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Nitrogen-15 Nuclear Magnetic Resonance Study of Benzenesulfonamide and Cyanate Binding to Carbonic Anhydrase†

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ABSTRACT: The binding of inhibitors, cyanate and benzenesulfonamide, to the active-site zinc of human carbonic anhydrase B was studied by ^{15}N nuclear magnetic resonance spectroscopy. The cyanate nitrogen resonance moved 34 ppm upfield on binding to the enzyme. The shielding is comparable to that reported for a zinc–isocyanate complex and strongly suggests complexation of cyanate to zinc through nitrogen. The proton-coupled ^{15}N resonance of the enzyme-bound benzenesulfonamide was a doublet. Hence, benzenesulfonamide is bound as $C_6H_5SO_2NH^-$. The proton-decoupled ^{15}N resonance of the bound benzenesulfonamide was observed 17 ppm upfield of that of free benzenesulfonamide anion. A model ligand, 2-aminobenzenesulfonamide anion, undergoes binding of zinc through the sulfonamide nitrogen which results in an 11.8 ppm shielding of the ^{15}N resonance. In contrast, *N*-(2-aminophenyl)benzenesulfonamide, which is reported to bind zinc through an oxygen, has its sulfonamide nitrogen deshielded by 4.3 and 1.2 ppm on complexation of zinc to the neutral and anionic ligands, respectively. Thus, coordination to the nitrogen causes shielding and to the oxygen deshielding of the sulfonamide resonance. The observed shielding of the enzyme-bound sulfonamide resonance strongly suggests that benzenesulfonamide binds primarily to zinc through the sulfonamide nitrogen. The implications of these results for the high affinity of association of the inhibitor are discussed.

Carbonic anhydrase, a zinc metalloenzyme widespread in nature, is a highly efficient catalyst for reversible hydration of CO_2:

$$CO_2 + H_2O \rightleftarrows HCO_3^- + H^+$$

The essential zinc ion at the active site has four tightly coordinated ligands with zinc–ligand distances of ~2 Å: three imidazolyl nitrogens of histidyl residues and a water molecule. There is also a more distant (2.9 Å) fifth ligand site. The possible sixth ligand site is sterically hindered by the protein (Nostrand et al., 1975; Kannan et al., 1977). The activity of human carbonic anhydrase B is governed by the ionization of a group with a pK_a of 7.3–7.6 or higher (Coleman, 1967b; Khalifah, 1971; Bauer et al., 1976). In one proposed model for the catalytic mechanism, this ionizable group is thought to be the zinc-bound water molecule, which on deprotonation to –ZnOH is postulated to act as a nucleophile to carbon dioxide (Pocker & Sarkanen, 1978; Pocker & Deits, 1982). A possible sequence for catalysis of the hydration of carbon dioxide by carbonic anhydrase is shown in Figure 1.

The activity of carbonic anhydrase is strongly inhibited by aromatic sulfonamides. A typical inhibitor, benzenesulfon-

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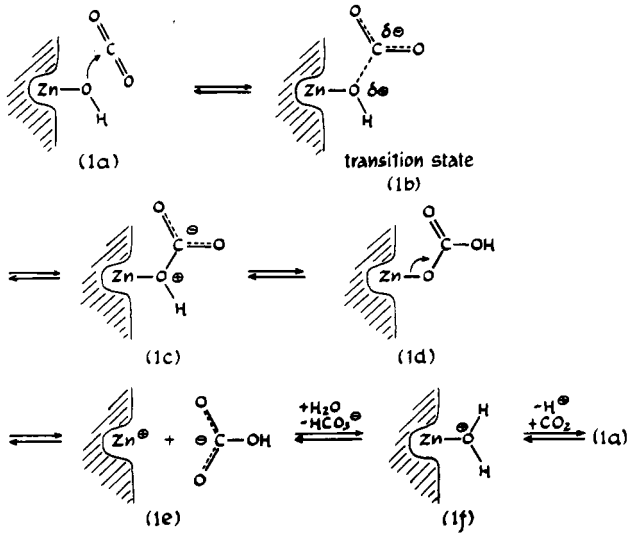

FIGURE 1: Possible sequence for catalysis of the hydration of carbon dioxide by carbonic anhydrase. Structure 1b seems most likely to be representative of the transition state for the rate-determining step.

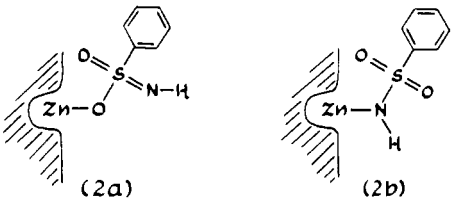

FIGURE 2: Possible modes of binding for the benzenesulfonamide–carbonic anhydrase complex.

