Enzyme Structure and Interaction with Inhibitors

by Robert E. London

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The metabolic studies described in the previous article represent a relatively straightforward application of NMR spectroscopy. The positions of the various resonances and their heights allow one to determine the presence and amount of metabolic intermediates and products and, in turn, the specific metabolic pathways involved. These tracer studies with stable-isotope labels are thus directly analogous to studies with radiolabels such as carbon- 14.

But NMR spectra contain a wealth of additional information, Changes in resonance position and shape under various conditions can reveal important structural and dynamic features of complex biological macromolecules. Here we will review some results of an extensive series of studies on the ^{13}C-labeled enzyme dihydrofolate reductase, or DHFR. The idea was to explore how much we could learn about structure and dynamics using NMR techniques in combination with isotopic labeling.

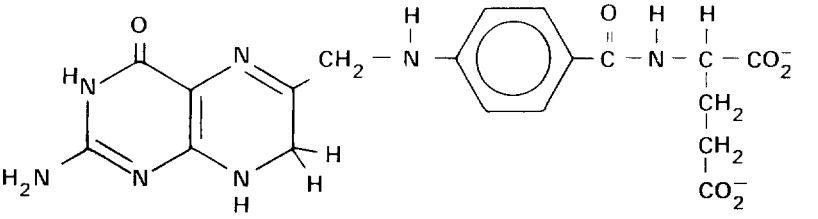
Enzymes are particularly interesting to study from this point of view since both their structure and dynamics may be important to their function of catalyzing biochemical reactions, In the familiar "lock and key" model of enzyme catalysis. a structurally rigid enzyme "lock" can bind only the structurally complementary substrate "keys." Extensive crystallographic data have also fostered this picture of enzymes as rigid structures. But recent evidence suggests that enzyme dynamics is also at work in recognition and catalysis.

We chose to study the enzyme DHFR because of its clinical relevance. Its function is to "activate" the vitamin folic acid by catalyzing the oxidation-reduction reaction

$$\text{dihydrofolate} + \text{NADPH} \overset{\text{DHFR}}{\rightleftharpoons} \text{tetrahydrofolate} + \text{NADP}^+ , \qquad (1)$$

where dihydrofolate is an inactive form of folic acid, and tetrahydrofolate is its activated form.*

Dihydrofolate



Methotrexate

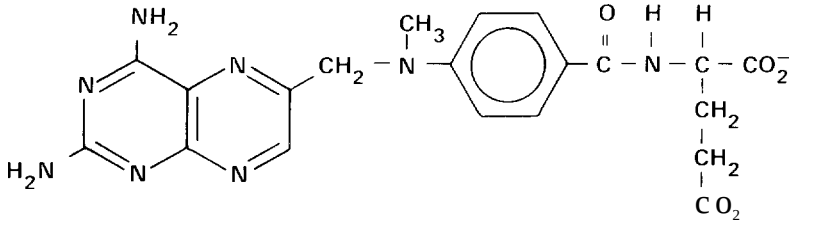


Fig. 1. Substrate dihydrofolate and inhibitor methotrexate of the enzyme dihydrefolate reductase (DHFR). Note that the left most regions of the substrate and the inhibitor are similar in structure. Because these regions presumably interact strongly with the enzyme, they were labeled with carbon-13 (at the locations indicated by gray circles) for the purpose of studying enzyme/substrate and enzyme/inhibitor interactions.

Tetrahydrofolate is required for the synthesis of thymidine, a component of DNA. Inhibiting the production of tetrahydrofolate therefore retards DNA synthesis and cellular growth. In fact, one treatment for the unregulated cellular replication that characterizes cancerous growth is to inhibit the production of tetrahydrofolate by administering drugs that bind with very high affinity to DHFR. These drugs, so-called antifolate inhibitors, are structurally quite similar to dihydrofolate (Fig, 1) and can bind at DHFR's "active site" far dihydrofolate, thereby preventing DHFR from catalyzing reaction 1. One goal of our studies was *to* understand, at the molecular level, why these inhibitors have a very high affinity for DHFR. Such information can be of value for the design of even more potent and specific inhibitors of the enzyme.

