A ^{13}C NUCLEAR MAGNETIC RESONANCE STUDY OF THE INTERACTION OF LIGANDS WITH ARGININE RESIDUES IN DIHYDROFOLATE REDUCTASE

Lennie Cocco, Raymond L. Blakley
The Department of Biochemistry
College of Medicine
University of Iowa
Iowa City, Iowa 52242

Thomas E. Walker, Robert E. London, and N. A. Matwiyoff
The Los Alamos Scientific Laboratory
University of California
Los Alamos, New Mexico 87545

Received March 14,1977

Summary: ^{13}C nmr spectra of Streptococcus faecium dihydrofolate reductase containing [^{13}C-guanidino] arginine and ligand complexes with the labeled enzyme are reported. The spectrum of the native enzyme shows 5 well-resolved resonances (the enzyme contains 8 Arg) with a chemical shift range of 1.2 ppm. Addition of ligands to the enzyme produces distinct changes in the enzyme spectrum, and demonstrates that ^{13}C nmr of labeled protein can be used in studies of protein-ligand interactions. An example of the use of ^{13}C-depleted material is also presented.

Several recent studies of enzymes (1-4) and proteins (5,6) have shown that the incorporation of ^{13}C labeled amino acids into macromolecules can provide an excellent, non-perturbing probe by which protein-ligand interactions can be studied. The role of particular amino acid residues in ligand binding can then be studied without the problems encountered in chemical modification studies. A major consideration in the design of such experiments is the selection of the site of ^{13}C labeling. Ideally, the labeled site should exhibit a narrow ^{13}C resonance whose chemical shift is very sensitive to chemical and conformational perturbations. However, even using the former criteria and ^{13}C nmr data obtained with model systems, it is not always a straightforward matter to select the optimum labeling site. Indeed, early attempts (1-3) at specific enrichment of amino acid residues in enzymes involved protonated carbons which generally gave broad lines, so that resolution of individual lines was not possible. In an extensive series of natural abundance ^{13}C nmr studies (7-9), Allerhand and co-workers have shown that in contrast to the case for protonated carbon atoms, non-protonated carbon atoms of tyrosine, histidine, tryptophan, and arginine do exhibit the narrow ^{13}C

ISSN 0006-291X

resonances (<5 Hz) suitable for high resolution ^{13}C nmr studies of proteins as large as hemoglobin. For the macromolecules (hemoglobin, myoglobin, cytochrome c and lysozyme) studied by Allerhand et al., it appeared that only the former three amino acids fulfilled the chemical shift criterion, the non-protonated guanido carbon resonance of arginine exhibiting a maximum chemical shift range of only 0.4 ppm in any of the individual macromolecules studied (8). Browne, et al. (4) have reported on alkaline phosphatase containing [γ-^{13}C] histidine (enriched at a non-protonated carbon), and show that a well-resolved spectrum can be obtained. However, they do not report any results of the effect of ligand binding on the ^{13}C nmr spectrum.

In this communication we report on the use of [^{13}C-guanidino] arginine to label dihydrofolate reductase from a methotrexate-resistant strain of Streptococcus faecium. This enzyme catalyzes the NADPH-linked reduction of dihydrofolate to tetrahydrofolate and is of interest because it is inhibited by such compounds as methotrexate which is used clinically in the treatment of neoplastic disease. We show that the guanidino carbon of arginine can provide a useful chemical shift range for studies of protein-ligand interaction, despite its narrow range in other proteins studied (8). In addition we show that the binding of ligands to the enzyme can produce marked changes in the ^{13}C nmr spectrum which demonstrate the potential of labeling specific sites in amino acids and subsequent incorporation into proteins as a probe for studying protein ligand interactions.

Materials and Methods

The enzyme was obtained from Streptococcus faecium var Durans Strain A grown on media containing [^{13}C-guanidino] arginine (90% enriched, Koch Isotopes, Cambridge, Mass.). It was purified by a modification of published methods (10).

Proton noise decoupled cmr spectra were obtained at 25° on a Varian XL-100-15 spectrometer operating in the FT mode. All spectra were obtained with a 250 Hz spectral width and 512 data points using a 70° pulse width and no pulse delay. All samples were in 0.05 M potassium phosphate buffer (pH 7.3) containing 20% D_2O which was used as the lock signal.