amide ($C_6H_5SO_2NH_2$), has an affinity constant of 4.9×10^6 M^{-1} toward human carbonic anhydrase (Pocker & Sarkanen, 1978). The aromatic ring of the inhibitor appears to be important for binding; X-ray studies show it to be within van der Waals contact with side chains on the hydrophobic wall of the active site (Kannan et al., 1977). Ultraviolet (King & Burgen, 1970), fluorescence (Chen & Kernohan, 1967), and resonance Raman spectra (Kumar et al., 1976) of the enzyme–inhibitor complexes suggest that the bound sulfonamide corresponds to a $C_6H_5SO_2NH$ group and not to $C_6H_5SO_2NH_2$ itself. Recent X-ray studies showed that in a *p*-aminobenzenesulfonamide–carbonic anhydrase complex, the sulfonamide group is located within the primary coordination sphere of the zinc, at the fourth ligand site which is occupied by a water molecule in the free enzyme. However, at the resolution reported so far for the crystal structure of the complex, it is not possible to decide whether the sulfonamide coordinates to zinc through one of the oxygen atoms (Figure 2a) (Taylor et al., 1970; Pocker & Sarkanen, 1978), through the nitrogen atom (Figure 2b) (Kannan et al., 1977), or through both, with one of the oxygen atoms occupying the more distant fifth ligand site (Kannan et al., 1977).

A recent cadmium-113 nuclear magnetic resonance study of the binding of aromatic sulfonamides to cadmium(II)-substituted carbonic anhydrase provides evidence for direct binding of cadmium to the sulfonamide nitrogen through observation of ^{113}Cd–^{15}N coupling (Evelhoch et al., 1981). However, compared to the native zinc enzyme, cadmium-substituted carbonic anhydrase has only 4% activity for the hydration of CO_2 (Coleman, 1967a) and up to 30% activity for the hydrolysis of esters with the pK_a of optimum activity shifted by 1.6 pH units to the alkaline side (Bauer et al., 1976). Consequently, it is important to investigate the mode of binding of the inhibitor to the native zinc enzyme, especially because

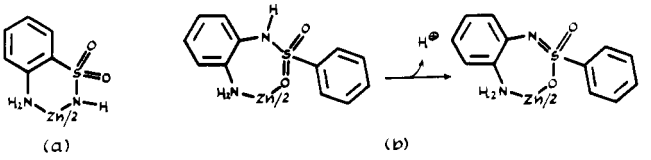

FIGURE 3: Zinc complexes of (a) 2-aminobenzenesulfonamide and (b) *N*-(2-aminophenyl)benzenesulfonamide.

it is not clear why sulfonamides have such a strong affinity for the active site of carbonic anhydrase as well as for that of alkaline phosphatase (Price, 1979, 1980), which is also a zinc enzyme.

Sulfonamide groups per se have a low affinity for metal ions, and other zinc enzymes such as carboxypeptidase and alcohol dehydrogenase do not bind sulfonamides. It has been proposed that, if the sulfonamide binds through the nitrogen (Figure 2b), its structure might be able to mimic the transition state for hydration of the substrate (Figure 1) and this may account in part for the high affinity of aromatic sulfonamides for the enzyme (Kumar et al., 1976).

Monoanions such as cyanide, sulfide, and cyanate are also powerful inhibitors of carbonic anhydrase. Among these, cyanate, which has an affinity constant of 2.5×10^6 M^{-1} for human carbonic anhydrase B (Maren & Couto, 1979), is of particular interest because of its structural similarity to the substrate carbon dioxide. Because the visible absorption spectrum of Co(II)-substituted human carbonic anhydrase B shows a striking change on binding of cyanate (Lindskog & Nyman, 1964), the inhibitor is thought to bind within the primary coordination sphere of the metal. However, no direct evidence of the nature of binding of the cyanate ion to the zinc atom of native carbonic anhydrase has been obtained.

^{15}N nuclear magnetic resonance (NMR) spectroscopy has proven very useful for studying the properties of the active sites of enzymes as well as the mode of inhibitor binding to selectively ^{15}N-enriched enzymes (Bachovchin & Roberts, 1978; Bachovchin et al., 1982). A ^{15}N NMR study of ^{15}N-enriched inhibitors, benzenesulfonamide and cyanate, bound to the native carbonic anhydrase offers the potential for obtaining quite specific information on the modes of binding through observation of ^{15}N–^{1}H coupling and comparison of the ^{15}N shifts of enzyme-bound inhibitors with those of model zinc complexes. For benzenesulfonamide, binding of zinc through the oxygen is expected to cause deshielding of the sulfonamide nitrogen because of increased sulfur–nitrogen double-bond character. By contrast, binding through the nitrogen is expected to cause a shielding of the sulfonamide nitrogen on the basis of the known effects of metal binding on amine nitrogens (Nee & Roberts, 1982). To obtain experimental evidence on the effect of the different modes of coordination on ^{15}N shifts, we have investigated zinc complexes of two benzenesulfonamide derivatives, 2-aminobenzenesulfonamide (Figure 3a) and *N*-(2-aminophenyl)benzenesulfonamide (Figure 3b).

We report here ^{15}N NMR studies of [^{15}N]cyanate and benzene[^{15}N]sulfonamide bound to native human carbonic anhydrase B, as well as studies of model zinc–sulfonamide complexes.

Experimental Procedures

Carbonic anhydrase B from human erythrocytes was purchased from Sigma Chemical Co. $^{15}NH_4Cl$ (95% enriched in ^{15}N) was purchased from Merck & Co. and K^{15}NCO (95% enriched in ^{15}N) from Koch Isotopes.

