Nuclear Magnetic Resonance Study of the State of Protonation of Inhibitors Bound to Mutant Dihydrofolate Reductase Lacking the Active-Site Carboxyl†

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ABSTRACT: ^{13}C nuclear magnetic resonance spectra have been obtained for complexes of [2-^{13}C]methotrexate and [2-^{13}C]trimethoprim with wild-type dihydrofolate reductase (DHFR) from *Escherichia coli* and with two mutant enzymes in which aspartic acid-27 is replaced by asparagine and by serine, respectively. In both the wild-type and mutated enzymes, exchange between the free inhibitor and the enzyme-complexed inhibitor is slow on the NMR time scale; hence, despite the considerably increased dissociation constants for binary complexes with the enzymes, the dissociation rate remains small relative to the frequency separation of the resonances. In all cases but one, the pK_a of an inhibitor that is complexed to enzyme differs greatly from that of the free inhibitor. However, while the pK_a of both inhibitors in complexes with the wild-type enzyme is elevated to above 10, the pK_a of the inhibitors complexed with the Asn-27 and Ser-27 enzymes is lowered to a value below 4. Exact determinations of bound pK_a values are limited by the solubility of the enzyme and the dissociation constants of the complexes. The single exception to these general conclusions is the ternary complex of the Ser-27 DHFR with trimethoprim and NADPH. In this complex, both free and enzyme-complexed trimethoprim exhibit similar pK_a values ($\approx$7.6). However, both the exchange between free and enzyme-complexed inhibitor and the protonation of the enzyme-complexed inhibitor are slow in the NMR time scale, so that the spectra reveal three resonances corresponding to free inhibitor, to protonated enzyme-complexed inhibitor, and to unprotonated enzyme-complexed inhibitor. Evidence for another slow conformational transition of the enzyme was also obtained in studies of the binary complex of Asn-27 DHFR with [2-^{13}C]methotrexate. These results are discussed in terms of crystallographic studies recently carried out on these mutated enzymes [Howell, E. E., Villafranca, J. E., Warren, M. S., Oatley, S. J., & Kraut, J. (1986) *Science (Washington, D.C.) 231*, 1123–1128].

There is strong evidence that when the inhibitor methotrexate (MTX)[1] is bound to wild-type dihydrofolate reductase (DHFR) it is protonated at N-1 of the heterocyclic ring. This protonation apparently results from ionic interaction between the protonated N-1 and the carboxylate of a buried acidic residue. The X-ray structures of two bacterial DHFR–MTX complexes have revealed that in crystals of the MTX–DHFR complex an aspartic acid side chain at the active site (Asp-27 in *Escherichia coli*) is close to the pteridine ring of bound MTX and forms a pair of hydrogen bonds with N-1 and the 2-amino group (Matthews et al., 1977, 1979; Bolin et al., 1982; Filman et al., 1982). An analogous interaction occurs between the carboxylate side chain of Glu-30 in chicken liver DHFR and the heterocyclic ring of MTX and other inhibitors (Volz et al., 1982; Matthews et al., 1985).

We demonstrated that the protonation state of N-1 of MTX can be determined very precisely by the introduction of ^{13}C at position 2 of the pteridine ring and the measurement of its ^{13}C nuclear magnetic resonance (NMR) chemical shift (Cocco et al., 1981a,b, 1983). This ^{13}C resonance shifts 6.2 ppm upfield when N-1 is protonated. It was further shown that MTX undergoes slow chemical exchange with the DHFR binding site, so that resonances of uncomplexed MTX and of MTX bound to DHFR can be observed simultaneously, and the protonation state of both species can be determined as a function of pH. Similar studies have been carried out with the diaminopyrimidine inhibitors trimethoprim (Cocco et al., 1983; Roberts et al., 1981) and pyrimethamine (Cocco et al., 1983). These studies indicated that the proton affinity of N-1 of each of the bound inhibitors is greatly enhanced by interaction with the enzyme. Thus, enzyme-bound inhibitor exhibits a pK_a of >10, compared with values for unbound inhibitors of 5.73 (MTX), 7.45 (trimethoprim), and 7.85 (pyrimethamine).

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[1] Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate, 4-amino-4-deoxy-10-methylpteroylglutamic acid; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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An opportunity to test the view that protonation of the bound inhibitors depends on interaction of N-1 with the active-site carboxylate was provided by the recent construction of two mutant forms of *E. coli* DHFR (Villafranca et al., 1983; Howell et al., 1986). in one of these Asp-27 is replaced by asparagine and in the other by serine. Both mutant enzymes were found to have low but readily measurable activity, and both were crystallized as their binary complexes with MTX. Determination of the X-ray structure at 1.9-Å resolution indicated that the substitutions do not change the conformation of the bound inhibitor or propagate distortions to other parts of the molecule, but that certain water molecules bound at the active site are moved slightly to accommodate the substitutions.

