Alkali-metal-ion- and H⁺-dependent activation and/or inhibition of intestinal brush-border sucrase

A model involving three functionally distinct key prototropic groups*

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For rabbit intestinal brush-border sucrase, a model based on classical Michaelis–Dixon theory cannot fully explain the peculiar antagonistic relationship existing between the substrate and one key proton, H_x, which at acid pH values behaves as a fully competitive inhibitor. In the same pH range, a second proton, H_y, is responsible for changes in catalytic activity and behaves as a mixed-type partially non-competitive inhibitor [Vasseur, Tellier & Alvarado (1982) *Arch. Biochem. Biophys.* **218**, 263–274]. Although involved in the same ionization reaction, these two protons have different kinetic functions, since they are responsible for affinity-type and capacity-type effects respectively. Depending on whether H_x is bound or not, we postulate the enzyme to alternate between two distinct forms differing in their binding properties. The alkali-metal ions Na⁺ and Li⁺ have a concentration-dependent biphasic effect on this equilibrium. At low concentrations they facilitate the release of H_x, resulting in K-type activation. At higher concentrations they favour enzyme reprotonation, causing K-type inhibition. On the basic side of the pH spectrum, our results confirm the existence of separate non-competitive effects of the alkali-metal ions, particularly Li⁺ [Alvarado & Mahmood (1979) *J. Biol. Chem.* **254**, 9534–9541]. To explain the molecular mechanisms underlying the alkali-metal-ion- and H⁺-dependent effects, we formulate a sucrase model, the three-protons model, in which the acid and basic ionization constants involve respectively two and one key prototropic groups that are functionally distinguishable. A global iterative fit of the relevant general equation to our whole set of data has permitted us to estimate the numerical value of each of the constants constituting the model.

INTRODUCTION

In the course of studies on the activation of transport systems by Na⁺, we singled out intestinal brush-border sucrase (sucrose α-D-glucohydrolase, EC 3.2.1.48) as an ideal model system. For sucrase, a key characteristic is that the addition of Na⁺ at pH 5.2 has a kinetic effect, allosteric affinity-type activation, equivalent to that found when, in the absence of Na⁺, pH is increased to 7 (Alvarado & Mahmood, 1979; Alvarado *et al.*, 1980; Vasseur *et al.*, 1982). In contrast, the V-type activation effect of Na⁺ appears to be ‘normal’, inasmuch as it is a simple function of pH in the range from 5.2 to 7. [The nomenclature of Monod *et al.* (1963) is used. We employ the term ‘allosteric’ in its most ample sense; indirect interactions between distinct specific binding sites that do not necessarily occur in separate monomers. Intestinal sucrase consists of a single chain containing one single substrate-binding site (Mosiman *et al.*, 1973) and presumably all the various metal-ion-binding sites distinguished in the present paper.]

The alkali-metal ions, however, have pH-dependent effects on sucrase far more complex than originally believed. Firstly, at acid pH values a biphasic effect occurs involving both K-type activation and, at high

concentrations, particularly of Li⁺, K-type inhibition. Secondly, above pH 7 non-competitive inhibition occurs and, again, Li⁺ is the strongest inhibitor (Alvarado & Mahmood, 1979; Vasseur *et al.*, 1982; Vasseur, 1985).

In an earlier investigation of the effects of Na⁺ and Li⁺ on brush-border sucrase as a function of pH (Vasseur *et al.*, 1982) we did not succeed in fitting our data to a general model based on classical concepts of pH-dependent enzyme activation (Michaelis & Davidsohn, 1911; Alberty, 1954; Segel, 1975; Cleland, 1977; Dixon & Webb, 1979; Tipton & Dixon, 1979). This failure we attributed to the fact that the acid ionization constant, which we call K₁, involves two key protons that have different kinetic functions. To obtain a good fit of all our data, we postulated that K₁ must be split to generate two distinct enzyme forms having an equal number of key protons but different binding properties (Vasseur *et al.*, 1982).

This postulate required the development of a new sucrase model involving three functionally distinct key prototropic groups: its description and application to sucrase are the subject of the present article. We show how a voluminous set of kinetic data involving sucrase and the variables [S], [H⁺] and [A] (where A is the allosteric modifier, Na⁺ or Li⁺) in the pH range 4.8–8.8

Abbreviations used: M.S.E., mean of squares of errors; M.S.L.F., mean of squares of lack of fit; M.S.P.E., mean of squares of pure error.

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can be fitted by the three-protons model. A preliminary account of this work has been reported in a thesis from our laboratory (Vasseur, 1985).

