NMR Studies of Carbonic Anhydrase–Fluorinated Benzenesulfonamide Complexes†

L. B. Dugad, C. R. Cooley, and J. T. Gerig*

Department of Chemistry, University of California, Santa Barbara, Santa Barbara, California 93106

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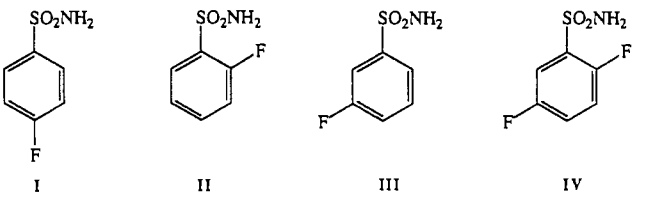
ABSTRACT: Fluorine NMR has been used to examine complexes formed by 2-fluoro-, 3-fluoro-, and 2,5-difluorobenzenesulfonamide and human carbonic anhydrases I and II. The results show that all three inhibitors form complexes with both isozymes that have 2:1 inhibitor/enzyme stoichiometry. The fluorine spectra observed for all inhibitor–isozyme combinations are consistent either with rapid rotation of the aromatic ring of the inhibitor in the complexes or with preferential binding of only one of the two possible conformations of the inhibitors that are isomeric by virtue of rotation about the C_1–C_4 bond of the fluoro aromatic ring. Because ring rotation is slow in the case of the pentafluorobenzenesulfonamide–CA I complex, selective binding of rotamers is the explanation of these observations presently favored. A computer graphics study shows that formation of 2:1 complexes of CA I is feasible without appreciable distortion of the protein tertiary structure found in the crystalline state.

Carbonic anhydrase isozymes catalyze the hydration of carbon dioxide and, depending on the isozyme and source, exhibit esterase and phosphatase activities. Two isozymes (CA I[1] and CA II) are readily available from erythrocytes of mammals, and the human forms of these proteins have been extensively studied by a variety of physicochemical techniques, including X-ray crystallography (Kannan, 1980). The literature related to the chemistry and structure of carbonic anhydrase is large and is summarized in recent reviews by Pocker and Sarkanen (1978), Bauers et al. (1980), Spiro (1983), Carter and Jeffry (1985), and Deutsch (1987). An entire volume of *Annals of the New York Academy of Sciences* is devoted to the biology and chemistry carbonic anhydrases (Vol. 429, 1984), and this collection represents a reasonable overview of current interests with regard to these enzymes.

Aromatic and heterocyclic primary sulfonamides are especially good competitive inhibitors of carbonic anhydrase (Maren & Sanyal, 1983). Such materials have considerable clinical utility, and the search for improved, side effect free carbonic anhydrase inhibitors based on these structures is an area of continuing activity (Hansch et al., 1985; Ponticello et al., 1987). Crystallographic studies of acetazolamide and sulfanilamide complexed with human CA I and CA II show a single molecule of these sulfonamides coordinated to the zinc atom of the enzymes at the ligand position that would be occupied by coordinated hydroxyl in an active enzyme (Kannan, 1980; Eriksson et al., 1986). NMR studies have confirmed direct interaction of the sulfonamide nitrogen with a metal ion at the active site (Blackburn et al., 1985) and shown that benzenesulfonamide is bound as the corresponding anion (Kanamori & Roberts, 1983). Similarly, pyruvamide, a substrate of CA with regard to carbonyl hydration, has been shown to bind to the enzyme as the corresponding deprotonated amide (Mukherjee et al., 1987).

We have recently described evidence that 4-fluorobenzenesulfonamide (I) forms complexes with CA I and CA II that involve binding of *two* molecules of inhibitor per molecule of enzyme and that both bound inhibitor molecules are present as anions (Dugad & Gerig, 1988). The two bound inhibitors appear to be in magnetically distinct environments, but the rate of interchange between these environments is sufficiently rapid that fluorine or nitrogen-15 NMR observations at room temperature report only exchange-averaged properties. In contrast, the rate of dissociation of the bound inhibitors from each isozyme was found to be in the slow-exchange limit.

