

Preparation and ^{13}C NMR Characterization of $[\epsilon\text{-}^{13}\text{C}]$ Methionine-192]- α -chymotrypsin

THE DEMETHYLATION OF $[\text{S-}^{13}\text{C}]$ METHYLMETHIONINE-192]- α -CHYMOTRYPSIN BY AN ACTIVE SITE-DIRECTED THIOL*

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Met-192 forms part of the binding crevice of α -chymotrypsin. The aim of this investigation was to find a nucleophile that would displace a methyl group from the sulfonium cation of $[\text{S-}^{13}\text{C}]$ methylmethionine-192]- α -chymotrypsin without disrupting the five disulfide bridges of the protein, thereby producing $[\epsilon\text{-}^{13}\text{C}]$ methionine-192]- α -chymotrypsin, an isotopically enriched version of the native enzyme desirable for ^{13}C NMR studies. Treatment of $[\text{S-methylmethionine-192-}]\alpha$ -chymotrypsin with mercaptoethanol and dithiothreitol failed to produce the latter protein, as deduced from elution profiles of reaction mixtures chromatographed on affinity columns of immobilized lima bean trypsin inhibitor. In contrast, when $[\text{S-methylmethionine-192-}]\alpha$ -chymotrypsin was incubated in a 3.0 mM solution of the active site-directed reagent 2-mercaptoacetyl-4'-methoxyanilide at pH 8.6 and 5 °C for 48 h, affinity chromatograms indicated the presence of a protein corresponding to native α -chymotrypsin. Upon repeating the experiment with $[\text{S-}^{13}\text{C}]$ methylmethionine-192]- α -chymotrypsin, we isolated in 40% yield a protein which was identified as $[\epsilon\text{-}^{13}\text{C}]$ methionine-192]- α -chymotrypsin by a combination of ^{13}C NMR and chemical criteria. This work represents the first active site-directed demethylation of an $\text{S-}^{13}\text{C}$ methylmethionine residue at the binding site of an enzyme.

Enrichment of the ^{13}C content of one or more carbons in an intact native protein provides ^{13}C NMR reporters of the microenvironments of these carbons without significant perturbation of the protein's structure. The methyl (ϵ) carbon of methionine residues is sometimes selected for ^{13}C enrichment (1-8). One (ϵ)- ^{13}C enrichment procedure employs the methyl exchange reaction, which consists of converting a methionine residue to its S-methylsulfonium cation by alkylation with ^{13}C methyl iodide, then displacing one of the pair of S-methyl groups with a strong nucleophile (1-7). If the demethylation is diastereotopically nonselective, the product is a native enzyme containing a methionine residue in which the S-

methyl group is ^{13}C -enriched to one-half of the isotopic enrichment of the alkylating agent. The advantages of this method are that it is in principle suitable for isotopic enrichment of any protein regardless of its source, and that it is sometimes possible to limit the number and locations of methylations by controlling the alkylation conditions. A practical disadvantage is that conditions usually used for demethylation (high pH, temperatures near 40 °C, and high concentrations of a strong nucleophile) constitute harsh treatment for many proteins. Moreover, mercaptoethanol and dithiothreitol (as the thiolate anions) are the only nucleophiles which have been used to effect demethylation. Since thiols reduce disulfides (9) these reagents hold little promise for demethylation of the S-methylmethionyl residues of a protein containing disulfide bridges unless, as with ribonuclease (5), the protein spontaneously reverts to its native structure upon removal of the reductant.

We recently reported the synthesis, purification, and characterization of $[\text{S-}^{13}\text{C}]$ methylmethionine-192]- α -chymotrypsin, the obligatory intermediate in the methyl exchange reaction leading to $\epsilon\text{-}^{13}\text{C}$ enrichment of Met-192 of α -chymotrypsin (10). The demethylation step is more challenging. Chymotrypsin contains five disulfide bridges, two of which are inter-chain (11). Scission of these bridges causes the product peptides to aggregate and precipitate as an irreversibly denatured mass (12). Despite these obstacles, we elected to attempt the demethylation of $[\text{S-}^{13}\text{C}]$ methylmethionine-192]- α -chymotrypsin.

