Journal of Biomolecular NMR, 10 (1997) 293–299
KLUWER/ESCOM

J-Bio NMR 487

Tritium NMR studies of the human carbonic anhydrase I–benzenesulfonamide complex

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Received 28 April 1997
Accepted 11 August 1997

Keywords: Tritium; Enzyme; Carbonic anhydrase; Enzyme inhibitor; Aromatic ring rotation

Summary

Tritium NMR spectroscopy has been used to examine the complex formed by [4-^{3}H]benzenesulfon-amide and human carbonic anhydrase I. The results show that in solution the inhibitor forms a 1:1 complex with the enzyme. A 100-spin computational model of the system, constructed with reference to crystallographic results, was used to interpret tritium relaxation behavior and $^3H\{^1H\}$ NOEs. The analysis shows that the rate of dissociation of the enzyme–sulfonamide complex is 0.35 s^{-1} and that the aromatic ring of the inhibitor undergoes rapid rotation while complexed.

The zinc-containing enzyme carbonic anhydrase (CA) has been found in virtually all plant and animal tissues and has been aggressively investigated since its discovery more than 60 years ago. Sly and Hu (1995) have recently reviewed current understanding of the seven known human forms of CA. Carbonic anhydrase activity in the human erythrocyte arises from CA I and the more potent CA II; CA II is widely distributed in human tissues, being found in kidney, brain, pancreas, gastric mucosa, skeletal muscle, retina and the lens (Dodgson et al., 1991).

Aromatic and heterocyclic sulfonamides are excellent competitive inhibitors of both the hydration of CO_2 by CA and the opposing reaction, the dehydration of carbonic acid (Lindskog et al., 1971; Maren and Sanyal, 1983). Systemically administered CA inhibitors are widely used to control intraocular pressure in the treatment of glaucoma, as well as other disorders (Feitl and Krupin, 1991). There have been continuing efforts to develop improved inhibitors (Boriack et al., 1995; Supuran et al., 1996), particularly topical inhibitors that may be delivered directly to ocular tissues, thereby minimizing systemic side effects (Hunt et al., 1994). A wide variety of structures have been examined for inhibitory activity and quantitative structure–activity relationships (QSARs) have been developed, these efforts being aided by interactive computer graphics (Vedani and Meyer, 1984; Hansch et al., 1985; Hansch and Klein, 1986) and molecular mechanics calculations (Menziani et al., 1989; Vedani et al., 1989). Maren (1987) has discussed CA inhibition with sulfonamides from the perspective of pharmacological treatment of glaucoma.

Crystallographic work has provided a trove of structural information for human CA I, II, and bovine CA as well as complexes of these enzymes with various ligands. Recent studies of human CA I (HCA I) have included a 0.16 nm structure of the product (bicarbonate)–enzyme complex (Kumar and Kannan, 1994) and structures of several inhibitor–protein complexes (Chakravarty and Kannan, 1994). Liljas et al. (1994) have summarized the crystallographic evidence regarding CA–inhibitor complexes. They note that tetrahedral or near-tetrahedral coordination to the metal is the expected structure for sulfonamide–enzyme systems, with three histidine residues of the protein and the anion of the sulfonamide being the metal ligands. However, there are examples of pentacoordination to the metal atom of a CA in the presence of small ligands such as formate or acetate.

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Abbreviations: HCA I, human carbonic anhydrase isoenzyme I; NOE, nuclear Overhauser effect; T_1, spin-lattice relaxation time; LSC, liquid scintillation counting.

Although there have not been a large number of studies of the stoichiometry of the interaction between sulfonamide inhibitors and these enzymes in solution, the crystallographic work indicates that a single molecule of sulfanilamide, acetazolamide, and other structurally related, good competitive inhibitors of the enzyme are coordinated to the Zn atom at the active site of the enzyme. A 1:1 stoichiometry of binding was found for acetazolamide in solution, consistent with crystallographic studies (Maren, 1967).

