

Molecular Recognition in Enzyme-Catalyzed Reactions of Ionic and Dipolar Substrates

INTRODUCTION

Molecular recognition seems to be apparent in all biological cell reactions. In the present Comment, a useful physical interpretation of the term recognition is outlined by using enzyme-catalyzed reactions of ionic and dipolar substrates as examples.

It is well known that enzyme catalysis is a multiphasic process: The initial step is the diffusional approach of the substrate to the enzyme surface, followed by the actual binding to the active site and the catalytic processes proper before the product is released.

The binding of the substrate and, on the same line, the associations of hormones or transmitters to their corresponding receptors and related processes, are frequently and unhesitatingly called recognition, discrimination or selection.^{1,2} We shall see, however, that the meaningful use of such anthropomorphic terms is very limited if more than binding alone is to be expressed.³ In particular, a quantitative physical-chemical characterization of specific molecular recognition will not only involve specific structural aspects but also dynamic principles of ligand-binding site interactions.

Clearly, the limit for the rate of substrate binding is the diffusion. However, electrostatic, polyelectrolyte-like properties combined with two-dimensional surface diffusion may appreciably increase the association rate of a substrate. It is trivial that recognition and

selection of the correct substrate must involve transient binding; recognition and selection by themselves are dynamic processes which can only be meaningfully specified in kinetic terms.

Recently, the novel concept of preselection during diffusional approach has been introduced.³ In this context the phenomenon of preselection of ionic and dipolar species in the inhomogeneous electric fields arising from polyionic structures will be discussed in some detail.

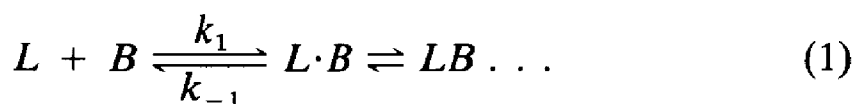
Indeed, specificity of molecular interactions arises from specific structural details between ligand and binding site. The dynamic principles of recognition and selection processes, however, appear to be general, based on weak noncovalent, short-lived interactions and may be viewed as sequential multiphasic association-dissociation steps involving several atoms of a substrate and a binding site. Two limit cases of a general conformational scheme for selection may be differentiated: the induced fit concept and the case of selective binding to a low concentration conformer.

These general aspects of molecular recognition are clarified by using specific examples. The nerve enzyme acetylcholinesterase (E.C. 3.1.1.7) represents a case where unusual electrostatic properties enormously accelerate the diffusional approach of the substrate to the active site. Kinetic data suggest that not only the active sites themselves but, perhaps, the entire surface is operative in entrapping the substrate. General features of recognition and selection become apparent in the discussion of the acetylcholine binding to the acetylcholine receptor and of the ligand-induced conversion of trypsinogen to trypsin.

In this Comment on recognition, the selection of substrates by enzymes will be discussed within the general framework of preselection and discrimination principles underlying the binding of specific ligands to specific macromolecular binding sites.

GENERAL ASPECTS OF MOLECULAR RECOGNITION

The initial association phase in the effective binding of a ligand, L , to a binding site, B , is the formation of the diffusional encounter complex, $L \cdot B$, before further (selective) processes can occur within the complex LB .⁴



The bimolecular reaction step of the encounter complex formation is always rate-limited by diffusion, driven by Brownian motion. The diffusional encounter process may, however, be appreciably accelerated when the ligand is charged, dipolar or polarizable and when the macromolecular binding site has polyelectrolyte-like properties. Furthermore, polyionic surfaces near the binding site may electrostatically entrap counterionic and dipolar ligands which then reach the specific binding site by surface diffusion. It is, however, less well recognized that the electrostatic field forces of oligo- and polyionic surfaces exert a kind of *electric preselection* before actual binding contact occurs.

ELECTRIC PRESELECTION OF LIGANDS

In this context it is recalled that a considerable proportion of macromolecular cell components carry electric charges. The ionized groups of macromolecular structures create strong electric fields in their immediate environment. These local fields not only affect the macromolecular conformation itself, but also the local ionic and dipolar milieu as well as chemical reactions involving low molar mass reaction partners in the immediate vicinity of polyionic structures. Polyionic field effects on chemical processes are especially pronounced when the density of ionized groups is high and when the majority of the fixed macromolecular charges are of the same sign (polyelectrolytes).

It should be mentioned that electric features are generally important for reactions between ionic and dipolar reaction partners. If polyions or polyionic parts of macromolecular structures such as biomembranes and cytoskeletal networks participate in such reactions, the electric contributions may become particularly large.⁵

The local electric field originating from ionic groups is inhomogeneous, decaying in intensity with increasing distance, r , from the charge centers. The mean electric field force, $\bar{E}(r)$, acting on charged and dipolar species is related to the mean electric potential

$\bar{\psi}(r)$ of the force field by

$$\bar{\mathbf{E}}(\mathbf{r}) = -\nabla\bar{\psi}(\mathbf{r}) \quad (2)$$

where ∇ is the vector derivative (gradient).

Electrodiffusion

It is well known that the electric field forces of a polyionic structure with predominantly like charges accumulate counterions and repel co-ions; the concentration of the ions, c_j , is dependent on the distance from the fixed charge center:

$$c_j(\mathbf{r}) = \bar{c}_j \cdot \exp[-z_j \cdot e \cdot \bar{\psi}(\mathbf{r})/kT] \quad (3)$$

where \bar{c}_j is the bulk concentration, z_j the charge number of ion j , e the (positive) elementary charge, k the Boltzmann constant and T the Kelvin temperature.

