Bioelectrochemistry and Bioenergetics, 8 (1981) 89—101
A section of *J. Electroanal. Chem.*, and constituting Vol. 128 (1981)
Elsevier Sequoia S.A., Lausanne — Printed in The Netherlands

412 — POLYELECTROLYTE FIELD EFFECTS *

EBERHARD NEUMANN and HANS-JÜRGEN NOLTE
Max-Planck-Institut für Biochemie, D-8033 Martinsried/München (G.F.R.)
(Manuscript received June 14th 1980)

SUMMARY

Polyelectrolyte field effects are indicated by particularly large variations of thermodynamic and kinetic constants with ionic strength. Some fundamental principles of local electric field effects in the microenvironment of polyelectrolyte structures are discussed, aiming at a reliable analysis of shifts in equilibrium and rate constants of ionic reaction partners with ionic strength. It is shown that the analytical expressions, within certain limitations, are suitable to determine effective charges involved in polyionic field effects on ionic reactions in the immediate neighborhood of a polyelectrolyte structure. An instructive example for such an observation is the neuro-enzyme acetylcholinesterase. The results of a relaxation—kinetic titration of this anionic enzyme (which hydrolyzes the cationic neuro-activator acetylcholine) with a cationic ligand suggest that the micro environment of the enzyme-active site consists of at least six anionic groups. A large effective, negative charge number is also reflected in the comparatively large association rate coefficients.

These results suggest that an enzyme surface area considerably larger than the ligand binding site itself is effective in trapping a cationic ligand. This larger surface area may include peripheral anionic sites from which ligand would move to the active site by surface diffusion.

INTRODUCTION

A considerable proportion of biological cell components are macromolecules, carrying electrical charges and interacting in aqueous environment containing low-molecular-weight ionic, dipolar or neutral species. The ionized groups of macromolecules (such as, for example, nucleic acids or proteins) which are either free in the cytoplasma or are an integral part of membranes and cytoplasmatic network structures, create strong electric fields in the immediate environment. These local fields influence not only the macromolecular conformation and interactions between polyions but also affect the local ionic milieu and chemical reactions which occur in the immediate vicinity of polyionic structures. Such polyionic field effects, charge screening and ionic compe-

* Invited lecture at the 5th International Symposium on Bioelectrochemistry, 3—8 September 1979, Weimar (D.D.R.)

0 302-4598/81/0000—0000/$02.50

tition will be especially pronounced if the density of the ionized groups is high and if all groups are of the same charge sign.

It is recalled that ionic—electrostatic features are generally encountered in reactions of ionic and dipolar reaction partners. If polyions participate in such reaction, either directly or indirectly, electrostatic contributions may become particularly large.

Ionic contributions to kinetic and equilibrium parameters of ionic and dipolar reactions are traditionally analyzed in terms of (electric) activity coefficients or of shifts in the apparent equilibrium constants (pK-shifts). Whereas activity coefficients are expressed in terms of a mean potential, equilibrium and rate constants may be readily formulated as a function of the electric field; see, e.g., Ref. 1. For polyions and polyelectrolytic environments, activity coefficients and pK-shifts are considerably larger than those of simple ions.

The prevalence of electrostatic interactions is usually indicated by a characteristic dependence of equilibrium and rate parameters on ionic strength. Analytically, variation of ionic strength may be used as a tool to investigate ionic properties of charged molecules, as well as electrostatic details of reactions between ionic or dipolar species. For instance, the quantitative analysis of equilibrium and rate constants as a function of the ionic strength mainly gives the effective charge of a macromolecular binding site and its environment.

The following account touches on some fundamental concepts for a practical and reliable analysis of pK-shifts and of rate constants from ionic strength dependencies; this analysis aims at the determination of effective charges involved in polyionic field effects. An instructive example for this type of analytical approach is the isolated neuro-enzyme acetylcholinesterase. The results of relaxation—kinetic titrations suggest that the micro-environment of the enzyme-active site has several anionic groups which create a local electric field equivalent to a point charge number of −6 to −7 [2]. Since the enzyme macromolecule has four active sites, the introduction of this charge number into the expression for the association rate constant suggests that perhaps the entire enzyme surface area is able to electrostatically trap cationic substrate acetylcholine, which is then channeled by "surface diffusion" to the active sites proper.