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 from 4.8 to 8.8 was prepared as previously described (Alvarado & Mahmood, 1979; Vasseur *et al.*, 1982). A mixture of n-butylamine as base and various organic acids (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 adjusted to the desired pH (measured at 37 °C) with HCl. 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 (Mosiman *et al.*, 1973; 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 by a four-step procedure developed in our laboratory (Vasseur, 1985). The procedure involves: (i) chromatography in DEAE-Sepharose CL-6B in the presence of Triton; (ii) papain treatment to render the sucrase-isomaltase complex water-soluble; (iii) hydrophobic chromatography in octyl-Sepharose CL-4B; (iv) gel filtration in Ultrogel AcA-22. In this last step the sucrase-isomaltase complex was fully separated from a peak of about double the M_r exhibiting maltase but not sucrase activity. The purified enzyme was stored at 4 °C in 10 mM-sodium phosphate buffer, pH 7.4, containing 100 mM-NaCl and 0.02% NaN₃. Immediately before use, the stock enzyme was exhaustively dialysed against the n-butylamide 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 activity was assayed by measuring its product D-glucose 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 as units/mg of protein (1 unit is defined as 1 μ mol of glucose formed/min under standard conditions). For each different pH (in the range pH 4.8–8.8) and alkali-metal ion concentration (in general 0, 50 and 300 mM, with exceptions, e.g. Fig. 4) 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 (Cleland, 1977). Preliminary estimations of the kinetic parameters V_{max} and K_m for each given saturation curve were performed by non-linear regression analysis (Vasseur *et al.*, 1982).

Mathematical techniques

The general equation derived from our sucrase model was used to seek, in a single run, the best fit of our entire set of kinetic data by non-linear least-squares regression analysis. Briefly, we followed the procedure of Fletcher & Powell (1964) as modified by Robinson *et al.* (1983), in which a set of uncorrected velocity data can be written as:

$$\log\{v_{\text{obs.}}([S], [A], [H^+])\} = \log\{v_{\text{theor.}}([S], [A], [H^+]) + \delta([S], [A], [H^+])\}$$

where $v_{\text{obs.}}$ is the observed v value for each given $[S]$, $[A]$ and $[H^+]$, $v_{\text{theor.}}$ is the predicted v value and δ reflects the random variation of the observations. The regression function can then be fitted to the complete data set by minimizing the sum of squares of errors. Comparison of a 'lack of fit' component with a 'pure error' one is finally accomplished to obtain an F value that provides a quantitative assessment of the goodness of fit.

In a second step, by imposing appropriate restrictions in the regression function, the significance of each estimated parameter was re-evaluated. Examples of the procedure used to evaluate our results are given in the text. All calculations, including the iterations, were performed with a Hewlett-Packard model HP-85 micro-computer.

THEORETICAL CONSIDERATIONS

In brush-border sucrase the acid ionization constant, K_1 , involves two protons because the $\log(V_{\text{max.}}/K_m)$ -versus-pH, $\log V_{\text{max.}}$ -versus-pH and $\text{p}K_m$ -versus-pH profiles have been found to yield limiting slope values of +2, +1 and +1 respectively (see Vasseur *et al.*, 1982). As a consequence of this, a model (Fig. 1a) based on classical concepts of pH-dependent enzyme activation (Segel, 1975; Tipton & Dixon, 1979) proved insufficient to explain our results on the pH-dependent activation of sucrase by the alkali-metal ions. To solve this problem, we proposed (Vasseur *et al.*, 1982) the splitting of K_1 into two separate ionization reactions, based on the rules of dibasic acid dissociation (Adams, 1916):

Protonation levels:

where H_x and H_y are the two protons forming K_1 , the acid ionization constant, and H_z is the single component of K_0 , the basic ionization constant. In contrast with classical models where, rather than only K_1 , the entire enzyme is treated as a dibasic acid (Tipton & Dixon, 1979; Cornish-Bowden, 1979), the three-protons model exhibits five different enzyme forms arranged in four protonation levels, three of which correspond to K_1 .

Although the splitting of K_1 is necessary to interpret sucrase correctly, it is insufficient to explain the distinct

Fig. 1. Two-dimensional representation of the interaction between a monomeric enzyme, H^+ and the substrate, S , according to either a classical two-protons model (a) or our three-protons model (b)

(a) The two-protons model. Because the enzyme contains only two key protons, the various enzyme forms can be arranged into three protonation levels: it is assumed that only the monoprotonated enzyme at L_1 (ovals) is catalytically active. However, direct application of this model to sucrase is prevented by the fact that, with this enzyme, the forms at L_2 contain three rather than two key protons, and these, furthermore, have different kinetic functions. The two-protons model makes no distinction between the protons involved in the deprotonation reaction linking L_2 to L_1 . (b) The proposed three-protons model for sucrase. To distinguish between the two protons just mentioned, the acid ionization constant, K_1 , has been split into two distinct pathways. As a result of this, and because K_1 and K_0 are independent, the model contains five different enzyme forms distributed into four protonation levels. Enzyme forms EH_3 and EH_{2x} , both of which bear the proton H_x , have no affinity for S . The S -binding forms at L_{2y} and L_0 (which either carry proton H_y or lack proton H_2) have no catalytic activity. Loss of both H_x and H_y (and retention of H_2) are postulated to be required to yield the fully active monoprotonated form, EH , at L_1 .

kinetic function of the two prototropic groups involved: the four enzyme forms participating in K_1 must also have different binding properties. Indeed, whether all four enzyme forms constituting K_1 bind substrate or whether only EH_z is capable of substrate binding, the limiting slopes of double-logarithmic plots will not yield the pattern found by us. It needs to be postulated that the enzyme forms containing H_x (EH_3 and H_xEH_z) have no affinity for S , whereas those without H_x (H_yEH_z , $E'H_z$ and E) are able to bind. In turn, S must prevent H_x from binding, in accord with the observation that S and H_x are reciprocal fully competitive inhibitors (Vasseur, 1985). The second proton, H_y , behaves as a mixed inhibitor and its release causes the enzyme to become catalytically active. The resulting model is illustrated in Fig. 1(b). It is clear that loss of both H_x and H_y (and gain of H_2) is required to generate the fully active enzyme form EH_z .

