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NMR Studies of Carbonic Anhydrase–4-Fluorobenzenesulfonamide Complexes†

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ABSTRACT: Binding of 4-fluorobenzenesulfonamide to human carbonic anhydrases I and II has been studied by proton, fluorine, and nitrogen-15 nuclear magnetic resonance spectroscopy. All three types of experiments provide evidence that the stoichiometry of the interaction of this inhibitor with both enzymes is 2 mol of inhibitor bound per mole of enzyme. Observations which suggest that the bound forms are involved in an exchange process that is rapid at room temperature but slower at 2 °C are described. Nitrogen-15 shift data show that the bound inhibitors are present at the active site as anions. The proton experiments indicate appreciable reorganization of the tertiary structure of the protein upon binding. Saturation-transfer experiments to determine the rate of dissociation of the inhibitor–enzyme complex lead to the conclusion that the dissociation process is more complicated than a simple free–bound equilibrium.

The presence of carbonic anhydrase in human erythrocytes has been known for over 50 years. Subsequent efforts have shown that the erythrocyte anhydrase activity arises from two distinct zinc-containing metalloproteins, which will be referred to here as CA I and CA II.[1] The molecular masses of these isozymes are nearly identical (30 kDa), although there is only a 60% sequence homology, and both contain a single zinc ion per molecule. CA II has very high activity toward CO_2 hydration and appears to be an almost perfectly evolved enzyme in that turnover is limited primarily by substrate availability (Fersht, 1977; Pocker & Janjic, 1987). The amino acid sequence of CA II has been highly conserved throughout evolution (Tashian et al., 1983). Although significantly more abundant than CA II in the red cell, CA I has an activity toward CO_2 that is about a thousandfold less than CA II. Curiously, CA I is nearly completely inhibited by Cl^- at the concentration of this anion found in the red cell, and a biochemical role for this high-abundance protein is not yet clear (Chapman & Maren, 1978).

Recent work has revealed that virtually all plant and animal tissues contain one or more carbonic anhydrase isozymes. CA II is apparently the more widely distributed of the erythrocyte isozymes, being found in kidney, brain, pancreas, gastric mucosa, skeletal muscle, retina, and the lens. A form of carbonic anhydrase (CA III) has been found at high concentrations in red muscle and in liver, although small amounts of this isozyme are also present in erythrocytes (Deutsch, 1987). A membrane-bound form of carbonic anhydrase (CA IV) has been detected in lung and kidney (Whitney & Briggle, 1982; Henry et al., 1986). This enzyme appears to be similar to the other isozymes but is conjugated to polysaccharide so that the molecular mass is increased to 52–68 kDa. Forms of carbonic anhydrase, also glycoproteins, have been found in saliva of humans and sheep and in sheep parotid glands (Fernley et al., 1984; Murakami & Sly, 1987).

Aromatic and heterocyclic primary sulfonamides are particularly potent competitive inhibitors of carbonic anhydrase (Maren & Sanyal, 1983). A wide variety of structures have been examined for inhibitory activity, and quantitative structure–activity relationships (QSAR) have been developed, these efforts being aided recently by interactive computer graphics (Vedani & Meyer, 1984; Hansch et al., 1985).

NMR studies have demonstrated that sulfonamide inhibitors coordinate to the metal ion at the active site of carbonic anhydrase through the nitrogen atom of the SO_2NH_2 group (Evelhoch et al., 1981; Blackburn et al., 1985) and that the sulfonamide is present at the active site as an anion (Kanamori & Roberts, 1983).[2]

Formation and dissociation of sulfonamide–carbonic anhydrase complexes are kinetically more complicated than a simple bimolecular process (King & Burgen, 1976). The associative step(s) is (are) pH dependent while dissociation of the complexes is pH independent (King & Maren, 1974; Taylor, P. W., et al., 1970). Given the small values of the dissociation constant (K_D), the half-life for dissociation of a sulfonamide complex is expected to be long; values determined for simple benzenesulfonamides range from 40 to 1100 ms. The complexity and slow time course for binding of aromatic sulfonamides is reflected in the leisurely and complex time

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[1] Abbreviations: CA I and CA II, isozymic forms of human erythrocyte carbonic anhydrase, referred to in earlier literaure as the B and C forms; NMR, nuclear magnetic resonance; fid, free induction decay; NOE, nuclear Overhauser effect; ^1H NMR, proton magnetic resonance; kDa, kilodalton(s); QSAR, quantitative structure–activity relationship(s), Tris, tris(hydroxymethyl)aminomethane; TMS, tetramethylsilane.

[2] Other sulfonamides apparently bind as the neutral molecule (Petersen et al., 1977).

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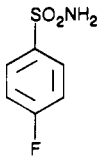
course for equilibration of binding of these compounds to carbonic anhydrase (Maren, 1967).