A preliminary investigation confirmed that demethylation of $[\text{S-methylmethionine-192-}]\alpha$ -chymotrypsin by mercaptoethanol and dithiothreitol cannot compete with disulfide bridge cleavage and other deleterious effects on the protein. In a pilot study the thiols were incubated in degassed aqueous solution with small samples of chromatographically pure $[\text{S-methylmethionine-192-}]\alpha$ -chymotrypsin in an argon atmosphere. The initial enzyme concentration was 4 mg ml⁻¹ (~0.15 mM); thiol concentration was varied from 1.0 mM to 0.50 M, temperature from 5-40 °C, and pH from 7.0 to 10.5. Each reaction mixture was analyzed for the appearance of native α -chymotrypsin by periodically removing a 1.0-ml aliquot from the mixture and chromatographing it on a lima bean trypsin inhibitor-Sepharose column (1 cm \times 25 cm) (13); the column capacity was >10 mg of native α -chymotrypsin. Eluted proteins were detected by their absorption at 280 nm. Thiols and denatured proteins eluted from the affinity column in the break-through fractions with 0.05 M Tris, pH 8.0; $[\text{S-methylmethionine-192-}]\alpha$ -chymotrypsin eluted with 0.10 M Tris, pH 8.0, containing 0.20 M KCl; native α -chymotrypsin eluted with pH 2.0 HCl containing 0.10 M KCl. At each set of reaction conditions we obtained chromatograms at zero time and every 12 h thereafter up to 48 h. The elution profiles showed losses of $[\text{S-methylmethionine-192-}]\alpha$ -chymotrypsin ranging from none to complete, depending on the thiol and the reaction conditions. Dithiothreitol was more destructive of the protein than mercaptoethanol. At pH 8.6 and 5 °C, for example, losses of $[\text{S-methylmethionine-192-}]\alpha$ -chymotrypsin after 48 h were negligible with 3.0 mM mercaptoethanol; at these conditions the same concentration of dithiothreitol completely degraded the protein. No protein peak characteristic of α -chymotrypsin was observed with either thiol in any of the elution profiles.

Since mercaptoethanol and dithiothreitol were inadequate

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for the task at hand, we contemplated the design of a thiol that would bind to the active site of [S-methylmethionine-192]- α -chymotrypsin with the thiol function oriented toward the S-methylmethionine-192 side chain. An ideal reagent could be used in low enough solution concentrations to minimize disulfide bond cleavage yet attain a sufficiently high concentration at the reaction site to effect demethylation of the S-methylmethionine residue. A recent report by Lawson and Rao immediately suggested a likely compound (14). These workers showed that for a series of alkyl bromides RBr which serves as active site-directed S-alkylating agents of Met-192 of α -chymotrypsin, the rate of alkylation is very sensitive to the structure of R. The half-life for the deactivation of the enzyme by 2-bromoacetyl-2'-methoxyanilide is 2700 min, whereas that for deactivation by 2-bromoacetyl-4'-methoxyanilide is only 12 min. Accordingly, we synthesized 2-mercaptoacetyl-4'-methoxyanilide (15) and tested it with [S-methylmethionine-192]- α -chymotrypsin at mild conditions as described for mercaptoethanol and dithiothreitol. Affinity chromatography on lima bean trypsin inhibitor-Sepharose was carried out with 1.0-ml aliquots of reaction mixtures taken from a 0.15 M aqueous solution of [S-methylmethionine-192]- α -chymotrypsin in pH 8.6, 0.05 M Tris buffer maintained under argon at 5 °C and containing 3.0 mM thiol. The elution profiles at 12-h intervals showed a progressive disappearance of [S-methylmethionine-192]- α -chymotrypsin, which was accompanied by the appearance and increase of a protein peak which eluted from the lima bean trypsin inhibitor-Sepharose column at pH 2.0. Within 48 h the [S-methylmethionine-192]- α -chymotrypsin had completely disappeared. The protein eluted with pH 2.0 HCl was estimated to be 30% of the starting material. The experiment was repeated with 64 mg of chromatographically pure [S- ^{13}C]methylmethionine-192]- α -chymotrypsin at the same conditions. After 48 h the reaction mixture was chromatographed on a lima bean trypsin inhibitor-Sepharose column (2.5 cm \times 100 cm) with a binding capacity of >350 mg of native α -chymotrypsin. The fractions containing protein eluted with pH 2.0 HCl were combined, dialyzed in the cold against pH 5.0 HCl, and lyophilized to give 26 mg (40% yield) of fluffy protein. A small sample of this protein exhibited the same specific activity as affinity purified native α -chymotrypsin in a rate assay with *p*-nitrophenyl 3-phenylpropionate (13). We tentatively concluded from its chromatographic behavior and specific activity that the isolated protein was [ϵ - ^{13}C]Met-chymotrypsin.¹ Since subsequent ^{13}C NMR experiments fully support this conclusion, we henceforth refer to it as such.

