Solid-state NMR Observation of Cysteine and Lysine Michael Adducts of Inactivated Estradiol Dehydrogenase*

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The inactivation of estradiol dehydrogenase by enzyme-generated 3-hydroxy-14,15-secoestra-1,3,5(10)-trien-15-yn-17-one is accompanied by the formation of a lysine enammine. The experiments leading to this conclusion involved degradation of the inactivated enzyme and subsequent analysis by solution-state $^{13}$C NMR. The present paper reports solid-state $^{13}$C NMR experiments on lyophilized intact inactivated enzyme which are free from problems due to Pronase digestion. These experiments combine conventional cross-polarization and magic-angle spinning with selective irradiation of resonances arising from a $^{13}$C double label in the steroid. Magnetization transfer between neighboring $^{13}$C nuclei is used to simplify the spectra and to identify peaks due to label. The formation of cysteine and lysine Michael adducts of the enzyme is established by comparisons with chemical shifts of solid model adducts.

We recently described (1) the conversion of 14,15-secoestra-1,3,5(10)-trien-15-yn-17-one (1) to ketone 2 by human term placental 17β,20α-hydroxysteroid dehydrogenase (estra-diol dehydrogenase). Following conversion, ketone 2 inactivates the enzyme. A solution-state $^{13}$C NMR study of Pronase-digested inactivated enzyme established the formation of a Michael adduct between ketone 2 and an amino acid side chain of the enzyme (2). Solution-state $^{13}$C NMR experiments could not detect the $^{13}$C labels in the undigested inactivated enzyme because of line broadening associated with the long correlation times that characterized the mobility of the large dimer in solution. Comparison of spectra of sodium dodecyl sulfate-solubilized peptide(s) from inactivated enzyme with spectra of model amino acid-acetylenic ketone adducts suggested that a lysine residue reacted with the steroid. Poor mobility of the $^{13}$C labels in the solubilized digest sample raised concerns that Michael adducts other than lysine enamminones were present but remained undetected by solution-state NMR because of their excessive line widths. In general, proteolytic digestion of the inactivated enzyme prior to NMR analysis is undesirable since rearrangement or loss of labile adducts could arise during sample preparation.

In this paper, we show that the complications with the solution-state NMR experiments described above are overcome by performing CPMAS $^{13}$C NMR on lyophilized intact enzyme inactivated by enzyme-generated $[^{13}$C$^2]$ketone 2. Differences of CPMAS spectra obtained with and without selective irradiation show only the irradiated peaks and peaks arising from magnetization transfer by spin diffusion. These difference spectra are compared to the corresponding difference spectra of model compounds to provide evidence for the formation of $^{13}$C$^2$-labeled cysteine and lysine Michael adducts.

**EXPERIMENTAL PROCEDURES**

Materials

99% [1,2-13C]acetylene gas was obtained from Cambridge Isotope Laboratories, Woburn, MA; and pyridine nucleotides and Reactive Red 120-agarose (type 3000-CL) were obtained from Sigma.

**Synthesis of $[^{13}$C$^2]$Alcohol 1 and $[^{13}$C$^2$]Ketone 2**

[1,2-13C$^2$]Acetylene gas (100 ml, 4.5 mmol) in dry tetrahydrofuran was treated with n-butyllithium (1.3 ml in hexanes, 4.5 mmol) under nitrogen at −78 °C (3). The clear solution was then treated with (2S,4aS,10aS)-1,2,3,4,4a,9,10,10a-octahydro-7-hydroxy-2-methyl-2-phenanthrenecarboxaldehyde (4) (244 mg, 1 mmol) in tetrahydrofuran. The crude product was purified on a column of 12-g silica gel with a stepwise gradient of ethyl acetate in hexanes to yield $[^{13}$C$^2]$alcohol 1. Crystallization from diethyl ether/hexanes provided 90 mg of a white powder. $[^{13}$C$^2]$alcohol 1 was oxidized with Jones reagent in acetonate to produce $[^{13}$C$^2$]Ketone 2 (4). Yields varied between 50 and 80%. $^{13}$C and H NMR data for $[^{13}$C$^2]$alcohol 1 and $[^{13}$C$^2$]ketone 2 are reported elsewhere (5).

