Substrate- and alkali-metal-ion-induced pK shifts in intestinal brush-border sucrase, according to the three-protons model

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To define adequately enzyme activation/inhibition mechanisms as a function of pH, it is necessary to characterize the effector-induced pK shifts on both the free enzyme and on the enzyme–substrate complex. On the basis of our recent three-protons model for sucrase [Vasseur, van Melle, Frangne & Alvarado (1988) *Biochem. J.* **251**, 667–675], we show how the ‘fundamental’ pK values, deduced from the classical double-logarithmic transformations, are insufficient to generate the required information. This insufficiency derives from the fact that, for sucrase, the acid ionization constant, K_1 , is a molecular constant that involves complex, V-type plus K-type, activatory and inhibitory kinetic effects. As a consequence, substrate-induced pK shifts cannot be interpreted correctly only by using the fundamental pK approach, because an unequal number of key protons is involved, depending on whether the free enzyme or the enzyme–substrate complex is considered. We demonstrate how this problem can be solved by using the ‘theoretical’ pK values, derived from the reciprocals of the Michaelis pH functions, i.e. Cha’s fractional concentration factors. The procedure we propose, which is general, has the advantage of yielding all the macroscopic pK values for any given model, as calculated from the microscopic pK values. Furthermore, it permits predicting pK shifts as a function of [S] and/or [A] (where S is the substrate and A is the allosteric modifier), an objective that cannot be attained by using the double-logarithmic plot approach. Finally, we describe the relation existing between the fundamental and the theoretical pK values.

INTRODUCTION

Intestinal brush-border sucrase (sucrose α -D-glucosylhydrolase, EC 3.2.1.48) is characterized by exhibiting asymmetrical pH profiles (Vasseur *et al.*, 1982, 1988; Vasseur, 1985). Whereas the basic ionization constant, K_0 , conforms to simple Michaelis–Dixon theory and yields respectively slopes of -1 , -1 and 0 in plots of $\log(V_{\max}/K_m)$ versus pH, $\log V_{\max}$ versus pH and pK_m versus pH, the acid ionization constant, K_1 , exhibits respectively slopes of $+2$, $+1$ and $+1$.

These observations indicate that K_1 involves two protons that we have defined as belonging to two functionally distinct, ‘V’ and ‘K’, proton families (see Vasseur *et al.*, 1988). This concept has led us to originate a new sucrase model, the three-protons model, which has been used successfully to fit a large body of kinetic data involving the three variables $[H^+]$, [S] and [A], where S is the substrate and A is the allosteric modifier, an alkali-metal ion (Vasseur *et al.*, 1988).

In the present paper we aim at defining, starting from basic principles, each of the key pK values characterizing the sucrase three-protons model. Firstly, we show how the standard procedure of deducing the ‘fundamental’ pK values from double-logarithmic transformations falls short of generating the sought-for information because, as concerns respectively the free enzyme and the ES complex, pK_1 implies the loss (or gain) of an unequal number of protons. Secondly, we demonstrate how this problem can be solved by using the reciprocals of the Michaelis pH functions, i.e. the fractional concentration

factors (Cha, 1968), from which the ‘theoretical’ pK values can be derived. The procedure we propose, which is general, has the advantage of yielding all the macroscopic pK values for any given model, as calculated from the microscopic pK values. Furthermore, it permits the prediction of pK shifts as a function of [S] and/or [A], an objective that cannot be attained by using the double-logarithmic-plot approach.

MATERIALS AND METHODS

Reagents and buffers

All chemicals used were of analytical-reagent grade. The n-butylamine, metal-ion-free universal buffer covering the pH range 4.8–8.8 (final composition: 1.8 mM-citric acid/1.8 mM-maleic acid/1.8 mM-phosphoric acid/1.8 mM-glycylglycine/1.8 mM-glycine/20 mM-n-butylamine) was prepared as described by Alvarado & Mahmood (1979). The alkali-metal ions were added as the chlorides and no attempt was made at keeping the ionic strength of the media constant, for reasons already explained (Alvarado & Mahmood, 1979).

