^{13}C-NMR studies of selectively carboxymethylated [*methyl*-^{13}C]methionine-labeled bacterial dihydrofolate reductase

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Dihydrofolate reductase *NMR* *Carboxymethylation* *Carbon-13 NMR*
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1. INTRODUCTION

Carboxymethylation of enzymes with bromo- or iodoacetate has been a popular method for the introduction of ^{13}C labels useful for NMR studies [1–5]. As part of a continuing series of studies of the enzyme dihydrofolate reductase utilizing isotopic labeling in combination with NMR detection [6–12], we report here studies of carboxymethylated *Streptococcus faecium* enzyme. In contrast with the usual labeling strategy, we have utilized unlabeled iodoacetate in combination with enzyme containing biosynthetically incorporated [*methyl*-^{13}C]methionine. This unique labeling strategy provides information on the degree and specificity of modification not available by the more usual approach. Such chemical modification data, in combination with analysis using traditional protein chemistry, also provides a basis for assigning resonances [13–15]. Under relatively mild conditions, iodoacetate will selectively carboxymethylate *S. faecium* dihydrofolate reductase, with the degree of modification strongly limited in the presence of the substrate analog, aminopterin [16,17]. These NMR studies confirm some conclusions [16,17] and provide assignment data for two of the methionine residues. The approach appears to be particularly valuable for cases such as [*methyl*-^{13}C]methionine-labeled DHFR, in which the resolution of the individual carbon resonances is limited.

2. MATERIALS AND METHODS

[*methyl*-^{13}C]Methionine was synthesized as in [18] from L-*S*-benzylhomocysteine (Sigma) and [90%-^{13}C]methyliodide. Yields of 90–93% based on methyliodide were obtained and the product was >99% pure as judged by amino acid analysis. [*methyl*-^{13}C]Methionine-labeled dihydrofolate reductase was biosynthesized by *Streptococcus faecium var.* Durans strain A as in [7].

Carboxymethylation of the enzyme was done as in [16,17]. A solution of the enzyme (2 mg/ml) in citric acid–Na_2HPO_4 buffer (pH 6.3) was made 0.2 M in iodoacetate and allowed to react at 0°C in the dark for 24 h. When aminopterin was present as a protective agent, the concentration was 1 mM. The samples were then passed through a Sephadex column (G-25, 1 × 55 cm). The columns were equilibrated and eluted with 0.05 M potassium phosphate buffer (pH 7.0) at 4°C in the dark. In the protected enzyme preparation, the aminopterin and iodoacetate were removed by passage through a column of similar size which was equilibrated and eluted with 0.01 M Tris–HCl, (pH 9.5).

Proton-decoupled ^{13}C-NMR spectra were obtained on a Varian XL-100-15 NMR spectrometer operating at 25.2 MHz and interfaced to a Data General Super Nova computer for Fourier transform operation. Typical spectral parameters were 512 spectral points, 500 Hz spectral width for an acquisition time of 1 s, and a 70° flip angle. This

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corresponds approximately to the Ernst angle based on the relaxation parameters in [7]. The buffer was made up to contain 5–10% D_2O to serve as the NMR lock. This concentration of D_2O is unlikely to cause significant changes in protein conformation or relaxation times, and the inclusion of D_2O in the sample has the advantage that the homogeneity can be optimized over the entire sample volume by tuning the lock channel. No exponential multiplication of the free induction decay was performed in order to retain maximum resolution.

Using the carboxymethylation procedure described above, the extent of modification of the 7 methionine residues in the *S. faecium* dihydrofolate reductase was reported to be 29% for the unprotected enzyme and 12% for the aminopterin protected enzyme [17]. Based on the fractional NMR intensities of the chemically-modified to the unmodified methionine methyl resonances, values of 37% and 14% were obtained, in reasonable agreement with the ^{14}C data.

3. RESULTS AND DISCUSSION

Dihydrofolate reductase derived from *S. faecium* is a globular protein of 167 residues (M_r = 20 000), containing 7 methionine residues. The proton-decoupled ^{13}C-NMR spectrum at 25.2 MHz of the enzyme isolated from *S. faecium* grown on a medium containing [*methyl*-^{13}C]methionine is shown in fig.1a. The chemical shift range for the 7 methionines of the uncomplexed enzyme spans only 1 ppm, so that no single carbon resonances can be resolved. *S. faecium* dihydrofolate reductase is inactivated as a consequence of treatment with iodoacetate [16,17]. Subsequent amino acid analysis of enzyme modified with iodo[1-^{14}C]-acetate demonstrated that only 4 of the 7 methionine residues were carboxymethylated (table 1). Following the conditions utilized here, Met-1, Met-5, and Met-42 show negligible modification, Met-163 is slightly modified (19%), Met-36 and Met-50 show about a 50% degree of modification, and Met-28 is almost completely carboxymethylated. Further, the modification of Met-28 and Met-50 is strongly limited if the iodoacetate treatment is carried out in the presence of the inhibitor aminopterin. This protective effect indicates that the accessibility of these two residues to the iodoacetate is limited by the addition of the inhibitor aminopterin prior to treatment with iodoacetate.

