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Apparent charge of binding site in ion-translocating enzymes: kinetic impact

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Abstract Recently, we presented a general scope for the nonlinear electrical properties of enzymes E which catalyze translocation of a substrate S with charge number z_S through lipid membranes (Boyd et al. *J. Membr. Biol.* 195:1–12, 2003). In this study, the voltage sensitivity of the enzymatic reaction cycle has been assigned to one predominant reversible reaction step, i.e. the reorientation of either E or ES in the electric field, leaving the reorientation of the alternate state (ES or E) electroneutral, respectively. With this simplification, the steady-state current–voltage relationships (*IV*) assumed saturation kinetics like in Michaelis–Menten systems. Here, we introduce an apparent charge number z_E of the unoccupied binding site of the enzyme, which accounts for the impact of all charged residues in the vicinity of the physical binding site. With this more realistic concept, the occupied binding site assumes an apparent charge of $z_{ES} = z_E + z_S$, and *IV* does not saturate any more in general, but exponentially approaches infinite or zero current for large voltage displacements from equilibrium. These nonlinear characteristics are presented here explicitly. They are qualtitatively explained in a mechanistic way, and are illustrated by simple examples. We also demonstrate that the correct determination of the model parameters from experimental data is still possible after incorporating z_E and its corollaries into the previous model of enzyme-mediated ion translocation.

Keywords Current–voltage relationship · Enzyme kinetics · Electro-enzymes · Voltage clamp · Voltage sensitivity

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Introduction

Current–voltage relationships of enzymes which catalyze the translocation of ions through lipid membranes between two compartments, e.g., inside “i” and outside “o”, have been discussed in some detail for systems with one voltage-dependent reaction step (class-I models, Hansen et al. 1981). Although this study focused on active transport, the formalism of class-I transporters can also be applied to uniporters and cotransporters (Läuger 1980, 1995; Gradmann and Boyd 2004). This formalism employs a four-state reaction cycle with a binding site E which can—occupied (ES) by substrate S or not (E)—alternate its orientation toward one or the other side of the membrane. In case of a single-file mechanism with *n* or *n*−1 substrate molecules bound as in the selectivity filter of K^+ channels (Bernèche and Roux 2001; Morais-Cabral et al. 2001), ES reflects ES_n, and E reflects $ES_{(n-1)}$. The concept of class-I models requires voltage sensitivity of the reversible reorientation either of $ES_I \leftrightarrow ES_o$, (model version 1 in Gradmann and Boyd 2004) or of $E_i \leftrightarrow E_o$ (model version 2 in Gradmann and Boyd 2004), whereas the rest of the reaction system is electroneutral, including binding/debinding of S with the charge *z* and reorientation of E in model version 1 or ES in model version 2. Since binding/debinding can be considered to be fast compared with the reorientation, the latter will become rate limiting for large voltage deflections from equilibrium.

This limitation is reminiscent of Michaelis–Menten kinetics, and it is tempting to assume equivalency of the kinetics of conventional enzymes and charge-transporting ones, simply substituting the substrate concentration *c* in conventional enzymes by the electrochemical activity $a = c\exp(zu)$ in charge-translocating enzymes, where $u = VF/RT$ is the reduced voltage with the voltage *V*, and *F*, *R*, and *T* have the normal thermodynamic meanings.

From a realistic point of view, however, the classification of the reorientation of either E or ES as electroneutral appears to be rather artificial. In fact, applications of these alternative approaches (e.g., Allen et al. 1998; Boyd et al. 2003) provided only approximate but not perfect descriptions of experimental data. Therefore, it has been suggested to introduce an apparent charge, z_E, of the unoccupied binding site E (Gradmann and Boyd 2004). This z_E is assumed to reflect the impact of all charged residues in the vicinity of the actual binding site. With this, both reorientation steps will become voltage-sensitive, i.e., $E_i \leftrightarrow E_o$ by z_E and $ES_i \leftrightarrow ES_o$ by $z_{ES} = z_E + z_S$, where z_S is the charge of the substrate S.