Chart I



In seeking to confirm these conclusions we have extended our studies to the fluorinated benzenesulfonamides II–IV, shown in Chart I. Besides permitting exploration of different regions of the active site by means of fluorine NMR observations, these inhibitors present an additional complication in that the aromatic ring of an inhibitor can bind to the enzyme in two ways that differ by rotation of the aromatic ring about its C_1–C_4 axis. There is reason to expect the rate of aromatic ring rotation in the bound state to be slow (Gerig & Moses, 1987) so that bound inhibitors could be represented by pairs of fluorine signals whose relative intensities will depend on the relative amounts of two rotationally isomeric structures that are present.

Experimental Procedures

Materials. 2-Fluoroaniline, 3-fluoroaniline, 1,4-difluorobenzene, and chlorosulfonic acid were purchased from Aldrich

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[1] Abbrevations: CA I and CA II, isozymic forms of carbonic anhydrase; NMR, nuclear magnetic resonance; IR, infrared; NOE, nuclear Overhauser effect.

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Chemical Co. Sulfur dioxide was obtained from Air Products, Inc. All other materials were as described previously (Dugad & Gerig, 1987). Isolation of CA I and CA II from outdated human blood by the affinity procedure of Khalifah et al. (1977) and Osborne and Tashian (1975) was carried out in the same way as indicated in the previous paper (Dugad & Gerig, 1987).

2-Fluorobenzenesulfonamide (II) was synthesized by treating a chilled solution of 4 g of 2-fluorobenzenesulfonyl chloride in 10 mL of tetrahydrofuran with 25 mL of chilled 50% NH_4OH solution, added dropwise with stirring over 30 min. The sulfonyl chloride was prepared from 2-fluoroaniline according to the procedure of Gerig and Roe (1974).

The reaction mixture was acidified to pH 1 by slow addition of 50% HCl whereupon the product precipitated. The solid sulfonamide was removed by filtration and recrystallized from water to give the product (mp 164–165 °C) in 40–50% yield. The mass spectrum showed a parent ion peak at m/e 175, and an infrared spectrum with frequencies characteristic of the aromatic sulfonamide group at 1151–1163 and 1306–1335 cm^{-1} (Bellamy, 1968). The product had proton and fluorine-19 NMR spectra consistent with the expected structure.

3-Fluorobenzenesulfonamide (III) (mp 128–130 °C) was prepared from 3-fluoroaniline by the same procedure as used for the 2-fluoro isomer. It exhibited a parent ion in the mass spectrum at m/e 175 and had infrared, proton, and fluorine NMR spectra consistent with the expected structure.

It was intended to prepare *2,5-difluorobenzenesulfonamide* (IV) from the corresponding sulfonyl chloride which, in turn, would be produced by treating 1,4-difluorobenzene with chlorosulfonic acid. In our hands, following a literature procedure (Gilman, 1932) and reasonable variations of this procedure, this approach produced only 2,2′,5,5′-tetrafluorodiphenyl sulfone. However, it was found that treatment of this sulfone with ammonia produced the desired sulfonamide in acceptable yield.

To 3 mL of chlorosulfonic acid contained in a small round-bottom flask at room temperature was added 1 mL of 1,4-difluorobenzene in 100-μL portions over a period of 1 h with magnetic stirring. The flask was kept closed to avoid contamination with moisture. After 3 h, 50 mL of CCl_4 was added and the reaction mixture poured onto 50 g of ice. The aqueous phase was separated immediately and the CCl_4 solution washed with 50 mL of $NaHCO_3$ solution. The CCl_4 solution was dried over anhydrous Na_2SO_4 and then transferred to rotary evaporator for removal of the solvent. The residue (60% yield, mp 124 °C) showed a parent ion in the mass spectrum at m/e 290 ($C_{12}H_6SO_2F_4$), consistent with the sulfone, but provided no evidence for the expected sulfonyl chloride (m/e 212, with Cl isotope cluster). The IR spectrum exhibited stretching frequencies in the 1358–1336- and 1169–1159-cm^{-1} ranges, again consistent with the sulfone but not the sulfonyl chloride (Bellamy, 1968).