**Purification of Estradiol Dehydrogenase**

The procedure of Murdock et al. (6) was modified after the heat treatment. The protein was precipitated with ammonium sulfate (0.313 g/ml supernatant), then resuspended and dialyzed against potassium phosphate buffer (50 mm, pH 7.0, 20% glycerol). Reactive

2 Portions of this paper (including part of "Experimental Procedures," Figs. S1–S6, Table S1, and Structures 4 and 5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Red 120-agarose was added, and the filtered resin was batch-washed in phosphate buffer to remove unbound proteins. The washed resin was poured into a column and eluted with 0.1 mM NADP⁺ and 0.1 mM estrone in phosphate buffer (10 mM, pH 7.0, 20% glycerol). The eluted enzyme was precipitated with ammonium sulfate (0.313 g/ml), then resuspended in and dialyzed against phosphate buffer. Each placenta afforded 5-10 mg of homogeneous estradiol dehydrogenase.

**Enzyme Inactivations for CPMAS NMR Experiments**

Estradiol dehydrogenase (100 mg, 250 units) was incubated with 100 μM NAD⁺ and 40 μM alcohol (13C-enriched or natural abundance) in 0.96 liter of 0.1 M sodium carbonate/bicarbonate buffer (pH 9.2, 20% glycerol, 0.5% ethanol) at 25 °C for 15-16 h (<10% of initial activity remained). The incubation mixture was concentrated by ultrafiltration to about 50 ml and dialyzed against 1 liter of 10 mM potassium phosphate buffer (pH 7.0, 1 g/liter bovine serum albumin) for 16-17 h with one change of buffer, then against the same buffer without bovine serum albumin (1 liter) for 19-20 h with one change (all at 4°C). The contents of the dialysis bag were filtered through Whatman paper and concentrated by suction ultrafiltration to 1-2 ml. The thick solution was filtered through cotton, diluted with 2 ml of water, and lyophilized. The dried samples weighed 81-85 mg.

**Preparation of Steroidal Model Adducts**

Model Lysine Adduct—A solution of [13C₂]ketone 2 (15 mg, 56 μmol) in acetonitrile (1 ml) was treated with N,N-dimethylethylene-diamine (Aldrich, 95%) (7 μl, 64 μmol) at room temperature. After about 15 min, the reaction mixture was concentrated to dryness and redissolved in 0.2 ml of CH₃CN. The product was precipitated with a few drops of water and lyophilized.

Model Cysteine Adduct—A mixture of [13C₂]ketone 2 (15 mg, 56 μmol) and N,N-dimethylaminoethanethiol hydrochloride (Aldrich, 90%) (12.8 mg, 50 μmol) was suspended in acetonitrile (1 ml) and treated with distilled triethylamine (10 μl, 72 μmol) at room temperature. After about 15 min, the reaction mixture was concentrated, precipitated from CH₃CN with water, and lyophilized.

**13C CPMAS NMR**

13C CPMAS spectra were obtained at 50.3 MHz using matched spin-lock cross-polarization transfers at 38 kHz with 2-ms contact times. The samples were spun at either 2300 or 2386 Hz in a double-bearing rotor. Detailed descriptions of cross-polarization and magic-angle spinning are given elsewhere (7-9).

Selective irradiation of 13C in solids was obtained with a CPMAS-DANTE (10) pulse sequence (Fig. S1, bottom, Miniprint Supplement). A DANTE pulse train of 65 0.7-μs pulses spaced 75 μs apart was applied at the rest frequency of the 13C peak to be selectively irradiated. Spectra were also obtained with the CPMAS-delay pulse sequence (Fig. S1, top, Miniprint Supplement) which omitted the DANTE pulses. When a CPMAS-DANTE spectrum is subtracted from the corresponding CPMAS-delay spectrum, the resulting DANTE difference spectrum shows only the effects of selective irradiation and subsequent magnetization transfer (see Miniprint Supplement).

