Resonance Raman Studies on Some Carbonic Anhydrase–Aromatic Sulfonamide Complexes†

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ABSTRACT: Resonance Raman spectra of 4-sulfonamido-4′-dimethylaminoazobenzene, 4-sulfonamido-4′-hydroxyazobenzene, and 4-sulfonamido-4′-aminoazobenzene bound to various isoenzymes of carbonic anhydrase were obtained by exciting into the sulfonamide absorption bands in the 400–500-nm region. In this way it was possible to obtain vibrational spectra of the sulfonamides in the active site unmasked by contributions from the vibrational modes of the protein and H_2O solvent. Direct evidence was obtained for the presence of $-SO_2NH^-$ in the complex, and it was possible to eliminate hydrophobic bonding and twisting in the Ph—N=N—Ph bonds as sources of the observed spectral changes. No detectable differences were found in the spectra of 4-sulfonamido-4′-dimethylaminoazobenzene bound to human carbonic anhydrase B and C or between these and the spectra of the sulfonamide bound to bovine carbonic anhydrase or Co(II) human carbonic anhydrase B. A new band appears in the spectra of the bound sulfonamides, and this is interpreted in terms of a change in geometry about the sulfonamido sulfur atom. A possible explanation for this change in geometry is that the bound sulfonamide group closely mimics the transition state of the reactants in the reversible hydration of CO_2. Sulfonamide binding at pH 12.0 was not detected in the resonance Raman spectrum. Sulfonamide replacement of CN^- in the binding site occurred over a period of minutes and could be monitored in the resonance Raman spectra.

Carbonic anhydrase (EC 4.2.1.1) (CA)[1] is a zinc metalloprotein whose function is to catalyse the reversible hydration of CO_2. Since the discovery by Mann and Keilin (1940) of the potent inhibition of carbonic anhydrase by sulfonamides, this enzyme-inhibitor interaction has attracted considerable interest. The sulfonamides have been of pharmacological and therapeutic value (Goth, 1972) because of their effect on various anion exchange reactions in the body ultimately involving HCO_3^-. Moreover, because of their highly specific interaction with the active site, they have proved extremely useful in work on the physicochemistry and mechanism of action of carbonic anhydrase (Coleman, 1973). While the sulfonamide structure does not suggest a metal complexing agent, recent x-ray data (Lindskog et al., 1971) show that in a sulfonamide-CA complex the $-SO_2NH_2$ group is probably bound within the coordination sphere of the protein's single Zn atom. However, the detailed geometry of the coordination is not yet known, and the precise relationships at the binding site may involve more subtle features than simple metal coordination. Most fluorescence and absorption spectroscopic studies (reviewed in Coleman, 1973) have indicated that the sulfonamide binds in a hydrophobic environment in the protein, and some workers (e.g., Chen and Kernohan, 1967; King and Burgen, 1970) have suggested that the sulfonamide binds in the anionic form.

Resonance Raman spectroscopy provides a sensitive tool for probing binding site structure in proteins and for obtaining detailed information on change occurring in the ligand upon binding (Carey et al., 1972). The technique provides a vibrational spectrum of the ligand in the protein binding site (Carey et al., 1973; Carey and Schneider, 1974; Kumar et al., 1974; McFarland et al., 1975). This is achieved by using laser excitation which lies in an absorption band of a chromophoric ligand, whereupon an intensity-enhanced resonance Raman spectrum is obtained from the ligand, while the normal Raman spectrum from the rest of the complex system is obscured by the spectral background. Comparison of the resonance Raman spectrum of the bound ligand with those of the free molecules in a variety of known states provides direct evidence on the changes taking place in the ligand upon binding and, by inference, the nature of the protein site. The sulfonamides, 4-sulfonamido-4′-dimethylaminoazobenzene (I), 4-sulfonamido-4′-hydroxyazobenzene (II), and 4-sulfonamido-4′-aminoazobenzene (III) (Figure 1), were selected for the present study because they met the criteria necessary for high association constants and because their resonance Raman spectra could, at least in part, be analyzed and understood. In contrast to many of the other spectroscopic methods applied to CA-sulfonamide binding, the resonance Raman spectra of the complex contain a wealth of detail. Certain spectral bands can be associated with the vibrational modes of submolecular groupings, enabling binding effects on these groupings to be studied. Moreover, because different spectral features are sensitive to different chemical effects, the results of sulfonamide group ionization and conformational changes in the aromatic nuclei can be separately monitored and distinguished from the effects of hydrophobic bonding.

A brief report on the binding of I and II to bovine CA has appeared (Kumar et al., 1974); the initial findings are confirmed in the present study and are extended to include an additional sulfonamide (III, Figure 1), binding to human CA(B) and CA(C) and binding to Co(II) human CA(B). Furthermore, binding studies at basic pH and competitive binding experiments with CN^- are reported. The appearance of a new band in the spectra of bound sulfonamides is also discussed.

Materials and Methods

The sulfonamides were prepared as follows: to ~50 ml of a solution of phenol (4 g) and aniline or dimethylaniline (4 ml)

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[1] Abbreviations used are: CA, carbonic anhydrase; EDTA, (ethylenedinitrilo)tetraacetic acid; CD, circular dichroism.

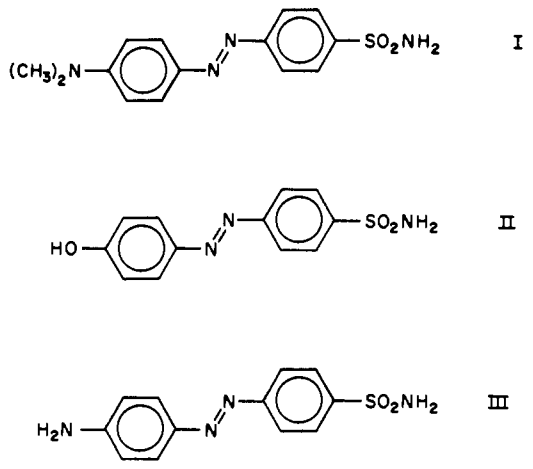


FIGURE 1: The structures of sulfonamides I, II, and III.

at pH 8 and 4 °C was added dropwise a solution in HCl at 4 °C of 7 g of sulfanilamide diazotized with HNO_2. The reaction mixture was kept cool in an ice-bath and kept at pH 8 by addition of KOH solution. When all the reactants had been added the mixture was stirred for 1 h and then filtered. The reaction with phenol or dimethylaniline goes directly to compounds II and I, so the residue was washed with cold water, then recrystallized from 1 l. of boiling water. The reaction with aniline proceeds as far as the N-substituted aniline, and the solid product is dried between sheets of filter paper, heated with aniline hydrochloride (2 g), aniline (8 ml), and acetic acid (10 ml) at 40 °C for 1 h, and left overnight to give III. The product was filtered, washed well with 50% acetic acid/water, and recrystallized from ethanol–water. Small amounts of impurity in III were removed by repeated recrystallization from ethanol–water. Purity of the samples was established by thin-layer chromatography.