Preliminary evidence regarding the protonation state of MTX bound to the mutant enzymes was obtained by difference spectroscopy (Howell et al., 1986). The protonation difference spectrum of free MTX is similar to the difference spectrum observed upon binding of MTX to wild-type DHFR, but considerably different from that obtained when MTX binds to the Asn-27 enzyme. This suggests that MTX bound to mutant enzyme is unprotonated, but the method is complicated by other interactions of the enzyme and inhibitor as witnessed by the existence of a significant difference spectrum observed when MTX binds to Asn-27 DHFR. In the present report, we have used ^{13}C NMR to examine the protonated state of [2-^{13}C]MTX and [2-^{13}C]trimethoprim bound to Asn-27 DHFR and Ser-27 DHFR. the most important conclusion is that MTX bound to the mutant DHFRs is not only totally unprotonated at neutral pH, but its interaction with the enzyme causes a major decrease in the affinity of N-1 for protons. However, in the case of Ser-27 DHFR this decrease is apparently not quite as great as for Asn-27 DHFR.

Experimental Procedures

Enzyme. The generation of the Asn–27 DHFR and Ser–27 DHFR genes has been described (Villafranca et al., 1983; Howell et al., 1986). The purified mutant or wild–type enzyme was concentrated on an Amicon ultrafilter to a concentration of 0.8–1.0 mM and then dialyzed for at least 16 h against 0.1 M potassium phosphate buffer, pH 6.9, containing 0.1 mM EDTA, 2 mM dithiothreitol, 0.02% sodium azide, and 20% (v/v) D_2O. The dialyzed solution (750 μL) was mixed with 100 μL of approximately 20 mM [2-^{13}C]MTX or [2-^{13}C]-trimethoprim and (in some instances) 50 μL of 20 mM NADPH (both in 98% D_2O). After each spectrum had been obtained, the sample pH was adjusted by transferring the sample to a 5-mL beaker, stirring in an ice bath, and cautiously adding a predetermined volume of 1 M H_3PO_4 (or 1 N NaOH). After pH adjustment, the sample was transferred back to the NMR tube for further collection of spectral data.

In most of the studies, and particularly in the studies suggesting the possibility of conformational changes of the enzyme (Figures 2A and 5), pH values were not varied monotonically. This permitted us the ascertain whether the observed changes were reversible, and complete reversibility was obtained in all cases.

Labeled Materials. We have previously reported the synthesis of [2-^{13}C]MTX (Cocco et al., 1981b) and [2-^{13}C]trimethoprim (Cocco et al., 1983).

NMR Studies. ^{13}C NMR studies were carried out on either a Nicolet NT-360 NMR spectrometer using a 5-mm fixed frequency ^{13}C probe or on a Nicolet QE-300 NMR spectrometer using a 5-mm dual $^1H/^{13}C$ frequency probe. The small difference in frequency, 90.0 MHz in the first case and 75.48 MHz in the second, is not expected to have an effect on the type of measurements reported, and this was checked in one case. Samples contained 20% D_2O for the lock, as well as a capillary containing diiodomethane and cyclooctane which served as a chemical shift reference (cyclooctane = 26.3 ppm) and as a temperature monitor (Vidrine & Peterson, 1976). Sample temperature was maintained at 21 ± 1 °C in the NT-360, but samples were run at ambient temperature in the QE-300, which lacks a temperature controller. Experience with this instrument indicates that sample temperature is maintained close to 21 °C, and this was checked by using a temperature-dependent standard.

In the series of studies with [2-^{13}C]methotrexate, spectra were obtained without proton decoupling since the labeled carbon position is quaternary, and we have previously found that decoupling leads to temperature gradients across the sample which can effectively broaden resonances which have a temperature-dependent chemical shift (London, 1984). However, there is a relatively large three bond coupling interaction between C-2 and H-6 of trimethoprim, and most studies of the latter were therefore carried out with proton decoupling.

Spectral parameters typically used were 80° pulse width of 13 μs on the NT-360, 15 μs on the QE-300; 16K data points (NT-360) or 32K data points (QE-300); 30 kHz sweep width (chosen in order to include the upfield diiodomethane resonance); and a recycle time of 0.76 s. Samples were typically run overnight, with the number of scans ranging from 50 000 to 100 000. A 5-Hz line-broadening function was used prior to transformation.

Results

We have demonstrated previously in studies of the binding of [2-^{13}C]-labeled inhibitors to DHFR derived from various sources (Cocco et al., 1981a,b, 1983) that the rate of chemical exchange of inhibitor free in solution with that complexed to the enzyme is slow on the NMR time scale. Hence, unless an excess of DHFR is present, separate resonances corresponding to free and enzyme-complexed inhibitor are observed simultaneously. Additionally, from observations of the ^{13}C chemical shift it has been concluded that bound inhibitors have N-1 protonated at all accessible pH values.

