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The enthalpimetric determination of inhibition constants for the inhibition of urease by fluoride ion

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Abstract

The hydrolysis of urea in phosphate buffer (pH 7.0) at 25°C in the presence of sodium fluoride as an urease inhibitor was studied. It was found that fluoride ion is a competitive slow-binding inhibitor of the enzyme and the constants of inhibition were measured.

The progress of the reaction was observed by means of a classical analytical method (increase of NH_3 concentration) and a calorimetric method using an isoperibol batch apparatus with an accuracy of 0.001 K.

Keywords: Enzyme; Inhibition constant; Inhibitor; Isoperibol; Urease

1. Introduction

Enthalpimetric methods have been reported for the determination of enzyme activity, substrate concentration, inhibitor concentration and kinetic constants. Native and immobilized enzymes have been applied [1–7].

Beezer et al. [8] discussed the advantages and limitations of applying flow-calorimetry to the determination of K_M, the Michaelis constant, for the urea–urease system. Lineweaver–Burk double-reciprocal plots were used in this procedure.

Grime et al. [9] determined the value of K_M from a single enthalpimetric progress curve and then fitted the data to the integrated Michaelis–Menten equation. Their

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studies were concerned with the system α-chymotrypsin–*N*-acetyl-L-tyrosine ethyl ester.

A similar procedure was used for the enthalpimetric determination of K_i, the inhibition constant for the competitive inhibition of serum cholinesterase by morphine, quinine and procaine. This method requires one experiment if K_M is already known [10].

Antonelli et al. [11] studied the inhibition of urease by the metal ions Hg(II), Ag(I), Cu(II), Zn(II), Cd(II), Co(II), Mn(II) and Mg(II) using the enthalpimetric technique.

In this study, an enthalpimetric method was applied for the determination of the set of inhbition constants for the system urease–urea–NaF which we have already studied analytically [12].

2. Theory

The theory of reactions involving enzymes and slow-binding inhibitors was presented by Morrison and Walsh [13] who developed equations supplementary to those already available [12]. The basic mechanism of the reactions is

$$\begin{aligned} &E + S \rightleftharpoons ES \rightarrow E + P \\ &E + I \rightleftharpoons EI \rightleftharpoons EI^* \end{aligned} \tag{1}$$

Inhibitor I and substrate S compete for the active site on the enzyme molecule E. The concentration of the complex EI is characterized by the dissociation constant

$$K_i = \frac{[E][I]}{[EI]} \tag{2}$$

It is formed and subsequently undergoes slow isomerization into a stable form EI*. The overall dissociation constant is defined as

$$K_i^* = \frac{[E][I]}{[EI] + [EI^*]} \tag{3}$$

The concentrations in Eqs. (2) and (3) are expressed in molar units. Constants K_i and K_i^* are inhibition constants. Eq. (4) gives the number of moles of the product $P(t)$ at time t

$$P(t) = V_s t + (v_0 - v_s)(1 - e^{-kt})\frac{1}{k} \tag{4}$$

$$v(t) = \frac{dP}{dt} = v_s + (v_0 - v_s)e^{-kt} \tag{5}$$

where k is the apparent rate constant. The reaction rate given by Eq. (5) and relevant values denoted by the main symbol v (with indices) have the dimension mol s^{-1} and are related to the known constant mass of the enzyme used in the

experiment. The initial reaction rate v_0 and the rate of the steady state reaction v_s are given by classical equations of competitive inhibition [14]

$$v_0 = \frac{v_{max}[S]}{K_M\left(1 + \frac{[I]}{K_i}\right) + [S]} \tag{6}$$

$$v_s = \frac{v_{max}[S]}{K_M\left(1 + \frac{[I]}{K_i^*}\right) + [S]} \tag{7}$$

where K_M is the Michaelis constant and v_{max} is the maximum velocity given by the Michaelis–Menten equation.