Enzyme preparation

Brush-border membranes from rabbit small intestine were obtained essentially as described by Kessler *et al.* (1978). This preparation was treated with Triton X-100 to solubilize the sucrase–isomaltase complex, which was then purified to electrophoretic homogeneity according to a four-step procedure developed in our laboratory

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(Vasseur, 1985, 1989). The purified enzyme was stocked at 4 °C in a 10 mM-phosphate buffer containing 100 mM-NaCl and 0.02% NaN₃. Immediately before use, the stock enzyme was exhaustively dialysed with the n-butylamine buffer at the appropriate pH. No change in enzyme stability (60 min at 37 °C) was observed in the pH range studied (Vasseur, 1985).

Determination of sucrase activity and expression of results

Sucrase was assayed by measuring its D-glucose product with glucose oxidase and peroxidase after stopping the reaction with Tris, as described previously (Vasseur *et al.*, 1982). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Velocities are given in units/mg of protein (1 unit is defined as 1 μ mol of glucose formed/min under standard conditions). For each different pH (ranging from pH 4.8 to 8.8) and alkali-metal ion concentration (in general, 0, 50 and 300 mM) used, kinetic experiments were performed in triplicate by using six sucrose concentrations covering from about 0.3 to 3 times the expected K_m value, as recommended by Cleland (1967). All calculations were performed with a Hewlett-Packard model HP-85 microcomputer.

THEORETICAL CONSIDERATIONS

To facilitate our presentation, we shall briefly recall the sucrase three-protons model, described in detail by Vasseur *et al.* (1988) (see Fig. 1). The key feature of this model consists of the splitting of K_1 into a two-pathway reaction encompassing three different protonation levels, formed by four enzyme species, each with a distinct kinetic function. These enzyme forms are: two containing the proton H_x and defined as having no affinity for S (forms EH_3 and EH_{2x}), and two lacking this particular proton (EH_{2y} and $E'H$). Precisely because they lack H_x , these last are the only enzyme forms capable of binding S. Inversely, S prevents H_x from binding, in accord with the observation that S and H_x are reciprocal fully competitive inhibitors (Vasseur *et al.*, 1982).

The second proton, H_y , behaves as a mixed inhibitor and its release causes the enzyme to become catalytically active. Loss of both H_x and H_y (and retention of H_z) is required to generate the fully active enzyme forms, $E'H$.

A final, important, feature of the model, is that the action of the alkali-metal ions is complex. At the acid side of the pH spectrum, they behave as either K-type activators (by binding to 'A' sites) or K-type inhibitors (by binding to 'I' sites). At the basic side, they behave as non-competitive inhibitors.

The general equation

The overall rate equation for the three-protons model has been given by Vasseur *et al.* (1988) and has the form of the Michaelis-Menten equation:

$$v = \frac{V\{1 + Q([A]/K'_{ar})\}/(\alpha) \cdot [S]}{[S] + K_{sr}(\beta/\alpha)} = \frac{V_{max} \cdot [S]}{[S] + K_m} \quad (1)$$

where, as we shall explain, $K'_{ar} = K'_{a1}$ and

$$\alpha = \sum_p (K_{sr}/K_{sp}) \cdot I'_p \cdot h_p \quad (2)$$

$$\beta = \sum_p I'_p \cdot h_p \quad (3)$$

For the theoretical analysis contained in the present paper, we have departed from the classical notation used by Vasseur *et al.* (1988), and utilize a new mathematical treatment, recently developed in our laboratory, based on using what we call intralevel and interlevel coefficients (Vasseur, 1985; Vasseur *et al.*, 1989). A brief description of our procedure follows.

The intralevel coefficients, I_p , serve to take into account all the various enzyme forms present at a given protonation level, L_p , and can be expressed in terms of intralevel sub-coefficients (I'_p, I''_p), such that:

$$I_p = I'_p + ([S]/K_{sp}) \cdot I''_p \quad (4)$$

where I'_p includes those enzyme forms not involving S (E , EA ; see Fig. 1) and I''_p includes those that contain S (SE , SEA); K_{sp} is the substrate dissociation constant at level L_p . For better illustration of the procedure, the complete set of coefficients constituting the sucrase three-protons model is listed in Table 1.