Benzenesulfonamide, 95% enriched in ^{15}N, was synthesized by addition of benzenesulfonyl chloride to a 6.3 M aqueous solution of $^{15}NH_4Cl$ in the presence of 6.3 N NaOH. Its purity

Table I: Sulfonamide ^{15}N Chemical Shifts of Free and Enzyme-Bound Benzenesulfonamide and of Model Compounds with and without Zinc

compound	solvent	^{15}N (ppm from 1 M HNO_3) alone neutral	alone anion	enzyme bound
benzenesulfonamide	H_2O	282.9	275.1	
	$(CH_3)_2SO$	279.7		
	$C_6H_5CH_3$	283.2		
benzenesulfonamide bound to carbonic anhydrase	H_2O			292.8

compound	solvent	alone neutral	alone anion	with $ZnCl_2$ 1 equiv	with $ZnCl_2$ 2 equiv
2-aminobenzenesulfonamide	$(CH_3)_2SO$	279.2	271.2	283.0	
N-(2-aminophenyl)benzenesulfonamide	$(OCH_2CH_2)_2$	266.4		263.7	262.1
			248.4		247.2
	$(OCH_2CH_2)_2/H_2O$	266.1 (2.4)[a]	252.9 (10.5)		
		267.3 (4.4)	247.6 (11.4)	266.8 (5.5)	
		267.3 (6.0)		265.8 (6.0)	
		265.5 (8.6)		266.6 (7.7)	
				247.2 (11.0)	

[a] Values in parentheses are pHs.

was checked by the melting point and by ^{15}N NMR spectroscopy. For the synthesis of 2-aminobenzenesulfonamide (95% enriched in ^{15}N at the sulfonamide nitrogen), *o*-nitrobenzenesulfonyl chloride was added to a 6.3 M aqueous solution of $^{15}NH_4Cl$ in the presence of 6.3 N NaOH. The resulting *o*-nitrobenzene[^{15}N]sulfonamide was crystallized from ethanol/H_2O and had a melting point of 190 °C. Reduction of the nitro group of this substance in refluxing ethanol/H_2O with iron and hydrochloric acid yielded 2-aminobenzene[^{15}N]sulfonamide (crystallized from H_2O, mp 152 °C). The sodium salt of 2-aminobenzene[^{15}N]sulfonamide anion was prepared by titration of an aqueous solution of the neutral compound with NaOH to pH 12.0 and lyophilization of the solvent.

N-(2-Aminophenyl)benzenesulfonamide was synthesized through the reaction of benzenesulfonyl chloride with *o*-phenylenediamine (Amundsen, 1937). For the synthesis of the same compound enriched in ^{15}N at the sulfonamide nitrogen, a mixture of the sodium salt of benzene[^{15}N]sulfonamide (0.2 M) and equimolar *o*-(nitrochloro)benzene was heated under reflux in *N*,*N*-dimethylacetamide in the presence of 0.2 M CuI. The resulting *N*-(2-nitrophenyl)benzenesulfonamide was reduced with iron and hydrochloric acid and yielded *N*-(2-aminophenyl)benzene[^{15}N]sulfonamide (crystallized from ethanol/H_2O, mp 168 °C).

p-Dioxane was distilled from 8-hydroxyquinoline to remove traces of paramagnetic metal ions. Dimethyl sulfoxide was dried over molecular sieves, while zinc chloride was "ultrapure" grade from Alpha Products.

The NMR sample of the carbonic anhydrase–benzene-[^{15}N]sulfonamide complex was prepared by adding benzene-[^{15}N]sulfonamide to a final concentration of 8 mM to a 2.2 mM aqueous solution of carbonic anhydrase containing 0.05 M tris(hydroxymethyl)aminomethane at pH 8.5. The NMR sample of the carbonic anhydrase–^{15}NCO complex was prepared by adding $K^{15}NCO$ to a final concentration of 10 mM to a 2 mM aqueous solution of carbonic anhydrase containing 0.05 M tris(hydroxymethyl)aminomethane at pH 8.8.

The ^{15}N NMR spectra of the enzyme–inhibitor complexes were obtained with a Bruker WM-500 spectrometer operating at 50.65 MHz. All chemical shifts are reported in ppm upfield of 1 M HNO_3. For the spectra of the enzyme–benzenesulfonamide complex, the operating conditions employed a 90° pulse and a 0.4–s repetition rate for proton-coupled spectra and a 1-s repetition rate for proton-decoupled spectra (decoupling during data acquisition only). The sample was maintained at 25 °C. For the spectra of the enzyme–cyanate complex, the operating conditions employed a 90° pulse and 0.53-s repetition rate, with the sample temperature at 2 °C.

Cyanate has been shown to react with functional groups of proteins, sulfhydryl and α-amino groups being the most reactive at pH 7–9 (Stark, 1972). In human carbonic anhydrase B, the single sulfhydryl group is buried and not susceptible to chemical modification (Rickli & Edsall, 1962). Amine groups of carbonic anhydrase undergo modification at high cyanate concentrations (Osborne & Tashian, 1975). However, rate measurements (Stark, 1972) show that 50% modification of an α-amino group of a peptide requires approximately 8 h at 30 °C at 10 mM concentration of cyanate. At 2 °C, this kind of reaction is expected to be significantly slower. Therefore, under our experimental conditions, little, if any, modification of the free α-amine or other functional groups of carbonic anhydrase is expected to occur.

