Direct Observation by Carbon-13 NMR Spectroscopy of the Regioselectivity and Stoichiometry of "Suicide" Enzyme Inactivation

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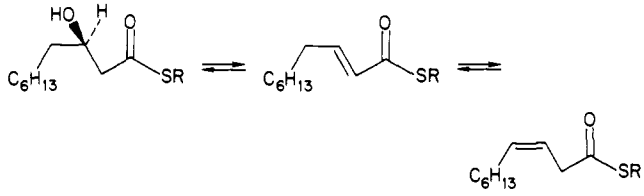
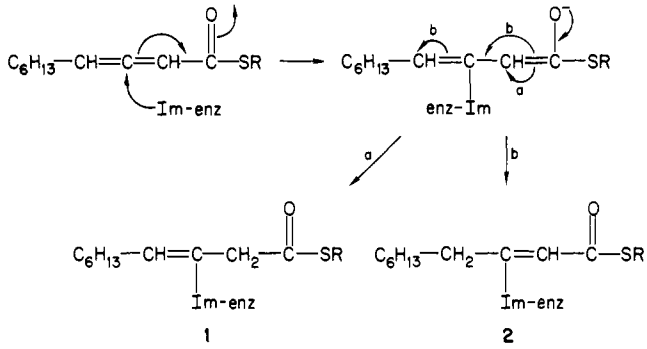
Mechanism-based, irreversible enzyme inactivation by "suicide" substrates[2] has become an important tool for the study of enzymatic reaction mechanisms and a focal point for drug design. The seminal demonstration of this phenomenon was made[3] by Bloch in studies on β-hydroxydecanoylthioester dehydrase,[4] an enzyme that catalyzes the reactions shown (R = acyl carrier protein, in



vivo). The dehydrase-catalyzed allylic rearrangement is a suprafacial process,[5] apparently mediated by a single active site histidine residue.[3b]

Inactivation of dehydrase by 3-decynoic acid *N*-acetylcysteamine thio ester (3-decynoyl-NAC) is believed to occur by a mechanism analogous to that of the "normal" allylic rearrangement. Thus, propargylic rearrangement of 3-decynoyl-NAC produces 2,3-decadienoyl NAC, a potent electrophile which alkylates the active site histidine.[3b] Nucleophilic attack on the allenic thio ester could in principle lead to one of two vinyl imidazole structures, **1** or **2**. We wish to report that this process is ac-



companied by protonation at *carbon-2*, giving the kinetically favored, nonconjugated vinyl imidazole **1**.

Acquisition of the ^{13}C NMR spectrum of homogeneous dehydrase[6] using WALTZ-16 proton decoupling[8] (Figure 1b) produced minimal sample warming and allowed rapid data collection.[9]