NMR observations made in our laboratory have indicated that some substituted benzenesulfonamide inhibitors of CA I and CA II form complexes in which the stoichiometry of the complexes is 2 moles of inhibitor bound per mole of enzyme (Dugad and Gerig, 1988; Dugad et al., 1989). Moreover, NMR studies show that the rate of rotation of the aromatic ring of the inhibitor in some of these cases is slow (Gerig and Moses, 1987; A. Chang, preliminary results). The present work was undertaken with the goal of defining the stoichiometry of complex formation for the parent inhibitor (I) and to provide information about the rate of aromatic ring rotation in such complexes. Advantage was taken in these studies of the abilities of tritium (^{3}H) NMR experiments with specifically enriched molecules to provide details of structure and dynamics in biological systems (Newmark et al., 1990; Gehring et al., 1991; Highsmith et al., 1993; O'Connell et al., 1993; Kubinec et al., 1996).

Tritium gas was purchased from E G & G Mound Laboratories and contained approximately 97% T_2 with the major contaminant being DT. Absolute ethanol came from Quantum Chemical Corp. and human carbonic anhydrase I was purchased from Sigma. Deuterium oxide (99.96 atom %D) and all other chemicals were purchased from Aldrich Chemical Co.

Tritium-labeled benzenesulfonamide was prepared by a catalytic tritiation of 4-bromobenzene sulfonamide. 4-Bromobenzenesulfonamide was synthesized by the procedure of Shupe (1942). Catalytic tritiation was carried out inside a glovebox using a custom-built microhydrogenation apparatus. The 5 ml reaction vessel was charged with 10.6 mg of 4-bromobenzenesulfonamide, 17.7 µl of anhydrous triethylamine, and 1 ml of dehydrated ethanol. The catalyst spoon was loaded with 10.3 mg of 10% palladium on carbon and the entire apparatus was degassed by three freeze-pump-thaw cycles. Tritium gas was admitted to the reaction vessel at 1 atm pressure. After thawing, the catalyst was added to the solution and the mixture was stirred at room temperature for 93 min. The reaction mixture was then frozen and, after evacuating to remove the remaining tritium gas, the system was flushed with nitrogen gas. The reaction mixture was lyophilized, 1 ml of methanol was added, and the mixture was again lyophilized. The product was purified by flash chromatography on a 0.7×10 cm column of silica gel (230–400 mesh, 60 µm) using 1:1 ethyl acetate–hexane as the eluant. The product mixture was applied to the column as a 0.5 ml suspension in the elution solvent. Eight milliliter of eluate was collected and evaporated by a stream of nitrogen gas to give the product as a white solid. [4-^{3}H]Benzenesulfonamide was dissolved in 1 ml of methanol-d_4 and a 150 µl aliquot was taken for analysis by ^{1}H and ^{3}H NMR. Liquid scintillation counting (LSC) showed that the product contained 0.995 Ci. The remaining methanol-d_4 solution was lyophilized and then dissolved in 1.37 ml of deuterium oxide containing 49.7 mM sodium sulfate. The concentration (16 mM) and specific radioactivity (26.2 Ci/mmol) of the [4-^{3}H]benzenesulfon-amide were determined by a combination of UV spectroscopy (Doub and Vandenbelt, 1947) and LSC. The extent of tritium incorporation was 94% T, calculated from the UV spectroscopic and LSC results. Analysis of [4-^{3}H]benzenesulfonamide by ^{1}H NMR using relaxation delays of 10 times the longest T_1 did not reveal any detectable protium at the 4-position.

Thin-layer chromatograms were developed on silica gel plates with an elution solvent system of 7:3 ethyl acetate–hexane. Sample pH was measured using a Markson digital pH meter with a 4.5 mm diameter electrode. UV spectra were acquired using a Hewlett-Packard 8452A diode array spectrometer. Liquid scintillation counting was done with a Packard 1500 Tri-Carb analyzer using Packard Optifluor scintillant. All NMR spectra were recorded on an IBM/Bruker AF-300 spectrometer equipped with 5 mm or 10 mm ^{3}H/^{1}H dual probes.