Since $\psi_i(\mathbf{r}) = z_i e / (4\pi\epsilon_0\epsilon r)$, where z_i is the effective charge number of the fixed charge at distance r , ϵ_0 the vacuum permittivity and ϵ the dielectric constant of the medium, it is readily seen that for counterions $c_j(\mathbf{r}) > \bar{c}_j$ and for co-ions $c_j(\mathbf{r}) < \bar{c}_j$. The higher the charge numbers z_j and z_i the stronger is the attraction of counterions. The diffusion of charged species then becomes an *electrodiffusion* and the driving force is the gradient $\nabla\bar{\mu}_j$ in the electrochemical potential $\bar{\mu}_j$:⁶

$$\bar{\mu}_j = \mu_j + F \int_0^{z_j} \bar{\psi} dz_j \quad (4)$$

Note that the Faraday constant is given by $F = N_A \cdot e$, where N_A is the Avogadro constant, μ_j is the ordinary chemical potential and a_j is the thermodynamic activity of j :

$$\mu_j = \mu_j^\ominus + RT \ln a_j, \quad (5)$$

where μ_j^\ominus is the standard potential, $\mu_j = \mu_j^\ominus$ for $a_j = 1$, $a_j = (c_j/c^\ominus)y_j$, where c_j is the molar concentration, $c^\ominus = 1 \text{ mol/dm}^3$ and y_j is the thermodynamic activity coefficient. As compared to the

diffusion of neutral species, the electrodiffusion of charged particles in the direction of higher field strengths results in an *acceleration* of the diffusional approach to a polyionic binding site. This is obvious by inspection of the expression for the rate v of diffusional drift, given by

$$v_{\text{diff}} = \frac{D}{RT} (-\nabla\mu_j) \quad (6)$$

where D is the diffusion coefficient and $R = k \cdot N_A$. In the case of electrodiffusion the gradient of the chemical potential $\nabla\mu$ is replaced by the electrochemical potential gradient. Thus:

$$\begin{aligned} v_{\text{electrodiff}} &= \frac{D}{RT} (-\nabla\hat{\mu}_j) \\ &= \frac{D}{RT} \left(-\nabla\mu_j - F \cdot \int_0^{z_j} \nabla\bar{\psi} dz_j \right) \\ &= \frac{D}{RT} [-\nabla\mu_j + F \cdot z_j \cdot \mathbf{E}(\mathbf{r})] \end{aligned} \quad (7)$$

It is readily seen that the electrodiffusion rate is enhanced in an inhomogeneous electric field. In a similar manner the diffusion of a dipolar or polarizable molecule is accelerated in an inhomogeneous electric field.

Dielectrodifffusion

The polyionic electric field affects not only ions, but also dipolar and polarizable species because the local electric fields are inhomogeneous. Therefore, electric dipoles like zwitterionic amino acids and peptides or any anisotropically polarizable species are oriented and move dielectrophoretically⁷ in the direction of higher field strength, thus approaching the fixed charges (see Fig. 1).

Extending Guggenheim's definition of an electrochemical potential for ions,⁶ we may introduce a *dielectrochemical potential*, $\hat{\mu}_j$, for the dipolar species j according to

$$\hat{\mu}_j = \mu_j - N_A \int_0^E \langle m_j \rangle dE \quad (8)$$

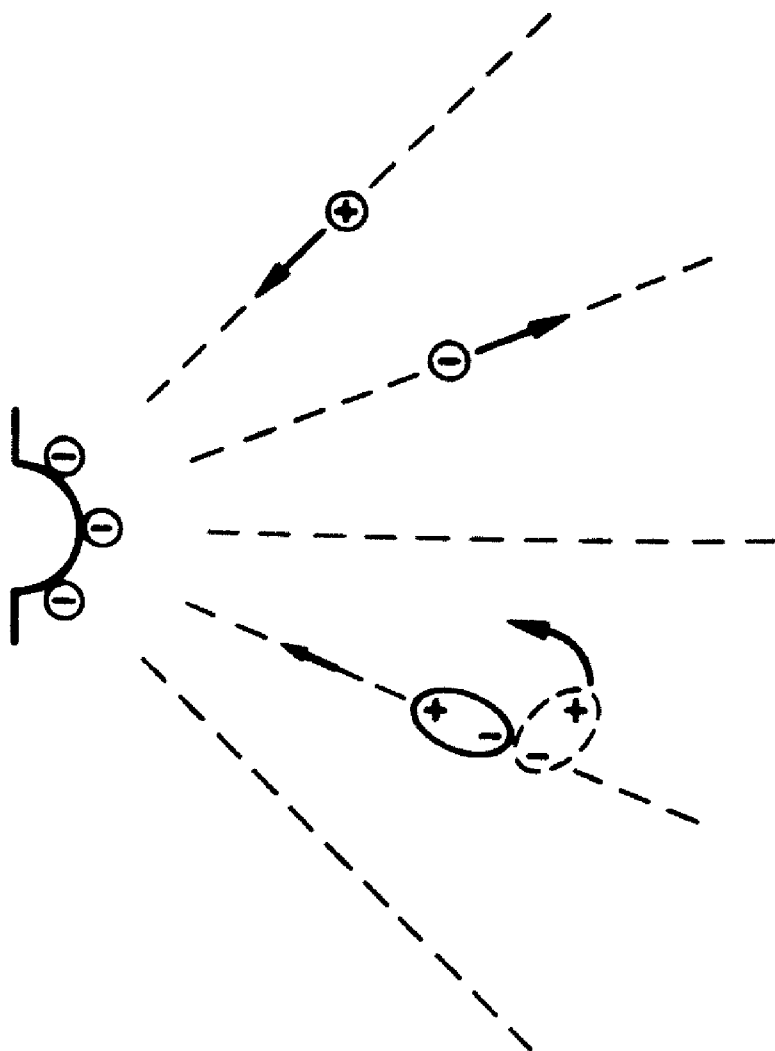


FIGURE 1 The inhomogeneous electric field originating from fixed charges (for instance, carboxylate or phosphate groups) affect ionic and dipolar species. Counter-ionic molecules are attracted, co-ions are repelled; dipolar species are oriented and then move dielectrophoretically in the direction of higher field intensity.

