LA-UR -78-1492

TITLE: ^{13}C NMR Studies of Bacterial Dihydrofolate Reductase Containing [Methyl-^{13}C]Methionine And [Guanido-^{13}C]Arginine.

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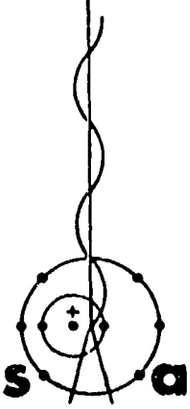
SUBMITTED TO: To be published in the Proceedings of the Third International Conference on Stable Isotopes to be held in Oak Brook, Ill. (May 23-26, 1978), R. Klein, Ed. (Academic Press, Inc., New York).

MASTER



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Form No. 836
St. No. 2629
1/75

UNITED STATES
ENERGY RESEARCH AND
DEVELOPMENT ADMINISTRATION
CONTRACT W-7405-ENG. 36

^{13}C NMR Studies of Bacterial Dihydrofolate Reductase Containing [Methyl-^{13}C]Methionine and [Guanido-^{13}C]Arginine*

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ABSTRACT

[Methyl-^{13}C]methionine and [guanido-^{13}C]arginine have been incorporated with high efficiency by Streptococcus faecium var. Durans strain A into dihydrofolate reductase isoenzyme 2 and ^{13}C NMR spectra have been obtained for the labeled enzymes and their complexes with substrates, co-factors, and inhibitors. The ^{13}C NMR spectra exhibit a high degree of discrimination -- up to six guanido-^{13}C resonances spanning a 1.2 ppm range have been resolved for the eight arginine residues and, under certain conditions, seven methyl-^{13}C resonances spanning a 3 ppm chemical shift range have been resolved for the seven methionine residues of the enzyme. The ^{13}C chemical shifts and spin lattice relaxation times of these distinct, relatively narrow resonances can be interpreted in terms of the conformational states of the enzyme and the interactions of the ^{13}C-labeled residues with bound ligands. In a larger context, the results reported here provide experimental data which bear on a central question in the use of ^{13}C NMR spectroscopy to probe the structure of labeled macromolecules, vis.: Where should the ^{13}C label be incorporated to ensure a relatively narrow resonance whose chemical shift is nonetheless sensitive to perturbations of the macromolecule? Contrary to one accepted view, this study demonstrates that a significant degree of internal motion for a class of amino acid residues is not necessarily incompatible with a large chemical shift dispersion within the class.

INTRODUCTION

It is becoming generally recognized that ^{13}C NMR spectroscopy in conjunction with the use of ^{13}C enriched amino acids to label specific sites has a great potential for providing unique insights into the structures of enzymes in solution. Unlike fluorescent probes or paramagnetic spin labels, ^{13}C enriched amino acids act as innocent reporter groups and do not perturb the structure of the protein. With respect to the NMR characteristics of ^{13}C itself, the chemical shift range is large and the favorable relaxation rates can result in relatively narrow lines, both characteristics preserving the high resolution nature of the spectra for even large molecules or heterogeneous systems. Indeed high resolution ^{13}C NMR spectra have been reported even for a variety of aqueous suspensions of ^{13}C enriched intact cells and virus particles (1-7).

Despite the considerable potential of the technique, there have been only a few ^{13}C NMR studies of proteins labeled by the incorporation of enriched amino acids, including: [2-^{13}C]histidine incorporated into tryptophan synthetase (8,9), α-lytic protease (10,11), and hemoglobin (12) by biosynthesis and into ribonuclease S' by synthesis of the labeled S peptide (13); [Me-^{13}C] methionine incorporated into cytochrome c (5) by biosynthesis and into myoglobin (14) and myelin basic protein (15) by semi-synthetic methods; and [4-^{13}C] histidine into alkaline phosphatase (16) by biosynthesis. The small number of these studies is due, in part, to the continued high cost and limited availability of ^{13}C enriched amino acids; but more important, perhaps is the fact that there is as yet no completely firm experimental or theoretical basis for selecting the site(s) of enrichment in the amino-acid which will yield the optimum structural NMR data.