The complete three-protons model for sucrase

The model shown in Fig. 1(b) is a framework upon which the interaction of sucrase with an alkali-metal ion, A (or I ; see below), can be studied. By considering all possible interactions, we obtain the complete sucrase model shown in Fig. 2, which distinguishes two opposing roles for the alkali-metal ions, activation and inhibition (further details are given below).

It should be emphasized that the three-protons model (Figs. 1b and 2) is the simplest one capable of explaining sucrase fully, and our data cannot be fitted by any model failing to account for the following. (i) Sucrase has three key prototropic groups: two for the acid ionization constant K_1 , and one for the basic ionization constant K_0 . (ii) The two protons pertaining to K_1 are either gained or lost as a block, a fact experimentally reflected by the free enzyme exhibiting a single pK_1 value with slope of $+2$ in $\log(V_{max}/K_m)$ -versus-pH plots. (iii) In spite of their concerted behaviour, the kinetic functions of the two protons are different. One is involved in V-type and the other in K-type activation effects, which explains the slopes of $+1$ in either $\log V_{max}$ -versus-pH or pK_m -versus-pH plots. (iv) Below pH 7 the alkali-metal ions have complex effects, indicating the existence of two different types of metal-ion-binding sites, namely activatory or A-sites and inhibitory or I-sites.

In addition, we take account of the following further facts (Alvarado & Mahmood, 1974; Mahmood & Alvarado, 1975; Semenza & von Balthazar, 1974; Semenza, 1981). (v) Sucrase is a monomeric enzyme, so that the various sites forming the three-protons model must occur in the same polypeptide chain. (vi) The ionization state of the substrate and the modifier, A , does not change throughout the pH range studied. We also

Fig. 2. Three-dimensional representation of the complete three-protons model for intestinal brush-border 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)

Also considered are the complex activating and/or inhibiting effects of the alkali-metal ions in the pH range 4.8–8.8. Below pH 7, at low concentrations, the interaction of the alkali-metal ions with the activatory A -sites stabilizes the monoprotonated enzyme forms, causing K -type activation. At high concentrations, these ions bind to the inhibitory I -sites, thereby stabilizing the inactive form at L_3 (but not at L_{2x} ; see the Results and interpretation section), which causes K -type inhibition. To simplify the Figure, only two I -sites have been drawn. Above pH 7, the alkali-metal ions (and Li^+ in particular) displace the equilibrium towards the inactive forms at L_0 , causing fully non-competitive inhibition.

assume the following. (vii) The substrate-binding, modifier-binding and proton-binding steps are all in rapid equilibrium. (viii) Only the monoprotonated enzyme form, $E'H_2$, is catalytically active and product formation is rate-limiting, so that the enzyme exhibits simple Michaelis–Menten kinetics throughout the pH scale. (ix) Finally, the acid and basic ionization constants are separated by more than 2 pH units and therefore behave independently.

The general equation

Since only the monoprotonated enzyme species are catalytically active:

$$v = k[E'H_2-S] + k'[A-E'H_2-S] \quad (2)$$

which, together with the state equation ($[E_T]$ is the sum of all enzyme complexes illustrated in Fig. 2), yields the following overall rate equation:

$$v = \frac{V_{\max} \cdot [S]}{[S] + K_m} = \frac{(V\{1 + Q([A]/k'_{a1})\}/(\alpha)) \cdot [S]}{[S] + K_{s1}(\beta/\alpha)} \quad (3)$$

where:

$$\alpha = \frac{[H^+]}{K_{1ys}} \left(1 + \frac{[A]}{K'_{a2}} \right) + \left(1 + \frac{[A]}{K'_{a1}} \right) + \frac{K_{0s}}{[H^+]} \left(1 + \frac{[A]}{K'_{a0}} \right) \quad (4)$$

$$\beta = \frac{[H^+]^2}{K_{2x} \cdot K_{1y}} \left(1 + \frac{[A]}{K_{13}} \right)^{n_{13}} + \frac{[H^+]}{K_{1x}} \left(1 + \frac{[A]}{K_{12}} \right)^{n_{12}} + \frac{[H^+]}{K_{1y}} \left(1 + \frac{[A]}{K_{a2}} \right) + \left(1 + \frac{[A]}{K_{a1}} \right) + \frac{K_0}{[H^+]} \left(1 + \frac{[A]}{K_{a0}} \right) \quad (5)$$

and

$$Q = k'[E_T]/k[E_T] = V'/V \quad (6)$$

Eqn. (3) is equivalent to that of Michaelis & Menten, $V_{\max}$ and K_m being complex functions of $[A]$ and $[H^+]$.