According to this notation, I_p is always fully defined because, when both S and A are absent or do not bind, I_p simplifies to 1. With sucrase, for instance, this particular situation is encountered at level L_{2x} (Fig. 1).

The interlevel coefficients, h_p , describe the interplay between levels connected by either one or several protonation/deprotonation reactions. A notation such as $h_{p,q}$ would express the amount of free enzyme at level L_p by reference to the corresponding amount of free enzyme at some other level, L_q . However, because of the obvious interest of using those pathways leading to $E'H$, the catalytically active enzyme species, we shall use this form as our reference. If $L_1 = L_r$ and, therefore, $K_{s1} = K_{sr}$, the shorter notation, h_p , will suffice.

pK DETERMINATIONS

The fundamental pK values

A classical way of calculating what we may call the 'fundamental' pK values is to use the double-logarithmic transformations, which, ideally, can be approximated to

Table 1. The interlevel coefficients, h , and the intralevel sub-coefficients, I' , and I'' , constituting the sucrase three-protons model

Protonation level, L_p	h	I'	I''
L_3	$\frac{[H^+]^2}{K_{2x} \cdot K_{1y}}$	$\left(1 + \frac{[I]}{K_{13}}\right)^{n_{13}}$	1
L_{2x}	$\frac{[H^+]}{K_{1x}}$	1	1
L_{2y}	$\frac{[H^+]}{K_{1y}}$	$1 + \frac{[A]}{K_{a2}}$	$1 + \frac{[A]}{K'_{a2}}$
$L_1(L_r)$	1	$1 + \frac{[A]}{K_{a1}}$	$1 + \frac{[A]}{K'_{a1}}$
L_0	$\frac{K_0}{[H^+]}$	$1 + \frac{[A]}{K_{a0}}$	$1 + \frac{[A]}{K'_{a0}}$

Fig. 1. Three-dimensional representation of the three-protons model for intestinal sucrase depicting the interaction between a monomeric enzyme, H^+ , the substrate, S , and an allosteric modifier A (or I)

For sucrase, A and I represent the same species, an alkali-metal ion (reproduced from Fig. 2 in Vasseur *et al.*, 1988).

three straight segments differing in slope. The two fundamental pK values, pK_1 and pK_0 , are given respectively by the points at which the central, pH-independent, horizontal branch is crossed by the ascending (acid) and the descending (basic) ones (Dixon & Webb, 1979; Cornish-Bowden, 1981). By using as an example the $\log V_{\max}$ -versus-pH function in the following we illustrate how this procedure can be applied to the complex situation represented by the three-protons model.

From eqn. (1), the logarithmic form of $V_{\max}$ can be written:

$$\log V_{\max} = \log Z - \log \left\{ \left(\frac{[H^+]}{K_{1ys}} \right) \cdot I''_2 + I''_1 + \left(\frac{K_{0s}}{[H^+]} \right) \cdot I''_0 \right\} \quad (5)$$

where $Z = V \{1 + Q([A]/K'_{ar})\}$.

At low pH values, i.e. those at which $[H^+] \gg K_{1ys} \gg K_{0s}$, eqn. (5) simplifies to give the equation for the acid segment:

$$\log V_{\max} = \log Z - \log \left\{ \left(\frac{[H^+]}{K_{1ys}} \right) \cdot I''_2 \right\} \quad (6)$$

As pH increases, and provided that $K_{1ys} \gg [H^+] \gg K_{0s}$, the pH-independent horizontal region will be attained and:

$$\log V_{\max} = \log Z - \log I''_1 \quad (7)$$

Finally, as pH increases further, the term $K_{0s}/[H^+]$ will become dominant, thereby yielding the equation for the basic branch:

$$\log V_{\max} = \log Z - \log \left\{ \left(\frac{K_{0s}}{[H]} \right) \cdot I''_0 \right\} \quad (8)$$

By equating eqns. (6) and (7), the left-hand crossing point, K_1 , is obtained:

$$pH = pK_{1ys} + \log (I''_2/I''_1) = pK_1 \quad (9)$$

Similarly, the right-hand intersection, pK_0 , will be obtained when eqns. (7) and (8) are made equal:

$$pH = pK_{0s} + \log (I''_1/I''_0) = pK_0 \quad (10)$$

Because all I'' coefficients equal 1 when $[A] = 0$, pK_{1ys} and pK_{0s} can be regarded as corresponding to the fundamental values of the enzyme-substrate complexes at either side of the pH spectrum, respectively pK_1 and pK_0 . The four fundamental $pK = f([A])$ functions derived from each $\log V_{\max}$ and $\log (V_{\max}/K_m)$, together with the limiting values attained at either $[A] = 0$ or $[A] \rightarrow \infty$, are listed in Table 2.