The same type of behavior has now been demonstrated for [2-^{13}C]MTX in the presence of wild-type *E. coli* DHFR (Figure 1), in either the presence or absence of NADPH. Thus, as in previous studies, the resonance arising from the free species undergoes typical titration behavior with pK_a close to 5.7, while the resonance assigned to the enzyme-complexes species remains unshifted with pH change. The chemical shift in the binary complex was 157.30 ppm and in the ternary complex with NADPH, 157.15 ppm.

Asn-27 DHFR. Studies carried out on the Asn-27 DHFR in the presence of [2-^{13}C]MTX similarly yielded two resonances which are assigned to the free and enzyme-complexed species (Figure 2). Thus, the inhibitor is concluded to be in slow exchange with the binding site on the mutant enzyme despite decreased affinity (Howell et al., 1986). In contrast to the result for the wild-type DHFR–MTX binary and ternary complexes, the chemical shift of the resonance for MTX in the binary and ternary complexes with the Asn-27 enzyme has constant chemical shift values characteristic of unprotonated MTX (162.69 and 163.00 ppm, respectively). Thus, in both binary and ternary complexes MTX is unprotonated not only at pH above the pK_a of free MTX (pH 5.7) but also at pH well below this pK_a.

Another respect in which the ^{13}C NMR spectrum of the binary complex with Asn-27 DHFR contrasts with that of the wild-type enzyme is that in the former the resonance for the

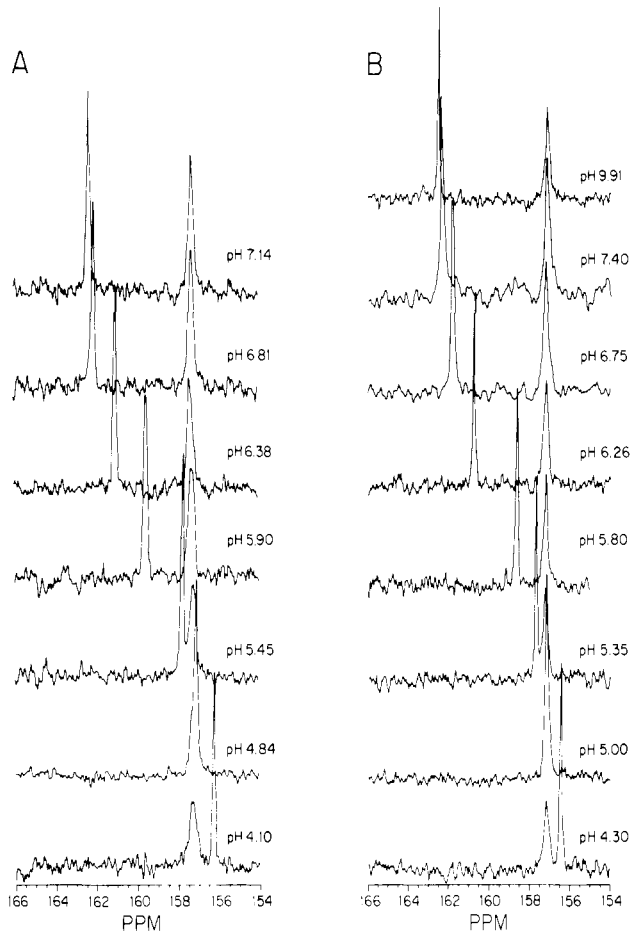

FIGURE 1: ^{13}C NMR spectra of [2-^{13}C]methotrexate in the presence of wild-type *E. coli* dihydrofolate reductase at various pH values. The resonance invariant with pH is due to bound MTX, that moving downfield with pH is due to free MTX: (A) binary complex; (B) ternary complex formed in presence of NADPH. For further details see Experimental Procedures.

bound inhibitor exhibits additional structure (Figure 2A). Thus, two component resonances are observed, at 162.79 and 162.55 ppm, with the fractional intensity of each varying as a function of pH. The resonances are of approximately equal intensity at pH 6.5, with the upfield component becoming more predominant at higher pH. This behavior suggests that a conformational equilibrium characterizes the enzyme–methotrexate complex, with an exchange rate that is slow compared to the chemical shift difference, i.e., $\tau \gg 1/\Delta\nu = 0.046$ s. Both conformational forms must contain unprotonated MTX. In the ternary complex no complex structure was visible in the resonance from MTX.

Ser-27 DHFR. Results of NMR observation of the MTX–NADPH–Ser-27 DHFR ternary complex were qualitatively similar to those obtained with the analogous complex of Asn-27 DHFR. Separate resonances are again observed for free and bound enzyme, despite still further decreased affinity of MTX for this mutant enzyme (Howell et al., 1986). However, the single resonance observed for enzyme-bound MTX shows a significant monotonic decrease with pH (Figure 3), which is not observed for resonances of MTX in binary or ternary complexes of Asn-27 DHFR or wild-type enzyme or for other complexed inhibitors previously studied. The significance of this is discussed later.

