CARBONIC ANHYDRASE–AROMATIC SULFONAMIDE COMPLEXES, A RESONANCE RAMAN STUDY

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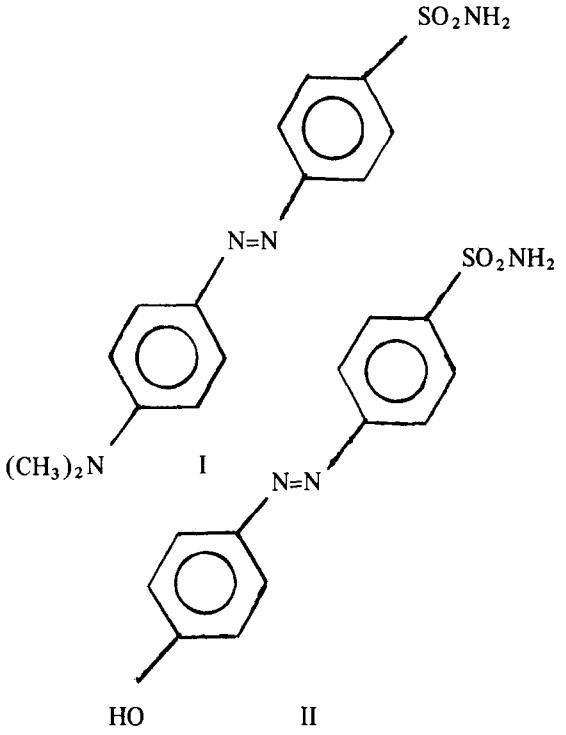
1. Introduction

The zinc metalloprotein carbonic anhydrase is strongly inhibited by a variety of aromatic sulfonamides [1–3]. This class of inhibitors is of great importance in studies of the physiological function of carbonic anhydrase [4]. X-ray crystallography [4] and spectroscopic studies [5,6] have shown that the inhibitor is bound in a hydrophobic cleft in the enzyme with the sulfonamido group probably within the co-ordination sphere of the zinc ion. However, the detailed geometry of the co-ordination is not yet known. It has been suggested by some workers [2,5] that the bound sulfonamide is present in the anionic state.

Resonance Raman spectroscopy provides a sensitive tool for probing binding site structure in proteins and for obtaining detailed information on changes occuring in the ligand upon binding [7]. It has been recently used to study 2,4-dinitrophenyl hapten–antibody interactions [8] and to follow critical substrate bonds during enzymolysis [9]. This report details an extension of this technique to study the enzyme inhibitor complexes between carbonic anhydrase and two sulfonamides,



4-sulfonamido-4′-dimethylaminoazobenzene (I) and 4-sulfonamido-4′-hydroxyazobenzene (II).

It was possible to distinguish effects due to sulfonamido group ionization, conformational changes in the aromatic nuclei and hydrophobic bonding. Direct evidence has been obtained for the anionic form ($-SO_2N^-H$) in the bound sulfonamido group. Slight distortion from planarity in the Ph-N=N-Ph moiety probably occurs on binding.

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2. Materials and methods

Bovine erythocyte carbonic anhydrase B was purchased from Miles Laboratories, Inc., Kankakee, III., USA, and used without further purification. The measured activity was 1430 units/g using *p*-nitrophenyl acetate as substrate [10]. Sulfonamides were prepared by one of us (R.W.K.) and purity was established by thin-layer chromatography.

The resonance Raman spectra were recorded using a Jarrell-Ash 25-400 laser Raman spectrophotometer equipped with a He-Cd laser (Spectra-Physics model 185). Line positions are accurate to ±2 cm^{-1}. Concentrations of enzyme and sulfonamides and the spectral conditions are reported in the figure captions. Spectra of the sulfonamide–enzyme complex were insensitive to increase in relative enzyme concentration, showing that unbound sulfonamides were not being detected. Neither photodecomposition nor photoisomerization were observed under the conditions employed.

In all the resonance Raman spectra of II reported herein the -OH group is present in the anionic form ($-O^-$). For the unbound sulfonamides the sulfonamido group is present as $-SO_2NH_2$ at pH ~8 and as SO_2N^-H at pH 13 (figs. 1 and 2). Since the resonance Raman spectrum of the inhibitor is much more intense than the normal Raman spectrum of the protein at the low concentrations used (~10^{-5} M), the protein contribution to the spectra of the complex can be ignored.

