THE BINDING OF THIOPHENOLS TO BOVINE CARBONIC ANHYDRASE

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Summary: In contrast to their oxygen analogs, the nitrophenols, 4-nitrothiophenol and 2,4-dinitrothiophenol have been found to be strong inhibitors of bovine carbonic anhydrase. Measurements of the inhibition constants, K_I, and the rate constants k_1 for the binding of the thiophenols to the enzyme are consistent with the hypothesis that enzyme-thiophenol complex formation occurs by the reaction of the ionized thiophenol (thiophenolate) with the form of the enzyme in which the water ligand bound to the active site Zn(II) ion is unionized.

Carbonic anhydrase, a well known zinc-containing enzyme (1) is eminently suitable as a prototype for studies of the state of complexation and mechanistic role of metal ions in metalloenzymes. Investigations of ligand binding to metal ions in metalloenzymes generally have employed either active site-directed complexing agents which probe the specificity site of the enzyme as well as the binding ability of the metal ion or, alternatively, very powerful chelators which usually produce total removal of the metal ion from the enzyme. Between these two extremes it would be advantageous to have a class of non-specific ligands which would not disrupt the existing metal ion-protein interactions,

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yet would be powerful enough to bind primarily to the metal ion. In particular, mechanistic studies of the interactions of such ligands with metalloenzymes should minimize the role of the protein and highlight the role of the metal ion in the binding phenomenon.

We wish now to report that compounds such as 4-nitrothiophenol and 2,4-dinitrothiophenol, which contain thiol functions known to be good ligands for Zn(II) ion (2), are strong inhibitors of the action of bovine carbonic anhydrase (A and B isozymes) while their oxygen analogs, the nitrophenols, are not (3). Extensive data exist on the binding of aromatic and heteroaromatic sulfonamides to carbonic anhydrase (1, 4). Because of the much stronger metal ion binding ability of the thiolates than that of the sulfonamides, however, mechanistic information obtained with the former class of compounds should reflect predominantly the binding properties of the active site Zn(II) ion while the same is probably not true for the latter category of inhibitor.

Upon binding to bovine carbonic anhydrase, the visible spectrum of 4-nitrothiophenolate undergoes a change similar to the spectral change accompanying protonation of the thiolate species. This change permits direct measurement of the kinetics of binding. From experiments in which equal initial concentrations of enzyme $[E]_o$ and thiol $[I]_o$ at pH 9.3 in Tris-Na_2SO_4 buffer, $\mu = 0.25$ were mixed in a Durrum stopped-flow spectrophotometer and the change in transmittance at 420 nm followed, the rate constant, k_1, for the binding of nitrothiophenol to bovine carbonic anhydrase (see equation 1) at 25.0° was found to be 1×10^7 M^{-1} sec^{-1}. Similar experiments at pH 9.3 gave a k_1 value of 2×10^7 M^{-1} sec^{-1} for 2,4-dinitrothiophenol (5).

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \qquad (1)$$

The rate constants, k_{-1}, for the dissociation of the bovine enzyme-thiophenol complexes were measured using the stopped-flow spectrophotometer by reacting solutions containing a mixture of the enzyme and excess thiophenol with solutions of the strong inhibitor acetazolamide (1) which were sufficiently concentrated that only the first-order kinetics of the enzyme-thiophenol dissociation was observed at 420 nm. The acetazolamide concentration was varied 13-fold to demonstrate that the measured rate constant was independent of the concentration of this inhibitor in the range used. At pH 7.7, Tris-Na_2SO_4 buffer, μ = 0.125, 25.0°, k_{-1} was 1.7×10^2 sec^{-1} for the 4-nitrothiophenol-enzyme complex, and at pH 8.0 it was 3.5×10^2 sec^{-1} for the 2,4-dinitrothiophenol-enzyme complex.

The dissociation constants, K_I, for the thiophenol-bovine carbonic anhydrase complexes were measured by inhibition experiments using 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone as the substrate (6). The only variation from our previous use of this technique (7) was that for 2,4-dinitrothiophenol conditions were employed such that this inhibitor was not in excess over the enzyme. The K_I value in this case was calculated as described by Lindskog and Thorslund (8) using equation 2 with k_{meas} and k_{spont} as defined earlier (7) and $k_{meas,o}$ being the rate constant measured for the enzyme catalyzed hydrolysis in the absence of inhibitor. At pH 7.5, Tris-Na_2SO_4 buffer, μ = 0.125, 25.0°, K_I was found to be 1.9×10^{-6} M for inhibition by 4-nitrothiophenol and at pH 7.7 it was 1.7×10^{-6} M for 2,4-dinitrothiophenol.* The pH dependence of the

