Spatial order as a source of kinetic cooperativity in organized bound enzyme systems

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ABSTRACT When enzyme molecules are distributed within a negatively charged matrix, the kinetics of the conversion of a negatively charged substrate into a product depends on the organization of fixed charges and bound enzyme molecules. Organization is taken to mean the existence of macroscopic heterogeneity in the distribution of fixed charge density, or of bound enzyme density, or of both. The degree of organization is quantitatively expressed by the monovariate moments of charge and enzyme distributions as well as by the bivariate moments of these two distributions.

The overall reaction rate of the bound enzyme system may be expressed in terms of the monovariate moments of the charge density and of the bivariate moments of charge and enzyme densities. The monovariate moments of enzyme density do not affect the reaction rate.

With respect to the situation where the fixed charges and enzyme molecules are randomly distributed in the matrix, the molecular organization, as expressed by these two types of moments, generates an increase or decrease of the overall reaction rate as well as a cooperativity of the kinetic response of the system. Thus both the alteration of the rate and the modulation of cooperativity are the consequence of a spatial organization of charges with respect to the enzyme molecules.

The rate equations have been derived for different types of organization of fixed charges and enzyme molecules, namely, clustered charges and homogeneously distributed enzyme molecules, clustered enzyme molecules and homogeneously distributed charges, clusters of charges and clusters of enzymes that partly overlap, and clusters of enzymes and clusters of charges that are exactly superimposed. Computer simulations of these equations show how spatial molecular organization may modulate the overall reaction rate.

INTRODUCTION

When enzyme molecules are embedded in a polyelectrolyte matrix the apparent kinetic properties of the bound enzyme are changed with respect to what these properties would be if the enzyme were free in solution. Diffusional resistances of the substrate and of the product may be a cause of this change of kinetic properties. Another cause of kinetic alterations is the existence of electrostatic interaction effects between the fixed charges of the matrix and the mobile charges of the solute. If the substrate is an ion, electrostatic repulsion between the fixed charges of the matrix and the mobile charges of the substrate mimics positive cooperativity, whereas electrostatic attraction mimics negative cooperativity. These effects are observed even with monomeric one-sited enzymes and are modulated by ionic strength (Engasser and Horvath, 1975; Ricard et al.; 1981; Ricard, 1987). Another type of alteration of the kinetic behavior of the bound enzyme is a shift of the pH-profile of the enzyme toward high or low pH values (Engasser and Horvath, 1974*a–d*).

These effects have been observed experimentally with enzymes cross-linked to polyanionic or polycationic resins, but also occur with enzymes bound to cell membranes. cell walls, or polynucleotides (Douzou and Maurel, 1977*a* and *b*; Maurel and Douzou, 1976). These electrostatic effects are thus likely to control the behavior of the enzymes in the living cell.

Physical theories developed so far allow one to explain roughly these effects but implicitly postulate that the enzyme molecules and the charges are homogeneously distributed within the matrix. This can be so only as a first approximation. There is experimental evidence, for instance, that this is not the case for acid phosphatase molecules buried in the plant cell walls (Ricard et al.; 1981; Crasnier et al.; 1985).

It is therefore important to develop a theory that allows one to understand how clustering and organization of fixed charges and enzyme molecules in a matrix may affect the overall reaction rate. More precisely one may wonder whether the overall enzyme reaction rate is unchanged when the same number of fixed charges and enzyme molecules are either randomly distributed within a unit volume of matrix, or clustered in that volume.

The aim of this paper is to offer the bases of such a

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theory. As biological membranes are polyanions and most of the charged substrates are anions, this theory will be restricted to electrostatic repulsion effects.

THEORY

1. Expression of the basic rate equation

Owing to the electrostatic repulsion of a mobile substrate anion by a polyanionic matrix, an electrostatic partition coefficient Π may be defined as

$$\Pi = \left(\frac{\gamma_o S_o}{\gamma_i S_i}\right)^{1/Z} = \exp(F\Delta\Psi/RT). \quad (1)$$

In this expression S_o and S_i are the bulk and the local substrate concentrations, γ_o and γ_i the corresponding activity coefficients, z is the valence of the substrate, F the Faraday constant, $\Delta\Psi$ the difference of electrostatic potential between the inside and the outside of the matrix and R and T have their usual significance. For a dilute solution of a monovalent substrate anion, this expression reduces to

$$\Pi = \frac{S_o}{S_i} = \exp(F\Delta\Psi/RT). \quad (2)$$

This electrostatic partition coefficient may be expressed in terms of bulk concentrations of mobile anions and of the fixed charge density, Δ. For the ideal case of the anionic substrate, S, and one cation, the electroneutrality equation assumes the form (Engasser and Horvath, 1975; Ricard et al., 1981)

$$\Pi^2 - \frac{\Delta}{S_o}\Pi - 1 = 0, \quad (3)$$

and the expression of Π is then equal to

$$\Pi = \frac{\Delta}{2S_o}\left\{1 + \sqrt{1 + \frac{4S_o^2}{\Delta^2}}\right\}. \quad (4)$$

For low ionic concentrations, one may write approximately,

$$\sqrt{1 + \frac{4S_o^2}{\Delta^2}} = 1 + \frac{2S_o^2}{\Delta^2}, \quad (5)$$

and the expression of the electrostatic partition coefficient is then

$$\Pi = \frac{\Delta^2 + S_o^2}{\Delta S_o}. \quad (6)$$

If the local charge density is much larger than the bulk substrate concentration, this expression reduces to

$$\Pi = \frac{\Delta}{S_o}. \quad (7)$$

Expressions 4, 6, and 7 show that the electrostatic partition coefficient declines as the bulk substrate concentration is increased.

It is important to define clearly at this stage what is meant by organization. The term of organization is taken to mean the lack of pure randomness in the spatial distribution of charges and enzyme molecules. Clustering of charges and clustering of enzyme molecules thus represents some form of spatial organization. Moreover organization may also involve the existence of a spatial correlation between the charge density and the enzyme density distributions. To express quantitatively how these different types of organization alter the overall rate equation of the bound enzyme system, one has to develop first the mathematical treatment of a simple and versatile model which postulates that the clusters of charges and the clusters of enzyme molecules are superimposed.

As we shall see later, the behavior of a bound enzyme system that comprises a large number of small clusters is indistinguishable from that of another system defined by a small number of large clusters, provided the charge and enzyme densities as well as the total volume of clusters be the same in the two cases. Therefore the concept of cluster may be applied to a spatial structure of a unit volume, whatever this structure is isolated in the matrix, or collected with other structures of similar density.

