Effect of Spontaneous Hydrolysis of *p*-Nitrophenylacetate in Aqueous Solutions on Kinetic Characteristics of Carbonic Anhydrase

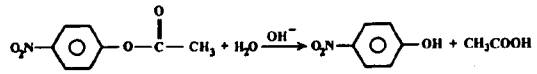
A. A. SABOURY[1], N. S. SARRAF[1] AND M. U. DAHOT[2]
[1]*Institute of Biochemistry and Biophysics*
University of Tehran, Tehran, Iran
[2]*Institute of Biotechnology and Genetic Engineering*
University of Sindh, Jamshoro, Pakistan

(Received 29th April, 2003, revised 3rd November, 2003)

Summary: The rate of hydrolysis of *p*-nitrophenylacetate to p-nitrophenol is measured by spectrophotometry and isothermal microcalorimetry in Tris buffer 50 mM, pH 7.5 at 27 °C. The rate constants from both techniques were obtained 3.05×10^{-4}min^{-1} and 3.00×10^{-4}min^{-1} by spectrophotometry and microcalorimetry, respectively, which are in good coincidence with each other. This rate must be considered in measuring the kinetic characteristics of carbonic anhydrase, including K_m and V_{max}.

Introduction

p-nitrophenylacetate (*p*-NPA;$C_8H_7O_4N$) is an aromatic ester and can be hydrolyzed to form an alcohol [1-2]. This reaction has been studied in detail and can be catalyzed by hydroxyl ion [1].



p-nitrophenol ($C_6H_5O_3N$, MW=139) is a primary alcohol produced from the above reaction, which is an acidic phenol with pK_a=7 [3]. This stable delocalized phenol is yellow with an extinction coefficient ε=13000 $M^{-1}cm^{-1}$ at 400 nm [3].

p-NPA is used as a substrate for some enzymes such as chymotrypsin [3] and carbonic anhydrase [4-7]. The latter catalyses the reversible hydration of CO_2 in solution to bicarbonate and a proton and also catalyses the hydrolysis of aromatic and aliphatic acids [5,8]. Carbonic anhydrase is involved in crucial physiological processes, connected with respiration and transport of CO_2/ bicarbonate between metabolizing tissues and the lung, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues, and many physiologic or pathologic processes [7,9]. The study of its kinetics and thermodynamic properties is important. Besides measuring the kinetic characteris-tics of such enzymes, it is necessary to know briefly about the rate of spontaneous (non-enzymatic) hydrolysis of this compound in aqueous solution. So we have carried out a spectrophotometry and calori-metry study on the kinetic spontaneous hydrolysis of *p*-NPA, and the effect of such rate on K_m and V_{max} of carbonic anhydrase.

Results and Discussion

Figure 1 shows the rate of spontaneous hydrolysis of *p*-NPA obtained by spectrophotometric method. This is a linear plot. So the production of p-nitrophenol from *p*-NPA can be considered as a first-ordered mechanism and the rate constant (k) can be obtained from the slope of this linear plot, which is 3.05×10^{-4} min^{-1}.

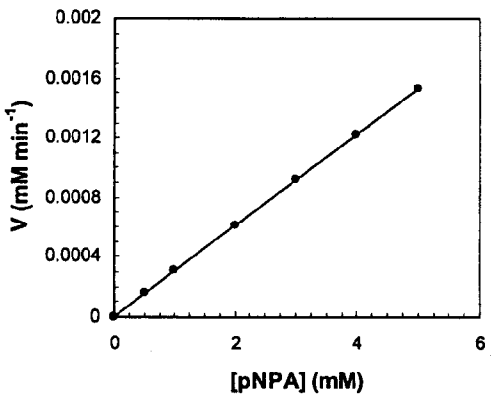


Fig. 1: The rate of production of p-nitrophenol as a function of concentration of *p*-NPA, in 50 mM Tris buffer, pH 7.5, at 27°C.

The heat-exchange curve due to spontaneous hydrolysis of *p*-NPA in Tris buffer solution is shown in Fig. 2. The heat exchange is going to reach a maximum value (q_{max}), which is related to the maximum concentration of *p*-nitrophenol. However, q_{max} can not be directly obtained from the data represented in Fig. 2, since complete hydrolysis of *p*-NPA requires longer incubation times.

