Determination of Ionization State by Resonance Raman Spectroscopy. Sulfonamide Binding to Carbonic Anhydrase†

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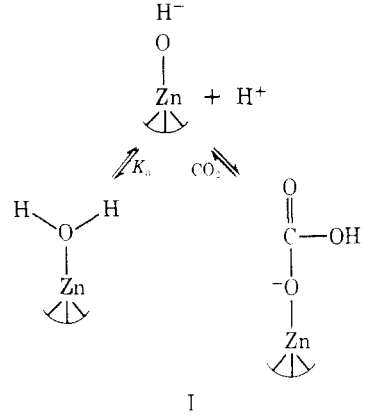
ABSTRACT: Resonance Raman (RR) spectroscopy has been used to study the ionization state of the sulfonamide, 4′-sulfamylphenyl-2-azo-7-acetamido-1-hydroxynaphthalene-3,6-disulfonate (Neoprontosil), bound to carbonic anhydrase. The correlation of effects of pH and deuteration on the spectra of model compounds with these effects on the Neoprotosil spectrum allows us to assign spectral bands in the 900–1000 and 1100–1200 cm^{-1} regions to the SO_2NH_2 group. Large shifts in these bands occur upon ionization of the sulfonamide. On the basis of the positions of bands in the enzyme complex, it was determined that the sulfonamide was bound to the enzyme as SO_2NH_2, rather than as SO_2NH^-. Rates of association and dissociation and the dissociation equilibrium constant were measured as a function of pH. The rate behavior for Neoprontosil is consistent with that observed for other sulfonamides and $k_{dissoc}/k_{assoc} = K_{dissoc}$, suggesting a one-step binding mechanism. Since RR spectroscopy establishes that the final ionization state of the sulfonamide in the enzyme complex is SO_2NH_2, protonated sulfonamide must bind directly to basic form of the enzyme. These conclusions suggest that sulfonamides form "outer-sphere" complexes with metal at the enzyme active site.

One potential application of resonance Raman (RR[1]) spectroscopy to biochemical problems is the determination of the ionization state of enzyme-bound chromophores. This paper reports the results of one such study.

Bovine carbonic anhydrase is a monomeric enzyme containing one gram-atom of zinc per mole of enzyme. Removal of the zinc results in loss of catalytic activity. X-ray studies for human carbonic anhydrase have shown that the zinc atom is located in a deep crevice which constitutes the active site and is coordinated to three histidinyl side chains and a water molecule (Liljas et al., 1972). A commonly accepted mechanism for the CO_2 hydration catalyzed by carbonic anhydrase is an "outer-sphere" mechanism which assigns a major role to the zinc-coordinated water molecule as shown in I.



I

Assuming this mechanism, the pK_a near 7, observed in the pH dependence of CO_2 hydration, is assigned to the coordinated water molecule. Recently, two other mechanisms have been proposed on the basis of NMR studies. The first, proposed by Pesando, involves a fourth histidinyl side chain near the active site, which ionizes at the enzyme pK_a to form a zinc-coordinated histidinyl species. The histidinyl species is then directly involved in the hydration reaction (Pesando, 1975). Alternatively, it has been proposed by Yeagle et al. that both an inner- and an outer-sphere bicarbonate ion take part in the catalytic process (Yeagle et al., 1975). The inner-sphere component of this mechanism involves direct substrate binding to zinc.

The catalytic activity of carbonic anhydrase is inhibited by aromatic sulfonamides, some of which form enzyme complexes with dissociation constants as low as 10^{-9} M. Both the aromatic ring and the unsubstituted SO_2NH_2 group are essential for strong binding (Linkskog, 1968). Studies of the binding of sulfonamides to the enzyme have suggested that: (1) binding takes place at a single site (Maren, 1967), (2) binding involves the metal atom (Coleman, 1967), and (3) binding involves significant contributions from aromatic interactions with the hydrophobic cleft (Chen and Kernohan, 1967). It has also been reported that sulfonamides bind as the anionic RSO_2NH^- on the basis of fluorescence (Chen and Kernohan, 1967), and difference ultraviolet-visible (King and Burgen, 1970) spectra, and the observation of a relationship between inhibitory activity and the sulfonamide pK_a (Miller et al., 1950). Since sulfonamides are specific competitive inhibitors for carbonic anhydrase, identification of direct metal–sulfonamide bonding is of interest in discussions of inner- vs. outer-sphere mechanisms.