If there exists in the matrix N clusters of fixed negative charge density and enzyme molecules, the overall rate of conversion of a monovalent substrate assumes the form

$$v = \sum_i \sum_j \frac{f_{ij} V_j S_o}{K\Pi_i + S_o}. \quad (8)$$

In this expression f_{ij} is the frequency of clusters that have an electrostatic partition coefficient, Π_i, and a maximum reaction velocity (proportional to enzyme density), V_j; f_{ij} is thus a measure of the true frequency of spatial structures and altogether a measure of their volume. K is the K_m of the reaction. If, as assumed above, the matrix is a polyanion and the substrate a monovalent anion, Eq. 8 assumes the form

$$v = \sum_i \sum_j \frac{f_{ij} \frac{V_j \Delta_i}{K + \Delta_i} S_o^2}{\frac{K\Delta_i^2}{K + \Delta_i} + S_o^2}. \quad (9)$$

Δ_i is then the fixed charge density of cluster i. If the charge density of the clusters is much higher than the

bulk substrate concentration, one must have

$$\Delta_i \gg K \qquad (i = 1, \ldots, n). \tag{10}$$

and Eq. 10 takes an even more simple form, namely,

$$v = \sum_i \sum_j \frac{f_{ij} V_j S_o^2}{K\Delta_i + S_o^2}. \tag{11}$$

One may define a new variable, σ_0, which has the dimension of a concentration, as

$$\sigma_o = S_o^2/K, \tag{12}$$

and Eq. 11 becomes

$$v = \sum_i \sum_j \frac{f_{ij} V_j \sigma_o}{\Delta_i + \sigma_o}. \tag{13}$$

Under this form it becomes obvious that any departure from Michaelis-Menten behavior, or cooperativity, with respect to the new variable σ_0, cannot be due to electrostatic repulsion effects but to a spatial heterogeneity, or organization, of the fixed charges and of enzyme molecules in the matrix. One may show after some lengthy algebra that the second derivative, with respect of $1/\sigma_o$, of the reciprocal rate, $1/v$, is always negative. This means that the cooperativity with respect to σ_0 can only be negative. As a matter of fact Eq. 13 is formally equivalent to that which describes the activity of a mixture of different enzymes acting on the same substrate (Δ_i would then correspond to the K_m of the enzymes and σ_0 to the substrate concentration). Under these conditions it has been demonstrated (Dixon and Webb, 1979) that the apparent cooperativity can only be negative.

Eq. 13, however, is too complex to be of any practical value. It should be reexpressed under a different form to take specific account of the degree of spatial order of the fixed charges and enzyme molecules.

2. Statistical formulation of spatial order of fixed charges and enzyme molecules in the matrix

One may express the charge density in the clusters, Δ_i, relative to its mean $\langle\Delta\rangle$, as

$$\Delta_i = \langle\Delta\rangle + \delta_i \qquad (i = 1, \ldots, n). \tag{14}$$

Similarly the maximum reaction velocity in the clusters, proportional to the enzyme density, may be expressed with respect to the corresponding mean, namely,

$$V_j = \langle V\rangle + \epsilon_j \qquad (j = 1, \ldots, n). \tag{15}$$

In expressions 14 and 15, δ_i and ϵ_j are the deviations about the corresponding means $\langle\Delta\rangle$ and $\langle V\rangle$.

The degree of spatial order and organization of fixed charges and enzyme molecules may be expressed by the monovariate and bivariate moments of these two normal distributions. The monovariate moments, centered on the zero value, are either equal to zero or express the degree of dispersion of charge and enzyme densities within the matrix. This degree of dispersion of charge and enzyme densities is a measure of the independent organization of fixed charges and enzyme molecules in the matrix. These monovariate moments do not take account of the degree of organization of charges with respect to enzyme molecules.

One has

$$\sum_i \sum_j f_{ij}\delta_i = \sum_i f_i\delta_i = N\mu_1(\delta) = 0$$

$$\sum_i \sum_j f_{ij}\delta_i^2 = \sum_i f_i\delta_i^2 = N\mu_2(\delta) = N \operatorname{var}(\delta)$$

$$\sum_i \sum_j f_{ij}\delta_i^3 = \sum_i f_i\delta_i^3 = N\mu_3(\delta) = 0$$

$$\sum_i \sum_j f_{ij}\epsilon_j = \sum_j f_j\epsilon_j = N\mu_1(\epsilon) = 0. \tag{16}$$

In these expressions $\mu(\delta)$ and $\mu(\epsilon)$ represent the monovariate moments of charge and enzyme density distributions.

The bivariate moments that associate the charge density, or the dispersion of that variate with the enzyme density, express how the charge density and its dispersion are organized in space with respect to enzyme density. The bivariate moments are defined as

$$\sum_i \sum_j f_{ij}\delta_i\epsilon_j = N\mu_{1,1}(\delta, \epsilon) = N \operatorname{cov}(\delta, \epsilon)$$

$$\sum_i \sum_j f_{ij}\delta_i^2\epsilon_j = N\mu_{2,1}(\delta, \epsilon)$$

$$\sum_i \sum_j f_{ij}\delta_i^3\epsilon_j = N\mu_{3,1}(\delta, \epsilon). \tag{17}$$

If the δ_i and the ϵ_j are normally distributed, the moments of odd degree are null. It is important to realize the intuitive significance of these monovariate and bivariate moments in term of spatial order and organization. The term of organization is taken to mean the lack of pure randomness, or the presence of macroscopic heterogeneity in the spatial distribution of charges and enzyme molecules. Clustering of charges and clustering of enzyme molecules thus represent some form of organization. The monovariate moments quantitatively express this form of organization. If the values of $\mu(\delta)$, for instance, are all equal to zero, the charges are either homogeneously distributed in the matrix or, if they are clustered, the local density is the same in the population of

clusters. Moreover, organization may also involve a correlation in the spatial distribution of charges and enzyme molecules. This type of organization is quantitatively expressed by the bivariate moments. Here again if $\mu(\delta, \epsilon) = 0$ no spatial correlation exists between the distribution of charges and that of enzyme molecules.

3. Spatial order and expression of the enzyme rate equation

Let us consider the rate equation defined for a cluster i:

$$v_i = f(\langle\Delta\rangle + \delta_i) = \frac{(\langle V\rangle + \epsilon_j)\,\sigma_o}{\langle\Delta\rangle + \delta_i + \sigma_o}. \tag{18}$$

Defining the following dimensionless quantities,

$$\sigma_o^* = \frac{\sigma_o}{\langle\Delta\rangle}$$

$$\delta_i^* = \frac{\delta_i}{\langle\Delta\rangle}$$

$$\epsilon_j^* = \frac{\epsilon_j}{\langle V\rangle}, \tag{19}$$

this equation may be cast into the following form (see Appendix)

$$v_i = \frac{\langle V\rangle\,(1 + \epsilon_j^*)\,\sigma_o^*}{1 + \sigma_o^*} \cdot \left\{1 - \frac{\delta_i^*}{1 + \sigma_o^*} + \frac{\delta_i^{*2}}{(1 + \sigma_o^*)^2} - \frac{\delta_i^{*3}}{(1 + \sigma_o^*)^3} + \cdots\right\}. \tag{20}$$

For the set of N clusters the overall rate equation is then (see Appendix)

$$\frac{v}{N\langle V\rangle} = \lim_{m\to\infty} \frac{\sigma_o^*}{1 + \sigma_o^*} \cdot \left\{1 + \sum_{r=1}^{m} (-1)^r \frac{\mu_r(\delta^*) + \mu_{r,1}(\delta^*,\epsilon^*)}{(1 + \sigma_o^*)^r}\right\}. \tag{21}$$

Eq. 21 shows that the cooperativity, with respect to σ_o^*, of the rate equation relies on the value of the monovariate moments $\mu_r(\delta^*)$ and of the bivariate moments $\mu_{r,1}(\delta^*,\epsilon^*)$. The monovariate moments $\mu_r(\epsilon^*)$ have no effect on the cooperativity. What basically controls this cooperativity is the distribution of charge density as well as its correlation with that of enzyme density. Moreover under the form of Eq. 21 it is evident that $N\langle V\rangle$ remains unchanged if spatial structures of enzyme molecules in the matrix are, by convention, split into clusters of unit volume, for $\langle V\rangle$ decreases and N increases accordingly.

