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Product Inhibition Study on Carbonic Anhydrase Using Spectroscopy and Calorimetry

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Kinetic and thermodynamic studies have been made on the effect of the *p*-nitrophenol product on the activity of bovine carbonic anhydrase in 50 mM Tris buffer pH 7.5, at 300 K using UV spectrophotometry and isothermal titration calorimetry (ITC). A competitive inhibition was observed for *p*-nitrophenol as a product of the enzymatic reaction. A graphical fitting method was used for determination of the binding constant and enthalpy of inhibitor binding using ITC data. The dissociation binding constant was 0.10 mM by the microcalorimetric method, which is in good agreement with the value of 0.11 mM for the inhibition constant obtained from the spectrophotometric method.

Keywords: Carbonic anhydrase; *p*-Nitrophenol; Product inhibition; Inhibition constant; Isothermal titration calorimetry

INTRODUCTION

Carbonic anhydrases (EC 4.2.1.1) are widespread zinc enzymes, present in archaeo and eubacteria, algae, green plants and animals.[1] The zinc ion in the structure of these enzymes is coordinated to three histidines (His 94, His 96, His 119) and to a water molecule, which is located at the bottom of a cavity.[2]

Carbonic anhydrase (CA) catalyses the reversible hydration of CO_2 in solution to bicarbonate and a proton and also catalyses the hydrolysis of aromatic and aliphatic esters.[3,4] CA exhibits outstanding dynamic properties, its most active isozymes reach turnover numbers[5] up to $10^6\,s^{-1}$.

Since this enzyme is involved in crucial physiological processes, connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and the lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues and organs, biosynthetic reactions (gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification, tumorigenicity and many other physiologic or pathologic processes,[6,7] the study of its kinetic and thermodynamic properties is important.

Several inhibitors of this enzyme have been studied. Some of them are important for understanding in detail the structure, catalytic and inhibitory mechanisms, and the others have led to the development of several classes of pharmacological agents.[1] Inhibitors such as imidazole,[8] cyanate and cyanide[5,9] and acetate[2] are from the first type and some other inhibitors such as sulfonamides are from the second type.[10] The CA inhibition by phenol has been reported by Lindskog's group.[11] Phenol is a competitive inhibitor of CO_2 hydration catalyzed by CA.

In this study, we investigated the inhibitory effect of *p*-nitrophenol (the product of esterase activity of the enzyme)[4] on the enzymatic reaction of CA by spectrophotometry and microcalorimetry.

MATERIAL AND METHODS

Materials

Erythrocyte bovine carbonic anhydrase and *p*-nitrophenylacetate were obtained from Sigma, *p*-nitrophenol was obtained from Merck. The buffer used in the assay was 50 mM Tris, pH = 7.5, which was obtained from Merck. All experiments were carried out in 300 K.

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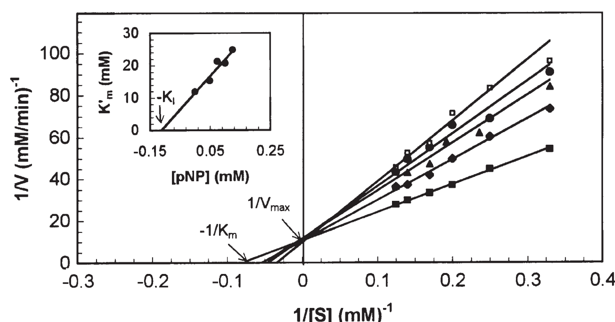

FIGURE 1 Double reciprocal Lineweaver–Burk plots for CA kinetics at pH = 7.5 and T = 300 K in the presence of different fixed concentrations of *p*-nitrophenol: 0 mM (■), 0.05 mM (◆), 0.075 mM (▲), 0.1 mM (●) and 0.125 mM (□). In the inset a secondary plot of 1/[S]-axis intercept versus [I] is shown; S and I are substrate and inhibitor, respectively.

Methods

Enzyme Assay

Enzyme activity was assayed using a spectrophotometer with jacketed cell holders by following the increase in absorbance at 400 nm due to the production of *p*-nitrophenol.[4] Its temperature was regulated by an external thermostated water circulator within ±0.05°C. A stock solution of *p*-nitrophenylacetate in acetonitrile (100 mM) and a stock solution of *p*-nitrophenol in Tris buffer (10 mM) were used as the enzyme substrate and enzyme inhibitor, respectively. The reference cell contained 1 ml of Tris buffer and the sample cell contained 1 ml Tris buffer, containing 10 μg of the enzyme. Before starting the enzymatic reaction, a required concentration of inhibitor was provided by injecting a small volume of the stock solution of inhibitor into both the reference and sample cells. The esterase activity of CA was measured by adding a small volume of the substrate solution into both the sample and reference cells. Rate of appearance of *p*-nitrophenol at 400 nm was determined by a previously reported procedure by Pocker and Stone.[4] Activities were measured over at least 6 different concentrations of *p*-nitrophenylacetate and the assays were repeated at least three times. The concentration of product was calculated using $\varepsilon = 13000\,M^{-1}\,cm^{-1}$ for *p*-nitrophenol. Care was taken to use adequate experimental conditions to keep the enzyme reaction linear during the first minute of reaction.