X-ray crystallographic studies of human CA I and II and complexes of these enzymes with sulfonamides have been carried out by Kannan and his co-workers (Kannan et al., 1977; Kannan et al., 1984). The isozymes have similar secondary and tertiary structures, and many of the amino acids close to the zinc atom are the same (Notstrand et al., 1974). In the resting state, the zinc atom is coordinated by three histidine imidazole nitrogen atoms with a fourth coordination site occupied by water or hydroxide, depending on pH. Consonant with the NMR results, the zinc-bound water molecule is displaced upon formation of complexes with the inhibitors acetazolamide or sulfanilamide, and nitrogen of the sulfonamide group enters the coordination sphere. There is a possibility that an oxygen of the sulfonamide group also coordinates to the zinc.

NMR spectroscopy remains a powerful tool for examination of protein structures in solution and for relating crystallographic results to observations made with solutions. Fluorine substitution on an inhibitor molecule provides a useful probe for NMR studies of enzymes (Gerig, 1978), and it was our goal to use this approach in examining the active sites of CA I and CA II. The prediction that fluorine substitution should enhance the inhibitory power of benzenesulfonamides (Hansch et al., 1985) added interest to our studies. We describe here initial results which bear on the nature of the binding of 4-fluorobenzenesulfonamide (I) to CA I and CA II.



I

Experimental Procedures

Materials. Deuterium oxide (D_2O, 99.8% deuterium atom), 4-fluorobenzenesulfonyl chloride, and sodium azide were purchased from Aldrich. Tris(hydroxymethyl)aminomethane (Tris buffer) was from Sigma Chemical Co. Sodium sulfate and potassium iodide were from Fisher Scientific. CM Bio-Gel A was obtained from Bio-Rad. All solvents were from Fisher and were of analytical reagent grade. Distilled, deionized water was used for making solutions. Outdated human blood was obtained from the local blood bank.

4-Fluorobenzenesulfonamide (I) was synthesized by treating a chilled solution of 4-fluorobenzenesulfonyl chloride (1 g) dissolved in 20 mL of chloroform with 10 mL of chilled 50% NH_4OH followed by stirring for 1 h. The solution was acidified with 6 M HCl to pH 1. After the product had precipitated, it was recrystallized from water (yield, 40%), mp 131 °C (lit. 126 °C; Weast & Astle, 1981). The product exhibited a parent ion at m/e 175 in the mass spectrum and the expected proton and fluorine-19 NMR spectra.

4-Fluorobenzene[^{15}N]sulfonamide was prepared by adding 110 mg of $^{15}NH_4Cl$ (MSD Isotopes, 99% enriched) to 80 mg of NaOH dissolved in 2 mL of water. A solution of 200 mg of 4-fluorobenzenesulfonyl chloride in 5 mL of chloroform was added, and the system was stirred overnight. After the separation of the aqueous layer, the chloroform layer was evaporated to afford 100 mg of crude product. After recrystallization from water, 30 mg of material, mp 131 °C, was obtained. The sample exhibited a parent ion at m/e 176 in the mass spectrum and had the expected fluorine-19 and nitrogen-15 NMR spectra.

The carbonic anhydrase isozymes were isolated from hemolysate according to the procedure of Khalifah et al. (1977) and Osborne and Tashian (1975). The isozymes were found to be about 90% homogeneous by gel electrophoresis. Enzyme concentrations were determined from the absorbance at 280 nm using $A^{1\%}$ = 16.3 and 18.7 cm^{-1} for CA I and CA II, respectively (Lindskog et al., 1970). Esterase activity of the enzymes was checked with *p*-nitrophenyl acetate and found to be 8 mol min^{-1} (mol of enzyme)$^{-1}$ (Armstrong et al., 1966).

Protein solutions were concentrated by ultrafiltration using an Amicon cell and a YM10 or PM10 membrane. For replacement of solvent water by deuterium oxide, protein solutions were reduced to 1–2 mL, diluted to 10 mL with D_2O, and reconcentrated, the process being repeated 4–5 times.

Instrumentation. Melting points were measured on a Thomas–Hoover capillary melting point apparatus. A Radiometer PHM 52 meter was used to record pH; values reported as "pH" are glass electrode readings not corrected for the isotopic composition of the solvent. For 5-mm o.d. NMR samples, the pH was recorded with a Markson digital pH meter equipped with a small-diameter electrode. Absorption spectra were recorded on a Hewlett–Packard 8452A spectrophotometer. Mass spectra were obtained with a Micromass ZAB-2F instrument with the use of electron-impact or chemical ionization. Methane was the reactant gas in the latter instance.

Proton NMR spectra were recorded on a General Electric GN-500 spectrometer operating at 500.1 MHz. Fluorine spectra were recorded on a Nicolet NT-300 instrument operating at 282.3 MHz or on General Electric GN-300 and GN-500 instruments operating at 282.7 and 470.5 MHz, respectively. Nitrogen-15 experiments were carried out at 50.6 MHz on the GN-500 spectrometer. The nitrogen chemical shift of 4-fluorobenzene[^{15}N]sulfonamide was found to be 159 ppm upfield from the signal from a solution of $NH_4{}^{15}NO_3$ in D_2O. Temperatures were regulated with the instrument controllers and are believed to be accurate to better than 1 °C.