where $\langle m_j \rangle$ is the orientational average of the (total) electric dipole moment including permanent and induced components in species j , and E is the local electric field interacting with the dipolar species. The driving force for *dielectrodifffusion* is the gradient, $\nabla \hat{\mu}_j$, of the dielectrochemical potential which, by definition, contains the electric field force term. It is obvious that dielectrodifffusion (dielectrophoresis) is faster for larger dipole moments. The rate of dielectrodifffusion is given by

$$v_{\text{dielectr}} = \frac{D}{RT} (-\nabla \hat{\mu}_j) \quad (9)$$

where $\nabla\hat{\mu}_j = \nabla\mu_j - N_A \cdot \nabla \int_0^f \langle m_j \rangle dE$.

It is remarkable to see that the dielectrochemical potential also describes the observation that the binding of dipolar or polarizable molecules to membrane components may be dependent on the membrane potential $\Delta\psi_m$ or the effective electric field E_m of the membrane at the site of binding. In the binding equilibrium the dielectrochemical potentials of the dipolar ligands $\hat{\mu}_j = \mu_j - N_A \times \int \langle m_j \rangle dE$ in the membrane phase $\hat{\mu}_j(m)$ is equal to that $\hat{\mu}_j(b)$ of the bulk. Because in the bulk $E_m = 0$, $\hat{\mu}_j(b) = \mu_j(b)$. For the equilibrium case, where $\hat{\mu}_j(m) = \hat{\mu}_j(b)$ we obtain

$$0 = \Delta\mu_j^0(m,b) + RT \ln[\bar{a}_j(m)/\bar{a}_j(b)] - N_A \int \langle m_j \rangle dE$$

The electric field dependence of the equilibrium ratio $\gamma_j = \bar{a}_j(m)/\bar{a}_j(b)$ (membrane bound versus free) is given by

$$\frac{\bar{a}_j(m)}{\bar{a}_j(b)} = \exp\left[\frac{-\Delta\mu_j^0(m,b)}{RT}\right] \cdot \exp\left[\frac{1}{kT_0} \int_0^{E_m} \langle m_j \rangle dE\right]$$

or (10)

$$\gamma_j(E) = \gamma_j(0) \cdot \exp\left[\frac{1}{kT} \int_0^{E_m} \langle m_j \rangle dE\right]$$

These electrodiffusive and dielectrodiffusive features of ionic and dipolar ligands in polyionic electric fields indicate a kind of *preselection potential*. Counterionic and dipolar species are already preferentially "selected" over neutral ligands, *before* the actual association-dissociation steps take place at the binding site. In the next section it is shown that this preselection phase finds its physical expression as an enhancement of the rate of the diffusional encounter process, as well as in an increase in the lifetime of the encounter complex.

RATE CONSTANTS

For neutral species, the rate constant k_1 of the diffusional encounter [see Eq. (1)] is given by^{4,8,9}:

$$k_1 = \Omega N_A \cdot 10^{-3} (D_L + D_B) d_{L \cdot B} \text{ M}^{-1} \text{ s}^{-1} \quad (11)$$

where Ω is the space angle of diffusional approach, D_L and D_B are the diffusion coefficients of L and B and $d_{L \cdot B}$ is the distance between the centers of mass of L and B . For spherical particles $\Omega = 4\pi$, whereas for membrane and protein surfaces the approximation $\Omega = 2\pi$ is appropriate.¹⁰

The association rate constant of an ionic encounter process contains an electrostatic factor.^{11,4} In the limit of zero ionic strength the association rate constant is given by

$$k_1^0 = \Omega N_A \cdot 10^{-3} (D_L + D_B) d_{L \cdot B} \phi_1^0 \quad (12)$$

where the electrostatic factor

$$\phi_1^0 = \frac{\eta}{e^\eta - 1} \quad (13)$$

is determined by the ratio η between electrostatic and thermal energies:

$$\eta = \frac{z_L z_B e^2}{4\pi\epsilon_0 \epsilon d_{L \cdot B} kT} \quad (14)$$

If in a counterionic association ($z_A > 0$, $z_B < 0$) the inequality $|z_A z_B| \gg 1$ holds, then $|\eta| \gg 1$ and $\phi_1^0 \approx \eta$. Substitution of Eqs. (13) and (14) into Eq. (12) yields⁵

$$k_1^0 = \Omega N_A \cdot 10^{-3} (D_L + D_B) \frac{|z_L z_B| e^2}{4\pi\epsilon_0 \epsilon kT} \quad (15)$$

where the encounter distance has dropped out. Therefore, if the charge number product $|z_L z_B|$ is large (say ≥ 5), then the isothermal

diffusional approach is directly proportional to, and solely determined by, the charges. The rate enhancement may reach a factor of up to 10.¹²

The corresponding expression for the dissociation rate constant, k_{-1} , of the encounter complex between oppositely charged species shows that the charge effects decrease the value of k_{-1} , thus increase the lifetime $t_e = 1/k_{-1}$.⁴

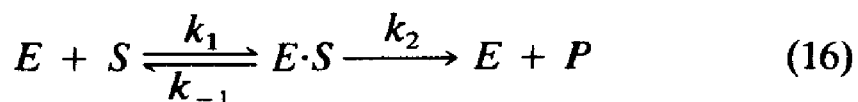
At finite ionic strengths, charge screening of course reduces the field effects between interacting ionic species. Polyionic field effects are indicated by particularly large variations of thermodynamic and kinetic constants with ionic strength. On the other hand, quantitative relations between equilibrium and rate constants and the ionic strength may be used to estimate the effective charge number z_B of binding sites with ligands of known charge number z_L .^{5,13}

A particularly instructive example of the determination of the effective macromolecular charge via ionic strength dependences is the nerve enzyme acetylcholinesterase which is discussed in the following section.