Calorimetric Measurement

The isothermal titration microcalorimetric experiments were performed with the 4-channel commercial microcalorimetric system, Thermal Activity Monitor 2277, (Thermometric, Sweden). Each channel consists of a twin heat-conduction calorimeter where the heat-flow sensor is a semiconducting thermopile (multi-junction thermocouple plates) positioned between the vessel holders and the surrounding heat sink. The insertion vessel was made from stainless steel. *p*-Nitrophenol solution (25 mM) was injected using a Hamilton syringe into the calorimetric stirred titration vessel, which contained 1.9 ml enzyme, 0.5 mg/ml, including Tris buffer (50 mM), pH = 7.5. Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of *p*-nitrophenol solution into the perfusion vessel was repeated 20 times, and each injection consisted of 40 μl reagent. The calorimetric signal was measured by a digital voltmeter that was part of a computerized recording system. The heat of each injection was calculated by the "Thermometric Digitam 3" software program (obtained from Thermometric, Sweden). The heat of dilution of the *p*-nitrophenol solution was measured as described above except enzyme was excluded. The enthalpy of dilution was subtracted from the enthalpy of enzyme-*p*-nitrophenol interaction. The enthalpy of dilution of enzyme was negligible. The microcalorimeter was frequently calibrated electrically during the course of the study.

In all calculations the molecular weight of CA was taken as 30000.[4]

RESULTS AND DISCUSSION

Figure 1 shows the double reciprocal Lineweaver–Burk plot for CA in different concentrations of *p*-nitrophenol, at pH = 7.5 and T = 300 K. The V_{max} value is unchanged by the *p*-nitrophenol, but the apparent Michaelis constant (K'_m) value is increased, which confirms the competitive inhibition by *p*-nitrophenol of CA. The values of K'_m at any concentration of *p*-nitrophenol were obtained from Figure 1 and plotted versus the concentration of *p*-nitrophenol, namely a secondary plot, from which the inhibition constant (K_I) was obtained. According to these plots, K_m and K_I are 11.9 mM and 0.11 mM, respectively. This value for the inhibition constant is very much smaller than the inhibition constant for phenol (≈ 10 mM).[11]

By titration of a solution containing an enzyme (E) with a solution of inhibitor (I), the equilibrium reaction moves toward increasing concentration of EI complex. The heat value of the reaction depends on the concentration of EI complex. Thus, the reaction under consideration can be written:

$$EI \Leftrightarrow E + I \quad K = [E][I]/[EI] \tag{1}$$

and also

$$[I]_{total} = [I] + [EI] \tag{2}$$

$$[E]_{total} = [E] + [EI] = (K[EI]/[I]) + [EI] \tag{3}$$

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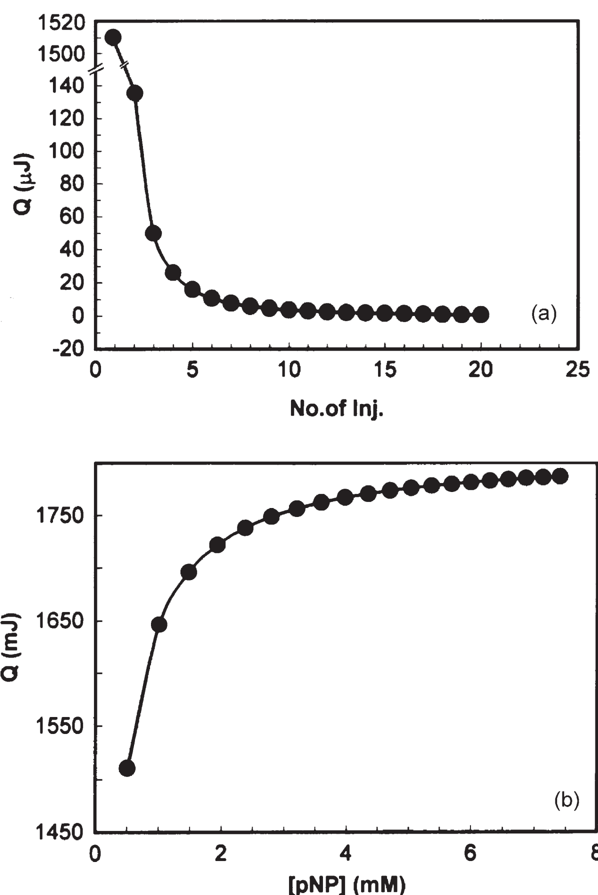