Studies with [2-^{13}C]Trimethoprim. As has previously been demonstrated by our group (Cocco et al., 1981a,b, 1983) and by Roberts et al. (1981), analogous studies can be carried out with [2-^{13}C]trimethoprim. Because of the lower association

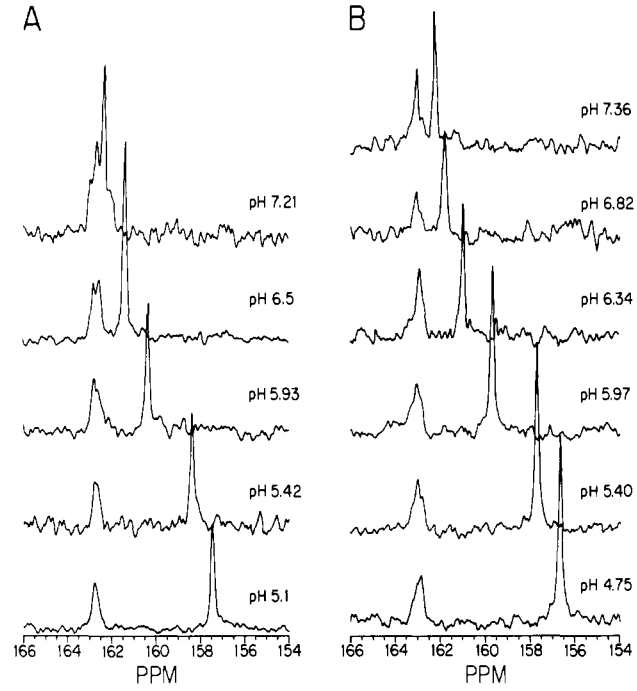

FIGURE 2: ^{13}C NMR spectra of [2-^{13}C]methotrexate in the presence of Asn-27 DHFR at various pH values: (A) binary complex; (B) ternary complex formed in presence of NADPH.

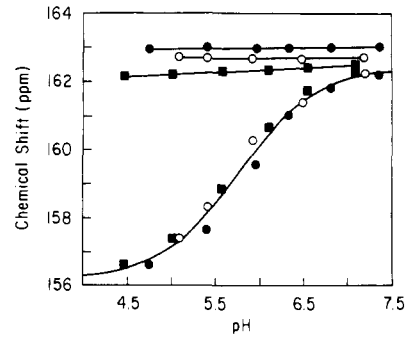

FIGURE 3: Effect of pH on the chemical shift of [2-^{13}C]methotrexate in the presence of Asn-27 DHFR and Ser-27 DHFR: (■) resonances of free and bound MTX in presence of Ser-27 DHFR and NADPH; (●) resonances in presence of Asn-27 DHFR and NADPH; (○) resonances in presence of Asn-27 DHFR (no NADPH). The sigmoid curve was obtained by computer fitting the chemical shifts for free MTX in all three experiments to the equation $pH = pK_a - \log[(\delta_B - \delta_{obsd})/(\delta_{obsd} - \delta_{HB})]$. This gave a pK_a of 5.77 ± 0.05.

constants of trimethoprim compared with methotrexate, we examined only the ternary complexes of trimethoprim (which have higher association constants than the binary complexes). Nevertheless, in NMR spectra for all three enzymes the free and enzyme-bound trimethoprim showed separate resonances, so that binding is sufficiently strong to give slow chemical exchange on the NMR time scale.

The ternary complex of the wild-type enzyme gave the anticipated NMR spectra under conditions of proton decoupling (Figure 4). One resonance exhibited titration behavior as the pH was adjusted, and a second resonance had a position invariant with pH and a chemical shift (155.5 ppm) indicating that the bound inhibitor was protonated. Spectra obtained with the ternary complex of Asn-27 DHFR showed that the bound inhibitor gives a resonance with a fixed chemical shift of 161.8 ppm (Figure 5). This is close to the chemical shift for free inhibitor at pH 9.23 where it is only 1.6% protonated. Thus, as in the case of bound MTX, trimethoprim bound to Asn-27 DHFR remains unprotonated even at pH values below

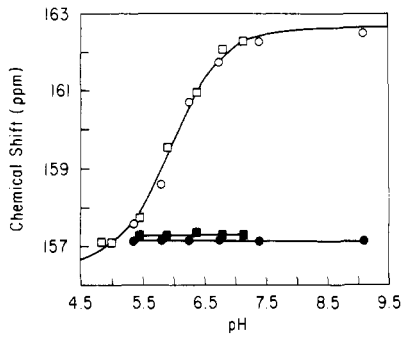

FIGURE 4: Effect of pH on the chemical shift of [2-^{13}C]trimethoprim in the presence of wild-type DHFR in the presence (circles) or absence (squares) of NADPH.