The remaining [ϵ - ^{13}C]Met-chymotrypsin, 25 mg, was dissolved in 1.0 ml of 30% (v/v) deuterium oxide in water to which 10 μl of acetonitrile had been added, and the solution was placed in a 1-cm NMR tube. Completely proton noise-decoupled ^{13}C NMR spectra were obtained on a Jeol FX-90Q-pulsed Fourier transform spectrometer operating at 22.5 MHz; the spectrometer was locked on the deuterium oxide of the solvent. Chemical shifts are reported in parts per million downfield from the natural abundance ^{13}C resonance of the methyl carbon of the internal acetonitrile. The following summarizes the ^{13}C NMR experiments and the results.

1. The solution containing [ϵ - ^{13}C]Met-chymotrypsin was brought to pH 5.0. The ^{13}C NMR spectrum was obtained and compared to that of native α -chymotrypsin taken at identical conditions (Fig. 1). Three peaks not observed in the α -chymotrypsin spectrum appeared in the [ϵ - ^{13}C]Met-chymotrypsin spectrum. Major peaks appeared at 14.7 and 16.3 ppm; a minor peak at 13.3 ppm was also present. The distinctive

methyl carbon resonance of the S-[^{13}C]methylmethionine residue of [S-[^{13}C]methylmethionine-192]- α -chymotrypsin at 23.8 ppm (10) was conspicuously absent. An aliquot taken from the NMR tube upon completion of the data acquisition had essentially the same specific activity in the *p*-nitrophenyl 3-phenylpropionate assay as the starting sample.

2. The [ϵ - ^{13}C]Met-chymotrypsin solution was immediately raised from pH 5.0 to pH 7.5 by addition of dilute NaOH and another ^{13}C NMR spectrum was taken. After 3.5 h of data acquisition the resonance at 14.7 ppm had increased in intensity at the expense of the 16.3 ppm resonance, which completely disappeared (Fig. 2). The resonance at 13.3 ppm remained sensibly constant after 3.5 h, but upon continuing the acquisition for an additional 10 h, the 14.7 ppm and 13.3 ppm resonances were of approximately equal intensity. Moreover, the background natural abundance ^{13}C resonances of the protein had narrowed appreciably. An aliquot taken from the NMR tube had about one-half of the original specific activity in the *p*-nitrophenyl 3-phenylpropionate assay.

3. The pH of the [ϵ - ^{13}C]Met-chymotrypsin sample was lowered from 7.0 to 4.0 with dilute HCl and 30% hydrogen peroxide was added to bring the peroxide concentration to 0.4 M. The ^{13}C NMR spectrum of the peroxide-treated protein was acquired following incubation at room temperature for 2 h (Fig. 3). The previously observed trio of peaks attributed to ^{13}C enrichment were replaced by a sharp, intense, single resonance at 35.2 ppm in this spectrum.