2,2′,5,5′-Tetrafluorophenyl sulfone (500 mg, 1.7 mmol) was dissolved in 5 mL of dichloromethane and cooled in a dry ice/acetone bath. To this solution was added 2–3 mL of chilled 15 M NH_4OH solution, and the mixture was stirred for 1 h. The pH of the reaction mixture was adjusted to 1 with 50% HCl and the solution allowed to stand until the product separated. The solid was removed by filtration, dried in air, and recrystallized from water to afford 100–150 mg (ca. 30% yield) of product (mp 132–134 °C). The IR spectrum showed bands at 1337 and 1166 cm^{-1} while the mass spectrum showed a parent ion at m/e 193. Proton and fluorine NMR spectra were consistent with 2,5-difluorobenzenesulfonamide.

Methods. Instrumentation and procedures for obtaining NMR spectral data were the same as those used in the previous study (Gerig & Dugad, 1987). Molecular graphics studies where carried out on an Evans & Sutherland PS300 system running BioGraf I software (Biodesign, Inc., Pasadena, CA). Coordinates for CA I were taken from the Brookhaven Protein Data Bank. Starting coordinates for sulfanilamide bound to CA I are given by Kannan et al. (1977); an apparent error in the coordinates for C_6 of the inhibitor was corrected by energy minimization of the aromatic ring of the inhibitor.

Results

Hansch et al. (1985) have derived the following expression relating molecular structure and the association constant for binding of simple sulfonamides to CA II:

$$\log K_A = 1.55\sigma + 0.62 \log P + 6.98 \quad (1)$$

where σ is the Hammett substituent constant for the group attached to the aromatic ring and log P is a hydrophobicity measure based on the octanol/water partition coefficient of the un-ionized form of the sulfonamide. (In writing eq 1 it has been assumed that the "indicator variables" for ortho and meta substituents used in the original Hansch equation can be omitted. These were previously included to take into account "position-dependent steric effects", but these likely will be small in the case of fluorine since the steric bulk of covalent fluorine and hydrogen are so similar.) Presuming that σ for the 2-fluoro and 3-fluoro substituents is 0.34 (Leffler & Grunwald, 1963) and that log P for a fluorine-substituted aromatic ring will be 0.15 larger than the value for the unsubstituted system (Hansch & Leo, 1979), one estimates that the association constants for inhibitors II–IV should be in the range 1×10^{-8} to 3×10^{-8} M.

If the association rate is near the values found for other aromatic sulfonamides, typically about 10^6 M^{-1} s^{-1} (Taylor et al., 1970; King & Burgen, 1976), then the dissociation lifetimes of the complexes of these inhibitors are expected to be in the range 30–100 s.

2-Fluorobenzenesulfonamide Complexes. In fluorine NMR spectra of samples containing CA I and 2-fluorobenzenesulfonamide at concentrations of inhibitor less than the concentration of enzyme, a single broad resonance is observed 1.63 ppm downfield of the position of the signal from the inhibitor in the absence of enzyme. As the amount of inhibitor relative to enzyme is increased, eventually a second signal appears in the spectrum at the position of the free inhibitor (Figure 1). Inhibitor molecules are, thus, in slow exchange between free and bound states, consistent with expectations for the dissociation rate indicated above. By considering peak intensities exhibited by a series of samples with different inhibitor/enzyme concentration ratios, it was determined that the stoichiometry of interaction of II with CA I is 2 mol of inhibitor bound per mole of enzyme.

With CA II a slightly larger shift difference (1.72 ppm) between the free and bound forms of II was observed (Table I). This system was also in the slow-exchange limit and was found to have a 2:1 stoichiometry.

3-Fluorobenzenesulfonamide Complexes. The behavior of 3-fluorobenzenesulfonamide is similar to that observed with the 2-fluoro isomer except that in this case the resonance for the bound inhibitor molecules appears at 2.49 ppm *upfield* (Figure 1c) of the shift of the free inhibitor. The chemical shift change on binding is virtually identical for the CA I and CA II complexes, but spin–lattice relaxation in the CA II complex of the 3-fluoro compound appears to be detectably more efficient than in the CA I complex (Table I). In both

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

fluoro substituents and enzyme	chemical shift (ppm)[b] free	chemical shift (ppm)[b] bound	excess line width (Hz)[c]	T_1 (s) free	T_1 (s) bound	$^{19}F[^1H]$ NOE bound
2-fluoro						
CA I	6.40	4.77	48 (84)	4.0	0.40	−0.90
CA II	6.40	4.68		4.0	0.35	
3-fluoro						
CA I	4.68	7.17	25 (70)	4.0	0.35	−0.75
CA II	4.68	7.18		4.0	0.23	
2-fluoro,3-fluoro mixture[d]						
CA I		4.78	30 (120)			
		7.08	15 (60)			
2,5-difluoro[e]						
CA I	12.18	11.18	75 (210)	4.5	0.35	−0.75
	10.68	13.98	35 (75)	4.5	0.35	−0.75
CA II	12.18	6.96		4.5	0.20	
	10.68	12.88		4.5	0.20	