The RR spectrum has recently been shown to be a useful method of studying the interaction of a chromophoric molecule with the active site of a metalloenzyme (McFarland et al., 1975). Therefore, we have chosen to use this method to study the interaction of a chromophoric sulfonamide with carbonic anhydrase. During the course of this work, the results of a RR study of the interaction of 4′-substituted 4-sulfonamidoazobenzenes with carbonic anhydrase were published (Kumar et al., 1974, 1976). That study concluded that sulfonamide binds in the anionic form.

We report a study of the binding of the compound 4′-sulfamylphenyl-2-azo-7-acetamido-1-hydroxynaphthalene-3,6-disulfonate (Neoprontosil, II) with carbonic anhydrase.

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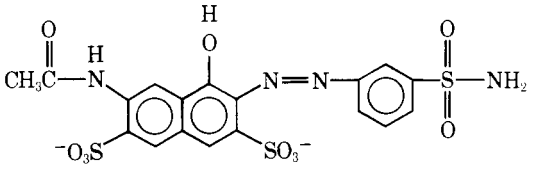
[1] Abbreviations used are: RR, resonance Raman; NMR, nuclear magnetic resonance; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.



II

Experimental Section

Preparation of Carbonic Anhydrase. Bovine carbonic anhydrase was obtained from Worthington Biochemicals, Freehold, N.J. Solution ultraviolet-visible spectroscopy showed a residual hemoglobin absorption at 410 nm, indicating a very small hemoglobin impurity in the commercial preparation. Carbonic anhydrase crystals were dissolved in the desired buffer and generally used without further purification. Where necessary to free enzyme of hemoglobin impurity, carbonic anhydrase was further purified by chromatography on DEAE-Sephadex A-50 from Pharmacia Fine Chemicals, Piscataway, N.J. All enzyme solutions were stored at 4 °C.

Preparation of Cobalt Carbonic Anhydrase. Apozinc carbonic anhydrase, prepared by the method of Coleman (1965), was dialyzed against a 250-fold excess of 1×10^{-4} M $CoCl_2$ solution in 0.05 M Tris buffer, pH 7.06. After 24 h, the solution was dialyzed against buffer to remove excess metal ions. The resulting solution displayed a visible absorption spectrum characteristic of bovine cobalt carbonic anhydrase (Coleman, 1967).

Activity Assays for Carbonic Anhydrase. Protein concentration was determined from absorbance measurements at 280 nm using a molar absorbtivity of 57 000 M^{-1} cm^{-1} for bovine carbonic anhydrase. The esterase activity toward *p*-nitrophenyl acetate was determined by the procedure of Armstrong et al. (1966). A fresh solution of 3.3 M *p*-nitrophenyl acetate (Aldrich) was prepared in 3 ml of acetone and 97 ml of water. The increase in absorbance at 348 nm over a 3-min period was measured for a mixture of 1.00 ml of this *p*-nitrophenyl acetate solution and 2.00 ml of 0.05 M Tris buffer (pH 7.06). Twenty microliters of carbonic anhydrase solution was added (with rapid mixing), and the increase in absorbance over another 3 min was recorded. The rate of the enzyme-catalyzed esterase reaction was proportional to the carbonic anhydrase concentration over the concentration range 1-10 μM.

Sulfonamide-inhibitor binding capacity was of greater interest than catalytic activity in this study. The sulfonamide, ethoxzolamide (a gift from Upjohn Co., Kalamazoo, Mich.), binds extremely tightly ($K_{dissoc} = 10^{-9}$ M) to carbonic anhydrase. Furthermore, it has been shown to form 1:1 complexes with carbonic anhydrase (Lindskog, 1966). These properties and the change in enzyme tryptophan fluorescence upon binding of ethoxzolamide to bovine carbonic anhydrase were the basis for a convenient active-site titration method for the enzyme. Carbonic anhydrase in pH 7.06, 0.05 M Tris buffer was titrated by ethoxzolamide; the decrease in fluorescence ($\lambda_{ex} = 280$, $\lambda_{em} = 340$ nm) due to complex formation was observed. Ethoxzolamide concentration was determined at pH 7.06 using $\epsilon_{300} = 9200$ M^{-1} cm^{-1}.

Stopped-Flow Kinetics of Sulfonamide Binding to Carbonic Anhydrase. Kinetic studies were carried out on a Durrum stopped-flow spectrophotometer using either a xenon arc or tungsten lamp as a light source. Mixing was initiated from syringes operated by an air-actuated pushing device.

Neoprontosil (a gift from Sterling-Winthrop Research Institute, Rensselaer, N.Y.) and ethoxzolamide both gave appreciable quenching of carbonic anhydrase tryptophan fluorescence. Sulfanilamide did not quench enzyme fluorescence.