Enzyme solutions for NMR experiments were prepared by dissolving 75 mg of HCA I in 1.8 ml deuterium oxide containing 49.7 mM sodium sulfate. Sample pH was adjusted to 6.6–7.0 with 0.1 M NaOH or HCl solutions; the concentration of the enzyme in solution was determined by UV spectroscopy using $A^{1\%} = 16.3$ cm^{-1} at 280 nm (Lindskog et al., 1970). The enzyme sample was contained within a Teflon liner for a 10 mm NMR tube (Wilmad). The sample was sonicated in an ultrasound bath for 2 min to displace dissolved gases. The Teflon plug was removed and the tube headspace was flushed with dry nitrogen gas. The sonication/degassing procedure was repeated an additional 2 times. The Teflon liner was then placed in a 10 mm NMR tube for all experiments. For binding stoichiometry studies, [4-^{3}H]benzenesulfonamide was added to the enzyme solution using a 50 µl syringe, with the sonication/nitrogen gas flushing procedure repeated after each addition.

All tritium NMR spectra were obtained with sample spinning and ^{1}H decoupling gated on during data acquisition. The sample temperature was regulated at 298 K by the instrument controller unless otherwise stated; temperatures were calibrated by means of a standard methanol sample. Data from the NMR spectrometer in Berkeley were transferred to a SUN workstation in Santa

Barbara via the Internet for analysis using FELIX 2.3 (BioSym Technologies, San Diego, CA, U.S.A.). The two-dimensional (2D) nuclear Overhauser effect (NOE) spectra were analyzed using the TRIAD section of SYBYL 6.1a (Tripos Inc., St. Louis, MO, U.S.A.). The Bruker spectrometer data files were converted to FELIX-compatible files using the *ASNET* program supplied with FELIX 2.3 whereas the conversion to files compatible with SYBYL used *bruker2nmri* supplied by Tripos.

The procedures of O'Connell et al. (1993) were used for the determination of tritium spin-lattice relaxation times, equilibrium (steady-state) $^{3}H\{^{1}H\}$ NOEs, NOE build-up, and transient $^{3}H\{^{1}H\}$ NOEs A recycle delay of at least 11 times the 'bound' tritium signal T_1 was used between each accumulation. Signal line widths reported were corrected for magnetic field inhomogeneity by subtracting the observed line width of the HOD signal in the proton spectrum after apodization from observed line widths. Integrations were done by fitting a collection of Gaussian lines to the observed spectra using the FELIX 2.3 software. Two-dimensional $^{3}H\{^{1}H\}$ heteronuclear NOE spectra were obtained using the TPPI method to provide quadrature detection in the t_1 dimension. Typically, 300–350 transients were collected for each of 64 t_1 values. The 2D data sets spanned 12–13 ppm in the ^{1}H dimension, represented by 64 points. During data processing, the ^{1}H dimension was zero-filled to 512 or 1024 points. The ^{3}H dimension in the NOESY spectra was referenced to the chemical shift of water (4.8 ppm) in the corresponding ^{1}H NMR spectrum.

The tritium NMR spectrum of [4-^{3}H]benzenesulfonamide shows a single resonance at 7.75 ppm, a position consistent with the shift for the para hydrogen of the inhibitor found in the proton NMR spectrum of the normal (protio) compound. When the inhibitor is in concentration-excess over the enzyme, a signal is observed at the chemical shift of the free inhibitor as well as a broader signal 0.18 ppm upfield of this position that is assigned to the tritiated enzyme–inhibitor complex. The tritium NMR signal for the protein-bound inhibitor is about 5 Hz broader than the signal for the free inhibitor. The system is thus in the slow exchange regime, at least as regards chemical shifts, a condition not unexpected given the small dissociation constant ($K_D = 2 \times 10^{-7}$ M) for this system (Kanamori and Roberts, 1983). Tritium NMR titration of CA I with the labeled inhibitor shows that the enzyme becomes saturated with inhibitor at a concentration ratio of 1:1 (Fig. 1).