[a] Data obtained at 282.3 MHz unless otherwise noted. Sample temperature was 25 °C, "pH" 7.0, in D_2O. [b] Chemical shifts are measured relative to the chemical shift of free 4-fluorobenzenesulfonamide. Positive numbers correspond to upfield shifts. [c] Line width in excess of the line width of the free inhibitor in the absence of enzyme. Values in parentheses were observed at 470 MHz. All line width changes are approximate as the spectra were obtained without proton decoupling or correction for spinning artifacts (Gerig & Hammond, 1984). [d] The first set of values given are for the 2-fluoro substituent; the second, for the 3-fluoro group. [e] The first set of values given are for the 2-fluoro substituent; the second, for the 5-fluoro group.

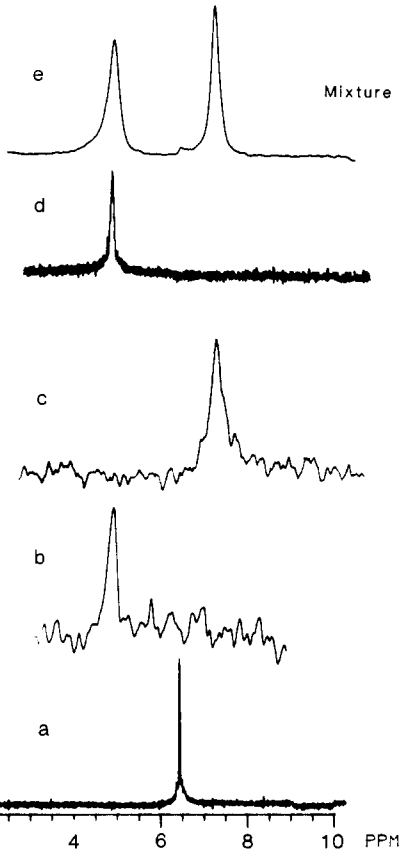


FIGURE 1: Fluorine NMR spectra at 282.3 MHz of (a) 2-fluorobenzenesulfonamide, (b) 2-fluorobenzenesulfonamide (1 mM) in the presence of CA I (0.5 mM), (c) 3-fluorobenzenesulfonamide (1 mM) in the presence of CA I (0.5 mM), (d) 3-fluorobenzenesulfonamide, and (e) a mixture of 2-fluorobenzenesulfonamide (0.5 mM) and 3-fluorobenzenesulfonamide (0.5 mM) with CA I (1 mM). All samples were in D_2O, "pH" 7.3, 25 °C.

the CA I and CA II complexes of III a 2:1 binding stoichiometry is observed.

Mixed Complexes. Both 2-fluoro- and 3-fluorobenzenesulfonamide are good inhibitors of carbonic anhydrase, and mixtures of these compounds should also be inhibitory. If each inhibitor binds to the enzyme with a stoichiometry of 2 molecules of inhibitor per molecule of enzyme and the association constants are closely matched, it should be possible to form a mixed complex in which one molecule of each inhibitor is bound to the enzyme. Figure 1 shows evidence that such a complex can be formed. When equimolar amounts of CA I, 2-fluorobenzenesulfonamide, and the 3-fluoro inhibitor are mixed together, the fluorine spectrum shown in the top trace of Figure 1 is obtained. It is clear from the chemical shifts and line widths that both inhibitors are bound to the enzyme. However, the chemical shifts observed are not exactly those found for either the 2-fluoro and 3-fluoro complex alone and suggest the formation of a discrete, new complex.

Attempts were made to observe changes in fluorine signal intensity in the spectrum of this complex by saturating in turn each signal of the spectrum. There were no reliably detected indications of magnetization transfer either by spin diffusion or generation of a fluorine–fluorine nuclear Overhauser effect.

