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Laboratory Exercises

Optimization of Kinetic Parameters of Enzymes*

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Enzymes are a product of evolution, having achieved a high specificity and catalytic performance by means of natural selection. Because catalytic mechanisms are very complex, a detailed and rigorous analysis of their optimization functions, as it has been developed in our previous work, is rather difficult for teaching purposes. In this paper we present a summarized and simplified view of our results by means of analyzing the optimization of kinetic parameters of a two-step enzyme mechanism under a set of several constraints. This analysis is presented in a way that makes it useful for teaching, as students can understand it easily, make the calculations, and even develop it by exploring new environmental conditions. The analysis of the optimization processes is presented through two calculation tools, analysis and computer programming.

In the last 15 years evidence has been accumulated that natural selection has worked at the molecular level to produce highly optimized structures in metabolic design [1–5]. Metabolism is specifically different from the whole set of chemical reactions that can occur among materials on earth, because life chemistry is a specifically and highly efficient catalyzed chemistry. It is clear, therefore, that a basic fact in biological evolution was the selection and improvement of enzymes as catalytic tools. This subject has been studied in depth by Heinrich and co-workers [6–9]; see also Refs. 10 and 11. Heinrich and Hoffmann [7] stated that, in contrast to previous assumptions of a number of authors, states of maximal activity are not always those with the highest possible values of the affinity constant. On the contrary, the functional dependence that relates the velocity of an enzymatic reaction with the parameters (rate constants and concentrations of the reactants) is a complex optimizable function that has several maxima depending on the concentration of the substrates and products; different specific sets of parameter values account for states of maximal activity, and so it is normally expected that one of them has been achieved in each enzyme as a result of natural selection.

The key of the optimization function is that the rate constants cannot have any value, for two reasons, the chemical reaction itself and the catalytic mechanism. Let us consider the simple enzymatic mechanism depicted in Fig. 1. The equilibrium constant is fixed, as it is a thermodynamic constraint that depends on the nature of substrates and products of the reaction but not on the properties of the catalyst. This reduces the possible rate constants to values fulfilling the condition $(k_1 \cdot k_2)/(k_{-1} \cdot k_{-2}) = \text{constant}$. On the other hand k_1 (the binding rate constant) and k_2, the catalysis rate constant (also called k_{cat}), cannot have any value as they depend on the chemical possibilities of the enzyme to bind the substrate and to catalyze the reaction, which must obviously have upper limits. Thus, although the mechanism of evolution and natural selection can be extremely efficient, the velocity of the catalyzed reaction cannot be made infinite.

An accurate study of natural selection requires a rigorous analysis of optimization functions that operate in the evolution of biological structures. Such functions are usually very complex because of the high number of variables involved in any biological structure. Therefore there are only a few examples where the operation of natural selection can be demonstrated in a simple and clear way. In this paper we present a case that we think fulfills these needs. It deals with the optimization of the affinity constant of an enzyme to achieve maximal activity under given substrate and product concentrations. This is also a good opportunity to demonstrate an example of an optimizable function, whose physical and chemical meaning is easily understood, as enzyme mechanisms are familiar to teachers and students of biochemistry.

Here, this concept is presented as a practical exercise in which students can calculate the optimized states that arise from exploring a broad number of cases. We also describe a computer program that calculates steady states in enzyme-catalyzed reactions. The program can be useful in introducing students to numerical integration techniques and computer programming.

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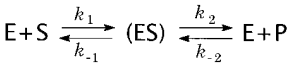

FIG. 1. **Two-step mechanism of an enzymatic reaction on which the analysis presented in this work has been made.**

DESCRIPTION AND DEVELOPMENT OF THE MODEL

The simplest model of an enzyme-catalyzed reaction mechanism is given in Fig. 1. The mechanism consists of two steps; the first one is the binding of enzyme to substrate, and the second one includes both the catalysis of the reaction and the release of the product. The binding of the substrate to the enzyme is determined by the affinity constant $K_A = k_1/k_{-1}$; the Michaelis constants are $K_S = (k_2 + k_{-1})/k_1$ for the substrate and $K_P = (k_2 + k_{-1})/k_{-2}$ for the product. The maximum velocity for the forward reaction is $V_{max} = k_2 e_T$, where e_T is the total amount of enzyme, and k_2 is the catalytic rate constant, which is also called k_{cat}.