The temperature dependence of the spectra was examined over the range 5–35 °C. The chemical shift difference between the free and bound signals increased slightly with increasing temperature, changing from 0.16 ppm to 0.19 ppm over this range. Both lines in the spectrum sharpened somewhat with increasing temperature. Given the observed chemical shift difference (~58 Hz), the rate of exchange of ligand between free and bound states must be less than ~80 s^{-1}. The analysis given below shows that the exchange is much slower than this. The contribution of exchange broadening to the observed line widths of the free and bound inhibitor signals is negligible at these rates and the changes in line width observed probably reflect an increase in rotational correlation time and reduction in sample viscosity with increasing temperature.

The tritium spin-lattice relaxation time (T_1) for free [4-^{3}H]benzenesulfonamide is 12 s. For samples in which the inhibitor was present with the enzyme at greater than the stoichiometric concentration, so that a separate signal is observed for free and bound inhibitor molecules, the apparent T_1 relaxation times for the two signals were approximately 2 and 0.5 s, respectively. Thus, the rate of exchange between free and bound states for the inhibitor is rapid enough to be nearing the fast exchange limit as regards spin-lattice relaxation. On this basis, the rate of dissociation of the CA–benzenesulfonamide complex must be greater than 0.08 s^{-1} (~$1/T_1$).

Various 1D and 2D $^{3}H\{^{1}H\}$ nuclear Overhauser experiments were done with this system. Since these effects are

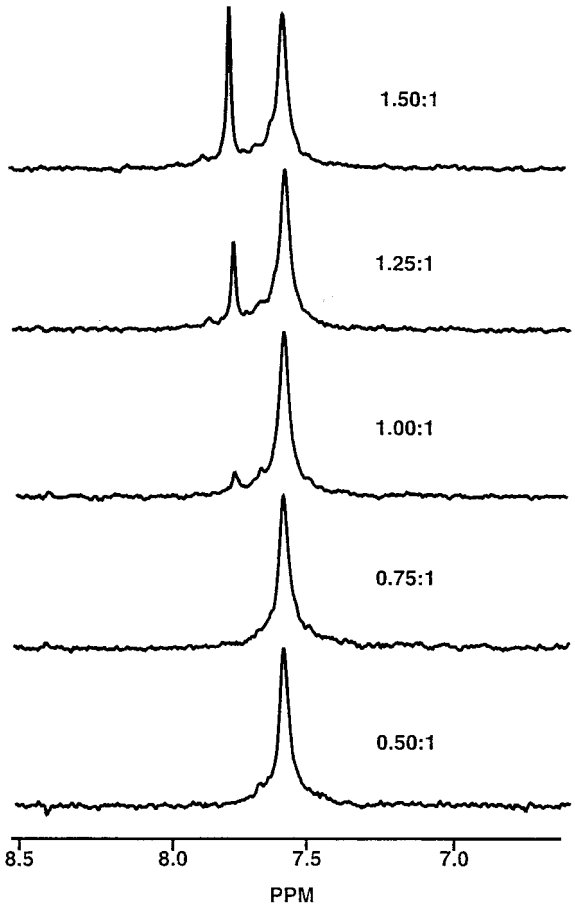

Fig. 1. Tritium NMR titration at 320 MHz of HCA I. The enzyme concentration was 1.06 mM and the samples contained 50 mM Na_2SO_4 in D_2O at pH 7 and 25 °C. The ratio of inhibitor to enzyme concentrations is indicated at each spectrum.