2,5-Difluorobenzenesulfonamide Complexes. The monofluorinated benzenesulfonamides I–III exhibit 2:1 stoichiometry of binding to CA I and CA II, and the effects exerted by the protein on the chemical shifts of the bound flurine nuclei are very similar for each isozyme, with regard to both direction and magnitude. In a sense, 2,5-difluorobenzenesulfonamide (IV) combines the structural features of II and III, but in this case the complexes formed with CA I and CA II are quite distinct. The fluorine spectrum of the free inhibitor exhibits two multiplets of equal intensity at 10.68 and 12.18 ppm (Figure 2). On the basis of shift comparisons to the monofluorinated inhibitors and the expected shift effect of the additional fluorine on the ring (Emsley et al., 1966), the signal at 10.68 ppm is assigned to the 5-fluorine while the signal at higher field arises from the 2-fluorine. At a sample temperature of 25 °C and in the presence of CA I, two broad signals are observed for the bound form of IV, one of which lies under the signal for the 2-fluorine position of the free inhibitor (Figure 2a,b). The bound shifts are temperature sensitive, and the situation is clearer at 2 °C. If the signal at low field is assigned to the bound 2-fluorine, then the direction and magnitude of the observed protein-induced shifts upon binding for the 2-fluorine and the 5-fluorine are similar to those found in the 2-fluorobenzenesulfonamide and 3-fluorobenzenesulfonamide complexes.

In the complex of VI with CA II two broad signals are again observed (Figure 2c,d), one at about the position of the bound 5-fluorine indicated above. By implication, the other signal arises from the 2-fluoro substituent, but regardless of the

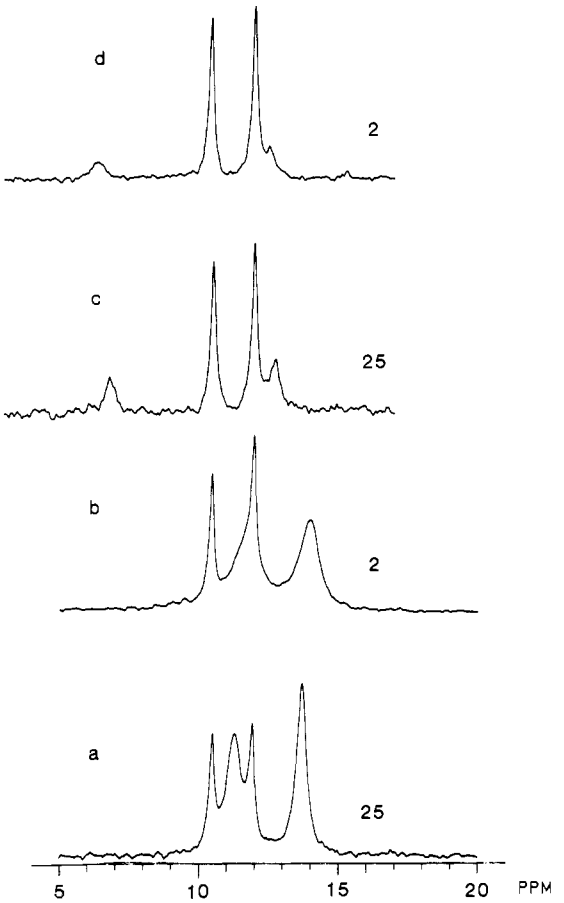


FIGURE 2: Fluorine spectra at 470 MHz of 2,5-difluorobenzenesulfonamide with CA I (a and b) and CA II (c and d). For the CA I spectra the enzyme concentration was 0.5 mM with the inhibitor 1.5 mM while for the CA II complexes the enzyme concentration was 0.3 mM with 0.9 mM inhibitor present. Samples were in D_2O, "pH" 7.2, 25 °C, with temperatures as indicated.

assignment of signals in this spectrum, it is clear that the protein environment for this nucleus in the CA II isozyme is very different from that experienced in CA I. Again, in this complex the chemical shifts of bound inhibitor are temperature dependent.

Attempts to clarify the tentative assignments of the shifts of bound 2,5-difluorobenzenesulfonamide in its CA I and CA II complexes by saturation-transfer experiments were not successful. While it was possible to saturate selectively an appropriately resolved bound or free signal, there was no detectable transfer of saturation to another signal in the spectrum. These observations are consistent with a slow rate of exchange between free and bound forms and indicates that spin diffusion from fluorine to fluorine is not rapid.

