Elucidation of the Catalytic Mechanism of Carbonic Anhydrase[1]

Sir:

Since its proposal by Quastel,[2] the "strained molecule" hypothesis has been assumed by many investigators to account for the relatively low activation energy of enzymatic reactions. By using accurate differential infrared spectrometry, we have detected the absorption at 2341 cm^{-1} (precision ± 0.5 cm^{-1}, accuracy ± 1 cm^{-1}) due to the asymmetric stretching of the CO_2 molecule bound in a hydrophobic cavity at the active site of bovine carbonic anhydrase. Because this value is very close to the corresponding observed frequency of 2343.5 cm^{-1} for CO_2 dissolved in water, we conclude that the CO_2 bound at the active site of carbonic anhydrase is not under appreciable strain.

In a typical experiment, the infrared sample cell with 0.075-mm path length and CaF_2 windows was filled with 33% by weight aqueous bovine carbonic anhydrase (Worthington) solution at pH 5.5 under equilibrium CO_2 pressure. The reference cell, which was adjusted by interference method to the same path length (within ± 0.1 μ), was similarly filled with the same enzyme solution containing either the stoichiometric amount of Ethoxyzolamide (Upjohn, 6-ethoxybenzothiazo-2-sulfonamide) or an excess of sodium azide. During each measurement, the Perkin-Elmer 125 spectrometer was continually flushed with N_2 and the cells were protected by germanium cut-off filters and water cooling jackets. After spectral measurements, the enzyme solutions were found to retain the initial concentration of soluble protein (within ± 2% OD at 280 mμ) and, for the uninhibited solution, the original enzyme activity (within ± 4% by esterase assay[3]). The intensity of this difference infrared absorption peak gives a direct measure of the concentration of carbonic anhydrase–CO_2 complex (E–CO_2). The enzyme concentration was determined by Ethoxyzolamide titration of the diluted sample.[4] A linear plot of $1/[E\text{–}CO_2]$ *vs.* $1/p_{CO_2}$ shows that each enzyme molecule binds only one CO_2 at its active site with a dissociation constant of 3 ± 0.5 atm in 33% solution at pH 5.5 and 25°, which is eight times the literature value of K_m in very dilute solutions.[5] Similar experiments with CO_2-equilibrated aqueous solutions of α-chymotrypsin and ovalbumin, respectively, did not detect any differential infrared spectrum.

The infrared spectra of carbonic anhydrase solutions equilibrated with $CO_2 + N_2O$ mixtures show that these two gases compete with similar affinity for the same binding site. Consequently, in view of the similar size, shape, small dipole moment, and weak ligand field of these two molecules, we infer that the CO_2 (or N_2O) is bound in a hydrophobic cavity of the protein, not coordinated to the Zn(II) of the enzyme. This conclusion is also consistent with the observation that the binding of CO_2 to the Co(II)-substituted carbonic anhydrase at low pH does not affect the visible spectrum of the latter.

Through the same infrared "window" in the spectrum of water–protein mixtures, we also observed the following absorption peaks due to the asymmetric stretching of azide ions in aqueous environment: free N_3^- in water, 2049 cm^{-1}; N_3^- in inert protein solution, 2046 cm^{-1}; N_3^- bound to carbonic anhydrase, 2094 cm^{-1}; N_3^- bound to the Co(II)–enzyme, 2082 cm^{-1}; N_3^- bound to the diethylenetriamine–zinc(II) complex, DETA–Zn(II), 2085 cm^{-1}; N_3^- bound to DETA–Co(II), 2067 cm^{-1}; HN_3 in water, 2147 cm^{-1}. These frequencies show that the N_3^- bound to carbonic anhydrase is coordinated to the Zn(II) of the enzyme. The concentration of the azide bound to carbonic anhydrase in an equilibrium mixture can be calculated directly from the intensity of the 2095-cm^{-1} peak, which exists side by side with the 2046-cm^{-1} peak due to the free azide in the mixture.

For a given enzyme solution at constant temperature, CO_2 pressure, and pH, the difference peak at 2341 cm^{-1} due to bound CO_2 was observed to decrease as the 2095-cm^{-1} peak due to bound N_3^- increases in such a way that there is a 1:1 correspondence between the per cent of Zn(II) coordinated to N_3^- and the per cent of CO_2 displaced from the hydrophobic cavity. Knowing that the N_3^- is coordinated to the Zn(II), we deduce that the hydrophobic cavity must be right next to the Zn(II) so that the ligand N_3^- can protrude into the cavity and sterically displace the CO_2.

Nitrate and bicarbonate were found in these infrared studies to displace both the N_3^- from the Zn(II) and the CO_2 from the hydrophobic cavity. Consequently we conclude that the bicarbonate ion must be coordinated to the Zn(II) through its negatively charged oxygen atom such that its relatively neutral oxygen atom and hydroxyl group are placed in the hydrophobic cavity, as illustrated by II in Figure 1.

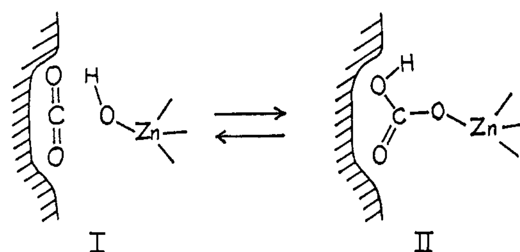

Figure 1. The catalytic mechanism of carbonic anhydrase.

Therefore in the dehydration reaction proton transfer must accompany the breaking of the C–O bond to leave an OH^- coordinated to the Zn(II), because we already know from the above results that only CO_2 is to be left in the hydrophobic cavity as a result of the reaction. Conversely, because of the principle of detailed balancing, it must be the OH^- on the Zn(II) which attacks the bound CO_2 and converts the latter to HCO_3^- in the reverse hydration reaction.

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