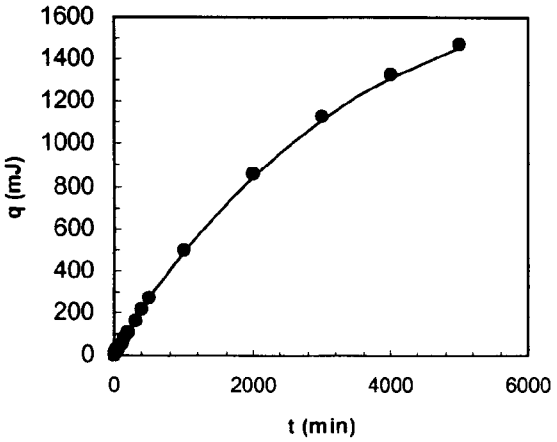


Fig. 2: The rate of heat exchange of a 2 cm^3 sample of *p*-NPA, at 0.99 mM concentration, in 50 mM Tris buffer, pH 7.5, at 27°C. Both the experimental data (•) and the calculative data ( ) are shown.

Suppose the transition of p-nitrophenylacetate →p-nitrophenol, or simply S→P, is a first-order reaction with the rate constant of k. So the kinetics of this reaction can be expressed by

$$d[S]/dt = k[S] \quad (1)$$

Integrating from Eq. (1) yields:

$$\int_{[S]_o}^{[S]} d[S]/[S] = k\int_{o}^{t} dt$$

or

$$\ln [S]/[S]_0 = kt$$

$$\ln ([S]_0-[P])/[S]_0 = kt \quad (2)$$

where $[S]_0$ is the initial concentration of *p*-NPA.

Replacing concentrations with heat exchange yields:

$$\ln (q_{max} - q)/q_{max} = kt$$

$$\text{or } \ln (1 - q/q_{max}) = kt \quad (3)$$

Therefore, a plot of ln $(1- q/q_{max})$ versus t is linear and the rate constant can be obtained from the slope of the plot.

Figure 3 shows the plot of ln $(1-q/q_{max})$ versus t which the rate constant is 3.00×10^{-4} min^{-1}, which is in good coincidence with spectrophotometric result. The value of 1870 mJ is considered as q_{max}, which is obtained using experimental data and provided the best linear plot. By using values of $k=3.00\times10^{-4}$ min^{-1} and q_{max} =1870 mJ in equation (3), the total heat exchange at any time can be calculated. In Fig. 2 and Fig. 3, data are experimental; however, plots were drawn with calculation data, which are in good coincidence with each other. Contrary to the most other non-enzymatic reaction of a substrate interchange to a product, the rate of non-enzymatic hydrolysis of pNPA is somewhat considerable. However, there are some reports without considering the rate of non-enzymatic hydrolysis of *p*-NPA into the obtaining of kinetic parameter [10].

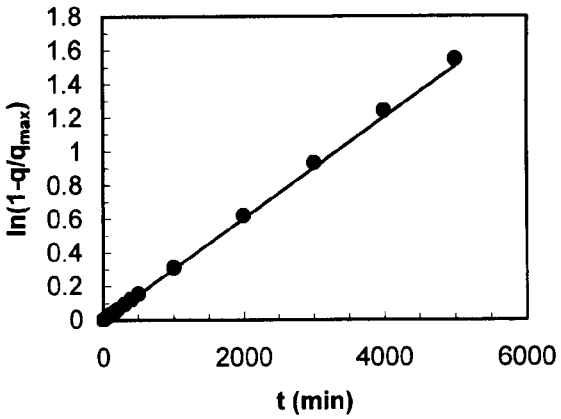


Fig. 3: The plot of ln(1 q/q_{max}) versus time using data from figure 2. Both the experimental data (•) and the calculative data ( ) are shown.

Figure 4 is a Lineweaver-Burk plot for the rate of enzymatic reaction of hydrolysis of *p*-NPA, both with and without subtracting non-enzymatic reaction rate. When subtracting the rate of non-enzymatic reaction, K_m and V_{max} are 12.1 mM and 0.09 mM min^{-1}, respectively. However, without subtracting the spontaneous rate, K_m and V_{max} are 13.5 and 0.103 mM min^{-1}, respectively.

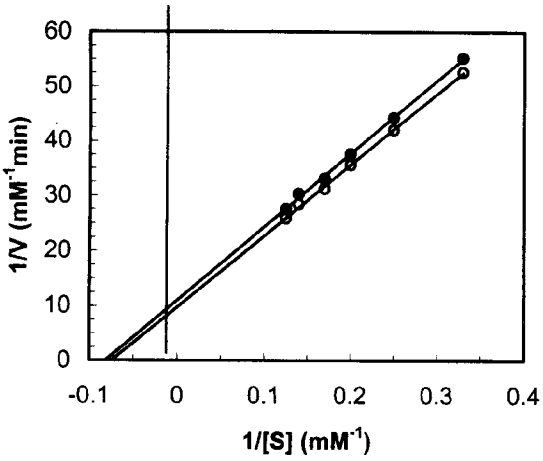


Fig. 4: The Lineweaver-Burk plot, for kinetics of bovine carbonic anhydrase on *p*-NPA at pH 7.5, 50 mM Tris buffer and 27°C, with (•) and without (o) subtracting non-enzymatic rates.