The rate of sulfonamide binding to carbonic anhydrase was followed by observing fluorescence emission at 340 nm (λ_{ex} = 280 nm). The rate of Neoprontosil binding was also followed by observing the absorbance at 480 nm (λ_{max} for the difference spectrum of Neoprontosil vs. Neoprontosil-bovine carbonic anhydrase complex).

The association rates for Neoprontosil and ethoxzolamide were determined under pseudo-first-order conditions using about 5 μM carbonic anhydrase and at least a fivefold excess of the sulfonamide. In the presence of excess inhibitor, the slope of a plot of ln $(F_t - F_{eq})$ (the difference between fluorescence at time t and at equilibrium) vs. time is $k_{assoc}(S_0) + k_{dissoc}$ (S_0 is the initial sulfonamide concentration). The off rate, k_{dissoc}, is much less than $k_{assoc}(S_0)$ under the conditions used and is determined separately from the dissociation-rate experiments.

The dissociation rates for the sulfonamides were determined by pushing a large excess of sulfanilamide against the carbonic anhydrase-sulfonamide complex. Fluorescence changes observed as a function of time indicated a single-exponential process. The reaction may be represented as follows:

$$S_2 + CA\text{-}S_1 \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} CA + S_2 + S_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} CA\text{-}S_2 + S_1$$

If the displacing sulfonamide is present in large excess such that $k_2(S_2) \gg k_1(S_1)$, the reaction kinetics will be first order and independent of S_2 concentration. The observed rate should then be k_{-1}, the dissociation rate of S_1. The equilibrium constant for binding of Neoprontosil to carbonic anhydrase was measured using a titration method which depended upon observation of quenching of enzyme fluorescence caused by binding of Neoprontosil. Excitation of enzyme fluorescence was 280 nm and emission was measured at 340 nm; fluorescence measurements were made on a Farrand spectrofluorimeter.

Visible-Ultraviolet Spectroscopy. Absorption spectra in the ultraviolet and visible regions were recorded on either a double-beam Beckman Acta V or Cary 17 spectrophotometer. Optical density at a single wavelength for concentration or binding-constant measurements were made on a Cary 16 spectrophotometer. The Cary 16 was equipped with a Varian recorder to measure absorbance as a function of time in the steady-state kinetic experiments.

Raman Spectroscopy. Raman spectra were recorded on a Spex 1401 laser Raman spectrophotometer equipped with a cooled ITT FW 130 phototube. A Coherent Radiation Model 52 argon ion laser was used for monochromatic excitation. Spectra were obtained using the 514.5-, the 488-, and the 476.5-nm laser wavelengths. Spike filters were employed to screen out all laser lines except the exciting frequency. Although the quality of the spectrum sometimes depended on which excitation frequency was employed, in no case did the line position or relative band intensity show significant excitation frequency dependence. Spectral slit widths for all spectra reproduced as figures were 5 cm^{-1} or less, unless otherwise noted in the figure. Line positions are accurate to at least ± 5 cm^{-1}. Spectra were generally obtained using melting-point capillary cells and did not vary with the time of irradiation.

Results and Discussion

Spectroscopic Studies. Neoprontosil has been shown to be an inhibitor of carbonic anhydrase esterase activity and to bind the enzyme with a dissociation constant of 1.5×10^{-6} M

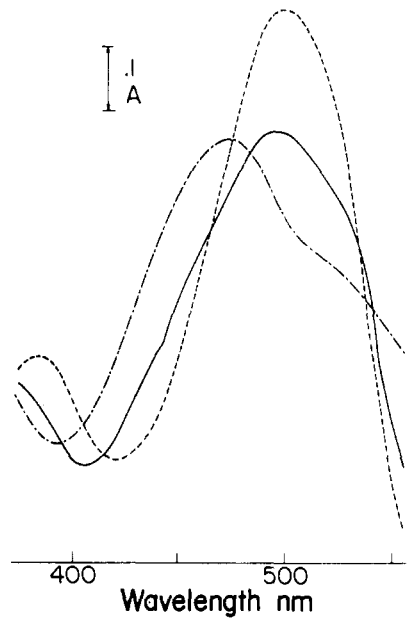


FIGURE 1: Visible-ultraviolet spectra of Neoprontosil solutions at pH 8.5 (- - -), pH 11.1 (—), and pH 12.2 (- • -). [Neoprontosil] = 3.9×10^{-5} M.