NMR analysis of the [*methyl*-^{13}C]methionine-labeled *S. faecium* DHFR is consistent with the conclusions in [16,17]. If the enzyme is modified in the presence of aminopterin, and the inhibitor subsequently removed by dialysis, the ^{13}C-NMR spectrum is nearly unchanged (fig.1b). The resonance intensity appearing ~10 ppm downfield of the methionine methyl resonances near 24 ppm corresponds to the methionine methyl groups of the carboxymethylated enzyme. From [16,17], these resonances correspond primarily to Met-36 (44%) and Met-163 (27%) (table 1). The qualitative similarity between the spectra of unmodified enzyme and enzyme modified in the presence of aminopterin which is subsequently removed suggests that the partial modification of the Met-36 and Met-163 do not lead to major conformational rearrangements which would perturb the resonance shifts of the unmodified methionine residues. In contrast with these results, treatment of the enzyme with iodoacetate in the absence of aminopterin leads to a more significant degree of modification as judged from the intensity of the resonances near 24 ppm (fig.1c). These are accompanied by more significant changes in the spectrum of the remaining unmodified methoinine residues. These changes can represent a combination of loss of intensity corresponding to those methionine residues which are modified and hence shifted about 10 ppm downfield, as well as chemical shift changes of unmodified methionine residues which result from conformational changes characterizing the partially carboxymethylated enzyme. The latter effect represents an important potential limitation of the chemical modification technique as a basis for making chemical shift assignments.

Some evaluation of the seriousness of this problem can be made by direct examination of the ^{13}C difference spactra of the modified, relative to the unmodified enzyme. The difference spectrum obtained from spectra 1a and 1b did not provide sufficient signal/noise to permit unequivocal interpretation. This reflects the small degree of modification of the enzyme in the presence of aminopterin. Alternatively, subtraction of spectrum 1c from 1b shows two distinct resonances. Based on the protective effects of the aminopterin as deduced in [17], this difference spectrum should give two reso-