By applying the well-known theories of Alberty (1954), Cleland (1977) and Tipton & Dixon (1979) concerning the effects of pH on the relevant kinetic parameters, the limiting values of the slopes derived from double-logarithmic transformations versus either $\log(V_{\max}/K_m)$, $\log V_{\max}$ or pK_m permit a direct assessment of the number of protons involved in a proposed model. The α factor

(eqn. 4), which only concerns $V_{\max}$, is relatively simple as it involves a single proton at either side of the pH scale. On the other hand the β function (eqn. 5), which concerns the ratio $V_{\max}/K_m$, is complex because it involves two protons on the acid side and one single proton on the basic side. Mechanistically, the three-protons model is the only model capable of yielding the slope pattern that we have found for sucrase, which implies the participation of distinct V and K effects in the same acid ionization reaction.

The β function has been arranged in eqn. (5) to show the diverse roles played by A, which, as our results show, behaves differently at the various protonation levels. At L_1 , for instance, A assumes its 'normal' (activatory) role, which we previously described as Model I (see Vasseur *et al.*, 1982). In contrast, at level L_3 , similar to H^+ , A is a competitive inhibitor rather than an activator. Furthermore, at this level, A appears to interact with several (inhibitory) binding sites, whose number is represented by the exponent n_{13} .

In a first stage, we considered the possibility that several distinct inhibitory sites exist at levels L_3 and L_{2x} , so that a family of inhibition constants (K_{131} , K_{132} , ..., K_{13n}) could occur at each of these levels. However, since this diversity of inhibitory sites would yield exceedingly complex equations that would serve no useful purpose, we have derived eqn. (5) on the simplifying assumption that all inhibition constants belonging to the same level have identical values, so that each of these constants will appear only once, although raised to the power of the

Table 1. The R parameters constituting the complete sucrase model in Fig. 2

$$\begin{aligned} R_1 &= K_{s1}/K'_{s1} = K_{a1}/K'_{a1} \\ R_2 &= K_{s2}/K'_{s2} = K_{a2}/K'_{a2} \\ R_3 &= K_{s1}/K_{s2} = K_{1y}/K_{1ys} \\ R_4 &= K'_{s1}/K'_{s2} = K'_{1ya}/K'_{1yas} \\ R_5 &= K_{a1}/K_{a2} = K_y/K_{1ys} \\ R_6 &= K'_{a1}/K'_{a2} = K'_{1ys}/K'_{1yas} \\ R_7 &= K_{2x}/K_{1x} = K_{2y}/K_{1y} \\ R_8 &= K_{12}/K_{13} = K_{2y}/K_{2y1} \\ R_9 &= K_{s0}/K'_{s0} = K_{a0}/K'_{a0} \\ R_{10} &= K_{s0}/K_{s1} = K_{0s}/K_{0as} \\ R_{11} &= K'_{s0}/K'_{s1} = K_{0s}/K_{0as} \\ R_{12} &= K_{s0}/K_{s1} = K_{0s}/K_{0as} \\ R_{13} &= K_{a0}/K'_{a1} = K_{0s}/K_{0as} \end{aligned}$$

number of sites involved [e.g. $(K_{13})^{n_{13}}$]. We have nonetheless allowed for the possibility that $K_{13} \neq K_{12}$ and $n_{13} \neq n_{12}$. As discussed below, however, our results indicate that n_{12} equals zero for both Na^+ and Li^+ , so that eqn. (5) can be simplified because the term $(1 + [A]/K_{12})^{n_{12}}$ can be assumed to equal 1.

RESULTS AND INTERPRETATION

Overall fit of the three-protons model to the sucrase results

Given the availability of microcomputers and sophisticated mathematical techniques (Fletcher & Powell, 1964;

Table 2. Kinetic parameters estimated for the metal-ion-independent interaction of sucrase with S and H^+ , according to the three-protons model

Eqn. (3) was used to obtain in a single run a fit of the three-protons model to the entire body of sucrase data covering the pH range 4.8–8.8. To obtain the eight metal-ion-independent estimates shown in **bold type**, three separate runs were performed, namely one with only the control data (no metal ions present: $n = 876$ data points with 128 different [S] values) and two with the control data supplemented with those obtained in the presence of either Li^+ ($n = 468$ with 133 [S] values) or Na^+ ($n = 355$ with 102 [S] values). For the last two columns, only the eight metal-independent estimates out of the whole set of 27 estimates are shown here. Standard deviations ($\pm$ S.D.) are given for the estimated parameters only; the remaining parameters were calculated from these by applying the R definitions in Table 1. Dissociation constants are given in molar units; V is expressed in units/mg of protein. Microscopic ionization constants are also expressed (parentheses) as pK values. The F test includes the mean of squares of errors (M.S.E.), the mean of squares of lack of fit (M.S.L.F.) and the mean of squares of pure error (M.S.P.E.) (Robinson *et al.*, 1983). The degrees of freedom are given in square brackets. Further details are given in the text.