When the substrate is a dipole the term ϕ_1^0 in Eq. (12) measures the energy integral of the dipole moment/electric field interaction [see Eq. (9)]. Here, too, the dependence of k_1 on ionic strength may be used to determine surface charge effects.

POLYIONIC FIELD EFFECT IN ENZYMES

In the framework of the Michaelis–Menten formalism for the parametrization of enzyme catalysis in terms of the Michaelis constant K_m and the catalytic turnover constant k_{cat} ,



where E represents the enzyme active site, S is the substrate and P the product, the optimum condition for the catalytic turnover may be approximated by¹²:

$$k_1 c_S \approx k_{-1} \approx k_2 \approx k_{cat} \quad (17)$$

The condition $k_2 \approx k_{-1}$ physically means that there is no (unnecessary) accumulation of substrate in intermediate complexes. For conditions where $k_1 c_S < k_2$, the turnover of substrate into product, k_2 , is determined by the influx of the substrate, $k_1 c_S$, which itself depends on substrate concentration c_S and the bimolecular rate coefficient k_1 .

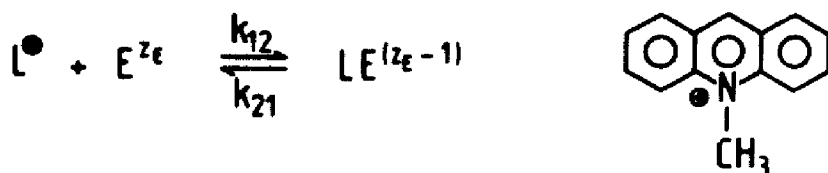
As outlined above, ionic and dipolar substrates and a polyionic active site are favorable properties for a rapid substrate association and selection due to intrinsic molecular electric field effects.

If the enzyme is membrane-bound or associated with cytoskeletal fiber structures, the substrate may at first be loosely bound to (unspecific) peripheral surface sites and then diffuse along the surfaces to the enzymatically active site. This reduction of three-dimensional diffusion to two-dimensional surface diffusion may enormously enhance the effective association rate of a substrate.¹²

Acetylcholinesterase

The catalytic parameters of acetylcholinesterase (E.C. 3.1.1.7) from electric eel are known to be strongly dependent on ionic strength.^{13,14} The enzyme itself can be isolated as a globular protein of relative molar mass of about 290,000 (11 S) with an isoelectric point of $pI = 4.5$. The protein is thus anionic under experimental conditions of pH 7 to 8. The turnover constant for the catalytic decomposition of the natural substrate acetylcholine in 0.1 M NaCl, pH 8 and 298 K is $k_{cat} = 1.6 \times 10^4 \text{ s}^{-1}$; the 11 S molecule has four apparently independent, catalytically active sites.

In order to explore ionic-electrostatic aspects of substrate binding, fluorescent nonsubstrates can be used, which bind specifically to the catalytic sites and are cations like acetylcholine, but are not hydrolyzed. Particularly suited is the compound N-methylacridinium (Fig. 2), the fluorescence of which is totally quenched when bound to the enzyme, thus providing an optical signal to monitor very rapid concentration changes. Primary kinetic data are the relaxation spectra caused by very rapid temperature jumps (of $\approx 3.3 \text{ K}$) in solutions of enzyme and fluorescent ligand at various ionic strengths. Relaxation times and amplitudes have been analyzed in terms of total (analytical) concentrations of ligand and protein.¹⁵



$$\lg k_{12} = \lg k_{12}^0 + \frac{2A z_E z_L \sqrt{I_c}}{1 + B\bar{a} \sqrt{I_c}} + C \cdot I_c$$

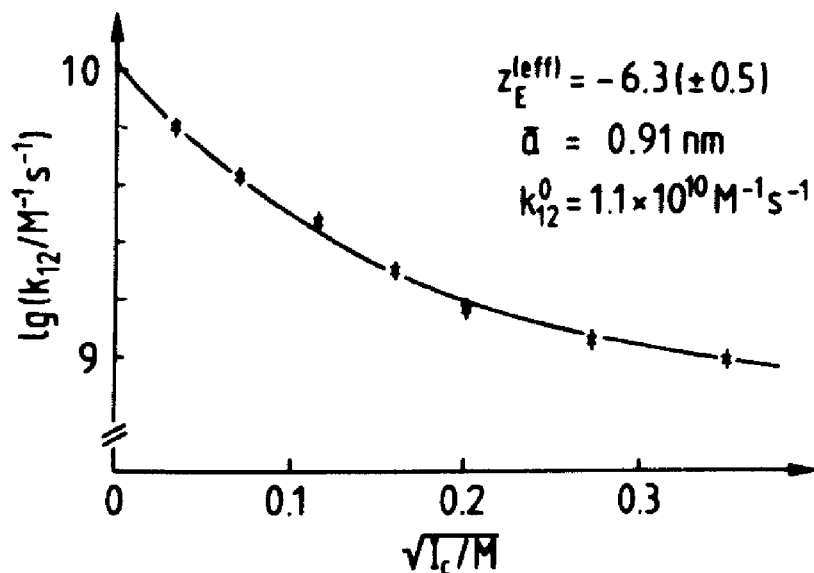


FIGURE 2 Ionic strength (I_c) dependence of the association rate constant k_{12} of the equilibrium of electric eel AcChE and N-methylacridinium, analyzed in terms of the semi-empirical Brønsted-Debye-Hückel equation resulting in $k_{12}(I_c \rightarrow 0) = k_{12}^0$, the numerical value of the effective point charge equivalent z_E of an enzyme active site and the approach distance $\bar{a} = d_{L-B}$ (see text).