The stoichiometry of inhibitor to enzyme was calculated from the integrated intensity S of 13C CPMAS spectra (S = k²n₁C₀, where k is a constant, n₁ is the number of molecules of type i, and C₀ is the number of carbon atoms/molecule with the fractional 13C abundance f₁). One enzyme subunit and one ketone 2 molecule contain 1502 and 18 carbon atoms, respectively. The integrated spectral intensities for the unenriched (S₀) and enriched (Sₑ) inactivated enzyme samples are

\[ S₀ = k(0.011) (18) nₐ + (0.011) (1502) nₑ \]
\[ Sₑ = k((0.99) (2) + (0.011) (16)) nₐ + (0.011) (1502) nₑ \]

where nₐ and nₑ are the respective number of ketone 2 molecules and enzyme subunits. Substituting the integrated spectral intensities into the above equations gives the number of molecules of ketone 2/ enzyme subunit.

**RESULTS**

**Solid-state 13C NMR of Inactivated Enzyme—50.3-MHz CPMAS 13C NMR spectra were obtained for samples of enzyme inactivated with natural-abundance alcohol 1 and with [15,16-13C₂]alcohol 1 (Fig. 1). Spectra obtained with total side band suppression (11) (to suppress spinning side bands) indicate that the 13C labels give rise to olefinic resonances in the 90-160-ppm region. Spectra obtained for analytical purposes were obtained without total side band suppression since this technique only partially suppresses the large side bands arising from 13C-13C dipolar coupling. The spectrum obtained from the difference of the CPMAS spectra of labeled enzyme (Fig. 1, bottom right) and natural-abundance inactivated enzyme (not shown) contains at least three resonances and their spinning side bands (Fig. 1, top right). A stoichiometry of 4.2 ± 0.3 eq of steroid/enzyme subunit was calculated from the integrated intensities of these spectra.

The overlap of resonances complicates the difference spectrum of Fig. 1. A simpler spectrum is obtained by selectively irradiating specific 13C resonances with a DANTE pulse se-

![Fig. 1. CPMAS 50.3-MHz 13C NMR spectra of estradiol dehydrogenase inactivated with 13C₂-ketone 2 or natural abundance ketone 2. Spectra of the sample spinning at 3205 Hz were obtained both with (left) and without (right) side band suppression. The difference spectrum (containing spinning side bands, top right) was obtained by subtracting the CPMAS spectrum of the natural abundance sample (also containing spinning side bands, but not shown) from the CPMAS spectrum of the 13C₂-labeled sample (bottom right).](image-url)
quence and then subtracting the resulting spectrum from one obtained without DANTE irradiation. Resonances in this difference spectrum result either from selective irradiation or from magnetization transfer to the selectively irradiated nucleus.

The labeled inactivated enzyme was selectively irradiated at 148.6 ppm with the CPMAS-DANTE pulse sequence (see Miniprint Supplement) while spinning at 3205 Hz. The resulting spectrum (Fig. 2, bottom left), which was obtained with the variable delay \( r \) set to 30 \( \mu \)s, appears similar to the normal CPMAS spectrum (Fig. 1, bottom right) except for reduced intensity near 150 ppm. The difference obtained by subtracting this spectrum from the corresponding spectrum obtained without DANTE irradiation consists of a 160-ppm center band and its side bands (Fig. 2, middle left). When the experiment was repeated with \( r = 10 \) ms, the appearance of a newly formed 117-ppm peak and the reduction of the 150-ppm peak (Fig. 2, top left) result from magnetization transfer from the carbon associated with the 117-ppm resonance to the carbon associated with the 150-ppm resonance. This rapid magnetization transfer indicates that the 117- and 150-ppm peaks arise from directly bonded \(^{13}\)C nuclei (12).