The ^{15}N NMR spectra of benzenesulfonamide derivatives and their zinc complexes were obtained on a Bruker WH-180 spectrometer operating at 18.25 MHz. The operating conditions employed a 70-μs pulse width (90° flip angle) and a 5-s delay. The spectra of *N*-(2-aminophenyl)benzenesulfonamide in dioxane and *N*-(2-aminophenyl)benzene[^{15}N]-sulfonamide in dioxane/H_2O were taken at 60 and 70 °C, respectively, to maintain sufficient concentration of the solutes.

Results

The ^{15}N chemical shift of benzenesulfonamide has been measured at the natural-abundance level in organic solvents (Schuster et al., 1978). However, because of its low solubility in water, ^{15}N enrichment was required to obtain the shift in 5 mM aqueous solution (see Table I). Benzenesulfonamide has a pK_a of 9.95 (Kakeya et al., 1969). Its neutral and anionic forms have ^{15}N shifts of 282.9 and 275.1 ppm, respectively. In the proton-coupled ^{15}N spectra, these peaks are singlets because the sulfonamide protons exchange rapidly with water protons on the NMR time scale. However, in dimethyl sulfoxide solution, where proton exchange is slow, a $^1J_{N-H}$ of 80.0 Hz has been observed for benzenesulfonamide (Schuster et al., 1978).

Enzyme–Benzenesulfonamide Complex. Typical ^{15}N spectra of the benzenesulfonamide–carbonic anhydrase complex at pH 8.5 are shown in Figure 4. Two peaks were observed in the proton-decoupled spectrum (Figure 4a). The

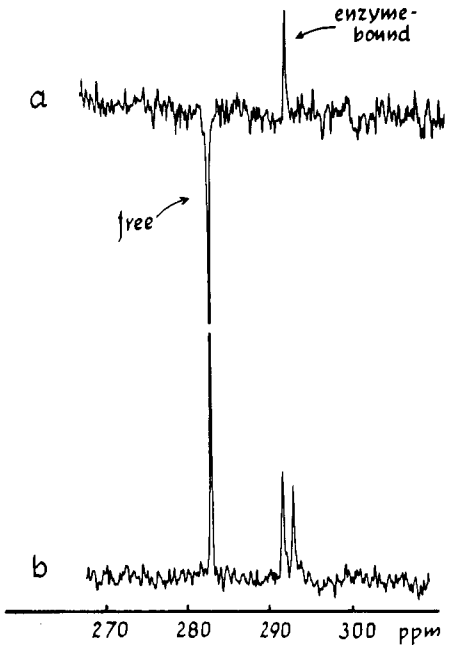

FIGURE 4: ^{15}N spectra of free and enzyme-bound benzene[^{15}N]-sulfonamide obtained at 50.65 MHz of a 2.2 mM aqueous solution of human carbonic anhydrase B containing 8 mM benzene[^{15}N]-sulfonamide at pH 8.5. (a) Proton-decoupled spectrum after 3472 scans; (b) proton-coupled spectrum after 86 460 scans.

peak at 282.3 ppm is very close to that of free neutral benzenesulfonamide in water and is assigned to unbound inhibitor. The peak at 292.8 ppm represents the enzyme-bound benzenesulfonamide. The shielding is not caused by the hydrophobic environment at the active site, because in a nonpolar solvent such as methylbenzene, the ^{15}N shift of benzenesulfonamide is very close to that in water (Table I). When the protons are coupled to nitrogen (Figure 4b), this peak splits into a doublet with a $^1J_{N-H}$ of 73 Hz while the unbound benzenesulfonamide peak remains a singlet. The doublet structure clearly shows that the enzyme-bound inhibitor has an –NH– group at pH 8.5, although the free inhibitor is very largely in the neutral form at this pH. Compared to free benzenesulfonamide anion (^{15}N = 275.1 ppm), binding to the enzyme causes a 17.7 ppm shielding of the benzenesulfonamide nitrogen. Furthermore, the N–H coupling observed for the enzyme-bound inhibitor shows that the sulfonamide proton exchanges slowly on the NMR time scale with water protons, in contrast to the unbound benzenesulfonamide protons which exchange rapidly.

Model Sulfonamide–Zinc Complexes. Figure 3 shows the structures of zinc complexes of two benzenesulfonamide derivatives, 2-aminobenzenesulfonamide and *N*-(2-aminophenyl)benzenesulfonamide. These bidentate ligands were selected because benzenesulfonamide itself does not form a zinc complex.