A key result of the relaxation kinetic study is that the observed relaxations are bimolecularly controlled throughout the whole concentration range of ligands. Thus the overall scheme $L + B \rightleftharpoons LB$ applies and may be used to estimate the effective charge controlling the approach of the ligand to an active center. In the simplest case, the four active centers of the enzyme macromolecule considered here are treated as single, freely diffusible reaction centers with reduced diffusion coefficients ($D_E \ll D_L$). We therefore apply the semi-empirical Brønsted-Debye-Hückel equation (Fig. 2).

It is seen in Fig. 2 that the bimolecular rate constants between 10^{10} and $10^9 \text{ M}^{-1} \text{ s}^{-1}$ are unusually high for enzyme-ligand interactions. In addition, the association rate constants are very strongly dependent on the ionic strength of the solution. An increase in

the ionic strength, I_c , from 1 mM to 100 mM decreases the association rate constant by a factor of about 10. The dissociation rate constant $k_{21} = 152 \pm 10 \text{ s}^{-1}$ is practically independent of I_c .¹³

The experimental values of k_{12} have been analyzed according to the expression for $\log k_{12}$ given in Fig. 2. The relationship between k_{12} and the ionic strength, I_c , is *semi-empirical*⁴; it is valid for the assumptions involved in the extended Debye-Hückel approximation of the activity coefficients. At the experimental temperature $T = 298 \text{ K}$, we have $\epsilon = 79$, $A = 0.509 \text{ M}^{-1/2}$, and $B = 0.329 \times 10^{10} \text{ M}^{-1/2} \text{ m}^{-1}$; \bar{a} is now the mean distance of closest approach between the enzyme active site E and the counterions, $z_L = +1$ is the charge number of ligand N-methylacridinium and $z_B = z_E$ is the effective charge number associated with a ligand binding site of the macromolecule.

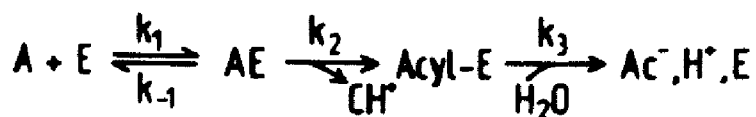
Treating the data in terms of the total concentration of active sites, least-squares analysis yields:

$$k_{12}^0 = 1.1 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1},$$

$$z_E e = -10.08 \pm 0.8 \times 10^{-19} \text{ C}, \quad \bar{a} = 0.91 \text{ nm}$$

Because for acetylcholinesterase there is no evidence for polyvalent charged groups, $|z_E| = 6.3(\pm 0.5)$ is the effective number of monovalent anionic groups involved in the association of cationic ligands to the active site. It is now of physiological importance that virtually the same ionic strength dependence is observed for a catalytic parameter proportional to k_{12} of acetylthiocholine, $k_{\text{cat}}/K_{\text{app}}$, a substrate whose structure and kinetic properties are very similar to those of acetylcholine¹³; K_{app} is the apparent Michaelis-Menten constant (Fig. 3).

The value $z_E = -6.3(\pm 0.5)$ aggravates the problems involved in viewing the macromolecular charges around an active site as an equivalent point charge. Furthermore, the number values of k_{12} classify the reaction as close to diffusion controlled. Hence coupling between the chemical relaxation and the relaxation of the ionic atmosphere must actually be taken into account; this is, however, still an unresolved theoretical problem.^{4,16} It must be assumed that the relatively high concentrations ($>1 \text{ mM}$) of inert strong electrolytes, compared to the μM concentrations of L and



$$\frac{k_{\text{cat}}}{K_{\text{app}}} = \frac{k_1 \cdot k_2}{k_{-1} + k_2} < k_1$$

$$k_{\text{cat}} \approx k_3 = 10^4 \text{ s}^{-1}, k_2 \approx 10^5 \text{ s}^{-1} \rightarrow k_1 > 2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1} (0.1 \text{ M}, \text{pH} 7; I_c = 0.1 \text{ M})$$

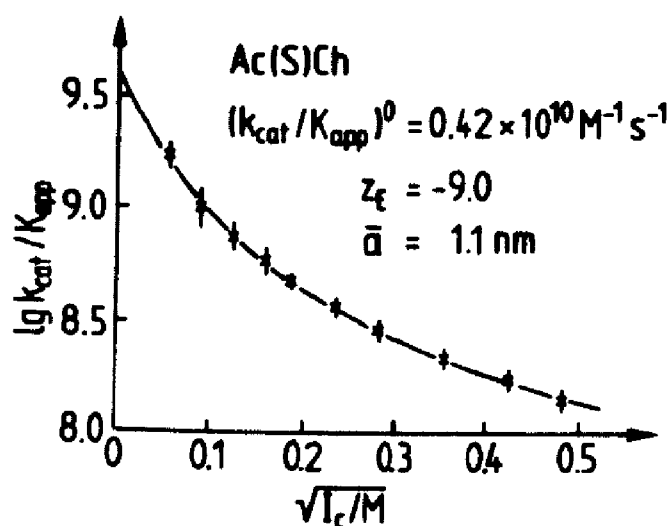


FIGURE 3 Ionic strength dependence of the ratio $k_{\text{cat}}/K_{\text{app}}$, according to the acylation-deacylation scheme, proportional to k_1 .