The stoichiometry of binding of IV to CA I and CA II was examined by using the relative intensities of the free and bound signals and was found in both systems to be 2 molecules of inhibitor bound per molecule of enzyme.

Fluorine Relaxation. Table I records fluorine T_1 relaxation data for each of the complexes examined in this work. As was the case with I, there is a dramatic shortening of fluorine T_1 upon binding of an inhibitor to carbonic anhydrase. Fluorine–proton nuclear Overhauser effect data were also determined for the CA I complexes and were found to be −0.8 to −0.9, consistent with results obtained with the 4-fluoro inhibitor, and indicating that fluorine–proton dipolar interactions are the dominant feature of relaxation in these complexes. Line width changes upon binding to the enzyme are also listed in Table I. The rather limited amounts of CA II that were

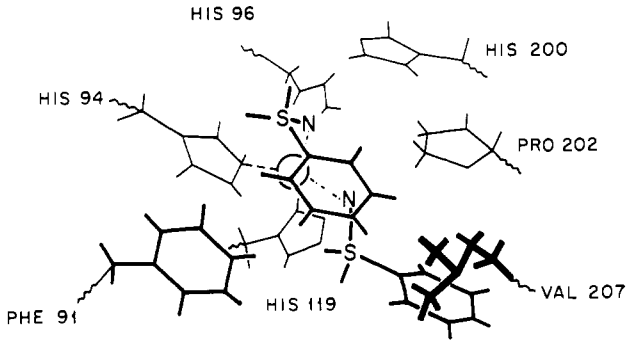


FIGURE 3: Schematic representation of the structure of a 2:1 inhibitor/CA I complex deduced by molecular graphics studies. A photograph of a screen display was the starting point for preparation of this drawing.

available during the course of these investigations precluded determination of all of these parameters for CA II.

Computer Graphics Studies. Observations made with the 4-fluoro system indicated that both bound benzenesulfonamides were present at the active site as anions and that likely both coordinated to the active site metal (Kanamori & Roberts, 1983; Blackburn et al., 1985; Dugad & Gerig, 1988). A reasonable question is whether or not such a structure is feasible within the context of the known tertiary structure of the protein. This question was addressed by molecular graphics. We first examined the published structure of the CA I–sulfanilamide complex and then sought to find a bivalent inhibitor complex in which (1) the basic tertiary structure of the enzyme is retained, (2) two molecules of sulfonamide are within coordination distance of the zinc of the active site, and (3) the geometry of coordination around the zinc is chemically reasonable.

X-ray crystallographic results indicated that sulfonanilamide (4-aminobenzenesulfonamide) forms a 1:1 complex with CA I. The inhibitor is bound to the active site region of the protein with the sulfonamide nitrogen within coordination distance of the active site zinc. One of the sulfonamide oxygen atoms is also proximate enough to the metal to suggest that a coordinate interaction of this atom with the metal is possible (Kannan et al., 1977). By use of the atomic coordinates available the energy minimization feature of the BioGraf software was used to make small adjustments of the protein tertiary structure and the location of the inhibitor in order to minimize the potential energy of this structure. At the completion of this process the empirical potential energy (due mostly to van der Waals and electrostatic terms) was reduced from $>10^6$ to 38 kcal/mol. The average change in the coordinates of atoms within 1 nm of the active site zinc after this process was 0.017 nm, well within the experimental error of the placement of the atomic coordinates by the crystallography.

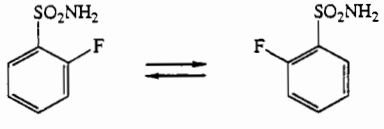
Two molecules of benzenesulfonamide were then generated, and the position of each was adjusted manually until clashes of the inhibitors with themselves and with the protein were minimum. Energy minimization by computer followed, finally producing the structure shown schematically in Figure 3. In this structure the empirical energy is 26 kcal/mol and the average change in the coordinates of the atoms within 1 nm of the zinc was 0.032 nm, relative to the values for the structure of the protein stored in the Brookhaven Protein Data Bank. In this structure a trigonal bipyrimidal coordination for zinc is observed, although *this was not forced in any way* by the process of construction of this model for the inhibited enzyme. The conclusion from these graphics studies is that formation of complexes between CA I and two benzenesulfonamide

molecules *is* feasible although it remains, of course, for crystallographic work to determine whether the structure postulated by Figure 3 is correct or not.