Procedures. Spin–lattice relaxation times were determined by using the inversion-recovery method and fit to the appropriate three-parameter function by routines provided with the instrument software, as described previously (Gerig & Hammond, 1984). The spin–lattice relaxation times obtained were generally reproducible to better than 5% and thought to be accurate to within 10%. Line widths were obtained by fitting the observed line width to a Lorentzian curve using instrument software. The fitted line widths at half-maximum height were corrected for line broadening arising from exponential multiplication of fid and were generally reproducible to within 2 Hz. However, the spectra were generally obtained without proton decoupling, and no corrections were made for the effects of spinning side bands on the line shape, as discussed previously (Gerig & Hammond, 1984). Thus, the line widths obtained by the fitting procedure are wider than the true line width. However, differences between line widths are regarded as reliable.

$^{19}F\{^1H\}$ NOE experiments were performed with alternate acquisitions of spectra with and without irradiation during the preacquisition period in order to minimize the effects of thermal and instrumental drifts. A delay of at least $10T_1$ was used between each accumulation. The NOEs could be reproducibly measured to within 2% using difference spectra.

Selective saturation experiments by the DANTE method were carried out as described by Gerig and Hammond (1984) using a series of 4K to 16K short pulses (ca. 5°). Control

Table I: Fluorine NMR Data for 4-Fluorobenzenesulfonamide Complexes[a]

enzyme	T (°C)	chemical shift (ppm) bound	excess line width (Hz) bound	T_1 (s) free	T_1 (s) bound	$^{19}F\{^1H\}$ NOE free	$^{19}F\{^1H\}$ NOE bound
Ca I	25	6.21	82	2.8	0.42	−0.40	−0.80
	7		167				
	1		210				
CA II	25	6.16	45	2.8	0.40	−0.35	−0.80
	7		50				
	1		72				

[a] Data obtained at 282.3 MHz. Chemical shifts are upfield of the signal for the free inhibitor, which was set at 0 ppm. Excess line widths are the difference between the apparent line widths of the free and bound signals in the presence of enzyme. The data in each column labeled "free" refer to the signal in the spectrum at the chemical shift of I when enzyme is present. T_1 for the inhibitor in the absence of enzyme was 7.3 s, and there was no detectable H–F NOE.

spectra were obtained with the same series of pulses but altering the position of the carrier frequency. Achievement of equilibrium was verified by increasing the number of pulses in the sequence until no additional changes in peak intensities were noted.

The pH of each sample was measured in the sample tube before and after each NMR experiment. The pH was adjusted with dilute NaOD or D_2SO_4. For stoichiometry experiments, small aliquots (50 or 100 μL) of a concentrated solution (15 mM) of 4-fluorobenzenesulfonamide were added sequentially to the enzyme solutions of known concentration.

Results

Fluorine NMR Observations. The fluorine spectrum of 4-fluorobenzenesulfonamide consists of a multiplet (due to proton–fluorine coupling) centered at 56.75 ppm downfield of the resonance of 4-fluorophenylalanine, a convenient internal reference. When either CA I or CA II is present with this inhibitor, a second, much broader signal about 6 ppm upfield of this position is observed (Figure 1). If the concentration of the sulfonamide is in excess, separate signals for enzyme-bound and unbound sulfonamide are observed. Thus, interaction of the inhibitor with either of these enzymes is in the NMR slow-exchange regime. The association constant for complexes of I with CA I or CA II can reasonably be expected to be in the range 5×10^6 to 5×10^7 M^{-1} (Kakeya et al., 1969; Taylor, P. W., et al., 1970; King & Burgen, 1976), and, given enzyme association rates previously determined for similar complexes (ca. 10^6 M^{-1} s^{-1}) and the large fluorine chemical shift difference between free and bound forms of I, the observation of slow-exchange conditions is not unexpected.

In addition to the large enzyme-induced chemical shift effect when I is bound to CA, there are appreciable relaxation effects as well (Table I). In the absence of protein, the fluorine T_1 relaxation time of the inhibitor is 7.3 s, and this is decreased to 0.4 s in the bound state. A substantial (negative) proton–fluorine NOE is observed on the signal for the bound inhibitor. These observations and the large line width difference between the signals assigned to the "free" and bound forms are consistent with assignment of the upfield signal to inhibitor molecules that are protein-bound.

Interestingly, the signal appearing at the chemical shift of the free inhibitor in samples where the inhibitor concentration is in excess of the enzyme concentration also shows a slight reduction in T_1, some line broadening, and a negative NOE when proton signals of the system are irradiated.