Bovine erythrocyte carbonic anhydrase B was purchased from Miles Laboratories Inc., Kankakee, Ill., in the form of a salt-free, freeze-dried powder. Human carbonic anhydrases B and C were prepared as described in Armstrong et al. (1966). Apocarbonic anhydrase B was prepared by dialysis against 1,10-phenanthroline (pH 5.5, 40 mM acetate) for 1 week. The dialyzed solution was then passed down a column of Sephadex G-25 and equilibrated with 50 mM Tris-SO_4, pH 8.0, containing 1 mM EDTA, to remove phenanthroline and chelated Zn. The column eluate was loaded onto a sulfanilamide–Sepharose affinity column and equilibrated with the same buffer solution, which retained all the remaining holoenzyme (5–20%) while eluting the apoenzyme. The cobalt(II) enzyme was then prepared by carefully titrating back the full activity of the enzyme with $CoSO_4$ solution. Excess cobalt chelated with EDTA was removed by dialysis.

Protein concentrations were determined from measurements of the optical densities at 280 nm using molar absorption coefficients 4.90×10^4 M^{-1} cm^{-1} for human CA(B), 5.34×10^4 M^{-1} cm^{-1} for human CA(C), and 5.6×10^4 M^{-1} cm^{-1} for bovine CA (Coleman, 1967).

Esterase activities were determined using *p*-nitrophenyl acetate as a substrate (Armstrong et al., 1966) and following the absorbance changes at 348 nm. The reaction mixture contained 1 mM *p*-nitrophenyl acetate in 4% acetone and 0.01 M Tris-SO_4 buffer, pH 7.6, at 23 °C. The hydrolysis was followed by recording the rate of increase of optical density at 348 nm. The molar extinction coefficient, ϵ, at 348 nm for *p*-nitrophenol or *p*-nitrophenolate was determined to be 4800 M^{-1} cm^{-1}. The unit of activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 mM *p*-nitrophenyl acetate/min at 23 °C and the activity was calculated as follows: U/mg = $\Delta\epsilon_{348nm/min}/(4.8 \times$ mg enzyme/ml of reaction mixture). The measured activity was 1489 U/mg.

The absorption spectra were recorded using a Cary 15 spectrophotometer. The concentrations and conditions of the spectra are indicated in the figure captions.

Raman spectra were recorded with a Jarrell-Ash 25-400 laser Raman spectrophotometer equipped with argon ion, krypton ion, and helium–cadmium ion lasers. The laser lines used from these sources are indicated in the figure captions. The spectra of enzyme–inhibitor complexes were recorded using a vertical capillary cell mounted on a goniometer head. The laser beam entered along the long axis of the tube and was focused inside. For the enzyme complexes laser power was kept below 30 mW to avoid photodecomposition. No photodecomposition or photoisomerization was detected under the conditions employed (see figure captions). In the resonance Raman experiments on azosulfonamide complexes several concentrations of sulfonamide and excess enzyme were employed; no concentration dependence was detected. Raman line positions were calibrated using emission lines from argon, neon, and xenon lamps. Spectra were repeated at least three times. The spectral slit widths were ~9 cm^{-1} or less. Intensities were measured as peak heights. Solutions containing the hydroxysulfonamide were kept at pH 9.0, ensuring that only spectra of the O^- form were recorded. The affinity constants were measured by fluorescence quenching titrations and were found to be $>5 \times 10^8$ M^{-1}, ~10^8 M^{-1} and ~2×10^8 M^{-1} for human CA(C) binding sulfonamides I, II, and III, respectively. Therefore, at the concentrations used for the experiments and in the absence of competitive inhibitors, more than 95% of the sulfonamides were bound, and unbound sulfonamides were not detected.

Results and Discussion

Comparison of the resonance Raman spectrum of the bound sulfonamide with the spectra of the sulfonamide in various reference states provides information on the structure of the bound ligand and the nature of the binding site. Solid compounds and solutions in organic solvents show how changes in dielectric constant, and thus a hydrophobic environment, affect the Raman spectra. Aqueous solutions at pH ~13 are used to characterize the Raman spectra of sulfonamides containing the $-SO_2NH^-$ moiety. Furthermore, studies on the vibrational spectra of other azo derivatives show how changes in the backbone structure of the azobenzene entity affect the Raman spectrum.

Because the resonance Raman spectra of the azosulfonamides contain many features and each band reflects changes in the dielectric constant of the medium, ionization of the SO_2NH_2 group, and backbone conformation in a different manner, these effects can be separately characterized in the spectra of the bound ligands. This situation is in contrast to that found in most absorption and fluorescence studies on sulfonamide–CA complexes where in spite of the most careful studies it has not always been possible to assign unambiguously the cause of a shift in a single spectral band to one of several possible chemical effects.

A systematic Raman spectroscopic study of the sulfonamides used in this work has been undertaken at the same time as the carbonic anhydrase binding studies reported herein. However, most of the conclusions on the nature of the enzyme–inhibitor interaction can be reached by an empirical comparison of the spectra of the enzyme-bound sulfonamide

with the spectra of the sulfonamide in solvents of differing dielectric constants and the sulfonamide in its anionic form. Thus, this paper will emphasize the direct empirical approach, and detailed spectroscopic interpretation will only be used as supportive evidence.

Evidence for the $-SO_2NH^-$ Form in the Active Site. There are two major changes in the resonance Raman spectra of I, II, and III upon binding to carbonic anhydrase (Figures 2-4). A peak near 1140 cm^{-1} moves to higher frequency and a change takes place in the intensity pattern of the peaks between 1350 and 1450 cm^{-1}. These changes are seen upon complexing to the Zn enzymes, human CA(B) and CA(C) and bovine CA, and to Co(II)-human CA(B) (e.g., Figures 2-4, Table I).

The shift in the band near 1140 cm^{-1} upon binding is most clearly seen for compound I, 1139 → 1146 cm^{-1} (Figure 2), and compound III, 1145 → 1150 cm^{-1} (Figure 4). Moreover, in compound I the 1139-cm^{-1} band shifts to 1146, 1147, and 1145 cm^{-1} upon binding to human CA(B), bovine CA, and Co(II)-human CA(B), respectively. For compound II the band shifts from 1134 to 1138 cm^{-1} upon binding to bovine CA (Figure 3). Although the presence of incompletely resolved bands in the 1140-cm^{-1} region of Figure 3a renders interpretation for II less clear than the preceding five cases, the error in this spectral region is ±1 cm^{-1}; thus, a significant shift occurs for all bound sulfonamides.