Discussion

The stoichiometry of binding of the anions acetate and bicarbonate to bovine CA I has been found to be 2 anions per molecule of enzyme (Lanir & Navon, 1974; Yeagle et al., 1975). The evidence suggests that both anions in each complex are bound at or near the metal atom of the active site but that the bound anions are not in chemically or magnetically equivalent environments. Aromatic sulfonamides likely bind to carbonic anhydrase as anions (Kanamori & Roberts, 1983), but until our report of the 2:1 binding of 4-fluorobenzenesulfonamide (I) to CA I and CA II, experimental investigations heretofore have indicated a 1:1 binding stoichiometry for these complexes. In the present work we provide evidence that three other fluorinated benzenesulfonamides (II–IV) also bind to both of these isozymes with a 2:1 stoichiometry. The simultaneous binding of two molecules of inhibitor is particularly strongly indicated by the experiment wherein one molecule of 2-fluorobenzenesulfonamide and one molecule of 3-fluorobenzenesulfonamide, per molecule of enzyme, are bound; in this case chemical shift changes can most reasonably be explained by the formation of a ternary complex.

All of the relaxation data (T_1, line widths, and NOE) reported in Table I are consistent with strong interactions between the enzyme and bound inhibitor molecules. We have previously reported that in the complex formed between pentafluorobenzenesulfonamide and CA I these interactions are sufficiently potent that rotation of the pentafluoro aromatic ring about its C_1–C_4 axis is strongly hindered (Gerig & Moses, 1987). Inhibitors II–IV can present two conformations to the active site of carbonic anhydrase, as indicated below. Given the very large protein-induced fluorine chemical shift effects that we have observed with I–IV, it seems likely that if significant quantities of each of the respective rotationally isomeric structures were bound to the enzyme, one would observe two bound signals for each fluorine of an inhibitor molecule. In compounds II–IV only one set of bound signals is observed, implying that one rotational isomer is preferentially bound to the enzyme to the extent that detection of the other isomer is not feasible. Since the bound fluorine chemical shift effects of the 2-fluoro and 3-fluoro inhibitors are in opposite directions, we thought it possible that when both of these substituents were present in the same molecule, the energy difference between bound forms of the two rotational isomers would be reduced and inhibitor IV would provide NMR evidence for binding of the two rotamers. This was not the case, however, and a careful search for additional signals in the fluorine spectrum of IV produced no indication of a second bound form. Our observations with II–IV are, to be sure, equally consistent with rapid aromatic ring rotation of the bound inhibitors, but in light of the results with the pentafluorinated inhibitor, this seems unlikely.



IIa IIb

This work indicates that the 2:1 binding stoichiometry observed with inhibitor I is not peculiar to that particular system. Although many of the aromatic sulfonamide complexes with CA I and CA II that we have examined including those described herein show the same 2:1 stoichiometry, we have found that other, structurally very similar molecules have a 1:1 stoichiometry (L. B. Dugad and D. Veenstra, preliminary work). There seems to be a rather delicate balance of features in the inhibitor molecule that determines which stoichiometry will be observed; we hope additional work will define these factors and produce reliable means of predicting what stoichiometry will be exhibited by a given inhibitor and isozyme combination. The relationship between these binding interactions detected by NMR and the inhibition of enzymic activity also remains to be demonstrated.

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Resonance Raman Assignment and Evidence for Noncoupling of Individual 2- and 4-Vinyl Vibrational Modes in a Monomeric Cyanomethemoglobin†

Klaus Gersonde,*,‡ Nai-Teng Yu,*,§ Shun-Hua Lin,§ Kevin M. Smith,∥ and Daniel W. Parish∥

Hauptabteilung Medizintechnik, Fraunhofer-Institut für zerstörungsfreie Prüfverfahren, D-6600 Saarbrücken, West Germany, Fachrichtung Medizintechnik, Universität des Saarlandes, D-6650 Homburg/Saar, West Germany, School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332, and Department of Chemistry, University of California, Davis, California 95616