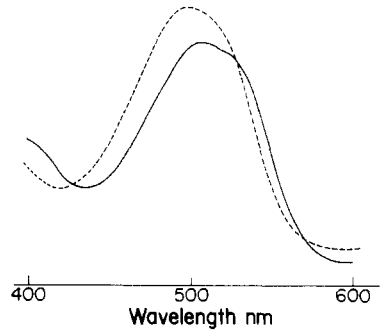


FIGURE 2: Visible-ultraviolet spectra of free Neoprontosil (···) and carbonic anhydrase bound Neoprontosil (—). [Neoprontosil] = 2.2×10^{-5} M. [Carbonic anhydrase] = 2.4×10^{-5} M. pH 7.0.

(Coleman, 1968). The visible absorption spectrum of Neoprontosil at three pH's is shown in Figure 1 and the spectrum of the complex with carbonic anhydrase is compared with that of free Neoprontosil in Figure 2. There are two proton ionizations in the pH range represented by Figure 1 as demonstrated by the absence of isosbestic points. Visible spectral titrations show pK_a values near pH 10.5 and 11.8. A pK_a at pH 10.4 had previously been assigned to a sulfonamide proton (Coleman, 1968) but no mention of the second pK_a, due to the naphthol proton, had been made.

Since the visible spectra of each ionic form of Neoprontosil and of the enzyme-bound compound show very strong absorptions at 514 and 488 nm, the laser wavelengths used for Raman excitation with our spectrometer, they were potential RR scatterers. All forms of the compound produce a strong RR spectrum at concentrations $<10^{-4}$ M using both 488.0- and 514.5-nm laser excitation. A RR titration established that three different ionic forms of Neoprontosil exist between pH 8.5 and 13.0, as demonstrated in Figure 3. The RR spectral changes on proceeding from pH 9.0 to 11.0 are dominated by the shift in the 918 cm^{-1} band to 933 cm^{-1}, the appearance of a new medium intensity band at 1156 cm^{-1}, and the disappearance of bands at 1196 and 1176 cm^{-1}. (These bands are marked (*) in Figure 3.) The spectral changes on going from

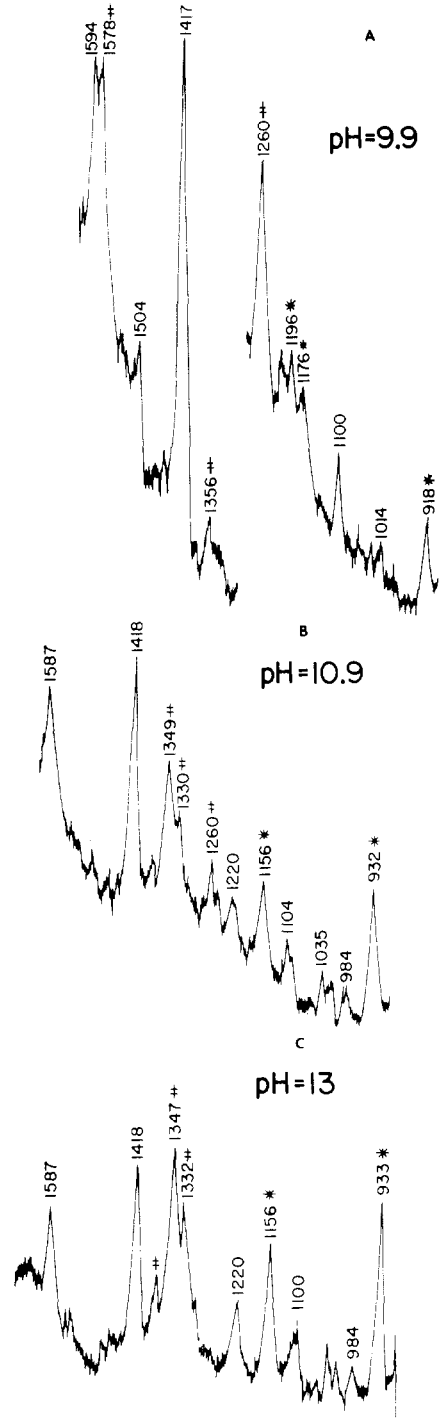
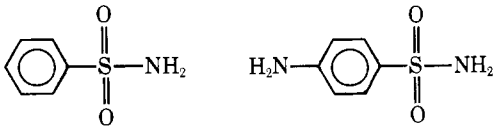


FIGURE 3: Resonance Raman spectra of Neoprontosil at (A) pH 9.9, (B) 10.9, and (C) 13. Solutions are unbuffered. Spectra were obtained using 488-nm excitation.

pH 11.0 to 13.0 include the disappearance of a band at 1578 cm^{-1}, the intensity enhancement of a band at 1347 cm^{-1} which becomes the strongest band in the spectrum, new bands at 1375 and 1332 cm^{-1}, and loss of a peak at 1260 cm^{-1}. (These bands are labeled (∓) in Figure 3.)