Upon binding, the intensity pattern in the 1400-cm^{-1} region shows similar changes for I, II, and III (Figures 2-4 and Table I). In particular the ratio of the band intensities for the features near 1420 and 1390 cm^{-1} increases for the bound sulfonamides. The change is most apparent for II where intensity (~1420/~1390) goes from 0.97 (unbound) to 1.33 (bound). The increase in intensity (~1420/~1390) for the bound form is also substantiated for I and III by the quantitative measurements (Table I). For I intensity (~1420/~1390) is 1.43 (unbound) and 1.61 (bound), and for III it is 1.59 (unbound) and 2.11 (bound).

Compared with the bound ligands the resonance Raman spectra of the anionic form of the unbound sulfonamides I, II, and III at pH ≳13.0 in aqueous solution show frequency shifts in the same direction in the 1140-cm^{-1} band and similar intensity patterns for intensity (~1420/~1390) (Figures 2-4, Table I). This is strong empirical evidence that the sulfonamides are bound in the complex as the $-SO_2NH^-$ moiety. Since the $-SO_2NH_2$ group is not strongly conjugated with the benzenoid ring, intense features from this substituent are not expected in the resonance Raman spectra. In the 1100-cm^{-1} region, where sulfonamide contributions are expected, *p*-amino- and *p*-dimethylaminoazobenzenes give spectra which are essentially identical with those of III and I (Kumar and Carey, 1976). However, changes in the $-SO_2NH_2$ chemistry are transmitted to the aromatic system via the σ and π electrons; thus, changes in the $-SO_2NH_2$ group upon binding are reflected in the main resonance Raman bands. Moreover, any increase in conjugation through the Ph-S bond may result in the appearance of a new band and this case is discussed in the Discussion section.

Hydrophobic Bonding. The effect of a hydrophobic environment can be explored by examining the resonance Raman spectra of sulfonamide I in the solid phase and in solutions of varying dielectric constant. Sulfonamide II is not suitable in this respect because of complications ensuing from the ionization of the OH group and the low solubility of the -OH form in H_2O. The resonance Raman spectra of I were recorded in the solid phase, in CH_3CN, in C_2H_5OH (not shown), in $CHCl_3$ (Figure 5A), in $(CH_3)_2CO$ (Figure 2c), and in 50:50 v/v

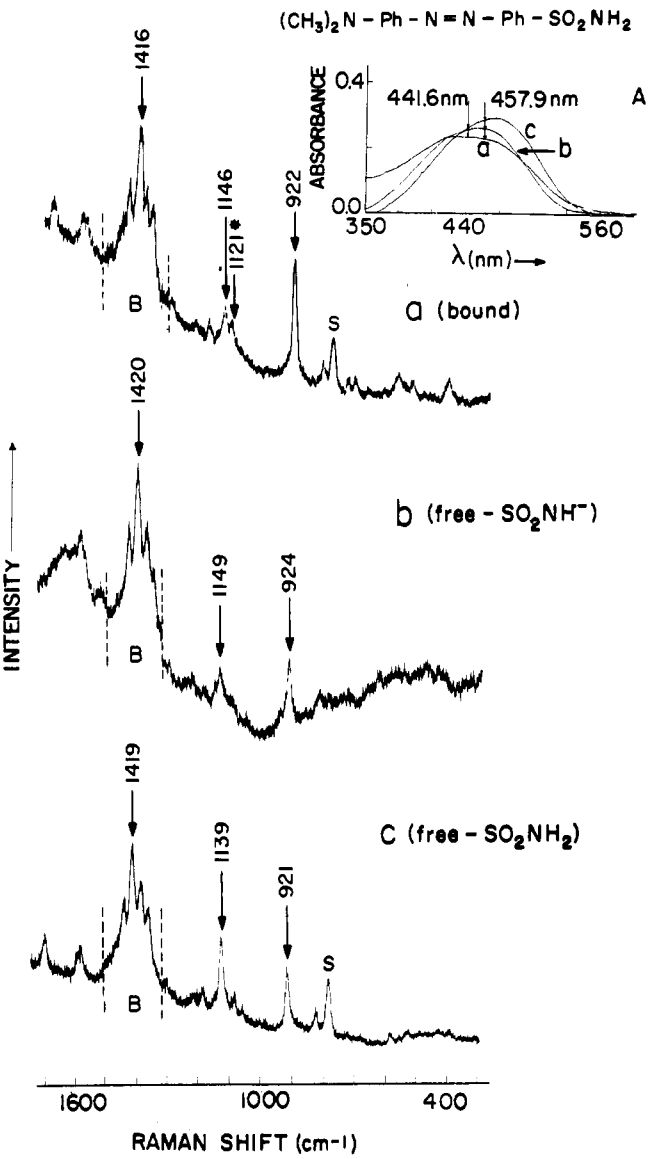


FIGURE 2: The resonance Raman spectra (441.6-nm excitation) of sulfonamide (I): (a) bound to HCA(C), acetone-Tris-SO_4 buffer (1:10 v/v), pH 9.0, sulfonamide ~6×10^{-5} M, enzyme ~10^{-4} M; (b) free anionic sulfonamide (SO_2NH^-) ~2×10^{-4} M, 0.1 N KOH, pH 13.0; (c) free $-SO_2NH_2$ form, ~1.0×10^{-2} M in acetone. Spectral slit width ~9 cm^{-1} in all cases and power ~20 mW for the bound and ~60 mW for the unbound sulfonamide spectra. The asterisk indicates a new band. S denotes acetone solvent peaks. The experimental conditions for the inset (A) absorption spectra: (c) free ($-SO_2NH_2$) form, 6.6×10^{-5} M, pH 8.0; (b) free (SO_2NH^-) form, 6.6×10^{-5} M, pH 13.6; (a) bound to BCA, sulfonamide (I) 6×10^{-5} M, enzyme ~1×10^{-4} M, pH 8.0. All the spectra were recorded in 1 cm cells and the compound was dissolved in acetonitrile-Tris-SO_4 buffer (32:68 v/v). Experimental conditions for inset B resonance Raman spectra (457.9-nm excitation): (a) bound to BCA, CD_3CN:Tris-SO_4 buffer (5:95 v/v), pH 8.8, sulfonamide ~4.5×10^{-4} M, enzyme ~1×10^{-3} M; (b) free anionic ($-SO_2NH^-$) form, ~1×10^{-3} M, CD_3CN:1 N KOH (50:50 v/v); (c) free ($-SO_2NH_2$) form, ~1×10^{-3} in CD_3CN. Slit width ~9 cm^{-1} in all cases. Power ~15 mW for unbound ($-SO_2NH_2$) and bound sulfonamide spectra and ~30 mW for unbound ($-SO_2NH^-$) spectra. Due to the overlapping with an acetone peak ~1430 cm^{-1}, the 1400 cm^{-1} region (inset B) is presented using CD_3CN as a solvent.