Given the slow exchange conditions, the stoichiometry of the interaction between I and the anhydrase isozymes is conveniently determined by simply adding increasing amounts of inhibitor to a known amount of enzyme. Figure 1 shows the result of such an experiment. Such experiments clearly showed that for both CA I and CA II the stoichiometry of

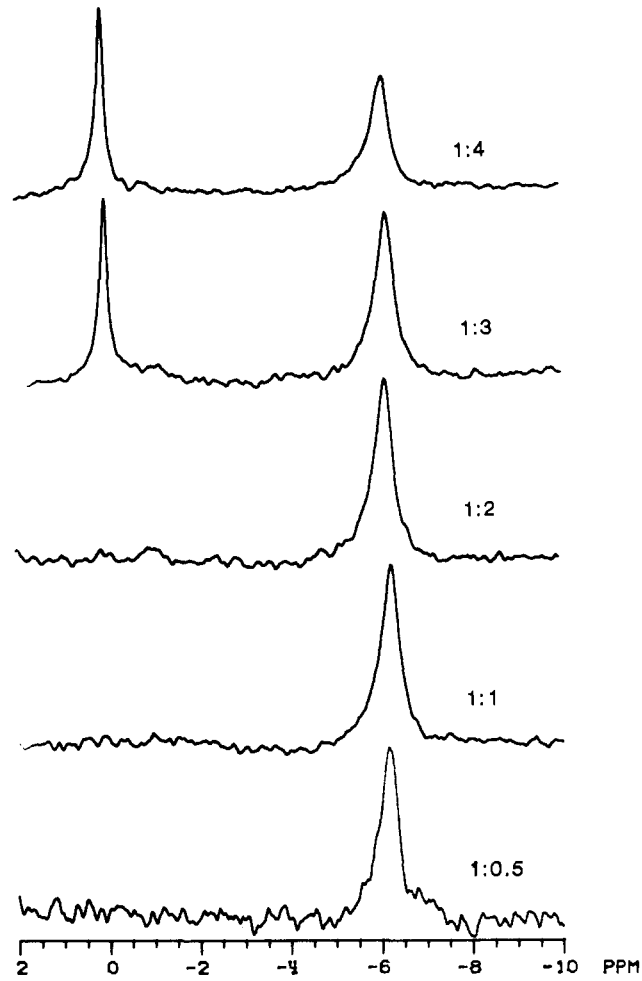


FIGURE 1: Fluorine NMR spectra at 282 MHz of the 4-fluorobenzenesulfonamide–CA I system at various ratios of inhibitor to enzyme, indicated on the traces. The enzyme concentration was 1 mM, and samples were at "pH" 7.2 in D_2O at 25 °C.

binding 4-fluorobenzenesulfonamide is 2 molecules of inhibitor per molecule of enzyme.

That only a single broad resonance represents the two bound inhibitor molecules in the fluorine spectrum indicates either that the magnetic environments of the bound inhibitors are extremely similar or that the bound molecules are exchanging environments rapidly enough that only an averaged signal is observed. We have observed that the line width for the bound species is strongly temperature dependent, increasing 130 Hz when the sample temperature is changed from 25 to 1 °C in the case of CA I (Figure 2). As indicated below, these line-width changes cannot be accounted for by exchange broadening involving the free-to-bound process. These effects also cannot be due to viscosity changes, for the signal at the

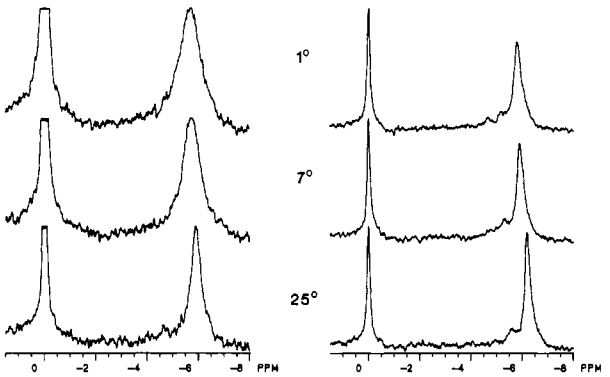


FIGURE 2: Fluorine NMR spectra at 282 MHz of complexes of 4-fluorobenzenesulfonamide with CA I (left) and CA II (right) at 25, 7, and 1 °C at "pH" 7.2 in D_2O. For the CA I spectra, the enzyme concentration was 1 mM, and the inhibitor concentration was 4 mM while for the CA II spectra these concentrations were 0.5 and 2 mM, respectively. Note that the free inhibitor signal for the CA I spectra is truncated.

chemical shift of free I is unaltered by lowering the temperature. Moreover, the behavior of the bound signal from the CA II complex is different from that of the CA I complex whereas the same percentage change would be expected in both cases if viscosity changes were the primary determinant of the line-width changes. The observations are consistent with an exchange process affecting only the bound inhibitor molecules that becomes slower as the temperature decreases, leading to exchange broadening.