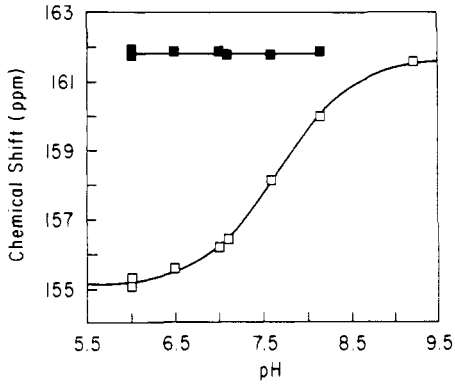

FIGURE 5: Effect of pH on the chemical shift of [2-^{13}C]trimethoprim in the presence of Asn-27 DHFR.

the pK_a for free inhibitor (7.69 from the data in Figure 4).

When NMR spectra were obtained from the ternary complex of NADPH and [2-^{13}C]trimethoprim with the Ser-27 enzyme, a result was obtained that is unparalleled in our previous investigations (Figure 6). At pH values between 7.3 and 8.0 the spectra exhibit *three* resonances rather than the usual two. A large resonance peak corresponding to free [2-^{13}C]trimethoprim is accompanied by two small peaks, both corresponding to bound drug, one at a chemical shift (153.98 ppm) close to that for protonated unbound trimethoprim and the other at a chemical shift (161.03 ppm) near the position for the unprotonated unbound drug. The shifts of the two resonances corresponding to bound drug are invariant with pH, but their relative intensity changes. As pH increases the resonance corresponding to protonated bound drug decreases in size, becoming undetectable at pH 9. Conversely, that corresponding to unprotonated bound drug increases as the pH is raised but is undetectable at pH 2. The pK corresponding to this pH dependence is similar to that for unbound trimethoprim. Since separate resonances are observed for bound protonated and unprotonated trimethoprim, the interconversion of these species must be surprisingly slow ($\ll$2110 s^{-1}). In these studies, pH was not varied monitonically, and the spectral variations with pH were found to be fully reversible.

Discussion

Rates of Chemical Exchange. A clear result from this study is that despite much higher dissociation constants for inhibitor complexes of the mutant enzymes the ligands were always in slow exchange between the bound and free states. The complexes of MTX with Asn-27 DHFR, Ser-27 DHFR, and wild-type enzyme and the complexes of trimethoprim with Asn-27 DHFR and wild-type enzyme gave ^{13}C NMR spectra

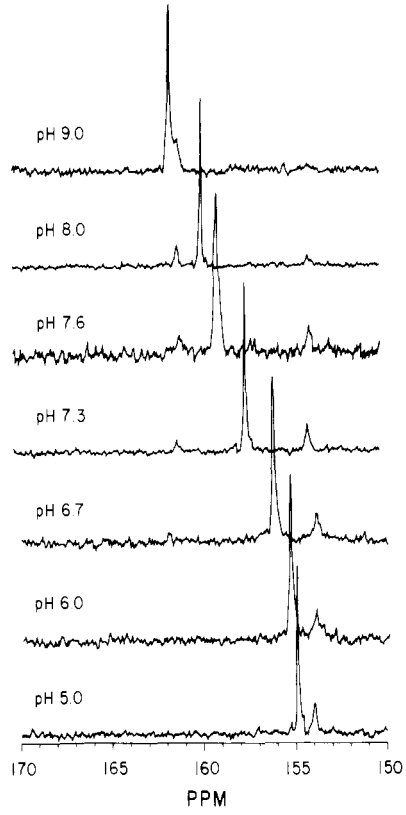

FIGURE 6: ^{13}C NMR spectra of [2-^{13}C]trimethoprim in the presence of NADPH and Ser-27 DHFR at various pH values. The resonances for bound trimethoprim are smaller because of the relatively high dissociation constant for this complex.

with separate resonances for free uncomplexed inhibitor and for enzyme-bound inhibitor. The rate of chemical exchange between the bound and free states was therefore slower than the frequency separation of the resonances, which was in the range 22–500 s^{-1}.

The dissociation constants at pH 7.0 and 4 °C for binary complexes of MTX to Asn-27 DHFR and Ser-27 DHFR are 27 times and 3000 times higher than for the wild-type complex, respectively (Howell et al., 1986). This probably reflects not a decreased rate of complex formation but an increased rate of dissociation since association rate constants (k_{on}) for a series of inhibitors forming ternary complexes with *Lactobacillus casei* DHFR were found to be remarkably constant (Dunn & King, 1980). Similarly, Baccanari et al. (1982) found that differences in dissociation constants (K_D) of ternary complexes of trimethoprim and its congeners with *E. coli* DHFR are due to differences in effective dissociation rate constants (k_{off}). It must be concluded that if the dissociation rates are greatly increased in complexes of the mutant enzymes, they are still low enough to limit the rate of chemical exchange to the slow class on the NMR time scale. This is consistent with the considerable amount of data indicating that the inhibitor–DHFR complexes initially formed undergo one or more isomerizations to conformers that cannot release inhibitor and that the rates of dissociation of the complexes are limited by the very slow reversal of these isomerizations (Williams et al., 1979, 1980; Stone et al., 1984; Blakley & Cocco, 1985b). Stone et al. (1984) give a rate constant of 0.026 min^{-1} for reversal of the slow isomerization of the ternary complex of MTX and NADPH with wild-type *E. coli* DHFR. Even if this rate is increased 3000-fold in the Ser-27 enzyme, the value (1.3 s^{-1}) would still limit chemical exchange enough to permit the observation of two peaks. The effective dissociation rate