H_2O:CH_3CN (Kumar et al., 1974). No detectable frequency shifts were observed for any of the intense peaks; the increased resolution obtained in $CHCl_3$ solutions is due to a sharpening of the bands. Furthermore, none of these spectra showed an increase in the relative intensity of the band near 1420 cm^{-1} of the kind seen upon binding to the enzyme. Thus, it seems

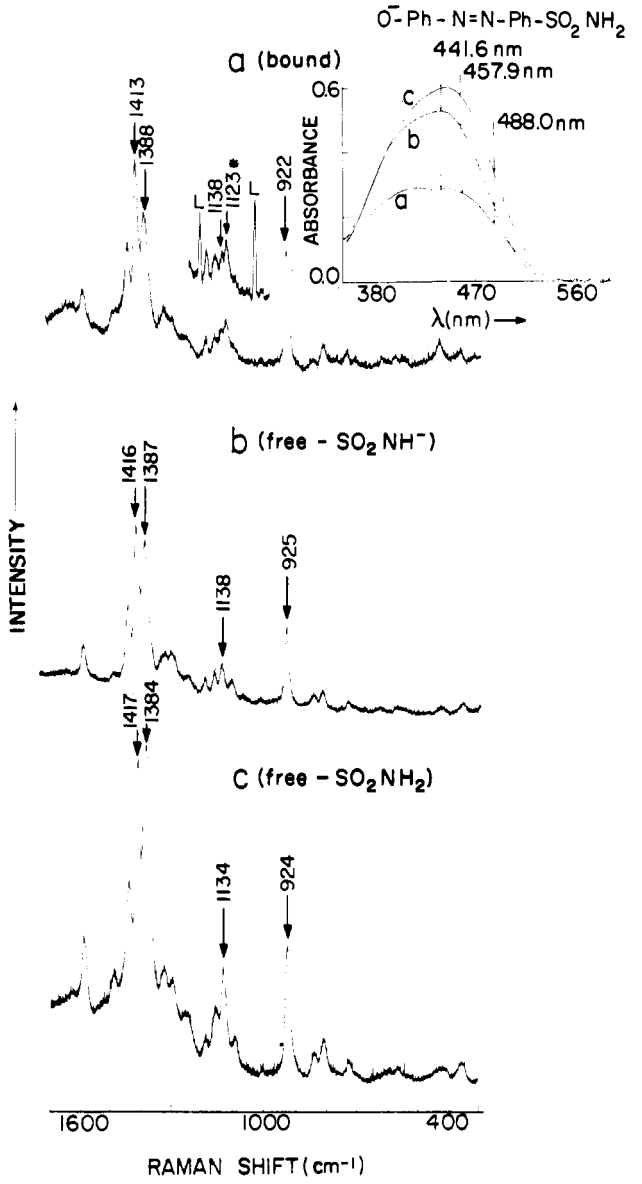


FIGURE 3: The resonance Raman spectra (441.6 nm excitation) of sulfonamide (II): (a) bound to BCA, Tris-SO_4 buffer, pH 9.0, sulfonamide (II) ~3.8×10^{-5} M, enzyme ~1×10^{-4} M; (b) free anionic ($-SO_2NH^-$) form in 0.1 N KOH, pH 13.0, ~7.7×10^{-5} M; (c) free (SO_2NH_2) form, ~7.7×10^{-5} M, pH 9.0. Spectral slit width ~9 cm^{-1}, and power ~20 mW in all cases. The experimental conditions for the superimposed spectrum of bound (II) (457.9-nm excitation) Tris-SO_4 buffer, pH 8.8, sulfonamide (II) ~5.5×10^{-5} M, enzyme ~1.6×10^{-4} m, spectral slit width ~7 cm^{-1}, and power ~15 mW. The asterisk indicates a new band. The letter L denotes laser plasma lines. The experimental conditions for the inset absorption spectra: (c) free (SO_2NH_2) form, 1.6×10^{-5} M, Tris-SO_4, pH 9.3; (b) free anionic ($-SO_2NH^-$) form, 1.6×10^{-5} M, pH 13.3, (a) bound to BCA sulfonamide (II), 1.4×10^{-5} M, enzyme 7×10^{-5} M, Tris-SO_4 buffer, pH 9.2. The spectra were recorded in a 1 cm cell. The resonance Raman spectra obtained using 488.0-nm excitation showed similar intensity changes upon binding and ionization.