Saturation-transfer experiments with the complex of 4-fluorobenzenesulfonamide with CA I were carried out to determine the exchange rate between the free and bound inhibitor (Figure 3). Spectra were obtained in which the resonances of the free and bound inhibitor were saturated, along with control spectra where the effective position of irradiation was at the same distance to low or high field of these signals as the chemical shift difference between them. Saturation of the free signal led to a 25% ± 5% reduction in the bound signal while saturation of the latter signal reduced the intensity of the free signal 15% ± 5%. Assuming that this exchange situation can be represented by the simple equilibrium

$$[\text{free}] \underset{k_{BF}}{\overset{k_{FB}}{\rightleftarrows}} [\text{bound}]$$

then the rate constants can be determined from

$$k_{BF} = \frac{B_0/B_E - 1}{T_1^B}$$

and

$$k_{FB} = \frac{F_0/F_E - 1}{T_1^F}$$

where the symbols F and F_0 respectively represent the magnetization of the free molecules in the presence and the absence of saturation of the bound molecules and B and B_0 have the same meaning for the magnetization of the bound species. Using the spin–lattice relaxation data given in Table I, it can be computed that $k_{BF} = 0.6\ s^{-1}$ and $k_{FB} = 0.02\ s^{-1}$. However, the ratio of these two rate constants must be consistent with the observed concentration ratio of free and bound molecules. Even with a generous allowance for experimental errors, this was not the case for the CA I complex of I, and one must conclude that the simple equilibrium model given above is inadequate and a more complex kinetic situation, involving more than one form of the free or bound molecules, is indicated. The Appendix discusses this situation in more detail.

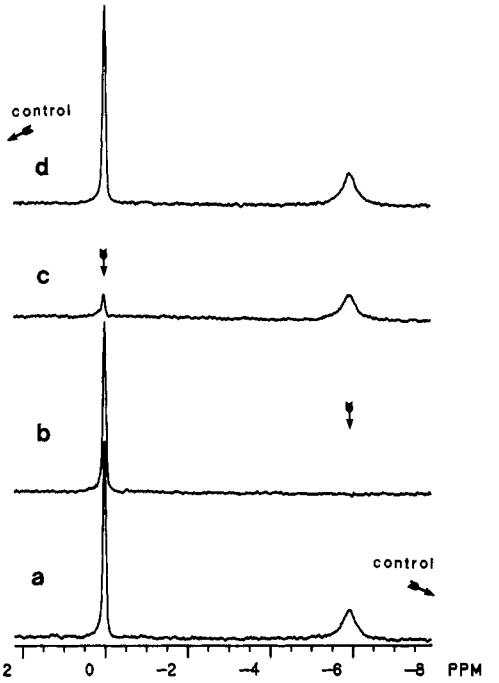


FIGURE 3: Saturation-transfer studies of the complex of I with CA I at 25 °C. (a and d) Control spectra; (b) irradiation of the resonance from bound inhibitors; (c) irradiation of the signal from free inhibitor. All spectra were obtained under identical conditions with the exception of the position of the carrier frequency. Protein concentration was 1 mM; the concentration of I was 4 mM, at "pH" 7.1 in D_2O. The steady-state selective saturation was achieved by applying 16 000 pulses of 1.50-μs duration with a 1-ms interval between them, with 20 s between scans.

The saturation-transfer experiments were less successful with the CA II complexes in that transfer of saturation from the free to the bound signal could be demonstrated but not the reverse. The data lead to $K_{FB} = 0.024\ s^{-1}$, a result similar to that found with the CA I complex. However, k_{BF} must be considerably larger than the value found with the other isozyme in that no saturation transfer was detectable.

The magnitudes of the dissociation rate constants of the complexes estimated by using the simple model outlined above are not inconsistent with previous observations made by optical spectroscopic methods (King & Burgen, 1976) or by other NMR saturation-transfer measurements (Gerig & Moses, 1987). Assuming equal amounts of free and bound forms of the inhibitor, a rate constant for dissociation of $0.6\ s^{-1}$ corresponds to an exchange broadening of the free and bound signals of less than 0.1 Hz (Caldin, 1964). Thus, even a considerably more rapid dissocation process than is expected would produce a negligible contribution to the line widths observed in these systems.

Proton NMR Studies. The formation of the enzyme–inhibitor complexes of CA I and CA III with 4-fluorobenzenesulfonamide was also monitored by 500-MHz ^1H NMR spectroscopy. The ^1H NMR spectrum of the enzymes with no inhibitor present shows a number of resonances in the region above the TMS reference position as well as resolved signals arising from histidines, aromatic amino acids, and amide linkages in the 5–8 ppm region. These spectral regions are distinctive for CA I and CA II. Addition of inhibitor produces many changes in both portions of the ^1H NMR spectrum of these isozymes; most readily apparent are those