2-Aminobenzenesulfonamide is known, on the basis of infrared studies, to form a 2:1 complex with zinc, in which the metal ion is coordinated to both sulfonamide and amine nitrogens (Kidani et al., 1973) (Figure 3a). The ^{15}N chemical shifts in dimethyl sulfoxide solution of the sulfonamide nitrogen of the neutral and anionic forms of this sulfonamide and on addition of 1 equiv of zinc chloride to the anionic form are shown in Table I. Representative ^{15}N spectra are shown in Figure 5. For the neutral form, the sulfonamide nitrogen is observed at 279.2 ppm in the proton-decoupled spectrum (Figure 5a). With proton coupling, the peak splits into a triplet with a $^1J_{N-H}$ of 81 Hz (Figure 5b). In the anionic form, the

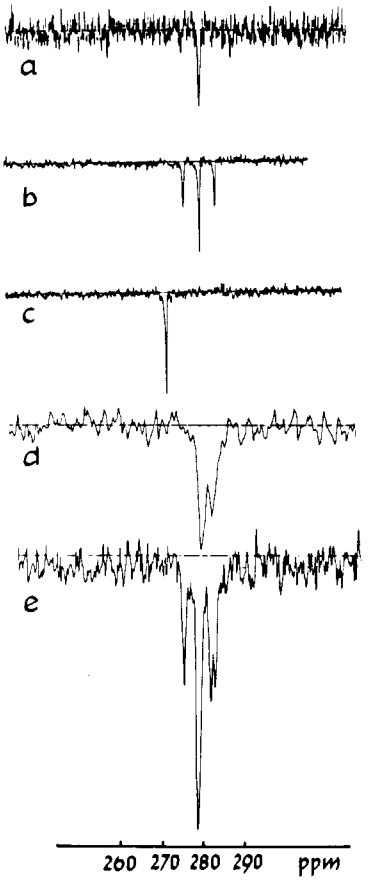

FIGURE 5: ^{15}N spectra of 5 mM 2-aminobenzene[^{15}N]sulfonamide and its zinc complex in dimethyl sulfoxide solution at 18.25 MHz. (a) Free neutral 2-aminobenzene[^{15}N]sulfonamide, with proton decoupling. (b) The same as (a), with proton-coupling. (c) Free 2-aminobenzene[^{15}N]sulfonamide anion, with proton decoupling. (d) On addition of 1 equiv of zinc chloride to (c), with proton decoupling. (e) The same as (d), with proton coupling.

sulfonamide nitrogen is deshielded to 271.2 ppm (Figure 5c) and is a singlet with protons coupled. A singlet is expected when there is rapid exchange of the single sulfonamide proton with water catalyzed by hydroxide ion. On addition of 1 equiv of zinc chloride to the anion, the proton-decoupled spectrum shows two peaks, at 280.0 and 283.0 ppm (Figure 5d). With protons coupled, the 280 ppm peak splits into a triplet ($^1J_{N-H}$ = 83 Hz), and the 283 ppm peak remains a singlet (Figure 5e). The 280 ppm peak can, on the basis of its chemical shift and its triplet structure, be assigned to free neutral ligand formed by protonation of the anion. The protons are probably those released by reaction of zinc chloride with traces of water present in the dimethyl sulfoxide and zinc chloride, although the solvent was dried with molecular sieves and the sample prepared under dry nitrogen. The 283 ppm peak is 3–4 ppm upfield of the free neutral ligand and can most reasonably be assigned to the sulfonamide nitrogen complexed to zinc. Thus, coordination of zinc to the free anion (^{15}N = 271.2 ppm) causes a *shielding* of 11.8 ppm in the sulfonamide nitrogen under these conditions. Although the magnitude of shielding is smaller than that observed for the enzyme-bound benzenesulfonamide (17.7 ppm relative to the free anion), the shift change demonstrates that coordination of zinc to the anionic sulfonamide nitrogen causes a substantial upfield shift and the shift is greater than that for protonation.

The singlet structure of the zinc-bound sulfonamide nitrogen resonance in the proton-coupled spectrum indicates that the sulfonamide proton in the complex undergoes a rapid base-catalyzed exchange with water in contrast to the slowly ex-

changing protons of the free neutral sulfonamide. The fast exchange is probably the result of an increased acidity of the sulfonamide group on coordination with zinc. For ammonium ion, replacement of one of the protons with a metal ion has been shown to lower the pK_a by 2 pH units (Grunwald & Fong, 1972). At a given pH, protons on more acidic groups exchange faster with water protons (Blomberg et al., 1976).

The second model compound, *N*-(2-aminophenyl)benzenesulfonamide, is expected to form a 2:1 zinc complex, coordinating through the sulfonamide oxygen and the amine nitrogen (Figure 3b). The mode of metal binding has been demonstrated on the basis of infrared studies for its close structural analogue, *N*-(2-aminophenyl)-4-methylbenzenesulfonamide, which forms a green zinc complex in dioxane/H_2O (Betteridge & Rangaswamy, 1968). Table I shows the ^{15}N shifts of 5 mM *N*-(2-aminophenyl)benzenesulfonamide in the absence and presence of zinc chloride at various pH values in 50% (v/v) dioxane/H_2O. The acid dissociation constants can reasonably be expected to be close to those of *N*-(2-aminophenyl)-4-methylbenzenesulfonamide whose NH_3^+ group has a pK_a of 2.7 and whose $-SO_2NHR$ group has a pK_a of 9.8 in dioxane/H_2O. Therefore, at pH 4–8, N-(2-aminophenyl)-benzenesulfonamide is neutral with the sulfonamide ^{15}N resonance at 267.3 ppm. On deprotonation of the sulfonamide group in the pH range 8.5 to ~11.4, the sulfonamide nitrogen shifts approximately 20 ppm downfield. Solutions of the free ligand in its acidic and neutral forms are colorless and yellow when in the anionic form.