This experiment was repeated with DANTE irradiation at 156.5 ppm and with magic-angle spinning at 2398 Hz. The spinning speed was changed to prevent overlap between a side band resonance of the irradiated nucleus and the center band resonance of the neighboring \(^{13}\)C nucleus. (See the discussion in the Miniprint Section associated with Fig. S3). The resulting difference spectra (Fig. 2, middle and top right) indicate that magnetization was transferred from the carbon associated with the 86-ppm resonance to the carbon associated with the 157-ppm resonance. The minor 86-ppm peak in the \( r = 30 \mu \)s difference spectrum results from magnetization transfer during the 4-ms period of DANTE irradiation (see Miniprint Supplement). Chemical shifts and shift anisotropies associated with \(^{13}\)C-labeled pairs of covalently bound carbon atoms of the inactivated enzyme are presented in Table I.

![Fig. 2. CPMAS-DANTE and DANTE difference 50.3-MHz plots of the \(^{13}\)C NMR spectra of estradiol dehydrogenase inactivated with \(^{13}\)C in ketone 2. CPMAS-DANTE spectra (\( r = 30 \mu \)s) were obtained with sample spinning at 3205 Hz and selective irradiation at 148.6 ppm (bottom left); and with sample spinning at 2398 Hz and selective irradiation at 156.5 ppm (bottom right). The DANTE difference spectra for \( r = 30 \mu \)s or 10 ms (middle and top) were obtained by subtracting the CPMAS-DANTE spectra from the corresponding CPMAS-delay spectra. The vertical display of the middle and top spectra is expanded by a factor of two relative to that of the bottom spectra. Arrows mark the frequency of DANTE irradiation. Triangles mark frequency differences that arise directly from selective irradiation, and the circle (left) and stars (right) mark frequency differences that result from magnetization transfer.](image)

\(^{13}\)C NMR of Model Compounds—To mimic possible adducts formed with ketone 2, a series of model adducts was generated by Michael reaction of a nonsteroidal ketone with nucleophilic side chain moieties representative of those found in amino acids (see Miniprint Supplement). In particular, the acetylenic ketone, 2,2-dimethyl-4-(1-oxo-2-propynyl)-1,3-dioxolane (3), was prepared with 99% \(^{13}\)C at both acetylenic carbon atoms. Chemical shifts from solution-state spectra of olefinic carbons of various nonsteroidal model adducts are presented in Table II. The olefinic carbon chemical shifts of the cysteine and lysine model adducts match, respectively, the shifts of the 117- and 150-ppm pair and the 86- and 157-ppm pair of coupled resonances in the solid-state spectra of the \(^{13}\)C-labeled enzyme. In addition, both the isotropic chemical shifts and the relative spinning side band intensities obtained from solid-state spectra of the \(^{13}\)C-labeled steroidal model adducts (Fig. 3) also match those of the inactivated enzyme (Table I).

Composition of Pronase Digest—CPMAS \(^{13}\)C NMR selective irradiation experiments were performed on the lyophilized inactivated enzyme under the same conditions used for the undigested inactivated enzyme. The resulting difference spectra (not shown) obtained with MAS at 3205 Hz and DANTE irradiation at 148.6 ppm show no evidence of the magnetization transfer that is characteristic of a cysteine adduct. However, the difference spectra (not shown) obtained with MAS at 2398 Hz and DANTE irradiation at 156.5 ppm

| Sample            | Magic-angle spinning | DANTE irradiation | Isotopic chemical shift (Hz) ppm | Relative intensities, side band number |
|-------------------|----------------------|-------------------|---------------------------------|---------------------------------------|
| **Ketone-enzyme** | 3205                 | 148.6             | 0.3                             | 1.0                                   |
| **Ketone-enzyme** | 2398                 | 156.6             | 0.3                             | 1.0                                   |
| **Cysteine mimic**| 3205                 | 148.6             | 0.3                             | 1.0                                   |
| **Lysine mimic**  | 2398                 | 156.5             | 0.45                            | 1.0                                   |