PRIMARY EFFECTS OF ELECTRIC FIELDS

The local electric fields originating from ionic structures are 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 field force by

$$\bar{E}(r) = -\nabla \bar{\psi}(r) \qquad (1)$$

The primary effect of electric fields on interacting reaction partners is fairly well understood [1]: orientation of dipolar species, deformation of polarizable systems (and subsequent orientation of induced dipoles) and movement of ionic species in the direction of the field vector. Less well explored is how these primary effects are specifically coupled to the various chemical transformations, such as conformational transitions, or dipolar and ionic association—dis-

sociation equilibria or steady states. In general, we know that polar structures tend to orient in the field direction; conformations or molecules with larger dipole moments increase in concentration at the expense of those configurations with smaller electric moments; finally, electric fields increase the dissociation of weak acidic and basic groups and promote the separation of ion pairs into the respective dissociated ions or ionic groups (second Wien effect).

A primary aspect of electric field effects, which is, perhaps, of general functional relevance for biopolymers and for biomembranes deserves particular attention. Here, because of steric restriction, ionic and dipolar subgroups of chain molecules have only restricted mobility for ion-pair separations and for orientational changes [3].

It is physically plausible that the strong electric fields of polyions with prevailing like charges accumulate counter-ions and repel co-ions. Therefore, the local ion concentrations (inclusively the local pH-value) in a polyelectrolyte microenvironment can differ considerably from that of the bulk solution. Furthermore, bimolecular reactions between molecules which are counter-ionic relative to the polyionic structure are accelerated, whereas reactions between co-ionic species are decelerated.

It appears that the large number of ionic reactions which have been investigated in the presence of dissolved polyelectrolytes, see, e.g., Ref. 4, can be analytically treated in the same way as the catalytic reactions of enzymes covalently coupled to polyelectrolyte networks, see, e.g., Refs. 5 and 6: the primary effects of the local electric fields causing concentration changes and orientational *fixations* of dipolar complexes, may either favor or disfavor the formation of the activated complexes between the ionic and dipolar species. Recently, Ise et al. provided evidence that dehydration is an important mechanistic factor in the electrostatic interaction between polyions and smaller ion complexes [4,7].

SIMPLE IONIC REACTIONS

The initial chemical reaction step of a large number of complicated processes in living organisms is the association of an ionic, low-molecular-weight ligand (substrate, hormone, transmitter, metal ions, etc.) to a specific macromolecular binding site B, on an enzyme or a receptor protein. Frequently the binding sites have one or more ionized groups of opposite charge sign compared to that of the ligand. Furthermore, such sites are often in the neighborhood of membrane surfaces or are surrounded by a network of microfilaments or other cytoskeleton structures.

The state of ionization of a macromolecular microenvironment will therefore influence ionic bimolecular reactions such as

$$L^{+} + B^{-} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} LB \qquad (2)$$

where, for simplicity, the charge numbers are taken as $z_L = +1$ and $z_B = -1$, and where k_{12} and k_{21} are the (apparent) association and dissociation rate constants respectively. The thermodynamic equilibirum constant K^0 can be expressed as a

product:

$$K^0 = K_c \Pi f \tag{3}$$

where

$$K_c = \Pi \bar{c}_j^{\nu_j} \quad \text{and} \quad \Pi f = \Pi f_j^{\nu_j} \tag{4}$$

are the apparent equilibrium constants and Πf is the ratio of the activity coefficients; $\bar{c}_j$ and ν_j are the equilibrium concentration and the stoichiometric coefficient of species j respectively. Applied to reaction (2) where all $|\nu_j| = 1$ we obtain:

$$K_c = \frac{\bar{c}_L \cdot \bar{c}_B}{\bar{c}_{LB}} = \frac{k_{21}}{k_{12}} \quad \text{and} \quad \Pi f = \frac{f_L \cdot f_B}{f_{LB}}$$

Generally, both K_c and Πf are dependent on the ionic strength I_c, defined by

$$I_c = \tfrac{1}{2} \sum_i c_i z_i^2 \tag{5}$$

where the sum covers all ionic species i.

