbon in dihydroisoxazole inhibitors is very sensitive to the substitution on the carbon and that the formation of an imino thioether in the enzyme–inhibitor complex should be observable on the basis of the chemical shift value of this carbon.

We first measured the ^{13}C MAS spectra of the complex between transglutaminase and the bromodihydroisoxazole inhibitor labeled with ^{13}C at the C-8 position (Figure 2). This allowed us to determine if the difference spectra, obtained by subtracting the spectrum of the complex between transglutaminase and the unlabeled inhibitor from the spectrum of the complex with labeled inhibitor, would permit detection of a signal due to a single ^{13}C-labeled carbon in this 76-kDa complex. The enzyme–inhibitor complex was studied as a lyophilized powder at room temperature. The difference spectrum, shown in Figure 2C, reveals a line at 172 ppm, with a width of about 250 Hz. The chemical shift of this peak is similar to that observed for the C-8 resonance of the free inhibitor in solution and in the solid state. This result indicates that the structure of the inhibitor is not significantly altered at the C-8 position. Moreover, it is clear from the quality of the spectra presented in Figure 2 that reliable chemical shift

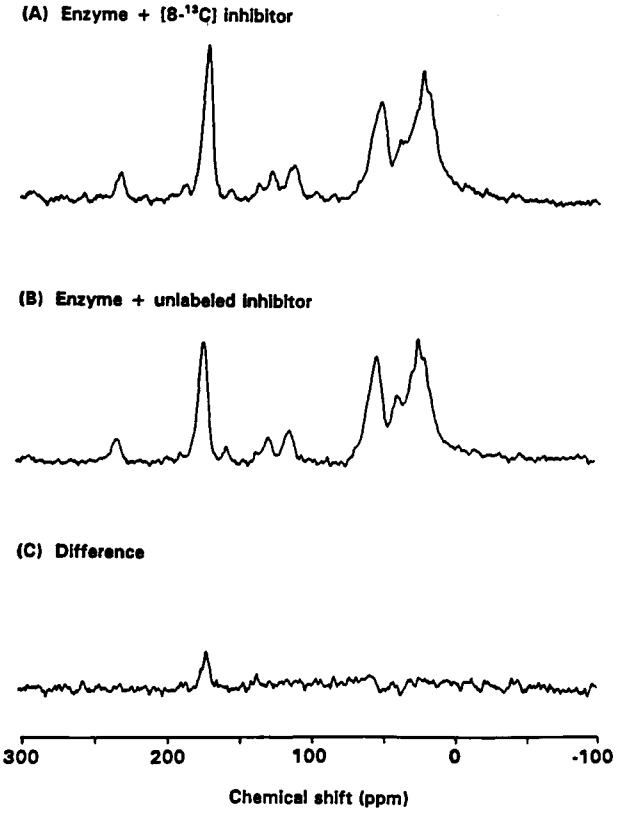


FIGURE 2: ^{13}C MAS spectra of (A) the complex between transglutaminase and the 3-bromo-4,5-dihydroisoxazole inhibitor labeled with ^{13}C at the C-8 position and (B) the complex between transglutaminase and the unlabeled bromodihydroisoxazole inhibitor, and (C) the difference spectrum (A minus B) showing only the ^{13}C-labeled C-8 carbon resonance of the inhibitor. The complex was studied as a lyophilized powder. The spectra were obtained at room temperature and at a spinning speed of 4.7 kHz. Each spectrum represents approximately 6000 scans.

values can be obtained from the difference spectra.

Pictured in Figure 3 are the ^{13}C MAS spectra of the complex between transglutaminase and the chlorodihydroisoxazole inhibitor labeled with ^{13}C at the C-3 position. The complex was first studied as a lyophilized powder at room temperature. The difference spectrum in Figure 3 reveals a broad peak (about 1 kHz) at 156 ppm, as well as rotational side bands separated by the spinning frequency (4.7 kHz). The chemical shift of this peak is very close to that observed for the C-3 resonance of the 3-ethylthio model compound and thus suggests the formation of a stable imino thioether enzyme adduct.