constant given by Baccanari et al. (1982) for the ternary complex of trimethoprim, NADPH, and *E. coli* DHFR (isoenzyme 1 of RT500) is 0.009 s^{-1}, and the inhibition constant is 1.3 nM (Baccanari et al., 1981). Since the Asn-27 and Ser-27 enzymes have K_i values for trimethoprim at pH 7.0 of 0.56 and 1.2 μM, respectively (E. E. Howell and M. S. Warren, unpublished results), the dissociation rate constants for the ternary complexes of these enzymes may be of the order of 3.8 and 8.3 s^{-1}, respectively, which is also compatible with slow exchange on the NMR time scale.

Unprotonated State of MTX Bound to Mutant Enzyme. The chemical shift of MTX bound to Asn-27 DHFR and to Ser-27 enzyme clearly indicates that the bound ligand is in the unprotonated form. In fact, the chemical shift of the bound ligand resonance is slightly further downfield than that of the free unprotonated ligand (Figure 3). This result completely confirms the conclusion based on difference spectroscopy (Howell et al., 1986). What is also evident from our present results, however, is that the bound ligand remains unprotonated at pH values one unit or more lower than the pK_a for the free ligand. This decreased affinity of N-1 of the bound ligand for protons must result from the interactions between the bound unprotonated ligand and the mutant enzyme. The X-ray crystallographic results suggest that N-1 of bound MTX is hydrogen bonded to the side-chain amide of Asn-27 in the binary complex with Asn-27 DHFR. The side-chain amide is also hydrogen bonded to Thr-113, to two fixed water molecules (403 and 405), and to the 2-amino group of MTX (Howell et al., 1986). This network of hydrogen bonds is apparently very unfavorable for addition of a proton to N-1 of MTX.

It may be seen in Figure 3 that the pH-invariant chemical shift of [2-^{13}C]MTX bound to Asn-27 DHFR is further downfield than that computed for free unprotonated MTX. The differences in chemical shift are 0.55 and 0.24 ppm for the ternary and binary complexes, respectively. These presumably arise from local effects of ring currents and/or electric fields in the active site. When [2-^{13}C]MTX is bound to the wild-type enzyme (Figure 1), the chemical shift is again downfield of the computed shift for free protonated MTX by 0.68 and 0.82 ppm for the ternary and binary complexes, respectively. In this case, the difference in bound and free shifts could arise in two ways. Probably there are again local effects similar to those experienced in the cavity of the Asn-27 enzyme but supplemented by a significant field effect due to the carboxylate group of Asp-27. In addition, the interaction between the carboxylate of Asp-27 and MTX may diminish the charge on N-1 of MTX with lengthening of the bond between N-1 and the hydrogen.

The resonance from [2-^{13}C]MTX bound to Ser-27 DHFR shows a small but measurable change in chemical shift with pH (Figure 3). This slight pH dependence is significantly more than we have observed for [2-^{13}C]MTX bound to other DHFR species and suggests a slight degree of protonation of N-1 of the bound inhibitor at low pH. X-ray crystallographic data indicate that in the MTX binary complex with Ser-27 enzyme the Ser-27 side chain is not hydrogen bonded either to N-1 or to the 2-amino group of MTX (Howell et al., 1986). Instead, OG (the hydroxyl oxygen) of Ser-27 is hydrogen bonded to a new bound water molecule (Wat-885), which in turn is hydrogen bonded to N-1 of MTX. OG of Ser-27 is also strongly hydrogen bonded to the side-chain hydroxyl of Thr-113, to which the Asp-27 and Asn-27 side chains are also hydrogen bonded in their respective enzymes. It appears that the hydrogen bond network via Wat-885 in the Ser-27 enzyme is not quite so restrictive for proton donation to MTX as the different network in the Asn-27 enzyme, although the electrically neutral environment of the binding site still raises a considerable energy barrier.

In the case of wild-type enzymes where bound MTX is protonated, the association rate constant for unprotonated MTX is considerably lower than for protonated MTX, the factor ranging from 7-fold for *L. casei* DHFR to 1000-fold for *Streptococcus faecium* DHFR isoenzyme 2 (Blakley & Cocco, 1985a). The resistance to protonation of MTX bound to mutant DHFR may therefore imply very unfavorable kinetics and equilibrim for binding of protonated MTX to these enzymes. This is in contrast to the conclusions reached about the binding to these enzymes of protonated dihydrofolate (Howell et al., 1986). In study of the variation of k_{cat} with pH it was found that for the mutant enzymes k_{cat} rapidly increases with decreasing pH and becomes comparable with k_{cat} for wild-type enzyme at pH 5. This observation indicates that binding of protonated dihydrofolate at low pH is able to give almost as rapid an enzymic reaction as binding of unprotonated dihydrofolate to wild-type DHFR at neutral pH followed by protonation by the enzyme. This different ease of binding protonated MTX and protonated dihydrofolate can be understood in terms of the flipped-over orientation of the pteridine ring for bound dihydrofolate compared with MTX (Fontecilla-Camps et al., 1979; Charlton et al., 1979) and the fact that MTX is protonated at N-1, whereas dihydrofolate is protonated at N-5.