The central question in the selection is: "Which ^{13}C site in the amino acid exhibits chemical shifts that are most sensitive to structural perturbations but does not experience extensive broadening by nuclear dipolar coupling with directly-bonded or adjacent protons in a slowly tumbling protein?" Experimental and theoretical (17) studies suggest that in proteins in which an amino acid residue is tightly locked into the structure with little freedom of internal motion, those ^{13}C atoms bearing directly bonded protons frequently will exhibit broad resonances containing little structural information. For example, the thirty-eight [2-^{13}C]histidine residues of the hemoglobin tetramer (12) give rise to a single, broad (half-width, 85 Hz) 2-^{13}C-H resonance. On the other hand, the ^{13}C-H dipolar interaction varies as the inverse sixth power of the C-H distance and quaternary carbons like [4-^{13}C]histidine which does not bear directly bonded protons, should exhibit much narrower lines. In the case of alkaline phosphatase selectively enriched with [4-^{13}C]histidine, eight narrow 4-^{13}C resonances corresponding to the 8-9 histidine residues per identical sub-unit were resolved (16). An interesting aspect of the latter study is that the 4-^{13}C resonances exhibited an extremely large sensitivity to chemical environment, having spanned a 14 ppm chemical shift range. At this writing it is not clear what structural features of the enzyme caused this large shift variation or indeed whether all histidine residues are tightly locked into the alkaline phosphatase structure. In any case, the experimental data and theoretical treatments available now show that if one wants to probe the structure of a protein at the site of a tightly locked amino acid residue by ^{13}C NMR, then a quaternary carbon is the ^{13}C labeling site preferred provided that the chemical shift or relaxation time at the site is sensitive to structural perturbations. In the case of ^{13}C at the present stage of development of the technique, this is usually not known

and is difficult to predict; but, even in the case of a small sensitivity of shift to perturbation, relaxation effects provide useful information (vide infra).

The situation is not so clear cut with respect to those amino acid residues having segments which are not tightly locked into the protein structure and which have a considerable freedom of motion. This rapid segmental motion can narrow the ^{13}C resonances of the segment as predicted theoretically (17) and observed experimentally, for example, in the ^{13}C resonances of the $N(^{13}CH_3)_3^+$ group of the N-trimethyl lysine residue of cytochrome c biosynthetically enriched with [Me-^{13}C]methionine as the substrate (5). Although the motional narrowing is highly desirable in the high resolution ^{13}C NMR spectroscopy of these macromolecules, it has been suggested (17) that the equally desirable chemical shift non-equivalence "caused" by the folding of the protein into its native conformation is probably diminished because these segments are not locked and are free to rotate. There are important assumptions about the nature of the chemical shift and relaxation processes inherent in this suggestion and they are probably incorrect as we discuss in the following description of the ^{13}C NMR spectra of dihydrofolate reductase (DHFR) containing [Me-^{13}C]methionine and [guanido-^{13}C]arginine.

The work described is part of a general program on the ^{13}C NMR spectroscopy of DHFR specifically enriched with ^{13}C labeled amino acids. The enzyme was selected for NMR investigations because its structure is currently under intensive study in a number of laboratories. General interest in DHFR derives in part from its clinical significance, being the target of methotrexate, a drug used in the treatment of various types of cancer. In addition, the bacterial enzyme is specifically inhibited by trimethoprim which, in combination with sulfa drugs,

is used extensively for the treatment of some infectious diseases.

EXPERIMENTAL

The preparation of the ^{13}C enriched enzyme from S. faecium and the NMR procedures are discussed in detail elsewhere (18,19).

RESULTS AND DISCUSSION

The ^{13}C NMR spectrum of [guanido-^{13}C]arginine labeled dihydrofolate reductase (DHFR) in the guanido carbon region only is shown in Figure 1a. Four resonances are clearly resolved and have integrated intensities of 1:1:5:1 (in the order low to high field for the resonances designated 1, 2, 3, 4). These narrow resonances (expected for a quaternary carbon) correspond to the eight arginine residues of DHFR from S. faecium and they span a chemical shift range of 1.2 ppm, somewhat larger than that observed in other proteins (20). The chemical shifts of the guanido carbon resonances in the free enzyme and in several of its binary and ternary complexes are summarized in Table 1. As illustrated in Figure 1b, when the enzyme is denatured with [^{12}C-99.999%]urea, all the resonances collapse to a single line with a chemical shift near that of peak 3 whose chemical shift in turn is near that of the guanido carbon in free arginine.