Metal ion ...	None	Li^+	Na^+
V	9.20 ± 1.02	9.21 ± 1.29	9.20 ± 1.11
K_{s2}	$(8.16 \pm 1.53) \times 10^{-3}$	$(9.26 \pm 1.64) \times 10^{-3}$	$(8.00 \pm 1.33) \times 10^{-3}$
K_{s1}	$(13.03 \pm 2.14) \times 10^{-3}$	$(12.95 \pm 2.04) \times 10^{-3}$	$(13.03 \pm 1.93) \times 10^{-3}$
K_{s0}	$(44.41 \pm 8.35) \times 10^{-3}$	$(44.54 \pm 7.91) \times 10^{-3}$	$(44.41 \pm 7.45) \times 10^{-3}$
K_{2x}	$(3.55 \pm 0.66) \times 10^{-7}$ (6.45)	$(4.08 \pm 0.71) \times 10^{-7}$ (6.39)	$(3.48 \pm 0.37) \times 10^{-7}$ (6.46)
K_{1y}	$(9.77 \pm 1.78) \times 10^{-6}$ (5.01)	$(8.48 \pm 1.43) \times 10^{-6}$ (5.07)	$(9.97 \pm 1.58) \times 10^{-6}$ (5.00)
K_{1x}	$(6.69 \pm 1.26) \times 10^{-5}$ (4.17)	$(3.44 \pm 0.61) \times 10^{-4}$ (3.46)	$(5.88 \pm 0.98) \times 10^{-5}$ (4.23)
K_{2y}	5.18×10^{-8} (7.29)		
K_1			
K_{1ys}	6.12×10^{-6} (5.21)		
K_0	$(8.77 \pm 1.65) \times 10^{-9}$ (8.06)	$(8.88 \pm 1.58) \times 10^{-9}$ (8.05)	$(8.77 \pm 1.47) \times 10^{-9}$ (8.06)
K_{0s}	2.57×10^{-9} (8.59)		
R_7	5.30×10^{-3}		
F test:			
M.S.E.	0.035 [868]	0.032 [1327]	0.028 [1214]
M.S.L.F.	0.070 [120]	0.057 [244]	0.048 [213]
M.S.P.E.	0.029 [748]	0.026 [1083]	0.024 [1001]
F	2.39	2.21	2.02

Robinson *et al.*, 1983), we sought to obtain in a single run a global iterative fit to the three-protons model of our entire set of sucrose kinetic data. To do this, we prepared a computer program to solve eqn. (3) by non-linear least-squares regression analysis.

Among the 28 different parameters constituting the full sucrose model in Fig. 2, some are related by the *R* parameters listed in Table 1. By eliminating those parameters that are redundant, we obtain 17 independent parameters. The computer program therefore has been directed to estimate the relevant independent parameters, from which the remaining, dependent, ones can be calculated by using the appropriate *R* values. Standard deviations are therefore given only for the estimated parameters. It should be pointed out that the whole set of 17 estimates reduces to either: (i) eight metal-ion-independent estimates when interactions with A do not apply (as in Fig. 1b) or (ii) nine metal-ion-dependent estimates when only the alkali-metal ions are present.

The results obtained by applying this program to the entire body of our sucrose $v = f([S], [A], [H^+])$ data, covering the pH range 4.8–8.8, are summarized in Tables 2 and 3.

As concerns the statistical significance of the results obtained, it is important to note that the numerical values of constants obtained for the metal-ion-independent estimates were essentially the same when the iterations were performed with either (i) data obtained in the absence of any metal ion, meaning that the regression function contains only eight estimates (Table 2, first column), or (ii) data that included either $[Na^+]$ or $[Li^+]$ as a variable, where the whole set of 17 estimates was involved (Table 2, the last two columns).

Equally stable were the metal-ion-dependent estimates, which were essentially identical (Table 3), regardless of whether or not the metal-ion-independent estimates had been fixed before each iteration was attempted (the regression function thereby containing either nine or 17 estimates). This stability of the results and its independence from the total number of estimates involved strongly supports the validity of our mathematical approach.

Double-logarithmic plots: comparison between global-fit profiles and the experimental data

An overview of the goodness of fit to the three-protons model of our entire set of data is given in Fig. 3. By using classical double-logarithmic plots, we illustrate how the control data (in the absence of metal ions) compare with those obtained with 300 mM concentrations of either Li^+ or Na^+ and how V_{max} and K_m data estimated by non-linear regression analysis of individual sucrose-saturation curves are fitted very well by theoretical curves computed by using eqn. (3) and the numerical constants derived from the global fit. Furthermore, Fig. 3 illustrates the dissymmetry exhibited by the slopes of the $\log V_{max}$ -versus-pH and the $\log(V_{max}/K_m)$ -versus-pH plots in the acid side of the pH spectrum.

Dual effect of the alkali-metal ions at acid pH values

Our results show that both Li^+ and Na^+ have complex effects on K_m at acid pH values. At low concentrations they induce it to decrease (K-type activation) but can cause it to increase again (K-type inhibition) if present in sufficiently high concentrations. This biphasic effect is especially evident with Li^+ (Fig. 4).

Table 3. Kinetic parameters computed for the various activatory and inhibitory effects of the alkali-metal ions on sucrose, according to the three-protons model

Eqn. (3) was used to obtain a single-run fit to the entire body of sucrose data, obtained in the presence of each of the indicated cations, after fixing the eight metal-ion-independent estimates to the values shown in Table 2, first column. The regression function therefore contains nine metal-ion-dependent estimates ($\pm$ S.D.), indicated in **bold** type. The other eight parameters were derived from the estimated ones by applying the *R* definitions in Table 1. Further explanation and details are given in the text.