The impossibility of interpreting mechanism in terms only of the fundamental pK values appears evident from the definitions in Table 2. For instance, in the absence of A , whereas pK_1 is a group ionization constant for the ES complex (it involves the one-step passage from L_2 to L_1 : $pK_1 = pK_{1ys}$), for the free enzyme pK_1 is a molecular pK involving the complex jump from L_3 to L_1 : $pK_1 = \frac{1}{2}(pK_{2x} + pK_{1y})$.

The theoretical pK values

A second well-established method for pK calculation is based on using the Michaelis-Dixon pH functions, f_p

Table 2. The 'fundamental' pK values derived from the double-logarithmic transformations, according to the sucrose three-protons model: theoretical limits as a function of the alkali-metal-ion concentration

Values in parentheses indicate the pK values computed for sucrose by using the data in Tables 2 and 3 of Vasseur *et al.* (1988).

Fundamental pK (definition)	Limiting values of pK _i	
	At [A] = 0	At [A] → ∞
log V _{max.} -versus-pH plot: pK ₁ = pK _{1ys} + log (I'' ₂ /I'' ₁)	pK _{1ys} (5.21)	pK _{1yas} (Li ⁺ : 5.68) (Na ⁺ : 5.38)
pK ₀ = pK _{0s} + log (I'' ₁ /I'' ₀)	pK _{0s} (8.59)	pK _{0as} (Li ⁺ : 6.62) (Na ⁺ : 7.63)
log (V _{max.} /K _m)-versus-pH plot: pK ₁ = ½[pK _{2x} + pK _{1y} + log (I'' ₃ /I'' ₁)]	½(pK _{2x} + pK _{1y}) (5.73)	+ ∞*
pK ₀ = pK ₀ + log (I'' ₁ /I'' ₀)	pK ₀ (8.06)	pK _{0a} (Li ⁺ : 6.89) (Na ⁺ : 7.18)

* When [A] tends to infinity, pK₁ may exhibit two different limits, which depend on the value of n₁₃. These limits are: pK₁ → + ∞ when n₁₃ > 1 (as we have found for sucrose) and pK₁ → - ∞ if n₁₃ = 0.

(Dixon & Webb, 1979). We shall show that this method is particularly suitable for analysis of the complex situation represented by the three-protons model because it permits us to define what we call the 'theoretical' pK values, the number of which (see list in Table 3) is larger and bears more information than those of the fundamental pK values (Table 2). Before proceeding, however, it seems worth recalling that this approach consists in plotting 1/f_p = f(pH), i.e. the reciprocals of the pH functions versus pH. However, 1/f_p is equivalent to F_p,

Cha's fractional concentration factor (Cha, 1968), meaning that the function we shall consider here is F_p = f(pH).

As previously defined (Vasseur, 1985), if [ET] is the sum of all possible enzyme forms constituting a model, and [ET]_p is the sum of all the forms present at each given protonation level, L_p, the state equation is:

$$[ET] = \sum [ET]_p \quad (11)$$

which permits us further to define:

$$F_p = \frac{[ET]_p}{[ET]} = l_p \cdot h_p / \sum l_p \cdot h_p \quad (12)$$

where $\sum l_p \cdot h_p = 1$ and $100 \times F_p$ is the percentage of the total amount of enzyme found at level L_p.

According to this notation, F_p is a function of each [S] and [A] through the intralevel coefficients, l_p, and of pH through the interlevel coefficients, h_p (see Table 1). Consequently, if [S] and [A] are fixed, F_p will be a function only of pH.