State of Trimethoprim Bound to Mutant Enzyme. The X-ray crystallographic structure of the trimethoprim complex with *E. coli* DHFR shows a similar positioning of the 2,4-diaminopyrimidine ring in the active site to that of the corresponding moiety of MTX (Matthews et al., 1985). Although the crystal structures of the complexes of the mutant enzymes with trimethoprim are not yet available, it seems reasonable to assume that in the Asn-27 DHFR complex trimethoprim is positioned in a very similar manner to MTX, so that a network of hydrogen bonds similar to that found in the MTX complex (Howell et al., 1986) would account for the similar low affinity of bound trimethoprim for protons.

The NMR data clearly indicate that the ternary trimethoprim complex of the Ser-27 enzyme differs significantly in some structural respects from those of trimethoprim with both the wild-type enzyme and the Asn-27 enzyme and also from the complex of MTX with Ser-27 DHFR. Whereas trimethoprim is bound to various wild-type bacterial enzymes in the protonated form and to the *E. coli* Asn-27 enzyme in the unprotonated form, Ser-27 DHFR forms a mixture of complexes of protonated and unprotonated trimethoprim. At each pH value the ratio of these two forms of the complex reflects the ratio of the protonated and unprotonated forms of the unbound trimethoprim with which the enzyme is in equilibrium. However, the complexes of protonated and unprotonated trimethoprim are in very slow equilibrium (exchange rate $\ll 210$ s^{-1}), since separate resonances are observed. This unexpected observation indicates that although either form of trimethoprim can be accommodated in the active site of the Ser-27 enzyme, proton uptake or release by bound trimethoprim is extremely difficult.

One explanation of this result is suggested by the fact that the mutant enzymes, in contrast to the wild-type enzyme, are unable to catalyze proton transfer to bound dihydrofolate (Howell et al., 1986). This suggests that they may also be unable to assist, or even permit, protonation or deprotonation of bound inhibitors. Inhibitor would then remain in the charge

state in which it binds to the mutant enzyme, unless it dissociates from the complex, a relatively slow process. This is consistent with the unaltered pK of the bound inhibitor. Such behavior would, however, be in contrast to the wild-type DHFR from *S faecium* and *L. casei*, where, although binding of unprotonated MTX is much slower than binding of the protonated inhibitor, the unprotonated inhibitor when once bound is immediately protonated (Blakley & Cocco, 1985a). However, these wild-type enzymes, like wild-type *E. coli* DHFR, have the active site aspartate to catalyze proton addition to bound ligand. To further explain the unique result with the trimethoprim complex of Ser-27 DHFR, it must also be assumed that protonated and unprotonated trimethoprim bind equally well to Ser-27 DHFR, whereas binding of protonated MTX to either of the mutant enzymes and binding of protonated trimethoprim to Asn-27 DHFR are insignificant. An observation difficult to reconcile with this view is the slight change with pH of the chemical shift of [2-^{13}C]MTX bound to Ser-27 DHFR.

Conformational Heterogeneity. The ^{13}C NMR resonance of [2-^{13}C]MTX bound to Asn-27 DHFR consists of two components with chemical shifts at 162.79 and 162.55 ppm. This strongly suggests that the binary complex is undergoing exchange between conformation states in which the local magnetic or electric fields at the C-2 of MTX are different. This observation of conformational heterogeneity for DHFR has numerous precedents.

We have previously found (Groff et al., 1984; London et al., 1982) that two of the tryptophan residues in *S. faecium* DHFR are involved in conformational equilibria. For one of these residues, tentatively identified as Trp-6, a broadened ^{13}C resonance is observed, indicative of intermediate–fast exchange between conformers, while in a second resonance, tentatively assigned to Trp-22, two components are seen, the relative intensity of which depends on the concentration of KCl in the buffer. Slowly exchanging conformers have also been observed by Birdsall et al. (1982) in studies of the ternary *L. casei* DHFR–[carboxamido-^{13}C]NADP$^+$–folate complex. Slowly interconverting conformers of the ternary complex between *L. casei* DHFR, NADP$^+$, and trimethoprim have been extensively studied by Roberts and his colleagues (Gronenborn et al., 1981; Birdsall et al., 1984). In the present case, the observed pK suggests the possible involvement of a histidine residue in the conformational process, although as is well-known, the pK values for titrable side chains in enzyme systems can be perturbed considerably from their values in the isolated amino acids, so that an identification on this basis alone is precarious. Regardless of the particular amino acid involved, the conformational process being reported by the C-2 of methotrexate is not a simple protonation, since such processes are typically rapid on the NMR time scale. Thus, a more likely interpretation would be a slow conformational equilibrium which is influenced by the titration of a nearby group with a pK of 6.5. This behavior was not observed for the ternary complex of Asn-27 DHFR with MTX and NADPH (or for the ternary complex of Ser-27 DHFR), so that in this respect it is different from the interconversion observed with the trimethoprim–*L. casei* DHFR–NADP$^+$ complex.