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ABSTRACT: We have investigated the resonance Raman spectra of monomeric insect cyanomethemoglobins (CTT III and CTT IV) reconstituted with (1) protohemes IX selectively deuterated at the 4-vinyl as well as the 2,4-divinyls, (2) monovinyl-truncated hemes such as pemptoheme (2-hydrogen, 4-vinyl) and isopemptoheme (2-vinyl, 4-hydrogen), (3) symmetric hemes such as protoheme III (with 2- and 3-vinyls) and protoheme XIII (with 1- and 4-vinyls), and (4) hemes without 2- and 4-vinyls such as mesoheme IX, deuteroheme IX, 2,4-dimethyldeuteroheme IX, and 2,4-dibromodeuteroheme IX. Evidence is presented that the highly localized vinyl C=C stretching vibrations at the 2- and 4-positions of the heme in these cyanomet CTT hemoglobins are noncoupled and inequivalent; i.e., the 1631- and 1624-cm^{-1} lines have been assigned to 2-vinyl and 4-vinyl, respectively. The elimination of the 2-vinyl (in pemptoheme) or the 4-vinyl (in isopemptoheme) does not affect the C=C stretching frequency of the remaining vinyl. Furthermore, two low-frequency vinyl bending modes at 412 and 591 cm^{-1} exhibit greatly different resonance Raman intensities between 2-vinyl and 4-vinyl. The observed intensity at 412 cm^{-1} is primarily derived from 4-vinyl, whereas the 591-cm^{-1} line results exclusively from the 2-vinyl. Again, there is no significant coupling between 2-vinyl and 4-vinyl for these two bending modes.

The 2- and 4-vinyl groups of iron protoporphyrin IX (protoheme IX) in hemoglobins play an important role in modulating heme reactivity through protein-heme interactions (Gersonde, 1983; Gersonde et al., 1986). The orientation of each vinyl (relative to the heme plane) and the nature of protein-vinyl interactions have been probed by nuclear magnetic resonance (NMR) spectroscopy (La Mar et al., 1978). On the other hand, resonance Raman spectroscopy provides useful information pertaining to the electronic and bonding nature of the vinyl groups in various ligated and unligated hemoglobins (Kerr et al., 1985; Gersonde et al., 1986, 1987; Tanaka et al., 1987).

The vibrational modes associated with the 2- and 4-vinyl groups have been assigned by Spiro and co-workers (Choi et al., 1982a,b; Choi & Spiro, 1983) in their resonance Raman studies on nickel(II) protoporphyrin IX dimethyl ester and its derivatives deuterated at the α-carbon as well as β-carbon atoms of the 2- and 4-vinyl groups. The identification of vinyl vibrational modes has been already made in monomeric insect (Kerr et al., 1985; Gersonde et al., 1986) and tetrameric human (Rousseau et al., 1983) hemoglobins. However, in these studies nonselective deuteration of vinyls in positions 2 and 4 did not allow the assignment of vibrational modes of individual vinyl groups. It is of fundamental interest to know whether or not the two vinyls in hemoglobins are vibrationally coupled.

In this paper, we demonstrate that certain Raman lines in the spectra of cyanomet CTT III can be assigned to 2- and 4-vinyl, respectively. Most importantly, we found that there is no significant coupling between the vibrational modes of 2- and 4-vinyl. The highly localized vinyl C=C stretching vibrations at the 2- and 4-positions are inequivalent. These assignments were made possible by the availability of the following synthetic hemes: 4-vinylprotoheme IX-α,β,β-d_3; monovinyl-truncated hemes, such as pemptoheme (2-vinyl is substituted by hydrogen) and isopemptoheme (4-vinyl is replaced by hydrogen); and symmetric hemes, such as protoheme III (with 2- and 3-vinyl) and protoheme XIII (with 1- and 4-vinyl). In addition, we compare resonance Raman spectra of hemoglobins with identical substituents at postions 2 and 4 (proto-IX, meso-IX, deutero-IX, 2,4-dimethyldeutero-IX, and 2,4-Br_2-deutero-IX CTT IV). We believe that an understanding of the vibrational properties of individual 2- and 4-vinyl is an essential step toward elucidating their role in mediating heme-protein interactions.

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* To whom correspondence should be addressed.
‡ Fraunhofer-Institut für zerstörungsfreie Prufverfahren and Universität des Saarlandes.
§ Georgia Institute of Technology.
∥ University of California.

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