All F_p functions are bell-shaped and will exhibit a maximum-occupancy peak that, depending on the distance separating the relevant pK values, may approach 100% [ET] for only some of them (see Fig. 3). The intersection of any two pH functions gives the pK of the protonation/deprotonation reaction(s) joining the corresponding two levels. Thus, for any two levels, L_p and L_q, we can define a theoretical pK, pK_{t(p,q)}, that equals that pH value at which F_p = F_q.

From eqn. (12), $l_p \cdot h_p = l_q \cdot h_q$, which, by taking logarithms, gives:

$$-\log (h_p/h_q) = \log (l_p/l_q) \quad (13)$$

To illustrate our procedure, in the following we shall explicitly compute the solution for pK_{t(3,1)}, which is the most complex. By using eqn. (13):

$$-\log ([H^+]^2/K_{2x} \cdot K_{1y}) = \log (l_3/l_1)$$

so that, because of the redundancy implied in any

Table 3. The value of D and the 'real' pK values (pK_{r(p,q)}) derived from the Michaelis pH functions, according to the sucrose three-protons model: limits of the 'theoretical' pK values (pK_{t(p,q)}) as a function of the substrate concentration

Values in parentheses indicate the pK_{t(p,q)} values computed for sucrose by using the data in Tables 2 and 3 of Vasseur *et al.* (1988).

Protonation levels			D	Limiting values of pK _{t(p,q)}	
L _p	L _q	pK _{r(p,q)}		At [S] = 0	At [S] → ∞
3	2y	pK _{2x}	1	(6.45)	- ∞
3	2x	pK _{2y}	1	(7.29)	*
3	1	pK _{2x} + pK _{1y}	2	(5.73)	- ∞
3	0	pK _{2x} + pK _{1y} + pK ₀	3	(6.51)	- ∞
2y	1	pK _{1y}	1	(5.01)	(5.21)
2y	0	pK _{1y} + pK ₀	2	(6.54)	(6.90)
2x	1	pK _{1x}	1	(4.17)	- ∞
2x	0	pK _{1x} + pK ₀	2	(6.12)	- ∞
1	0	pK ₀	1	(8.06)	(8.59)

* pK_{t(3,2x)} is a substrate-independent function.

model:

$$\begin{aligned} \text{pH} &= \frac{1}{2}[\text{p}K_{2x} + \text{p}K_{1y} + \log(I_3/I_1)] \\ &= \frac{1}{2}[\text{p}K_{2y} + \text{p}K_{1x} + \log(I_3/I_1)] = \text{p}K_{t(3,1)} \end{aligned}$$

From this derivation it can be deduced that the theoretical pK values will all exhibit an identical form, namely:

$$\text{p}K_{t(p,q)} = D^{-1} \cdot [\text{p}K_{r(p,q)} + \log(I_p/I_q)] \quad (14)$$

where the divisor D indicates the number of protons linking levels L_p and L_q . For lack of a better name, the entity $\text{p}K_{r(p,q)}$, necessary to obtain $\text{p}K_{t(p,q)}$, is named here the 'real' pK. All the real pK and D values constituting the three-protons model are listed in Table 3. By applying eqn. (14) and this information, together with the numerical data estimated previously for sucrase (Tables 2 and 3 in Vasseur *et al.*, 1988), all the theoretical pK values constituting the model have been computed. Their limiting values for either the free enzyme or for the ES complex are listed in Table 3 (last two columns). Let us emphasize in closing that, through the term $D^{-1} \cdot \log(I_p/I_q)$ (see eqn. 14), the theoretical pK values are functions of each $[A]$ and $[S]$. This information is not furnished by the fundamental pK values, which are a function only of $[A]$ (Table 2).

INTERPRETATION

Fundamental pK values

pK₀ as a function of $[A]$. Because it is the simplest one to explain, we shall begin by discussing the $\text{p}K_0 = f([A])$ function, which, according to the three-protons model, involves four group ionization constants linking L_1 to L_0 (Fig. 1).

For the free enzyme, both Li^+ and Na^+ inhibit by inducing $\text{p}K_0$ to decrease by 1.2 and 0.9 pH units respectively (Fig. 2b). In contrast, S causes activation by displacing $\text{p}K_0$ upwards ($\text{p}K_{0s} = 8.6$; Fig. 2a). Although these results reveal Li^+ to be a stronger inhibitor than Na^+ , the quantitative difference between the two ions is even greater when the whole picture is considered, as we explain below.

