Acta Biochim Biophys Sin 2011, 43: 409–417 | © The Author 2011. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmr014.
Advance Access Publication 2 April 2011

Original Article

A novel approach for estimating the relationship between the kinetics and thermodynamics of glycoside hydrolases

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A series of experiments were performed, in which *p*-nitrophenyl-β-D-cellobioside (PNPC) was hydrolyzed by 1, 4-β-D-glucan-cellobiohydrolase (CBHI: EC 3.2.1.91), and *O*-nitrophenyl-β-D-galactoside (ONPG) was hydrolyzed by β-galactosidase (EC 3.2.1.23) under different combinations of temperature and time period. The combined effects of temperature and time on *p*-nitrophenyl and *O*-nitrophenyl formation were characterized as the change of the instantaneous reaction velocity occurrence per temperature range termed as $v_{inst}\cdot T^{-1}$. This parameter was used as a stable index to evaluate the apparent activation energy (E_a) based on the Arrhenius approach, instead of the reaction velocity constant, *k*. It was found that E_a for PNPC hydrolysis by CBHI first decreased with temperature increase and then slightly increased at higher temperature, and its minimum value was obtained just at the maximum point of v_{inst}. In addition, E_a for PNPC hydrolysis by dilute sulfuric acid was not a constant, but was continuously increased with temperature. The present studies demonstrated that E_a obtained by Arrhenius approach for the hydrolysis reaction of β-hydrolases appears to be only an empirical kinetic parameter for the dependence of the reaction velocity on temperature and time, and has no meaning in the sense of thermodynamic energy.

Keywords β-galactosidase; apparent activation energy; Arrhenius approach

Received: September 20, 2010 Accepted: January 30, 2011

Introduction

The effects of temperature on enzyme-catalyzed reactions are generally as follows: the reaction is slow at low temperatures, then the reaction velocity increases rapidly with temperature, and reaches a maximum value at the optimal temperature. The changes in enzyme-catalyzed reaction velocity before the temperature reaches the optimal one may be caused by the inhibition of the products formed during catalytic process to the catalytic activities of the enzyme. When temperatures go beyond the optimal temperature, the decrease in the reaction velocity with increasing temperature arises primarily from the thermal inactivation of the enzyme and the irreversible change in enzyme structure [1–3]. These complex changes suggest that the entire reaction process can not be described only by the initial velocity. Thus, it is necessary to estimate the reaction velocities within a wide range of temperature [4,5]. Indeed, from the physical point of view, the effects of temperature on reaction velocity is time dependent [6,7], and consequently, the time–temperature equivalency principle may be appropriate to analyze the effects of temperature and time. The essential idea of this principle is that certain results obtained at short time and at high temperature before enzyme inactivation can be used to predict the results at long time and at low temperature. This phenomenon is similar to the thermodynamic restrictions of the temperature and pressure to reaction velocity constants in chemical reaction kinetics [8]. Thus, estimating the intrinsic enzyme-catalyzed reaction velocity by establishing the genuine relationship between kinetics and thermodynamics is very complex [9–12]. The present analysis focuses on how to estimate the combined effects of temperature and time on enzyme-catalyzed reaction velocity. This analysis may provide a theoretical base for the application of the Arrhenius approach. The key concept here is that a new parameter, $v_{inst}\cdot T^{-1}$, was constructed to quantitatively express the effects of time and temperature on the velocity of enzyme reaction. In this way, the instantaneous velocity may be correlated with thermodynamics and therefore it can be used as an index to evaluate the apparent activation energy (E_a) based on the Arrhenius equation. The assays for determining enzyme-catalyzed velocity usually involve a series of time-course measurements at a given interval under a fixed temperature. Under these conditions, the catalytic velocity is a mean value and is obtained from the formula: $\bar{v} = (y_{i+1} - y_i)/(t_{i+1} - t_i)$. Mathematically, $\bar{v}$ equals the slope of the line linking two points (y_{i+1} and y_i) on the