The arginine residues in DHFR can be divided into two classes on the basis of their chemical shifts. Residues of the first class which give rise to the components of peak 3 (Table 1), appear to be solvent accessible whereas the second class comprised of peaks 1, 2, and 4 corresponds to residues subject to significant environmental perturbations such as proximity to the ring currents of aromatic residues and involvement in salt bridges and hydrogen bonds. The relative spin lattice relaxation times for these residues (Table 2) are con-

sistent with these conclusions. In contrast to the data for peaks 1, 2, and 4, the longer T_1 values and the complex temperature dependence of the peak 3 T_1 imply significant internal motion for the residues comprising that resonance. The T_1 values for the arginines 1, 2, and 4, together with line width and Nuclear Overhauser Enhancement data (19), indicate that these three residues are tightly locked into the DHFR structure and that the motion responsible for their relaxation is the slow tumbling of the protein. From the relative values of T_1 in D_2O and H_2O solvents for peaks 1, 2, and 4, a rotational correlation time for the protein of 2×10^{-8} sec (diffusion coefficient for tumbling of 8.33×10^6 sec^{-1}) was calculated, a reasonable value for a protein of M.W. 20,000.

Thus the data for the arginine labeled enzyme are consistent with the suggestion (17) that it is the residues which are restricted in their internal motion as a result of protein folding that give rise to chemical shift inequivalence. The data for the [Me-^{13}C]methionine labeled enzyme provide an interesting contrast. The ^{13}C chemical shifts of the proton bearing methyl group of the seven methionine residues in the labeled enzyme and several of its complexes are summarized in Table 3 (18). The labeled enzyme denatured with [^{12}C-99.999%]urea exhibits a single resonance centered at 15.32 ppm, whereas the resonance of free methionine occurs at 15.04 ppm. It is evident from the data in the table the methionine methyl groups in the labeled enzyme complexes exhibit a large chemical shift dispersion to both the low and high field side of the single resonance in the denatured enzyme, the total chemical shift range for the methyl group spanning more than 3 ppm. The surprising result is that all the lines are quite narrow (2.5 - 5 Hz width at half-height) indicating that there is rapid rotation about the CH_3-S bond. The measured T_1

values are consistent with this conclusion. For example, the peak at 15.34 ppm in the free enzyme exhibits a T_1 of 590 msec and a line width of 3.2 Hz. Using these values, the measured Nuclear Overhauser Enhancement of 2, and the diffusion coefficient of 8.33×10^6 sec^{-1} for tumbling of the protein, a diffusion coefficient of 4×10^{10} sec^{-1} was calculated for the rotation about the CH_3-S bond (18,21). It is significant that the latter data could not be fit using a model based on isotopic diffusion of the protein and free segmental motion of the methionine side chain. Instead, it was necessary to develop one based on isotropic diffusion, free rotation about the CH_3-S bond where the angular excursions about the CH_2-S axis are restricted to $\sim 90°$. Thus, although protein folding certainly affects the relaxation and chemical shifts in the side chains of amino-acids in proteins, the observation of narrow lines and large chemical shift dispersions for proton bearing ^{13}C atoms in such side chains are <u>not</u> necessarily mutually exclusive.

REFERENCES

* This work performed under the auspices of the U.S. Department of Energy and was supported in part by a National Institutes of Health Research Grant 1P07 RR-00962-02 from the Division of Research Resources, DHEW (NAM) and in part by the U.S. Public Health Research Grant CA 13840 from the National Cancer Institute of the National Institutes of Health (RLB). RLB gratefully acknowledges support by a John Simon Guggenheim Memorial Foundation Fellowship during the period in which this work was carried out.

(1) Matwiyoff, N.A. and Needham, T.E. Carbon-13 NMR Spectroscropy of Red Blood Cell Suspensions. Biochem. Biophys. Res. Commun. 49:1158-1164, 1972.

(2) Morrow, J.S. Keim, P., Visscher, R.B., Marshall, R.C., and Gurd, F.R.N. Interaction of $^{13}CO_2$ and Bicarbonate with Human Hemoglobin Preparations. Proc. Natl. Acad. Sci. 70:1414-1418, 1973.

(3) Metcalfe, J.C. Birdsall, N.J.M., and Lee, A.G. ^{13}C NMR Spectra of Archoleplasma Membranes Containing ^{13}C Labeled Phospholipids. FEBS (Fed. Eur. Biochem. Soc.) Lett. 21:335-340, 1972.

(4) London, R.E., Kollman, V.H., and Matwiyoff, N.A. ^{13}C Fourier Transform Nuclear Magnetic Resonance Studies of Fractionated Candida Utilis Membranes. Biochem. 14:5492-5500, 1975.

(5) Eakin, R.T., Morgan, L.O., and Matwiyoff, N.A. Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Whole Cells and of Cytochrome c from Neurospora crassa Grown with [S-Me-^{13}C]Methionine. Biochem. J. 152:529-535, 1975.