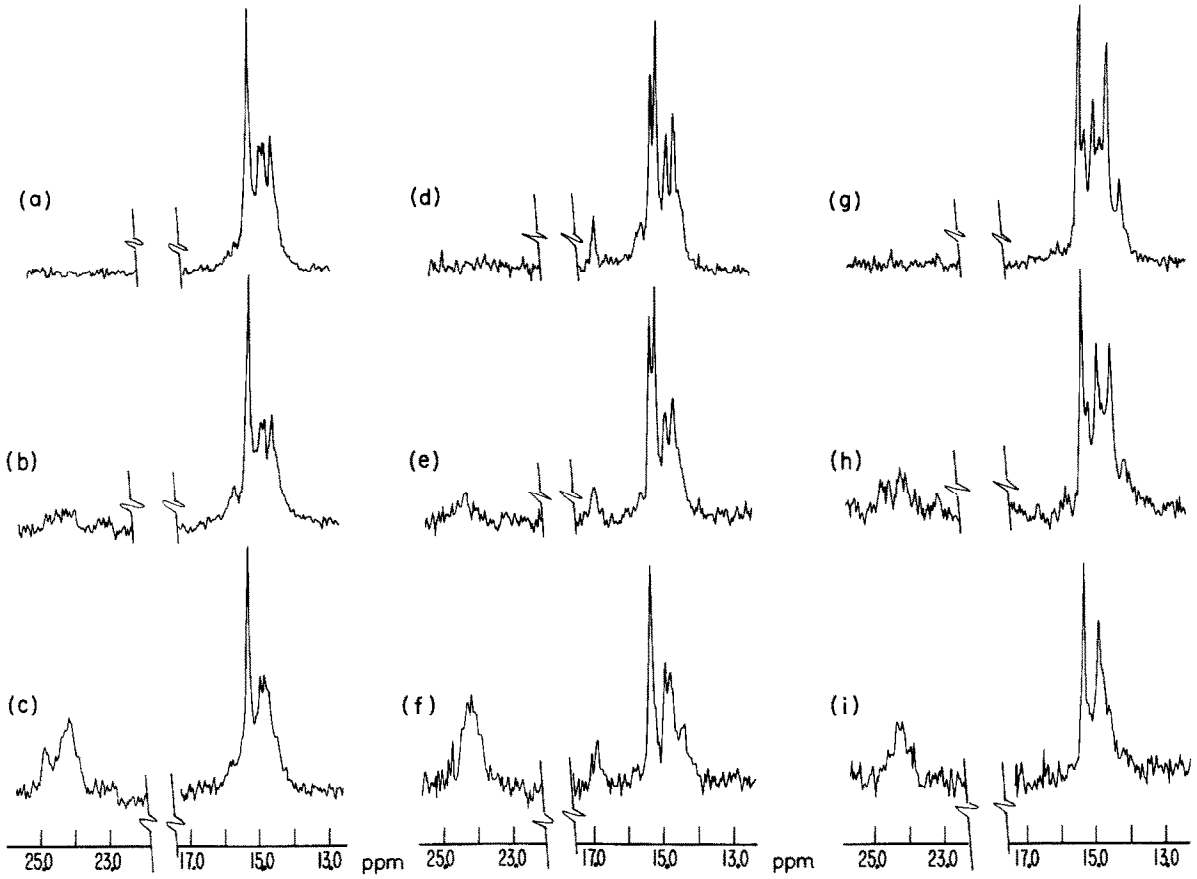

Fig.1. Proton decoupled ^{13}C-NMR spectra of [*methyl*-^{13}C]methionine DHFR derived from *S. faecium:* (a) uncomplexed enzyme; (b) uncomplexed enzyme which was exposed to iodoacetate as in [16] in the presence of aminopterin; (c) uncomplexed enzyme which was treated with iodoacetate in the absence of aminopterin (resonances near 24–25 ppm correspond to carboxymethylated methionines); (d) binary enzyme–NADP$^+$ complex; (e) binary complex of NADP$^+$ with enzyme pre-treated with iodoacetate in the presence of aminopterin; (f) binary complex of NADP$^+$ with enzyme pre-treated with iodoacetate in the absence of aminopterin; (g) binary complex of enzyme with methotrexate; (h) binary complex of methotrexate with enzyme which had previously been treated with iodoacetate in the presence of aminopterin; (i) binary complex of methotrexate with enzyme pre-treated with iodoacetate in the absence of aminopterin.

nances with intensities: (92%–0%) = 92%, corresponding to Met-28, and (48%–10%) = 38%, corresponding to Met-50. The difference spectrum, fig.2(b–c) exhibits about the expected pattern, allowing identification of Met-28 and Met-50 as indicated. The difference spectrum obtained is consistent with the conclusion that modifications of Met-28 and Met-50 do not lead to significant perturbations of the chemical shifts of the remaining methionine residues, since such perturbations would probably lead to a more complex difference spectrum with both negative and positive bands.

Studies similar to those described above for the uncomplexed enzyme were also carried out with the binary enzyme–NADP$^+$ and the binary enzyme–methotrexate (MTX) complexes. The NADP$^+$ binary complex was chosen for study since in this case there is a unique downfield shift of one of the methionine resonances (fig.1d). As in the above example, fig.1d,g correspond to the complexes with the unmodified enzyme; fig.1e,h correspond to the NADP$^+$ and MTX complexes with the enzyme which had been modified in the presence of aminopterin, but with the aminopterin

Table 1

Degree of modification of *S. faecium* methionine residues by iodo[1-^{14}C]acetate[a]

Residue	Study 1 (no inhibitor)	Study 2 (1 mM aminopterin)
Met-1	0	0
Met-5	0	0
Met-28	0.92	0
Met-36	0.46	0.44
Met-42	<0.01	<0.02
Met-50	0.48	0.10
Met-163	0.19	0.27

[a]Based on data included in table 6 of [17]

removed prior to the addition of the ligands; fig.1f and 1i correspond to the NADP$^+$ and MTX complexes, respectively, with enzyme that had been modified in the absence of protective ligands. As in the case of the uncomplexed enzyme, difference spectra obtained by subtracting spectra for the unmodified enzyme from spectra for the enzyme modified in the presence of aminopterin did not provide sufficient sensitivity for resonance assignments. However, difference spectra between the enzymes modified in the presence and absence of aminopterin yielded spectra with the expected intensities corresponding to Met-28 and Met-50, fig.2(e–f) and fig.2(h–i).