It is interesting to note that the line width obtained for the ^{13}C-labeled C-3 carbon in the enzyme–inhibitor complex is significantly broader than that obtained for the complex with the ^{13}C-labeled C8 inhibitor (1 kHz compared to 250 Hz). This line width is much broader than what is normally observed in disordered polymers (Vanderhart et al., 1981) and might be explained by the presence of conformational disorder at the active site of the enzyme, resulting in a broadened spectrum. Another hypothesis could be a quadrupolar broadening due to the direct attachment of the C-3 carbon to a nitrogen atom. The effect of quadrupolar nuclei is, however, unlikely to be the sole contribution to the broadening since there is no significant broadening observed for the resonance of the C-8 carbon, which is also directly attached to a nitrogen atom.

The large line width observed for the C-3 resonance in the enzyme–inhibitor complex also raises the question of non-specific binding of the hydrophobic inhibitor. However, it has been shown that the activity of the enzyme does not return

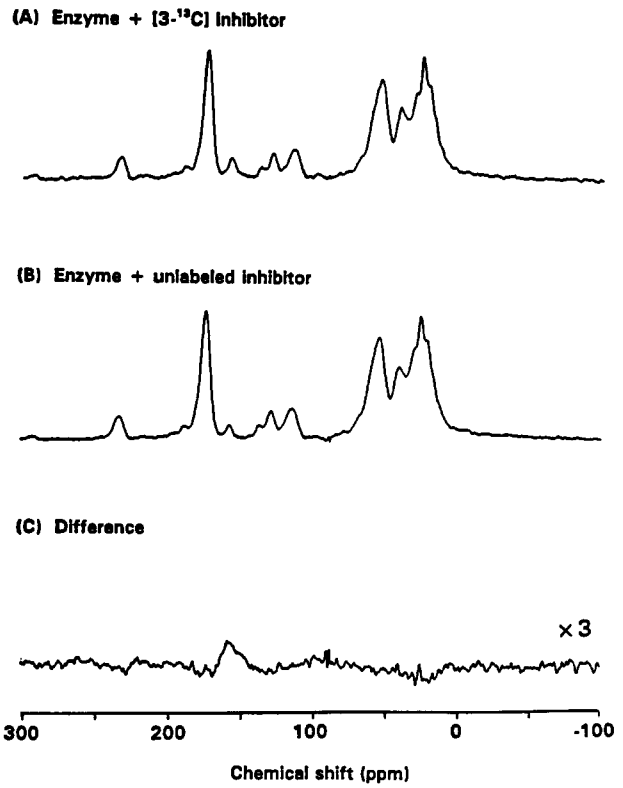


FIGURE 3: ^{13}C MAS spectra of (A) the complex between transglutaminase and the 3-chloro-4,5-dihydroisoxazole inhibitor labeled with ^{13}C at the C-3 position and (B) the complex between transglutaminase and the unlabeled chlorodihydroisoxazole inhibitor, and (C) the difference spectrum (A minus B) showing only the ^{13}C-labeled C-3 carbon resonsance of the inhibitor. The complex was studied as a lyophilized powder. The spectra were obtained at room temperature and at a spinning speed of 4.7 kHz. Each spectrum represents approximately 15 000–20 000 scans.