4. Spatial order and kinetic cooperativity

Expression 21 clearly shows that the reaction rate cannot follow, in all generality, Michaelis-Menten kinetics. One may expect the reaction rate to display a cooperative kinetics with respect to the dimensionless variable σ_o^*. More importantly, one may predict this cooperative kinetics not to be the consequence of electrostatic interaction effects between the charges of the matrix and the charged substrate. The cooperative behavior of Eq. 21 appears as a consequence of charge and enzyme organization in the matrix, for the expression of the reaction rate becomes that of a rectangular hyperbola if the monovariate and bivariate moments $\mu_r(\delta^*)$ and $\mu_{r,1}(\delta^*,\epsilon^*)$ are all equal to zero. Thus, the kinetic cooperativity displayed by Eq. 21 is clearly the consequence of order and organization of fixed charges and enzyme molecules.

It is thus important to analyze this cooperativity, which may be called organizational cooperativity, to understand its precise nature. This study may be performed by analyzing the rate behavior of Eq. 21 when the Taylor series converges rapidly enough as to generate a negligible error when $m = 2$. The reaction rate then assumes the form

$$\frac{v}{N\langle V\rangle} = \frac{\sigma_o^*}{1 + \sigma_o^*} - \frac{\mathrm{cov}\,(\delta^*, \epsilon^*)\,\sigma_o^*}{(1 + \sigma_o^*)^2} + \frac{\mathrm{var}\,(\delta^*)\,\sigma_o^*}{(1 + \sigma_o^*)^3} + \frac{\mu_{2,1}(\sigma^*, \epsilon^*)\,\sigma_o^*}{(1 + \sigma_o^*)^3}. \tag{22}$$

Reducing this expression to the same denominator yields

$$\frac{v}{N\langle V\rangle} = \frac{\sigma_o^{*3} + \{2 - \mathrm{cov}\,(\delta^*, \epsilon^*)\}\,\sigma_o^{*2} + \{1 + \mathrm{var}\,(\delta^*) + \mu_{2,1}(\delta^*, \epsilon^*) - \mathrm{cov}\,(\delta^*, \epsilon^*)\}\,\sigma_o^*}{(1 + \sigma_o^*)^3}. \tag{23}$$

Clearly if

$$\mathrm{var}\,(\delta^*) = 0$$

$$\mathrm{cov}\,(\delta^*, \epsilon^*) = 0$$

$$\mu_{2,1}(\delta^*, \epsilon^*) = 0, \tag{24}$$

Eq. 23 reduces to

$$\frac{v}{N\langle V\rangle} = \frac{\sigma_o^*}{1 + \sigma_o^*}. \tag{25}$$

There are two important questions that must be answered about the way organization of charge and enzyme density modulate the reaction rate. The first one is to know how charge clustering and enzyme organization increase or decrease the reaction rate, and the second one is to determine how these parameters of molecular organization generate a kinetic cooperativity.

The first question may be answered by comparing the actual reaction rate (Eq. 23), to what the rate would be if no organization of charges and enzyme molecules were

occurring, namely to Eq. 25. The difference between these two equations allows one to define a function, the function of organizational rate modulation, $\Xi(\sigma_o^*)$, which expresses how molecular clustering and organization affect the reaction rate. One has

$$\Xi(\sigma_o^*) = -\frac{\operatorname{cov}(\delta^*, \epsilon^*)\,\sigma_o^{*2} - \{\operatorname{var}(\delta^*) + \mu_{2,1}(\delta^*, \epsilon^*) - \operatorname{cov}(\delta^*, \epsilon^*)\}\,\sigma_o^*}{(1 + \sigma_o^*)^3}. \quad (26)$$

Depending on the sign of the bivariate moments and the value of σ_o^*, this function may take positive or negative values. Fig. 1 shows some types of variation of this function.

The important conclusion of this analysis is that the organization of charges and enzyme molecules may enhance or decrease the reaction rate.

The extent of cooperativity of an enzyme system is usually described by the so-called Hill function. In the case of a rate equation of a 3:3 type (Eq. 23) is

$$\frac{v}{N\langle V\rangle} = \frac{\sigma_o^{*3} + \Psi_2\sigma_o^{*2} + \Psi_1\sigma_o^* + \Psi_o}{\sigma_o^{*3} + \Psi_2'\sigma_o^{*2} + \Psi_1'\sigma_o^* + \Psi_o'}, \quad (27)$$

where the coefficients, Ψ and Ψ' have the significance given in Eq. 23, the Hill function may be expressed as

$$h(\sigma_o^*) = 1 + \Omega(\sigma_o^*), \quad (28)$$

where the function $\Omega(\sigma_o^*)$ assumes the form

$$\Omega(\sigma_o^*) = \frac{\lambda_3\sigma_o^{*3} + \lambda_2\sigma_o^{*2} + \lambda_1\sigma_o^*}{\lambda_4'\sigma_o^{*4} + \lambda_3'\sigma_o^{*3} + \lambda_2'\sigma_o^{*2} + \lambda_1'\sigma_o^* + \lambda_o'}. \quad (29)$$

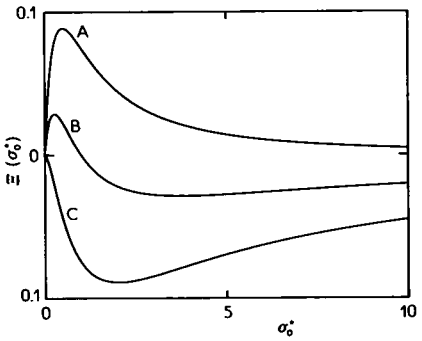

FIGURE 1 Modulation of enzyme catalysis through organization of fixed charges and enzyme molecules. The $\Xi(\sigma_o^*)$ function is plotted versus the dimensionless variable σ_o^* for different values of cov (δ^*, ϵ^*) and a fixed value of var $(\delta^*) = 0.6$ and $\mu_{2,1}(\delta^*, \epsilon^*) = 0$. (*Curve A*) cov $(\delta^*,\epsilon^*) = 0$; (*curve B*) cov $(\delta^*, \epsilon^*) = 0.3$; (*curve C*) cov $(\delta^*, \epsilon^*) = 0.6$. The simulated results show that the clustering of fixed charges tends to enhance the overall reaction rate ($\Xi[\sigma_o^*] > 0$) if no spatial organization of charges with respect to enzyme molecules occurs. If such an organization does exist, the overall reaction velocity is decreased.