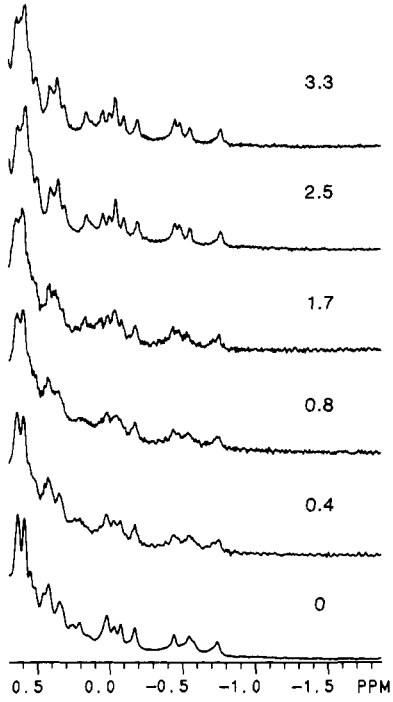


FIGURE 4: Proton NMR spectra at 500 MHz showing changes during the formation of the CA I–4-fluorobenzenesulfonamide complex at 35 °C, with the ratio of inhibitor to enzyme concentrations shown. The enzyme concentration was 1.5 mM at "pH" 7.0 in D_2O. The residual HOD signal was saturated for 2 s before data acquisition. Chemical shifts were referenced to the HOD signal set at 4.8 ppm. Spectral changes in the 0.1 and −0.55 ppm regions are not complete until 2 mol of inhibitor per mole of enzyme is present.

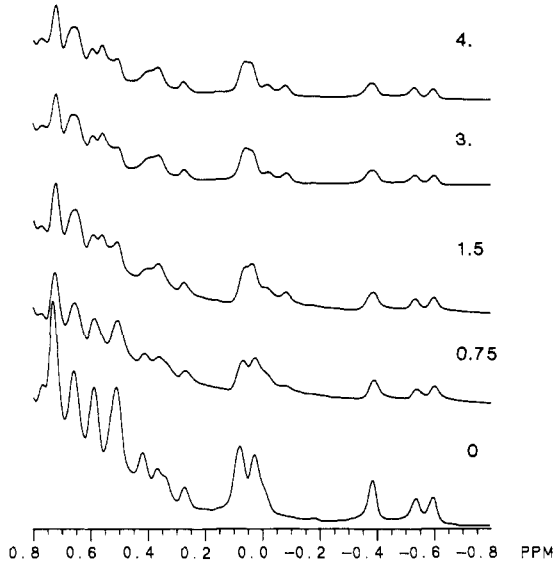


FIGURE 5: Proton NMR spectra at 500 MHz showing formation of the CA II–4-fluorobenzenesulfonamide complex at 35 °C at the inhibitor:enzyme concentration ratios shown. The enzyme concentration was 0.7 mM, "pH" 7.0, in D_2O. Acquisition and referencing were the same as for Figure 4. Changes around 0.5 and 0.0 ppm are not complete until a 2:1 ratio of inhibitor to enzyme is achieved.

changes that take place in the high-field region (Figures 4 and 5). Study of the high-field region as well as the aromatic region as increasing amounts of inhibitor were added to a fixed amount of enzyme confirmed the 2:1 stoichiometry indicated by the fluorine NMR observations described above. A number of resonances shift position or change shape (possibly because some sets of signals are in intermediate exchange) with increasing amounts of inhibitor until a 2:1 concentration ratio is achieved, at which point no further changes in the ^1H NMR

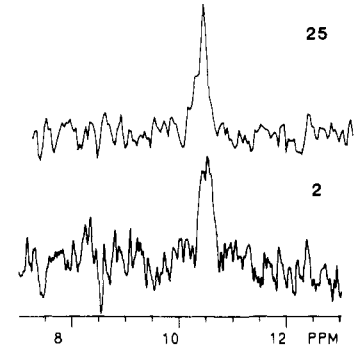


FIGURE 6: Proton-decoupled ^{15}N NMR spectra at 50.6 MHz of enriched 4-fluorobenzenesulfonamide and the CA I–inhibitor complex. (Top) Spectrum of the enzyme–inhibitor complex at 25 °C. The enzyme concentration was 1 mM with the inhibitor present at 4 mM, in 10% D_2O and "pH" 7.1. 28K scans were collected with 16K data points and a repetition rate of 1 s. (Bottom) Same as top spectrum but with the sample at 2 °C. 25K scans were collected with 16K data points and 1.5-s delay between the acquisitions. The proton decoupler was on only during data acquisition. The chemical shift of I in the absence of enzyme was set to 0 ppm; positive numbers correspond to upfield shifts. Data at both temperatures were apodized with an exponential line-broadening function that increased the line width 2 Hz.

spectrum of the protein are observed. Again, the spectra of each isozyme saturated with inhibitor are distinctive.

Changes of several resonances previously identified as arising from histidine residues (Campbell et al., 1975; Pesando, 1975) were observed when inhibitor binds. The effects upon binding are only complete after achievement of a 2:1 ratio of inhibitor and enzyme.

Acetazolamide, a heteroaromatic sulfonamide, binds to CA II with a 1:1 stoichiometry (Maren, 1967). As a check on our procedure, binding of this inhibitor was followed by observation of the upfield proton signals; a 1:1 stoichiometry was found (L. B. Dugad, unpublished observations).