As an alternative to the hypothesis offered in the previous section, the slow interconversion between protonated and unprotonated trimethoprim in the ternary complex of Ser-27 DHFR (Figure 6) is also explicable by assuming that it is associated with a conformational transition. On this view, the conformation of the protonated trimethoprim complex must differ significantly from that of the other inhibitor complexes involving the Asn-27 and Ser-27 enzymes. In the case of the MTX complex with Ser-27 DHFR such a conformational transition must not be required for protonation of the inhibitor, so that protonation, although not thermodynamically favored, is a fast reversible process.

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Registry No. DHFR, 9002-03-3; MTX, 59-05-2; L-Asn, 70-47-3; L-Ser, 56-45-1; L-Asp, 56-84-8; trimethoprim, 738-70-5.

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Characterization of Elapidae Snake Venom Components Using Optimized Reverse-Phase High-Performance Liquid Chromatographic Conditions and Screening Assays for α-Neurotoxin and Phospholipase A_2 Activities†

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ABSTRACT: The vast majority of Elapidae snake venoms, genus *Naja*, includes three classes of toxic polypeptides: α-neurotoxins, phospholipases A_2, and cardiotoxins. A new experimental approach using reverse-phase high-performance liquid chromatography in particular has been developed, allowing their respective resolution, identification, and quantitation from milligram quantities of venom. First, definition of optimal chromatographic conditions for *Naja mossambica mossambica* toxins has been ascertained. Different column packing and solvent systems were compared for their efficiency, with particular attention to the ionic strength of the aqueous solvent. A medium-chain alkyl support (octyl) in conjunction with a volatile ammonium formate (0.15 M, pH 2.70)/acetonitrile solvent system was found to be particularly effective. All the components known until now from this venom could be resolved in a single step, and the elution order was α-neurotoxins, phospholipases A_2, and cardiotoxins with a total recovery of absorbance and toxicity. Then, with these suitable conditions, we describe a new major cardiotoxin molecule in this venom by hydrophobic and not ionic-charge discrimination. Second, specific assays were designed to detect α-neurotoxin and phospholipase A_2 activities in chromatographic fractions: α-neurotoxin activity was determined by competition for the binding of a radiolabeled α-neurotoxin to the acetylcholine receptor of the ray electric organ, and phospholipase A_2 activity was defined by the enzymatic activity of these toxins with a fluorescent phospholipid as substrate. Finally, the applicability of these new methods to study other *Naja* snake venoms was demonstrated.

Considerable progress has been made concerning the molecular mechanisms of the pharmacology of some animal venoms and their toxins [reviewed in Bettini (1978) and Lee (1979)] as well as, more recently, the antigenic structures implicated in their immunological neutralization for an appropriate serotherapy (Boulain et al., 1982; El Ayeb et al., 1983), thereby requiring the purification and identification of a number of analogous toxins. The isolation of such polypeptidic toxins is consistently a thorny problem in biochemistry, and our laboratory has been actively engaged in this work since the 1960s (Miranda & Lissitzky, 1961; Miranda et al., 1970).

Elapidae snake venoms, genus *Naja*, can be separated into several active components. Among them α-neurotoxins, phospholipases A_2, and cardiotoxins are recognized as the main factors involved in the pharmacology and the lethality of these venoms. They are low molecular weight polypeptides, chemically very stable. Only the mode of action of α-neurotoxins is currently well-known at the molecular level. They bind to the postsynaptic AcChR[1] with a very high affinity (K_D about 0.01–1 nM). Through a competitive inhibition of the response to the acetylcholine of the neuromuscular junction, they cause a flaccid paralysis (Lee & Chang, 1966; Changeux et al., 1984). The toxicity of phospholipases A_2 is generally related to their basicity (pH_i) and to their esterase-type activity. Their mechanism of action nevertheless remains totally unknown (Karlsson et al., 1979). Cardiotoxins have been defined as such because of their pharmacological action on heart muscle, causing considerable cardiovascular depression (Sarkar, 1947).

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[1] Abbreviations: RP-HPLC, reverse-phase high-performance liquid chromatography; AcChR, acetylcholine receptor; NTX, neurotoxin; PH, phospholipase A_2; CTX, cardiotoxin; PMSF, phenylmethanesulfonyl fluoride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetate; TFA, trifluoroacetic acid; R_t, retention time.

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