The RR spectral changes that occur on passing through the pK_a at pH 10.5 bear close similarity to those that are observed in the normal Raman spectrum of two aromatic sulfonamides upon acid ionization of the sulfonamide group. The Raman frequencies assigned to sulfonamide group vibrations for benzenesulfonamide, III, and sulfanilamide, IV, are listed in

Table I together with the pertinent Neoprontosil frequencies.

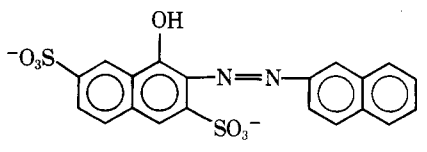


III IV

In each case, a band in the 900 cm^{-1} region shifts to higher frequency and a peak above 1150 cm^{-1} shifts to lower frequency upon ionization of the proton. Bands near 900 and 1170 cm^{-1} have been previously assigned to ν(S–N) and symmetric ν(S–O) vibrations, respectively, in several simple sulfonamides (Colthup et al., 1964; Goldstein et al., 1969). We have investigated several other azosulfonamides, including one of those studied by Carey and co-workers (Kumar et al., 1974, 1976). In none of these did we observe the set of bands which we have assigned to the sulfonamide subgroup. Neoprontosil was uniquely suited to our experiments, since the λ_{max} for all forms, including the enzyme complex, falls in the 480–510 nm region where our most intense laser excitation frequencies are available. At these wavelengths, we are able to excite with ~100 mW power without any apparent photodecomposition problems. Thus, the RR spectrum which we obtained for Neoprontosil may have been more intense than for other sulfonamides which have been studied by this method.

The resonance enhanced behavior of the bands near 900 and 1150 cm^{-1} further supports our assignment of these to ν(S–N) and ν(S–O), respectively. The acid ionization at pK_a = 10.5 results in a considerable increase in the intensity of the band near 900 cm^{-1} for the anionic form relative to the acid form of the sulfonamide. This could be the result of increased coupling of the S–N bond into the conjugated system, as is suggested by the fact that ν(S–N) increases in frequency upon ionization, indicating increased double-bond character in the S–N bond. In addition, in the normal Raman spectrum, obtained by excitation with a tunable laser operating near 600 nm, the relative intensity of bands assigned as ν(S–O) and ν(S–N) is much greater than in the RR spectrum obtained by excitation near 500 nm. This would be expected if the sulfonamide group vibrations are only weakly resonance enhanced, while most of the strong bands in the RR spectrum, such as ν(N=N) and aromatic-ring vibrations, are strongly enhanced.

Since those bands which shift upon passing through the pK_a at 10.5 are vibrations of the sulfonamide group, then the second pK_a at 11.8 must be associated with ionization of the napthol proton. The changes in RR spectral features on passing through pH 11.8 must then be associated with the napthol ring and/or the azo linkage. To further test this thesis, we have studied a model compound, 2-azonaphthyl-1-hydroxynaphthlene-3,7-disulfonate (Azorubin-S), V.



V

This compound contains the 1-hydroxy-2-azonaphthalenic group but no other ionizable group. The RR spectrum of azorubin-S is shown in Figure 4 both above and below the pK_a for ionization of the naphthol proton. The changes observed upon acid ionization are very similar to shifts observed for Neoprontosil near pH 11.8. The affected bands are probably