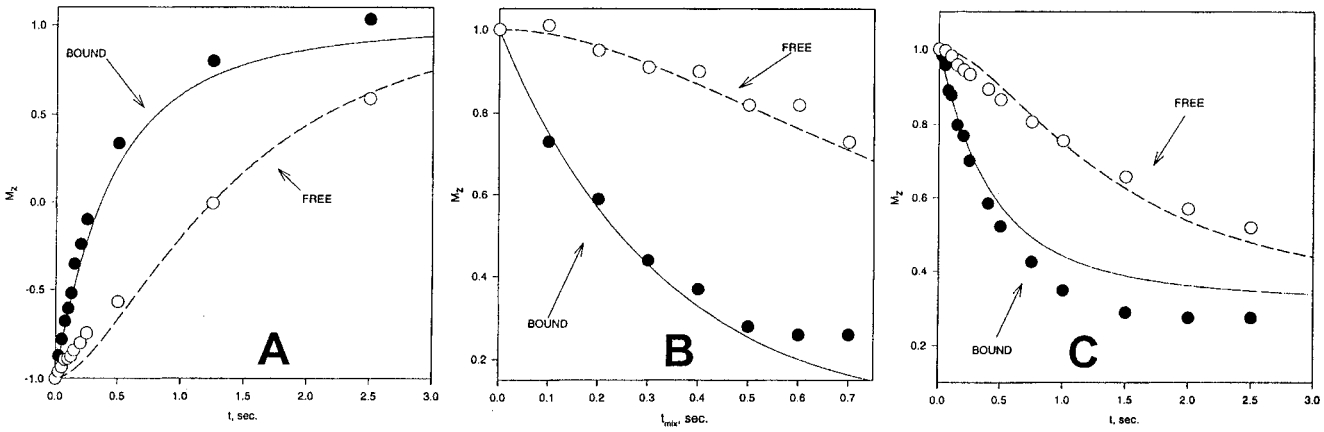

Fig. 2. Comparisons of observed and calculated tritium T_1 relaxation behavior in an inversion-recovery experiment (A), the transient $^3H\{^1H\}$ NOE as a function of mixing time (B), and time development of the $^3H\{^1H\}$ NOE (C) in a sample in which HCA I concentration was 1.06 mM and the ratio of [4-^{3}H]benzenesulfonamide to HCA I was 1.35:1. The sample conditions were the same as described in the caption for Fig. 1. The points represent experimental observations while the lines are calculated using the model and parameters described in the text.

basically spin-lattice relaxation phenomena, any efforts to interpret them must take into consideration the rate of exchange of the tritium spin between its free and protein-bound states. For analysis of the relaxation and NOE data collected, we developed a computational model of the interaction of tritiated benzenesulfonamide with HCA I. The approach was the same as used in previous studies from our lab (O'Connell et al., 1993; Sylvia and Gerig, 1995). It was assumed that this interaction can be described in terms of the equilibrium

$$EI = E + I \qquad (1)$$

where EI represents the enzyme–inhibitor complex, E is the free enzyme, and I is the free inhibitor. The observed tritium spin attached to I is thus assumed to either reside in the environment offered by EI or to be free in solution. The model assumes that there is only a single binding locus for I on the protein that is significantly populated and that there is only a single 3D arrangement of the atoms of EI. The free and bound forms of I are assumed to be in equilibrium, with the rate constant for dissociation of EI given by k_{diss}. It is also assumed that dipole–dipole interactions provide the only significant relaxation mechanism for any of the hydrogen spins in the system.

The available X-ray structure of HCA I complexed with 4-aminobenzenesulfonamide (Kannan et al., 1977) was used to develop a model for the benzenesulfonamide binding site. The 4-aminobenzenesulfonamide inhibitor in that structure was modified by the removal of the amino group and addition of hydrogens to all heavy atoms. The energy of the resulting structure minimized using the facilities of SYBYL (Tripos Inc., St. Louis, MO, U.S.A.). The 99 hydrogens closest to the tritium nucleus of the inhibitor ring in this structure were selected for inclusion in the model of EI. It was assumed that this collection of spins tumbles isotropically with a correlation time $\tau_{c,EI}$. However, the aromatic ring of the inhibitor was assumed to undergo two-site jump-rotation within the binding site at a rate characterized by the residence time $\tau_{i,EI}$.

A six-spin model was developed for the aromatic ring of the free inhibitor I. These spins included the tritium nucleus, the four protons on the aromatic ring, and a fifth proton located at a distance r_H away from the tritium along the C_1–C_4 axis of rotation. Motion of free I was characterized by an overall correlation time ($\tau_{c,I}$) and a correlation time ($\tau_{i,I}$) for rotation about the C_1–C_4 axis. The same 99 hydrogens in the model for EI were present in the model for E, where the relaxation behavior was assumed to be controlled by the same correlation time present in EI ($\tau_{c,E} = \tau_{c,EI}$).