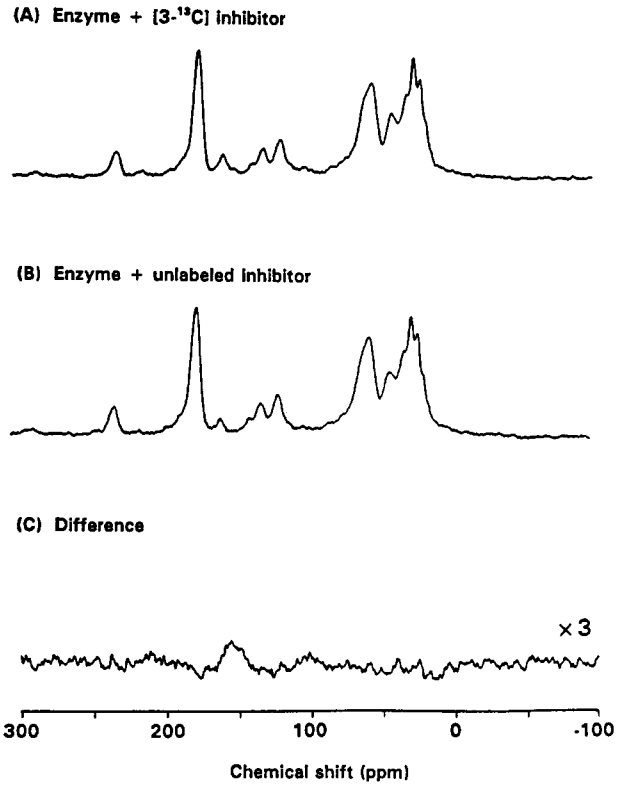


FIGURE 4: ^{13}C MAS spectra of (A) the complex between transglutaminase and the 3-chloro-4,5-dihydroisoxazole inhibitor labeled with ^{13}C at the C-3 position and (B) the complex between transglutaminase and the unlabeled chlorodihydroisoxazole inhibitor, and (C) the difference spectrum (A minus B) showing only the ^{13}C-labeled C-3 carbon resonance of the inhibitor. The complex was studied as a frozen solution in the presence of 1 mM Ca^{2+}. The temperature was –30 °C, and the spectra were acquired at a spinning speed of 4.7 kHz. Each spectrum represents approximately 15 000–20 000 scans.

after gel filtration or dialysis, indicating that the inhibitor is bound in a covalent fashion (Pliura et al., unpublished results). Moreover, the stoichiometry of binding between transglutaminase and radiolabeled dihydroisoxazole inhibitor has been determined to be 1:1 (Pliura et al., unpublished results). In addition, solution NMR studies have been performed before and after removal of excess inhibitor labeled at the C-8 and C-3 positions. In both cases, no evidence of any remaining labeled small molecules can be observed. On the other hand, spectral simulations of two broad lines, with chemical shifts corresponding to those observed for the chloride (149 ppm) and SCH_2CH_3 (156 ppm) model compounds, indicate that the two lines could still be resolved in a resultant peak with a line width of about 1 kHz.

In order to investigate if the lyophilization procedure may partially disrupt the active site of the enzyme, resulting in greater disorder, we also studied the complex between transglutaminase and 3-chloro-4,5-dihydroisoxazole inhibitor as a frozen solution. The low temperature has the additional advantage of providing enhanced signal-to-noise for the NMR experiment. The enzyme–inhibitor complex was first dissolved in water and then frozen at a temperature of about –30 °C. The difference spectrum obtained for the complex between transglutaminase and the 3-chloro-4,5-dihydroisoxazole inhibitor labeled at the C-3 position (results now shown) is very similar to that obtained for the sample prepared as a lyophilized powder. The line is also broad (line width $\approx$ 1 kHz) and centered at 156 ppm. These results indicate that the lyophilization procedure does not seem to significantly alter the structure of the active site of the enzyme.