E , provide sufficient electrostatic screening to permit estimation of the activity coefficients for the nonequilibrium states during the relaxation in the same formal way as for equilibrated ionic atmospheres. Therefore the value of z_E must be viewed as an approximation. The number value clearly refers to one active site, but it includes contributions of the entire macromolecule; the relatively low isoelectric point of 4.5 suggests a considerable net negative charge at pH 7.

The value of $k_{12}^0 = 1.1 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$, at zero ionic strength, for N-methylacridinium is the highest reported for the interaction of a small ligand with a specific protein binding site.¹⁷

Enzyme Surface Diffusion

The comparatively large values of k_{12} observed with cationic ligands and acetylcholinesterase led to the suggestion that an enzyme surface area larger than the ligand binding site itself is effective in

trapping a ligand in the encounter complex.¹⁵ This larger surface area might include peripheral anionic sites from which a ligand would move to the active site by surface diffusion. The high negative charge number z_E supports this concept. The charged groups contributing to an effective charge of about 6 would be expected to be dispersed over an enzyme surface area greater than the immediate catalytic site. In any case, from Eq. (15) it is apparent that the size of the encounter cross section represented by d_{E-L} becomes irrelevant if the charge number product $|z_E \cdot z_L|$ becomes as high as 6.

Recently, Hofer *et al.*¹⁸ have confirmed that the alkali metal ion (Na^+ , K^+) effect on the catalytic activity of *Torpedo marmorata* acetylcholinesterase is a pure ionic strength effect which can be described up to $I_c = 25$ mM with the extended Brønsted-Debye-Hückel equation; Ca^{2+} exerts a more specific effect.^{13,18} It should be mentioned, however, that it is a *rate coefficient* which is proportional to the ratio $k_{\text{cat}}/K_{\text{app}}$, and *not solely* k_{cat} which is subject to the I_c dependence! The I_c dependence of $k_{\text{cat}} \approx k_3$ is *different* from that of $k_{\text{cat}}/K_{\text{app}}$ because k_3 is a dissociation rate constant.⁴

According to Smissaert,¹⁹ who has measured the NaCl effect on bovine erythrocyte acetylcholinesterase, Na^+ binds specifically to this enzyme, associated with the (unusually high) association constant $\approx 150 \text{ M}^{-1}$ (!). The data on electric eel and *Torpedo* acetylcholinesterase cannot be satisfactorily analyzed as a specific ion effect.^{13,18}

The data of Hasinoff²⁰ suggest that, indeed, surface diffusion may be responsible for the measured dependence of $k_{\text{min}} = k_1 \cdot k_2 / (k_2 + k_{-1})$; this results from a short form of the actual, more complicated reaction scheme. In Hasinoff's approach it is assumed (*ad hoc*) that about 1% of the enzyme surface is involved in the actual binding of the substrate. The ionic strength dependence is solely associated with a dissociation rate constant. Among the various examples of surface diffusion effects is the enzyme cytochrom *c*.²¹

Meanwhile there is appreciable progress in the theory of bimolecular processes such as substrate-enzyme encountering.²¹⁻²⁷ In particular the formalisms developed by Shoup and Szabo²⁷ and Berg and Ehrenberg²⁶ appear to be promising new tools for more realistic analyses than those applied up to now. An impressive example of facilitated diffusion has been recognized in the repressor-operator DNA system by von Hippel *et al.*²⁸

At present, attempts have been started to chemically change the number of charged groups on the enzyme surface (A. Aaviksaar and E. Neumann, in preparation) in order to test the model at various values of z_E .

The actually available data and analysis of acetylcholinesterase lead to the conclusion that the high bimolecular association rate constants and the unusually strong ionic strength dependence of the kinetic and the thermodynamic parameters have their physical origin in a predominantly anionic surface structure of this enzyme. Physiologically, the polyionic enzyme acetylcholinesterase appears to be a powerful electrostatic sink for trapping and decomposing the acetylcholine cation, A . The maximum rate with which the hydrolysis products of acetylcholine can appear is $k_{\text{cat}} = 1.6 \times 10^4 \text{ s}^{-1}$ at 298 K and $I_c = 0.1 \text{ M}$. This high turnover is actually only achieved when the condition $k_{12}[A] > k_{\text{cat}}$ holds. If for acetylcholine k_{12} is indeed $\approx 10^9 \text{ M}^{-1} \text{ s}^{-1}$ the activator concentration, $[A]$, may decrease to 10^{-5} M , yet an efficient hydrolytic removal of acetylcholine is guaranteed. This physiological aspect is discussed elsewhere in the context of molecular processes involved in neuronal information transfer.^{29,30}