Relaxation behavior of the collection of species present in the equilibrium given by Eq. 1 was described in terms of the Solomon equations (Neuhaus and Williamson, 1989). Spectral densities for spin pairs undergoing isotropic rotation were computed using standard equations (Neuhaus and Williamson, 1989); spectral densities for dipolar interactions between the nuclei of the inhibitor ring and nuclei of the enzyme where jump-rotation modulates internuclear distances were calculated using equations based on the work of Tropp (1980). Spectral densities for interactions between the five spins on the aromatic ring of the inhibitor (jump-rotation but no change in internuclear distances) were computed using the equations given by London (1980). Methyl groups of both EI and E were assumed to rotate freely, with rotation characterized by an internal correlation time of 20 ps. Spectral densities for dipolar interactions between spins of the methyl groups and other spins of the model were computed by procedures of Olejniczak (1989) as described previously (O'Connell et al., 1993). Since all experiments reported were done with D_2O solutions, all interactions of

solvent-exchangeable hydrogens with other hydrogens of the EI and E models were calculated using the gyromagnetic ratio of deuterium.

The parameters $\tau_{c,I}$, $\tau_{i,I}$, and r_H for the free inhibitor were adjusted until tritium T_1 and steady-state $^3H\{^1H\}$ NOEs computed from the model were in agreement with experimental results. A good fit was obtained with the values 0.395 ns, 5 ps, and 0.3375 nm, respectively. Moving to the complete system (free plus enzyme-bound inhibitor, Eq. 1), the values for $\tau_{c,I}$, $\tau_{i,I}$, and r_H found for the free inhibitor were fixed and the values for the parameters $\tau_{c,EI}$, $\tau_{i,EI}$, and k_{diss} were iteratively adjusted until agreement between observed and calculated tritium T_1, $^3H\{^1H\}$ NOE growth, steady-state $^3H\{^1H\}$ NOE, transient $^3H\{^1H\}$ NOE, and tritium line width data was obtained. The nature of the agreement between calculated and experimental data obtained when $\tau_{c,EI}$, $\tau_{i,EI}$, and k_{diss} are, respectively, 15 ns, 0.9 ns, and 0.35 s^{-1} is illustrated in Fig. 2. These parameters gave computed steady-state $^3H\{^1H\}$ NOEs for the tritium signals assigned to the free and bound inhibitor of –0.64 and –0.68, respectively, well within the errors for the experimental values, –0.62 and –0.65. The computed tritium line width in the EI complex was 4.6 Hz, in good agreement with the experimental value, ~5 Hz. Calculated steady-state and time-dependent NOEs were highly sensitive to the values for $\tau_{i,EI}$ and k_{diss} and it is believed that the values given for these parameters are reliable to within ±15%.

Heteronuclear 2D NOE experiments were done at several mixing times. Transections of the resulting 2D maps at the tritium chemical shift of the bound species are presented in Fig. 3. No cross peaks at the shift of the free signal were detected. These experiments show that at short mixing times the relaxation of the tritium spin in the enzyme–inhibitor complex is dominated by dipolar interactions with protons that have a chemical shift of 7.7 ppm, with somewhat weaker interactions (or spin diffusion effects) with protons with shifts of 7.9 ppm. At longer mixing times NOE cross peaks to protons with shifts in the aliphatic range become apparent; the modeling done suggests that these are likely the result of spin diffusion. The strongest $^3H\{^1H\}$ NOEs most likely arise from interactions of the tritium with the protons on the aromatic ring of the inhibitor and the structure of the enzyme–inhibitor complex must be such that distances from the tritium to protons of amino acid side chains of the protein are significantly greater than 0.25 nm, the H_3–T_4 distance in the inhibitor.