The aim of this study is to explore the kinetic consequences of this realistic extension from a strict class-I formalism to a more general formalism, where both z_E and z_{ES} may differ from zero and assume rational values.

Materials and methods

The four-state enzymatic reaction cycle used here (Fig. 1a) corresponds to previous versions (e.g., Gradmann and Boyd 2004) by the assumption of slow translocation reactions (small k_{12}, k_{21}, k_{34}, k_{43}) compared with fast, binding/debinding reactions (large k_{13}, k_{31}, k_{24}, k_{42}) forming the V-insensitive equilibria $K_1 = k_{31}/k_{13}$ and $K_2 = k_{42}/k_{24}$. The rational, apparent charge number z_E of the unloaded enzyme inside (state 3) and outside (state 4) is new. By this definition and the charge number z_S of the substrate, the substrate–enzyme complex ES bears the charge $z_{ES} = z_E + z_S$. In the previous model (Gradmann and Boyd 2004) a factor δ $(0 < \delta < 1)$ was accounted for by the shape of the Eyring barrier of the V-sensitive reaction. The corresponding shape factors δ_E and δ_{ES} are defined in Fig. 1a for the V-sensitive reorientation of the empty and of the loaded binding site, respectively.

In order to discuss the role of the new parameter z_E within the framework of the previous model (Gradmann and Boyd 2004), two features of this model are recalled briefly (Fig. 1b).

V gating

A simple gating mechanism with an active state and an inactive one is considered. The gating particle carries the charge z_g and alternates between its two states which differ in their position by the electric distance d_g $(0 < d_g < 1)$. The V-sensitive transition probabilities from the active state to the inactive one (k_{ai}) and the reverse (k_{ia}) are $k_{ia} = k_{ia}^0 \exp(d_{ia}u)$ and $k_{ai} = k_{ai}^0 \exp(d_{ai}u)$, where the superscript zero marks the k value at $V = 0$ again, and the voltage-sensitivity coefficients d_{ia} and d_{ai} comprise z_g, d_g and the shape factor δ_g for the gating barrier (not illustrated) as the products $d_{ia} = z_g d_g \delta_g$ and $d_{ai} = z_g d_g(\delta_g - 1)$. The mean steady-state probability of the gate to be in the active position will be