Results and Discussion

The spectra of the native enzyme and of the enzyme in the presence of a number of effectors and inhibitors is shown in Figures 1 a-f in the region

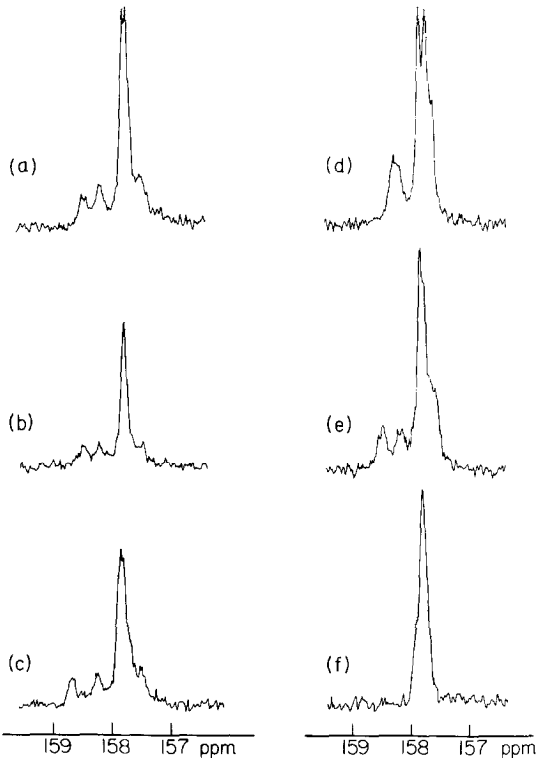

Figure 1. ^{13}C nmr spectra of [^{13}C-guanidino]arginine labeled dihydrofolate reductase and its complexes with various ligands. (A) Dihydrofolate reductase (0.66 mM) alone; 28,000 transients. (B) Dihydrofolate reductase (0.62 mM) and dihydrofolate (1.3 mM); 15,700 transients. (C) Dihydrofolate reductase (0.67 mM) and methotrexate (0.72 mM); 15,500 transients. (D) Dihydrofolate reductase (0.66 mM) and NADPH (1.30 mM); 25,500 transients. (E) Dihydrofolate reductase (0.65 mM), NADPH (1.24 mM) and methotrexate (0.73 mM); 22,500 transients. (F) Dihydrofolate reductase (0.4 mM) denatured with 6 M [^{12}C] urea; 21,000 transients.

where the guanido carbon of arginine is expected to resonate, ca. 158 ppm downfield of TMS. Spectra obtained using larger spectral widths exhibited no resonances due to the natural abundance carbons of the enzyme. The spectra exhibit a remarkably high degree of resolution* and the guanido resonances cover a useful shift range, 1.2 ppm. More interesting is the fact that large changes do occur in the spectra when effectors and inhibitors are bound,

*The spectrum of the native enzyme in D_2O buffer shows much narrower lines than that for enzyme in H_2O buffer as expected (cf. ref. 7).

TABLE 1

Chemical Shift of the Guanido Carbon of Arginine in Dihydrofolate Reductase[a]

Ligand bound to enzyme	Peak Position[b]				
	1	2	3	4	5
None (Fig. 1a)	158.40	158.16	157.75	157.70	157.46
Dihydrofolate (Fig. 1b)	158.45	158.20	157.76	---	157.46
Methotrexate (Fig. 1c)	158.64	158.20	157.79	157.75	157.48
NADPH (Fig. 1d)	158.23	158.23	157.82	157.72	157.60(s)[c]
NADPH + Methotrexate (Fig. 1e)	158.48	158.18	157.82	157.77(s)[c]	157.57(s)[c]
6 M [^{12}C] Urea (Fig. 1f)			157.78		

[a]Data taken from spectra in Figure 1.

[b]Peak positions are given in ppm downfield from TMS. The peaks are numbered starting with the furthest downfield peak.

[c]These peaks appear as shoulders on the larger center peak and, therefore, may appear further downfield than they actually are.

suggesting that the guanido site will provide a useful probe of conformational effects in proteins where the arginine residues are involved directly or indirectly in binding ligands (vide infra).

The protein contains eight arginine residues (11,12) and the integrated intensities of the resonances in Figures 1a and 1c correspond to the ratios 1:1:5 (two overlapping signals);1, proceeding from low to high field**. Their chemical shifts are summarized in the Table. The resonances numbered 3 and 4 have a chemical shift similar to that of free arginine and it is likely that they are due to arginines on the surface of the protein and are accessible to

**Preliminary measurements of spin-lattice relaxation times (T_1) and nuclear Overhauser enhancement (NOE) show that for all of the observed peaks, the T_1's are similar and the NOE values are approximately equal to one. Thus the integrated intensities are proportional to the number of carbons.

the solvent. Consistent with the latter is the fact that denaturation of the enzyme with [^{12}C-99.999%]urea*** causes the resonances to collapse to a single line near the center of gravity of the original 3 and 4 resonances.