It should be mentioned that the ions of a salt which is used to vary the ionic strength may compete with the ligand L for the same site B. In this case, the dependence on I_c of $K_c = K^0(\Pi f)^{-1}$ is, within the usual scatter of data points, often not discernible from the salt concentration dependence of a competition reaction with

$$K_c = K_c^0(1 + K_i^{-1}\bar{c}_i) = \frac{k_{21}}{k_{12}(1 + K_i^{-1}\bar{c}_i)^{-1}} \tag{6}$$

where K_i and $\bar{c}_i$ are the equilibrium constant and the concentration of a competing ion i, respectively; $K_c = K_c^0$ at $c_i = 0$. Therefore, proper choice of salt and buffer are necessary in order to differentiate between (unspecific) ionic strength effects and specific site binding of competing ions such as metal ions; see, e.g., Ref. 2. At higher salt concentrations and with di- and trivalent ions, specific site binding has to be taken into account.

Activity coefficients

The classical theoretical framework for the description of ionic strength dependencies of rate and equilibrium constants is the calculation of activity coefficients in terms of mean electric potentials $\bar{\psi}$ [8]. It is recalled that the theoretical expressions then cover only the *deviations* from the ideal Coulomb behavior, hence they only account for screening contributions (due to other ions). Therefore, extrapolation of K_c or k_{12} to zero ionic strength, where $\Pi f = 1$ yields the quantities K^0 and k_{12}^0 which contain the ideal, unscreened Coulomb terms.

This very important practical aspect will be outlined in slightly more detail by recalling that the electrochemical potential $\tilde{\mu}_j$ of an ion j is given by the chemical potential μ_j and an electrostatic term:

$$\tilde{\mu}_j = \mu_j + \int \bar{\psi}_j \, \mathrm{d}(z_j e) \tag{7}$$

charging process are from $z_j e = 0$ to the charge $z_j e$. For the isolated ion the integral is equal to $z_j e\bar{\psi}_j$. Following the definition:

$$\mu_j = \mu_j^0 + kT \ln c_j \tag{8}$$

where k is the Boltzmann constant and T is the absolute temperature, it is seen that all non-idealities (of short-range interactions) except for the purely electrostatic contributions are included in the standard chemical potential μ_j^0; $\mu_j^0 = \mu_j$ at $c_j = 1\ M$.

We may now introduce a standard electrochemical potential

$$\tilde{\mu}_j^0 = \mu_j + \int \psi_j^0 \, \mathrm{d}(z_j e) \tag{9}$$

as the limit value of $\tilde{\mu}_j$ at $c_i \to 0$, where

$$\psi_j^0 = \frac{z_j e}{4\pi\epsilon\epsilon_0 r} \tag{10}$$

is the ideal, unscreened Coulomb potential at the radial distance r from the charge center; ϵ_0 is the vacuum permittivity and ϵ is the dielectric constant. Inserting equations (8) and (9) into equation (7) yields:

$$\tilde{\mu}_j = \tilde{\mu}_j^0 + \int [\bar{\psi}_j(r) - \psi_j^0(r)] \, \mathrm{d}(z_j e) \tag{11}$$

The integral in equation (11) represents the contribution of the screening ion cloud to the potential of ion j at r in the presence of other ions; it therefore defines the (electric) activity coefficient f_j according to

$$kT \ln f_j' = \nu_j \int [\bar{\psi}_j(\bar{a}) - \psi_j^0(\bar{a})] \, \mathrm{d}(ez_j) \tag{12}$$

where $r = \bar{a}$ is the distance of closest approach of ions i to ion j; see, e.g., Ref. 8. At infinite dilution $\bar{\psi}_j = \psi_j^0$ and $f_j = 1$, as required.