On addition of 1 equiv of zinc chloride at pH 2.3, the solution is colorless but then turns green when the pH is increased to 5.5. At this point, the sulfonamide nitrogen, relative to that of the free ligand, shows a 1.5 ppm downfield shift at pH 6.0 (Table I). Thus, complexation through the sulfonamide oxygen causes a small downfield shift of the sulfonamide nitrogen. At this pH, the ligand is likely to bind in the neutral form (as shown on the left-hand side of Figure 3b). If the deprotonation of the sulfonamide had occurred on binding of zinc, a much greater deshielding of the sulfonamide ^{15}N resonance would be expected because, as will be shown shortly, the ^{15}N resonance of the zinc complex of *N*-(2-aminophenyl)benzenesulfonamide anion in dioxane is deshielded to 247.2 ppm (Table I). The downfield shift of the neutral ligand on binding to zinc is probably due to the increased contribution of the resonance structure $-HN^+{=}S(=O)-O-Zn-$. When the pH is increased to 7.7, the sulfonamide nitrogen shifts slightly upfield (Table I) and precipitation occurs. This is caused by partial dissociation of the zinc–sulfonamide complex as a result of competition of hydroxide ions for zinc and formation of $Zn(OH)_2$. Similar dissociation of the zinc complex of *N*-benenesulfonylglycine in 1:1 (v/v) water:ethanol solution above pH 7.8 has been observed by potentiometric titration (Ghosh & Majumder, 1963). When the pH is increased to 11.0, the sulfonamide nitrogen in the zinc mixture moves downfield as the result of deprotonation of the sulfonamide groups to 247.2 ppm, which is very close to that of the free anion (247.6 ppm). At this high pH, most of the zinc ions are likely to have dissociated from sulfonamide to form $Zn(OH)_2$.

The formation of the Zn–*N*-(2-aminophenyl)benzenesulfonamide complexes at pH 6.0 in dioxane/H_2O is probably incomplete because of the low concentration (0.005 M) employed and the competition of hydroxide ions for zinc. The observed ^{15}N shift thus represents a weighted average of bound and free ligands. To optimize complex formation, the ^{15}N chemical shifts of neutral *N*-(2-aminophenyl)benzenesulfonamide in the absence and presence of zinc chloride were investigated in dioxane in which the ligand is soluble to the extent of 0.67 M concentration. The results are listed in Table I. The solution of the neutral compound in dioxane is colorless, and the sulfonamide nitrogen resonance is observed at 266.4 ppm in a proton-decoupled spectrum and as a doublet ($^1J_{N-H}$ of 85 Hz) in a proton-coupled spectrum. The coupling demonstrates that the compound has a $-SO_2NH-$ group and not the $-S(=O)(OH)=N-$ group proposed for *N*-(2-aminophenyl)-4-methylbenzenesulfonamide in dioxane/H_2O (Betteridge & Rangaswamy, 1968). On addition of zinc chloride, the solution turned green, and the sulfonamide nitrogen resonance moved downfield by 2.7 and 4.3 ppm at 1:1 and 1:2 ligand:Zn ratios, respectively. These shifts show that formation of the zinc complex through the sulfonamide oxygen causes a signficant *downfield* shift of the sulfonamide nitrogen. The amine nitrogen resonance, on the other hand, which comes at 322.9 ppm in the free ligand, moves 4.2 and 6.4 ppm upfield on addition of 1 and 2 equiv of zinc chloride, respectively. Such shielding is expected if the zinc coordinates to the amine nitrogen as well as to the sulfonamide oxygen, because aniline-type nitrogens show upfield shifts on coordination to metal ions (Nee & Roberts, 1982).

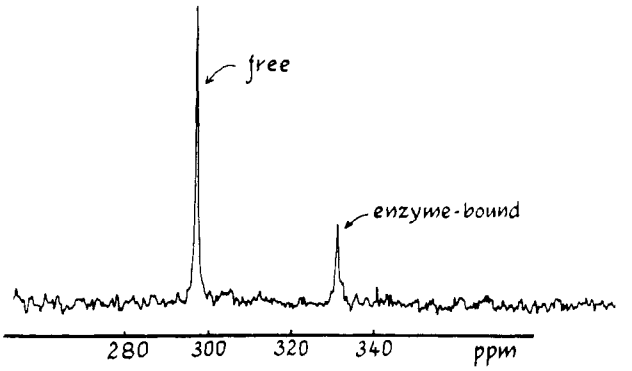

FIGURE 6: Proton-coupled ^{15}N spectrum of free and enzyme-bound [^{15}N]cyanate obtained at 50.65 MHz on a 2 mM aqueous solution of human carbonic anhydrase B containing 10 mM $K^{15}NCO$ at pH 8.8, after 82 303 scans.