Now let us study the effect of changing the values of certain rate constants to show that the reaction velocity is an optimizable quantity. The model shown in Fig. 1 is really much more complicated than it appears to be, because it involves six independent parameters, four rate constants and S and P concentrations, or five if it deals with a chemical reaction that has a fixed equilibrium constant. Thus, to present a simple case that can be analyzed easily, let us fix the values of some parameters in addition to K_{eq}, say s and p (the substrate and product concentrations), k_1 (which is the rate constant of enzyme-substrate binding), and k_2 (which involves the catalytic and product release constants). This choice has a clear chemical meaning, as both the binding of the enzyme to the substrate and the catalysis rate have a limit imposed by chemical constraints of the active site and substrate structure. Thus, the evolution of the enzyme toward an optimum set of values of rate constants can be summarized as follows.

Let us imagine a hypothetical sequence of evolutionary steps for an enzyme with fixed values of k_1 and k_2 (see Fig. 2). In a first stage the enzyme has a very low affinity for the substrate, yielding obviously a very low reaction rate, and then evolution and natural selection work thereafter, improving the affinity of the enzyme for its substrate, by decreasing k_{-1}. Because the equilibrium constant of the reaction $K_{eq} = (k_1 \cdot k_2)/(k_{-1} \cdot k_{-2})$ is fixed, as are k_1 and k_2 in this case, the decrease of k_{-1} must be accompanied by an increase of k_{-2}. This last effect is, in principle, unfavorable to the whole activity, but because the former stage yielded a very low reaction rate the positive effect of a decrease in k_{-1} is more important than the negative one of an increase in k_{-2}, and so the global result is that the second stage (Fig. 2) has a net increase in the reaction velocity. Now let us imagine that the affinity is additionally increased by further decreases of k_{-1}, again involving a corresponding increase of k_{-2}. At this stage the affinity of the enzyme for its substrate has been greatly increased, but instead of enhancing the reaction rate, it now decreases the catalytic efficiency. The price one has to pay for such a high affinity for the substrate is a high affinity for the product, as well; the enzyme tightly binds the substrate but also tightly binds the product. Because the product is only slowly released, the system now exhibits a poor net reaction velocity. This simple reasoning demonstrates that

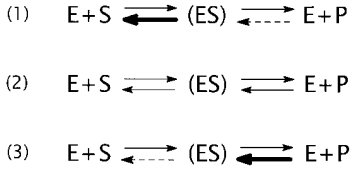

FIG. 2. **Three hypothetical evolutionary stages for optimizing the activity of the enzyme mechanism depicted in Fig. 1.** *1*, low affinities of the substrate and the product for the enzyme (high k_{-1}, low k_{-2}); *2*, intermediate values of the two affinities; 3, high affinities of the substrate and the product to the enzyme.

the value of the affinity constant has an optimum value to get the maximal activity of the enzyme. In other words, the mathematical function that relates the reaction velocity with the set of rate constant values of the enzyme is an optimizable function and so a clear target of natural selection.

Analytical Calculation

This sequence of evolutionary steps described above can be followed in detail mathematically making it possible to study the weight of each parameter on the global result. This can be done by two approaches. Here we present both, because we think that each has a different educational value. The first approach is an analytical procedure. The Michaelis-Menten equation for an enzyme reaction at steady state with a given concentration of the product is represented by Equation 1, as described by Alberty [12].

$$v = \frac{e_T(k_1k_2s - k_{-1}k_{-2}p)}{k_{-1} + k_2 + sk_1 + pk_{-2}} \qquad \text{(Eq. 1)}$$

According to the above-stated definitions, this equation is easily transformed to that represented by Equation 2, which is the general Michaelis-Menten equation for a reversible enzymatic reaction under conditions of non-initial velocity ($p > 0$).

$$v = \frac{\frac{V_{max}}{K_S}\left(s - \frac{p}{K_{eq}}\right)}{1 + \frac{s}{K_S} + \frac{p}{K_P}} \qquad \text{(Eq. 2)}$$

It is easy to see that under initial velocity conditions (*i.e.* $p = 0$) Equation 2 is reduced to that represented by Equation 3.

$$v = \frac{V_{max}s}{K_S + s} \qquad \text{(Eq. 3)}$$

For given values of s, p, k_1, and k_2, Equation 1 can be rewritten for studying the dependence of v on s, p, and K_A as follows in Equation 4.

$$v = \frac{e_T\left(s - \frac{p}{K_{eq}}\right)}{\frac{1}{K_Ak_2} + \frac{1}{k_1} + \frac{s}{k_2} + \frac{pK_A}{k_1K_{eq}}} \qquad \text{(Eq. 4)}$$

This formula can be studied analytically with a calculus program, such as *Mathematica* [13] or *Microsoft Excel* [14], by giving values to each parameter and plotting the results

