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^{13}C-Enriched *S*-Methyl Probe at the Active Site of an Enzyme: [*S*-[^{13}C]Methylmethionine-192]-α-chymotrypsin (MSMC)

Sir:

The serine protease α-chymotrypsin contains two methionine (Met) residues. The side chain of Met-192 lies at the outer edge of the active site crevice[1] where it is more vulnerable to S-alkylation[2] and S-oxidation[3] than the side chain of Met-180, which is buried in the interior of the tertiary structure of the enzyme. Met-192 is mechanistically interesting since it serves as a flexible hydrophobic lid over the active site and interacts with bound substrates and inhibitors.[4]

Others have shown that the facile detection of S-[^{13}C]-methylated Met residues by ^{13}C nuclear magnetic resonance spectroscopy (^{13}C NMR) may be exploited to gain insights into protein conformational changes induced by a variety of perturbing conditions. Thus far the *S*-methyl probe has been used to study apomyoglobin,[5] basic myelin protein,[6] ribonuclease,[7] and basic pancreatic trypsin inhibitor.[8] We now wish to report its selective incorporation into Met-192 of α-chymotrypsin. Our results show that an enzymatic *S*-methyl probe can be a particularly informative reporter when the labeled Met residue is near or involved in the enzyme's catalytic and binding functions.

Native α-chymotrypsin was S-methylated by stirring 1.0 g of the protein dissolved in 20 mL of pH 4.0 HCl containing 0.1 M KNO_3 with a 100-fold molar excess of 90 atom % $^{13}CH_3I$ for 16 h in the dark. Affinity chromatography of the dialyzed and lyophilized protein on a lima bean trypsin inhibitor—Sepharose column gave three protein peaks. The protein in the first peak ($\sim$10% of the total protein) eluted in the breakthrough fractions with pH 8.0, 0.05 M Tris—probably denatured chymotrypsin and its autolysis products, it was inactive in a rate assay with *p*-nitrophenyl 3-phenylpropionate (PNPP).[9] Amino acid analyses of samples of the inactive protein hydrolyzed in 3 M *p*-toluenesulfonic acid gave a 1.7:0.3 molar ratio of *S*-methylmethionine (SMM) to Met. The protein in the second peak ($\sim$75% of the total protein), eluted with pH 8.0, 0.1 M Tris containing 0.12 M $CaCl_2$ and 0.1 M KCl, exhibited 130% of the activity of the native enzyme in the PNPP assay.[10] Amino acid analyses of its hydrolysates gave equimolar quantities of SMM and Met. The protein in the third peak ($\sim$15% of the total protein), eluted with pH 2.0 HCl containing 0.1 M KCl, had a PNPP activity and amino acid composition identical with that of native α-chymotrypsin.

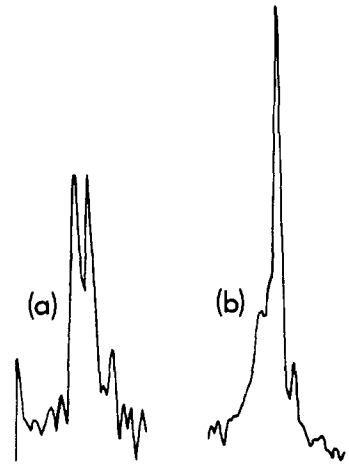

Figure 1. Methyl resonances of *S*-[^{13}C]methyl groups in the proton-decoupled 15.0-MHz Fourier transform ^{13}C NMR spectrum of MSMC after (a) 2 h (1.5×10^4 transients) and (b) 14 h (1.0×10^5 transients). Enzyme concentration 1.5 mM, temperature 30 °C, pH 7.0. Solvent composition is given in the text. Chemical shifts of the two resonances in spectrum a are 24.11 and 23.82 ppm downfield from the methyl carbon of internal acetonitrile, respectively; the resonance in spectrum b is at 23.82 ppm. Conditions of acquisition were 27.7° pulse, repetition rate 0.5 s, 8×10^3 data points, and 0.25-Hz digital resolution.

Chemical evidence confirms that the protein obtained in the second peak is MSMC: the methylated protein fails to react with α-bromo-4′-nitroacetophenone, a chromophoric alkylating agent which specifically alkylates Met-192,[11] and amino acid analyses of hydrolysates of methylated protein treated with H_2O_2 at conditions which give predominant S-oxidation of Met-192 in the native enzyme[12] revealed negligible amounts of methionine sulfoxide.

For ^{13}C NMR studies proteins were dissolved in pH 7.0 0.1 M phosphate buffer in 30% v/v deuterium oxide–water containing 1% v/v acetonitrile. Chemical shifts are referenced to the methyl carbon of the internal acetonitrile. The 15.0-MHz broadband proton-decoupled Fourier transform ^{13}C NMR spectrum of a 1.5 mM solution of MSMC equilibrated with the NMR solvent for 12 h is essentially identical with that of the native enzyme except for a narrow intense resonance of 23.82 ppm attributable to a ^{13}C-enriched SMM residue. No separation of resonances due to diastereotopic methyl groups was observed.[4] A study of the effects of active-site-directed inhibitors on the microenvironment of the *S*-methyl probe was initiated by converting MSMC to its phenylmethanesulfonyl (PMS) derivative. The phenylmethanesulfonylation doubtless occurs at Ser-195 as it does in the native enzyme.[13] The resonance assigned to the ^{13}C-enriched *S*-methyl carbon shifted downfield by 0.28 ppm in the ^{13}C NMR spectrum of PMS–MSMC, indicating an interaction between the probe and the inhibitor.