DHFR is a medium-sized enzyme with a molecular weight of about 20,000, and its NMR spectrum in the absence of labeling would show only a tow-intensity "background," due to naturally occurring carbon-13, of nearly 800 carbon resonances. Labeling specific portions of the enzyme with carbon-13 was therefore essential to

**NADPH (nicotinamide adenine dinucleotide phosphate], a derivative of the vitamin niacin, is an enzyme cofactor involved in biological oxidation-reductim reactions.*

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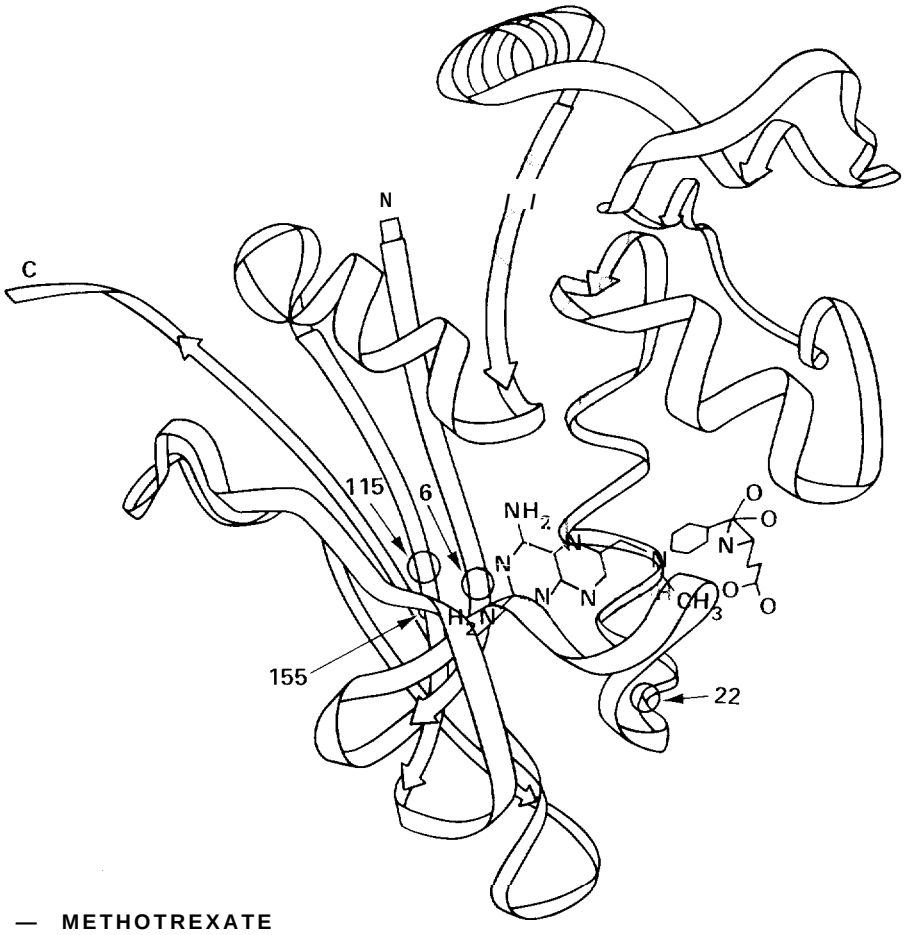

Fig. 2. Backbone ribbon drawing, showing the locations of the enzyme cofactor NADPH and of the inhibitor methotrexate, of the enzyme DHFR (a linked chain of 167 amino acid residues) derived from the microorganism Lactobacillus casei. *[Adapted from J. T.* ***Bolin, D. J. Filman, D.A. Matthews, R. C. Hamlin, and J.Kraut, Journal of Biological Chemistry 257, 13650 {1982).] By occupying the enzyme's active site for dihydrofolate, the inhibitor prevents DHFR from catalyzing the reaction that converts this inactive form of the vitamin folic acid to its active form. NMR spectra for DHFR derived from the microorganism*** Streptococcus faecium *and containing the* ^{13}***C-labeled residue of the amino acid tryptophan provided information about the dynamic of the enzyme and the interaction between enzyme and inhibitor. The approximate positions of the four tryptophan residues in DHFR derived from S.*** faecium ***are those indicated in the drawing, if it is assumed that DHFR derived from S.*** faecium ***and*** L. casei ***are homologous.***

enhance selected peaks and thereby reduce the complexity and increase the sensitivity of these studies.