(6) Stoffel, W. and Bister, K. ^{13}C Nuclear Magnetic Resonance Studies on the Lipid Organization in Enveloped Virions (Vesicular Stomatitis Virus. Biochem. 14:2841-2848, 1975.

(7) London, R.E., Hildebrand, C.E., Olson, E.S., and Matwiyoff, N.A. ^{13}C NMR Spectroscopy of Suspensions of Chinese Hamster Ovary Cells Specifically Enriched with [Me-^{13}C]Choline. Biochem. 15:5480-5486, 1976.

(8) Browne, D.T., Kenyon, G.L., Packer, E.L. Sternlicht, H., and Wilson, D.M. Studies of Macromolecular Structure by ^{13}C NMR. II. A Specific Labeling Approach to the Study of Histidine Residues in Proteins. J. Am. Chem. Soc. 95:1316-1323, 1973.

(9) Browne, D.T., Kenyon, G.L., Packer, E.L., and Wilson, D.M. A Study of the Dynamic State of Histidine Residues in Tryptophan Synthetase α Subunit by ^{13}C Nuclear Magnetic Resonance. Biochem. Biophys. Res. Commun. 50:42-47, 1973.

(10) Hunkapiller, M.W., Smallcombe, S.H., and Richards, J.H. Mechanism of Serine Protease Action. Ionization Behavior of Tetrahedral Adduct between α-Lytic Protease and Tripeptide Aldehyde Studied by ^{13}C NMR. Org. Mag. Res. 7:262-265, 1975.

(11) Hunkapiller, M.W., Smallcombe, S.H. Whitaker, D.R., and Richards, J.H. ^{13}C NMR Studies of the Histidine Residue in α-Lytic Protease. Implications for the Catalytic Mechanism of Serine Proteases. Biochem. 12:4732-4743, 1973.

(12) London, R.E., Gregg, C.T., and Matwiyoff, N.A. Nuclear Magnetic Resonance of Rotational Mobility of Mouse Hemoglobin Labeled with [2-^{13}C]Histidine. Science 188:266-268, 1975.

(13) Chaiken, I.M., Cohen, J.S., and Sokoloski, E.A. Microenvironment of Histidine 12 in Ribonuclease S as Detected by ^{13}C NMR. J. Am. Chem. Soc. 96:4703-4705, 1974.

(14) Jones, W.C. Rothgeb, T.M., and Gurd, F.R.N. Nuclear Magnetic Resonance Studies of Sperm Whale Myoglobin Specifically Enriched with ^{13}C in the Methionine Methyl Groups. J. Biol. Chem. 251:7452-7460, 1976.

(15) Deber, C.M., Moscarello, M.A., and Wood, D.D. Conformational Studies on ^{13}C Enriched Human and Bovine Myelin Basic Protein in Solution and Incorporated into Liposames. Biochem. 17:898-903, 1978.

(16) Browne, D.T., Earl, E.M., and Otvos, J.D. Selectively Enriched [γ-^{13}C] Histidine as an NMR Probe of Enzyme Structure and Function Synthesis and Incorporation into E. coli Alkaline Phosphatase. Biochem. Biophys. Res. Commun. 72:398-403, 1976.

(17) Oldfield, E., Norton, R.S., and Allerhand, A. Studies of Individual Carbon Sites of Proteins in Solution by Natural Abundance ^{13}C NMR Spectroscopy. Relaxation Behavior. J. Biol. Chem. 250:6368-6380, 1975.

(18) Blakley, R.L., Cocco, L., London, R.E., Walker, T.E., and Matwiyoff, N.A. NMR Studies on Bacterial Dihydrofolate Reductase Containing [Me-^{13}C] Methionine. Biochem., in press, 1978.

(19) Cocco, L., Blakley, R.L., Walker, T.E., London, R.E., and Matwiyoff, N.A. NMR Studies on Bacterial Dihydrofolate Reductase Containing [Guanido-^{13}C] Arginine. Biochem., in press, 1978.

(20) Oldfield, E., Norton, R.S., and Allerhand, A. Studies of Individual Carbon Sites of Proteins in Solution by Natural Abundance ^{13}C NMR Spectroscopy. Strategies for Assignment. J. Biol. Chem. 250:6381-6402, 1975.

(21) London, R.E. Calculated NMR Relaxation Parameters for a Restricted Internal Diffusion Model. Application to Methionine Relaxation in Dihydrofolate Reductase. J. Am. Chem. Soc., in press, 1978.