reaction curve of y versus t. The value of $\bar{v}$ depends not only on the changes in the concentrations of reactants, but also on the time interval [13,14]. Thus, the accurate estimation of the changes of reaction velocity with time course can not be determined, which can then yield a variety of the temperature-dependent properties; therefore, terming it as the apparent velocity, v_{app}, may be more suitable. To overcome the uncertainty in the measurement of the reaction velocity, the concept of v_{inst} has been proposed and used to characterize the velocity of the enzyme-catalyzed reaction in our previous report [15]. v_{inst} is the first derivative of y_i with respect to t_i and it is calculated from the formula: $v_{inst} = \lim_{\Delta t \to 0} (\Delta y / \Delta t)$. Mathematically, v_{inst} is the slope of a tangent at any point p_i (y_i, x_i) on the curve. Since the velocity obtained is at the instantaneous scale, the value of v_{inst} is not affected by the time interval (Δt), and can be derived directly from the experimental data by numerical differentiation [16]. In this manner, the changes in reaction velocity with time course can be accurately determined. For kinetic studies, using v_{inst} as an index is sufficient; furthermore, for thermodynamics, the effect of temperature should be calculated in different temperature range. This is because the effect of per unit temperature increase on reaction velocity is different at different temperature levels. This may be a key approach to rationally estimate the relationship between the kinetics and thermodynamics. However, simultaneously estimating the combined effects of temperature and time in the instantaneous scale is quite difficult [3–8]. Mathematically, these effects can be expressed as a compound function of velocity with respect to temperature and time and calculated as the algebraic sum of the first-order partial derivative of temperature and time based on differential calculus: $c - v_{inst} = (\partial p/\partial T)t \cdot dT + (\partial p/\partial t)T \cdot dt$, where p represents the substrate decreased or the product produced during the reaction, and T and t are the temperature and time, respectively [13,14,16]. Recently, investigations carried out in our laboratory have shown that because the instantaneous rate is quite sensitive, a small experimental error may result in a large deviation from the theoretical curve [17,18], and the calculation of the sum of the partial derivatives for each of the cases is quite time consuming and errors are inevitable [19,20]. This difficulty may be overcome using the parameter, $v_{inst} \cdot T^{-1}$, to express the reaction velocity. The assays were performed for different times at a series of temperatures to obtain a series of v_{app} for each temperature with different time. A series of curves of v_{inst} versus time were recorded for each assay temperature, and then $v_{inst} \cdot T^{-1}$ can be denoted as v_{inst} divided by each assay temperature, respectively. Finally, a series of curves of $v_{inst} \cdot T^{-1}$ versus temperature was established, which can be used to calculate the apparent E_a based on the Arrhenius equation [21]. In this way, the combined effects of temperature and time can also be estimated. The classical Arrhenius equation $k = A \exp(-E_a/RT)$ is an empirical equation that describes the relationship between reaction rate constant and temperature in the elementary reaction. The optimum temperature and the enthalpy of denaturalization of enzyme systems have been widely used to investigate biochemical processes. However, because the kinetics of enzyme-catalyzed reactions differs from that of the elementary reaction and are quite complex, some questions about the adaptability of the Arrhenius equation have long been raised for enzyme-catalyzed reactions [22–24]. One of the key problems is that different reaction velocity parameters were used empirically as reaction rate constants k for evaluating the E_a. From the physicochemical point of view, k is a proportionality coefficient of the reaction velocity and it is mathematically equal to the slope of the reaction curve [25,26]. In Arrhenius's original approach, k is the increase in the reaction rate when the temperature increases by 10 K, and it is a constant only under a certain temperature range [27]. As mentioned above, this recompilation is not adaptable to the enzymatic reaction system. Several approaches have been proposed to determine k by simplifying the kinetics of enzyme-catalyzed reactions. In most cases, v_{app} has been used as an objective function to evaluate E_a based on the assumption that when the concentration of the reactants is constant, v_{app} is proportional to k, and thus, v_{app} can be used to replace k [21]. However, this assumption may only be suitable under very short time duration. In fact, the value of v_{app} should be different at different time intervals in the assay [15,16]. As mentioned above, this difficulty may be overcome using $v_{inst} \cdot T^{-1}$ as an index to evaluate the apparent E_a. In the present study, three parameters (v_{app}, v_{inst}, and $v_{inst} \cdot T^{-1}$) were, respectively, used to evaluate the apparent E_a for 1, 4-β-D-glucan-cellobiohydrolase (CBHI) and β-galactosidase [28–30].

Materials and Methods

Chemicals and enzymes

1, 4-β-D-glucan-cellobiohydrolase, a major component of the cellulase system, was purified by a previously described method [15,17] and β-galactosidase, which is used commonly as reporters of gene expression in molecular biology, were purchased from Sigma Chemical Co. (St Louis, MO, USA). The remaining reagents including *p*-nitrophenyl-β-D-cellobioside (PNPC), *O*-nitrophenyl-β-D-galactoside (ONPG), *p*-nitrophenyl (PNP), and *O*-nitrophenyl (ONP) were of analytical grade.

Spectrophotometric assays for the enzymatic hydrolysis

To obtain complete information on the combined effects of temperature and time on the enzyme's catalytic reaction, all enzymatic hydrolysis experiments were performed within a wide range of temperatures and over time periods. A high

ratio of [S] to [E] was used for providing a sufficient number of substrate for hydrolysis. The final concentrations of PNPC and ONPG were 200 μM and the concentrations of CBHI and β-galactosidase were 1 μM in 50 mM buffer (pH 4.8 for CBHI and pH 7.0 for β-galactosidase). For CBHI, experiments were carried out at eight temperatures (10, 20, 30, 40, 50, 55, 60, and 65 ± 0.2°C) for different time periods (from 2.5 to 20 min at 2.5 min intervals). Experiments involving the hydrolysis of ONPG by β-galactosidase were also performed within a wide range of temperatures (from 25 to 60°C at 5°C intervals) and time periods (from 2.5 to 17.5 min at 2.5 min intervals). The ultra violet spectra were measured with a UV-3100 UV-VIS-NIR recording spectrophotometer, equipped with a temperature controller (Shimadzu, Kyoto, Japan), over the region of 200–500 nm, at a scan speed of 240 nm min^{-1} using a 1.0 cm path-length quartz cell.