X-ray crystallographic studies have shown that enzymes are long strings of peptide-linked amino acids (that is, the amino group of one acid residue binds to the carboxylic acid group of the next). These long strings of amino acids fold in a complex way to form a globular structure (Fig. 2). To study the sensitivity of NMR spectra to structure, we labeled selected amino acid residues of DHFR with carbon-13 and measured the spectra of the labeled enzyme in its globular form and again after its structure had been changed into a random coil by the addition of urea.

The carbon- 13 labeling was accomplished by first labeling the amino acids methionine, arginine, and tryptophan and then growing the microorganism *Streptococcus faecium* in media containing one of these labeled amino acids. S. *faecium,* which is a good source of the enzyme DHFR, incorporates the labeled amino acids into the DHFR molecules. The labeled DHFR was then isolated from the microorganism, and its NM R spectra were obtained for the globular and random coil configurations. Figure 3 shows the results for DHFR labeled with [3-^{13}C]tryptophan. The spectrum for the globular form of DHFR shows several carbon- 13 resonances corresponding to different positions along the polypeptide backbone and therefore to different chemical environments of the individual tryptophan residues within the enzyme. Note that most of these so-called chemical shifts disappear when the enzyme structure is disrupted into a random coil. Thus NMR spectra are sensitive to structure.

Looking more closely at the spectrum for the globular structure, we note that it has five resolved resonances although there are only four tryptophan residues in each enzyme molecule. Evidently a single tryptophan residue is responsible for the two adjacent peaks near 110 ppm. This splitting probably indicates that the enzyme takes on two dif-

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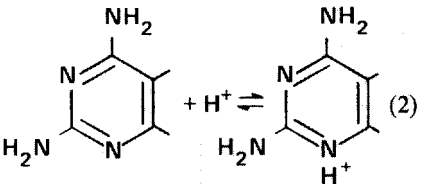
ferent configurations in the region of that particular residue.

The resonance at 106 ppm is also noteworthy because it is much broader than the others, a fact suggesting that the residue responsible for the resonance is located in a portion of the enzyme that undergoes confirmational changes with time. Spectra taken at different temperatures (Fig. 4) confirmed this suggestion. The resonance at 106 ppm (resonance 4)exhibits a strongly temperature-dependent linewidth believed to be associated with "breathing" of the enzyme. That is, the enzyme adopts an ensemble of molecular conformations leading to temperature-dependent dynamic behavior. Figure 4 also shows that this "breathing'" phenomenon disappears when the enzyme is complexed with the inhibitor 3',5'-dichloromethotrexate, (The structure of this inhibitor is like that of methotrexate in Fig. 1 except that chlorine replaces hydrogen at positions 3 and 5 on the benzene ring.) The resonance then becomes sharp, indicating that a part of the binding energy between enzyme and inhibitor stabilizes a particular subset of enzyme conformations. For DHFR derived from S. *faecium* we found that the substrate dihydrofolate and each of the inhibitors studied lead to sharpening of resonance 4. In contrast, NADPH, the enzyme cofactor in reaction 1, does not significantly sharpen the resonance. Presumably, the NADPH binds to a portion of the enzyme molecule more remote from the particular tryptophan residue responsible for resonance 4.

The addition of substrate or inhibitor molecules can also lead to changes in resonance position, or chemical shifts. For example. the spectra at 15 degrees Celsius in Fig. 4 show that dichloromethotrexate causes a slight shift of resonance 1 to the right. Such chemical shifts reflect interactions between the enzyme and the bound molecules. In general, our results demonstrate the sensitivity of NMR spectra to the precise folded enzyme structure. Interactions among specific pairs of residues are important in producing chemical shifts, and these shifts are altered when the enzyme binds other molecules. Dynamic behavior such as "breathing" is also observable, but whether this behavior observed in DHFR is significant for catalysis (for example, whether it helps the enzyme to "recognize" the substrate dihydrofolate) is still an open question.