FIGURE 2 (a) The heat of *p*-nitrophenol binding with CA for 20 automatic cumulative injections, each of 40 μl of *p*-nitrophenol solution, 25 mM, into the sample cell containing 1.9 ml CA solution at a concentration of 0.5 mg/ml. (b) The heat of binding versus total concentration of *p*-nitrophenol, calculated from Figure 2a.

Equation (2) can be solved for [I] and this can then be substituted into equation (3), which can then be rearranged to give the quadratic equation its only real root is:

$$[EI] = \{(B + K) - [(B + K)^2 - C]^{1/2}\}/2 \quad (4)$$

where

$$B = [E]_{total} + [I]_{total} \quad C = 4[E]_{total}[I]_{total} \quad (5)$$

The sum of heat evolutions following the ith titration step, Q_i, can be expressed as

$$Q_i = \Delta H\, V_i[EI]_i \quad (6)$$

where V_i is the volume of the reaction solution and ΔH is the enthalpy of binding. Combination of equations (4) and (6) leads to

$$\Delta H = 1/A_i\{(B_i + K) - [(B_i + K)^2 - C_i]^{1/2}\} \quad (7)$$

where

$$A_i = V_i/2Q_i \quad (8)$$

A_i, B_i and C_i can be calculated in each injection, so equation (7) contains two unknowns, K and ΔH.

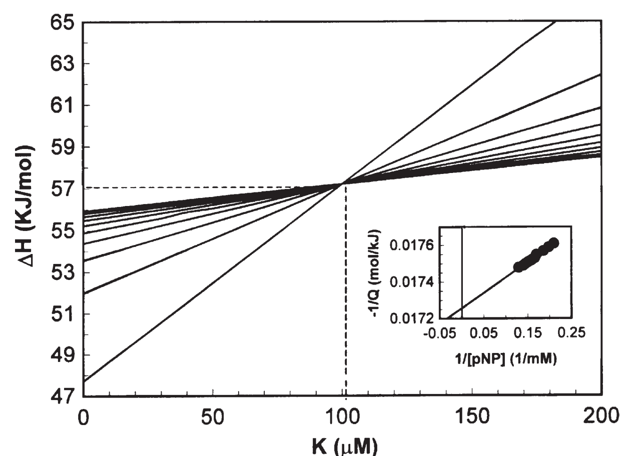

FIGURE 3 ΔH versus K for the first 10 injections using reasonable values of K, according to equation (7). The coordinates of the intersection point of curves give true values for ΔH and K. A plot of 1/Q versus 1/[pNP] for the last 10 injections is shown in the inset.

A series of reasonable values for K is inserted into equation (7) and the corresponding values for ΔH are calculated and a graph ΔH versus K is constructed. Curves of all titration steps will intersect at one point, which represents the true value for ΔH and K.

The data obtained from isothermal microcalorimetry of the CA interaction with *p*-nitrophenol is shown in Figure 2. Figure 2a shows the heat of each injection and Figure 2b shows the heat related to each total concentration of *p*-nitrophenol.

The plots of ΔH versus K, according to equation (7), for the first 10 injections are shown in Figure 3. The intersection of curves gives $K = 0.10$ mM and $\Delta H = -57.2$ kJ/mol.

According to our previous calorimetric data analysis, a simple linear plot of 1/Q versus 1/[I] can also be used for determination of the inhibitor binding constant and the enthalpy of binding by using the equation:[12–14]

$$1/Q = (K/\Delta H)1/[I] + 1/\Delta H \quad (9)$$

This double reciprocal linear plot is shown in the inset to Figure 3 for the last 10 injections, where it can be assumed that the total and free concentrations of inhibitor are approximately equal. The values of K and ΔH obtained from the axis intercept and slope are $K = 0.098$ mM and $\Delta H = -57.8$ kJ/mol.

The results obtained by two methods of graphical fitting and simple linear plot are almost the same because the assumption of $[I]_{total} = [I]$ is true. However the graphical fitting method is general and useful and does not involve any assumption.

It is concluded that CA is inhibited by *p*-nitrophenol in a competitive manner with an inhibition constant of 0.10 mM. Moreover, the proposed graphical fitting method for determination of binding constant and enthalpy of binding on

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the basis of isothermal titration microcalorimetry data is very simple with good accuracy and can be used in other systems.

Acknowledgement

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