From the progress curves (4) found experimentally for a given initial substrate [S] and a given inhibitor concentration [I], the values of v_0 and v_s were determined. The first is equal to the slope of the tangent at $t = 0$ and the second to the slope of the asymptote. From Eqs. (6) and (7), the inhibition constants K_i and K_i^* were calculated.

Values of the basic constants, K_M and v_{max}, were determined independently using a standard procedure in the absence of inhibitor.

The value of k was determined from the intersection point of the tangent to the progress curve at $t = 0$ with the asymptote of the curve. The abscissa gives the value of $1/k$. Similarly, the intercept of the asymptote gives $(v_0 - v_s)/k$.

3. Experimental

3.1. Materials

The jack bean urease used was Sigma type III. Its specific activity was 27.2 units per mg of active protein. One unit is the amount of enzyme that liberates 1.0 μmol of NH_3 from urea per minute at pH 7 and 25°C. The molecular weight is 590 000 and the equivalent weight 96 6000 [15]. Urea, sodium fluoride and all chemicals (AnalaR grade) were obtained from POCH, Gliwice, Poland.

3.2. Enzymatic reaction

The hydrolysis of urea catalyzed by urease was studied in phosphate buffer pH 7.0 (22 mmol dm^{-3}, 1 mmol dm^{-3} EDTA, ionic strength 0.07) at 25°C. The initial concentration of urea 50 mmol dm^{-3}. The concentration of NaF in the successive experiments was 0, 1, 2 and 5 mmol dm^{-3} respectively. The reaction was initiated by addition of 1 cm^3 of the urease solution (3 mg cm^{-3}) into 110 cm^3 of the assay mixtures.

3.3. Methods

The progress of the reaction of urea hydrolysis in the presence of sodium fluoride was observed using two methods: one analytical and the other calorimetric.

In the analytical method the reaction took place in an open vessel. After every 30 s, 1 cm^3 aliquat was removed and the amount of ammonia analytically determined. The phenol–hypochlorite colorimetric method, measuring the absorbance at 625 nm [16], was applied. In the calorimetric method the reaction took place under the same conditions and the rate of change of temperature was measured.

3.4. Calorimetric equipment

The calorimetric apparatus was an Ecolab (Krakow, Poland) mod. MKR 0.001 isoperibol (constant temperature environment) batch microcalorimeter. The 110 cm^3 reaction vessel was equipped with a stirrer, ampoule (1 cm^3) and ampoule breaker. A thermistor sensor was used to measure the temperature with an accuracy of 0.001 K. The built-in digital meter allowed a read-out of the temperature every second along with the time from the start of the reaction. The data were stored in a microprocessor memory. In the experiments presented, the temperature was registered in 10 s intervals. The Joule heating calibration set was equipped with a digital current regulator within the range of 0–250 mA, an internal time of 1–250 s, for a heater resistance of 20–50 Ω. The standard RS-232 interface transmitted the data to an IBM-PC computer. The temperature stabilization of the reaction cell was realized with an accuracy of 0.001°C using a Peltier semiconductor junction thermostat controlled by an MRTP microprocessed temperature regulator. The system was housed in a thermostated room and all measurements were carried out at 25 ± 0.001°C. The progress of the enzymatic reactions was observed over a period of 15 min; the total increment of temperature did not exceed 0.06°C. The observed differential increments ΔT were corrected to account for heat exchange by applying the recurrence formula

$$\Delta T'(t+10) = \Delta T'(t) + \Delta T(t+10) - \Delta T(t) + \frac{\Delta T(t+10) + \Delta T(t)}{2} 10\beta$$

where the heat loss modulus β was found to be equal to 0.0003127 s^{-1}. The corrected values $\Delta T'$ are presented in the figures.

4. Results

The results from the calorimetric studies of the hydrolysis of urea at various concentrations of sodium fluoride are illustrated in Figs. 1 and 2. The experimental points obtained in the first minute of the reaction are shown in Fig. 1B. Linear least-squares analysis of the points yielded the parameters m and n listed in Table 1.