A second set of studies with DHFR was designed to investigate the basis for the high affinity between the inhibitor methotrexate and DHFR. Rather than labeling the enzyme, we chose to label the inhibitor. The label was placed in that portion of methotrexate thought to interact strongly with DHFR (see Fig. I, where the position of the carbon- 13 label is marked by a gray circle). We found that the NMR spectrum of a solution containing labeled methotrexate and DHFR exhibits two carbon-13 resonances, one corresponding to inhibitor molecules that are free in solution and one corresponding to inhibitor molecules that are tightly associated with the enzyme. The fact that the single carbon- 13 label exhibits two resonances is a reflection of the very high affinity of methotrexate for the enzyme.

We then varied the pH of the solution containing the enzyme and the labeled inhibitor and found that the two resonances behave very differently (Fig. 5). The resonance ascribed to the uncompleted inhibitor undergoes a large shift in position at a pH of about 5.7, This so-called titration behavior indicates that the uncormplexed inhibitor accepts a proton (H+) in the following reversible reaction:



The pH at which the curve's large shift is centered (5.7) is called the pK of this proc-

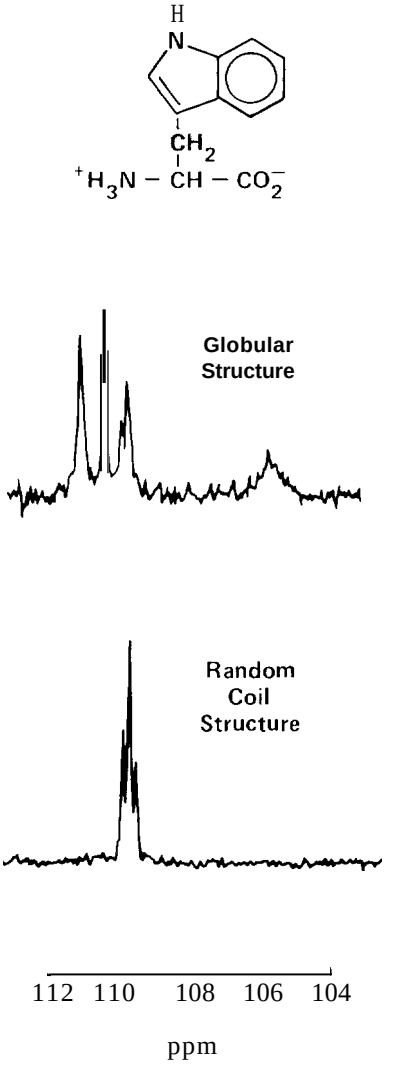
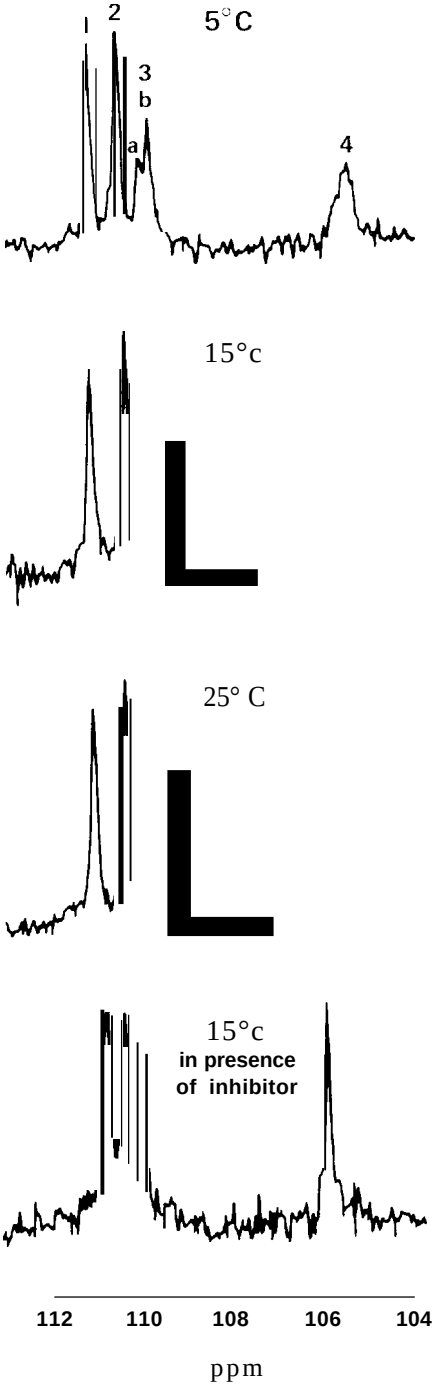