Results

UV spectra characteristics of PNPC, PNP, ONPG, and ONP

As shown in **Figures 1** and **2**, beyond 360 or 400 nm, the absorbance of PNP or ONP was not affected by the presence of other fractions in the solution and thus, spectral output over the range 370–400 nm or 450–500 nm only reflects the presence of PNP or ONP, respectively. Therefore, in the present study, the total absorbance in these wavelength ranges was used to quantify the PNP or ONP concentrations generated during hydrolysis process by the area under curve (AUC) method [31]. AUC is one of the most commonly used methods in pharmacokinetics and chromatography. Although the absorbance of a single point on the spectral curve can reflect the influence of all the factors in a system, the wavelength interval can influence experimental errors to a large extent, as the larger the interval the smaller the error. Therefore, the error will be smaller when the AUC is used. The AUC can be directly derived by a definite integral of absorbance to the initial and final wavelength. The numerical integration equation may be written as: AUC $= \int_0^i A_i(\lambda)\, d\lambda$, here i is the wavelength ($i = 1, 2, \ldots,$ n) and A_i denotes the absorbance at wavelength i. Compared with the classical absorbance method, the AUC method appears to be more accurate, and it can reflect the trend of the whole spectrum in chromatography, reducing the error caused by choosing only one single wavelength as the objective function.

Kinetic features of PNPC hydrolysis by CBHI under different combinations of temperature and time course

PNPC (200 μM) was hydrolyzed by CBHI (1 μM) for different times (from 2.5 to 20 min at 2.5 min intervals) at eight different temperatures (283–338 K at 5 K intervals)

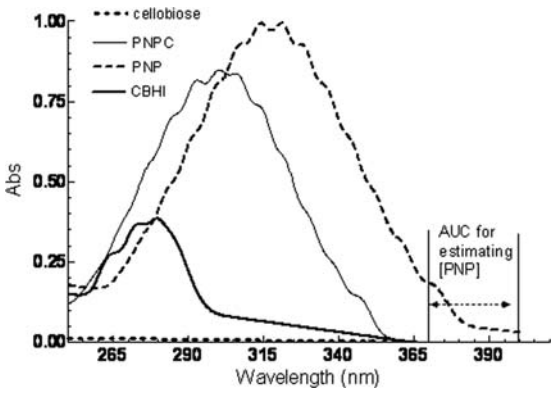

Figure 1 Comparison of UV-spectra CBHI (25 μM), cellobiose, PNPC, and PNP (100 μM) in acetate buffer, pH 4.8

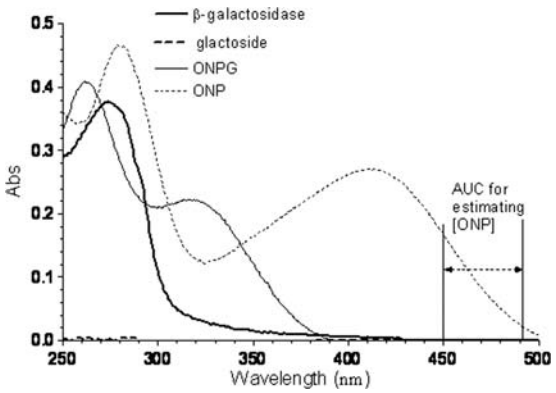

Figure 2 Comparison of UV-spectra of ONPG, ONP, and galactoside (100 μM) and β-galactosidase (1 μM)

in acetate buffer at pH 4.8. **Figure 3(A)** showed the kinetics of PNPC hydrolysis by CBHI under different combinations of temperature and time. When the reaction velocity was expressed as v_{app}, those kinetic curves all appeared approximately in the shape of Gaussian distributions. However, when the reactive velocity was expressed as v_{inst} and $v_{inst} \cdot T^{-1}$, different kinetic patterns were observed as shown in **Fig. 3(B,C)**.

Figure 4 showed the semi-logarithmic plot of ln ($v_{inst} \cdot T^{-1}$) versus $1/T$. The slope of the Arrhenius plot was not a straight line, but appeared as a curve that continuously changed as the temperature and time course increased. The result was different from that of a simple chemical reaction, such as the acid hydrolysis of sucrose. **Figure 5(A)** showed the calculation of the apparent E_a based on the Arrhenius equation [$E_a = -$ slope $\times R$], where R is the gas constant and equals $-$ 8.31 J mol^{-1} K^{-1} [24].

When the E_a was calculated by v_{app} or v_{inst}, the results obtained were different from those shown in **Fig. 4**. For each of the curves, the direction of the slope changed continuously, turning from positive to negative at a certain temperature (data not shown). **Figure 5(B,C)** showed the plot of apparent E_a obtained by v_{app} or v_{inst}, respectively.