The spectrum of the binary complex of enzyme and NADPH is shown in Figure 1d. Clearly evident is the upfield shift of the resonance centered at 158.4 ppm and the larger separation between peaks 3 and 4. A spectrum obtained from half-saturated enzyme shows that the NADPH is in slow exchange. These changes suggest the direct involvement of at least one arginine residue in the binding of NADPH and/or a large conformational change in the enzyme. It is noteworthy that the peak centered at 158.4 ppm has several features which distinguish it from others: 1) it is the resonance furthest removed from the central peak; 2) it shows the largest chemical shift range of any of the resonances; 3) the resonance is seen to shift both upfield (Figure 1d) and downfield (Figure 1c) from the position in the free enzyme. The ternary complex between enzyme, NADPH and methotrexate (Figure 1e) exhibits a spectrum which is surprisingly similar to that of the native enzyme and may reflect a true conformational similarity or a fortuitous combination of competing effects.

Direct involvement of arginine in NADPH binding seems reasonable in light of a recent study of dihydrofolate reductase from L. casei, where NADPH was found to protect two arginines from modification by phenylglyoxal (13). It is also consistent with the fact that in the two reductase sequences known, those of the enzymes from E. coli (14) and S. faecium (15), there are two species-invariant arginine residues. In addition, arginine has been implicated in NADH binding in a variety of dehydrogenases (16-18). The production of a large conformation change by NADPH binding is consistent with the fact that the enzyme has an ordered sequential reaction mechanism with NADPH binding first (19).

***The use of urea depleted in carbon-13, a gift of Dr. T. W. Whaley of the Los Alamos Scientific Laboratory, eliminates the dynamic range problem, among others which arise in the use of large concentrations of natural abundance reagents and solvents.

Studies are now in progress to assign the observed resonances to specific arginine residues and to elucidate the details of the interactions of the various arginine residues with the ligands and with other residues during binding.

Acknowledgment

This work performed under the auspices of the U. S. Energy Research and Development Administration and was supported in part by Grant CA 13840 (RLB) from the National Cancer Institute of the National Institutes of Health, and the National Institutes of Health Research Grant 1P07 RR-00962-01 (NAM) from the Division of Research Resources (DHEW). T. E. Walker gratefully acknowledges a Postdoctoral Fellowship (5 F22 CA00971-02) from the National Cancer Institute. We wish to thank Dr. Gerald A. Pearson for his help in performing the initial nmr experiments.

References

(1) M. W. Hunkapiller, S. H. Smallcombe, D. R. Witaker, and J. H. Richards, (1973) Biochemistry, 12, 4732-4743.
(2) D. T. Browne, G. L. Kenyon, E. L. Paker, H. Sternlicht, and D. M. Wilson, (1973) J. Amer. Chem. Soc., 95, 1316-1323.
(3) I. M. Chaiken, M. H. Freedman, J. R. Lyerla, Jr., and J. S. Cohen, (1973) J. Biol. Chem., 248, 884-891.
(4) D. T. Browne, E. M. Earl and J. D. Otvos, (1976) Biochem. Biophys. Res. Comm., 72, 398-402.
(5) R. E. London, C. T. Gregg, and N. A. Matwiyoff, (1975) Science, 188, 266-268.
(6) R. T. Eakin, L. O. Morgan, and N. A. Matwiyoff, (1975) Biochem. J., 152, 529-535.
(7) E. Oldfield, R. S. Norton, and A. Allerhand, (1975) J. Biol. Chem., 250, 6368-6380.
(8) E. Oldfield, R. S. Norton, and A. Allerhand, (1975) ibid, 6381-6402.
(9) E. Oldfield and A. Allerhand, (1975) ibid, 6403-6407.
(10) P. F. Nixon and R. L. Blakley, (1968) J. Biol. Chem., 243, 4722-4731.
(11) J. M. Gleisner, D. L. Peterson, and R. L. Blakley, (1975) J. Biol. Chem. 250, 4937-4944.
(12) D. L. Peterson, J. M. Gleisner, and R. L. Blakley, (1975) ibid, 4945-4954.
(13) G. A. Vehar and J. H. Freisheim, (1976) Biochem. Biophys. Res. Commun., 68, 937-941.
(14) C. D. Bennett, (1974) Nature, 248, 67-68.
(15) J. M. Gleisner, D. L. Peterson, and R. L. Blakley, (1974) Proc. Nat. Acad. Sci., USA, 71, 3001-3005.
(16) P. C. Yang and G. W. Schwert, (1972) Biochemistry, 11, 2218-2224.
(17) L. G. Lang, J. F. Riordan, and B. L. Valler, (1974) Biochemistry, 13, 4361-4370.
(18) K. M. Blumenthol and E. L. Smith, (1975) J. Biol. Chem., 250, 6555-6559.
(19) R. L. Blakley, M. Schrock, K. Sommer, and P. F. Nixon, (1971) Ann. N. Y. Acad. Sci., 186, 119-130.