Now, the Gibbs free energy change for a chemical reaction between ions j may be written as

$$\Delta G = \sum_j \nu_j \tilde{\mu}_j = \sum_j \nu_j \tilde{\mu}_j^0 + kT \ln \Pi f \tag{13}$$

where, according to equation (11),

$$\sum \nu_j \tilde{\mu}_j^0 = \sum \nu_j \mu_j^0 + \sum \nu_j \int \psi_j^0 \, \mathrm{d}(z_j e) + kT \sum \nu_j \ln c_j \tag{14}$$

and with equation (4), the decadic logarithm of Πf is given by

$$\log \Pi f = \frac{0.4343}{kT} \sum \nu_j \int [\bar{\psi}_j(\bar{a}) - \psi_j^0(\bar{a})] \, \mathrm{d}(z_j e) \tag{15}$$

At equilibrium where all $c_j = \bar{c}_j$, $\Delta G = 0$. Since $\Sigma\ \nu_j \ln \bar{c}_j = \ln K_c$ equations (13)—(15) lead to the expression:

$$-\frac{1}{kT}\left(\sum \nu_j [\mu_j^0 + \int \psi_j^0(\bar{a}) \, \mathrm{d}(z_j e)]\right) = \ln(K_c \Pi f) = \ln K^0 \tag{16}$$

in accord with equation (3). Equation (16) clearly shows that K^0 contains the ideal unscreened Coulomb potentials. Applying equation (10) to reaction (2), the unscreened Coulomb contribution is, as expected, given by

$$\sum \nu_j \int \psi_j^0(\bar{a})\, \mathrm{d}(z_j e) = \frac{z_L z_B e^2}{8\pi\epsilon_0 \epsilon \bar{a}} \tag{17}$$

Ionic strength dependence of Πf *and* K_c

If the reaction partners L and B are free spherical ions, then at low ion concentrations ($\leqslant 0.01\ M$) the general relationship of equation (15) may be very well approximated by the conventional Debye—Hückel expression which analytically relates Πf with I_c [8]. For higher ion concentrations c_i and higher charge numbers z_i, Monte-Carlo calculations and equivalent empirical approximations (using expressions for $\bar{\psi}_j$ in the presence of ions i which have the form of the Debye—Hückel potential but are corrected for electroneutrality [9]) have shown that it is usually *preferable to use semi-empirical extensions of the Debye-Hückel expression*; for a review, see Ref. 10. For ion concentrations not too high ($\leqslant 0.2\ M$), equation (15) may be applied to reaction (2) in the form:

$$\log \Pi f = \frac{2A_f z_L z_B \sqrt{I_c}}{1 + B\bar{a}_{LB}\sqrt{I_c}} + C \cdot I_c \tag{18}$$

where A_f and B are the Debye—Hückel constants for a given temperature and dielectric constant [8]. The last term on the r.h.s. of equation (18) is more correctly expressed in terms of the molality m_{LB} of the complex LB [10]. If, however, as very often encountered, $m_{LB} << I_c$, we may use equation (18) with C as an adjustable parameter.

Finally, the combination of equation (18) with equation (3) results in the practically applicable relationship:

$$\log K_c = \log K^0 - \frac{2A_f z_L z_B \sqrt{I_c}}{1 + B\bar{a}_{LB}\sqrt{I_c}} - C \cdot I_c \tag{19}$$

from which number values for the product $z_L \cdot z_B$, $\bar{a}_{LB}$ and C may be obtained.

pK-shift

Equation (3) may be rewritten as

$$\log \Pi f = \mathrm{p}K_c - \mathrm{p}K^0 = \Delta \mathrm{p}K \tag{20}$$

where the dependence of K_c on I_c is expressed as a pK-shift.

In the presence of ions competing with L for B, or vice versa, the measured total pK-shift, $\Delta \mathrm{p}K_T$ has two contributions:

$$\Delta \mathrm{p}K_T = \Delta \mathrm{p}K + \Delta \mathrm{p}K_c \tag{21}$$

where, according to equation (6),

$$\Delta \mathrm{p}K_c = \mathrm{p}K_c - \mathrm{p}K_c^0 = -\log(1 + K_i^{-1} c_i) \tag{22}$$

being zero for $c_i = 0$. Note that it is only the difference $\Delta \mathrm{p}K = \Delta \mathrm{p}K_T - \Delta \mathrm{p}K_c$ which can be analyzed according to equations (18) and (19).