In order to convert the values of the slope $m/(\mathrm{K\ s^{-1}})$ into the first stationary rate $v_0/(\mathrm{mol\ urea\ s^{-1}})$, and similarly the slope of the asymptotes presented in Fig. 1A into the second stationary rate v_s, the enthalpy of the reaction had to be determined.

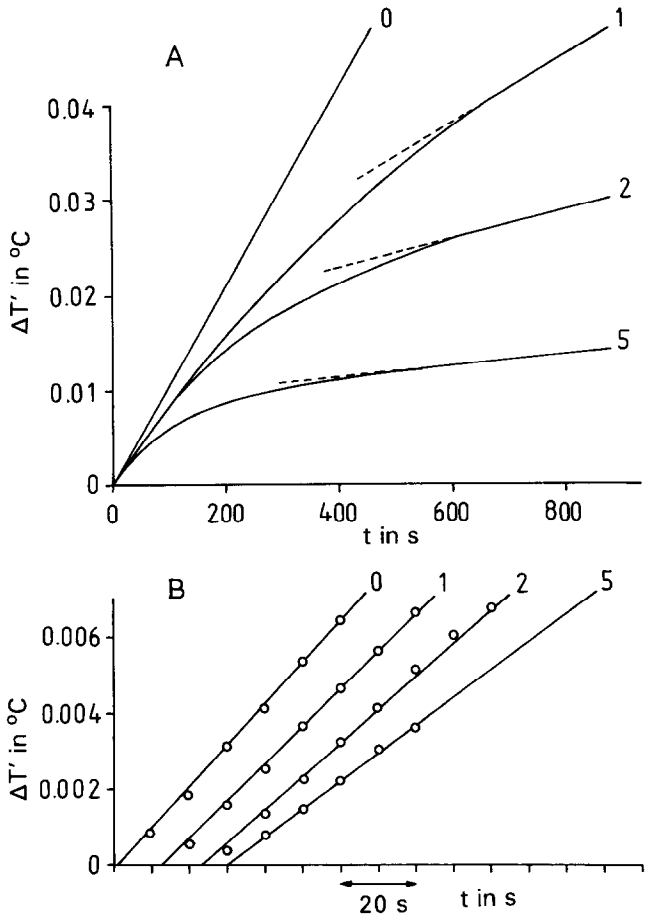

Fig. 1. Enthalpimetric progress curves for urease in the presence of sodium fluoride. Numbers indicate the millimolar concentration of NaF. A. Experimental points are not shown on the plots; the distance between them is 10 s. B. $\Delta T'$ versus time for the first stage of reaction; the experimental points (○) determine the straight lines whose parameters are given in Table 1.

4.1. Enthalpy of urea hydrolysis

The enthalpy ΔH of the hydrolysis of urea (yielding NH_4^+ and HCO_4^- ions) was measured by comparison of two independently registered progress curves; namely the calorimetric and analytical curves (Fig. 2). The solid line represents the increment of temperature (left scale) and the circles the increasing contents of ammonia determined analytically (right scale). At any time t, the increment $\Delta T'$ is proportional to the number of moles of ammonia n_{NH_3}