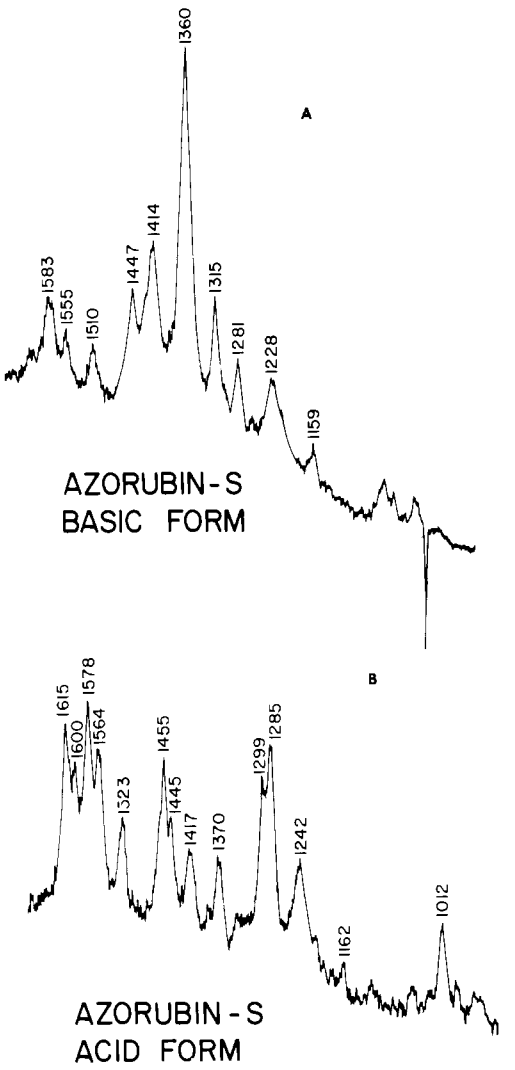


FIGURE 4: Resonance Raman spectra of Azorubin-S (A) above and (B) below the ionization pK. Spectral resolution, 10 cm^{-1}; excitation wavelength, 488 nm.

naphthol-ring and azo-group vibrations, since these appear to be very sensitive to ionizations of hydroxy groups ortho to the azo linkage (McFarland et al., 1975; Petersen et al., unpublished data). These observations confirm our assignment of the ionizable group with pK_a = 11.8 as the naphtholic hydroxyl group.

The RR spectrum of the inhibitor complex formed between Neoprontosil and carbonic anhydrase could be easily obtained at concentrations below 1×10^{-4} M. Throughout the pH range 6.0–11.2, the spectrum of the bound Neoprontosil is indistinguishable from that of free Neoprontosil in the protonated form. Under the enzyme and azosulfonamide concentrations employed in these spectroscopic experiments, the Neoprontosil is quantitatively bound to carbonic anhydrase ($K_{dissoc} < 1 \times 10^{-7}$ M). The RR spectrum of free Neoprontosil at pH 11.2 is compared with that of the enzyme complex at the same pH in Figure 5. These results indicate that the inhibitor binds in the *protonated form only*. The free dye spectrum is typical of the anionic form of the sulfonamide, SO_2NH^-, while the complex gives a spectrum typical of protonated form, SO_2NH_2. Furthermore, the displacement of this azosulfonamide inhibitor by another tight-binding sulfonamide, ethoxzolamide, causes the Neoprontosil spectrum to revert to that of the anionic form of the free ligand at pH 11.2 shown in

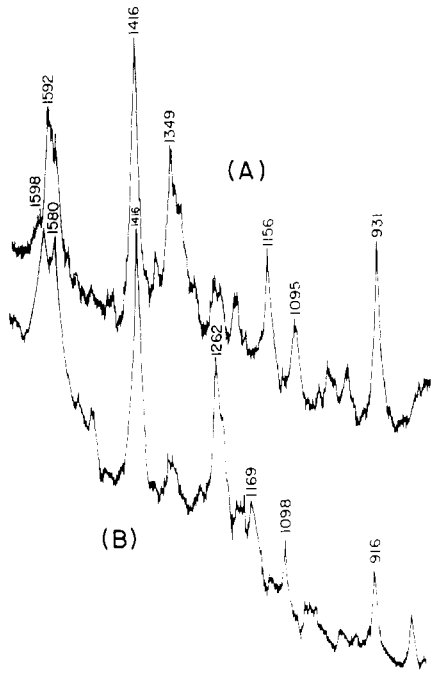


FIGURE 5: Resonance Raman spectra of free Neoprontosil (A) and carbonic anhydrase bound Neoprontosil (B); pH = 11.2. [Neoprontosil] = 4×10^{-5} M. [Carbonic anhydrase] = 5.3×10^{-5} M. Addition of 8×10^{-5} M ethoxlamide to the Neoprontosil-carbonic anhydrase complex produced the spectrum shown in A. Spectra were obtained using 488-nm excitation. Solutions were unbuffered.

TABLE I. Raman Frequencies[a] Assigned to Sulfonamide Group Vibrations for Model Compounds and for Neoprontosil.