The parameters λ and λ' are defined in terms of the coefficients Ψ and Ψ' of the rate Eq. 23. One has

$$\begin{aligned}
\lambda_3 &= \Psi_1' - \Psi_1 - \Psi_2\,(\Psi_2' - \Psi_2)\\
\lambda_2 &= 2\Psi_o' - 2\Psi_1\,(\Psi_2' - \Psi_2)\\
\lambda_1 &= \Psi_2\,\Psi_o' - \Psi_1\,(\Psi_1' - \Psi_1),
\end{aligned} \quad (30)$$

and

$$\begin{aligned}
\lambda_4' &= \Psi_2' - \Psi_2\\
\lambda_3' &= \Psi_1' - \Psi_1 + \Psi_2\,(\Psi_2' - \Psi_2)\\
\lambda_2' &= \Psi_o' + \Psi_2\,(\Psi_1' - \Psi_1) + \Psi_1\,(\Psi_2' - \Psi_2)\\
\lambda_1' &= \Psi_2\,\Psi_o' + \Psi_1\,(\Psi_1' - \Psi_1)\\
\lambda_o' &= \Psi_1\,\Psi_o'.
\end{aligned} \quad (31)$$

Because the coefficients Ψ and Ψ' of Eq. 41 depend on the values of monovariate and bivariate moments of charge and enzyme density distributions, it is obvious that the function $\Omega(\sigma_o^*)$ also depends on these parameters that express the degree of organization of charges and enzyme molecules. Because the sign and the extent of cooperativity rely on this function, $\Omega(\sigma_o^*)$ may be termed function of ionic-charge-organization cooperativity. The explicit formulation in terms of moments, of coefficients λ and λ' is found to be

$$\begin{aligned}
\lambda_3 &= -\overline{\operatorname{var}}(\delta^*) + \operatorname{cov}^2(\delta^*, \epsilon^*)\\
\lambda_2 &= -2\{\overline{\operatorname{var}}(\delta^*) + \operatorname{cov}(\delta^*, \epsilon^*)\,\overline{\operatorname{var}}(\delta^*) + \operatorname{cov}^2(\delta^*, \epsilon^*)\}\\
\lambda_1 &= -\overline{\operatorname{var}}(\delta^*) + \{\operatorname{cov}(\delta^*, \epsilon^*) - \overline{\operatorname{var}}(\delta^*)\}^2,
\end{aligned} \quad (32)$$

and

$$\begin{aligned}
\lambda_4' &= 1 + \operatorname{cov}(\delta^*, \epsilon^*)\\
\lambda_3' &= 4 - \overline{\operatorname{var}}(\delta^*) + 2\operatorname{cov}(\delta^*, \epsilon^*) - \operatorname{cov}^2(\delta^*, \epsilon^*)\\
\lambda_2' &= 6 - \overline{\operatorname{var}}(\delta^*) - 2\operatorname{cov}^2(\delta^*, \epsilon^*) + 2\operatorname{cov}(\delta^*, \epsilon^*)\,\overline{\operatorname{var}}(\delta^*)\\
\lambda_1' &= 4 + \overline{\operatorname{var}}(\delta^*) - 2\operatorname{cov}(\delta^*, \epsilon^*) - \{\operatorname{cov}(\delta^*, \epsilon^*) - \overline{\operatorname{var}}(\delta^*)\}^2,\\
\lambda_o' &= 1 + \overline{\operatorname{var}}(\delta^*) - \operatorname{cov}(\delta^*, \epsilon^*)
\end{aligned} \quad (33)$$

where

$$\overline{\operatorname{var}}(\delta^*) = \operatorname{var}(\delta^*) + \mu_{2,1}(\delta^*, \epsilon^*). \quad (34)$$

In fact $\mu_{2,1}(\delta^*,\epsilon^*) = 0$ for normal distributions and if these distributions are close to normality the value of the bivariate moment is negligible relative to the variance of δ^*. This is a consequence of the fact that the regression of δ_i^{*2} v ϵ_j^* is parabolic in shape and symmetrical about the δ axis. Because both var(δ^*) and cov(δ^*,ϵ^*) are smaller than unity, the nonlinear terms in the expressions 32 and 33 must be smaller than the linear ones. Therefore the terms of the numerator of the $\Omega(\sigma_o^*)$ function must all be negative and those of the denominator of this function are positive. This implies that the type of cooperativity that may be expected from Eqs. 28 and 29 is only negative.

This conclusion is quite consistent with a more general statement made previously (see Eq. 13). As σ_0^* is increased, the cooperativity, as expressed by the Hill function, decreases at first, then increases (Fig. 2).

This variation of the negative cooperativity as a function of σ_0^* depends on the value of the variance of charge density. This phenomenon occurs even if the bivariate moments are equal to zero, that is, when there is no organization of charges with respect to enzyme molecules. As the variance increases, the minimum of the Hill function is shifted toward higher σ_0^* values. At the same time, cooperativity becomes more and more negative.

If at constant variance, the covariance values are increased and the minimum of the Hill function is shifted toward low σ_0^* values. This is illustrated in Fig. 2. The modulation of cooperativity by the variance and covariance thus corresponds to adverse effects. The shift of the minimum of the Hill function brought about by increasing the values of the covariance results in an increase or a decrease of negative cooperativity depending on whether the values of σ_0^* are low or high.

5. Spatial localization of charges and enzyme molecules as a source of kinetic cooperativity

We have considered so far that the fixed charges and enzyme molecules are clustered at the same specific places. Therefore clusters of fixed charges and enzyme molecules exist. This may indeed be viewed as a highly organized system (Fig. 3 *E*). But it is obvious that other types of organization may not involve such a high degree of order (namely exact superimposition of charge and enzyme clusters). From a mathematical viewpoint however they may be considered as special cases of the model discussed above and schematized in Fig. 3 *E*.

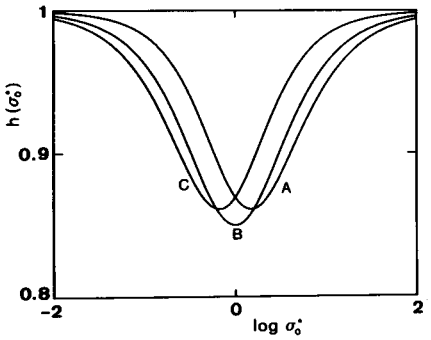

FIGURE 2 Modulation of enzyme cooperativity through organization of fixed charges and enzyme molecules. The Hill function is plotted versus $\log \sigma_0^*$ for different values of cov (δ^*, ϵ^*) and at a fixed value of var $(\delta^*) = 0.6$ and $\mu_{2,1}(\delta^*, \epsilon^*) = 0$. (*Curve A*) cov $(\delta^*, \epsilon^*) = 0$; (*curve B*) cov $(\delta^*, \epsilon^*) = 0.3$; (*curve C*) cov $(\delta^*, \epsilon^*) = 0.6$. The simulated results show that increasing the covariance value, results in a shift of the minimum of the Hill function toward low value of σ_0^*.