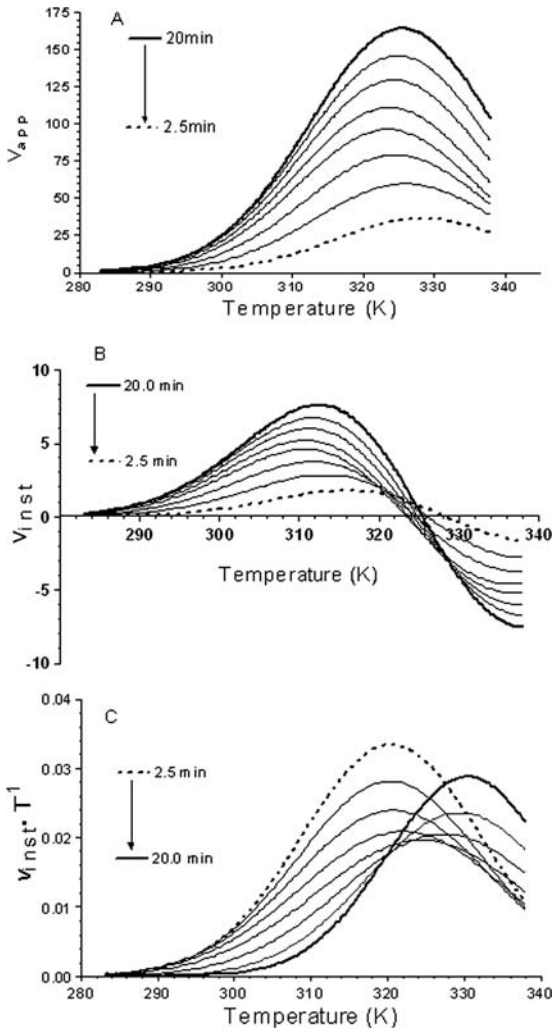

Figure 3 Dependence of PNP formation during PNPC hydrolysis (200 μM) by CBHI (1 μM) over an assay time from 2.5 to 20 min at temperatures range from 283 to 338 K (A) The reaction velocity was expressed as v_{app}. (B) The reaction velocity was expressed as v_{inst}, and (C) the reaction velocity was expressed as $v_{inst} \cdot T^{-1}$.

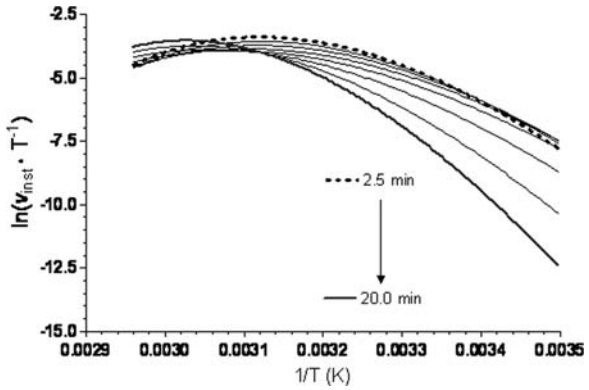

Figure 4 Arrhenius plot for PNPC hydrolysis by CBHI Data obtained from **Fig. 3(C)**

The above results demonstrated that E_a of PNPC catalysis by CBHI derived from the Arrhenius equation is not a constant, and appeared as a crescent-shaped curve

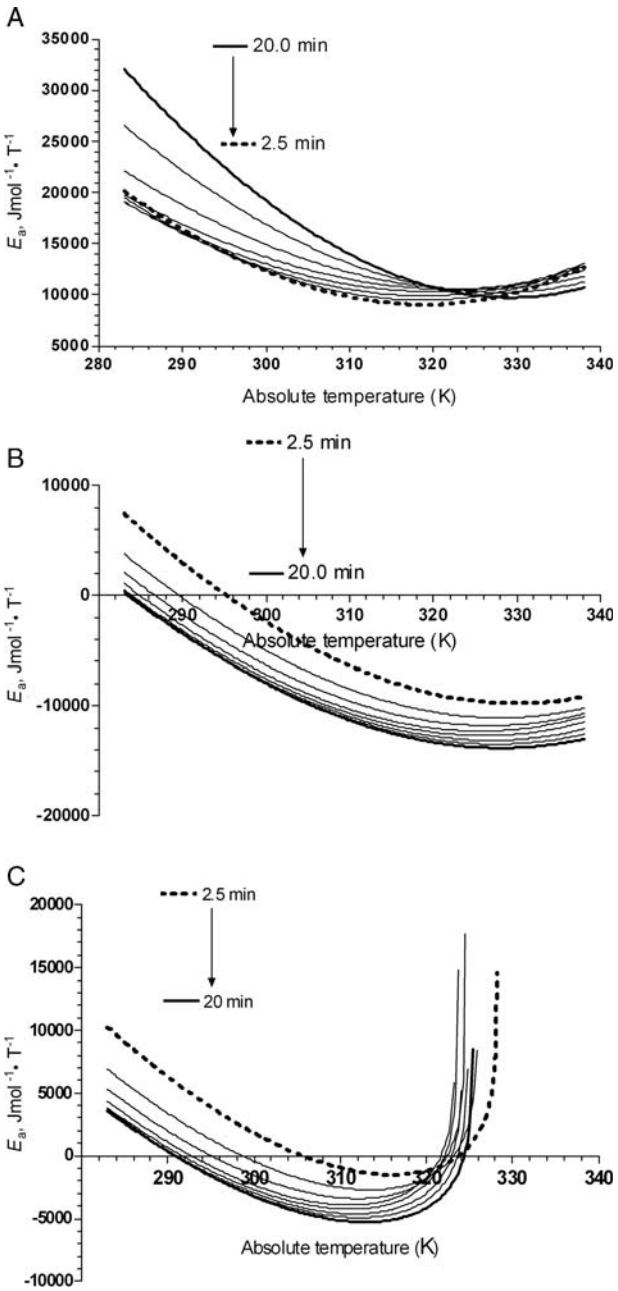

Figure 5 Estimation of the apparent activation energy from the Arrhenius plots as $E_a = -\text{slope} \times R$, based on $v_{inst} \cdot T^{-1}$, v_{app} and v_{inst}

changing with temperature. In the original concept proposed by Svante Arrhenius [27,32], it is assumed that E_a is an energy barrier for chemical reactions. It is very difficult to relate our observations to this definition. Particularly, the appearance of the negative E_a **[Fig. 5(B,C)]** can not be explained rationally when E_a is assumed as the critical energy necessary for the occurrence of reaction, or as E_a is defined as the difference between the critical energy and the mean value of the reactants [33–36]. The results in **Fig. 5** indicated that E_a is a phenomenological quantity determined by the slope of the curve of ln$k$ versus 1/T.

