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THE ANALYSIS OF KINETIC DATA FOR REGULATORY ENZYMES AS A FUNCTION OF pH

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Summary

A new method is described for the analysis of kinetic data for regulatory enzymes as a function of pH. The method makes use of the limiting hyperbolae predicted by the exponential model for a regulatory enzyme. The pH dependence of the parameters that describe these hyperbolae can be analysed with the Michaelis pH functions to give the pK values of the enzyme-substrate complex and those of the free enzyme in its limiting conformational states. The procedure is illustrated by application to data for the effect of pH on the kinetic properties of aspartate transcarbamylase (carbamoylphosphate: L-aspartate carbamoyltransferase, EC 2.1.3.2).

Introduction

The effects of pH on those enzymes the reaction velocity of which can be described by the Michaelis-Menten equation have been well documented in the literature. In many cases the dependence of the maximal velocity V and the Michaelis constant K_m on pH can be explained by assuming that the activity of each enzyme form is determined by the ionization of two groups which are operationally linked to the active site; the active form of the enzyme being the half-ionized species [1—3]. The pK values of groups involved in substrate binding and catalysis can be determined by graphical methods [4,5] or by non-linear regression [6].

Unfortunately, there is no comparable theory that describes the effects of pH on the behaviour of regulatory enzymes. The kinetic properties of these

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enzymes are usually represented by the Hill equation:

$$v = \frac{VA^{\lambda}}{S_{0.5} + A^{\lambda}} \tag{1}$$

Where A is the substrate concentration, $S_{0.5}$ the apparent dissociation constant at half-saturation, λ is the Hill coefficient and V the maximum velocity.

However, no simple expressions exist that describe the Hill parameters $S_{0.5}$ and λ as functions of pH. Consequently, the physical meaning of the pK values estimated from plots of $S_{0.5}$ and λ against pH is obscure and the identification of specific residues which may be implicated in cooperativity is uncertain.

Alternatively, the behaviour of regulatory enzymes may be described by the exponential model [7]. The properties of the exponential model equation make it particularly suitable for the analysis of kinetic data for regulatory enzymes as a function of pH; the pH dependence of the exponential model parameters being given by analogy with the Michaelis pH functions.

The purpose of this paper, therefore, is to describe the application of the exponential model to the study of pH effects on regulatory enzymes. The procedure is illustrated by application to data from the literature for the effect of pH on the kinetic properties of aspartate transcarbamylase.

The exponential model

The exponential model [7,8] assumes that the initial velocity of the enzyme-catalysed reaction may be represented by an equation of the form of the Michaelis-Menten equation:

$$v = \frac{VA\alpha_i}{1 + A\alpha_i} = Vp_a \tag{2}$$

The association binding constant α_i is defined for a particular value of the fractional saturation $p_A = i$ as:

$$\alpha_i = \alpha_0 \text{ exp. } kp_A \tag{3}$$

Here, k is the interaction free energy in units of RT and α_0 is the association binding constant that characterizes the original state of the enzyme. Combining Eqns. 2 and 3 yields the exponential model equation:

$$v = \frac{VA\alpha_0 \text{ exp. } kp_A}{1 + A\alpha_0 \text{ exp. } kp_A} \tag{4}$$

Eqn. 4 predicts two limiting hyperbolae as p_A approaches its limiting values of 0 and 1 defined by:

$$v = \frac{VA\alpha_0}{1 + A\alpha_0} \tag{5}$$

lim. $p_A \rightarrow 0$

and

$$v = \frac{VA\alpha_1}{1 + A\alpha_1} \tag{6}$$

lim. $p_A \rightarrow 1$

where $\alpha_1 = \alpha_0$ exp. k.

The pH dependence of the exponential model parameters

Enzyme activity typically displays a bell-shaped profile as a function of pH. Behaviour of this type, for enzymes displaying hyperbolic kinetics can be simply explained by assuming that the enzyme exists in three states of ionization, i.e., EAH_2^+, EAH and EA^-, of which only the half-ionized species EAH is active [1,2].

The ionizations of a given species, say the enzyme-substrate complex (EA), are described by the following equilibria:

$$EAH_2^+ \underset{K_{EA,1}}{\rightleftharpoons} EAH \underset{K_{EA,2}}{\rightleftharpoons} EA^- \tag{7}$$

where $K_{EA,1}$ and $K_{EA,2}$ are ionization constants of the enzyme-substrate complex. The fractional concentration ϕ of the active half-ionized form is then given by:

$$\phi_{EA} = \frac{EAH}{EA_t} = \left[\frac{H}{K_{EA,1}} + 1 + \frac{K_{EA,2}}{H}\right]^{-1} \tag{8}$$

In a similar manner we can obtain an expression for the fractional concentration of the half-ionized form of the free enzyme, EH. However, when considering a regulatory enzyme it is necessary to distinguish between the different conformational states of the free enzyme. For this purpose we can make use of the limiting behaviour predicted by the exponential model and examine the pH dependence of the parameters that describe the limiting hyperbolae given in Eqns. 5 and 6.