Some calculations of 2D $^3H\{^1H\}$ NOEs were done using the model described above for analysis of the relaxation data. The chemical shifts for the protons of the enzyme that correspond to the spins in the model are

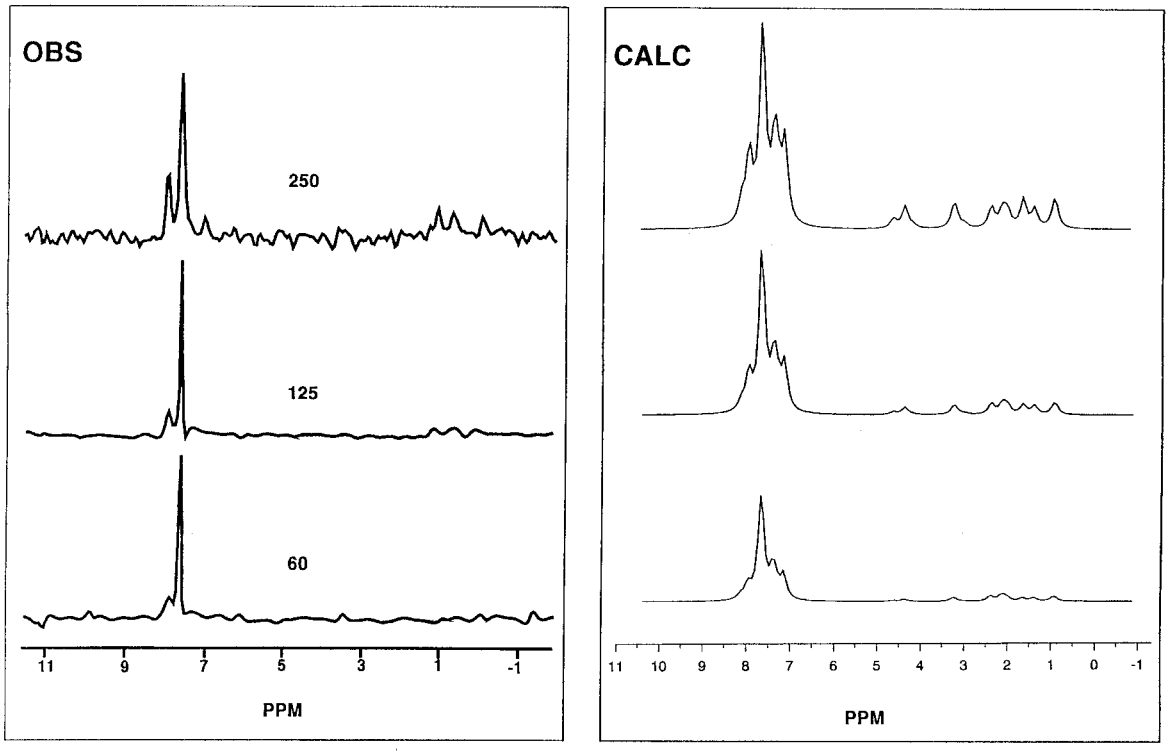

Fig. 3. Observed and calculated transections at the bound inhibitor chemical shift of maps obtained in 2D heteronuclear $^3H\{^1H\}$ NOE experiments. In the sample the ratio of [4-^{3}H]benzenesulfonamide to HCA I was 1.5:1 and sample conditions were the same as described in the caption for Fig. 1. Mixing times (ms) for the experimental spectra are indicate by each trace. The calculated curves at the same mixing times were obtained using the 100-spin model of the environment around the tritium spin used in the analysis of relaxation and NOE data and the correlation times and rate of exchange developed in that analysis.

unknown and the random coil shifts were used. The results of these simulations are shown in Fig. 3. The computed features in the 7–8 ppm range arise from the protons on the inhibitor as well as side-chain protons of His^{67}, Phe^{91}, and His^{94}. If the chemical shifts of these side-chain protons were closer to 7.7 ppm (rather than the random coil values), the relative intensities of the low-field part of these calculated transections to the intensity of the spin diffusion peaks in the higher field parts would be in reasonably good agreement with experiment. These calculations support the model used and parameters derived from it in the analysis of the relaxation data, confirming the conclusion that tritium relaxation is dominated by interactions between spins of the inhibitor. The rapid rate of spin diffusion suggested by the calculations is consistent with the size of the protein.