Table 1

^{13}C Chemical Shifts of the Guanido Carbon of Arginine in [Guanido-^{13}C]Arginine Labeled DHFR (ppm with respect to external TMS).

	Peak Position			
Complex	1	2	3	4
Enzyme (E)	158.24	157.86	157.46; 157.40	157.17
E• NADPH	158.10	157.84	157.5; 157.38	-
E• $NADP^+$	158.11	157.81	157.48; 157.40	157.18
E• Dihydrofolate	158.29	157.84	157.45	157.16
E• Aminopterin	153.40	157.85	157.52; 157.45; 157.33	157.20
E• NADPH•Aminopterin	158.30	157.84	157.55; 157.48; 157.33	157.20

Table 2

Effect of Temperature on T_1 Values (Sec.) of the Guanido Carbons of Arginine Residues in the Ternary Complex of [Guanido-^{13}C]Arginine Labeled DHFR with NADPH and Methotrexate in D_2O.

Peak	Temperature, °C		
	5	15	25
1	1.14	0.96	0.91
2	1.21	1.06	0.85
3	1.14	1.37	1.31
4	-	0.94	0.84

Table 3

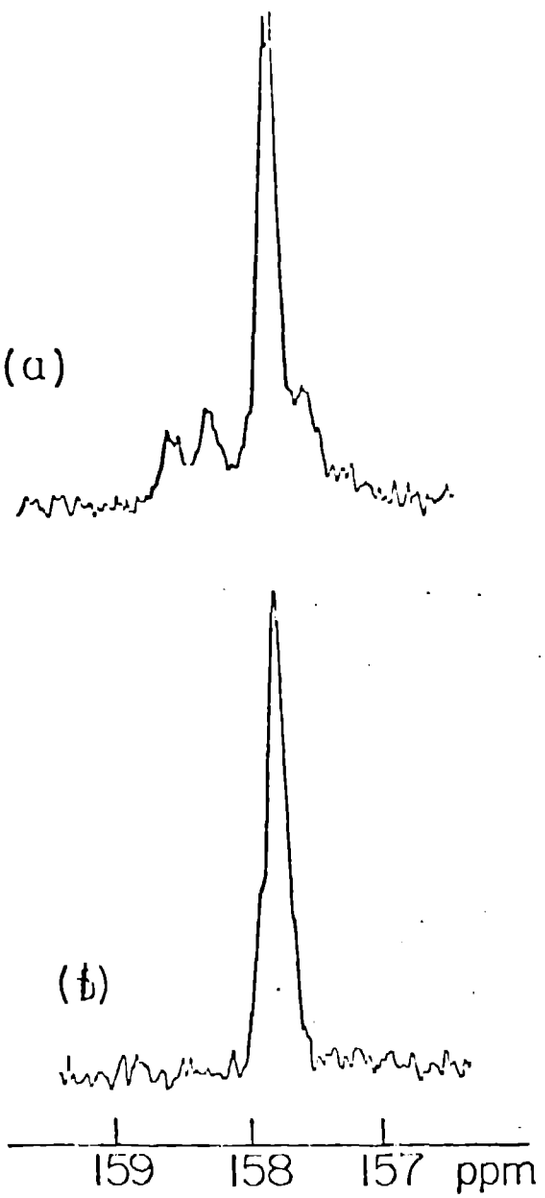
^{13}C Chemical Shifts of the Methyl Carbon of Methionine in [Me-^{13}C] Methionine Labeled DHFR (in ppm with respect to external TMS).

Complex	Chemical Shifts								
E			15.35		14.99	14.66			
E•NADPH			15.40	15.11		14.75	14.55		
E•$NADP^+$	17.08	15.68	15.39	15.24	14.95	14.73			
E•Dihydrofolate			15.41		14.98	14.77			
E•Methotrexate			15.37	15.24	14.95	14.74	14.57	14.18	
E•$NADP^+$•Dihydrofolate			15.40	15.17	15.01	14.74	14.62		
E•$NADP^+$•Methotrexate			15.34	15.08			14.53	14.09	13.94

Figure 1

a. Proton Noise Decoupled ^{13}C NMR Spectrum (25.2 mHz) in the Guanido Guanido ($N\text{-}^{13}C(=NH_2^+)\text{-}NH_2$) Carbon Region for Aqueous (1.1 mM) Dihydrofolate Reductase Labeled with [Guanido-^{13}C]Arginine.

b. Proton Noise Decoupled ^{13}C NMR Spectrum of a Denatured with [^{12}C-99.999%] Urea.


(a)
(b)
159
158
157 ppm