Ionic strength dependence of kinetic constants

According to concepts of Brønsted and Bjerrum the association step $L^+ + B^- \rightarrow LB$ is viewed as

$$L^+ + B^- \rightleftharpoons X \rightarrow LB$$

where X is the activated complex; see e.g., Ref. 8. Analogous to Scatchard's treatment of the primary salt effect we may specify the apparent association rate constant by

$$k_{12} = k_{12}^0 \Pi f \tag{23}$$

where $\Pi f = f_L \cdot f_B / f_X \cong f_L \cdot f_B / f_{LB} \cong f_L \cdot f_B$; $f_X \cong f_{LB} = 1$, because X and LB are neutral.

According to Eigen (1954) a more realistic scheme for an ionic reaction comprises at least two steps [11,12]:

$$L^+ + B^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} L^+ \cdot B^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} LB \tag{24}$$

where the encounter complex $L^+ \cdot B^-$ represents an ion pair with still intact hydration shells (outer-sphere complex).

The ionic strength of the solution primarily affects the diffusional encounter step $L^+ + B^- \rightleftharpoons L^+ \cdot B^-$; at equilibrium,

$$k_1 = k_1^0 \Pi f \tag{25}$$

where the species $L^+ \cdot B^-$ is considered neutral.

Experimentally, the rate coefficients are determined from the concentration changes with time. The expression (25) applies only if during the changes in $c_j (j = L, B, LB)$, the activity coefficients can be considered constant. For small concentrations of j and small concentration changes δc_j (as encountered under chemical relaxation conditions), and if the activity coefficient are primarily determined by the presence of an inert salt, equations (23) and (25) may be applied; see, however, Ref. 12.

When the condition $c_{L \cdot B} \ll c_{LB}$ holds, the steady-state assumption applies and equations (2) and (24) are related by

$$k_{12} = \frac{k_1 \cdot k_2}{k_{-1} + k_2} \quad \text{and} \quad k_{21} = \frac{k_{-1} \cdot k_{-2}}{k_{-1} + k_2} \tag{26}$$

For $k_2 >> k_{-1}$, $k_1 \cong k_{12}$; in this case the overall complex formation is termed "diffusion-controlled".

Provided that the experimental conditions permit the use of equations (23) or (25), together with equations (18), then, for instance, from the expression:

$$\log k_1 = \log k_1^0 + \frac{2A_f z_L z_B \sqrt{I_c}}{1 + B\bar{a}_{LB}\sqrt{I_c}} + C \cdot I_c \tag{27}$$

effective charge z_B and $\bar{a}$, but also the adjustable parameter C may be determined.

Effective charge numbers from k_1^0

It should be mentioned that the absolute value of k_1^0 directly involves the charge numbers and for $|z_L \cdot z_B| >> 1$, permits a good estimate for this product. The theoretical expression for k_1^0 may be written as

$$k_1^0 = \frac{\Omega N_A}{10^3}(D_L + D_B)\, d_{L \cdot B} \cdot \phi_1^0 \tag{28}$$

where Ω is the solid angle of diffusional approach ($\Omega = 4\pi$ for spherically symmetric ions), N_A Avogadro's number, $D_L + D_B$ the sum of the diffusion coefficients of L and B and $d_{L \cdot B}$ the mean distance between the centers of L and B in the encounter complex; the electrostatic factor at $I_c = 0$,

$$\phi_1^0 = \eta(e^{\eta} - 1)^{-1} \tag{29}$$

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

$$\eta = \frac{z_B z_L e^2}{4\pi\epsilon_0 \epsilon d_{L \cdot B} kT} \tag{30}$$

Similarly, the dissociation rate constant is expressed [11,12] as:

$$k_{-1}^0 = \frac{3}{2d_{L \cdot B}^2}(D_L + D_B)\, \phi_{-1}^0 \tag{31}$$

where

$$\phi_{-1}^0 = \eta(1 - e^{-\eta})^{-1}$$

Introducing now equation (30) into equation (29), it is seen that for $|\eta| >> 1$, $\phi_1^0 \cong \eta$ and that equation (28) is reduced to the simple form

$$k_1^0 = \frac{\Omega N_A}{10^3}(D_L + D_B)\frac{|z_L \cdot z_B| e^2}{4\pi\epsilon_0 \epsilon kT} \tag{32}$$

where the encounter distance has dropped. Therefore, if the charge number product $|z_L \cdot z_B|$ is large, then the isothermal diffusional approach between L and B is determined solely by the charges. On the other hand, extrapolation of $k_1 = f(I_c)$ to $I_c = 0$ should permit a good estimate of $z_L \cdot z_B$ from equation (32).