The visible absorption spectra of the free, bound and ionized sulfonamides are shown in fig. 3, laser excitation frequencies are marked by arrows. Intensity enhancement of the ligand's Raman spectrum is due to coupling between electronic and vibrational states and is achieved by exciting into the ligand's absorption spectrum. For II experiments using 4880 Å excitation yielded similar changes upon binding to those using 4416 Å.

3. Results and discussion

The nature of the protein binding site and the structure of the bound sulfonamide can be elicited from the changes taking place in the resonance Raman spectrum of the ligand upon binding [8]. Changes in the structure of the sulfonamide upon binding can

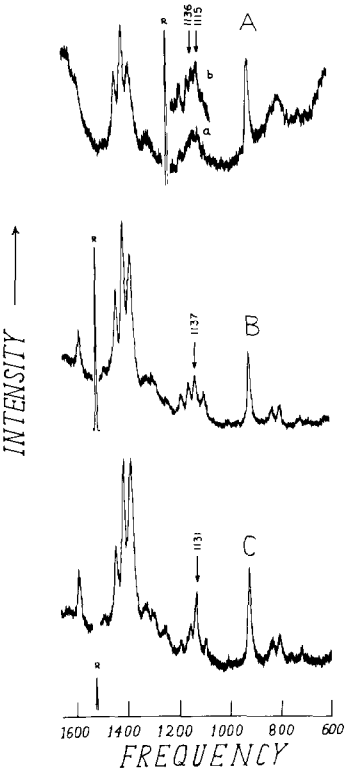

Fig. 1

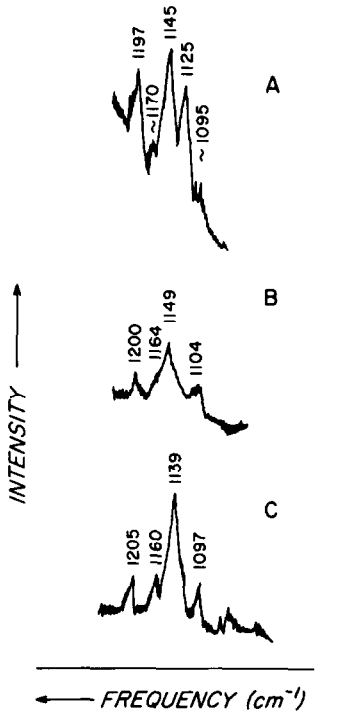

Fig. 2

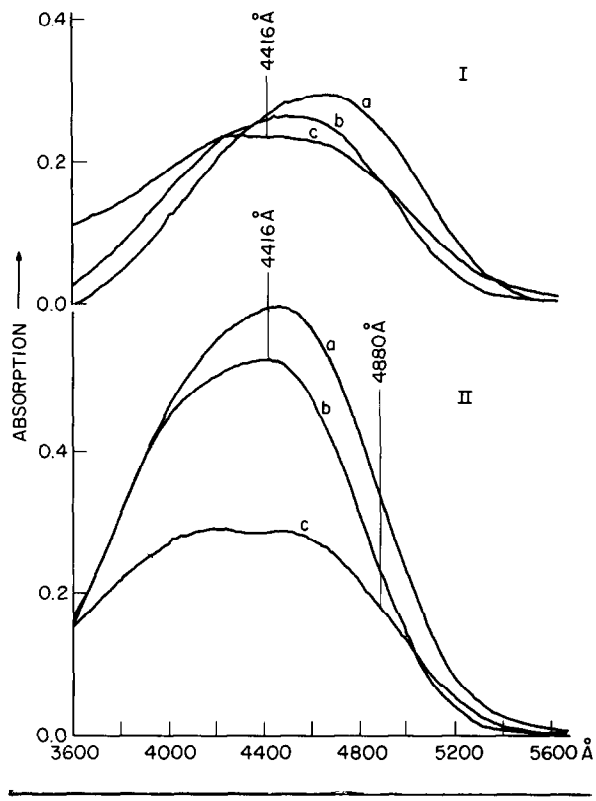

Fig. 1. Resonance Raman spectra of 4-sulfonamido-4'-hydroxyazobenzene, 441.6 nm excitation: (A) bound to enzyme, Tris–SO_4 buffer, pH 8.0, (a) sulfonamide 1.6×10^{-5} M, enzyme 3.5×10^{-5} M, (b) sulfonamide 5×10^{-5} M, enzyme 6.6×10^{-5} M, (B) SO_2N^-H form 3.9×10^{-5} M in 0.1 N KOH, pH 13.0, (C) SO_2NH_2 form 1.1×10^{-4} M, Tris–SO_4 buffer, pH 8.2. R denotes calibration lines from Xe lamp. Instrumental conditions: slit width ~10 cm^{-1} [(A) (a)], slit width ~5 cm^{-1} [(A) (b), (B), (C)], scan speed 0.5 cm^{-1}/sec [(A)(a,b), (B), (C)], time constant 5 sec. [(A) (b)], time constant 2 sec [(A)(a), (B), (C)], power ~35 mW [(A)(a), (B)], power ~100 mW (C). Ratemeter setting 1000 counts/sec in all cases.