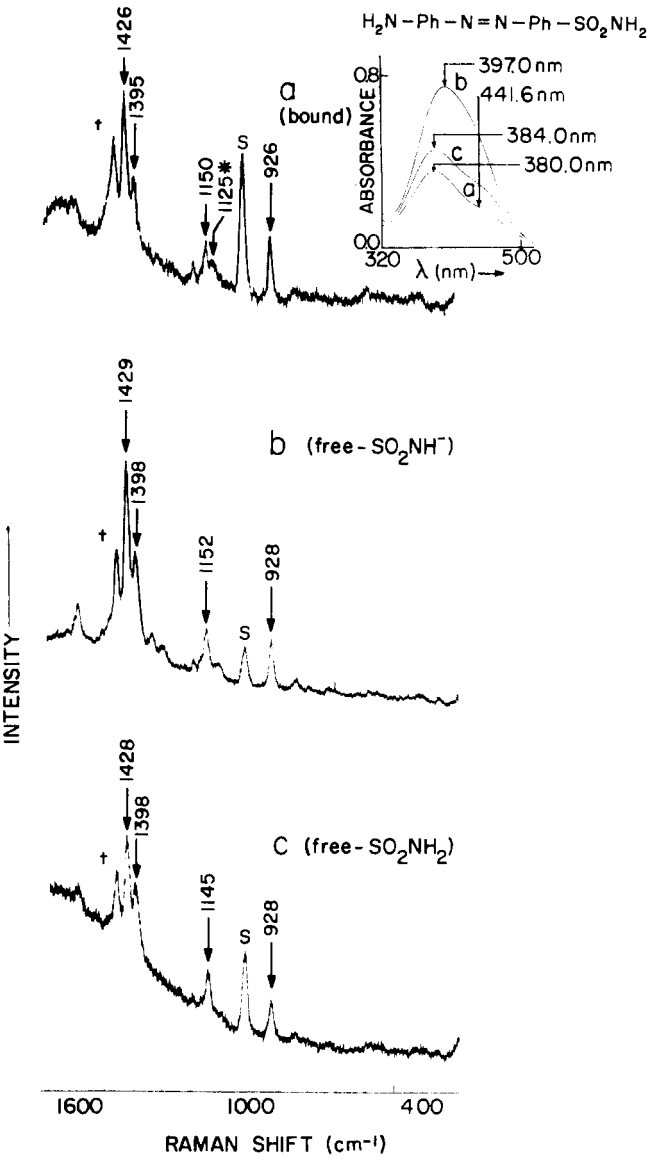


FIGURE 4: The resonance Raman spectra (441.6-nm excitation) of sulfonamide (III): (a) bound to BCA, methanol: Tris-SO_4 buffer (1:3 v/v), pH 7.0. Sulfonamide ~3.5×10^{-5} M, enzyme ~1.2×10^{-4} M; (b) free anionic ($-SO_2NH^-$) form, ~4×10^{-5} M, methanol: 1.0 N KOH (1:3 v/v); (c) free ($-SO_2NH_2$) form, ~4×10^{-5} M, methanol: Tris-SO_4 buffer (1:3 v/v), pH 7.0. Slit width ~9 cm^{-1} and power ~20 mW for bound and ~40 mW for unbound sulfonamide spectra. The asterisk indicates the new band and the dagger indicates some overlapping in this region with the methanol feature. S denotes solvent methanol peak. The experimental conditions for the inset absorption spectra: (b) free ($-SO_2NH^-$) form, ~2×10^{-5} M, pH 13.0; (c) free (SO_2NH_2) form, ~2×10^{-5} M, pH 7.6; (a) bound to BCA, sulfonamide ~2×10^{-5} M, enzyme ~5×10^{-5} M, pH 7.6. All the spectra were recorded in 1 cm cells and the compound was dissolved in methanol-water (buffer) mixed solvent (25:75 v/v).

possible to eliminate a hydrophobic environment as a source of the frequency changes in the 1140-cm^{-1} band and the intensity changes in the 1400-cm^{-1} region. This is not to say that hydrophobic bonding is not important, only that the resonance Raman spectra are insensitive to such effects. This is in contrast to the absorption spectral data where the position of λ_{max} is sensitive to dielectric constant (Figure 6). It is interesting to note that the blue shift in the absorption spectrum of I upon binding to the enzyme (Figure 2, inset A) is mimicked in the unbound ligand by going to higher concentrations of CH_3CN in a CH_3CN/H_2O mixture (Figure 6). Thus, in contrast to the Raman evidence, the absorption data of this compound gave no compelling indication that any factor apart from a change in dielectric constant has to be taken into account in a model for the interaction.

Conformational Changes About the Ph—N=N—Ph Bonds. The extensive studies of the Raman spectra of substituted azobenzenes (Hacker, 1968) together with the new data reported below identify a band in the resonance Raman spectra of I, II, and III which has a large contribution from a Ph—N stretching vibration and suggest an assignment for the -N=N- stretching mode. Any change in about the Ph—

TABLE I: The Frequencies[a] and Intensities[b] of the Raman Bands of Sulfonamides I, II, and III in the Region 800–1700 cm^{-1}.

Sulfonamide I (cm^{-1} (Int.))			Sulfonamide II (cm^{-1} (Int.))			Sulfonamide III cm^{-1} (Int.))		
Free	Anionic	Bound	Free	Anionic	Bound	Free	Anionic	Bound
1593w	1596w	1595w	1592m	1588w	1590w	~1595w	1595w	~1596w
			1488w	~1489vw	~1487vw			
1446m (5.4)	1446s (5.8)	1444s (5.8)	1445s (5.0)	1443m (4.6)	1442s (5.2)	1464s	1462s	1461s
1419vs (10)	1420vs (10)	1416vs (10)	1417vs (10)	1416vs (10)	1413vs (10)	1428vs (10)	1429vs (10)	1426vs (10)
1392s (7.0)	1392s (6.2)	1391s (6.2)	1384vs (10.3)	1387vs (8.6)	1388vs (7.5)	1398s (6.3)	1398s (5.0)	1395s (4.7)
1370m (5.0)	1370m (3.9)	1369m (5.2)	1328w	~1325w	~1325w		1342w	
~1318vw	~1317vw	~1319vw	1306w	~1305w	~1303w		1310w	
			1250w	~1245w	~1245w			
1202vw	~1200vw	1196vw				~1200vw	1196vw	1190vw
~1160sh	~1164sh	~1166sh	1188w	1194w	1188w	~1175sh	~1173sh	~1175sh
			1157w	1162w	1160w			
1139m	1149m	1146m	1134m	1138m	1138w	1145m	1152m	1150m
-	-	1121w	-	-	1123m	-	-	1125w
1097w	~1104vw		1098w	1105w	~1105w		1113w	
921m	924m	922m	924m	925m	922m	928m	928m	926m
826w	825vw	827w	843w	840w	~840w	846w	845w	~850w
			811w	807w	804w			

[a] The experimental conditions for compounds I, II, and III are those given in the captions of Figures 2, 3, and 4. [b] Band intensities in the 1400-cm^{-1} region are given in parentheses relative to ~1420 cm^{-1} as 10. The ~1400-cm^{-1} bands for III are overlapped by solvent. The abbreviations are: vs, very strong; s, strong; m, medium; vw, very weak; w, weak.

N=N—Ph bonds leading to a reduction in planarity would lead to a decrease in the bond order of the Ph—N's and an increase in the N=N bond order; this would be reflected in a shift in Ph—N to lower frequency and a shift in N=N to higher frequency. Thus, if significant twisting takes place in the azo bonds upon binding, it would be detected by frequency changes in a known direction in the Ph—N and N=N bond stretching modes.