POLYELECTROLYTE MICROENVIRONMENT

As outlined in the previous Section, the analysis of shifts in pK and rate constants with ionic strength is straightforward for small spherical ions. When, however, one of the reaction partners, say B, is a part of macromolecular or membraneous surface, it will generally be accessible only from one side; i.e. the solid angle of approach may be approximated by $\Omega = 2\pi$; see equations (28) and (32). For the same reason the ion cloud is certainly not spherical. A further degree of complexity appears when the neighborhood of site B is polyionic. As long as the exact position of the fixed charges is not known, any theoretical approach to describe details of a polyelectrolyte microenvironment remains highly approximate, see also Ref. 6. Formally, we may separate the observed pK-shift, ΔpK_T, into two contributions because the mobile ions as well as the

ionized fixed groups determine the mean potential $\bar{\psi}_B(\bar{a})$ at the site of interaction with the ligand L:

$$\Delta pK_T = pK_c - pK^0 + \Delta pK' \tag{33}$$

where

$$\Delta pK' = \frac{0.4343}{kT} z_L \cdot e\Delta\bar{\psi}_P \tag{34}$$

covers the *contribution* $\Delta\bar{\psi}_P$ of the polyelectrolyte environment.

Two limiting cases are now of particular interest.

At low bulk ionic strength the polyelectrolyte contribution may be considered dominant, therefore:

$$\Delta pK_T \cong \Delta pK' \tag{35}$$

and the local concentration c'_L of L may be expressed as

$$c'_L = \bar{c}_L \exp[-z_L e\, \Delta\bar{\psi}_P/kT] \tag{36}$$

It is readily seen that counter-ionic L is accumulated, $c'_L > \bar{c}_L$ relative to the bulk, and co-ionic L is repelled ($c'_L < \bar{c}_L$) from the microenvironment.

At high ionic strengths and if the average distance between the neighboring fixed charges and the site B is larger than $\bar{a}$, small counter-ions may screen the contribution $\Delta pK'$ to a large extent. Then:

$$\Delta pK_T \cong pK_c - pK^0 \tag{37}$$

may be analyzed according to equations (20) and (18). Indeed for immobilized enzymes it has been found that the polyelectrolyte contribution is practically non-existent at high ionic strengths [5].

In the case where pK-shifts and variations of rate constants are caused by the presence of linear polyelectrolyte structures, theory predicts that Πf depends on the logarithm of I_c rather than $\sqrt{I_c}$ at low ionic strength, see, e.g., Refs. 4, 13—15. Thus, the shape of the I_c-dependence may be used as an additional diagnostic tool.

If, however, large variations of equilibrium and rate parameters are more similar to a $\sqrt{I_c}$-dependence, then it is always tempting to start the analysis in the framework of equations (18), (19) and (27). An instructive example of this type of approach is the neuro-enzyme acetylcholinesterase which catalyzes the hydrolysis of the neuro-activator acetylcholine.

POLYIONIC FIELD EFFECT IN BIOCATALYSIS

The catalytic parameters of acetylcholinesterase (E.C. 3.1.1.7) from the electric eel are known to be strongly dependent on the ionic strength; see, e.g., Ref. 2. The enzyme itself can be isolated as a globular protein of a molecular weight of about 290 000 daltons (11 S), and has 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 $k_{cat} = 1.6 \times 10^4\ s^{-1}$; the 11 S molecule has four apparently independent, catalytically active sites.

In order to explore ionic—electrostatic aspects of substrate binding, fluorescent non-substrates 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, the fluorescence of which is totally quenched when bound to the enzyme, thus providing an optical signal to resolve very rapid concentration changes [16]. Primary kinetic data are the relaxation spectra caused by very rapid temperature jumps (of 3.3 K) in solutions of enzyme and fluorescent ligand at various ionic strengths. Relaxation times and amplitudes have been analyzed in terms of total concentrations of ligand and protein [16].