The tritium NMR titration shows cleanly that in solution the complex formed between benzenesulfonamide and HCA I has the stoichiometry of 1 inhibitor molecule per molecule of enzyme. Previous NMR experiments have shown that this inhibitor binds as an anion (Kanamori and Roberts, 1983) and that the anion very likely interacts with the Zn^{2+} ion at the active site (Blackburn et al., 1985). Although the benzenesulfonamide–enzyme complex has not been studied by crystallographic techniques, these conclusions are in agreement with studies of inhibitors of similar structure. The apparent formation of 2:1 inhibitor–enzyme complexes when fluorine substituents are present must be the result of additional interactions between the fluorine and the protein that remain to be elucidated.

Our analysis of tritium relaxation data apparently provides the first direct measurement of the rate of dissociation of the benzenesulfonamide–HCA I complex. The rate constant found ($k_{diss} = 0.35\ s^{-1}$) is similar to values for the dissociation of the benzenesulfonamide–HCA II complex (0.16–0.18 s^{-1}; King and Burgen, 1976; Maren, 1992). Dye displacement studies of aromatic sulfonamide binding to HCA II have suggested that the binding to that isoform is more complex than the two-site situation indicated by Eq. 1 in that several forms of bound inhibitor may be present (King and Burgen, 1976):

$$\cdots = EI_2 = EI_1 = E + I \qquad (2)$$

The tritium NMR observations of the HCA I complex are consistent either with the presence of only a single dominant enzyme–inhibitor structure, or with rapid exchange between the forms of the complex if several are present. These possibilities cannot be distinguished by the observations we have reported.

Assuming that HCA I can be represented by a sphere whose effective volume is 7.9×10^4 Å^3 (Creighton, 1984), the Stokes–Einstein treatment leads to an expected rotational correlation time (τ_c) for HCA I of 17 ns. Electron spin resonance spectra of nitroxide-containing inhibitors suggest an overall rotational correlation time for the protein in the range 20–50 ns (Mushak and Coleman, 1972) while fluorescence experiments with various carbonic anhydrases have led to estimates of 11 ns (quoted in Yguerabide et al., 1970), 14.7 ns (Kask et al., 1987), and 29 ns (Chen and Kernohan, 1967). The value for τ_c found in our analysis of the relaxation data (15 ns) suggests that the tumbling of the C_1–C_4 axis of enzyme-bound benzenesulfonamide is very close to the tumbling behavior of the protein as a whole. An unexpected result is that the aromatic ring of the inhibitor is rotating rapidly about this axis. Rapid ring rotation is consistent with the 2D NOE experiments which indicate that the protons on the inhibitor ring that are adjacent to the tritium have the same chemical shift, a situation that could be the result of shift averaging by rapid ring rotation. The 2D NOE results also suggest that interactions between the inhibitor and the protein are relatively weak, in that strong contacts with the protein at the binding site would be expected to slow ring rotation. The contrast with the slow inhibitor ring rotation observed in the cases of the 2,3,4,5,6-pentafluorobenzenesulfonamide and 2,6-difluorobenzenesulfonamide complexes of HCA I is striking (Gerig and Moses, 1987; A. Chang, preliminary results) and suggests that interactions between the fluorine atoms in these complexes and the binding site or the slightly larger steric requirements of fluorine provide a significant barrier to aromatic ring rotation in the fluorine-containing complexes.

In summary, the tritium NMR results described support the conclusion that the structure of the benzenesulfonamide–HCA I complex is very similar to that observed in crystal structures of related complexes of this enzyme. While the entire inhibitor is tightly bound to the protein, the aromatic ring of the inhibitor is sufficiently removed from interactions with the binding site that it undergoes rotation at a rate of approximately $10^9\ s^{-1}$. It remains for further studies to illuminate the reasons that fluorine substitution in the inhibitor so drastically alters the stoichiometry and aromatic ring dynamics of the complex.

Acknowledgements

We thank Mr. H. Morimoto for his invaluable assistance with the tritiation reactions and Dr. E. Olejniczak for providing a copy of his code. Work at UCSB was supported by the U.S. Public Health Service through NIH Grant GM 25975. The NTLF was supported by the Biomedical Research Technology Program, National Center for Research Resources, under Grant P41 RR01237, through Department of Energy Contract DE-AC03-76SF-000098, with the University of California. P.G.W. is an adjunct faculty member of the Department of Pharmaceutical Chemistry, University of California, San Francisco.

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