$$p_a = 1/(1 + k_{ai}/k_{ia}), \tag{1}$$

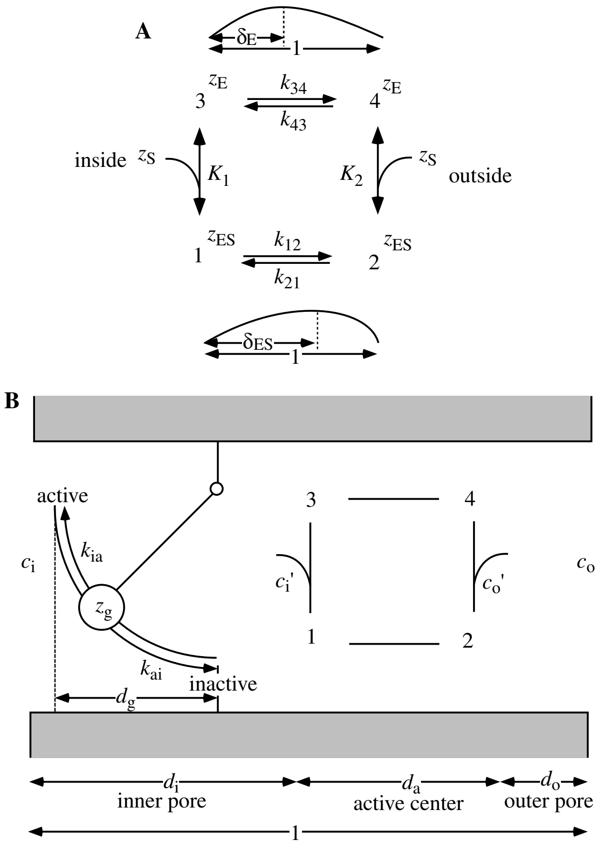

Fig. 1 Model with definitions. **a** Four-state reaction scheme for enzymatic translocation of substrate with charge z_S through a membrane (active center: k_{ij}, transition probabilities in units of per second; $K_{1,2}$, stability constants of states 1,2; z_E, apparent charge of empty binding site; z_{ES}, charge-loaded binding site; δ_E and δ_{ES}, electric distances of barrier peaks for reorientation of binding site compared with a barrier width equal to 1). **b** Active center (four-state scheme) embedded in the context of gating and substrate access through pore sections: z_g, charge of mobile gating particle; d_g, electrical distance of gating particle between functional (active) and blocking (inactive) position; k_{ai} and k_{ia}, transition probabilities for inactivation and activation; c_i and c_o substrate concentrations in internal and external bulk solution; c_i' and c_o', substrate concentrations at the inner and outer boundary of binding site; d_i, d_a, and d_o, electrical distances of inner pore section, active center, and outer pore section compared with a total transmembrane distance equal to 1. Note that the two V portions d_a for translocation and d_g for gating are not necessarily in series, as the scheme might infer; these two V portions may rather overlap as in Shaker-type channels (Jiang et al. 2003)

and upon a V change from V_0 to V_1, p_a will relax in time, t, from $p_a(V_0)$ to $p_a(V_1)$ with

$$p_a(t) = p_a(V_1) + \Delta p_a \exp\{-t[k_{ai}(V_1) + k_{ia}(V_1)]\}, \tag{2}$$

with $\Delta p_a = p_a(V_0) - p_a(V_1)$.

V partitioning

The V drop across the entire transporter is assumed to be subdivided into three sections: an inner and an outer pore section (d_i and d_o) in which fast electrodiffusion takes place, and a section d_a of the active center. In this configuration, the substrate concentrations at the active center inside (c_i') and outside (c_o') differ from the corresponding bulk concentrations (c_i and c_o) by the relationships

$$c_\mathrm{i}' = c_\mathrm{i} \exp(d_\mathrm{i} z_\mathrm{S} u) \quad \text{and} \quad c_\mathrm{o}' = c_\mathrm{o} \exp(d_\mathrm{o} z_\mathrm{S} u), \tag{3a, b}$$

and the V-sensitive transition probabilities of the enzymatic cycle are

$$k_{12} = k_{12}^0 \exp(d_{12} u), \tag{4a}$$

$$k_{21} = k_{21}^0 \exp(d_{21} u), \tag{4b}$$

$$k_{34} = k_{34}^0 \exp(d_{34} u), \tag{4c}$$

$$k_{43} = k_{43}^0 \exp(d_{43} u), \tag{4d}$$

with the V-sensitivity coefficients

$$d_{12} = d_\mathrm{a} \delta_\mathrm{ES} z_\mathrm{ES}, \tag{5a}$$

$$d_{21} = d_\mathrm{a} (\delta_\mathrm{ES} - 1) z_\mathrm{ES}, \tag{5b}$$

$$d_{34} = d_\mathrm{a} \delta_\mathrm{E} z_\mathrm{E}, \tag{5c}$$

$$d_{43} = d_\mathrm{a} (\delta_\mathrm{E} - 1) z_\mathrm{E}. \tag{5d}$$

Using standard algebra, the steady-state occupancy probabilities p_i ($0 < p_i < 1$) of the four states can be calculated from the system of four differential equations $\mathrm{d}p_i/\mathrm{d}t = fk_{ij}$ ($i,j = 1,...,4$; $\sum p_i = 1$). For thermodynamic reasons, only five of the six fundamental system parameters (k_{12}^0, k_{21}^0, k_{34}^0, k_{43}^0, $K_1^0 = k_{31}^0/k_{13}^0$, $K_2^0 = k_{42}^0/k_{24}^0$) are independent. Here, $K_2^0 = K_1^0 k_{12}^0 k_{43}^0 / k_{21}^0 / k_{34}^0$ has arbitrarily been chosen to be dependent.