Hacker's extensive work on the normal (non-intensity-enhanced) Raman spectra of 4,4′-substituted azobenzenes strongly suggests the assignment of an intense band near 1150 cm^{-1} to a mode associated with the Ph—N stretching vibration and a band close to 1420 cm^{-1} to the N=N vibration. Our data on a series of azobenzenesulfonamides fully substantiate Hacker's findings; e.g., Figure 5A compares the resonance Raman spectrum of I with its normal Raman spectrum; similarly, Figure 5B compares the normal Raman spectrum of solid II with its preresonance spectrum (the preresonance region is where the exciting line lies within the absorption band but far from λ_{max}). The normal Raman spectrum of III is not shown. For both I and II the normal Raman spectra are dominated by bands near 1140 cm^{-1}. In view of Hacker's work and the expectation that the high polarizability of the Ph-N bond will lead to an intense normal Raman feature, the band near 1140 cm^{-1} in I, II, and III can be assigned to modes associated with the Ph-N stretching vibration. It is particularly important to note that C-H bending vibrations which are known to occur in this region (Colthup et al., 1964) would not give rise to such an intense band, and thus can be eliminated as a possible source for the bands near 1140 cm^{-1}. The Ph-N bands are still present in the resonance Raman spectra of I and II but are of reduced relative intensity. In II it undergoes a frequency change upon going to aqueous solutions which reflects the effect of the OH group ionizing to O^-. Solution spectra of II were recorded in CH_3OH and frequencies were coincident with those in the spectra obtained from the solid.

On the basis of Hacker's work, which included ^{15}N-substituted azobenzenes, it is possible to assign the bands near 1420 cm^{-1} in the resonance Raman spectra of aqueous I, II, and III to the N=N stretching vibration.

Upon binding, the band near 1140 cm^{-1} in I, II, and III moves to higher frequency, i.e., in the opposite direction to the change expected if twisting occurs in the Ph—N=N—Ph linkages. Thus, the frequency change cannot be ascribed to conformational change in the azobenzene backbone. However, a small (probably <10°) twist may occur in the Ph-N bonds, leading to a decrease in the 1140-cm^{-1} position, and be masked by the $-SO_2NH_2$ ionization causing a large increase in frequency. The overall increase in the Ph-N frequency would then be small. The observed increase which is 4–8 cm^{-1} is always reproducible and is outside the limits of experimental error which, for sharp well-resolved bands in this spectral region, is ±1 cm^{-1}.

The reported decreases in the N=N stretching frequency (1419 → 1416 cm^{-1} for I and 1417 → 1413 cm^{-1} for II) upon binding are at the limit of experimental error for this region (±2 cm^{-1}) and are only significant in that they are consistent with the finding that pronounced twisting is not taking place in the Ph—N=N—Ph moiety. If it were, the frequency of the N=N would increase in the complex. Although unlikely, it is possible that another band in the 1350–1450 cm^{-1} is the N=N stretching vibration; however, since in I, II, or III none of the bands in this region moves significantly to higher frequency upon binding, the "no-twisting" conclusion does not depend on the exact assignment. Although the cause of the shift in the band near 1140 cm^{-1} is clearly the ionization of the SO_2NH_2, the band does not shift as far in the complex (4–8 cm^{-1}) as for the unbound aqueous anionic form (4–10 cm^{-1}). A probable reason for the reduced shift is the interaction between $-NH^-$ and Zn^{2+}; however, another possibility for molecule I mentioned in our initial communication (Kumar et al., 1974) is that slight twisting about Ph-$N(CH_3)_2$ bonds may occur upon binding and reduce the net shift of the ~1140-cm^{-1} band.

Comparison of Sulfonamide I Bound to Bovine CA, Human

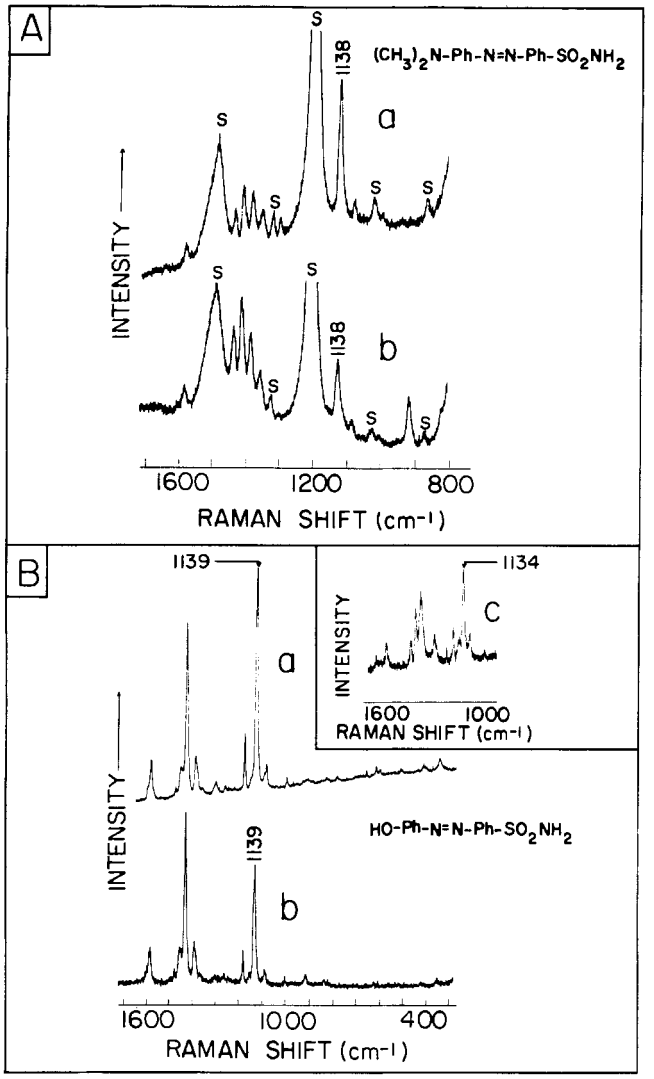


FIGURE 5: The Raman spectra of sulfonamide I and II: (A); (a) normal Raman spectrum of I dissolved in chloroform saturated solution, 647.1-nm excitation; (A)—(b) resonance Raman spectrum of I dissolved in chloroform saturated solution, 441.6-nm excitation. S denotes solvent peaks. (B)—(a) normal Raman spectrum of II as solid (OH-form) in KBr matrix, 647.1 nm excitation; (B)—(b) resonance Raman spectrum of II as solid (OH-form) in KBr matrix, 441.6-nm excitation; (B)—(c) normal Raman spectrum of II in Tris-SO_4 buffer (pH 9.1), ~2.5×10^{-3} M, 647.1-nm excitation.