The ^{15}N shifts of anionic *N*-(2-aminophenyl)benzenesulfonamide in dioxane were obtained at 5 mM concentration with the ^{15}N-labeled compound because of its low solubility. On addition of 2 equiv of zinc chloride, a 1.2 ppm downfield shift of the sulfonamide nitrogen was observed (Table I). The smaller extent of deshielding than for the neutral complex is probably due to incomplete complexation at the low concentration.

In summary, coordination of zinc to the sulfonamide oxygen of *N*-(2-aminophenyl)benzenesulfonamide results in a green complex with concomitant deshielding of the sulfonamide nitrogen for either neutral or anionic ligand.

Enzyme–Cyanate Complex. The ^{15}N spectrum of the cyanate–carbonic anhydrase complex at pH 8.8 is shown in Figure 6. The peak at 297.6 ppm is very close to that of free cyanate in aqueous solution (Table II) and is assigned to unbound inhibitor. The peak at 331.6 ppm represents that of the enzyme-bound cyanate. Thus, the cyanate nitrogen resonance moves 34 ppm upfield on binding to the enzyme. Table II shows these chemical shifts and, for comparison, the ^{14}N shifts of model zinc isocyanate, alkyl isocyanates, and alkyl cyanates obtained by Chew et al. (1970). The shift of the enzyme-bound cyanate nitrogen is close to that in $[(C_2H_5)_2NH_2^+]_2[Zn(NCO)_4]^{2-}$ at 325 ± 2 ppm. For this compound, X-ray and infrared studies have shown the NCO^-

Table II: Nitrogen Chemical Shifts of Enzyme-Bound Cyanate, Model Cyanates, and Isocyanates

compound	solvent	N (ppm from 1 M HNO_3)
enzyme-bound cyanate	H_2O, pH 8.8	331.6[a]
enzyme-bound cyanate	H_2O, pH 7.0	331.8[a]
KNCO	H_2O, pH 8.8	298.6[a]
$[(C_2H_5)_2NH_2^+]_2[Zn(NCO)_4]^{2-}$	$(CH_3)_2CO$	325 ± 2[b]
HNCO	$(C_2H_5)_2O$	351 ± 2[b]
C_2H_5NCO	neat	346 ± 1[b]
$NCOC_2H_5$	$(C_2H_5)_2O$	222 ± 1[b]

[a] ^{15}N chemical shifts obtained in the present work. [b] ^{14}N chemical shifts from Chew et al. (1970).

to be coordinated to zinc through nitrogen in a tetrahedral configuration with linear Zn–NCO groupings (Forster & Goodgame, 1963, 1964). Thus, the observed ^{15}N shift of the enzyme-bound inhibitor can most reasonably be interpreted as due to complexation of the cyanate to zinc through the nitrogen. Although the nitrogen in HNCO (^{14}N = 351 ppm) is also shielded relative to cyanate ion (Table II), it seems highly unlikely that the observed shielding of the enzyme-bound cyanate at pH 8.8 is caused by partial protonation of the inhibitor at the active site because HNCO is acidic with a pK_a of 3.7. Formation of a zinc complex through cyanate oxygen is ruled out for the enzyme-bound inhibitor because such complexation is expected to move the nitrogen resonance substantially downfield on the basis of the observed resonance position of ethyl cyanate (222 ppm).

At pH 7.0, the resonance position of the enzyme-bound cyanate nitrogen is the same as that at pH 8.8 (Table II). If the cyanate binds to zinc at its fifth ligand site, with a water molecule bound at the fourth ligand site, the deprotonation of the zinc-bound water which has a pK_a of 7.3–7.6 is expected to perturb the ^{15}N shift of the zinc-bound cyanate over the pH range 7.0–8.8. Such perturbation attendant on ionization of zinc-bound water has been observed for the C2 proton chemical shifts of zinc-bound imidazole ligands (Campbell et al., 1974; Pesando, 1975). Therefore, the observed pH independence of the ^{15}N shifts of the enzyme-bound cyanate nitrogen suggests that the cyanate binds at the fourth ligand site, replacing the activity-linked water molecule.

Discussion

The model sulfonamide studies provide strong evidence that the coordination of zinc to a sulfonamide group through its nitrogen causes shielding of its ^{15}N resonance, while coordination through the oxygen results in deshielding of the nitrogen nucleus. Therefore, the large shielding observed in the enzyme-bound inhibitor strongly indicates that benzenesulfonamide binds to the catalytic zinc of human carbonic anhydrase B through its sulfonamide nitrogen.

The ^{15}N–^{1}H doublet structure of the enzyme-bound nitrogen resonance shows that the bound benzenesulfonamide is in $-SO_2NH-$ form. This could not have been determined from the ^{15}N chemical shift alone, because deshielding resulting from deprotonation is masked by shielding due to complexation with zinc. The deprotonation state of the bound sulfonamide is in accordance with the results obtained previously by difference UV spectroscopy for a carbonic anhydrase complex of *p*-nitrobenzenesulfonamide and by resonance Raman spectra for phenylazo derivatives of benzenesulfonamide. With the latter technique, the presence of the azo substituent was essential for distinguishing the anionic from the neutral state. ^{15}N NMR provides a simple and unequivocal method for determining the protonation state of any enzyme-bound sulfonamides and amines through the N–H coupling, provided that the proton exchange is slow on the NMR time scale.