Fig. 2. Resonance Raman spectra of 4-sulfonamido-4'-dimethylaminoazobenzene, 441.6 nm excitation: (A) bound to enzyme, CH_3CN:Tris–SO_4 buffer (25:75 v/v), pH 7.9, sulfonamide 1.75×10^{-5} M, enzyme 2.5×10^{-5} M, (B) SO_2N^-H form 2×10^{-4} M, 0.1 N KOH, pH 13.0, (C) SO_2NH_2 form 2.5×10^{-5} M in CH_3CN:Tris–SO_4 buffer (50:50 v/v), pH 7.9. Instrumental conditions: slit width ~10 cm^{-1} (A), slit width ~5 cm^{-1} [(B),(C)], scan speed 0.5 cm^{-1}/ sec. [(A),(B),(C)], time constant 5 sec. (A), time constant 2 [(B),(C)], power ~20 mW [(A),(C)], power ~80 mW (B). Ratemeter 1000 counts/sec in all cases. Enzyme activity was unchanged after one hr in CH_3CN: Tris–SO_4 buffer (25:75 v/v).

Fig. 3. Visible absorption spectra: compound I a) 6.6×10^{-5} M pH 8.0, b) 6.6×10^{-5} pH 13.6 c) bound to carbonic anhydrase 1.6×10^{-5} M enzyme ~1×10^{-4} M pH 8.0. Compound II a) 1.6×10^{-5} M pH 9.3, b) 1.6×10^{-5} M pH 13.3 c) bound to carbonic anhydrase II 1.4×10^{-5} M enzyme 7×10^{-5} M pH 9.2. Compound I was dissolved in CH_3CN–H_2O (buffer) mixed solvent (32:68 v/v). All the spectra were recorded in 1 cm path length cell.

be understood by comparing the spectra (figs. 1 and 2, table 1) of the bound molecules with the free sulfonamide ($-SO_2NH_2$) and free ionized form of the sulfonamide ($-SO_2N^-H$). An increase in frequency (cm^{-1}) of the 1139 (I) and 1131 cm^{-1} (II) bands (probably Ph-N stretches) upon binding and ionization strongly suggests that the bound sulfonamido group is present as SO_2N^-H (figs. 1 and 2). This is supported by a close resemblance between the spectra of the bound and free ionized sulfonamides in the region 1350–1450 cm^{-1} (fig. 1). The presence of the $-SO_2N^-H$ form is strong evidence that the sulfonamido group remains in the co-ordination sphere of the Zn in going from the crystalline [4] to the aqueous complex.

The hydrophobic nature of the sulfonamide site cannot account for the above frequency and intensity changes. The requencies and relative intensities of the bands in I are insensitive to increasing CH_3CN concentration in a CH_3CN–H_2O mixed solvent and remain the same within experimental error in a spectrum of solid I (not shown) obtained under the same spectral conditions.

Changes in the planarity of the Ph-N=N-Ph moiety upon binding can, at best, be only very slight and cannot explain the observed frequency and intensity changes. The 1350–1450 cm^{-1} region contains the N=N stretching vibration (probably the peak near 1420 cm^{-1} [12]) and no frequency changes are observed in this region upon binding, any significant reduction in planarity would increase the N=N frequency. The Ph-N stretch can be assigned to 1139 (I) and 1131 (II) cm^{-1} on the basis of [12] and the fact that these bands are the strongest features in the normal Raman spectra (not shown) excited at 6471 Å. If binding reduced the planarity in the Ph-N=N-Ph moiety the Ph-N= stretching vibration would be expected to shift to lower wave numbers. However, for molecule I slight twisting about the Ph-N= or

Table 1
The fequencies of the Raman lines of 4-sulfonamido-4′-dimethylaminoazobenzene (I) and 4-sulfonamido-4′-hydroxyazobenzene (II)