With these p_i's, the steady-state current through the active system is

$$I_\mathrm{a} = e z_s (p_1 k_{12} - p_2 k_{21}), \tag{6}$$

with the elementary charge $e = 6.2 \times 10^{-19}$ A s.

Temporal relaxations of I_a will take place with

$$\Delta I_\mathrm{a}(t) = \Delta I_\mathrm{a}^0 \exp[-t(k_{12} + k_{21} + k_{34} + k_{43})], \tag{7}$$

and are not discussed here in more detail because they are assumed to be much faster than the relaxations of p_a.

So the actual, V- and t-dependent current here will be

$$I = I_\mathrm{a} p_\mathrm{a}, \tag{8}$$

with steady-state $I_\mathrm{a}(V)$ and non-steady-state $p_\mathrm{a}(V,t)$.

Results and discussion

The main issue of this study, i.e., the impact of z_E, on I_a, can be discussed by the simplified model version of Fig. 1a, which corresponds to the extended model Fig. 1b with $p_\mathrm{a} = 1$ and $d_\mathrm{a} = 1$. Another simplification is the assumption of symmetric energy barriers, i.e., $\delta_\mathrm{E} = \delta_\mathrm{ES} = 0.5$.

With this configuration, Fig. 2 shows a family of steady-state current–voltage curves of the reaction system with a common set of fundamental parameters ($k_{ij}^0 = 1\ \mathrm{s}^{-1}$, and $K_i = K_i^0$), and seven different values of z_E (−1.5, −1, −0.75, −0.5, −0.25, 0.0, and 0.5). Owing to the simple configuration of the fundamental parameters, all the curves presented are symmetric about the origin, and show coincidences of several pairs, namely for $z_\mathrm{E} = -1.5/0.5$, $-1/0$, $-0.75/-0.25$.

Only the sigmoid curves for $z_\mathrm{E} = -1$ ($z_\mathrm{ES} = 0$) and $z_\mathrm{E} = 0$ ($z_\mathrm{ES} = 1$) represent typical class-I IV curves, where the voltage-independent reaction steps define constant saturation currents at large voltage deflections from equilibrium (Hansen et al. 1981; Gradmann and Boyd 2004).

All other cases obey the following pattern: if $z_\mathrm{E}/z_\mathrm{ES} < 0$, i.e., if z_E and z_ES have different signs, I_a rises exponentially with V, and if $z_\mathrm{E}/z_\mathrm{ES} > 0$, i.e., if z_E and z_ES have the same sign, I_a falls exponentially with V toward zero current. Qualitatively, this behavior can be explained as follows.

Let us assume $-1 < z_\mathrm{E} < 0$, and $z_\mathrm{S} = 1$ as for the monovalent cation K^+. This configuration yields $0 < z_\mathrm{ES} = z_\mathrm{E} + z_\mathrm{ES} < 1$ or $z_\mathrm{E}/z_\mathrm{ES} < 0$, meaning that with increasing $V \gg 0$, the transition from state 4 to state 3 ($E_\mathrm{o} \to E_\mathrm{i}$) in Fig. 1 is accelerated exponentially by $k_{43} = k_{43}^0 \exp(d_{43} u)$, with $d_{43} = -z_\mathrm{E}/2 > 0$ (Eq. 5d), and by the same time the transition from state 1 to state 2 ($\mathrm{ES_i} \to \mathrm{ES_o}$) is accelerated also by $k_{12} = k_{12}^0 \exp(d_{12} u)$, with $d_{12} = z_\mathrm{ES}/2 > 0$ (Eq. 5a), resulting in a cooperative acceleration of the reaction cycle in a counterclockwise