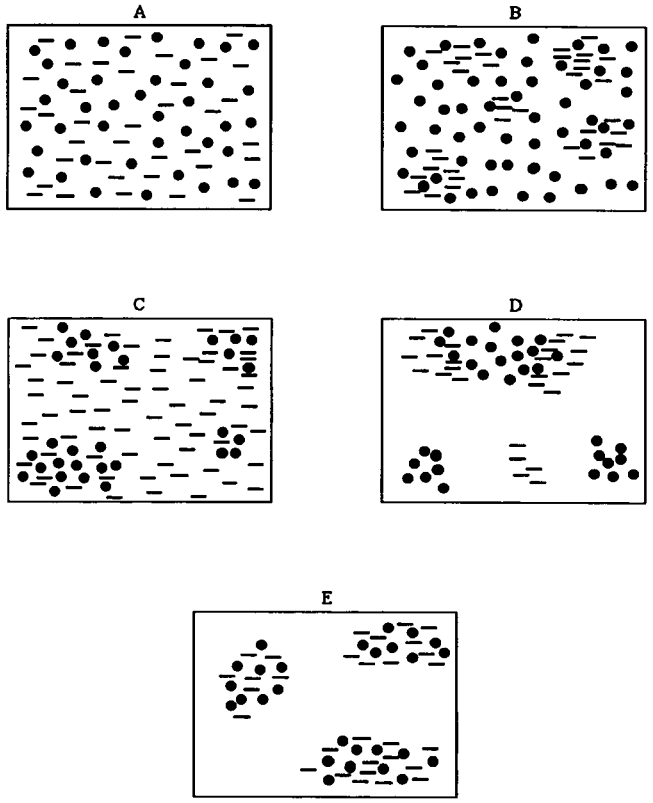

FIGURE 3 Models of organization of fixed negative charges and enzyme molecules. (*A*) The enzyme molecules and fixed negative charges are randomly distributed in the matrix. (*B*) The enzyme molecules are randomly distributed but the charges are clustered. (*C*) The enzyme molecules are clustered and the charges are randomly distributed. (*D*) There exists in the matrix clusters of charges and clusters of enzyme molecules that partly overlap. (*E*) The clusters of enzyme molecules and of fixed charges are superimposed.

In line with this model, there are five broad types of organization of fixed charges and enzyme molecules.

The first one is shown in Fig. 3 *A*. The charges and enzyme molecules are randomly distributed in the polyanionic matrix. With respect to the reduced variable σ_0^*, no cooperativity occurs. There is therefore no organization of charges and enzyme molecules. Then the rate curve is sigmoidal with respect to substrate concentration, and the corresponding rate equation assumes the form

$$v = \frac{V S_0^2}{K\Delta + S_0^2}. \tag{35}$$

The second type of distribution of charges and enzyme molecules involves some form of organization. The

charges are supposed to be clustered but the enzyme molecules are randomly distributed in the matrix (Fig. 3 *B*). Therefore the enzyme molecules that are located outside the charge clusters are involved in a reaction that follows Michaelis-Menten kinetics and where the substrate is not submitted to electrostatic repulsion effects. The substrate of the enzyme molecules located within the charge clusters are submitted to electrostatic repulsion effects. The monovariate moments of the charge distribution cannot all be equal to zero, and this generates a negatively cooperative response with respect to the reduced variable σ_o^*, of the enzyme molecules located in the charge clusters. However, because the enzyme molecules are randomly distributed in the matrix, the bivariate moments $\mu(\delta^*,\epsilon^*)$ are all equal to zero and there is no organization of charges with respect to enzyme molecules. The corresponding rate equation is rather complex. With respect to the bulk substrate concentration, S_o, it assumes the form

$$v = \frac{V_1 S_o}{K + S_o} + \frac{N\langle V\rangle_2 S_o^2/K\langle\Delta\rangle}{1 + S_o^2/K\langle\Delta\rangle} \cdot \left\{1 + \sum_{r=1}^{m} (-1)^r \frac{\mu_r(\delta^*)}{(1 + S_o^2/K\langle\Delta\rangle)^r}\right\}, \quad (36)$$

where V_1 is proportional to the enzyme density randomly distributed in the matrix and $\langle V\rangle_2$ is proportional to the enzyme density clustered in that matrix. An example of the kinetic behavior obtained from this type of organization is shown in Fig. 4. A particular case implying less order, that is, more homogeneity in the distribution of fixed charges, occurs if the monovariate moments are all equal to zero. This implies that the charge density in the clusters is about the same. The corresponding rate equation then reduces to

$$v = \frac{V_1 S_o}{K + S_o} + \frac{N\langle V\rangle_2 S_o^2/K\langle\Delta\rangle}{1 + S_o^2/K\langle\Delta\rangle}. \quad (37)$$

A simulation of the type of mixed positive-negative cooperativity to be expected from Eq. 36 and 37 is also shown in Fig. 4.

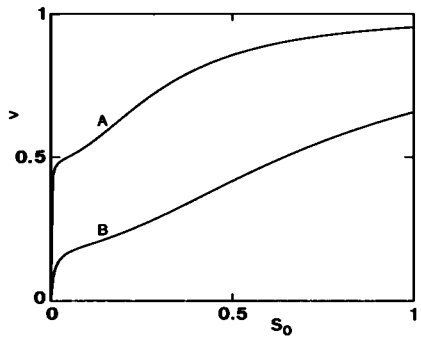

FIGURE 4 Mixed positive and negative cooperativity generated by a random distribution of enzymes and a clustering of charges (Fig. 3 *B*). The two curves are simulated from Eqs 36 and 37 with the approximation that $m = 2$. The parameter values are the following: (*curve A* [Eq. 37]) $V_1 = 0.5$; $\langle V\rangle_2 = 0.0005$; $K = 0.01$; $N = 1000$; $\langle\Delta\rangle = 100$; var $(\delta^*) = 0$; cov $(\delta^*,\epsilon^*) = 0$; $\mu_{2,1}(\delta^*,\epsilon^*) = 0$; (*curve B* [Eq. 36]) $V_1 = 0.2$; $\langle V\rangle_2 = 0.0008$; $K = 0.01$; $N = 1000$; $\langle\Delta\rangle = 100$; var $(\delta^*) = 0.6$; cov $(\delta^*,\epsilon^*) = 0$; $\mu_{2,1}(\delta^*,\epsilon^*) = 0$.

Another type of spatial organization occurs when the enzyme molecules are clustered, whereas the fixed charges are not (Fig. 3 *C*). Then the corresponding reaction rate follows Michaelis-Menten kinetics with respect to σ_o^* and follows sigmoidal kinetics relative to S_o. This situation is thus indistinguishable from the first one considered above (Fig. 3 *A*).