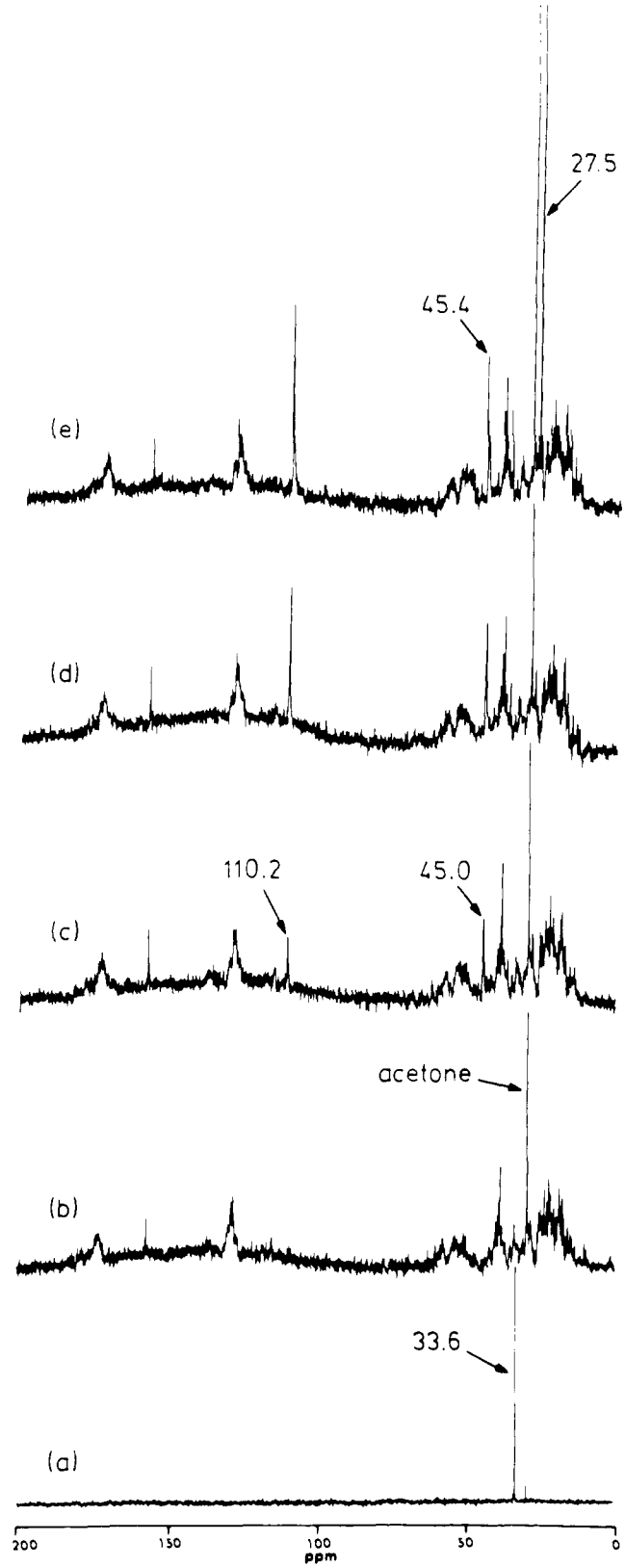


Figure 1. $^{13}C\{^1H\}$ NMR spectra were obtained on a Varian XL-400 at 100.6 MHz, using 3.0-mL samples in 10 mM KPO_4, pH 7.0, in 10-mm tubes. Sample temperatures reached only 27 °C, obviating external cooling. Data points, 32 K, were collected over 200 ppm (acquistion time = 0.8 s, digital resolution = 1.22 Hz). Chemical shifts are referenced to internal acetone at 29.8 ppm. Short T_1 values for the resonances of interest allowed the use of a 90° pulse (24 μs), with no pulse delay. Except as noted, spectra were processed with 3.0-Hz line broadening (LB). (a) 3-[2-^{13}C]Decynoyl-NAC (0.34 mg, 1.26 μmol), 2500 scans, LB = 1.5 Hz; (b) dehydrase (51 mg, 1.42 μmol), 75000 scans; (c) dehydrase (50 mg, 1.39 μmol) plus 1.0 subunit equivalent of 3-[2-^{13}C]-decynoyl NAC, 75 000 scans; (d) as in (c), but with a total of 2.0 equiv of inactivator; (e) as in (c), but with a total of 3.0 equiv of inactivator.

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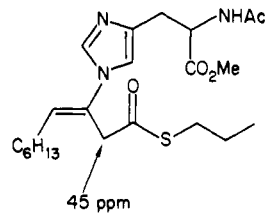
To a 50-mg (1.39 μmol) sample of dehydrase (determined gravimetrically) was added 1.0 subunit equivalent of 3-[2-^{13}C]decynoyl-NAC.[10] (Dehydrase is a dimer, MW = 36000.) Following virtually instantaneous[11] enzyme inactivation, a $^{13}C\{^1H\}$ NMR spectrum of the mixture (Figure 1c) showed the absence of free inhibitor. Instead, two new signals were evident, at 45.0 (LW = 36 Hz)[9] and 110.2 ppm (LW = 28 Hz),[9] in a ratio of ca. 2:1. Further insight came from a preliminary experiment in which aging of inactivated enzyme (2 weeks at 4 °C) caused the ratio of integrated areas of the peaks at 45 and 110 ppm to become *reversed*. This suggests that the 45 ppm signal is due to the initially formed adduct, which undergoes slow conversion to the species exhibiting the 110 ppm resonance.