Saturating the EA complex with S has diametrically opposite kinetic effects, depending on the nature of A. With Na^+ , $\text{p}K_0$ increases from 7.2 (Fig. 2b) to 7.6 (Fig. 2a), indicating that S partially overcomes Na^+ inhibition. In contrast, with Li^+ , S further increases the metal-ion inhibition: $\text{p}K_0$ decreases from 6.9 to 6.6. The ionization constants underlying these effects being related by an R parameter ($R_{11} = K_{0a}/K_{0as}$; Vasseur *et al.*, 1988), it is useful to discuss the situation in these terms. The interaction of S with the EA complex exhibits either negative or positive co-operativity, depending on whether A is Li^+ ($R_{11} = 0.54$) or Na^+ ($R_{11} = 2.9$). Further insight into the quantitatively different effects of Li^+ and Na^+ on $\text{p}K_0$ comes from considering the interaction of these ions with the ES complex (the R coefficient playing a role here is $R_{13} = K_{0s}/K_{0as}$). Our experimental results yield $R_{13} = 0.011$ and $R_{13} = 0.11$ for Li^+ and Na^+ respectively, meaning that Li^+ is a 10-fold stronger inhibitor. The overall result, illustrated in Fig. 2(a), is that Na^+ induces $\text{p}K_0$ to decrease by 1 pH unit (from 8.6 to 7.6), whereas Li^+ induces a decrease of 2 pH units (from 8.6 to 6.6). The 1-pH-unit difference in the effect of the two metal ions explains the observation that Li^+ is the strongest sucrase inhibitor above pH 6.5 (Alvarado & Mahmood, 1979).

Fig. 2. Evolution of the fundamental pK values as a function of the alkali-metal-ion concentration

The $\text{p}K_i = f([A])$ functions for the ES complex and for the free enzyme are illustrated in panels (a) and (b) respectively. As a reference, the pK values corresponding to either $\text{p}K_{0s}$ and $\text{p}K_{1ys}$ (upper diagram) or $\text{p}K_0$ and $\text{p}K_1$ (lower diagram) at $[A] = 0$ are illustrated by the horizontal lines (numerical data on the left-hand side). Their respective limiting values as $[A]$ tends to infinity are given by the values in parentheses on the right.

pK₁ as a function of $[A]$. Let us consider first the overall value of $\text{p}K_1$ at $[A] = 0$ (horizontal continuous lines in Fig. 2). Substrate binding causes activation by inducing $\text{p}K_1$ to decrease from 5.73 to 5.21. However, this simple analysis does not fully describe the situation because, as explained above, $\text{p}K_1$ is defined quite differently when derived from either $\log V_{\max}$ or $\log(V_{\max}/K_m)$ plots (Table 2). We conclude that, when an ionization reaction is complex, as is the case with K_1 for sucrase, the correct interpretation of S-induced pK shifts cannot be reached simply by using the fundamental pK values (further below we shall show how the theoretical pK values provide a solution to this problem).

For the ES complex, $\text{p}K_1$ involves the group ionization constants, K_{1ys} and K_{1yas} , connecting L_2 and L_1 . Each Li^+ and Na^+ inhibits by making $\text{p}K_{1yas} > \text{p}K_{1ys}$ (Fig. 2a and Table 2). It seems interesting that, for each $\text{p}K_1$ and $\text{p}K_0$, the alkali-metal ions are always inhibitory, although the effects seen on $\text{p}K_1$ are negligible when compared with those produced on $\text{p}K_0$. The direct consequence of these inhibitory effects of the alkali-metal ions on sucrase is that the distance separating $\text{p}K_1$ from $\text{p}K_0$ decreases from 3.4 pH units at $[A] = 0$ to 0.9 and 2.2 pH units respectively in the presence of saturating concentrations of either Li^+ or Na^+ (see Fig. 2a).