It has been suggested that calcium is required to preserve the conformational integrity of the active enzyme (Folk & Gross, 1971). In order to determine if the absence of calcium is responsible for the broadening observed for the C-3 carbon resonance of the inhibitor in the enzyme–inhibitor complex, we studied the complex as a frozen solution in the presence of 1 and 10 mM calcium. The results obtained for the two different concentrations of calcium are very similar, and the spectra obtained in the presence of 1 mM calcium are shown in Figure 4. The difference spectrum reveals a broad peak (line width $\approx$ 1 kHz) centered at 157 ppm, as well as rotational sidebands separated by the spinning frequency (4.7 kHz). Comparison of these spectra with those measured in the absence of calcium (both as a lyophilized powder and as a frozen solution) indicates that the presence of calcium does not seem to have an effect on the observed resonance. Moreover, the chemical shift shift value obtained for the C-3 resonance in the complex in the presence of calcium is also very close to the value measured for the model ethylthio compound, again suggesting the formation of a stable imino thioether adduct.

It is interesting to compare the spectra presented in Figures 3 and 4 with those obtained by solution NMR on an aqueous solution of the complex between the enzyme and the inhibitor labeled at the C-3 position. These ^{13}C solution NMR spectra are shown in Figure 5. It should be noted that these spectra are the result of about 100 000 accumulations, compared to about 20 000 accumulations for the solid-state spectra presented in Figures 3 and 4. The cross-polarization technique

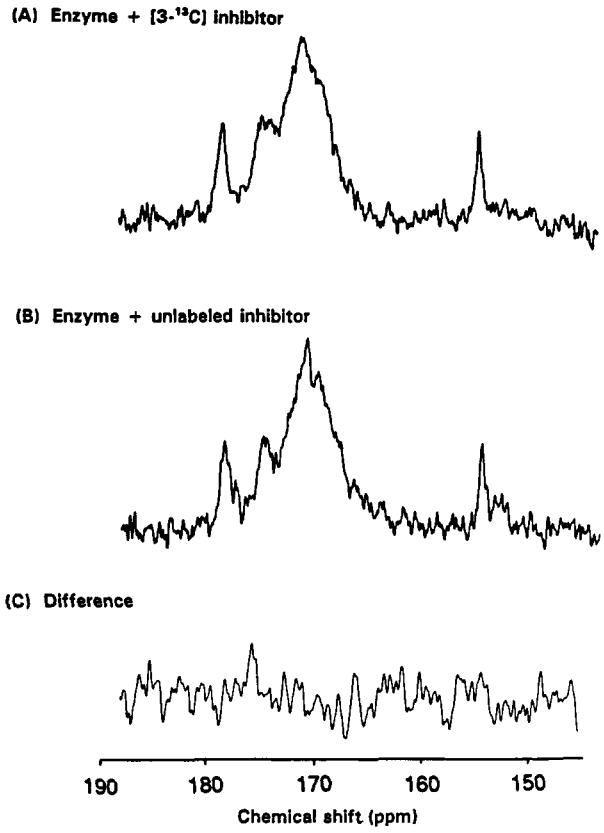


FIGURE 5: 125-MHz ^{13}C solution NMR spectra of (A) the complex between transglutaminase and the 3-chloro-4,5-dihydroisoxazole inhibitor labeled with ^{13}C at the C-3 position and (B) the complex between transglutaminase and the unlabeled chlorodihydroisoxazole inhibitor, and (C) the difference spectrum (A minus B). The spectra were acquired at room temperature. Each spectrum represents approximately 100 000 scans. The difference spectrum does not show any evidence of an adduct signal.

used to acquire the solid-state spectra results in a large increase in the signal-to-noise ratio compared to that of the solution spectra. The low signal-to-noise ratio obtained for the solution spectra, in addition to severe line broadening, yields a poor quality difference spectrum in which it is not possible to detect the signal for the labeled carbon on the inhibitor.

CONCLUSIONS

The present study demonstrates that solid-state MAS NMR can be used to elucidate the structure of an uncharacterized enzyme–inhibitor complex. The results confirm that the binding of dihydroisoxazole inhibitors to the enzyme transglutaminase results in the formation of a stable imino thioether enzyme adduct. The results described above also demonstrate the utility of the solid-state NMR technique in structural investigations of high molecular weight proteins, for which solution NMR spectroscopy has limited utility.

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