The tendencies of $v_{inst}\cdot T^{-1}$ and E_a obtained from $v_{inst}\cdot T^{-1}$ were visualized by three-dimensional graphs (**Fig. 6**). Clearly, there is nearly a negative relationship between both parameters.

Kinetic features of ONPG hydrolysis by β-galactosidase at different combinations of temperature and time course

Experiments involving the hydrolysis of ONPG (200 μM) by β-galactosidase (1.0 μM) were performed under conditions of a wide range of temperature and time course in different combinations (from 2.5 to 15.0 min at 2.5 min intervals and from 283 to 333 K, at 5 K intervals). Similar result was obtained as shown in **Fig. 7**.

PNPC hydrolysis by dilute sulfuric acid at different temperatures and time courses

Figures 8 and **9** showed the patterns for PNPC (100 μM) hydrolysis by 2 M sulfuric acid at different temperatures (over a temperature range of 303–363 K at 10 K interval) and time courses (2.5–50 min at 5 min interval). The rates of glycoside hydrolysis are so slow at low temperatures that the formed PNP can not be detected by UV spectroscopy, and can only be accurately detected at very high temperatures, i.e. >333 K. The increase in the amount of PNP formed could be approximated by an exponential function of temperature and time course. Furthermore, the exponential model was used to determine the reaction rate constant k for the overall process. The linear least-squares analysis revealed that there was a linear correlation between ln k and $1/T$ with a negative slope, and appeared to be fit for the data well (**Fig. 10**; $R^2 = 0.96$).

E_a can then be calculated from the slope of the plot of ln k versus the reciprocal of the absolute temperature, i.e.

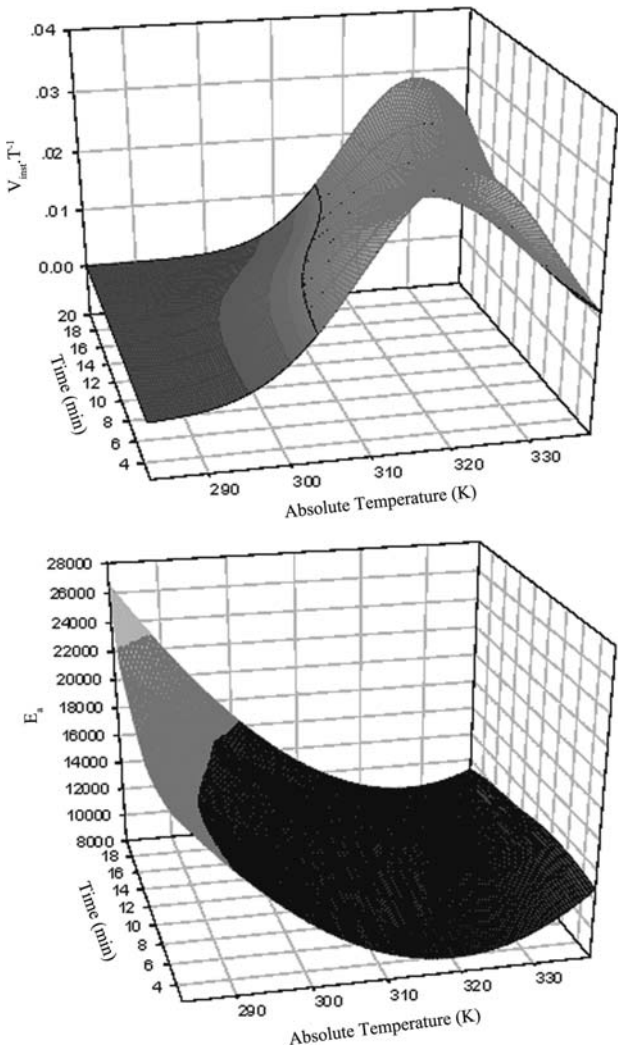

Figure 6 Three-dimensional graph for the dependence of $v_{inst}\cdot T^{-1}$ and E_a on different combination of temperature and time course during PNPC hydrolysis by CBHI