Hence we have:

$$\phi_E^0 = \left[\frac{H}{K_{E_0,1}} + 1 + \frac{K_{E_0,2}}{H}\right]^{-1} \tag{9}$$

and

$$\phi_E^1 = \left[\frac{H}{K_{E_1 1}} + 1 + \frac{K_{E_1 2}}{H}\right]^{-1} \tag{10}$$

ϕ_E^0 and ϕ_E^1 are the fractional concentrations of the half-ionized forms of the free enzyme in the conformations, α_0 and α_1, characteristic of the unbound and fully-bound forms, respectively. The ionization constants, K_{E_0} and K_{E_1}, that enter into the definitions of ϕ_E^0 and ϕ_E^1 correspondingly refer to the unbound and fully-bound conformations.

It should also be noted that the substrate itself may ionize over the range of pH values that are employed. The fractional concentration of the substrate form that binds to the enzyme is then given by an equation analogous to Eqns. 8, 9 and 10; i.e., for AH we have:

$$\phi_A = \frac{AH}{A_t} = \left[\frac{H+1}{K_{A,1}} + \frac{K_{A,2}}{H}\right]^{-1} \tag{11}$$

The parameters V, α_0 and α_1 can then be expressed as functions of pH in a manner identical to that employed for the Michaelis-Menten parameters [3,9]:

$$V = \overline{V} \cdot \phi_{EA} \tag{12}$$

$$\alpha_0 = \bar{\alpha}_0 \cdot \frac{\phi_E^0 \phi_A}{\phi_{EA}} \tag{13}$$

and

$$\alpha_1 = \bar{\alpha}_1 \cdot \frac{\phi_E^1 \phi_A}{\phi_{EA}} \tag{14}$$

These equations depend on the assumption that the maximum velocity does not change with saturation by A, at constant pH, and therefore that substrate-bound active sites have a single conformation described by one set of ionization constants. Here, $\bar{V}$ is the specific maximum velocity of the half-ionized form and $\bar{\alpha}_0$ and $\bar{\alpha}_1$ are the specific association constants of the half-ionized form in the conformations characteristic of the unbound and fully-bound enzyme, respectively.

The pK values of the enzyme-substrate complex can be determined directly from an analysis of V as a function of pH. However, it will be observed that α_0 and α_1 are more complex functions of pH that involve the ionizations of the free enzyme and substrate, and the enzyme-substrate complex. The ionization of the substrate is generally known or can be determined and hence ϕ_A may be calculated as a function of pH. The effects of ionizations of the enzyme-substrate complex EA on α_0 and α_1 can be removed by combining Eqns. 13 and 14 with Eqn 12.

Hence we have:

$$F_0 = \frac{V\alpha_0}{\phi_A} = \bar{V}\bar{\alpha}_0\phi_E^0 \tag{15}$$

and

$$F_1 = \frac{V\alpha_1}{\phi_A} = \bar{V}\bar{\alpha}_1\phi_E^1 \tag{16}$$

F_0 and F_1 are proportional to the fraction of the enzyme in unbound and fully-bound conformations that are in the half-ionized state, respectively. The pK values of these free enzyme species can now be determined from an analysis of F_0 and F_1 as functions of pH.

Finally, the interaction free energy, k as a function of pH is given by:

$$\text{exp. } k = \frac{\bar{\alpha}_1}{\bar{\alpha}_0} \cdot \frac{\phi_E^1}{\phi_E^0} \tag{17}$$

The pH dependence of the reaction catalyzed by aspartate transcarbamylase

The velocity of the reaction catalysed by aspartate transcarbamylase as a function of L-aspartate concentration has been determined at a number of different pH values [10]. Values of the Hill coefficient λ and the substrate concentration at half-saturation $S_{0.5}$ as a function of pH, (given in Table II of Ref. 10) were used to obtain approximate values of the exponential model parameters k and α_0.

The relationship between the interaction free energy, k and the Hill coefficient, λ [11,7] is given by:

$$k = 4(\lambda - 1)/\lambda, \tag{18}$$

TABLE I

The kinetic parameters for the reaction catalysed by aspartate transcarbamylase as a function of pH. Carbamyl phosphate was maintained at a saturating concentration throughout.