A more elaborate type of organization occurs if both charges and enzyme molecules are clustered and if some of the clusters partly overlap (Fig. 3 *D*). Then some enzyme molecules are not surrounded by fixed negative charges and display a response with respect to the substrate concentration that follows Michaelis-Menten kinetics. But other enzyme molecules are surrounded by fixed charges and display an apparent negative cooperativity with respect to the dimensionless parameter σ_o^*. Then the monovariate and bivariate moments may well not be all equal to zero, and the resulting rate equation assumes the form

$$v = \frac{V_1 S_o}{K + S_o} + \frac{N\langle V\rangle_2 S_o^2/K\langle\Delta\rangle}{1 + S_o^2/K\langle\Delta\rangle} \cdot \left\{1 + \sum_{r=1}^{m} (-1)^r \frac{\mu_r(\delta^*) + \mu_{r,1}(\delta^*,\epsilon^*)}{(1 + S_o^2/K\langle\Delta\rangle)^r}\right\}. \quad (38)$$

Some examples of the rate curves generated by this equation are shown in Fig. 5, An additional simplification occurs if the monovariate and bivariate moments are all close to zero, that is, if the charge density is nearly the same for all the clusters. Then the resulting rate equation is again Eq. 37.

A higher degree of organization occurs if the charge and enzyme clusters are exactly superimposed. Then the rate equation displays a negative cooperativity with respect to σ_o^*, and one has

$$v = \frac{N\langle V\rangle S_o^2/K\langle\Delta\rangle}{1 + S_o^2/K\langle\Delta\rangle} \cdot \left\{1 + \sum_{r=1}^{m} (-1)^r \frac{\mu_r(\delta^*) + \mu_{r,1}(\delta^*,\epsilon^*)}{(1 + S_o^2/K\langle\Delta\rangle)^r}\right\}. \quad (39)$$

The situation is thus identical to the one described previously. With respect to S_o, the rate curve displays a

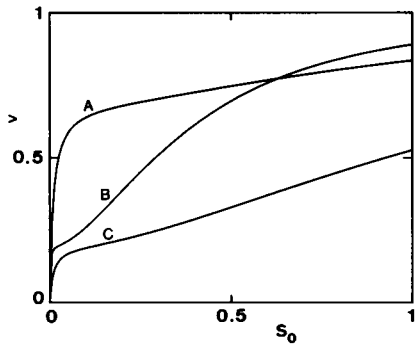

FIGURE 5 Mixed positive and negative cooperativity generated by a partial overlap of enzyme and charge clusters (Fig. 3 *D*). The curves are simulated from Eq. 38 with the approximation that $m = 2$. The parameter values are the following: (*curve A*) $V_1 = 0.7$; $K = 0.01$; $\langle V \rangle_2 = 0.0003$; $N = 1000$; $\langle \Delta \rangle = 100$; var $(\delta^*) = 0.4$; cov $(\delta^*, \epsilon^*) = 0.3$; $\mu_{2,1}(\delta^*, \epsilon^*) = 0$; (*curve B*) $V_1 = 0.2$; $K = 0.001$; $\langle V \rangle_2 = 0.0008$; $N = 1000$; $\langle \Delta \rangle = 100$; var $(\delta^*) = 0.5$; cov $(\delta^*, \epsilon^*) = 0.6$; $\mu_{2,1}(\delta^*, \epsilon^*) = 0$; (*curve C*) $V_1 = 0.2$; $K = 0.01$; $\langle V \rangle_2 = 0.0008$; $N = 1000$; $\langle \Delta \rangle = 100$; var $(\delta^*) = 0.5$; cov $(\delta^*, \epsilon^*) = 0.6$; $\mu_{2,1}(\delta^*, \epsilon^*) = 0$.

sigmoidicity that is not present when enzyme molecules are not submitted to electrostatic repulsion effects. Results of Fig. 6 show how this type of organization of charges and enzyme molecules affects the rate curve.

DISCUSSION

The overall response of an enzyme buried in a polyanionic matrix is different, depending on whether the charges and

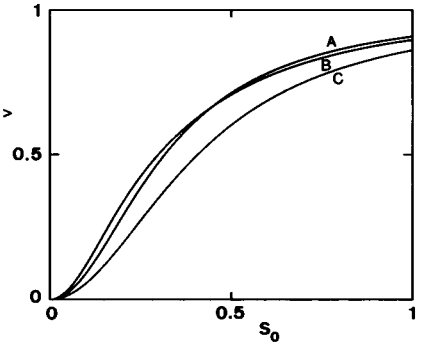

FIGURE 6 Sigmoidicity of the rate curve generated by an exact superimposition of charge and enzyme clusters. The curves are simulated from Eq. 39 with the approximation that $m = 2$ and the following parameters values: (*curve A*) $\langle V \rangle = 0.001$; $K = 0.001$; $N = 1000$; $\langle \Delta \rangle = 100$; var $(\delta^*) = 0$; cov $(\delta^*, \epsilon^*) = 0$; $\mu_{2,1}(\delta^*, \epsilon^*) = 0$. (*curve B*) $\langle V \rangle = 0.001$; $K = 0.001$; $N = 1000$; $\langle \Delta \rangle = 100$; var $(\delta^*) = 0.6$; cov $(\delta^*, \epsilon^*) = 0.2$; $\mu_{2,1}(\delta^*, \epsilon^*) = 0$; (*curve C*) $\langle V \rangle = 0.001$; $K = 0.001$; $N = 1000$; $\langle \Delta \rangle = 100$; var $(\delta^*) = 0.2$; cov $(\delta^*, \epsilon^*) = 0.6$; $\mu_{2,1}(\delta^*, \epsilon^*) = 0$.

the enzyme molecules are randomly distributed or clustered in that matrix. The spatial organization of fixed charges, as well as that of the enzyme molecules with respect to the charges, may modulate the cooperativity of the bound enzyme system. To appreciate quantitatively how molecular order may generate some form of cooperativity of the bound enzyme, one has to express the kinetic cooperativity with respect to a dimensionless variable, $\sigma_o^* = S_o^2/K\langle \Delta \rangle$. In the absence of organization of charges and enzyme molecules, the kinetics of the bound enzyme system should be Michaelian with respect to this dimensionless variable. The cooperativity that may possibly be observed with respect to this variable is indicative of an organization of fixed charges and enzyme molecules with respect to the charges. It is therefore important to distinguish the cooperativity with respect to this variable σ_o^* from the overall cooperativity expressed with respect to the bulk substrate concentration S_o. As outlined above, cooperativity with respect to σ_o^*, which can only be negative, is indicative of the spatial organization of the charges and enzyme molecules and does not reflect substrate repulsion effects. Cooperativity with respect to the bulk substrate concentration, S_o, results from the interplay between substrate repulsion effects and spatial organization of fixed charges and enzyme molecules. If this spatial organization does not exist there is no cooperativity with respect to σ_o^* but a strict positive cooperativity with respect to S_o (Eq. 35). This effect is the consequence of substrate repulsion effects (Engasser and Horvath, 1975; Ricard et al., 1981). For such a sigmoidal curve its slope must be maximum for the substrate concentration which yields half maximum velocity. If there is both substrate repulsion and spatial organization, cooperativity with respect to σ_o^* is negative, but cooperativity with respect to S_o is more complex. The corresponding rate curve looks sigmoidal but its slope is not maximum at the substrate concentration which pertains to the half-maximum velocity. Organization of charges and the enzyme molecules results in a distorsion of the sigmoidal curve (Fig. 6). It is therefore possible to distinguish in the overall cooperative response of a bound enzyme the contribution of the spatial order of fixed charges and enzyme molecules. More precisely, if the same number of fixed charges and enzyme molecules are either randomly distributed or clustered in the same volume of the matrix, the overall response of the enzyme system will be different in the two cases. Spatial organization of charges and enzyme molecules may enhance or decrease the reaction rate and, as stated above, may modulate the kinetic cooperativity of the system.