$$\Delta T' = \frac{1}{2}\frac{\Delta H}{R_{cal}} n_{NH_3}$$

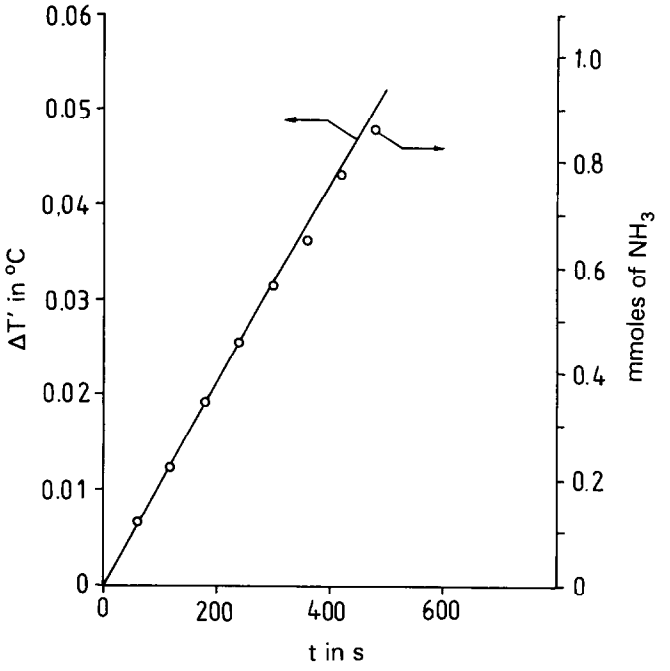

Fig. 2. The determination of the enthalpy of hydrolysis of urea into NH_4^+ and HCO_3^- ions as final products of the reaction in the absence of inhibitor.

Table 1
The parameters of the straight lines $\Delta T' = mt + n$ (Fig. 1B)

[NaF]/ (mol dm^{-3} × 10^3)	m/ (K s^{-1} × 10^6)	n/ (K × 10^6)
0	109	170
1	96.7	236
2	87.6	300
5	73.4	0

where the heat capacity of the calorimeter R_{cal} is 534.93 J K^{-1}. With $\Delta H = 59\,580$ J per mol urea the best fit of the analytical points to the calorimetric curve in its initial, linear range, $0 < t < 300$ s, was obtained. This value is slightly lower than that found by Jespersen [17], namely 61 300 ± 800 J per mol urea.

4.2. Calculation of the kinetic constants

The inhibition constants K_i and K_i^* were calculated from Eqs. (6) and (7) converted into the forms (6′) and (7′)

$$K_i = \frac{[I]}{\frac{v_{max} - v_0}{v_0}\frac{[S]}{K_M} - 1} \quad (6')$$

$$K_i^* = \frac{[I]}{\frac{v_{max} - v_s}{v_s}\frac{[S]}{K_M} - 1} \quad (7')$$

The values of the basic constants, i.e. the Michaelis–Menten constant K_M and the terminal reaction rate v_{max}, were measured in an independent experiment in the absence of inhibitor. Under the conditions of the experiments these values were: $K_M = (6.5 \pm 0.2)10^{-3}$ mol dm^{-3} and $v_{max} = (1.0 \pm 0.05)10^{-6}$ mol urea s^{-1}.

The reaction rate depends strongly on the amount of enzyme. The above value of v_{max} and the values of v_0 and v_s presented below are values measured directly in the presence of a constant, arbitrarily chosen mass of enzyme (3 mg). The mass of enzyme, however, does not influence the dimensionless expressions $(v_{max} - v_0)/v_0$ and $(v_{max} - v_s)/v_s$ appearing in Eqs. (6') and (7') not, consequently, the kinetic constants K_i and K_i^*. The apparent rate constant k is also independent of the mass of enzyme used. The initial concentration of urea was $[S] = 50 \times 10^{-3}$ mol dm^{-3}.

The ratio $[S]/K_M$ appearing in Eqs. (6') and (7') was constant and equal to 7.69. The first stationary rate v_0 was determined from the first ($0 < t < 60$ s) linear segment of the calorimetric progress curve (Fig. 1B). The parameter m/(K s^{-1}) of the line multiplied by the factor

$$1\ \mathrm{K} = \frac{R_{cal}}{\Delta H} = \frac{534.93}{59580} = 0.008978\ \text{mol urea}$$

yields the rate v_0/(mol urea s^{-1}) related to a given inhibitor concentration (Table 2).

Two independent techniques, calorimetric (Fig. 1A) and analytical (Fig. 3), were applied to determine the second stationary rate v_s. The slope of the second linear segment appearing on the progress curves in the range $600 < t < 800$ s was converted into the searched rate v_s/(mol urea s^{-1}). The values obtained are listed in Tables 3 and 4.

