2.12: pH Dependence of Inhibitor Binding

The ease with which electronic spectra can be obtained provides a simple way of determining the affinity constants of inhibitors for the cobalt-substituted enzymes. An aliquot of enzyme is diluted in a spectrophotometric cell up to a fixed volume, and the spectrum is measured. Then the spectra are remeasured on samples containing the same amount of enzyme plus increasing amounts of inhibitor in the same cell volume. The pH is rigorously controlled. If solutions of enzyme and inhibitor have the same pH, the pH should be verified after the spectral measurements, in order to avoid contamination from the electrode salt medium. Both absolute values and pH dependences of affinity constants obtained from electronic spectra are the same as those obtained from inhibition measurements, where known, and are comparable to those obtained on the native enzyme.

Although affinity constant values reported in the literature were measured under different experimental conditions of, e.g., pH, buffer type, and buffer concentration, several pH-dependent trends are apparent. According to such dependences, three classes of inhibitors can be identified[48] (Figure 2.15).

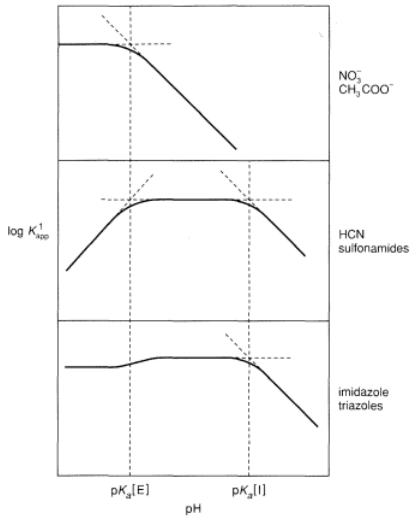


Figure 2.15 - Types of pH dependences observed for the affinity constants of inhibitors for cobalt(II)-substituted carbonic anhydrases. $pK_a[E]$ represents the main pK_a value of the enzyme, $pK_a[I]$ that of the inhibitor, if present.[48]

In the first class, the affinity constant, expressed as log K, decreases linearly with increasing pH. Anions that are weak Lewis bases (Cl^-,N_3^-,CH_3COO^-, NO_3^-, etc.) behave in this manner, as do neutral ligands like CH_3OH and aniline. An example is shown in Figure 2.16. A qualitative fit to such curves can be obtained using a single pK_a. This behavior could be accounted for by assuming that the ligand binds only the low-pH form of the enzyme, in a simplified scheme in which only one pK_a value determines the species distribution in CA. We know, however, that the picture is more complex.

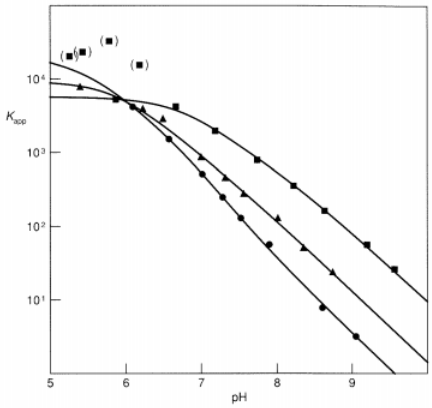


Figure 2.16: *pH dependence of the apparent affinity constants of nitrate for human I ■, bovine II (▲), and human II (•) carbonic anhydrases. The curves are best-fit curves obtained assuming non-zero affinity of the anion for species I and 3 of Figure 2.9. The best-fit parameters are reported in Table 2.6. Points in parentheses for HCA I reflect possible binding of a second nitrate ion and have been excluded from the fit.*[57]

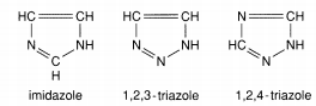
If the species distribution calculated according to the scheme of Figure 2.9 is assumed to hold, and if it is assumed that only the two water-containing species (1) and (3) can be bound by the ligand, then actual affinity constants can be evaluated for both species (1) and (3)[57] (see Table 2.7). Such constants are similar for the three isoenzymes, whereas the apparent affinity constants at pH 7, for example, mainly depend on the pK_a's of the coordinated water according to the values of Table 2.5. Therefore, the low-activity species CA I has larger affinity for anions like nitrate (and bicarbonate) than do the high-activity forms at pH 7.

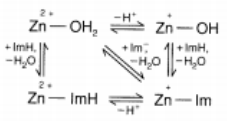
Table 2.7: Affinity constants of nitrate for species 1 and 3a of cobalt(II)-substituted carbonic anhydrases.[57] * As defined in Figure 2.9

	HCA I	BCA II	HCA II
log K_1	3.74 ± 0.04	4.01 ± 0.02	4.34 ± 0.04
log K_3	2.62 ± 0.06	2.56 ± 0.04	2.61 ± 0.05

A second type of behavior occurs for weak acids like HCN, H_2S, and aromatic sulfonamides ($ArSO_2NH_2$).[76,77] Assuming that the anions (conjugated bases) bind the low-pH species of the enzyme, the bell-shaped plot of log K versus pH (Figure 2.15) can be accounted for. In fact, at low pH, the inhibitors are in the protonated form, which is not suitable for metal binding. At high pH the concentration of the low-pH species of the enzyme decreases. The maximal apparent affinity is experimentally halfway between the pK_a of the inhibitor and the "pK_a" of the enzyme, treated as if it were only one. The same type of curve is also expected if the high-pH species of the enzyme binds the weak acid. Indeed, kinetic measurements seem to favor this hypothesis for sulfonamides.[78]

A third type of behavior obtains for inhibitors like imidazole and triazoles, which bind the enzyme with similar affinities over a large range of pH (Figure 2.15),[79,80] because both the imidazolate anion and the neutral imidazole can bind to the aquo forms of the enzyme with essentially the same affinity,[48,80,81] and the reaction of imidazole with the Zn—OH species cannot be distinguished thermodynamically from the reaction of imidazolate with the aquo forms:





It is possible that the noncoordinated nitrogen can interact with a group in the protein via a hydrogen bond. A candidate could be the NH group of His-200 in HCA I or the hydroxyl group of Thr-200 in HCA II. Indeed, only imidazole and triazoles, which have two nitrogens in 1,3-positions, seem to have this ability.[213]

In summary, from cobalt substitution we have learned:

1. the coordination geometry of the high- and low-pH forms by means of electronic spectroscopy;
2. the values of the pK_a's from the pH dependence of the electronic spectra;
3. the four and five coordination of the various derivatives with exogenous ligands;
4. the affinity constants of exogenous ligands and their pH dependence;
5. a fingerprint in the ^1H NMR spectra that can be used to monitor structural variations.

Most of these conclusions can be safely transferred to the native zinc enzyme, although minor differences can occur, for example, in the position of the equilibrium between four- and five-coordinate species.