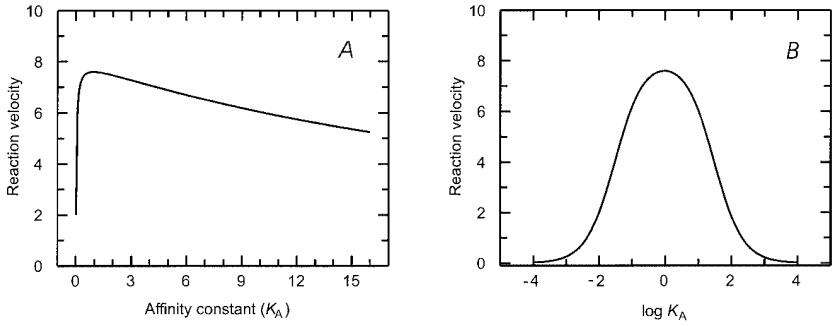

FIG. 3. *A*, plot showing the relationship between the reaction velocity *v* and the affinity constant for the substrate K_A. The maximum shows that it is an optimizable function. *B*, the same dependence using a logarithmic scale for K_A, to better show the maxima. Parameter values, $k_1 = 3$, $k_2 = 9$, $K_{eq} = 27$, $s = 30$, $p = 10$.

of the velocity *v versus* K_A; see first appendix. In a similar way, one can plot the variation of *v* at changing k_{cat}/K_M, etc. It must be remembered that the equilibrium constant is fixed, so if one is studying the influence of a certain number of parameters on a same reaction, all sets of values for the elementary rate constants must obey this condition. Of course, the global value of K_{eq} can also be varied if one wants to observe the effect of the equilibrium constant on the velocity of the reaction.

Computer Simulation

The same problem can be studied by means of computer simulations without using an explicit expression for the rate equation. This is a very powerful approach and may be the only possible one for more complicated cases, *e.g.* for reactions with more than one substrate or product. Here a value of the reaction rate that corresponds to each K_A value is obtained by means of calculating it according to the formula represented by Equation 5.

$$v = k_2 \cdot (es) - k_{-2} \cdot e \cdot p \qquad \text{(Eq. 5)}$$

The time-dependent changes of the concentration of the enzyme-substrate complex is determined by the differential equation, shown in Equation 6,

$$\frac{d(es)}{dt} = k_1 \cdot e \cdot s + k_{-2} \cdot e \cdot p - (es) \cdot (k_{-1} + k_2) \qquad \text{(Eq. 6)}$$

where the concentration of the free enzyme is shown in Equation 7.

$$e = e_T - (es) \qquad \text{(Eq. 7)}$$

Using Equations 6 and 7 we may calculate the variation of the concentration of the enzyme-substrate complex, which increases from the starting time until the steady state is reached when it becomes constant.

The differential equation (Equation 6) can be solved by numerical integration, for example by using the simplest technique to do so, namely Euler's method. Equation 6 can be approximated by the following difference equation, Equation 8.

$$\Delta(es) = \Delta t \cdot \{k_1 \cdot [e_T - (es)] \cdot s + k_{-2} \cdot [e_T - (es)] \cdot p - (es) \cdot (k_{-1} + k_2)\} \qquad \text{(Eq. 8)}$$

Now the differential progress of the reaction, described by Equation 6 (the increase of (*es*)), with time can be followed by calculating Equation 8, taking for Δt very small quantities and then recalculating the reaction rate *v* with Equation 5 with the new values of (*es*). The first calculation yields a certain value of $\Delta(es)$, obviously very small, as is Δt. After each step we adjust the value of (*es*) by adding this increase to its former value, represented by Equation 9,

$$es + \Delta(es) \rightarrow (es) \qquad \text{(Eq. 9)}$$

and recalculate the concentration of the free enzyme and the reaction velocity according to Equations 5 and 7. The repeated application of this algorithm gives us the variation of (*es*) with time. The procedure is followed until (*es*) approaches its steady state. This calculation becomes more accurate by reducing Δt to smaller values. In the example given in the second appendix we have used $\Delta t = 0.001$, which is small enough for the present case, but it might be necessary to use a lower value in calculations involving more complicated systems. The procedure obviously involves a large number of calculations, which, 250 years ago, Euler had to carry out by hand to resolve systems of differential equations that had no explicit solution. Fortunately, we now have computers that can do it so rapidly. The second appendix shows a program written in *Quick BASIC* [15] to calculate the steady state values in a range of K_A. It stores the results in a file to be processed later by any graphical plotting software, and it can also plot them on a computer screen for a quick view. A broad range of parameter values can be explored by changing the corresponding parameter values in the program.