Metal ion...	Li^+	Na^+
V'	20.67	16.05
Q	2.25 ± 0.33	1.75 ± 0.18
$*K'_{s2}$	0.10×10^{-6}	1.01×10^{-6}
$*K_{s2}$	632.70 ± 101.6	5.00 ± 0.52
K_{s2}	$(7.53 \pm 1.21) \times 10^{-3}$	$(0.62 \pm 0.07) \times 10^{-3}$
R_2	8.41×10^4	0.81×10^4
K'_{s1}	10.28×10^{-3}	6.89×10^{-3}
K_{s1}	$(28.09 \pm 4.51) \times 10^{-3}$	$(1.71 \pm 0.18) \times 10^{-3}$
K_{s1}	$(22.17 \pm 3.36) \times 10^{-3}$	$(0.91 \pm 0.09) \times 10^{-3}$
R_1	1.25	1.89
K'_{s0}	5.54×10^{-3}	19.78×10^{-3}
K_{s0}	$(1.93 \pm 0.31) \times 10^{-3}$	$(0.22 \pm 0.02) \times 10^{-3}$
K_{s0}	$(0.24 \pm 0.04) \times 10^{-3}$	$(0.10 \pm 0.01) \times 10^{-3}$
R_0	8.01	2.25
K_{i3}	$(406.1 \pm 55.59) \times 10^{-3}$	$(65.88 \pm 6.91) \times 10^{-3}$
n_{i3}	6.47 ± 0.76	2 ± 0.21
K_{1ya}	0.22	(0.66)
K_{1yas}	2.08×10^{-6}	(5.68)
K_{0a}	1.28×10^{-7}	(6.89)
K_{0as}	2.37×10^{-7}	(6.63)
F test:		
M.S.E.	0.025 [459]	0.011 [346]
M.S.L.F.	0.045 [124]	0.019 [93]
M.S.P.E.	0.018 [335]	0.007 [253]
F	2.46	2.59

* The very large value for K_{s2} and, consequently, the very low one for K'_{s2} come from the assumption that the model is symmetrical.

The results for the $K_m = f([Li^+])$ function (curve a) reveal a certain discrepancy between the theoretical curve calculated from the global fit and the experimental data. However, this is the only instance of lack of fit in the present study. It is technically difficult to measure K_m when most of the enzyme is in the low-affinity form EH_3 , and this difficulty is compounded when a competitive inhibitor such as Li^+ is present. In addition, the n-butylamine present in the buffer may not be totally inert here. In previous studies we found indications that t-butylamine can interfere with Li^+ at pH 5.2, although with this amine there was no evidence of inhibition with Li^+ concentrations up to 100 mM (Alvarado & Mahmood, 1979; Alvarado *et al.*, 1980). In experiments where the n-butylamine in the universal buffer was replaced by LiOH (M. Vasseur, G. van Melle, R. Frangne & F. Alvarado, unpublished work), the enzyme gave $K_m = 22$ mM, a value that remained constant at Li^+ concentrations ranging from 20 to 120 mM. The K-type inhibition

Fig. 3. Plots of $\log V_{\max}$ and $\log (V_{\max}/K_m)$ versus pH in the absence of any metal ion (●) and in the presence of either 300 mM- Li^+ (■) or 300 mM- Na^+ (▲)

The experimental points shown were calculated by non-linear regression analysis as applied individually to the appropriate set of saturation curves. The theoretical curves fitting these data were calculated by using eqn. (3) and the parameters estimated from the global fits (Tables 2 and 3).

Fig. 4. Variation of the apparent K_m as a function of either $[\text{Li}^+]$ (■) or $[\text{Na}^+]$ (▲), at pH 5.2

Theoretical curves (—; a for Li^+ and b for Na^+) were calculated by using eqn. (3) and the constants obtained from the global fits (Tables 2 and 3). As explained in the text, to illustrate the activatory effect of Li^+ more clearly, its inhibitory effect was masked by making $n_{13} = 0$ when theoretical curve a' (-----) was calculated.

caused by Li^+ became evident only at concentrations of 200 mM or higher (e.g. $K_m = 80$ mM at 420 mM- Li^+ ; compare with the data in Fig. 4). Finally, another explanation for the lack of fit of the Li^+ inhibition results could be that, in the equation used to fit the data, we assume that all Li^+ -binding sites have identical K_i values, which is probably an oversimplification.

We therefore conclude that the lack of exact fit of the Li^+ inhibitory effects illustrated in Fig. 4 does not invalidate the premise that this alkali-metal ion is a competitive inhibitor of sucrase at high concentrations, although this effect is clearly evident only when n-butylamine is present.

Estimating the number of metal-binding inhibitory sites in sucrase

We showed previously that the data cannot be fitted if the splitting of K_i is not taken into account (Model II proposed by Vasseur *et al.*, 1982), which indeed is the reason why the three-protons model needed to be developed. Another problem concerns the number of inhibitory metal-ion-binding sites needed to explain the inhibitory effect of the alkali-metal ions at acid pH values. One observation warranting detailed analysis was that Li^+ , not Na^+ , behaves as a strong inhibitor at pH 5.2 (Fig. 4). This finding, which suggested the existence of a qualitative difference between the two alkali-metal ions, led us to enlarge the model to include the metal-ion-binding inhibitory I-sites at L_3 and, perhaps, L_{2x} .