For the free enzyme $\text{p}K_1$ is a macroscopic constant that, at $[A] = 0$, has the simple solution $\text{p}K_{1(\text{overall})} = 5.73$ (Table 2). In contrast, in the presence of the alkali-metal ions $\text{p}K_1$ becomes a complex function of $[A]$. Firstly, it is

Fig. 3. Evolution with pH of the six pH functions constituting the sucrase three-protons model, both in the absence and in the presence of a 300 mM concentration of both the substrate and the indicated alkali-metal ions

As a reference, the four vertical lines identify the fundamental pK values corresponding to either the free enzyme (left) or the ES complex (right). The horizontal arrows point out the pK_i shifts induced by A.

biphasic, as illustrated in Fig. 2(b). At relatively low ion concentrations pK_1 decreases, indicating a K-type activation effect that reflects formation of the E-A complex at L_1 . At higher concentrations a fully competitive inhibitory effect sets in that involves enzyme reprotonation and formation of the E-(I) $^{n_{13}}$ complex at L_3 . Secondly, even if these two opposite kinetic effects are treated separately, the limiting values of the pK_1 function when [A] tends to infinity remain complex. If we retain for consideration the inhibitory component, meaning that we apply the experimental observation that $n_{13} > 1$ for each Li^+ and Na^+ (see Table 2), then the limit $pK_1 = +\infty$ is obtained. However, if we eliminate from consideration the inhibitory component, which can be done simply by making $n_{13} = 0$, then the limit is $pK_1 = -\infty$.

Because, as demonstrated, the fundamental pK values cannot yield all the information that is necessary to interpret correctly the effects of each S and A on a three-key-protons enzyme, the theoretical pK values need to be used, as we explain below.

Theoretical pK values

The sucrase three-protons model contains five different enzyme species distributed into four protonation levels, a situation yielding six different pH functions: EH_3 , EH_{2x} , EH_{2y} , $EH_{2x} + EH_{2y}$, $E'H$ and E (see Fig. 3, upper left panel). The EH_{2x} form represents only a small part of the total and disappears at high concentrations of either S or A or both: under such conditions the EH_{2y} and $EH_{2x} + EH_{2y}$ functions superimpose, thus diminishing the effective number of pH functions to consider (see the remaining panels in Fig. 3).

The effect of S on either the free or the metal-bound enzyme is different, depending on whether $pK_{(3,1)}$ or $pK_{(2y,1)}$ is considered. If we analyse first $pK_{(2y,1)}$, the shifts induced by S at [A] = 0 (notation: $\Delta pK_{(p,q)}$) are given by:

$$\Delta pK_{(2y,1)} = pK_{1ys} - pK_{1y} + \log \left\{ \frac{(K_{s2} + [S])}{(K_{s1} + [S])} \right\} \quad (15)$$

meaning that $\Delta pK_{(2y,1)}$ equals $(pK_{1ys} - pK_{1y})$ when [S] tends to infinity. Here, therefore, the key effect of S is inhibitory, as a result of its favouring enzyme reprotonation back to L_{2y} : the pertinent pK (pK_{1y} ; Table 3) increases by 0.2 pH unit. For the E-A complex, S inhibits further: $pK_{(2y,1)}$ increases respectively by 5.01 and 4.03 pH units when A is either Li^+ or Na^+ (Table 4).

As concerns $pK_{(3,1)}$, the shifts induced by S are given by:

$$\Delta pK_{(3,1)} = \frac{1}{2} \cdot \log \left\{ \frac{K_{s1}}{(K_{s1} + [S])} \right\} \quad (16)$$

which means that $\Delta pK_{(3,1)}$ will exhibit negative values. As [S] increases, $pK_{(3,1)}$ will decrease monotonically and approach $-\infty$ as [S] tends to infinity (compare eqns. 15 and 16). Here, therefore, the key effect of S is activatory since it favours enzyme deprotonation from EH_3 to $E'H$. It induces $pK_{(3,1)}$ to decrease from an initial 5.73 value to approach $-\infty$ (line 3, Table 3). To show better the difference between the theoretical and the fundamental pK values, we list in Table 4 the limiting values for $pK_{(3,1)}$ and $pK_{(2y,1)}$, derived from our experimental data as a function of [A] and/or [S].