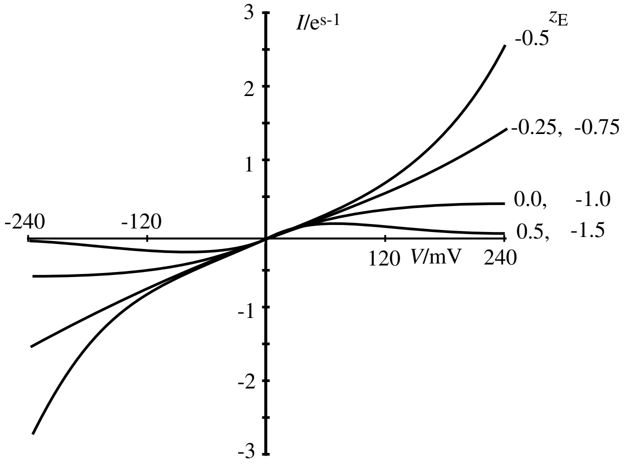

Fig. 2 Effect of z_E on the shape of the steady-state current–voltage relationship of the enzymatic reaction cycle for ion translocation through membranes (Fig. 1a) with the simplistic parameter configuration as listed in column A of Table 1. The major result is saturation only in special cases ($z_\mathrm{E} = 0$ or -1), otherwise there is an exponential increase or decrease of current (in elementary charges per second) with large V deflections from equilibrium. The minor results are pseudolinearity in the case of $z_\mathrm{E} = -0.25$ or -0.75, and the maximum exponential rise is at $z_\mathrm{E} = -0.5$

direction. This will cause I to rise exponentially with V at large V displacements from equilibrium. The exponential slope will be limited by the smaller of the two exponential coefficients d_{43} and d_{12}. Correspondingly, there will be maximum slope in case of $d_{43} = d_{12}$ (0.5 in our simple case) and zero slope if either d_{43} or d_{34} is zero—which means a constant saturation current for $V \gg 0$. Equivalent relations hold for $V \ll 0$ correspondingly.

In a formal way, we can extend the discussion of limiting exponential slopes to cases when either d_{43} or d_{12} becomes less than 0, which will consequently result in negative exponential slopes, namely exponential inhibition of the currents toward zero with increasing $V \gg 0$. And this is exactly what is illustrated in Fig. 2 by the coinciding records for $z_E = -1.5$ and $z_E = 0.5$ when the currents approach zero at large V displacements.

This inhibition in the case of $z_E/z_{ES} > 0$ (equal sign of z_E and z_{ES}), can be explained in a mechanistic way: at large V displacements (e.g., $V \gg 0$; $z_E > 0$ and $z_{ES} > 0$), the occupancies p_1 and p_3 of the internal states 1 (ES_i) and 2 (E_i) will be depleted in parallel by large k_{12} and k_{34} pushing the positive charges from inside to outside. Thus, the availability of empty binding sites inside (p_3) will vanish and will consequently suppress the "maximum" current at large V deflections toward zero current.

Equivalent relationships hold for $V \ll 0$ correspondingly, of course, especially in our symmetric example. The question is, however, whether the mentioned effects of z_E are only evident in extremely degenerated versions of the model such as used for our illustration purposes (Fig. 2), or also under more general conditions. To answer this question we chose a completely asymmetric configuration of the model (parameters x_0 listed in Table 1, column B) and calculated *dIVt* records as they would result from comparisons of the *IVt* curves in the absence and in the presence of external substrate (0.5 and 5 mM in our example), as recorded by V-clamp experiments with a triangular V protocol (Gradmann and Boyd 2004). Then the parameters x_0 were changed by arbitrary factors to x_{start} values (Table 1, column C) which were used as start parameters for fitting the model to the hypothetical data by a least-squares algorithm, as described previously (Gradmann and Boyd 2004). This previous study has shown that the parameters K_1 and k_{34} have an almost complementary impact on the system when no changes in c_i are considered—an absolutely plausible result. Therefore, one of these parameters has to be kept constant in order to determine the other one. Here we chose K_1 to be constant and k_{34} to be fitted.