Although visual inspection of the Na^+ data in Fig. 4 seems to reveal only an activatory effect, the computer fits indicate the existence of a significant inhibition, meaning that, most probably, the difference between the two metal ions is merely quantitative. The affinity of Na^+ for the inhibitory sites seems to be greater than that of Li^+ ($K_{i3} = 66$ mM for Na^+ , 406 mM for Li^+ ; Table 3). However, as we see next, there appear to be only two inhibitory Na^+ -binding sites, as compared with seven for Li^+ .

If Li^+ first causes K_m to decrease but at concentrations over 50 mM to increase, the exponential shape of the curve (Fig. 4) indicates that several, rather than only one, inhibitory Li^+ -binding sites exist. The best computer fits of our data, based on the simplifying assumption that all of these sites have identical K_i values, gave $n_{13} = 6.47$ and 2.0 for Li^+ and Na^+ respectively (Table 3). To find the integer value best fitting the Li^+ data, this result was further analysed by fixing n_{13} to the series of values indicated in Table 4. It appears that the F function exhibits a minimum at $n_{13} = 7$. In contrast, for Na^+ this minimum was found to occur at $n_{13} = 2$ (results not illustrated). We therefore conclude that the best fits of our sucrase data indicate the existence at L_3 of seven and two inhibitory sites for Li^+ and Na^+ respectively. Why protonation of a certain enzymic group should unmask an unequal number of inhibitory sites for these two metal ions cannot be answered at present.

For illustration purposes, the pure K-type activating effect of Li^+ can be separated from the inhibitory one by giving to n_{13} a value of zero (instead of the estimated one, Table 3). The theoretical curve obtained in this way (a' in Fig. 4) shows that the limiting value of K_m as $[\text{A}]$ tends to infinity is 2.5 and 2.8 mM for Li^+ and Na^+ respectively. Nevertheless, it should be emphasized that these calculations were made only to illustrate the pure K-type activatory effect of the alkali-metal ions when binding to

Table 4. Statistical analysis performed to estimate the number of Li⁺-binding inhibitory sites in sucrase, according to the three-protons model

Eqn. (3) was used to obtain in a single run a fit to the entire body of sucrase data obtained in the presence of Li⁺: N indicates the number of parameters free to float. The first line ($N = 9$) shows the result obtained after fixing the eight metal-ion-independent parameters, as in Table 3. By fixing, in addition, the number of inhibitory binding sites, n_{13} , the regression function simplifies to $N = 8$. For the special case where $n_{13} = 0$, the regression function reduces further to $N = 7$ because the K_{13} parameter has no meaning.

N	Fixed n_{13} value	M.S.E.	M.S.L.F.	M.S.P.E.	F
9	(Free)	0.025 [459]	0.045 [124]	0.018 [335]	2.46
7	0	0.174 [461]	0.588 [126]	0.018 [335]	32.49
8	1	0.091 [460]	0.283 [125]	0.018 [335]	15.61
8	2	0.030 [460]	0.063 [125]	0.018 [335]	3.50
8	3	0.027 [460]	0.0498 [125]	0.018 [335]	2.75
8	4	0.0256 [460]	0.0458 [125]	0.018 [335]	2.53
8	5	0.0253 [460]	0.0446 [125]	0.018 [335]	2.46
8	6	0.0252 [460]	0.044214 [125]	0.018 [335]	2.442966
8	7	0.0252 [460]	0.044213 [125]	0.018 [335]	2.442918
8	8	0.0252 [460]	0.0444 [125]	0.018 [335]	2.45
8	9	0.0253 [460]	0.0446 [125]	0.018 [335]	2.46

A sites: if n_{13} is fixed at zero, the fit is rejected according to an F test (see Table 4), and this is true for both Li⁺ and Na⁺. In contrast, neither Na⁺ nor Li⁺ binds at L_{2z} ($n_{12} = 0$ for both); all fits attempted on the premise that metal-ion binding occurs at this level were also rejected.

DISCUSSION

The global iteration procedure used to fit in a single step our brush-border sucrase data can supply the best values for all the kinetic constants forming the model, an objective that cannot be attained by using double-logarithmic plots that are functions of $[H^+]$, not of $[A]$ (Cleland, 1979; Viola, 1984). Moreover, instead of yielding apparent (macroscopic, molecular; Dixon & Tipton, 1973) ionization constants, the overall iteration procedure goes directly to estimate the best values for each of the microscopic (group) ionization constants, from which the macroscopic ones are deduced. To the best of our knowledge, this approach is the only one giving direct access to group ionization constants, thus making it unnecessary to use cumbersome methods involving graphical procedures and the replots of intercepts and slopes.

From our results, an explanation of the complex effects of the alkali-metal ions on sucrase emerges. The binding of S at L_0 is confirmed, making sucrase a V-type enzyme on the basic half to the pH-activity curve. Activation by the alkali-metal ions at levels L_1 and L_{2y} according to our previous pH-independent model (see Vasseur *et al.*, 1982) is demonstrated by the values greater than 1 found for the parameters R_1 and R_2 (Table 3).