The values of the inhibition constants K_i and K_i^* calculated from the presented data using Eqs. (6') and (7') are shown in Table 5.

The final, average value of the first inhibition constant is $(K_i)_{av} = (1/3)(0.95 + 0.97 + 1.21) \times 10^{-3} = (1.04 \pm 0.14) \times 10^{-3}$ mol dm^{-3}. Upon calcula-

Table 2
The first stationary rate v_0 measured calorimetrically (Fig. 1B)

[NaF]/ (mol dm^{-3} × 10^3)	v_0/ (mol urea s^{-1} × 10^6)	$\frac{v_{max} - v_0}{v_0}$
1	0.868	0.267
2	0.786	0.399
5	0.659	0.669

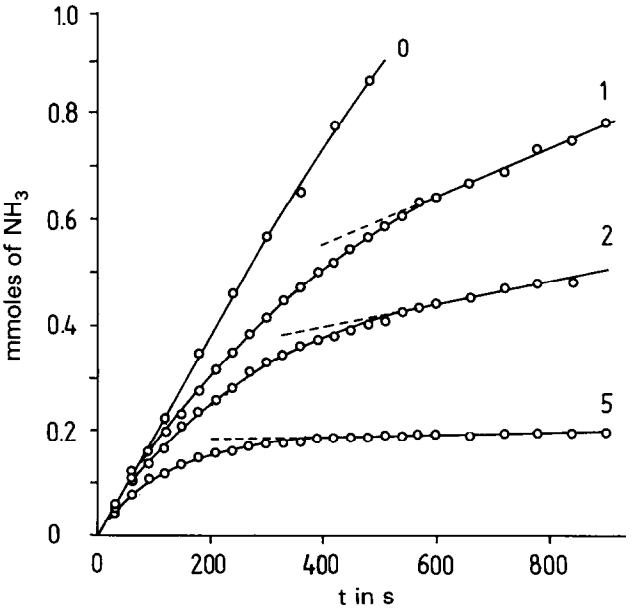

Fig. 3. Analytical progress curves for urease in the presence of sodium fluoride.

tion of the average value of the second inhibition constant K_i^*, it was assumed that both the calorimetric and analytical methods are equally accurate. When the reaction rate v_s vanishes at higher concentrations of inhibitor, the expression (7′) becomes indefinite. One value of K_i^*, in parentheses in Table 5, was therefore

Table 3
The second stationary rate v_s measured calorimetrically (Fig. 1A)

[NaF]/ (mol dm^{-3} × 10^3)	v_s/ (K s^{-1} × 10^6)	v_s/ (mol urea s^{-1} × 10^6)	$\frac{v_{max} - v_s}{v_s}$
1	35.5	0.319	2.45
2	14.6	0.131	7.40
5	6.23	0.056	18.6

Table 4
The second stationary rate v_s measured analytically (Fig. 3)

[NaF]/ (mol dm^{-3} × 10^3)	v_s/ (mol NH$_3$ s^{-1} × 10^6)	v_s/ (mol urea s^{-1} × 10^6)	$\frac{v_{max} - v_s}{v_s}$
1	0.452	0.226	3.87
2	0.220	0.110	9.00
5	0.0194	0.00969	113.0

Table 5
The experimental inhibition constants K_i and K_i^*

[NaF]/ (mol dm^{-3} × 10^3)	K_i/(mol dm^{-3} × 10^3)		K_i^*/(mol dm^{-3} × 10^3)	
	Calor.	Anal.	Calor.	Anal.
1	0.95	–	0.056	0.035
2	0.97	–	0.036	0.029
5	1.21	–	0.035	(0.006)

Table 6
The experimental values of the apparent rate constant k for different concentrations and final (average) values

[NaF]/ (mol dm^{-3} × 10^3)	k/(s^{-1} × 10^3)	k_{av}/(s^{-1} × 10^3)	
	Calor.	Anal.	
1	3.7	3.4	3.55
2	4.2	4.3	4.25
5	7.5	7.3	7.4

rejected: $(K_i^*)_{av} = (1/5)(0.056 + 0.036 + 0.035 + 0.035 + 0.029) \times 10^{-3} = (0.03 \pm 0.010) \pm 10^{-3}$ mol dm^{-3}.