Our previous study (Gradmann and Boyd 2004) showed significant differences between the two model versions ($z_E = 0$ or $z_E = -1$) and reliable fits of δ_{ES} or δ_E. These well-distinguished cases gave rise to the assumption δ_{ES} and δ_E might be determined in the present model when z_E and z_{ES} are not equal to 0. However, this expectation turned out to be wrong, as at least one of these parameters diverged from x_o during the fits. This finding is explained by the similar composition of the V-sensitivity coefficients (Eqs. 5a, 5b, 5c, 5d). So δ_{ES} and δ_E were kept constant at an arbitrary value of 0.5 to enable fits to converge to $x_{act} = x_0$. An example of such a fit to theoretical data is documented by the data in Table 1, column D. Unpublished fits to experimental data (e.g., of Boyd et al. 2003) indicate, however, that in reality not all parameters discussed here can be unambiguously determined, of course, when additional

Table 1 Sets of independent model parameters; for definitions see Fig. 1; *column A*: set for example calculations in Fig. 2 with $c_i = c_o = 1$ mM; *column B*: identification of parameters x by fitting the model with parameters x_{act} (for definitions, also see Fig. 1) to hypothetical experimental data constructed with parameters x_0 (column B), $z_S = 1$, $c_i = 1$ mM, $c_o = 0.5$ and 5 mM, triangular V protocol 0 to −240 mV to +240 to 0 mV, in steps of 80 mV and 40 ms; start parameters x_{start} either 0.5 or 2 times x_o (*column C*), except z_E, which was numerically fitted as $\exp(z_E)$; fit with fixed δ_E, δ_{ES} and K_1^0 according to Gradmann and Boyd (2004); start increment 1×10^{-4}; stop when all parameters x_{act} (*column D*) have converged toward x_o with increment $< 1 \times 10^{-6}$

Symbol/unit	Description	A Fig. 2	B Fit	C	D
			x_0	x_{start}/x_0	x_{act}
z_E	Apparent charge number of empty site	−1.5 to 0.5	−0.1	−9	−0.0993
δ_E	Shape factor of reorientation barrier for E	0.5	0.5	1	0.5
δ_{ES}	Shape factor of reorientation barrier for ES	0.5	0.5	1	0.5
δ_i	Portion of V drop in inner pore section	0	0.1	0.5	0.1016
δ_o	Portion of V drop in outer pore section	0	0.2	2	0.2003
k_{12}^0 (s^{-1})	Transition probability $ES_i \rightarrow ES_o$ at $V = 0$	1	3	0.5	2.765
k_{21}^0 (s^{-1})	Transition probability $ES_o \rightarrow ES_i$ at $V = 0$	1	4	2	3.824
k_{34}^0 (s^{-1})	Transition probability $E_i \rightarrow E_o$ at $V = 0$	1	5	0.5	4.525
k_{43}^0 (s^{-1})	Transition probability $E_o \rightarrow E_i$ at $V = 0$	1	6	2	5.508
K_1^0 (mM^{-1})	Equilibrium k_{31}/k_{13} for state ES_i at $c_i' = 1$ mm	1	7	1	7
k_{ai}^0 (s^{-1})	Transition probability active → inactive at $V = 0$	0	0.1	0.5	0.1007
k_{ia}^0 (s^{-1})	Transition probability inactive → active at $V = 0$		0.2	2	0.2641
d_{ai}	V-sensitivity coefficient in $k_{ai} = k_{ai}^0 \exp(d_{ai}u)$	0.3	0.5	0.2988	
d_{ia}	V-sensitivity coefficient in $k_{ia} = k_{ia}^0 \exp(d_{ia}u)$	−0.4	2	−0.4132	

mechanisms may be effective, such as instrumental noise or gating.

In conclusion, the concept of an apparent charge number z_E of the binding site is a realistic extension of the previous model. However, it introduces an ambiguity as d_a, z_E , δ_E and δ_{ES} in the V-sensitivity coefficients (Eqs. 5a, 5b, 5c, 5d) can no longer be determined separately by analyzing electrophysiological data only.

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