Results

A key result of the relaxation kinetic study is that the relaxations observed are bimolecularly controlled throughout the whole concentration range of ligands. Thus, the overall scheme of equation (2) applies.

It is seen in Fig. 1 that the bimolecular rate constants between 10^{10} and 10^9 M^{-1} 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 $k_{21} = 153 \pm 10$ s^{-1} is practically independent of I_c.

The experimental values of k_{12} have been analyzed according to equations (18) and (23); see also equation (27). At the experimental temperature $T = 298$ K, we have $\epsilon = 79$, $A = 0.509$ $M^{-1/2}$, and $B = 0.329 \times 10^{10}$ $M^{-1/2}$ m^{-1}; a is now the mean distance of closest approach between the enzyme active site E and counter ions; $z_L = +1$ 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 [2,16],

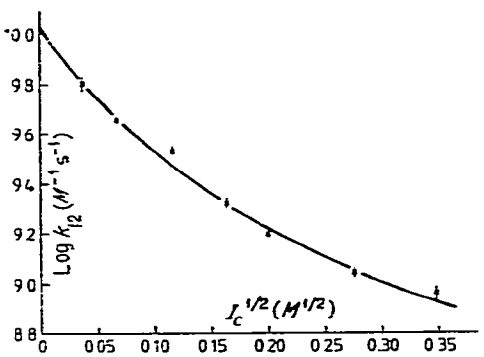

Fig. 1. Association rate constant k_{12} for the reaction of acetylcholinesterase with N-methylacridinium at pH 7 and 298 K, as a function of ionic strength, I_c (see Ref. 2).

least-squares analysis yields:

$k^0_{12} = 1.1 \times 10^{10}\ M^{-1}\ s^{-1}$, $z_E \cdot e = -10.08 \pm 0.8 \times 10^{-18}$ C, $\bar{a} = 0.91$ nm

Because for acetylcholinesterase there is no evidence for polyvalent charged groups, $|z_E| = 6.3$ (± 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_{cat}/K_{app}, a substrate whose structure and kinetic properties are very similar to those of acetylcholine [2]; K_{app} is the apparent Michaelis—Menten constant.

Discussion

The application of equation (27) to the acetylcholinesterase data requires some comment. The value $z_E = -6.3$ (± 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, equation (24), 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, a still unresolved theoretical problem [11,12]. It must be assumed that the relatively high concentrations (>1 mM) of inert strong electrolytes, compared to the μM concentrations of L and E, provide sufficient electrostatic screening to permit estimation of the activity coefficients for the non-equilibrium 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 which has to be considered with care for several reasons. 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. Furthermore, the possibility that the enzyme-active site may be partially buried in a hydrophobic area of lower dielectric constant could lead to an overestimate of z_E because both the constants A and B in equation (27) are inversely proportional to ϵ at the active site surface [2].

The value of $k^0_{12} = 1.1 \times 10^{10}\ M^{-1}\ s^{-1}$, at zero ionic strength, for N-methylacridinium is the highest reported for the interaction of a small ligand with a specific protein binding site. To assess the question of diffusion control the overall reaction has been analyzed in terms of scheme (24). Because the relaxation spectrum shows no evidence for a second relaxation with a respective characteristic dependence of time constant and amplitude on ligand concentration, $E^- \cdot L^+$ must be present only in very low steady-state concentration, and equations (26) can be applied. Further analysis continues with equations (28) and (29). Using $\Omega = 2\pi$, the estimate for the sum $D_E + D_L \cong D_L = 10^{-5}$ cm^2 s^{-1}, $e = 1.6 \times 10^{-19}$ C, $\epsilon_0 = 8.85 \times 10^{-12}$ C^2 J^{-1} m^{-1}, $\epsilon = 79$ and $kT = 4.12 \times 10^{-21}$ J at 298 K, $z_E = -6.3$ and $\bar{a} = d_{E \cdot L} = 0.91$ nm, we obtain $\eta = -4.9$, hence $\phi^0_1 = 4.9$ and finally $k^0_1 = 1.7 \times 10^{10}\ M^{-1}\ s^{-1}$.

