Aromatic Ring Dynamics in a Carbonic Anhydrase–Inhibitor Complex

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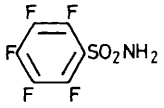
The aromatic ring of pentafluorobenzenesulphonamide rotates slowly when this inhibitor is bound at the active site of human carbonic anhydrase II but the rotation is much more rapid than dissociation of the enzyme–inhibitor complex.

Carbonic anhydrase is an enzyme found in a wide variety of plant and animal tissues and in several isozymic forms.[1] A major role for these proteins is catalysis of the hydration of carbon dioxide to carbonic acid and the most active of these have been perfected by evolution to the extent that their turnover numbers correspond to about 10^6 catalytic events per second.[2]

Primary benzenesulphonamides are good inhibitors of carbonic anhydrase[3] and find clinical utility as diuretics[4] and in the treatment of glaucoma, pulmonary disorders, and gastroduodenal ulcers.[5—7] Molecular features which contribute to the specificity of sulphonamide–enzyme interactions have been examined in detail.[8] N.m.r. experiments show that binding of sulphonamides to carbonic anhydrase involves direct co-ordination of the monoanion derived from the sulphonamide to the zinc atom at the active site of the enzyme.[9,10] These conclusions are consistent with the results from other spectroscopic experiments and crystallographic studies.[11]

Pentafluorobenzenesulphonamide (**1**) is an effective inhibitor of carbonic anhydrase (K_I 2×10^{-8} M).[12] We have now shown that rotation of the pentafluoroaromatic ring in the complex formed between (**1**) and the enzyme is slow (k 4 s^{-1}), although ring rotation is much more rapid than the rate of dissociation of the complex (k 0.02 s^{-1}). Thus, conformational excursions of the enzyme large enough to permit dissociation of the inhibitor complex occur much less frequently than those which expand the protein sufficiently to permit ring rotation, and both of these processes are very much slower that those which release product during the hydration reaction.

Pentafluorobenzenesulphonamide was prepared using a procedure based on that of Robson *et al.*[13] while human carbonic anhydrase II was obtained from Sigma Chemical Co. Figure 1(a) shows the ^{19}F n.m.r. spectrum of the inhibitor. Based on precedent the three signals that are observed can be assigned to fluorine nuclei *ortho*, *para*, and *meta* to the sulphonamide group of (**1**) as indicated in Figure 1.[14] When enzyme is present in a concentration in excess of the inhibitor,



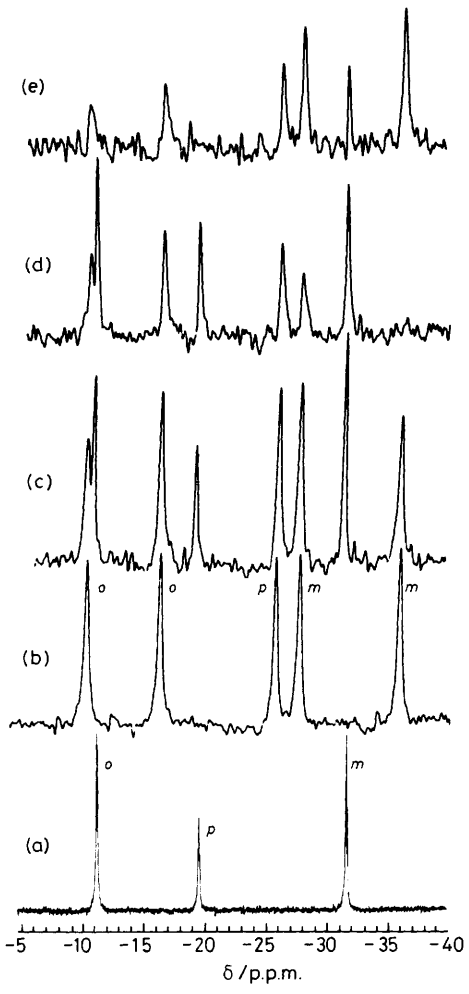


Figure 1. ^{19}F N.m.r. spectra of pentafluorobenzenesulphonamide obtained at 282 MHz, 25 °C. (a) 12 mM (**1**) in a solution composed of 0.025 M Tris and 0.05 M Na_2SO_4 in deuterium oxide at an apparent pH of 7.2. (b) 0.42 mM Human carbonic anhydrase II and 0.30 mM (**1**) in the same solvent and at the same apparent pH as for (a). (c) 0.42 mM Enzyme and 0.55 mM (**1**), same conditions as for (a). (d) Same as for (c), except that the resonance at highest field was saturated by the DANTE method. (e) Difference spectrum formed by subtracting (d) from (c), so that reduction of signal intensity is represented as positive. Traces (c), (d), and (e) are plotted on the same vertical scale. The extent of peak saturation did not change beyond experimental uncertainty when the length of the DANTE sequence was doubled. Experiments were carried out on a Nicolet NT-300 spectrometer using 10 mm sample tubes.