pH	$S_{0.5}$ (mM)	λ	$\ln \alpha_0$ ($\ln mM^{-1}$)	k	$V \cdot 10^{-3}$ (mol h^{-1} mg^{-1})	F_0	F_1
6.1	7.0	1.01	−1.97	0.04	2.01	0.281	0.293
6.6	6.7	1.11	−2.10	0.40	3.00	0.366	0.549
7.1	6.1	1.36	−2.34	1.06	6.57	0.637	1.826
7.6	5.9	1.61	−2.52	1.52	10.39	0.821	3.76
7.8	7.7	1.88	−2.98	1.87	17.00	0.867	5.63
8.3	10.1	1.96	−3.30	1.97	29.5	1.092	7.818
9.1	14.0	1.96	−3.62	1.96	30.00	0.810	5.70
9.7	15.2	1.82	−3.62	1.80	27.58	0.745	4.468
10.0	14.9	1.39	−3.26	1.12	26.01	0.988	3.069
10.7	13.2	1.07	−2.71	0.26	4.85	0.325	0.417

$\ln \alpha_0$ is given by:

$$\ln \alpha_0 = -(\ln S_{0.5} + k/2) \tag{19}$$

Values of V were used as given in Table II of Ref 10. These data were used to calculate values of F_0 and F_1 according to Eqns. 15 and 16 in which ϕ_A for aspartate was set equal to 1 [10]. The kinetic parameters so obtained are listed in Table I. The values of V, F_0 and F_1 were analysed as functions of pH by a non-linear regression procedure [6]. The computed curves together with the data points are shown in Fig. 1 and the resulting pK values are listed in Table II.

It is apparent that the pK values for the free enzyme are significantly shifted as the conformational state of the enzyme varies from α_0 to α_1. $pK_{E,1}$ is shifted

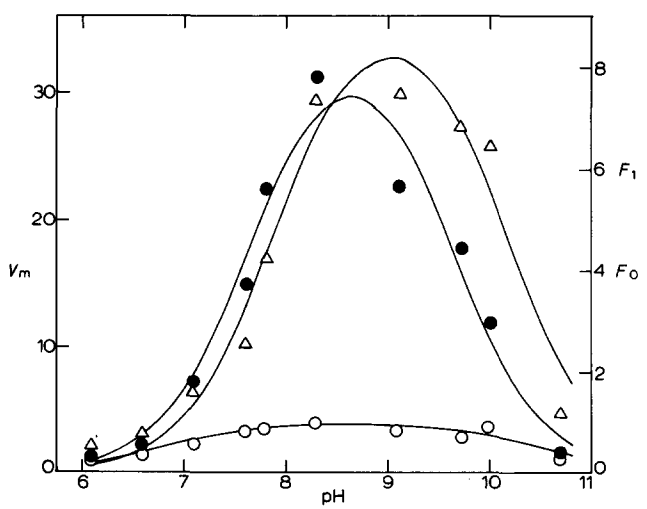

Fig. 1. The effect of pH on the reaction catalysed by aspartate transcarbamylase; the parameters V (△——△), F_1 (●——●) and F_0 (○——○) were analysed as functions of pH according to Eqns. 12, 16 and 15, respectively. The curves are calculated with the pK values given in Table II together with the values: $\overline{V} = 37.32 \pm 3.31$, $\overline{V\alpha_1} = 8.92 \pm 0.89$ and $\overline{V\alpha_0} = 0.97 \pm 0.07$.

TABLE II

THE pK VALUES FOR ASPARTATE TRANSCARBAMYLASE DETERMINED FROM THE ANALYSIS OF V, F_0 AND F_1 AS FUNCTIONS OF pH.

Enzyme species	pK_1	pK_2
EA (V)	7.87 ± 0.11	10.18 ± 0.14
E_1 (F_1)	7.64 ± 0.12	9.63 ± 0.12
E_0 (F_0)	6.76 ± 0.18	10.57 ± 0.21

by +0.88 ± 0.22 pH units, while $pK_{E,2}$ is shifted by −0.94 ± 0.24 pH units. These shifts reflect changes in the local environment of ionizable groups within the active site. It is interesting that the shifts in the pK values observed here very nearly compensate each other, so that the maximum in the pH response appears at the same pH value for both conformational forms:

$$pH_{max} = \frac{pK_1 + pK_2}{2} = 8.65 .$$

Formally, at least, these results may be explained by assuming that the conformational changes that accompany the binding of aspartate to the enzyme result in a redistribution of charge between the ionizable groups. For the scheme shown below:

$$\begin{bmatrix} XH \\ e^- \\ YH^+ \end{bmatrix} \overset{pK_1}{\rightleftharpoons} \begin{bmatrix} X^- \\ e^- \\ YH^+ \end{bmatrix} \overset{pK_2}{\rightleftharpoons} \begin{bmatrix} X^- \\ e^- \\ Y \end{bmatrix}$$

the withdrawal of electrons from a basic group Y to an acidic group X would result in a decrease in the value of pK_2 and an increase in the value of pK_1. It is apparent that the analytical approach described here is able to provide parameter values the physical significance of which can be readily appreciated and interpreted in terms of changes in the electronic environment caused by conformational changes.

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