To conclude, in Fig. 4 we illustrate the pK shifts induced by A on each $pK_{(3,1)}$ and $pK_{(1,0)}$, in the absence and in the presence of increasing S concentrations. This

Fig. 4. Evolution of the two key theoretical pK values as a function of both the alkali-metal-ion and the substrate concentrations

Depending on whether they refer to either Li⁺ (left) or Na⁺ (right), the variation of each pK₀ (pK_{t(1,0)}) and pK₁ (pK_{t(3,1)}) as a function of [A] is illustrated for the indicated concentrations of substrate.

Table 4. S- and A-dependent pK shifts according to the sucrase three-protons model: comparison of the limiting values of pK_t exhibited by the 'theoretical' pK values pK_{t(3,1)} and pK_{t(2y,1)}, when [A] and/or [S] are zero or tend to infinity

Values in **bold type** signify that the theoretical pK values and the fundamental pK values are identical (see the text).

Limiting [A] value:	[A] = 0		[A] → ∞	
	[S] = 0	[S] → ∞	[S] = 0	[S] → ∞
pK _{t(3,1)}	5.73	−∞	+∞	+∞
pK _{t(2y,1)}	5.01	5.21	(Li ⁺) 0.67	5.68
			(Na ⁺) 1.35	5.38

information, as mentioned above, cannot be obtained by using the fundamental pK values. As concerns pK_{t(3,1)}, the effect of S is to decrease it, meaning that S is always activatory.

CONCLUSION

Relationship between the fundamental and theoretical pK values

It seems interesting to consider the relationship existing between the fundamental and the theoretical pK values: particularly when a different number of protonation levels is involved in either the free enzyme or the E_s complex.

At S = 0, the situation is simple: I_p simplifies to I'_p and eqn. (14) becomes:

$$pK_{t(p,q)} = D^{-1} \cdot [pK_{r(p,q)} + \log(I'_p/I'_q)] \quad (17)$$

It appears that, among the entire series of theoretical pK values, only pK_{t(3,1)} and pK_{t(1,0)} are equivalent to the corresponding fundamental pK values deduced from $\log(V_{\max}/K_m)$. Furthermore, both are functions of [A] (see Tables 2 and 3).

When [S] → ∞, the situation is quite different, the two intralevel coefficients, I_p and I_q, being defined quite differently (Table 1). According to the three-protons model, binding of S does not occur at levels L₃ and L_{2x}, meaning that K_{sp} tends towards infinity or, simply, that I_p = I'_p at these particular levels. Two different cases can therefore be considered.

Case 1. The two coefficients are defined differently as, for instance, pK_(3,q) and pK_(2x,q), where L_q would be different respectively from either L₃ or L_{2x}. From eqn. (14):

$$pK_{t(p,q)} = D^{-1} \cdot [pK_{r(p,q)} + \log(I'_p/I'_q \cdot [S]/K_{sq})] \quad (18)$$

When [A] = 0 and [S] → ∞, the limiting values for each relevant pK_t tend towards −∞ (Table 3). Under these conditions, none of the theoretical pK values is equivalent to the fundamental pK values.

Case 2. The two intralevel coefficients, I_p and I_q, are defined in the same way as is the case for pK_(3,2x), pK_(2y,1), pK_(1,0) and pK_(2y,0). Whereas pK_(3,2x) will be a substrate-independent function and therefore similar to eqn. (17), the three other theoretical pK values will vary with S as defined by eqn. (14). When [S] → ∞, eqn. (14) simplifies to:

$$pK_{t(p,q)} = D^{-1} \cdot [pK_{r(p,q)} + \log(K_{sq}/K_{sp}) + \log(I''_p/I''_q)] \quad (19)$$

which clearly shows that, when [S] → ∞, pK_t is a simple function of I''. Here, among the three theoretical pK values, only pK_(2y,1) = pK_{1ys} and pK_(1,0) = pK_{0s}, when [A] = 0, are identical with the fundamental pK values deduced from $\log V_{\max}$.

We conclude, therefore, that use of the fundamental pK values cannot always yield all of the information needed to define enzyme mechanism. This insufficiency becomes apparent in particular when two distinct proton families participate in a single ionization reaction, as is the case with sucrase. By using the theoretical pK values, on the other hand, the total information needed to define enzyme mechanism becomes accessible.

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