From the difference spectra obtained, it appears that Met-28 corresponds to a relatively sharp resonance with a chemical shift value of 15.4 ppm in the uncomplexed enzyme which is near the value for the denatured enzyme. These spectral parameters, in combination with the susceptibility of this residue to carboxymethylation, are consistent with the interpretation that Met-28 is a 'surface residue'; i.e., located at the enzyme–aqueous interface. Thus, its shift is not strongly perturbed by inter-residue interactions which would more probably be significant for residues located further from the enzyme surface. Met-50 fits less clearly into this category since it is shifted upfield significantly relative to the position of the denatured enzyme resonance, is somewhat broader than the Met-28 peak, and is subject to only partial modification by iodoacetate. Thus, it is concluded that this residue is in a somewhat more protected environment, most probably close to the face of an

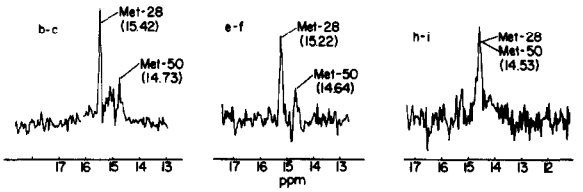

Fig.2.. Difference spectra of [*methyl*-^{13}C]methionine-labeled enzyme obtained by subtracting spectra corresponding to treatment with iodoacetate in the presence and absence of aminopterin. The labeling corresponds to that used in fig.1.

aromatic sidechain or charged residue which could produce the upfield shift.

Although the crystal structure of *S. faecium* dihydrofolate reductase has not as yet been solved, the crystal structures of the *E. coli* and *L. casei* enzymes have been solved to 1.7 Å resolution [19,20], and the question of sequence homology has been discussed in [21]. Based on this homology, the Met-28 and Met-50 residues correspond to Leu-27 and Phe-49 in the *L. casei* enzyme. From the crystal structure data of the *L. casei* enzyme complexed with methotrexate and NADPH [19], it is apparent that Leu-27 and Phe-49 lie on either side of the *p*-aminobenzoyl moiety of the methotrexate. Thus, the protection of the homologous Met-28 and Met-50 residues from modification by iodoacetate in the presence of aminopterin is readily explained. In addition, the upfield shift of Met-28, from 15.4 ppm and 15.2 ppm for the uncomplexed enzyme and NADP$^+$ complex, respectively, to 14.5 ppm in the methotrexate complex (fig.2) is readily explained by the proximity of Met-28 to the *p*-aminobenzoyl ring of the inhibitor. With the assumption that the orientation of this moiety in the folate binding site is similar for both the substrates and inhibitors, we can conclude that the chemical shift would also be similar in each case.

Based on the above assignments, it is apparent that the single resolved downfield methionine methyl resonance in the NADP$^+$ binary complex (fig.1d) is not significantly carboxymethylated and does not correspond to Met-28 or Met-50. As discussed in [21], the combination of X-ray diffraction data for the *L. casei* enzyme and the suggested sequence homology indicate that this resonance must be assigned to Met-5 (Leu-4 in the *L. casei* enzyme), since this is the only other methionine residue predicted to be in the active site cavity. As

proposed in [21], the large downfield shift of Met-5 resulting from the formation of the $NADP^+$ complex (1.7 ppm) can be explained in terms of a direct contribution of the bound ligand only if the orientation of the $NADP^+$ in the binding site differs significantly from the orientation of the NADPH. The most likely explanation for such a difference is an electrostatic attraction between the carboxylate anion of Asp-26 and the positively charged oxidized nicotinamide ring. This would draw the ring further into the cavity and closer to the Met-5 residue.

We conclude that this strategy represents a reasonable basis for interpretation of the extent and specificity of modification of a particular class of residues by carboxymethylating or related chemical agents. In contrast, analogous information is not directly available if the label is introduced by means of the chemical reagent itself. It is reassuring that the difference spectra are directly interpretable on the basis of predictions from iodo[1-^{14}C]acetate modification studies. The use of chemical probes of enzyme structure is predicated on the absence of the introduction of major conformational perturbations. The difference spectra obtained here indicate that on the basis of one measure of such perturbations (chemical shifts of unmodified methionine residues which would lead to complex difference spectra) no major conformational effects are apparent. This presumably reflects the fact that the reactive methionine residues 28, 36 and 50 which are accessible to the iodoacetate, are situated near the enzyme aqueous interface, so that the introduction of a negative charge to a solvated residue does not destabilize the enzyme structure: The specificity of carboxymethylation provides a useful basis for making resonance assignments.

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