Benzenesulfonamide		
pH 7[b]	1168 (s)[d]	925 (w, br)
pD 7	1152 (s)	
pH 13[c]	1121 (s)	976 (s)
pD 13	1124 (s)	976 (s)
Sulfanilamide		
pH 7[b]	1165 (s)	
pD 7	1148 (s)	
pH 12[c]	1114 (s)	973 (s)
pD 12	1117 (s)	973 (s)
Neoprontosil		
pH 9.9[b]	1196 (w)	918 (m)
	1176 (w)	
pD 9.9	1197 (w)	896 (w)
	1171 (w)	
pH 13[c]	1156 (m)	933 (s)
pD 13	1153 (m)	933 (s)
Neoprontosil-Carbonic Anhydrase Complex		
pH 9.7	1196 (w)	918 (m)
	1176 (w)	
pD 9.7	1197 (w)	895 (w)
	1171 (w)	

[a] Frequencies given in cm^{-1}. [b] pH values at which the sulfonamide is in the protonated form. [c] pH values at which the sulfonamide is in the anionic form. [d] s = strong, m = medium, w = weak, br = broad.

Figure 5. We obtained identical RR results for the Neoprontosil complex of cobalt carbonic anhydrase.

Previous studies have indicated that the vibrational spectrum of a metallated ligand can be very similar to the spectrum of the protonated ligand (Mansy and Tobias, 1974). Our observation of a Neoprontosil spectrum typical of the protonated form could be the result of zincation rather than protonation upon formation of the sulfonamide-enzyme complex. The desired model zinc complex of Neoprontosil could not be obtained due to the presence of azo and naphthol groups, which would preferentially chelate free zinc ion in solution. However, deuterium substitution discriminates between zincation and protonation as follows. Table I lists the frequencies obtained for benzenesulfonamide, sulfanilamide, and Neoprontosil in D_2O. Under these conditions, the sulfonamide and naphthol protons would be replaced by deuterons. As is seen in the table, the band at 918 cm^{-1}, associated with SO_2NH_2, is shifted to 896 cm^{-1} in D_2O where the sulfonamide exists as SO_2ND_2. At pH 11.0, where the predominant species is SO_2NH^-, deuteration causes no frequency shifts. As is seen in the table, this observation is consistent with the normal Raman spectrum of the simple sulfonamides we have studied (Table I). The Raman spectrum of the enzyme complex of Neoprontosil in D_2O is identical to the spectrum of the free acid form in D_2O (Table I). The identity of the RR spectrum of the enzyme-bound ligand to that of the acid form of Neoprontosil in both H_2O and D_2O eliminates the possibility that we are observing the zinc complex of the anionic form.

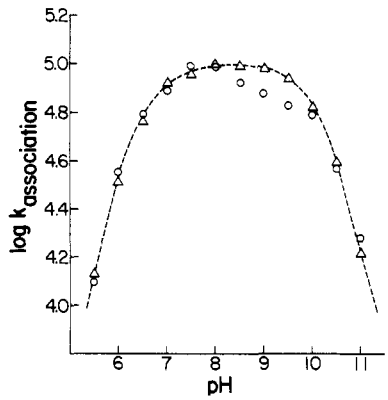
In summary, the pH dependence of the RR spectrum of Neoprontosil establishes the pK_a for sulfonamide ionization to be 10.5. The Neoprontosil-enzyme complex gives a RR spectrum identical to that of the low pH acid form even at pH's where the free sulfonamide is ionized. We conclude, therefore, that Neoprontosil binds to carbonic anhydrase in the protonated form.

Kinetic Studies. To determine whether the mechanism of sulfonamide binding for Neoprontosil is similar to the binding of other sulfonamides, we have investigated the pH dependence of the association and dissociation rates for Neoprontosil binding to carbonic anhydrase. The rate of the reaction was followed using the stop-flow method by monitoring changes in enzyme tryptophan fluorescence which are associated with complex formation.

The pH dependence of the association rate constants are shown in Figure 6. The rate of dissociation of sulfonamides from carbonic anhydrase has previously been shown to be pH independent (Taylor et al., 1970). We observe the same behavior for Neoprontosil. The rate of association for Neoprontosil binding exhibits a bell-shaped pH profile with a maximum between pH 7 and 9 (Figure 6). The behavior observed for Neoprontosil parallels the pH dependence of sulfonamide inhibition of the esterase activity of bovine cobalt carbonic anhydrase as previously shown (Linkskog and Thorslund, 1968). The steeply increasing slope of the profile at pH 6-7 corresponds to an enzyme pK_a in this region for cobalt carbonic anhydrase (Lindskog, 1962), while the decreasing profile from pH 10-11 corresponds to the sulfonamide pK_a.