The simple and versatile model presented in this paper allows one to express quantitatively how the degree of organization within clusters of charges and enzyme mole-

cules may modulate the kinetic cooperativity. This degree of modulation is called ionic-charge organizational cooperativity. The degree of spatial organization may be quantitatively expressed by monovariate and bivariate moments of charge and enzyme density distribution. If there is no organization of enzyme molecules with respect to charges, the bivariate moments are all equal to zero. The cooperativity generated by the spatial organization of fixed charges and enzyme molecules is, of necessity, negative.

The larger the extent of the ionic-charge organizational cooperativity and the greater is the number of moments that are significantly different from zero. If the cooperativity is not very large, an approximation to the second order for instance is sufficient to describe quantitatively this cooperativity. This is what has been shown in the computer simulations presented in this paper. However if the cooperativity is much larger, the approximation should be of a higher order.

Five types of spatial organization of charges and enzyme molecules may be distinguished and result in different types of kinetic behavior. The first one is defined by a random distribution of charges and enzyme molecules in the matrix. Then no type of organizational cooperativity is to be observed and the rate curve will be sigmoidal with respect to the substrate. The second type of organization is defined by a clustering of fixed charges but not of enzyme molecules that are randomly distributed in the matrix. With respect to substrate concentration, the rate curve is complex and displays a mixed positive and negative cooperativity. The corresponding equation must exhibit a Michaelian contribution plus another one which is modulated by the monovariate moments of the charge distribution. The organizational cooperativity is thus solely defined by the distribution of charge density in the clusters. A third type of organization involves the clustering of enzyme molecules, whereas charges are randomly distributed. Then no organizational cooperativity is to be expected. A fourth and fifth type of organization are defined by the clustering of both charges and enzyme molecules. Thus clusters may partly, or exactly, overlap. This is then the highest type of organization one may expect, for it may involve both the spatial organization of charges and enzyme molecules with respect to these charges. The corresponding reaction rate is then rather complex and relies on both the monovariate and the bivariate moments of charge and enzyme distributions.

Probably the most general idea that may be drawn from these studies is that kinetic cooperativity of polyelectrolyte-bound enzymes may be a systemic property and that some sort of spatial order in the distribution of charges with respect to enzyme molecules may modulate the fine tuning of enzyme behavior, in situ, either in the living cell or in man-made enzyme reactors.

APPENDIX

Derivation of the Eq. 21 of main text

Let us consider the rate equation defined for a cluster i, one has

$$v_i = f(\langle\Delta\rangle + \delta_i) = \frac{(\langle V\rangle + \epsilon_j)\,\sigma_o}{\langle\Delta\rangle + \delta_i + \sigma_o}. \tag{1'}$$

This equation may be expanded in Taylor series with respect to the variable δ_i. One obtains

$$\begin{aligned} v_i &= f(\langle\Delta\rangle + \delta_i) \\ &= (\langle V\rangle + \epsilon_j)\left\{\frac{\sigma_o}{\langle\Delta\rangle + \sigma_o}\right. \\ &\quad + \delta_i \frac{\partial}{\partial\delta_i}\left(\frac{\sigma_o}{\langle\Delta\rangle + \delta_i + \sigma_o}\right)_o \\ &\quad + \frac{\delta_i^2}{2}\frac{\partial^2}{\partial\delta_i^2}\left(\frac{\sigma_o}{\langle\Delta\rangle + \delta_i + \sigma_o}\right)_o \\ &\quad \left. + \frac{\delta_i^3}{6}\frac{\partial^3}{\partial\delta_i^3}\left(\frac{\sigma_o}{\langle\Delta\rangle + \delta_i + \sigma_o}\right)_o + \cdots\right\}. \end{aligned} \tag{2'}$$

Moreover one has

$$\begin{aligned} \frac{\partial}{\partial\delta_i}\left(\frac{\sigma_o}{\langle\Delta\rangle + \delta_i + \sigma_o}\right)_o &= -\frac{\sigma_o}{(\langle\Delta\rangle + \sigma_o)^2} \\ \frac{\partial^2}{\partial\delta_i^2}\left(\frac{\sigma_o}{\langle\Delta\rangle + \delta_i + \sigma_o}\right)_o &= \frac{2\sigma_o}{(\langle\Delta\rangle + \sigma_o)^3} \\ \frac{\partial^3}{\partial\delta_i^3}\left(\frac{\sigma_o}{\langle\Delta\rangle + \delta_i + \sigma_o}\right)_o &= -\frac{6\sigma_o}{(\langle\Delta\rangle + \sigma_o)^4}. \end{aligned} \tag{3'}$$

Therefore the approximate expression of the rate v_i of cluster i assumes the form

$$\begin{aligned} v_i &= (\langle V\rangle + \epsilon_j) \\ &\quad \cdot\left\{\frac{\sigma_o}{\langle\Delta\rangle + \sigma_o} - \frac{\delta_i\sigma_o}{(\langle\Delta\rangle + \sigma_o)^2} + \frac{\delta_i^2\sigma_o}{(\langle\Delta\rangle + \sigma_o)^3}\right. \\ &\quad \left. - \frac{\delta_i^3\sigma_o}{(\langle\Delta\rangle + \sigma_o)^4} + \cdots\right\}. \end{aligned} \tag{4'}$$

As it becomes clear below, there is an obvious advantage in expressing this equation in dimensionless form, by writing

$$\begin{aligned} \sigma_o^* &= \frac{\sigma_o}{\langle\Delta\rangle} \\ \delta_i^* &= \frac{\delta_i}{\langle\Delta\rangle} \\ \epsilon_j^* &= \frac{\epsilon_j}{\langle V\rangle}. \end{aligned} \tag{5'}$$

Eq. 4′ may be reexpressed as

$$v_i = \langle V\rangle\left(1+\frac{\epsilon_j}{\langle V\rangle}\right)\left\{\frac{\sigma_o/\langle\Delta\rangle}{1+\sigma_o/\langle\Delta\rangle} - \frac{(\delta_i/\langle\Delta\rangle)(\sigma_o/\langle\Delta\rangle)}{(1+\sigma_o/\langle\Delta\rangle)^2} + \frac{(\delta_i/\langle\Delta\rangle)^2(\sigma_o/\langle\Delta\rangle)}{(1+\sigma_o/\langle\Delta\rangle)^3} - \frac{(\delta_i/\langle\Delta\rangle)^3(\sigma_o/\langle\Delta\rangle)}{(1+\sigma_o/\langle\Delta\rangle)^4} + \cdots\right\}, \quad (6')$$

and as

$$v_i = \frac{\langle V\rangle(1+\epsilon_j^*)\sigma_o^*}{1+\sigma_o^*}\left\{1 - \frac{\delta_i^*}{1+\sigma_o^*} + \frac{\delta_i^{*2}}{(1+\sigma_o^*)^2} - \frac{\delta_i^{*3}}{(1+\sigma_o^*)^3} + \cdots\right\}. \quad (7')$$