In contrast with the inhibition constants, the apparent rate constant k depends on the concentration of the inhibitor. The values of k calculated separately for the calorimetric and analytical curves from the intersection point of the tangents and their average values are shown in Table 6.

5. Discussion

Dixon et al. [18] in 1980 studied the inhibition of the activity of urease by fluoride ion and found an irregular shape for the Lineweaver–Burk plot of $1/v$ vs. 1/[urea]. The average reaction rate v was defined as the ratio of the number of moles of the product created after time t to the number of seconds, $v = P(t)/t$. Contrary to numerous enzymatic reactions in which v was found to be constant, in the system studied v depends on t.

At a constant concentration of the inhibitor, two values of $1/v$ were assigned to a given value of 1/[urea]: the lower one relating to the first (higher) stationary rate measured over the short 30 s period and the higher corresponding to the second (lower) stationary rate of reaction measured over a large time range lasting several minutes. The first set of values of $1/v$ lies along the lines which intersect in the point $(0; 1/v_{max})$ which indicates that in the first 30 s reaction step sodium fluoride acts as a competitive inhibitor. Its activity is characterized by a constant K_i. The second set of values of $1/v$ yields a corresponding set of lines which focus in the point

$(-1/K_M; 0)$. Such L–B plots are related to systems with non-competitive inhibitors. It is evident that the mode of inhibition of urease by sodium fluoride varied as the reaction progressed. The authors introduced a hypothetical complex ESI, the dissociation constant of which, $K = [ES][I]/[ESI]$, controls the course of the second step of the reaction.

The experimental results presented here and in our earlier paper [12] are in full agreement with those of Dixon over the whole range of time. The properties of the studied system, however, may be explained differently. The reason why stronger inhibition and resultant deceleration were observed in the later stage of the reaction is that the complex EI converts into a more stable form EI*. Its dissociation constant defined by Eq. (3) and calculated above governs the kinetics of the second stage of inhibition. We have adopted the general model of slow-binding inhibition introduced by Morrison and Walsh [13]. The value of the first inhibition constant determined by Dixon et al. [18] K_i was 1.01 ± 0.08 mmol dm^{-3} and agrees very well with our value of 1.04 ± 0.14.

Measurements conducted recently in our laboratory [19] confirm the assumed scheme, Eq. (1). The hydrolysis of urea can be initiated in two ways, namely by addition of the enzyme E to a preconditioned mixture of S and I, or by addition of S to a preconditioned mixture of E and I. In the first case the initial rate of the reaction is high whereas in the second case it is very low. This finding strongly supports scheme (1) which postulates the formation of the inactive complex EI*.

6. Concluding remarks

In studies on the kinetics of enzymatic reactions the short-lived stages may be observed using calorimetric methods. In this work, temperature was registered every 10 s but this interval can be reduced down to 1 s. Calorimetry also enables the detection of secondary processes following the main reaction that are undetectable using analytical techniques.

The presented results confirm the assumption that the fluoride ion is a competitive "slow-binding" inhibitor of urease. The hydrolysis of urea runs through two stages proceeding according to zero-order kinetics. The first covers the period of the first minute ($0 < t < 1$ min), the second was observed between the 5th and 15th minute. The structure of the stable, catalytically inactive complex EI* remains unknown.

At high concentration of inhibitor when the hydrolysis of urea had stopped completely, a small but detectable production of heat was observed, possibly connected with a change in conformation of the inactive enzyme molecule. This phenomenon will be studied.

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