RESULTS AND DISCUSSION

Fig. 3*A* shows the influence of the affinity constant K_A on the reaction velocity *v* at steady state. The plot has a maximum, which means that such a relationship is an optimizable function *i.e.* either very low or very high values of K_A are unfavorable for enzyme activity, as it is indicated qualitatively in Fig. 2. Fig. 3*B* shows the same results plotted on a logarithmic scale, which provides a clearer indication of the optimization function. These results have been obtained both by the analytical method using Equation 4 (see first appendix) and by computer simulation with the program shown in the second appendix.

Fig. 4 shows the effect of product concentration on the reaction rate *v* as a function of K_A. It can be seen that higher product concentrations produce a sharper maximum of the curve, whereas for lower *p* values the maximum is extended to a plateau. If the concentration of the product is zero there is no local maximum. This effect indicates well that the optimization function depends strongly on the concentrations of the reactants. We can conclude that each enzyme in metabolism has to be opti-

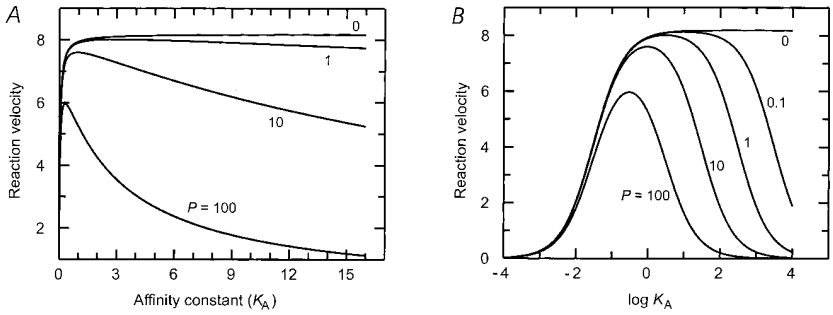

FIG. 4. **Effect of the product concentration on the relationship between reaction velocity and affinity constant.** *A*, reaction velocity *v versus* the affinity constant K_A; *B*, reaction velocity *versus* logK_A. Other parameter values are the same as those given in Fig. 3.

mized according to its specific environmental conditions, and so, the recognition of an optimized state in an enzyme requires the knowledge of concentration range of its reactant under which the enzyme usually functions; a detailed study of these relationships can be seen in the work of Heinrich and co-workers [6–9].

Considering these results, one can understand that for enzymes that have to function under very different concentrations of substrates and/or products, which is the usual case in metabolism, a unique optimized set of rate constants on a single enzyme to satisfy such requirements would be virtually impossible. Rather, a need can be met by duplicating the enzyme and optimizing each one for its particular conditions. This seems to be the reason behind the existence of isoenzymes.

The maximization of the ratio V_{max}/K_S (or k_{cat}/K_S) has been considered as an index of catalytic efficiency, and it has been claimed frequently to be the evolutionary target of enzyme activity with its only upper limit being the rate of diffusion in aqueous solution, for examples see Refs. 16 and 17. The basis of this statement is that when substrate concentration is in the same range of K_M, or lower, which is the normal situation in the cell, Equation 3 can be written as shown in Equation 10,

$$v = \frac{k_{cat}}{K_S} \cdot Q \qquad \text{(Eq. 10)}$$

with $Q = (e_T \cdot s)/2$ for $s \approx K_S$, or $Q = e_T \cdot s$ for $s \ll K_S$. This reasoning is, however, only valid for conditions of initial velocity ($p = 0$), which is clearly not the typical situation in metabolism. Equation 3 is very useful in determining the values of K_S and V_{max} in the laboratory, but it cannot be used to describe the behavior of an enzyme that is embedded in a chain of reactions, as there are no initial velocities there. Considering Equation 2, it is clear that the ratio k_{cat}/K_S has a more complex meaning, and the effect of its variation on the enzyme activity can also be explored by means of the approaches we have presented here. The results of this analysis are shown in Fig. 5. They demonstrate that under conditions where $p > 0$ this relationship is also an optimizable function that has a maximum for a certain value of k_{cat}/K_S. Thus, the target of natural selection on the enzyme parameters cannot be to maximize the ratio k_{cat}/K_S but to find its precise value to maximize the enzyme activity. The curves depicted in Fig. 5 demonstrate that, at fixed k_{cat}, maximization of the enzyme activity results in optimal K_S. More detailed conclusions concerning optimal K_S values as a consequence of the evolutionary optimization of elementary rate constants of enzyme