Basic ionization constant

An interpretation of K_0 is easy because this ionization reaction involves only microscopic constants. The results (Tables 2 and 3) show that ionization of the free enzyme ($pK_0 = 8.06$) is displaced to the right by S ($pK_{0s} = 8.59$), meaning substrate activation. In contrast the alkali-metal ions inhibit by displacing the corresponding pK to

the left ($pK_{0a} = 6.89$ and 7.18 for Li⁺ and Na⁺ respectively). Even more interestingly, this difference between the two ions (0.3 pH unit larger for Li⁺ than for Na⁺) is greater when S is present: pK_{0as} equals 6.63 with Li⁺ and 7.63 with Na⁺. This difference of a full pH unit in favour of Li⁺ explains why this metal ion is such a strong sucrase inhibitor above pH 7 (Alvarado & Mahmood, 1979).

Acid ionization constant

An analysis of K_1 is more difficult because it is a molecular ionization constant that involves complex (V-type plus K-type) kinetic effects. The difficulty of analysing K_1 derives also from the fact that the free enzyme and the ES complex cannot be compared directly because a different number of protonation levels is involved (see Fig. 2). For the free enzyme, as deduced from $\log(V_{\max}/K_m)$ -versus-pH plots (slope = +2), K_1 involves a two-level jump (L_3 to L_1), so that $pK_1 = \frac{1}{2}(pK_{2z} + pK_{1y}) = \frac{1}{2}(pK_{2y} + pK_{1x})$. On the other hand, with regard to the ES complex, deduced from $\log V_{\max}$ -versus-pH plots (slope = +1), the jump occurs only from L_2 to L_1 and involves one single (group) constant, pK_{1ys} .

Similar difficulties are encountered when the effects of the alkali-metal ions are analysed; these can be quite different depending, for instance, on whether the overall ionization constant, K_1 , or the microscopic one, K_{1y} , is considered. Thus Li⁺ activates by facilitating enzyme deprotonation, so that pK_{1y} falls drastically from a 5.01 value to give $pK_{1ya} = 0.66$ (at saturating Li⁺ concentrations; see Table 3). In contrast, after an initial decline at low concentrations, Li⁺ causes pK_1 steadily to increase, in agreement with the tenet that Li⁺ favours enzyme reprotonation. At moderately high concentrations (300 mM-Li⁺, Fig. 3) pK_1 increases from 5.73 to 5.97 but, more interestingly, the theoretical limit of this increase is $pK_1 = \infty$ at infinite Li⁺ concentrations.

A complete analysis of the acid ionization constant, which we have studied in detail as a function of each $[H^+]$, $[A]$ and $[S]$, has been developed (M. Vasseur, G. van Melle & F. Alvarado, unpublished work; see also

Vasseur, 1985), but would exceed the limits of the present paper.

Before closing, it is worth considering K_1 in terms of the relationship $R_7 = K_{2x}/K_{1x} = K_{2y}/K_{1y}$ (Table 1). An equivalent relationship has been applied to enzymes, although here it normally concerns K_1 and K_0 , the entire enzyme being treated as a dibasic acid (Tipton & Dixon, 1979). In such cases the relevant R parameter is generally large (negative co-operativity) because loss of the first proton normally hinders release of the second (Tipton & Dixon, 1979; Dixon & Tipton, 1973). Free amino acids give identical results: e.g. $R = 120$ for glycine (Mahler & Cordes, 1971). In sharp contrast, in our treatment R_7 concerns only K_1 . The results give $R_7 = 0.005$ for the free enzyme (Table 2), indicating a strong positive co-operativity. This co-operativity explains the practically simultaneous release of H_x and H_y and hence why sucrase exhibits a single pK_1 value: the pK of the second deprotonation in the sequence [which equals $pK_{1xy} = \log(K_{2x} + K_{2y})/(K_{2x} \cdot K_{1y}) = 5.07$] is smaller than that of the first [$pK_{2xy} = -\log(K_{2x} + K_{2y}) = 6.39$].

Let us now compare directly the K_1 constant in the three-protons model with the original model proposed by Adams (1916). For both free amino acids and enzymes, re-arrangement of the relationship forming the R_7 parameter permits defining a pH-independent ratio (Tipton & Dixon, 1979), P_1 , such that:

$$P_{1(\text{diprotic amino acid})} = [\text{zwitterion}]/[\text{un-ionized form}]$$

and

$$P_{1(\text{enzyme})} = [EH_{2y}]/[EH_{2x}]$$

which, in our nomenclature, gives:

$$P_1 = K_{2x}/K_{2y} = K_{1x}/K_{1y}$$

For typical amino acids P_1 is known to be very large [e.g. 2.2×10^5 for glycine (Mahler & Cordes, 1971)], meaning that the un-ionized species is virtually non-existent in aqueous solution. In contrast, from our results (Table 2) it can be calculated that P_1 is as low as 6.85 for sucrase, meaning that the diprotonated enzyme consists of a mixture of about 87.3% EH_{2y} and 12.7% EH_{2x} . We therefore conclude that, whereas for amino acids and most enzymes one of the four possible ionic species in Adams' (1916) model is usually non-existent, for sucrase the inactive EH_{2x} form constitutes a significant proportion of the total free enzyme, a fact fully justifying the three-protons model.

In conclusion, R_7 is an index of the co-operative nature of an enzyme's key deprotonation reaction (positive or negative), whereas P_1 determines whether or not the enzyme form at L_{2x} will be quantitatively negligible. However, application of the R_7 and P_1 tests requires knowing the numerical value of the microscopic ionization constants involved. As far as we can ascertain, global iteration is the best method available at present to obtain the necessary estimates.

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