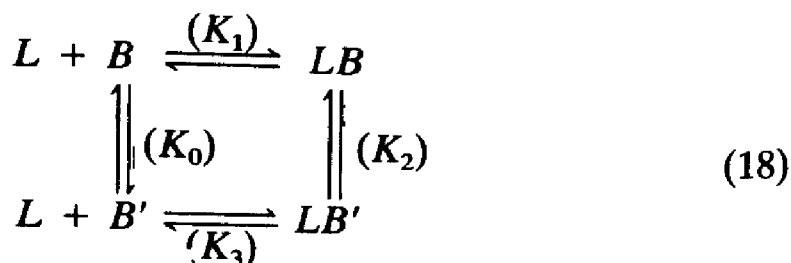
SEQUENTIAL SELECTION PROCESSES

If several chemically similar ligands compete for a macromolecular binding site in the same reaction compartment, recognition and selection of the correct ligand is of great functional importance. The effective ligand association may then involve a sequence of consecutive elementary steps during which more and more subsites of the ligand make contact with subsites of the macromolecular binding pocket. The effective rate constant of ligand binding is therefore a net quantity composed of the rate constants of all the steps involved. The lifetimes of the intermediary complexes are usually different for different ligands. Only if the next contact occurs within the lifetime of a given configuration will the ligand gradually be bound more tightly and selected in preference to ligands with weaker subsite binding. Of course, the necessary association-dissociation sequence between the contacted subsites requires time. The advantage of selection and discrimination has to be paid for by a smaller effective rate (constant) of ligand binding.

The binding of the neuroactivator acetylcholine to the acetylcholine receptor appears to be an example of such a sequential association via several subsites. The overall association rate constant for the effective binding of acetylcholine to the isolated protein is $k_1(R) = 2.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and the lifetime associated with this process is about 7 ms.³⁰⁻³² The number value of $k_1(R)$ suggests that it is a complex rate coefficient involving several, more rapid elementary steps.

CONFORMATIONAL SELECTION MODELS

A very instructive dynamic selection principle is observed in cases where a functionally active conformation, B' , of a macromolecular binding site increases in concentration only when a substrate or an activatory ligand is bound. In this case the minimum reaction scheme comprises two structural isomerizations, $B \rightleftharpoons B'$ and $LB \rightleftharpoons LB'$, and two bimolecular reactions according to the cyclic reaction scheme:



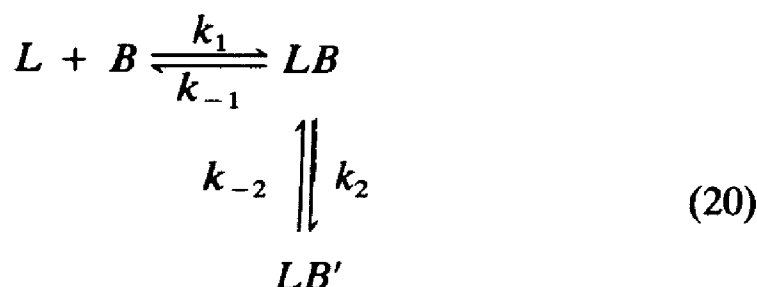
where in the absence of L the structural equilibrium is shifted far to the side of B , i.e., $\bar{c}_{B'} \ll \bar{c}_B$. If the affinity of L to structure B' is greater than to the conformation B , i.e., $K_1 \gg K_3$, then the ligand displaces the previous unfavorable equilibrium such that

$$\bar{c}_{LB'}/\bar{c}_{LB} = (\bar{c}_{B'}/\bar{c}_B)K_1/K_3 > \bar{c}_{B'}/\bar{c}_B \quad (19)$$

The distribution of states in the presence of the ligand is now determined by $K_2 = K_0K_1/K_3 > K_0$ and not by K_0 alone. The general reaction scheme (18) for a two state model includes two limiting cases, because the concentration of the complex LB' can increase by two pathways: by direct binding of L to B' and via the complex LB .

Induced Fit Model (Ref. 33)

If L binds selectively to the conformation B , scheme (18) reduces to



where the structural isomerization $LB \rightleftharpoons LB'$ is frequently slower than the bimolecular relaxation.

An important consequence of this model is that the ligand can only leave the binding site after the return of the conformation LB' to the conformer LB ; i.e., from the conformation LB .³⁴

It should be mentioned that many enzymes and receptors, B , have evolutionarily adjusted the rate constant k_2 characterizing the flux $LB \rightarrow LB'$ such that $k_2 \approx k_1 c_L$, the rate of ligand influx $L + B \rightarrow LB$, where $k_1 \approx 10^8 \text{ M}^{-1} \text{ s}^{-1}$ is usually determined by the diffusion limit. For instance, if the substrate concentration is $c_L = 10^{-4} \text{ M}$ then $k_2 \approx k_1 \cdot c_L = 10^4 \text{ s}^{-1}$. In this way no "waste" in terms of accumulation or depletion of L in the complex LB occurs.

Selective Binding to the Low Concentration Conformers

If the ligand has a sufficiently high affinity to the conformation B' only, this structure increases in concentration by selective binding of L to B' , according to the alternate limiting case:



Again, the structural isomerization $B \rightleftharpoons B'$ is usually rate-limiting. In the scheme (21), the ligand leaves the binding site on the same pathway on which it entered. Of course, any displacement of the bimolecular step is coupled to a reequilibration of the intramolecular process.

In the framework of scheme (18), which contains the two limiting cases (20) and (21), molecular recognition and selection is an at least two-phase sequence: ligand binding and conformational change. If the physiological ligand binds, the functionally relevant conformational transition may proceed along two pathways. An inhibitory ligand may only bind to the *B*-structure with no shift of the system to the set of alternate conformations *B'* and *LB'*.