*From these values and those for k_{-1} cited above, using the relationship $K_I = k_{-1}/k_1$, k_1 is calculated to be 9×10^7 M^{-1} sec^{-1} for 4-nitrothiophenol at pH 7.7 and 9×10^7 M^{-1} sec^{-1} for 2,4-dinitrothiophenol at pH 8.0. We have found that the observed inhibition is unaffected by the presence of excess Zn(II) in solution, indicating that the loss of carbonic anhydrase activity is not the result of the extraction of Zn(II) from the enzyme by the thiophenol.

$$\frac{[I]_o}{(1-\alpha)} = [E]_o + \frac{K_I}{\alpha} \quad \text{where } \alpha = \frac{(k_{meas} - k_{spont})}{(k_{meas,o} - k_{spont})} \qquad (2)$$

inhibition constant for 4-nitrothiophenol as displayed in Figure 1 can be accounted for by postulating that the enzyme-thiophenol complex is formed

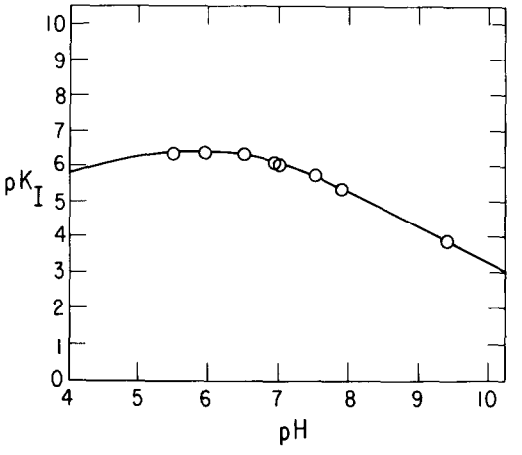

Figure 1. pH dependence of K_I for the inhibition of bovine carbonic anhydrase by 4-nitrothiophenol. All experiments were performed at μ = 0.125, 25.0° and in the presence of about 1 per cent acetone. The solid curve is a theoretical one calculated using pK_a = 4.6 for the ionization of the thiophenol at μ = 0.125 and pK_a = 6.8 (4,8) for the ionization of the activity-linked group on the enzyme. The dependence on the former pK could not be conclusively demonstrated experimentally since below pH 5.5 the bovine carbonic anhydrase samples precipitated Bis-Tris-Na_2SO_4 buffers were used from pH 5.5 to 6.9, and Tris-Na_2SO_4 buffers were employed from pH 7.0 to 9.4. Measurements in acidic solutions were done at 360 nm while those in neutral and alkaline solutions were carried out at 410 nm.

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by the reaction of the ionized thiophenol (thiophenolate) with the form of the enzyme in which the water ligand bound to the active site Zn(II) ion is unionized. The rate constants k_{calcd} listed in Table 1 were calculated from the k_1 values on the basis of this assumption. The k'_{calcd} values shown there were calculated according to an alternative hypothesis in which it is postulated that the unionized thiols react with the form of the enzyme in

TABLE 1. Summary of Calculated Rate Constants for the Binding of Thiophenols to Bovine Carbonic Anhydrase at 25.0°

Inhibitor	k_{calcd} M^{-1} sec^{-1}	k'_{calcd} M^{-1} sec^{-1}
4-Nitrothiophenol	7×10^{8} [a]	1×10^{11} [a]
	3×10^{9} [b]	5×10^{11} [b]
2,4-Dinitrothiophenol	2×10^{9} [a]	3×10^{12} [a]
	6×10^{9} [b]	1×10^{13} [b]

[a] These k_{calcd} and k'_{calcd} values were computed from k_1 values calculated from our measurements of k_{-1} and K_I. The measurements of k_{-1} were difficult to obtain because the reactions observed were so fast that they were on the limits of accurate detection by our stopped-flow instrument.

[b] These k_{calcd} and k'_{calcd} values are based on the direct measurement of k_1. Considering our comments in footnote (a) concerning the problems in obtaining accurate k_{-1} values, the correspondence between the k_{calcd} and k'_{calcd} values calculated from the directly or indirectly obtained values of k_1 is not bad.

which the water bound to the active site Zn(II) is ionized. The values obtained for the k'_{calcd} are greater than those anticipated for a diffusion controlled reaction (9). Therefore, we can rule out the latter hypothesis. While we have not demonstrated that the water coordinated to the active site Zn(II) is displaced by the thiophenols, the presently available data are compatible with the type of mechanism suggested for the formation of inorganic complexes of Zn(II) (10). That is, carbonic anhydrase-thiophenol complex generation may be described in terms of rapid pre-equilibrium formation of an outer sphere complex between the thiophenolate anion and the Zn(II) ion at the active site, which then loses water as the rate-determining step of inner sphere enzyme-inhibitor complex formation.

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