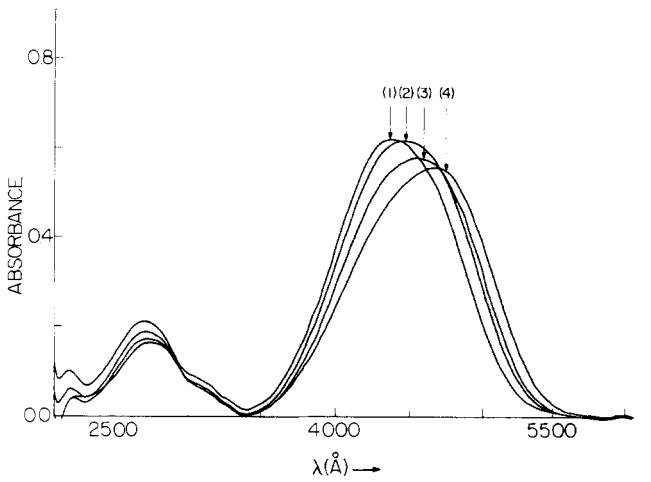


FIGURE 6: The absorption spectra of sulfonamide I in acetonitrile and acetonitrile-water mixed solvent system, sulfonamide ~1.3×10^{-4} M; (1) CH_3CN 100%, (2) CH_3CN:H_2O (70:30 v/v), (3) CH_3CN:H_2O (50:50 v/v), (4) CH_3CN:H_2O (30:70 v/v).

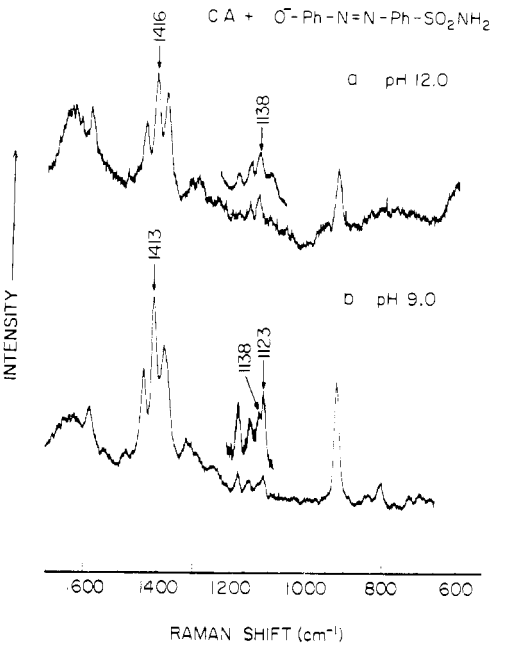


FIGURE 7: The resonance Raman spectra (441.6-nm excitation) of sulfonamide II-BCA complex at pH 9.0 and pH 12.0; (a) sulfonamide, 9.0×10^{-6} M, enzyme ~6×10^{-5} M, pH 12.0; in the superimposed spectrum, sulfonamide ~7×10^{-5} M, enzyme ~2×10^{-4} M; (b) sulfonamide ~7.7×10^{-5} M, enzyme ~2×10^{-4}; in the superimposed spectrum, sulfonamide ~2.5×10^{-4}, enzyme ~4×10^{-4} M.

CA(B) and CA(C), and Co(II)-Human CA(B). Resonance Raman spectra of I bound to Zn(II) bovine CA, Zn(II) human CA(C), and CA(B), and Co(II) human CA(B) are identical. All forms of the enzyme produce the same shift in the sulfonamide peak near 1140 cm^{-1} and the same intensity changes in the 1350–1450-cm^{-1} region. Within the limits of experimental error, there were no detectable differences between the spectra of I bound to the various forms of the enzyme. This situation contrasts with the considerable variations in the CD spectra of 2-(4-sulfamylphenylazo)-7-acetamido-1-hydroxynaphthalene-3,6-disulfonate in the complexes with several species and isozyme variants of the enzyme (Coleman 1973). However, the analogy between the latter sulfonamide and the sulfonamides I, II, and III cannot be too closely drawn since the naphthalene derivative is considerably more complex chemically and spectroscopically. Furthermore, as has been noted under hydrophobic bonding, the resonance Raman spectra are not as sensitive to changes in dielectric constant as the electronic transitions, and in particular it must be remembered that the positions of Raman bands are a property of the electronic ground state, whereas electronic spectra represent a difference in electronic ground- and excited-state properties.

Sulfonamide Binding as a Function of pH, and Competition between CN^- and Sulfonamide Inhibitors. A number of studies of the pH dependence of sulfonamide-CA interaction show binding to be maximum at about neutral pH, falling off rapidly at acid or alkaline pH. This is fully born out in the resonance Raman spectra. Figure 7 compares the spectrum of bovine CA + sulfonamide II at pH 9.0 and 12.0. At pH 9.0 the spectrum shows the characteristics of the bound form, the new peak at 1123 cm^{-1}, the shifted peak at 1138 cm^{-1}, and the intense 1413-cm^{-1} band; at pH 12.0 the peak at 1123 cm^{-1} disappears and the spectrum is identical with the unbound ionized sulfonamide ($-SO_2NH^-$) shown in Figure 3. Thus,

binding between CA and sulfonamide II is not detected at pH 12.0. The low intensity of the 1123-cm^{-1} peak makes this technique unsuitable for a pH titration, and none was attempted.

It was first pointed out by Kernohan (1966) and amplified by Lindskog and Thorslund (1968) that the dissociation rate of the bound sulfonamide inhibitor is slow enough such that equilibrium with a competing substrate molecule is achieved only slowly. It is known that one site on CA is involved in the inhibition by CN^- and sulfonamide and, as is shown in Figure 8, equilibrium is also achieved slowly for sulfonamide and CN^- competing for this site. Ten minutes after adding sulfonamide II to a solution of CA containing an excess of CN^- the resonance Raman spectrum shows that the sulfonamide is still predominantly in the unbound form (Figure 8a). After incubating for 20 min, however, the spectrum clearly shows that most of the sulfonamide is bound (Figure 8b). If considerable excess of KCN is subsequently added to the same solution, the spectrum of the sulfonamide reverts to that of the unbound molecule (Figure 8c). Although the low intensity of the new band at 1123 cm^{-1}, which characterizes the bound molecule, prevents an accurate estimate of bound sulfonamide concentrations, the present case does illustrate that in favorable examples specific complexation could be distinguished and monitored in a complicated set of reactions. Since the detection of intermediates in multistage reactions is often extremely difficult or impossible by conventional means, the resonance Raman technique may be able to resolve some of the problems presently encountered in kinetic studies of multistage reactions.

