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Research paper

Temperature dependence of binding and catalysis for human serum arylesterase/paraoxonase

Jean Debord [a,*], Jean-Claude Bollinger [b], Michel Harel [c], Thierry Dantoine [d]

[a] Service de Pharmacologie-Toxicologie, Hôpital Dupuytren, 2 Avenue Martin Luther King, 87042 Limoges, France
[b] Groupement de Recherche Eau, Sol, Environnement, Université de Limoges, Faculté des Sciences, 123 Avenue Albert Thomas, 87060 Limoges, France
[c] Institut de Mathématiques de Toulouse (UMR CNRS 5219), 31062 Toulouse, France
[d] Service de Gérontologie clinique, Hôpital Dupuytren, 2 Avenue Martin Luther King, 87042 Limoges, France

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ABSTRACT

The influence of temperature upon the hydrolysis of phenyl acetate, catalysed by purified human serum arylesterase/paraoxonase (E. C. 3.1.8.1), was studied in the temperature range 10 °C–40 °C by spectrophotometry in TRIS buffer, pH 8.0, using both initial rate analysis and progress curve analysis. The kinetic parameters (catalytic constant k_{cat}; Michaelis constant K_m; product inhibition constant K_p) were determined by nonlinear regression. All parameters increased with temperature, but the ratios k_{cat}/K_m and K_p/K_m remained practically constant. Binding of both substrate and reaction product (phenol) was exothermic. A negative entropic term accounted for about 50% of the enthalpy change for both the binding and catalytic steps. Thermodynamic analysis suggested that: (1) the rate-limiting step is the nucleophilic attack of the carbonyl group of the substrate by a water molecule, (2) the active site is preorganized with no induced fit, (3) the enzyme-bound calcium plays an important role in stabilizing both the substrate and the transition state. The practical implications of these results are discussed.

1. Introduction

Paraoxonase, also known as arylesterase (EC 3.1.8.1) is a calcium-containing enzyme linked to the high density lipoproteins (HDL). It can hydrolyze a wide range of substrates, including lactones, aromatic esters such as phenyl acetate, or phosphate esters such as paraoxon, the active metabolite of the insecticide parathion. This enzyme was first studied for its ability to detoxify organophosphorus pesticides, but since has been ascribed a protective role against several pathologies [1]. Structural studies have allowed to propose a mechanism of action [2,3], but the thermodynamics of the hydrolytic reaction remain poorly understood, in spite of the previous studies devoted to the hydrolysis of phenyl acetate catalyzed by sodium acetate [4] or the hydrolysis of nitrophenyl esters by serum albumin, a non-specific catalyst [5]. It was therefore interesting to study the influence of temperature on the kinetics of the enzyme, in order to investigate the thermodynamic basis of its specificity.

2. Theory

We assume that the reaction follows the simple mechanism:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_{cat}}{\rightarrow} E + P \quad (1)$$

where E, S, ES and P denote the enzyme, substrate, enzyme–substrate complex and reaction product, respectively. k_1 and k_{-1} are the association and dissociation rate constants, and k_{cat} is the catalytic constant. The Michaelis constant is defined by:

$$K_m = \frac{k_{-1} + k_{cat}}{k_1} \quad (2)$$

If $k_{cat} << k_{-1}$, $K_m \approx k_{-1}/k_1$ represents the dissociation constant of the enzyme–substrate complex.

The initial reaction rate v_0 is linked to the initial substrate concentration $[S]_0$ by the Michaelis equation:

$$v_0 = \frac{V_{max}[S]_0}{K_m + [S]_0} \quad (3)$$

* Corresponding author. Tel.: +33 (0)555056140.
E-mail address: jean.debord@unilim.fr (J. Debord).

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where:

$$V_{max} = k_{cat} e_{tot} \quad (4)$$

is the maximal velocity and e_{tot} the total enzyme concentration.

The association equilibrium constant ($1/K_m$) and the catalytic rate constant (k_{cat}) depend on the absolute temperature T by means of the classical equations:

$$\ln \frac{1}{K_m} = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (5)$$

$$\ln \frac{k_{cat}}{T} = \ln \frac{k_B}{h} - \frac{\Delta H^\ddagger}{RT} + \frac{\Delta S^\ddagger}{R} \quad (6)$$

where ΔH° and ΔS° denote the binding enthalpy and entropy, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ denote the activation enthalpy and entropy, k_B is Boltzmann's constant, h is Planck's constant and R is the gas constant.

If both $[S]_0$ and T are varied simultaneously, a global equation can be obtained by bringing eqs. (5) and (6) into eq. (3):

$$v_0 = \frac{k_B}{h} \cdot e_{tot} \cdot [S]_0 \cdot T \cdot \frac{\exp(-a/T + b)}{\exp(c/T - d) + [S]_0} \quad (7)$$

with $a = (\Delta H^\ddagger/R)$, $b = (\Delta S^\ddagger/R)$, $c = (\Delta H^\circ/R)$, $d = (\Delta S^\circ/R)$.