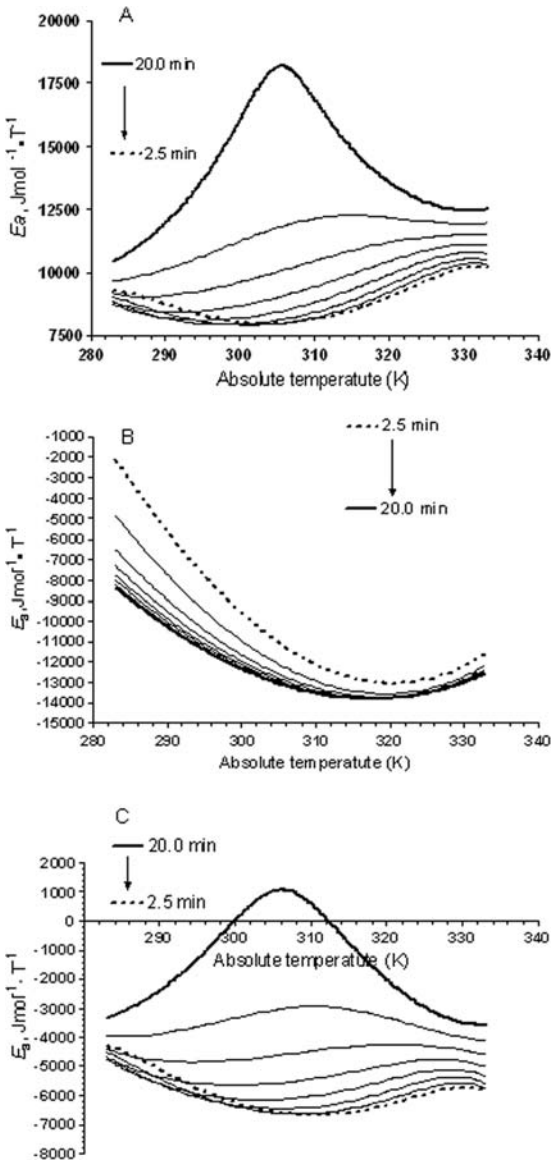

Figure 7 Estimation of the apparent activation energy for ONPG hydrolysis by β-galactosidase from the Arrhenius plot (E_a = − slope × R) based on $v_{inst}\cdot T^{-1}$ (A), v_{app} (B), and v_{inst} (C)

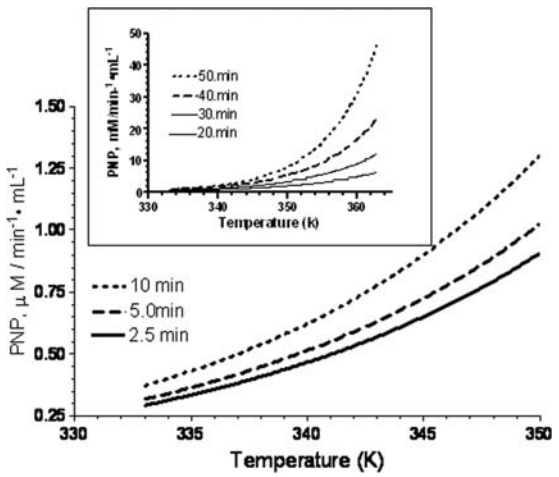

Figure 8 PNPC hydrolysis by 2 M sulfuric acid at different temperatures (333–363 K) and time (2.5 to 50 min).

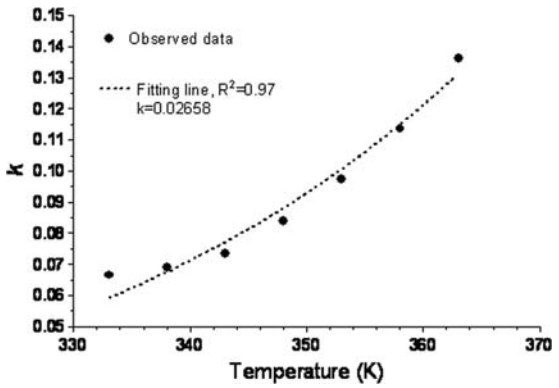

Figure 9 Dependence of the reaction rate constant *k* on temperature for PNPC hydrolysis by sulfuric acid Data obtained from Fig. 8 and fitted by an exponential growth equation.

$E_a = -\text{slope} \times R$. It is $\sim$23,000 $\text{Jmol}^{-1}\cdot\text{K}^{-1}$ over the temperature range 333–363 K. This result was similar to the disaccharides hydrolysis by dilute acid reported previously [29,30].

To explore the alternative analysis strategy, in the present study, $v_{inst}\cdot T^{-1}$, rather than the reaction rate constant k, was used to calculate E_a, as shown in **Fig. 11**. A straight line will appear when fitting the data by linear regression, the slope of which is different from each other and depends on the time course. E_a derived from the slope increased continuously with temperature. These results suggested that the value of E_a is also affected by the time course as calculated from $v_{inst}\cdot T^{-1}$.

Discussion

The consistency between chemical kinetics and thermodynamics plays an important role in almost all chemical and biochemical processes. In 1883, Arrhenius proposed an assumption for exploring this relationship based on sucrose

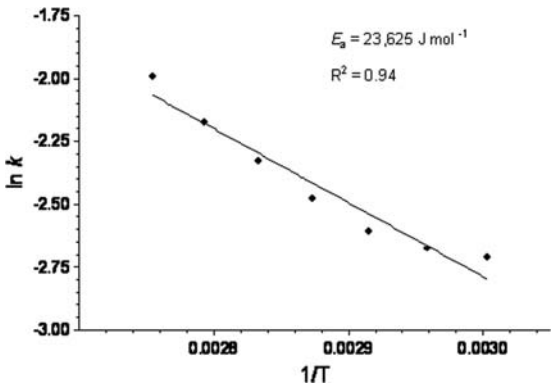

Figure 10 Arrhenius plot for PNPC hydrolysis by sulfuric acid Data obtained from **Fig. 9** and fitted by the first-order polynomial equation (straight line).