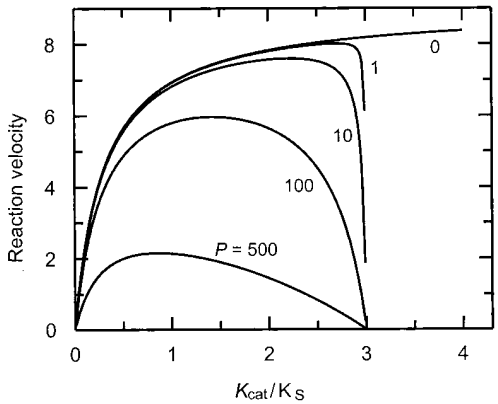

FIG. 5. **Reaction velocity *v versus* k_{cat}/K_S for different values of the product concentration.** This plot shows that this is also an optimizable function when the system functions under conditions of non-initial velocity, *i.e.* $p > 0$. Other parameter values are same as those given in Fig. 3.

mechanisms are described in Refs. 6–9.

The general problem of optimizing the rate constants of enzymes to achieve maximal activity is nevertheless much more complicated than what this short view can consider. The full theory is explained in detail in our previous work including more complicated cases, such as reactions with more than one substrate. Papers [6–9] and also a textbook [18] have discussions on the degree of optimization of some real enzymes with detailed investigations of the enzymes inorganic pyrophosphatase (EC 3.6.1.1) and triosephosphate isomerase (EC 5.3.1.1.).

APPENDIX

Program for Mathematica [13] to Study Analytically the Relationship between Reaction Rate and the Affinity Constant K_A.

The results of these calculations are shown in Fig. 3.

```
Clear ["Global'*"];
s = 30;
p = 10;
et = 1;
k1 = 3;
k2 = 9;
Keq = 27;
Plot [et * (s − p/Keq)/(1/(kA * k2) + 1/k1 + s/k2 + p * kA/(k1 * Keq)),
)kA, 0.01, 16*, PlotRange → )0, 10*, Frame → true];
Plot [et * (s − p/Keq)/(1/10[caret]a * k2) + 1/k1 + s/k2 + p * (10[caret]a)/(k1 * Keq)),
)a, −4, 4*, PlotRange → )0, 10*, Frame → true];
```

Computer Program Written in Quick BASIC [15] to Study the Relationship between Reaction Rate and the Affinity Constant K_A by Means of Numerical Integration of Elementary Rate Equations (See Equations 5–9 in the Text).

The results of these calculations are shown in Fig. 3*A*.

Initialization

```
CLEAR
CLS
SCREEN 12−
s = 30: p = 10: et = 1: e = et − es
k1 = 3: k2 = 9
dt = 0.001
DIM x(100): DIM y(100): DIM a(100): DIM b(100)
ymin = 10000
```

Calculation

```
FOR kr1 = 20 TO 5 STEP −1: REM Ka = 0.15 − 0.6
GOSUB StationaryState
NEXT kr1
FOR kr1 = 4.8 TO 0.8 STEP −0.3: REM Ka = 0.63 − 3.75
GOSUB StationaryState
NEXT kr1
FOR kr1 = 0.7 TO 0.1 STEP −0.1: REM Ka = 4.29 − 30
GOSUB StationaryState
NEXT kr1
LOCATE 21, 20: INPUT "Do you want to plot the graphic (y/n)"; graf$
IF graf$ = "y" OR graf$ = "Y" THEN GOTO Graphic
END
```

StationaryState

```
kr2 = 1/kr1
ka = INT(1000 * k1/kr1)/1000
FOR i = 1 TO 1000
des = dt * (k1 * e * s + kr2 * e * p − es * (kr1 + k2))
es = es + des
e = et − es
v = k2 * es − kr2 * e * p
REM Stationary State condition
IF des < 0.001 THEN
count = count + 1
x(count) = ka
IF x(count) > xmax THEN xmax = x(count)
y(count) = v
IF y(count) > ymax THEN ymax = y(count)
IF y(count) < ymin THEN ymin = y(count)
IF count < 19 THEN y = count ELSE y = count - 18:
x = 35
LOCATE y, 5 + x
PRINT "Ka = v = "
LOCATE y, 10 + x
PRINT USING "##.###"; ka
LOCATE y, 23 + x
PRINT USING "###.####"; v
RETURN
END IF
NEXT i
END
```

Graphic

```
xmult = xmax/630
ymult = (ymax − ymin)/460
FOR i = 1 TO count
a(i) = x(i)/xmult
b(i) = 470 + 2.05 * ymin − (y(i)/ymult)
PALETTE 0, 4144959
COLOR 1
PSET (a(i), b(i))
NEXT i
BEEP: BEEP
DO
LOOP WHILE INKEY$ = ""
```

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