The ^{13}C NMR spectrum of MSMC taken immediately after dissolution of the lyophilized protein in the NMR solvent is different from that taken after thorough equilibration. For example, the spectrum of the nonequilibrated protein after 2 h of data acquisition (1.5×10^4 scans) exhibited two *S*-methyl absorptions of equal intensity, one at 23.82 ppm corresponding to the peak seen in the spectrum of the equilibrated protein and the other a new, broader absorption at 24.11 ppm (Figure 1). Successive spectra obtained by continuing the data acquisition

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showed that the intensity of the upfield resonance increased as the downfield resonance disappeared; the eventual result was the spectrum of the equilibrated protein. The upfield resonance was not observed in spectra of PMS–MSMC taken immediately after dissolution, even at the minimum number of scans required to detect the ^{13}C-enriched *S*-methyl group (~2500 scans).

The changing spectrum of nonequilibrated MSMC solutions does not result from autolysis or denaturation of protein. During a typical NMR experiment, none of the original activity of the modified enzyme toward PNPP is lost in 9 h, and only 15% is lost in 14 h. A more attractive explanation for the ^{13}C NMR results is that in lyophilized MSMC the cationic side chain of the SMM-192 residue is tucked into the active site where it forms a salt with the anionic side chain of a nearby amino acid residue, but upon exposure of the modified enzyme to a solution containing competing counterions, it rotates into solution. The identity of the anionic group is uncertain, but Asp-194, a nearby residue involved in a buried salt bridge with Ile-16,[14] is a likely candidate. The greater half-bandwidth of the transient downfield resonance compared with that of the upfield resonance ($W_H \sim 3.5$ Hz vs. $W_H \sim 1.9$ Hz) supports this proposal, since it suggests a greater rotational constraint on the SMM residue in the nonequilibrated fraction of protein than in the equilibrated fraction.[15] The ^{13}C NMR spectra obtained immediately after dissolution of PMS–MSMC also support this view; evidently the interaction in lyophilized MSMC giving rise to the downfield resonance is blocked by the presence of the bulky PMS group at the active site of the enzyme. Although we are uncertain of the reasons for the sluggishness of the transformation undergone by the SMM-192 residue as lyophilized MSMC solutions equilibrate, a study of [*S*-phenacylmethionine-192]-α-chymotrypsin provides a precedent. At pH 7 the *S*-phenacyl group of this protein is irreversibly locked in a buried environment.[16] If, as proposed, lyophilization provokes movement of the SMM-192 side chain of MSMC from solution to a constrained environment within the active site, reduced steric or other requirements of *S*-methyl compared with *S*-phenacyl may allow the reverse process to occur, albeit slowly, upon dissolution of the protein at pH 7.

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Tetracoordinate Planar Carbon: A Singlet Biradical

Sir:

Coincident with the centenary of the van't Hoff and LeBel proposal that tetracoordinate carbon prefers a tetrahedral shape, the last 10 years has witnessed a persistent interest in the prospect of either foiling or exploiting nature in order to generate the planar tetracoordinate species.[1-9] Numerous theoretical studies have

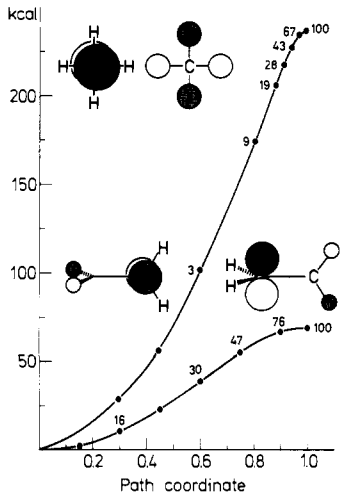
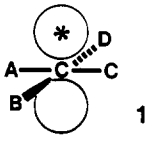


Figure 1. The PRDDO-GVB linear synchronous transit (LST) pathways connect planar ethylene and tetrahedral methane [path coordinate (pc) = 0.0] to perpendicular D_{2d} ethylene and planar D_{4h} methane (pc = 1.0), respectively. The percentage of biradical character along the isomerization pathways is indicated at points along each curve and corresponds to the degree of occupation of the least populated GVB natural orbital. The GVB split-pair orbitals, linear combinations of the singly occupied natural orbitals, are depicted for the $CH_2{=}CH_2$ and CH_4 biradicals (pc = 1.0).[17]

yielded estimates of 95–250 kcal/mol for the energy requirement to flatten CH_4.[3a,4-6] Simultaneously, a series of strategies have been recommended as a guide to the experimentalist in his search for the elusive planar structure **1**. These have drawn their inspiration from two main sources. The first pays tribute to the organic chemist's synthetic ingenuity and requires the CR_4 subunit to be nested in a larger frame designed to enforce the desired geometric distortion, for example, *trans*-fenestrane **2**.[1b,5c8,9]



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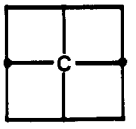

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The second and more rigorous approach is a consequence of single-determinant molecular orbital theory's prediction that the lowest singlet state of planar tetracoordinate carbon possesses a

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