Free ($-SO_2NH_2$)		Free Ionized ($-SO_2N^-H$)		Bound sulfonamide	
I cm^{-1} Int	II cm^{-1} Int	I cm^{-1} Int	II cm^{-1} Int	I cm^{-1} Int	II cm^{-1} Int
	717 w		716 w		723 w
	804 w		804 w		801 w
827^{-h_3} w	834 w	825 w	834 w	828^{-h_3} w	831 w
921^{-d_3} m	924 m	924 m	924 m	922^{-d_3} m	921 ms
1097^{-h_3} w	1095 w	1104 w	1101 w	~1095^{-h_3} vvw	1095 vw
				1125^{-h_3} w	1115 w
1139^{-h_3} m	1131 m	1149 w	1137 w	1145^{-h_3} w	1136 w
1160^{-h_3} w	1155 w	1164 sh	1161 w	~1170^{-h_3} w	1154 w
1205^{-h_3} w	1200 w	1200 w	1191 w	1197^{-h_3} w	1184 w
	~1254 w		~1248 vw		overlap
	1299 w		1302 vw		~1299 w
1371^{-d_3} m	1326 w	1317 w	1326 vw	1369^{-d_3} m	~1320 w
		1374 m			
1392^{-d_3} ms	1390 vs	1392 ms	1389 s	1369^{-d_3} m	1389 ms
1413 / 1419 $-d_3$ s	1416 s	1416 / 1422 vs	1416 s	1417 / 1422 d_3 vs	1413 s
1446^{-d_3} m	1443 m	1446 m	1443 m	1446^{-d_3} m	1443 m
	1491 vw		1488 vw		1491 vvw
1587^{-d_3} w	1593 m	1596 w	1590 mw	~1587^{-d_3} w	1593 vvw
1605^{-d_3} w		1605 w		~1602^{-d_3} w	

Abbreviations: w = weak, m = medium, s = strong, vw = very weak, vvw = very very weak, ms = medium strong, vs = very strong, $^{-d_3}$ = indicates frequency in CD_3CN-H_2O (buffer), and $^{-h_3}$ = indicates frequency in CH_3CN-H_2O (buffer).

Ph-$N(CH_3)_2$ linkages may occur since the 1145 cm^{-1} feature in bound sulfonamide lies between the values for free ($-SO_2NH_2$) and free ionized ($-SO_2N^-H$) forms. Thus the 1145 cm^{-1} position may represent the superposition of two effects, ionization tending to increase and twisting about Ph-N bonds tending to decrease the frequency. Although spectral changes in the 1100 cm^{-1} region are complicated due to overlapping bands, similar effects are seen for II upon binding. The intensity of the feature ~1590 cm^{-1} (a benzenoid ring mode) is sensitive to changes in conjugation [11] and further evidence for slight twisting for I (not shown) and II (fig. 1) is seen in the reduction of the intensity of this band upon binding. Consistent with the X-ray evidence for the shape of the cleft [4], perturbation from planarity is small since the -N=N- frequency does not shift significantly upon binding. The larger effect in the Ph-N= vibration follows from its greater sensitivity to changes in conjugation compared to the -N=N- stretching mode [13]. Slight twisting about Ph-N bonds could account for the relative enhancement of the high energy shoulder of the visible spectrum seen upon binding (fig. 3).

Vibrations associated with the $-SO_2NH_2$ group are vital for an understanding of the Zn, $-SO_2N^-H$ co-ordination geometry. In this respect an important change in the spectra of both bound sulfonamides is the appearance of a new feature at 1125 cm^{-1} in I and 1115 cm^{-1} in II. Comparison of this spectral region for the free inhibitors with their parent molecules *p*-dimethylaminoazobenzene and *p*-hydroxyazobenzene [12] does not reveal any band which can be attributed to a $-SO_2$ symmetric stretching vibration of the $-SO_2NH_2$ group. However, sulfonamide–zinc interaction in the

active site may result in the intensity enhancement of a mode not seen in the free molecules. Thus the appearance of this new band and the intensity changes in the band at ~920 cm^{-1} (fig. 1), where a contribution from a N-S stretching vibration is expected [11], are being studied in an attempt to gain further information on the mode of the zinc–ligand interaction.

These results show that resonance Raman spectroscopy is capable of yielding detailed structural information on a physiologically important enzyme–inhibitor complex. By the choice of suitable inhibitors the method may be extended to other enzyme systems.

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