**Table II**

| Amino acid mimics | Double-bond configuration | Isotopic chemical shifts ppm |
|-------------------|---------------------------|-----------------------------|
| Lys               | \( Z \)                   | 89.16                       |
| Ser/Thr           | \( E \)                   | 101.03                      |
| Cys               | \( E \)                   | 117.06                      |
| Cys               | \( Z \)                   | 115.69                      |
| Arg               | \( Z \)                   | 99.65                       |
| Tyr               | \( E \)                   | 105.55                      |
| His               | \( E \)                   | 108.95                      |
| Asp/Glu           | \( E \)                   | 108.78                      |
| Asp/Glu           | \( Z \)                   | 105.53                      |

**Table I**

| Sample              | Magic-angle spinning | DANTE irradiation | Isotopic chemical shift | Relative intensities, side band number |
|---------------------|----------------------|-------------------|------------------------|---------------------------------------|
| Ketone-enzyme       | 3205                 | 148.6             | 0.3                    | 1.0                                   |
| Ketone-enzyme       | 2398                 | 156.5             | 0.3                    | 1.0                                   |
| Cysteine mimic      | 3205                 | 148.6             | 0.3                    | 1.0                                   |
| Lysine mimic        | 2398                 | 156.5             | 0.45                   | 1.0                                   |

**Table II**

Olefinic \(^{13}\)C chemical shifts of nonsteroidal model adducts in CDCI₃
**Solid-state NMR of Dehydrogenase Inactivation**

**Mechanism of Inactivation**—Chemical experiments with nonsteroidal model compounds show that \( \beta \)-thioenones are converted to \( \beta \)-enaminones in the presence of an amine. These results suggest that there are two ways for lysine adducts to form during enzyme inactivation. First, lysine adduct formation may involve the initial bonding of ketone 2 to a cysteine residue followed by the transfer of the ketone 2 to the \( \epsilon \) nitrogen of a lysine residue via a Michael addition-elimination reaction. The cysteine residue would then be free to bond to a different ketone 2 molecule. The other possibility is that cysteine and lysine residues are independently modified by ketone 2. The experiments reported here make no distinctions between these two paths, each of which might reduce the enzyme's catalytic activity.

**Stoichiometry**—In the present study, a stoichiometry of 4.2 ± 0.3 eq of steroid/subunit was calculated from the NMR experiments. Previous experiments using tritiated alcohol 1 gave a stoichiometry of one to two Michael adducts/enzyme subunit (1). Since solid-state \( ^{13} \text{C} \) NMR measurements on proteins are, in general, quantitatively reliable (see Miniprint Supplement), systematic errors in the NMR stoichiometric measurement are restricted to chemical impurities in the enzyme sample. However, we were unable to find significant concentrations of impurities in our samples. The ultrafiltrate arising from a preparation of inactivated enzyme was examined by solution state \( ^{13} \text{C} \) NMR and found to contain only excess \([^{13}\text{C}_2]\)alcohol 1 (\( \delta_c = 83 \) and 84 ppm) and glycerol adducts with \([^{13}\text{C}_3]\)ketone 2 (\( \delta_c = 41 \) and 101 ppm). No significant intensities with these chemical shifts are present in the CPMAS difference spectrum of inactivated enzyme (Fig. 1, upper right). We conclude that no more than about 10% of the \( ^{13} \text{C} \) signal from label can arise from impurities.