Similarly ϕ^0_1 in equation (31) is 0.037 and hence $k^0_{-1} = 7 \times 10^7\ s^{-1}$.

Introducing now the number values of $k^0_{12} = 1.1 \times 10^{10}\ M^{-1}\ s^{-1}$ and of $k^0_{21} = 1.53 \times 10^2\ s^{-1}$ and those of k^0_1 and k^0_{-1} into equations (26), we find that $k^0_2 \geqslant$

(32), we estimate $|z_E| \geq 4$, constistent with the z_E value derived by equation (27).

It thus appears that the analytical treatment of the kinetic data of acetylcholinesterase according to equation (27) leads to a quite reliable estimate of the ratio $z_E e/\epsilon$. With $\epsilon = \epsilon_{H_2O}$, $z_E = -6.3$ (± 0.5) may be considered as a meaningful estimate for the effective charge of the active site.

The comparatively large values of k_{12} observed with cationic ligands 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 [2,16]. This larger surface area might include peripheral anionic sites from which the 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. From equation (32) it is apparent that the size of the encounter cross-section represented by $d_{E \cdot L}$ becomes in any case irrelevant, if the charge number product $|z_E \cdot z_L|$ becomes as high as 6.

In summary, we may conclude that the high bimolecular association rate constants and the unusually strong ionic strength dependence of kinetic and thermodynamic parameters have its physical origin in a dominantly 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. The maximum rate with which the hydrolysis products of acetylcholine can appear is $k_{cat} = 1.6 \times 10^4\ s^{-1}$ at 298 K and $I_c = 0.1\ M$. This high turnover is actually only achieved when the condition $k_{12}[A] \geq k_{cat}$ holds. If for acetylcholine k_{12} is indeed $\approx 10^9\ M^{-1}\ s^{-1}$ the activator concentration, [A], may decrease to $10^{-5}\ M$ yet an efficient hydrolytic removal of acetylcholine is guaranteed. This physiological aspect is further discussed in the context of molecular processes involved in neuronal information transfer [17].

It is finally remarked that the expressions in equations (19) and (27) respectively, appear to represent a useful framework to determine surface charges of globular polyionic systems by thermodynamic and kinetic titrations with proper ligands.

ACKNOWLEDGEMENT

We thank the Deutsche Forschungsgemeinschaft for financial support; grant NE 227.

REFERENCES

1 E. Neumann, Electro-optic Changes in Biopolymers — Chemical and Rotational Contributions, in: Electro-Optics and Dielectric Properties of Macromolecules and Colloids, B.R. Jennings (Ed.), Plenum Press, New York, 1979, pp. 233—245.

Mosbach (Ed.), 44 (1977) 397—443.
6 L. Goldstein, Y. Levin and E. Katchalski, Biochemistry, 3 (1964) 1913.
7 N. Ise, T. Maruno and T. Okubo, Polymer Bull., 17 (1978) 1.
8 G. Kortüm, Electrochemistry, Verlag Chemie, (1972) p. 178.
9 W. Olivares and D.A. McQuarrie, Chem. Phys. Lett., 46 (1977) 178.
10 K.S. Pitzer, Acc. Chem. Res. 10 (1977) 371.
11 M. Eigen, Z. Phys. Chem. N.F., 1 (1954) 176.
12 M. Eigen, W. Kruse, G. Maass and L. DeMaeyer, Progress in Reaction Kinetics, 2 (1964) 287.
13 A. Katchalsky, Pure Appl. Chem., 26 (1971) 327.
14 S. Lifson and A. Katchalsky, J. Polymer Sci., 13 (1954) 43.
15 G.S. Manning, Ann. Rev. Chem., 23 (1972) 117.
16 T.L. Rosenberry and E. Neumann, Biochemistry, 16 (1977) 3870.
17 E. Neumann, T.L. Rosenberry and H.W., Chang, in: Neuronal Information Transfer, A. Karlin et al., (Eds.), Academic Press, New York, (1978) pp. 183—210.