It is calculated that the rate of spontaneous transition of p-nitrophenylacetate → p-nitrophenol obtained from spectrophotometric and calorimetric techniques, which are in good coincidence with each other, must be considered in enzymatic reactions with this substrate.

Experimental

p-nitrophenylacetate (*p*-NPA) and erythrocyte bovine carbonic anhydrase were obtained from Sigma. Acetonitril was obtained from Merck. The buffer utilized throughout the study was Tris-HCl 50 mM, pH 7.5, which was obtained from Merck. All experiments were carried out at 300 K.

Spectrophotometric method

A recording spectrophotometer, UV-3100 Shimadzu model, was used for recording the rate of absorbance change at 400 nm wavelength (ΔA_{400}) and 1 cm^3 cuvettes thermostated to maintain the temperature at 27.0± 0.2 °C. 30-87 µl of pNPA (100 mM in acetonitril) was injected into the sample cell containing 1 ml of buffer solution. The increase in absorbance at 400 nm due to the conversion of pNPA to p-nitrophenol was monitored. The molar extinction coefficient of p-nitrophenol in this wavelength is 13000 $M^{-1}cm^{-1}$. The rate of reaction (V) was calculated by:

$$V = \frac{\Delta A_{400}/\text{min}}{(13000\ M^{-1}cm^{-1})(1\ \text{cm})}$$

While measuring the rate of enzymatic reaction, both sample and reference cells contained 1 ml Tris buffer, containing 10 µg of enzyme. The esterase activity of carbonic anhydrase was normally measured by adding a small volume of substrate solution (30-87 µl of pNPA 100 mM in acetonitril) in the sample cell; and then the rate of enzymatic reaction was determined by a previously reported procedure of Pocker and Stone[5].

Isothermal microcalorimetry methods

The isothermal microcalorimetry used in these studies was thermal activity monitor, TAM (Thermometric, Sweden). It was equipped with four channels, allowing for the rate of heat output. Each channel of the calorimeter was independently electronically calibrated with a precision of ±0.2%. Each channel is a twin heat-conduction calorimeter, where the heat-flow sensor is a semiconductor thermopile (multi-junction thermocouple plates) positioned between the vessels made from stainless steel. The rate of heat output from the sample cell with respect to the reference cell, displayed digitally, was recorded with an accuracy of 0.1 µW by a 'DIGITAM 3' computer program. The vessels contained 2 ml buffer and 20 µl of pNPA 100 mM (in acetonitril) was injected into the sample vessel and the rate of heat output was measured. The injection of acetonitril into the buffer gives a small heat of mixing, which nevertheless was subtracted as a small correction term in the final evaluation of the rate of heat output. The microcalorimeter was frequently calibrated electrically during the course of the study.

Acknowledgement

Financial assistance from the Research Council of the University of Tehran is greatfully acknowledged.

References

1. A. N. Nesmeyanov and N. A. Nesmeyanov, *Fundamentals of Organic Chemistry*, Page 219, Mir Publishers, Moscow (1981).
2. R. T. Morrison and R. N. Boyd, *Organic Chemistry*, Pages 832, Allyn & Bacon, Inc. (1983).

3. C. Walsh, *Enzymatic Reaction Mechanisms,* Pages 67, The Maple-Vail Book manufacturing group (1979).
4. Y. Pocker and J. T. Stone, *J. Am. Chem. Soc.*, **87**, 5497 (1965).
5. Y. Pocker and J. T. Stone, *Biochemistry* **6**, 668 (1967).
6. C. T. Supuran, A. Scozaffava, F. Mincione, L. Menabuoni, F. Briganti, G. Mincione and M. Jitianu, *Eur. J. Med. Chem.*, **34**, 585 (1999).
7. C. T. Supuran, F. Briganti, D. Tilli, W. R. Chegwidden and A. Scozzafava, *Bioorg. Med. Chem.*, **9**, 703 (2001).
8. W. S. Sly and Y. Hu. Peiyi, *Ann. Rev. Biochem.*, **64**, 375 (1995).
9. C. T. Supuran and A. Scozzafava, *Curr. Med. Chem-Imm. Endoc. Metab. Agents*, 1, 61 (2001).
10. F. Azari and M. Nemat-Gorgani, *Biotech. & Bioengin.*, **62**, 193 (1999).