Clearly, only C-2 of structure **1** (and *not* structure **2**) could produce the 45 ppm resonance. In fact, C-2 of the adduct formed



between *N*-acetylhistidine methyl ester and 2,3-decadienoic acid *n*-propyl thio ester (**3**,[12] C=C configuration unknown) resonates at 44.8 ppm ($CDCl_3$).[13,14]

Application of ^{13}C NMR spectroscopy[15] has also led to resolution of the question of whether one or two inactivator molecules are bound per dehydrase dimer.[16] Thus, to the sample giving the spectrum shown in Figure 1c was added a second equivalent of inactivator. While the proportions of the 45 and 110 ppm signals were changed (vide supra, Figure 1d), importantly, no free inhibitor was detected. Additionally, the total integrated area of the bound species was twice what had been observed in Figure 1c. With the addition of a third equivalent (Figure 1e), a sharp new resonance at 27.5 ppm (LW = 3 Hz, LB = 0)[9] appeared rapidly ($t_{1/2}$ = 10–15 min) at the expense of free thio ester (33.6 ppm). Control experiments showed that 3-[2-^{13}C]decynoyl-NAC is stable in buffer and that this 27.5 ppm resonance owes to the corresponding free acid, presumably formed by nonspecific, protein-catalyzed hydrolysis of the thio ester.[17] In addition, the small new signal at 45.4 ppm (LW = 13 Hz)[9] suggests that a small portion of the inactivator has become bound (as **1**) to a histidine on the periphery of the enzyme.[18]

In conclusion, inhibition of dehydrase by 3-decynoyl-NAC involves isomerization to 2,3-decadienoyl-NAC, followed rapidly by reaction of the latter with an active site histidine, forming species **1**. The stoichiometry of inactivation is clearly one molecule of inactivator for each dehydrase subunit, providing additional evidence that the subunits are identical and refuting the proposed "half-of-the-sites" reactivity.[16a,c,19]

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(6) The isolation and purification of dehydrase from *E. coli* DM51 A will be described elsewhere.[7]

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(9) See legend to Figure 1.

(10) 3-[2-^{13}C]Decynoyl-NAC was made from 1-octyne by the following steps: (a) EtMgBr;[20] (b) $^{13}CH_2O$;[20] (c) PBr_3, pyridine;[20a] (d) CuCN, LiBr, DMF;[21] (e) MeOH, HCl;[16b] (f) dilute NaOH;[16b] (g) $PhOPOCl_2$, Et_3N;[22] (h) $TlSCH_2CH_2NHCOCH_3$.[23] $^{13}C\{^1H\}$ NMR δ 33.6 (see Figure 1a).

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(13) K. Bloch and J. P. Stein have used a different approach to determine the structure of dehydrase inactivated by 3-decynoyl-NAC.[16a] (Stein, J. P., manuscript in preparation.)

(14) Preliminary experiments involving isomerization of **3** suggest that the 110 ppm signal can be assigned to adduct **2** or the derived carboxylic acid. Early in acquisition of the spectrum in Figure 1c the 45 ppm adduct (but *not* the 110 ppm adduct) was clearly evident, suggesting that the former is the only kinetically significant product of inactivation.

(15) For an excellent, up-to-date review of the application of ^{13}C NMR to the study of enzyme mechanisms, see: Mackenzie, N. E.; Malthouse, J. P. G.; Scott, A. I. *Science (Washington, D.C.)* **1984**, *225*, 883–889.

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(18) Amino acid analysis[16b] shows two histidines per dehydrase subunit.

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Direct, ESR Observation of the Localized Biradical 1,3-Dimethyl-1,3-cyclobutadiyl

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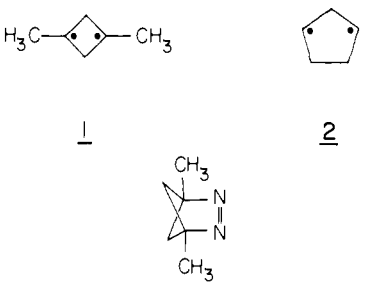
We describe herein the direct observation of the triplet state of 1,3-dimethyl-1,3-cyclobutadiyl (**1**) by ESR spectroscopy and a study of its thermal behavior. Buchwalter and Closs' landmark observation of triplet 1,3-cyclopentadiyl (**2**)[2] nearly 10 years ago



1 **2**

3

appeared to open the way for the direct spectroscopic study of localized biradicals, an important class of reactive intermediates.[3]

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