Fig. 3. Carbon-13 NMR spectra for [3-^{13}C]tryptophan-labeled DHFR in its active globular form and in its random coil form. The structure of tryptophan is also shown, with the position of the carbon-13 label indicated by a gray circle. Note that the globular structure produces many more distinct resonances than does the random coil structure.



Fig, 4. Carbon-13 resonances observed for [3-^{13}C]tryptophan-labeled DHFR at various temperatures and at 15 degrees Celsius in the presence of the inhibitor 3',5'-dichloromethotrexate. Resonance 4 exhibits a temperature-dependent linewidth that becomes a sharp resonance in the presence of the inhibitor. The tryptophan residue thought to be responsible for resonance 4 is labeled 6 in Fig. 2.

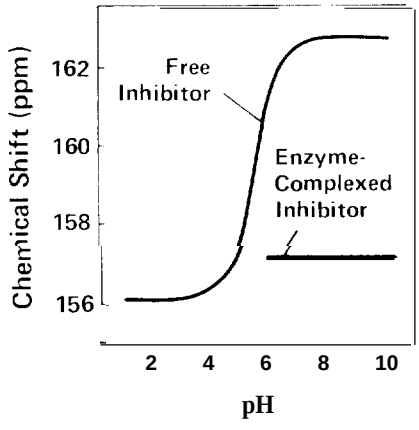


Fig. 5. Chemical shifts of the carbon-13 resonance for the inhibitor methotrexate (labeled as in Fig. 1) as a function of pH. When the enzyme DHFR is present (that is, at pH values above 5.5), two sets of resonances are observed, one corresponding to free methotrexate and one to the enzyme-complexed inhibitor. These results show that methotrexate in its protonated form has an extremely high affinity for the enzyme.

ess. It represents the pH value at which half of the molecules are protonated and half unprotonated. **Thus the one resonance ob**served for the uncompleted inhibitor is actually an average produced by its protonated and unprotonated forms. In contrast, the resonance ascribed to the enzyme-complexed methotrexate remains fixed near the resonance of the protonated form of the uncompleted inhibitor. These data indicate that the protonated form of methotrexate is the potent enzyme inhibitor: in other words, a strong interaction between protonated methotrexate and the enzyme must be the critical factor in making methotrexate an effective inhibitor. This conclusion is supported by the x-ray crystallographic data of Matthews and coworkers at the University of California, San Diego. They found that in the crystalline state the enzyme-complexed methotrexate is protonated and hydrogen-bonded to a negatively charged amino acid (aspartate-26) of the enzyme.

Figure 5 shows that the pK for protonation of the enzyme-complexed inhibitor is well above 10, the highest pH value used in the study. Such a large difference between the pK of the enzyme-complexed inhibitor and that of the free inhibitor is a measure of the binding energy between the inhibitor and the enzyme. The strength of binding was not accurately known prior to these studies and had in fact been incorrectly determined using conventional ultraviolet spectroscopic techniques. It is an impressive achievement of the isotopic labeling/NMR method that of all the interactions among amino acid residues and between amino acids and solvent molecules that stabilize the enzyme structure, we can probe a single one and quantify its strength. ■

Acknowledgment

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