It was observed that the ^1H NMR spectra of both the free enzyme and enzyme with 4-fluorobenzenesulfonamide bound are temperature dependent over the range 1–35 °C; further exploration of the changes observed may lead to elucidation of the dynamics of the protein's structural response to binding.

Nitrogen-15 NMR Studies. Previous nitrogen-15 studies of the benzenesulfonamide complex demonstrated that this inhibitor binds to CA I as an anion (Kanamori & Roberts, 1983). It was found that a ca. 10 ppm upfield shift relative to the free inhibitor signal accompanied complexation to the enzyme. We have examined the nitrogen-15 spectrum of isotopically enriched I bound to CA I using a sample in which the inhibitor is present in a greater than 2-fold excess. In consonance with the results of Kanamori and Roberts, we observed that at room temperature the signal from the enzyme-bound inhibitor appears 10.3 ppm upfield from the signal arising from the free sulfonamide (Figure 6). [Blackburn et al. (1985) reported a value for this shift difference of 8.2 ppm, obtained under somewhat different experimental conditions.] When the sample temperature was lowered to 2 °C, the spectrum exhibits a line shape suggestive of the presence of two signals of approximately equal intensity, separated by about 0.1 ppm (Figure 6).

Discussion

Although a 1:1 stoichiometry for binding other sulfonamides to CA has been demonstrated, it is clear from the fluorine, proton, and nitrogen-15 data presented here that 4-fluorobenzenesulfonamide binds to CA I and CA II with a 2:1 stoichiometry and, on the basis of the nitrogen chemical shift(s)

observed, that both bound inhibitor molecules are present at or near the zinc ion of the active site as anions.

Binding of two acetate anions has been found in bovine CA I (Lanir & Navon, 1974). It was found that only one acetate ion was involved in the inhibition of esterase activity, which led to the proposal of two distinct binding sites for this anion on the bovine system. The noninhibitory acetate ion was found to be untitratable by sulfonamide. Spin–lattice relaxation measurements using the Mn^{2+}-substituted enzyme indicated 0.43- and 0.48-nm distances for the two acetate ions from the metal ion. Thus, the two acetate binding sites are different, and likely only one acetate ion is directly coordinated to the metal ion. Similar results for the binding of bicarbonate ion to bovine CA I have been reported by Yeagle et al. (1975). On the basis of carbon-13 NMR studies, they proposed one loose and one tight binding site for this anion, one anion being directly coordinated to the metal ion and the other in the outer coordination sphere of the metal ion. The binding of two cyanide ions to Co(II) and Cu(II) carbonic anhydrases from human, monkey, and bovine sources has been demonstrated by Taylor, J. S., et al. (1970). However, the comparison of Co(II) or Cu(II) metalloenzymes with the native zinc enzyme may not be appropriate as far as the ligand coordination is concerned. Moreover, binding of small anions, which have no possibility of hydrophobic or other interactions with the residues at the active site of these enzymes, must be fundamentally different from the binding of aromatic sulfonamides even if these latter species are present as anions.

The nitrogen-15 results coupled with the observation of greatly increased fluorine line widths at low temperatures suggest that the two inhibitor molecules bound to CA I or CA II are rapidly exchanging between two magnetically distinguishable environments at 25 °C. These sites must be physically proximate to accommodate rapid exchange and, since the coordination chemistry of zinc is known to accommodate four or five ligands (Sigel, 1983; Wells, 1984), could involve simultaneous coordination of the three histidine residues which hold the zinc in the resting enzyme and the two sulfonamide molecules.

Fluorescence correlation spectroscopy indicates that the rotational correlation time for CA I should be 12–15 ns (Kask et al., 1987). If one assumes a standard geometry for the nuclei attached to the aromatic ring of I and assumes that a proton of the enzyme comes within van der Waals contact of the fluorine nucleus of the inhibitor, the relaxation of the fluorine nucleus of a bound inhibitor molecule should be similar to that deduced for [(4-fluorophenyl)sulfonyl]chymotrypsin (Ando et al., 1986) where the fluorine T_1 and NOE values and the rotational correlation time are all similar to those found for carbonic anhydrase. In the case of the chymotrypsin derivative, it was concluded that rotation of the aromatic ring holding the fluorine atom was significantly hindered, being characterized by a correlation time slightly smaller than that for overall tumbling of the protein. The line width of the (bound) fluorine signal for that system was about 38 Hz, much larger than expected from a dynamic picture that was consistent with the T_1 and NOE data at several frequencies; it was suggested that small conformational excursions around the fluorophenyl ring produce an exchange broadening of the signal. In the case of 4-fluorobenzenesulfonamide bound to CA, the line widths are larger still (nearly 20 times larger than expected) than the limited T_1 and NOE data available would suggest they should be. A detailed analysis of the ring dynamics of the anhydrase system will thus be of interest in defining both the dynamics of ring rotation and those factors that lead to such significant line broadening.