Stoichiometric determinations by radiometric methods usually involve separate protein and radioactivity measurements. Since only microgram quantities of enzyme were used in our radiolabeling experiments, significant errors in stoichiometry may have arisen either from sample loss due to nonspecific binding of the protein (possibly to glass surfaces) or from incomplete solubilization during scintillation counting. We plan to resolve uncertainties regarding stoichiometry by performing solid-state \( ^{13} \text{C} \) NMR and radiolabeling experiments on a single sample of enzyme inactivated with \( ^{13} \text{C}_2 \)- and \( ^{3} \text{H}_2 \)-labeled ketone 2.

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**FIG. 3.** CPMAS-delay and DANTE difference 50.3-MHz \( ^{13} \text{C} \) NMR spectra for the cysteine (left) and lysine (right) steroidal model adducts. Spectra were obtained under the conditions described in the legend to Fig. 2.
Selective irradiation in solids is obtained by combining DANTE pulse sequences with conventional CPMAS techniques. The DANTE-CPMAS pulse sequence (Figure 5D) provides a means to probe the chemical environment of specific $^{13}C$-$^{13}C$ linkages in complex materials. The $^{13}C$-$^{13}C$ linkage is a common feature in many natural products and drugs.

**Selective Irradiation in Solids**

**Figure 5D** shows the principle of the DANTE-CPMAS pulse sequence. The DANTE sequence consists of a series of DANTE pulses, each followed by a delay of 34.6 ms. After these delays, the DANTE pulses are halted, and the CPMAS sequence is started. The DANTE-CPMAS pulse sequence is repeated, and the CPMAS sequence is stopped after a series of delays.

**Selective Magnetic Decoupling**

**Figure 5E** shows the principle of the selective magnetic decoupling technique. The DANTE-CPMAS pulse sequence is repeated, and the CPMAS sequence is stopped after a series of delays.

**Conclusion**

The DANTE-CPMAS pulse sequence provides a means to probe the chemical environment of specific $^{13}C$-$^{13}C$ linkages in complex materials. This technique is particularly useful for studying the chemical environment of specific $^{13}C$-$^{13}C$ linkages in natural products and drugs. Future work will focus on optimizing the DANTE-CPMAS pulse sequence to obtain more accurate information about the chemical environment of specific $^{13}C$-$^{13}C$ linkages in complex materials.
Solid-state NMR of Dehydrogenase Inactivation

Figure 52. CPMAS 50.3-MHz $^{13}$C NMR spectra of natural-abundance hexamethylbenzene spinning at 3205 Hz. The DANTE difference spectrum (top) is obtained by subtracting the CPMAS-DANTE (middle) from the CPMAS-delay spectrum (bottom). The delay was 30 μsec.

Figure 53. CPMAS 50.3-MHz $^{13}$C NMR spectra of (a) natural-abundance hexamethylbenzene spinning at 3205 Hz, (b) 1,2-$^{13}$C$_2$glycine spinning at 3205 Hz, and (c) 1,2,3,4,5,6-$^{13}$C$_6$glycine spinning at 3205 Hz. The bottom spectra were acquired using the CPMAS-delay pulse sequence with a 30 μsec delay. The middle and top spectra were obtained using the CPMAS-DANTE pulse sequence with $\tau$ = 10 μsec and 100 μsec, respectively. Triangles mark the frequency of DANTE irradiation, and circles designate peaks arising from magnetization transfer.

Figure 54. Intensities of the methylene resonance in DANTE-difference 50.3-MHz $^{13}$C NMR spectra of 1,2-$^{13}$C$_2$glycine are plotted as a function of $\tau$ for samples spinning at 2702 Hz (circles) and 3205 Hz (triangles).

Figure 55. CPMAS $^{13}$C peak intensities of natural-abundance alpha (open squares), beta(γ) (open triangles), and gamma (open circles) protons of 1,2,3,4,5,6-$^{13}$C$_6$glycine. The measured carbon (solid triangles) of enzyme inactivated by 1,2,3,4,5,6-$^{13}$C$_6$glycine was plotted as a function of cross-polarization contact time. Each peak decays with a $T_2\text{P(Hi)}$ of 5.0±0.3 μsec.