In addition, we assume that the reaction product P can bind to the enzyme with a dissociation constant K_p:

$$E + P \underset{K_p}{\rightleftharpoons} EP \quad (8)$$

K_p can be determined by recording the complete time course of the reaction, assuming that the Michaelis equation (3) is valid at any time t:

$$v = -\frac{d[S]}{dt} = \frac{V'_{max}[S]}{K'_m[S]} \quad (9)$$

where K'_m and V'_{max} are the apparent kinetic parameters:

$$K'_m = K_m \frac{K_p + [S]_0}{K_p - K_m} \quad (10)$$

$$V'_{max} = V_{max} \frac{K_p}{K_p - K_m} \quad (11)$$

According to previous studies [6–8], the analytical solution of the differential equation (9) is the integrated Michaelis–Menten equation:

$$[P] = [S]_0 - K'_m \cdot W\left\{\frac{[S]_0}{K'_m} \exp\left(\frac{[S]_0 - V'_{max} t}{K'_m}\right)\right\} \quad (12)$$

where $[P]$ denotes the product concentration at time t. W is Lambert's function, which is such that, if $w = W(x)$, then $x = w \exp(w)$.

So, if K_m and V_{max} are known, K_p can be estimated by fitting eq. (12) to the whole progress curve. Alternatively, K_p can be determined by recording the initial rate v_0 in the presence of added product and using the classical equation for competitive inhibition:

$$v_0 = \frac{V_{max}[S]_0}{K_m(1 + [P]_0/K_p) + [S]_0} \quad (13)$$

where $[P]_0$ denotes the concentration of the added product at time 0. The binding enthalpy and entropy for the product P are estimated from eq. (5) by using K_p instead of K_m.

3. Material and methods

3.1. Enzyme

Human serum arylesterase has two isozymes A and B which differ by the aminoacid at position 192: glutamine (Q) in the A isozyme and arginine (R) in the B isozyme [9]. There are therefore three phenotypes: A, AB and B (respective genotypes QQ, QR and RR). These isozymes differ by their hydrolytic activity towards paraoxon(diethyl-4-nitrophenyl-phosphate), the B isozyme being the most active. This property allowed the determination of the phenotype of each subject, according to the method of Eckerson et al. [10], by computing the ratio of the hydrolysis rates of paraoxon, in the presence of 1 M NaCl, and phenyl acetate. We used this method to phenotype sera from healthy volunteers [11]. Sera from subjects having the same homozygous phenotype A were pooled, and the enzyme was purified by the method of Gan et al. [12]. Details of the purification procedure have been published elsewhere [13]. A molecular weight of 40 kDa for the proteic part of the glycoproteic enzyme was used to compute enzyme concentrations [14]. The enzyme and substrate solutions were prepared in TRIS buffer (9 mM) containing 1 mM $CaCl_2$ as activator. The pH of the buffer was adjusted to 8.0 at the desired temperature by NaOH titration in a thermostated cell.

3.2. Kinetic studies

The hydrolysis of phenyl acetate was followed by spectrophotometry as described previously [13]. At each temperature (10 °C–40 °C), a set of progress curves was recorded for different initial substrate concentrations (0.1–10 mM) by measuring the absorbance of the reaction product (phenol) as a function of time. For each curve, the initial rate v_0 was computed from the extinction coefficient of phenol, determined at the same temperature. The kinetic parameters V_{max} and K_m were estimated from the variation of the initial rate v_0 with substrate concentration $[S]_0$, according to eq. (3). The catalytic constant k_{cat} was computed from eq. (4), using a final enzyme concentration of 8.5 nM. Enthalpy and entropy for substrate binding, as well as activation enthalpy and entropy, were estimated from the linear least squares fit of eqs. (5) and (6) to the K_m and k_{cat} data, and were further refined by fitting eq. (7) to the whole data set of initial rates (65 points), using nonlinear regression.

The product inhibition constant K_p was determined by recording an additional progress curve at each temperature, with an initial substrate concentration equal to about two times the Michaelis constant at the same temperature. The integrated Michaelis equation (12) was fitted to each curve by nonlinear regression. The apparent K'_m value was used to compute the product inhibition constant K_p from eq. (10). Enthalpy and entropy for the binding of the reaction product phenol were determined by fitting eq. (5) with K_p instead of K_m.

An additional experiment to determine K_p was carried out at 25 °C by measuring the initial reaction rates at several substrate concentrations (0.25–4 mM) in the presence of added phenol (0.2–0.6 mM). The phenol concentration was limited to avoid an excessive increase of the initial absorbance. The results were analysed according to eq. (13).

4. Results

4.1. Initial rate analysis

The initial rates (v_0) of phenyl acetate hydrolysis are plotted in Fig. 1 as a function of initial substrate concentration ($[S]_0$) at several temperatures from 10 °C to 40 °C. Inspection of the graph revealed that both V_{max} and K_m increased with temperature (K_m is the

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