The signal that appears at the same fluorine chemical shift as the free inhibitor in systems where the inhibitor is present in excess over the enzyme shows a reduced T_1 relative to the T_1 of the inhibitor in the absence of protein. There is also a negative NOE on this signal when protons are irradiated. Calculations based on a two-site exchange model (Leigh, 1971) and the apparent dissociation rate constant (ca. 0.6 s^{-1}) estimated by the saturation-transfer experiments suggest that it is likely that the T_1 reduction is the result of exchange of inhibitor between the free and bound states. Similarly, calculations indicate that the NOE on the free peak that is observed can arise from transfer of the NOE generated on the bound molecules (Borzo & Maciel, 1981). A more detailed analysis is not possible at this point because, while it is clear that the two-site exchange model is inadequate, we do not know the correct model for the exchange situation.

The signals that appear in the 0 to −2 ppm region of the proton spectrum of CA probably arise from methyl groups that are close enough to aromatic rings in the tertiary structure to experience a ring current effect. Consideration of the crystal structures of CA I and CA II indicates that several valine, alanine, leucine, threonine, and isoleucine residues in these structures should experience +0.2 to −2.2 ppm ring current shifts (D. H. Gregory, unpublished calculations). Some of these methyl groups are within 0.5 nm of the zinc and, thus, can be considered part of the active site while the remaining methyl groups are scattered throughout the tertiary structure. It is clear from Figures 4 and 5 that binding of 4-fluorobenzenesulfonamide to either isozyme significantly alters the high-field spectral region, implying that binding produces structural responses within the enzyme. More detailed conclusions regarding these responses will have to await the results of efforts to assign the high-field signals to particular residues.

The overall free energy change which favors double coordination over single coordination of I likely is the result of favorable interactions between the aromatic ring(s) and the hydrophobic "side" of the active site (Hansch et al., 1985) and conceivably between the inhibitors themselves. It may be possible to design two complementary inhibitor molecules for these enzymes which, when cobound at the active site, are more inhibitory than either one is alone.

Appendix

That the forward and reverse rate constants measured by the saturation-transfer experiments are not consistent with the relative amounts of free and bound inhibitor molecules present at equilibrium implies that the simple model [free] $\rightleftharpoons$ [bound] does not correctly describe the binding process. If more than one free or bound form is present along the exchange itinerary and these forms are in rapid exchange, the nature of the exchange becomes more difficult to access by saturation-transfer methods. Consider the system

$$A \underset{k_{BA}}{\overset{k_{AB}}{\rightleftharpoons}} B \underset{k_{CB}}{\overset{k_{BC}}{\rightleftharpoons}} C$$

where species A and B are in rapid exchange and, therefore, represented in the NMR spectrum by a single line or set of lines. [This and more complex exchange situations have been treated by Ugurbil (1985) using a different formalism.] In terms of the Bloch equations, the z components of the magnetization vectors of each of these species are given by

$$\frac{dM^A}{dt} = \frac{-(M^A - M_0{}^A)}{T_1{}^A} - k_{AB}M^A + k_{BA}M^B \quad \text{(A1)}$$

$$\frac{dM^B}{dt} = \frac{-(M^B - M_0{}^B)}{T_1{}^B} + k_{AB}M^A - k_{BA}M^B + k_{CB}M^C - k_{BC}M^B \quad (A2)$$

$$\frac{d(M^A + M^B)}{dt} = \frac{-(M^A - M^B)}{T_1} + \frac{M_0{}^A + M_0{}^B}{T_1} + k_{CB}M^C - k_{BC}M^B \quad (A3)$$

where the k's represent rate constants for the various steps in the exchange, M^X represents the z components at time t, and $M_0{}^X$ represents these components at equilibrium. If the signal of component C is saturated and the system allowed to come to equilibrium, then $M^C = 0$ and

$$k_{BC} = \frac{-(M_E{}^A + M_E{}^B) + (M_0{}^A + M_0{}^B)}{T_1 M_E{}^B} \quad (A4)$$

where $M_E{}^X$ represents the values of the magnetization components after the system has come to equilibrium in the irradiating field and it has been assumed that T_1's for both A and B are equal. Since the signals for A and B are not resolved, one can experimentally measure only $M^A + M^B$: the equilibrium magnetization for a single component ($M_E{}^B$) is not accessible, and the calculation of k_{BC} is not possible within this formulation.

For binding to the CA isozymes, there are likely two forms of the free inhibitor important in the binding process, namely, the unionized form and the anion, and these are probably in rapid exchange. Our observations indicate that two molecules of inhibitor bind to the protein in what are probably magnetically distinguishable environments that are also in rapid exchange at room temperature. Thus, the overall free-to-bound exchange process involves at the least two pairs of sites, in rapid exchange within a pair, but slow exchange between pairs. Absent additional information, it does not appear that a saturation-transfer experiment can directly provide the correct rate constants for interchange between these pairs.

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