The ^{13}C NMR spectra of [ϵ - ^{13}C]Met-chymotrypsin show unequivocally that the protein is enriched in ^{13}C . Chemical shifts of the peaks present in the spectra of the ^{13}C -enriched enzyme but not in the isotopically normal enzyme are characteristic of the ϵ - ^{13}C carbon of methionine residue in proteins and related model compounds (1-5, 7, 8). The large downfield shift and collapse of these peaks to a single resonance in the

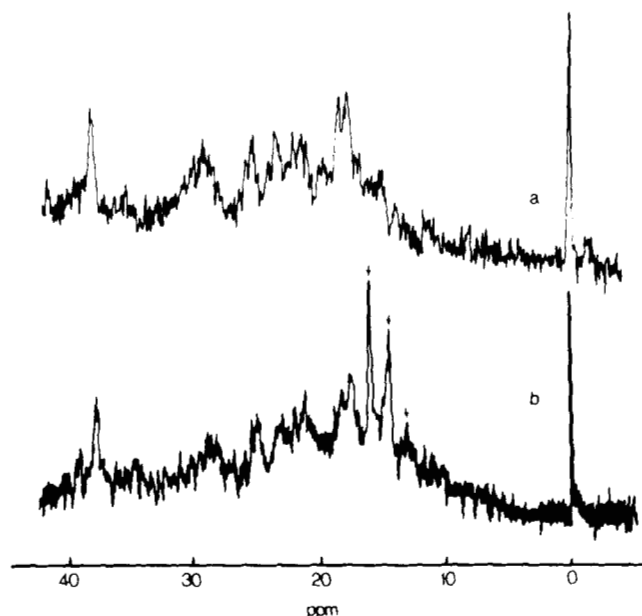


FIG. 1. Segments (1000 Hz) of the proton-decoupled 22.5 MHz Fourier transform-pulsed ^{13}C NMR spectra of (a) affinity purified native α -chymotrypsin and (b) [ϵ - ^{13}C]Met-chymotrypsin. Protein concentration 25 mg/ml (1.0 mM); pH 5.0; 25 °C. Solvent composition is given in the text. Arrows in spectrum b indicate peaks not present in spectrum a. These peaks are 13.3 ppm, 14.7 ppm, and 16.3 ppm downfield from the natural abundance ^{13}C resonance of the methyl carbon of 1% (v/v) acetonitrile which appears at zero ppm on the δ scale. The spectra are the result of 1.0×10^3 transients. Conditions of acquisition were: 27.7 ° pulse; repetition rate, 0.5 s; 8×10^3 data points, and 0.25 Hz digital resolution.

¹ The abbreviation used is: [ϵ - ^{13}C]Met-chymotrypsin, [[ϵ - ^{13}C]methionine-192]- α -chymotrypsin.

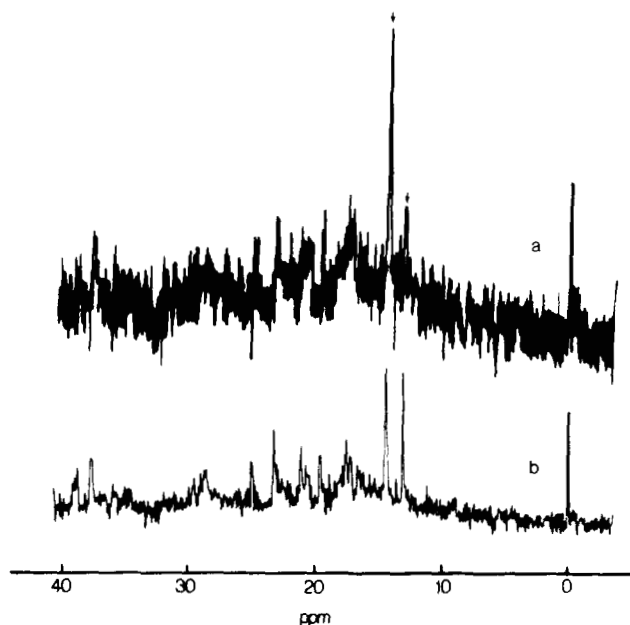


FIG. 2. The ^{13}C NMR spectra of [ϵ - ^{13}C]Met-chymotrypsin at pH 7.50 after (a) 2.5×10^4 transients (3.5 h) and (b) 1.0×10^5 transients (13.8 h). Arrows indicate peaks at 13.3 ppm and 14.7 ppm. Except for the pH, the conditions of acquisition and the presentation are identical with those given in Fig. 1.