The slow rate of the base-catalyzed exchange of the enzyme-bound sulfonamide proton with water suggests either that its rate constant for exchange is much smaller than that of free benzenesulfonamide or that the active site is highly hydrophobic or both. Coordination with zinc should make the sulfonamide group more acidic, resulting in faster proton exchange as was observed for the 2-aminobenzenesulfonamide–Zn complex. On the other hand, hydrogen bonding between the N–H and a neighboring threonine oxygen should make the proton less accessible for direct exchange with water. In this connection, it is interesting that the proton-coupled ^{15}N3(π) resonance of the active-site histidyl residue of α-lytic protease was a singlet from pH 4 to 9.5, which indicates rapid exchange of the imidazole N3(π) proton with water protons (Bachovchin & Roberts, 1978), even though (a) the N3(π)-H is hydrogen bonded to an aspartyl carboxylate, (b) an imidazole proton (with a pK_a of ~15 for deprotonation) is expected to exchange more slowly than a sulfonamide proton (pK_a ~10), and (c) this imidazole is known to be in a partially hydrophobic environment. These considerations suggest that the slow exchange rate of the carbonic anhydrase bound sulfonamide proton reflects a highly hydrophobic environment at the active site.

The high affinity of benzenesulfonamide toward carbonic anhydrase may not, in fact, be related to its being a transition-state analogue but rather may simply reflect the pK_a of benzenesulfonamide. The affinity of cyanide ion for the enzyme is relevant in this connection, because cyanide coordination can hardly be regarded as resulting in formation of a transition-state analogue. Hydrogen cyanide has a pK_a of 9.4 which is quite comparable to that of benzenesulfonamide (pK_a = 9.95), and cyanide forms a complex with carbonic anhydrase with an affinity constant of the order of 3×10^5 to $\sim 3 \times 10^7$ M^{-1} (Lindskog & Thorslund, 1968; Coleman, 1967b) compared to 4.9×10^6 M^{-1} for benzenesulfonamide. For the benzenesulfonamide–carbonic anhydrase complex, additional stability may arise from interactions of the phenyl ring of the inhibitor with hydrophobic residues of the enzyme.

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Registry No. Carbonic anhydrase, 9001-03-0; benzenesulfonamide, 98-10-2; cyanate, 661-20-1; Zn, 7440-66-6; 2-aminobenzenesulfonamide, 3306-62-5; *N*-(2-aminophenyl)benzenesulfonamide, 43200-31-3; KNCO, 590-28-3.

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pH-Dependent Polymerization of a Human Leukocyte Interferon Produced by Recombinant Deoxyribonucleic Acid Technology†

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ABSTRACT: The pH-dependent polymerization of human leukocyte interferon A produced by recombinant DNA technology was investigated by potentiometric hydrogen ion titration and sedimentation velocity analytical ultracentrifugation. The titration curve is completely reversible from pH 6.2 to 2.5. Analysis of the side-chain carboxyl residues by using the Linderstrøm-Lang equation yields a linear plot from pH 5.2 to 3.5. The resulting pK_{int} and W values are 4.66 ± 0.06 and 0.036 ± 0.004, respectively. The titration curve from pH ~9.6 to 7 is also reversible. However, the reverse titration from pH 7 to 6 results in a time-dependent variation of pH. Sedimentation velocity analysis yields an increase in the corrected sedimentation coefficient, $s_{20,w}$, from 2.0 to 4.0 S over a pH range of 2.5-9 at 0.38-0.25 mg/mL total protein concentration. The concentration dependence of the sedimentation coefficient at pH 7.0 over the range of 0.05-6.0 mg/mL results in a positive slope which is typical for self-aggregating systems. This aggregation phenomenon is discussed in terms of its ramifications for interferon therapy.

Interferons are an important class of proteins which exhibit antiproliferative and antiviral activity (Stewart, 1979). These proteins isolated from cell cultures are of insufficient purity and quantity to be studied by biophysical techniques. With the advent of recombinant DNA technology, we are now able to produce large amounts of protein of high purity. This paper deals with a study of the amino acid ionizations and possible linkage to self-polymerization of a human leukocyte interferon, designated LeIF-A,[1] produced by recombinant technology.

LeIF-A is one of eight related human leukocyte interferons (Mantei et al., 1980; Streuli et al., 1980; Goeddel et al., 1980, 1981) and has been characterized biochemically by determining amino acid composition, molecular weight (by NaDodSO$_4$-polyacrylamide gel electrophoresis), N-terminal sequence (first 35 residues)(Wetzel et al., 1981), and disulfide linkages (Wetzel, 1981). Recent circular dichroism and ultraviolet absorption measurements of LeIF-A have probed the well-known acid stability of the molecule in terms of changes in secondary and tertiary structures (Bewley et al., 1982).

The self-polymerization of proteins is a common occurrence which very often plays a role in the biological function of the

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[1] Abbreviations: LeIF-A, human leukocyte interferon A produced by recombinant DNA technology; K(H)PO$_4$, potassium orthophosphate, mono- and dibasic salts; NaDodSO$_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; I, ionic strength.

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