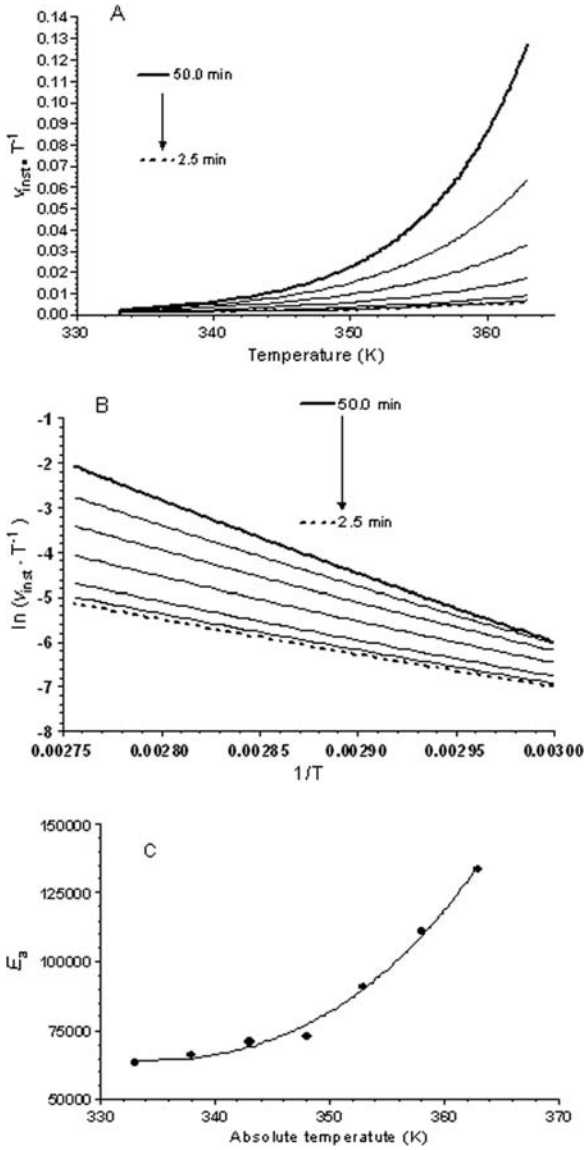

Figure 11 Estimation of the apparent activation energy from Arrhenius plots ($E_a = -\text{slope} \times R$) during PNPC (100 μM) hydrolysis by 2 M sulfuric acid at different temperatures (333–363 K) over different time courses (2.5–50 min)

hydrolysis by acid [27]. In the classical Arrhenius approach, the reaction velocity is only estimated from the effect of the temperature, while the effect of time is not considered. As mentioned above, that should lead to certain errors. In addition, there are still some questions, such as: Why were non-linear fits found in many cases to be statistically no better than simple linear fits? Why does the E_a not change with temperature, and why is the reaction rate constant temperature independent? Until now, there has been a lack of understanding about these phenomena. During the past years, the concept of variable E_a has been proposed [37–39]. There are at least two hypotheses attempting to define E_a [40–42]. One hypothesis is that E_a is defined as the critical energy necessary for the reaction to occur, as suggested by Lewis. The other is that E_a is considered as the difference between two statistical quantities, the critical energy and the mean value of the reactants, based on Tolman's theorey; i.e. the population of the states in the reactant manifold follows a Boltzmann distribution. A widely accepted concept for simple chemical reactions is that the reaction rate follows the form of $k \propto e\ [-E^{barrier}/k_BT]$, where k_B is the Boltzmann constant, and the energy barrier, $E^{barrier}$, is independent of temperature [43]. The present study has demonstrated, however, that E_a is a compound function of temperature and time rather than a constant not only for enzyme hydrolysis, but also for simple acid hydrolysis. Furthermore, there is no perfectly rational explanation for why E_a is sometimes negative. We believe that the problem is not due to experimental error, but arises from the Arrhenius assumption itself. Both critical energy barrier and energy barrier are microscopic quantities at the molecular scale, their values are usually greater than zero or equal to zero, but they can not be negative values. In the present study, the apparent is a macroscopic quantity calculated as the algebra sum of entire reaction process under certain reaction condition, when the slope of ln$K$ versus $1/T$ is a positive value, a negative E_a will be obtained. As Luo mentioned in his paper, the negative E_a has no intrinsic sense in physics [44]. If this result is used to explain the thermodynamic for enzyme catalytic reaction, it will lead to errors.

There are still some problems in understanding the Arrhenius approach when it is applied to estimate the consistency between chemical kinetics and thermodynamics. First, the reaction rate constant k is confused with the thermodynamic equilibrium constant K_{eq} in the Van't Hoff equation. In fact, The Van't Hoff equation is a thermodynamic equilibrium model that can be used to characterize the relationship between the standard equilibrium constant and temperature, and assumes that the enthalpy ΔH is a constant under the temperature range of the reaction [45]. It is similar to the Arrhenius equation only in the mathematical form of the expression, but different in its physical terms. According to the principles of physical chemistry, there is no direct numerical relationship between chemical kinetics and chemical thermodynamics. Traditionally, biochemical thermodynamics and biochemical kinetics are quite different; they are related only under equilibrium conditions for elementary reactions expressed as follows: $K_{eq} = k_{positive}/k_{negative}$. Here K_{eq} is the thermodynamic equilibrium constant, $k_{positive}$ is the positive reaction rate constant, and $k_{negative}$ is the negative reaction rate constant. This formula provides a quantitative relationship between the reaction rate constant and the thermodynamic equilibrium constant. In general, this mathematical relationship is only limited to the elementary reaction [45].