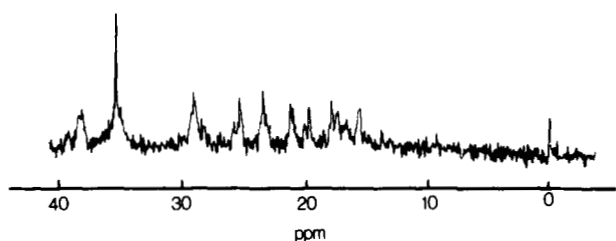


FIG. 3. The ^{13}C NMR spectrum of [ϵ - ^{13}C]Met-chymotrypsin following treatment of the protein with hydrogen peroxide. The spectrum is the result of 1.0×10^5 transients. The pH of the sample was 4.0; otherwise the conditions of acquisition were identical with those listed in Fig. 1.

peroxide-treated protein are consistent with this assignment. Cohen *et al.* observed a 22.6 ppm downfield shift of the methyl carbon resonance when [ϵ - ^{13}C]methionine was oxidized to [ϵ - ^{13}C]methionine sulfoxide (8). Similar shifts were observed upon sulfoxidation of accessible [ϵ - ^{13}C]methionine residues of β -galactosidase (8). The oxidation conditions employed here convert Met-192 of α -chymotrypsin to the sulfoxide; Met-180 is unaffected (16). From these results we can safely say that only the methyl carbon of Met-192 in our sample of [ϵ - ^{13}C]Met-chymotrypsin is ^{13}C -enriched.

The minor resonance at 13.3 ppm appearing in the ^{13}C NMR spectrum at pH 5.0, and increasing in intensity with time in the spectra at pH 7.50, doubtless comes from Met-192 residues of autolyzed or otherwise denatured [ϵ - ^{13}C]Met-chymotrypsin. The growth of this peak coincides with a loss of catalytic activity, and autolysis of the protein is faster at pH 7.5 than at pH 5.0. Diminished rotational constraints (17) on peptide chains which are denatured or hydrolyzed to shorter peptides account for the narrowing of the natural abundance ^{13}C resonances observed at long acquisition times at pH 7.5.

Beyond stating that the 14.7 ppm and 16.3 ppm peaks indicate at least two environments for the methyl carbon of Met-192 in intact [ϵ - ^{13}C]Met-chymotrypsin at pH 5.0, and possibly only one environment at pH 7.5, we are unable to

interpret these resonances with certainty. The environments giving rise to the 14.7 ppm and 16.3 ppm peaks are noninterconvertible on the NMR time scale at pH 5.0, but at pH 7.5 all Met-192 side chains of intact [ϵ - ^{13}C]Met-chymotrypsin attain the environment or environments represented by the 14.7 ppm resonance. We believe that this behavior is most consistent with the known isomerization of α -chymotrypsin from an inactive conformation predominating at extremes of pH to an active conformation predominating at pH values near neutrality (18–20). Thus, [ϵ - ^{13}C]Met-chymotrypsin may exist in both the active and inactive conformations at pH 5.0, whereas conversion to the active conformation is essentially complete at 7.5. We emphasize that this explanation may be complicated or obviated by other factors such as perturbation of monomer-dimer or monomer-polymer equilibria with changes in pH (21, 22).

This study demonstrates an active site-directed demethylation of an *S*-methylmethionine residue proximate to the binding site of an enzyme containing disulfide bridges. This approach may prove useful for other enzymes in which an *S*-methylmethionine residue is near a catalytic or other binding site whose topography is fairly well understood. Our ^{13}C NMR experiments demonstrate the potential of the ^{13}C -enriched methyl carbon of Met-192 in [ϵ - ^{13}C]Met-chymotrypsin as a probe for studying the dynamics of the Met-192 side chains as well as exploring the influence of such factors as pH, salt concentration, and the binding of substrates and inhibitors on active site conformations of α -chymotrypsin. Such studies are in progress.

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