the ^{19}F spectrum of the bound inhibitor consists of five signals of equal intensity, Figure 1(b). Fluorine–fluorine spin couplings are not resolved in this spectrum because of appreciable line broadening that is the result of slow tumbling of the enzyme–inhibitor complex. The molecular environment of the pentafluorophenyl ring in the complex is asymmetric[11] and observation of a magnetically distinct environment for each fluorine of enzyme-bound (**1**) indicates that rotation of the aromatic ring about its C(1)–C(4) axis is slow. When an excess of inhibitor is present, separate spectra are observed for free and protein-bound inhibitor, Figure 1(c), showing that exchange between these forms is also slow.

Selective saturation experiments using the DANTE pulse sequence[15] were carried out to determine assignments for the signals observed with complexed (**1**) and to provide an estimate of the rate constants for ring rotation and complex dissociation. An example of these experiments is given in Figure 1(d). Saturation of the highest field fluorine resonance from bound (**1**) leads to partial saturation of the remaining signals for bound (**1**) but with a significantly larger effect observed at the other *meta* signal for the bound inhibitor. There is also transfer of saturation to the *meta* fluorines of the free inhibitor. These spectral changes upon saturation are clearer in the difference spectrum given in Figure 1(e). Thus, the two fluorine signals from the complex that appear at highest field arise from the *meta* fluorines of the inhibitor. Similar experiments indicate that the two enzyme–inhibitor signals at lowest field are from the *ortho* positions of bound (**1**).

All experiments involving selective saturation of a particular resonance of the bound inhibitor showed substantial transfer of saturation to spins represented by the other chemical shifts. We believe nuclear Overhauser effects, complicated by spin diffusion and chemical exchange, account for the reduction of signal intensities observed.

Assuming that a spin can exchange between two enzyme-bound environments, a single protein-free environment, and that the n.m.r. signal corresponding to one of the bound environments is saturated until the system comes to equilibrium, the rate expressions (1) and (2) can be obtained. k_{BF} and k_{BB} are the rate constants for dissociation of the complex and for interchange of the two bound environments, respectively,

$$k_{BF} = \frac{r\left(1-\frac{F}{F_0}\right)\frac{1}{T_1^F}}{2\frac{F}{F_0}-\frac{B}{B_0}} \quad (1)$$

$$k_{BB} = \frac{\frac{1}{T_1^B}+k_{BF}\frac{F}{F_0}}{\frac{B}{B_0}} - \frac{1}{T_1^B} - k_{BF} \quad (2)$$

r is the ratio of the concentrations of free to bound inhibitor, F/F_0 is the ratio of the intensities of the signal for the free inhibitor with and without saturation, B/B_0 is the intensity ratio for the other (non-saturated) signal of the inhibitor, and T_1^B and T_1^F are spin–lattice relaxation times, found experimentally to be 0.6 and 1.8 s for fluorines at the *meta* position. Utilization of these equations in the present case is rendered somewhat uncertain because a correction for nuclear Overhauser effects or saturation transfer by spin diffusion must be made. Although probably an overestimate, we took the average reduction of signal heights at the *ortho* and *para* fluorines (when *meta* is saturated) to represent this correction. With these assumptions k_{BB}, corresponding to the rotation of the pentafluoroaromatic ring, is computed to be 4 s^{-1}, while k_{BF}, representing dissociation of the enzyme–inhibitor complex, is 0.02 s^{-1}. The latter rate constant can be compared to 0.08—0.11 s^{-1} found using other methods for (**1**) bound to bovine carbonic anhydrase I with experimental conditions identical to those employed in the present work.[12]

A more detailed analysis taking into account the effects of DANTE excitation in a multispin system and the fluorine–fluorine and fluorine–proton interactions that lead to spin–lattice relaxation will provide more accurate values for these parameters, but it is clear that the time scales for ring rotation and dissociation of the complex are very different. How the

rates of these processes and the much more rapid turnover of substrate are defined by the conformational dynamics of the enzyme at the active site will be interesting to unravel.

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