Second, there is a wrong idea about the estimate of the linear slope by using linear regression calculations and transforming the observed data to a semi-logarithmic plot [46,47]. An easy method for dealing a non-linear relationship is to transform the observed data into a straight line and then the linear regression is performed. The goodness of fit was expressed by the coefficient of determination, R^2; an example is shown in **Fig. 9**. In this way, the k value appears to follow an exponential growth equation ($y = A\ e^{[-Bx]}$) for PNPC hydrolysis by sulfuric acid at different temperatures. As shown in **Fig. 10**, the plot of the logarithm of each k value against the reciprocal of the absolute temperature appeared to be nearly straight lines. In general, many investigators would consider an R^2 value as a proof of linearity, or as a strong verification that a mathematical model is valid. However, a high R^2 value only shows a statistically good fit. It is a necessary, but not sufficient, condition for indicating the intrinsic sense [46,47]. This is because these non-equivalent transformations often lead to wrong construction of the relationship between the two variants, from an exponential relationship to a logarithmic one. Such analytical mistakes can be frequently found, because authors do not adhere to the simple rules for data transformation: the two sides of an equation must have the same dimensions and only dimensionless pure numbers have logarithms [48,49]. Third, mathematically, the linear results are obtained by distorting the original data distribution. E_a for ONPG hydrolysis by β-galactosidase appears irregular changes as shown in **Fig. 7**, and this may be caused by these logarithmic transformations. Because goodness of fit is calculated as the sum of squares of the vertical distances of the points from the assumed curve, the fit with lower sum of squares is not preferred. However, a high R^2 only shows that the assumed curve remains very close to a set of observed points, but that does not mean that the constructed assumptions are correct [46–49]. As illustrated in **Fig. 12**, there were significantly misleading results. Which equation better describes the data? It is difficult to answer only by examining the goodness of fit. In general, 'do not choose linear regression when you want to compute a correlation coefficient [46,47].

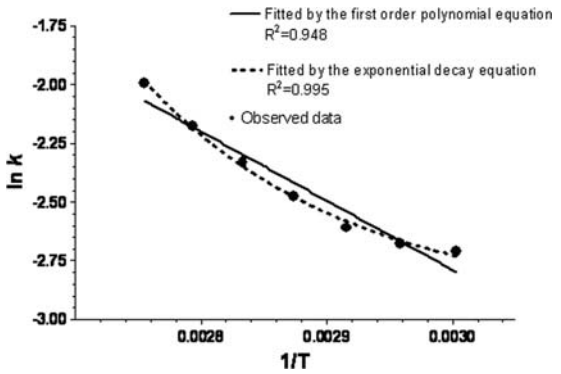

Figure 12 Arrhenius plot for PNPC hydrolysis by sulfuric acid Data obtained from **Fig. 9** and fitted by the first-order polynomial equation (straight line) and exponential decay equation.

Finally, from the physics point of view, thermo-physical property for a specific state is the compound function of a given temperature and time course. Therefore, the effect of temperature should correlate with a certain time period: the temperature–time equivalency principle [50,51], which is similar to the idea for determining the free energy by the combined effect of temperature and pressure [8]. The essential idea here is that certain results obtained from short time and high temperatures before enzyme inactivation can be used to predict that from long time and low temperatures. However, different combinations of temperature and time course have produced different kinetics that can not be expressed by v_{app}, as generally performed in enzyme assays. The $v_{inst}\cdot T^{-1}$ method may provide an effective approach to overcome this difficulty and the combined effects can directly visualized by contour plots.

Even though the E_a can be estimated by using $v_{inst}\cdot T^{-1}$, rather than the reaction rate constant k, as index, theoretical justification of the result is quite difficult. Thus, this suggested that the applicability of the Arrhenius equation in enzyme-catalyzed reactions obviously lacks theoretical justification or rationalization and its artifacts have also been indicated [52]. Unfortunately, until now the Arrhenius approach is generally used to describe the kinetic influence of temperature, and to analyze the mechanism in enzymatic reactions for many biochemical processes as is reported elsewhere. That appears to be due to either the theoretical complexities for recognizing the meaning of E_a, or because, in common, biologists may only take pains to select a classical existing and easily used model, but do not analyze rationally the errors in the used model. In particular, a simple method has been applied widely to obtain E_a [21]. That is, k_1 and k_2 are determined at two different temperatures, T_1 and T_2. Instead, they often appear as velocity v_1 and v_2, and follow the integrated form of the Arrhenius equation (similar to the Van't Hoff equation: $\ln k_2/k_1 = E_a/R\ (T_2 - T_1/T_2T_1)$. Despite a series of pitfalls present as mentioned above, because only a single E_a can be obtained by this method from the values of v_1 and v_2 estimated at T_1 and T_2, it may lead to more variable results, and unreliable conclusions for the enzyme-catalytic mechanism.

In summary, the results reported here clearly indicated that the combined effects of temperature and time on enzyme-catalyzed reaction velocity can be effectively calculated by $v_{inst}\cdot T^{-1}$. The results also demonstrated that there are a series of artifacts in the construction of the Arrhenius equation based on physico-chemical and mathematical analysis, and on a series of experiments of PNPC hydrolysis by glycosidases and dilute acid. Thus, E_a obtained from the Arrhenius equation seems to be only an empirical-kinetic or a phenomenological quantity, and has no meaning in the sense of thermodynamic energy.

The present results were only derived from the hydrolysis of two glycosidase. Can this new approach be applied to other enzymatic activities? Obviously, in order to assess the efficacy of this approach, there is a need to conduct further experiments. Same study is in progress in our laboratory based on the catalytic property of soybean peroxidase. Theoretically, the approach developed in the present study is based on the kinetic analysis of derivative curves and it was not related with enzyme structure, and so it may be consistent with a certain common sense for comparing the kinetic behavior of different enzymes.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (30870080) and National Basic Research Program of China (2011CB707401).

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