The kinetics of reaction scheme (18) are characterized by three relaxation modes; not all of them will usually be resolvable on the time scale. If only two relaxation modes are observed, then one of the two limiting cases may apply. Indeed, the induced-fit model and the case of selective binding to a conformational state of low concentration may be differentiated by kinetic methods. If only the concentration of bound ligand can be monitored, it is not possible to differentiate between the two models by thermodynamic techniques. Actually there are numerous ligand-binding studies which have been analyzed in terms of the induced-fit model, *ad hoc*. A few examples exist for the selective binding model.³⁵

The key to the mechanism of ligand mediated structural changes is the ligand dependence of both relaxation times and amplitudes. For instance, the ligand dependence of the relaxation time of a slower isomerization coupled to a more rapid binding step according to the induced-fit model is opposite to that of the selective binding; see, e.g., Refs. 3 and 34. One of the few examples where a clear-cut decision between the two alternate models was possible is the structural transition from trypsinogen to trypsin³⁶; see, also, Lancet and Pecht (1976).³⁷

In this context it is noted that the allosteric control of catalytically active structures is an important feature of enzyme function. This type of regulation appears to be involved in the conversion of trypsinogen to trypsin. The kinetics of such a structural conversion have been investigated by temperature-jump relaxation spectrometry, using the absorbance change at 280 nm associated with the interaction between p-guanidinobenzoate-trypsinogen and the isoleucine-valine dipeptide.³⁶ In detail, the dipeptide binds to its binding site and induces a conformation change that allosterically affects the substrate binding site which is some distance away from the dipeptide association pocket.

The concentration dependence of the relaxation parameters of this system is consistent with the conventional induced-fit model: rapid ligand binding coupled to a slower intramolecular change; some alternative mechanisms can be excluded. At 296 K, 0.1 M Tris·HCl, pH = 7.4, the dissociation equilibrium constant for the overall process is $\bar{K} = K_1(1 + K_2)^{-1} = 5.1 (\pm 0.2) 10^{-5}$ M; for the actual binding step $K_1 = 2.3 (\pm 0.3) 10^{-3}$ M and the rate constants for the structural change are $k_2 = 36 (\pm 6) \text{ s}^{-1}$ and $k_{-2} = 0.61 (\pm 0.4) \text{ s}^{-1}$; the overall dissociation reaction enthalpy is $\Delta H^0 = 26 \text{ kJ mol}^{-1}$ and the reaction entropy is $\Delta S^0 = 4 (\pm 20) \text{ J K}^{-1} \text{ mol}^{-1}$.³⁶

In combination with CD and x-ray crystallographic data of Bode and Huber,³⁸ the results of the kinetic study suggest that the binding of the dipeptide to a trypsinogen-like, partially disordered conformation *induces* a transition to a trypsin-like highly ordered structure. The dominant pathway for the dissociation of the complex therefore involves the *return* of the trypsin structure into the trypsinogen conformation *before* the dipeptide can leave the protein.

CONCLUSIONS

In this Comment on molecular recognition the kinetic aspects of molecular recognition and selection and the novel concept of electric preselection of ionic and dipolar ligands have been developed. With respect to enzyme catalysis, the scheme given in Fig. 4 appears to be a physically more realistic description of enzyme catalysis, where a nonequilibrium distribution of substrate and product $S \rightleftharpoons P$ is coupled to a conformationally flexible enzyme. The enzyme should exhibit at least two sets of different conformation substrates ($E \rightleftharpoons E'$), the one set favoring the binding of substrate and the other set associated with a higher affinity to the product. Hence, a kind of reaction cycle results.⁴ If products and substrates are sufficiently different in charge and dipole moment, a sort of preselection can already occur before binding. The ultimate recognition and selection certainly involves transient association and dissociation steps between subsites of the ligand and the active site.

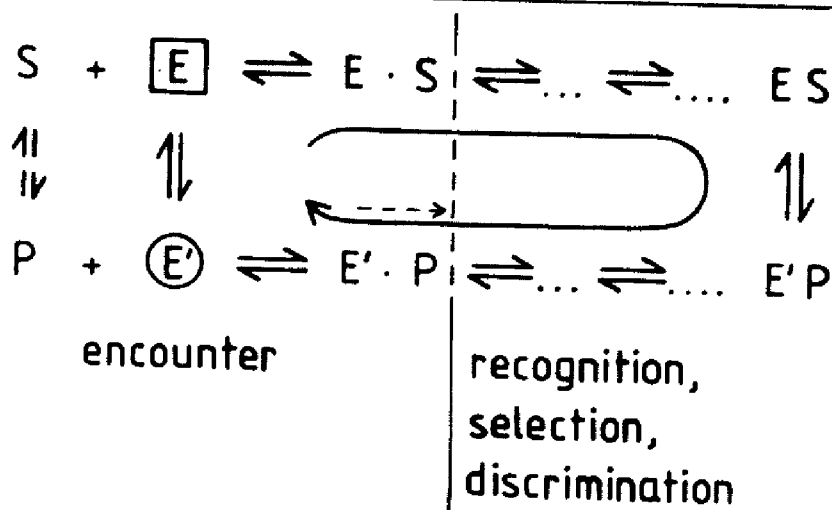
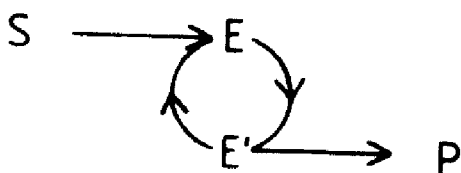
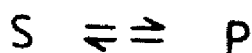


FIGURE 4 Enzyme catalysis schematically viewed as the coupling of an unfavorable distribution of substrate, S , and product, P , onto a structural enzyme equilibrium $E \rightleftharpoons E'$, where E favors the association with S , and E' associates with higher affinity with P . In the encounter phase ionic and dipolar preselection occurs, whereas the specific recognition and selection processes require transient, multiphasic association and dissociation steps between the subsites of the reaction partners.

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