The Appearance of a New Resonance Raman Feature and the Transition-State Analogy—a Possible Explanation for the High Sulfonamide–CA Binding Constants. A new band, near 1120 cm^{-1}, is seen in the resonance Raman spectra of the bound sulfonamides and is marked with an asterisk in Figures 2–4. Specifically, it occurs at 1121 cm^{-1} in bound I, 1123 cm^{-1} in bound II, and 1125 cm^{-1} in bound III. This feature appears in the bound species only; it is absent in the resonance spectra of unbound molecules in both the $-SO_2NH_2$ and $-SO_2NH^-$ forms (Figures 2–4). Moreover, it is absent in the normal (the nonresonance) Raman spectra of I, II, and III (e.g., Figure 5); this strongly suggests that the 1120-cm^{-1} band does not arise from a change in vibronic coupling which enhances a vibrational mode already present in the unbound molecules. Thus, the appearance of a new band in the resonance Raman spectrum is evidence that a structural change takes place upon binding, which results in an extra vibration being coupled into the electronic transition or transitions responsible for Raman intensity enhancement. Since structural changes in the Ph—N=N—Ph bonds have been effectively ruled out (above) and intense benzene ring modes remain essentially unperturbed, it is the $-SO_2NH_2$ moiety that must undergo distortion upon binding. Furthermore, the absence of the 1120-cm^{-1} peak in the unbound $-SO_2NH^-$ species means that the conformational change must be in addition to any change resulting from ionization.

A resonance-enhanced mode from the complexed $-SO_2NH^-$ indicates that conjugation has increased between the $-SO_2NH^-$ and azobenzene groups. This would result from a distortion which increased overlap between the S 3d and C 2p orbitals and requires protein interactions at one or both of the $-SO_2NH^-$ oxygen atoms in addition to the protein $-NH^-$ bonding.

There is evidence from x-ray data (Lindskog et al., 1971) that the sulfonamido group interacts with the -OH of a threonine side chain; this could be one source of the distortion about

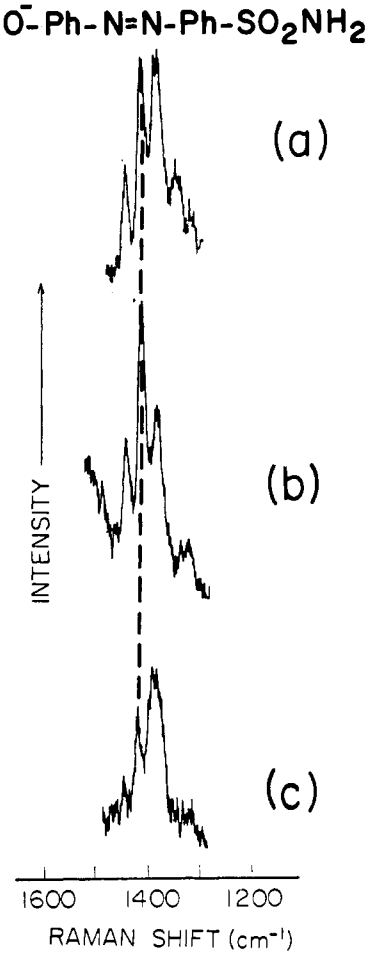
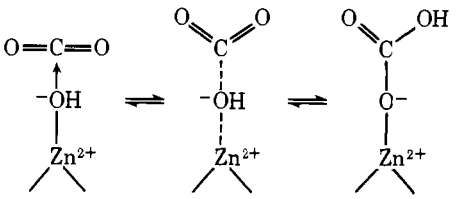


FIGURE 8: The resonance Raman spectra (441.6-nm excitation) of sulfonamide II showing the competitive binding of anion (CN^-) and sulfonamide II: (a) to a solution containing 25 μl. of $\sim 2 \times 10^{-4}$ M enzyme and 10 μl of $\sim 10^{-2}$ M KCN was added 32 μl of $\sim 7 \times 10^{-5}$ M of sulfonamide and the spectra of unbound sulfonamide was detected for ~10 min; (b) after sulfonamide had been added for ~20 min bound sulfonamide was detected; (c) then to the above solution was added ~10 μl of 2 M KCN and unbound sulfonamide was again detected.

the sulfur. However, the x-ray data are not sufficiently precise to detect relatively minor changes in sulfonamide geometry. Because further delocalization is taking place through the Ph–S linkage, the new band near 1120 cm^{-1} may be expected to have a large contribution from the Ph–S bond vibration with possible mixing with the $-SO_2-$ symmetric stretch. The assignment of bands near 1100 cm^{-1} to Ph–S stretching modes (Colthup et al., 1964) further supports the assignment of the 1120-cm^{-1} band to the Ph–SO_2NH^- group.

The avidity of sulfonamides for the active site of carbonic anhydrase has remained an enigma. One possible explanation is that the bound sulfonamide group closely mimics the transition state of the reactants in the reversible hydration of CO_2. A favored mechanism for this reaction involves the attack of a coordinated ^-OH on a CO_2 molecule (Coleman, 1973):



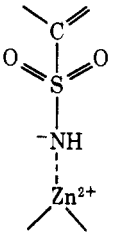
possible transition state

TABLE II:

	Bond Lengths (Å)	Geometry and Bond Angles
CO_2[a]	C=O 1.159	Linear
HCO_3^-[b]	C=O 1.28, 1.32, 1.33	Planar
		∠OCO 118.5°, 119.5°, 122°
$PhSO_2$-NH_2[c]	C-S 1.750	∠CSO 107.5°, 107.8°
	N-S 1.620	∠OSO 118.2°
	S-O 1.454, 1.448	∠OSN 107.2°, 105.5°
		∠CSN 110.7°

[a] Dennison, D. M. (1940), *Rev. Mod. Phys. 12,* 175. [b] In $KHCO_3$ Nitta, I., Tomiia, Y., and Koo, C. H. (1950), *Acta Cryst. 5,* 292. [c] In β-sulfanilamide O'Connor, B. H., and Maslen, E. N. (1967), *Acta Cryst. 22,* 134.

A similarity between the postulated transition state and bound sulfonamide



is apparent and may account, in part, for the high sulfonamide-protein binding constants. Similar arguments have been used to account for other high protein-inhibitor binding constants (see Wolfenden, 1972, and Lienhard, 1973, for recent reviews). Comparison of bond lengths and angles (Table II) is consistent with the idea that the $-SO_2NH^-$ is fitting into the CO_2-OH^- transition-state site and, in particular, the resonance Raman evidence that distortion takes place about the S atom upon binding can be interpreted in terms of the SO_2 group fitting into the CO_2 transition-state binding site. Although in the ground state there is ir evidence (Riepe and Wang, 1968) that the bound CO_2 is linear, in the transition state the CO_2 molecule is expected to be bent. Furthermore, in the transition state complex the OCO angle and bond lengths are unlikely to be exactly the same as the OSO dimensions in unbound sulfonamides (e.g., Table II). Thus $-SO_2-$ binding in the CO_2 site would result in a distorted OSO angle, leading to a possible increase in S(d) and C(π) overlap, increased conjugation into the $-SO_2NH^-$ group, and the appearance of a new resonance Raman feature.

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