The rate of dissociation, measured at two different pH values ($k_{dissoc} = 2.2 \times 10^{-3}\ s^{-1}$ at pH = 11.0 and $k_{dissoc} = 3.2 \times 10^{-3}\ s^{-1}$ at pH = 7.5), was used to obtain the ratio k_{dissoc}/k_{assoc}. This ratio is equal to the independently measured K_{dissoc} at both pH's ($K_{dissoc} = 2.8 \times 10^{-8}$ at pH 11 and $K_{dissoc} = 5.4 \times 10^{-8}$ at pH 7.5), indicating a single rate-limiting binding step in the formation of the enzyme-inhibitor complex.

Two mechanistic models have been proposed to explain the observed rate behavior for sulfonamide binding to carbonic anhydrase. In the first, the basic form of the enzyme reacts with the acidic form of the sulfonamide, while, in the second, the acid form of enzyme reacts with the basic form of the sulfon-



PIGURE 6: Plot of association rate constant vs. pH for the binding of Neoprontosil to bovine carbonic anhydrase. Experimental points (○); theoretical points based upon equations of Taylor et al., 1970 (△).

amide (Taylor et al., 1970). We have fit our Neoprontosil binding data to the equations developed (Taylor et al., 1970) for the mechanistic models cited above. The best fit of calculated to experimental rate profile is obtained with $pK_E = 6.3$ and $pK_S = 10.3$ (Figure 6). The pK_a of 6.3 for the enzyme is reasonably close to the pK_a of 7.0 ± 0.2 identified in the visible spectrum of cobalt carbonic anhydrase (Lindskog, 1962), while the pK_a value of 10.3 is in satisfactory agreement with our assignment of the sulfonamide pK_a at 10.5

Conclusions

The RR spectral study reported here provides very strong evidence that Neoprontosil, the sulfonamide studied, is bound to the enzyme surface in the acidic (protonated) form. Our results are not in agreement with the results of an earlier RR study of sulfonamide binding, which concluded that sulfonamides bind in the anionic form (Kumar et al., 1974, 1976). However, that conclusion was based upon small shifts of bands in the 1125–1160 cm^{-1} region, which were not identified as vibrations of the sulfonamide functionality but were, instead, assigned to Ph–N vibrations. We observe no bands for Neoprontosil, which display this pattern of shifts, but we do observe bands which, on the basis of their pH dependence, the study of model compounds, and shifts occurring upon deuteration, can be assigned to ν(S–O) and ν(S–N) vibrations of the sulfonamide group. It is the behavior of these bands which provides evidence that the sulfonamide group of Neoprontosil binds in the protonated form. The virtual identity of the vibrational spectrum of the acid form of the sulfonamide to the enzyme-bound sulfonamide provides the most unambiguous evidence currently available about the ionization state of sulfonamide bound to carbonic anhydrase. We have investigated several other chromophoric sulfonamides but none of them displayed a RR spectrum which contained the bands typical of the SO_2NH_2 group. Apparently, the electronic structure of Neoprontosil is ideally suited to RR studies employing the exciting laser frequencies available to us. An extremely intense RR spectrum, which includes the bands due to the sulfonamide group, is obtained only for this compound among the several which have been studied by us and other workers.

Our kinetic studies are consistent with a simple one-step binding mechanism, since the ratio of our measured association and dissociation rate constants agrees with the binding equilibrium constant. This and the pH–rate profile show that the binding of Neoprontosil to carbonic anhydrase is the same as the binding of other sulfonamides and, thus, strongly suggests that other sulfonamides also bind in the protonated form. Our observation that the enzyme-bound sulfonamide is protonated demands a two-step process for sulfonamide binding if the initial reactants are anionic sulfonamide and protonated enzyme

$$E{-}BH + SA \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E\begin{matrix}BH\\SA\end{matrix} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E\begin{matrix}B\\SAH\end{matrix} \quad (1)$$

On the other hand, eq 2 shows a one-step mechanism leading to the same final state involving a reaction of protonated sulfonamide with the basic form of the enzyme

$$E{-}B + SAH \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E\begin{matrix}B\\SAH\end{matrix} \quad (2)$$

Our data support the mechanism shown in eq 2, since the agreement of k_{dissoc}/k_{assoc} with K_{eq} indicates a one-step binding mechanism. Earlier kinetic studies on sulfonamide binding to carbonic anhydrase supported the mechanism of eq 2 (Taylor et al., 1970).

Finally, our observation that sulfonamides (which are potent and competitive inhibitors of substrate binding to carbonic anhydrase) bind in the protonated form is not consistent with direct inner-sphere complexation to active-site zinc. This is interesting in light of the fact that, although many previous studies have suggested direct inner-sphere sulfonamide complexation, few have invoked inner-sphere binding of substrate in CO_2 hydration.

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