If δ_i and ϵ_j are normally distributed, δ_i^* and ϵ_j^* are normally distributed as well and δ_i^* and ϵ_j^* are of necessity smaller than unity. Therefore under the form of Eq. 7′ it becomes evident that the Taylor series is convergent. The overall rate equation for the all set of clusters is obtained by summing up the elementary rates for all the clusters, namely,

$$v = \sum_i\sum_j \frac{\langle V\rangle f_{ij}(1+\epsilon_j^*)\sigma_o^*}{1+\sigma_o^*}\left\{1 - \frac{\delta_i^*}{1+\sigma_o^*} + \frac{\delta_i^{*2}}{(1+\sigma_o^*)^2} - \frac{\delta_i^{*3}}{(1+\sigma_o^*)^3} + \cdots\right\}. \quad (8')$$

This expression may be rewritten as

$$v = \frac{\left\{\sum_i\sum_j f_{ij} + \sum_i\sum_j f_{ij}\epsilon_j^*\right\}\langle V\rangle\sigma_o^*}{1+\sigma_o^*} - \frac{\left\{\sum_i\sum_j f_{ij}\delta_i^* + \sum_i\sum_j f_{ij}\delta_i^*\epsilon_j^*\right\}\langle V\rangle\sigma_o^*}{(1+\sigma_o^*)^2} + \frac{\left\{\sum_i\sum_j f_{ij}\delta_i^{*2} + \sum_i\sum_j f_{ij}\delta_i^{*2}\epsilon_j^*\right\}\langle V\rangle\sigma_o^*}{(1+\sigma_o^*)^3}. \quad (9')$$

This expression may also be rewritten in terms of monovariate and bivariate moments, centered on the zero value, of charge and enzyme density distributions. One must have

$$\begin{aligned}
&\sum_i\sum_j f_{ij} = N\\
&\sum_i\sum_j f_{ij}\delta_i^* = N\mu_1(\delta^*) = 0\\
&\sum_i\sum_j f_{ij}\epsilon_j^* = N\mu_1(\epsilon^*) = 0\\
&\sum_i\sum_j f_{ij}\delta_i^{*2} = N\mu_2(\delta^*) = N\,\mathrm{var}(\delta^*)\\
&\sum_i\sum_j f_{ij}\delta_i^*\epsilon_j^* = N\mu_{1,1}(\delta^*,\epsilon^*) = N\,\mathrm{cov}(\delta^*,\epsilon^*)\\
&\sum_i\sum_j f_{ij}\delta_i^{*2}\epsilon_j^* = N\mu_{2,1}(\delta^*,\epsilon^*).
\end{aligned} \quad (10')$$

The bivariate moment $\mu_{2,1}(\delta^*,\epsilon^*)$ is equal to zero if δ_i^* and ϵ_j^* are normally distributed. In these expressions it is important to stress that

$$\begin{aligned}
&\mathrm{var}(\delta^*) < 1\\
&\mathrm{cov}(\delta^*,\epsilon^*) < 1\\
&\mathrm{var}(\delta^*) > \mu_{2,1}(\delta^*,\epsilon^*).
\end{aligned} \quad (11')$$

This may easily be shown in the following way. If δ_i is normally distributed,

$$\langle\Delta\rangle > \sqrt{\mathrm{var}(\delta)}, \quad (12')$$

and this implies that

$$\frac{\mathrm{var}(\delta)}{\langle\Delta\rangle^2} < 1. \quad (13')$$

This expression is equivalent to the first of inequalities 11′, for one has

$$\frac{\mathrm{var}(\delta)}{\langle\Delta\rangle^2} = \mathrm{var}\left(\frac{\delta}{\langle\Delta\rangle}\right) = \mathrm{var}(\delta^*). \quad (14')$$

Similarly,

$$\frac{\mathrm{cov}(\delta,\epsilon)}{\sqrt{\mathrm{var}(\delta)}\ \sqrt{\mathrm{var}(\epsilon)}} \le 1, \quad (15')$$

and

$$\begin{aligned}
&\sqrt{\mathrm{var}(\delta)} < \langle\Delta\rangle\\
&\sqrt{\mathrm{var}(\epsilon)} < \langle V\rangle.
\end{aligned} \quad (16')$$

Therefore inequality 15′ implies that

$$\frac{\mathrm{cov}(\delta,\epsilon)}{\langle\Delta\rangle\langle V\rangle} < 1, \quad (17')$$

and this is equivalent to the second expression of the inequalities 11′, for

$$\frac{\mathrm{cov}(\delta,\epsilon)}{\langle\Delta\rangle\langle V\rangle} = \mathrm{cov}\left(\frac{\delta}{\langle\Delta\rangle},\frac{\epsilon}{\langle V\rangle}\right) = \mathrm{cov}(\delta^*,\epsilon^*). \quad (18')$$

Furthermore one has

$$\mu_{2,1}(\delta^*,\epsilon^*) = \frac{1}{N}\sum_i\sum_j f_{ij}\delta_i^{*2}\,\epsilon_j^* \quad (19')$$

Since $\epsilon_j^* < 1$ and

$$\mathrm{var}(\delta^*) = \frac{1}{N}\sum_i\sum_j f_{ij}\delta_i^{*2}. \quad (20')$$

The last expression of inequalities 11′ must of necessity be fulfilled. For normal distributions this is even more obvious because $\mu_{2,1}(\delta^*,\epsilon^*) = 0$.

With the definition of monovariate and bivariate moments given in expression 10′, Eq. 9′ may be rewritten as

$$v = \frac{N\langle V\rangle\sigma_o^*}{1+\sigma_o^*} - \frac{N\langle V\rangle\mu_{1,1}(\delta^*,\epsilon^*)\sigma_o^*}{(1+\sigma_o^*)^2} + \frac{N\langle V\rangle\{\mu_2(\delta^*)+\mu_{2,1}(\delta^*,\epsilon^*)\}\sigma_o^*}{(1+\sigma_o^*)^3}. \quad (21')$$

This expression may be rewritten in more compact form as

$$\frac{v}{N\langle V\rangle} = \lim_{m\to\infty} \frac{\sigma_o^*}{1+\sigma_o^*} \left\{1 + \sum_{r=1}^{m}{}' (-1)^r \frac{\mu_r(\delta^*) + \mu_{r,1}(\delta^*, \epsilon^*)}{(1+\sigma_o^*)^r}\right\}. \quad (22')$$

G. Mulliert is a recipient of a fellowship from the Consejo Nacional de Ciencia y Tecnología, Mexico.

Received for publication 11 October 1988 and in final form 1 May 1989.

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