pH-activity curves for enzyme-catalysed reactions in which the hydron is a product or reactant

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A reaction in which the hydron [International Union of Pure and Applied Chemistry (1986) Chem. Int. 8 (4), 21] is a reactant will have a more acidic pH optimum than the reverse reaction in which it is a product. Simple mechanistic models, made by postulating various ways in which the hydron is supplied for the forward reaction, lead to the prediction that the pK that characterizes the fall in activity on the alkaline side for the forward reaction will be equal to that characterizing the fall on the acid side for the reverse reaction. It is not possible to identify what is responsible for this pK without additional information; as is well known, different possible dispositions of hydrons in transition states cannot usually be distinguished solely by kinetics.

INTRODUCTION

The present paper examines how pH-activity curves for forward and reverse reactions may be related in enzyme-catalysed reactions in which the hydron [as the International Union of Pure and Applied Chemistry (1986) suggests that the ion H^+ be called generally, irrespective of isotope] is taken up in one direction and given out in the reverse direction. It is common and convenient to write such a reaction with H^+ as reactant or product. By considering possible ways in which the reactant hydron may be supplied, however, the problem is reduced to that of the hydronic state of the enzyme required for activity. Standard treatment then leads to a general conclusion, and its limitations are discussed after the treatment.

1. GENERAL FEATURES OF THE MODELS TREATED

Many enzymes show a reversible dependence of activity on pH that has the same form as the concentration of the monohydrated species of a dibasic acid (for review see Tipton & Dixon, 1979). There are two obvious reasons why there may be exceptions. The first is that hydration of each of several groups might cause successive changes in activity. Since, however, many enzymes follow the simple relationship, it seems worth exploring models in which one form of a group gives full activity, and the other no activity. The second obvious cause for breakdown of the simple relation is that hydration of either of two groups of close pK values could activate or deactivate. This can usually be ignored in the present context, because it causes only slight distortion of the bell-shaped curves (Brocklehurst *et al.*, 1983), but it will be discussed in section 2 in connection with Fig. 2 (below).

The forward direction is considered to be that in which a hydron is taken up, and the reverse direction that in which it is released.

It is useful to determine the pH-dependences of two constants. One is k_o , the catalytic constant ($= V/[E]_o$, where V is the limiting velocity and $[E]_o$ is the total

enzyme concentration). The second is k_A , the specificity constant, which is defined by the equation:

$$v = k_A \cdot [A][E]$$

where v is the rate of reaction, and $[A]$ and $[E]$ are the concentrations of unligated substrate A and unligated enzyme E. It is also equal to k_o/K_m , where K_m is the Michaelis constant [for definitions, see Nomenclature Committee of the International Union of Biochemistry (1983)]. The pH-dependence of the catalytic constant provides pK values of the enzyme-substrate complex (or complexes), and that of the specificity constant can provide pK values of the free enzyme (or substrate) (see Brocklehurst & Dixon, 1976, 1977). The pH-dependence of rate at arbitrary fixed substrate concentration is of little value (Knowles, 1976). The treatment that follows applies equally well to the variation with pH of catalytic and specificity constants.

2. FIRST MODEL: INDEPENDENT GROUPS

In the first model treated, the enzyme has three ionizing groups of relevance, and they are considered to be independent, i.e. their pK values do not change as the others (or other ionizing groups in the molecule) gain or lose hydrons. The second model will relax this condition; even in the first model it can be relaxed to the extent that it does not matter if the pK values should be affected by gains and losses of hydrons that occur outside the pH range at which the enzyme is active.

Groups A and B need to be dehydrated (as $-A^-$) and hydrated (as $-BH$) respectively for the enzyme to be active. They will be called the 'structural groups'. The structural groups may be required in particular forms in order to give the enzyme a particular conformation, or because these forms are needed close to the active centre to assist catalysis, substrate binding, substrate access, etc. The treatment applies equally to both situations.

Group C is the acidic group that donates the hydron for the forward reaction and whose conjugate base accepts it for the reverse reaction, e.g. the imidazole group of a histidine residue in lactate dehydrogenase

Fig. 1. Dependences of concentrations of particular enzyme forms on pH

(a) Logarithms of fractional concentrations of required forms of structural groups A and B; (b) logarithms of fractional concentrations of required forms of the participating group C; (c) logarithms of fractional concentrations of required forms of the enzyme molecule; (d) fractional concentrations of required forms of the enzyme molecule. The enzyme requires groups -A and -B to be in the forms $-A^-$ and $-BH$ for activity. The forward reaction requires group -C in the form $-CH$ to supply a hydron, whereas the reverse reaction requires it in the form $-C^-$ to receive one. The pK values of A, B and C have been arbitrarily set at 4.5, 10, and 8 respectively. Graph (c) is composed by adding the lines from graph (a) to one from graph (b); graph (d), which gives the pH-dependences of the two directions, by taking antilogarithms of graph (c). The arrow marks the pK that is common to the two bells, in this case the pK of group C.

(Holbrook *et al.*, 1975). This group will be called the 'participating group'. Not every enzyme that has a hydron as product or reactant need have such a group, and enzymes that do not will be considered after those that do. It can obtain a hydron from buffer species, and do so more quickly than from the low natural concentration of H_3O^+ of about 100 nM (Cornish-Bowden, 1976) (postulating that H^+ is obtained from water does not help to explain high rates, because the reverse reaction would then require OH^- , which is also present in only low concentrations).

Figs. 1(a) and 1(b) show how the logarithms of the fractional concentrations of the relevant forms of the groups vary with pH. Arbitrary pK values have been chosen. The logarithmic plots are useful, because each curve is composed of regions of slope 1 and 0, or 0 and -1 ; hence they can easily be added by eye (Dixon, 1949, 1953) to give the curves of the relevant products of concentrations shown in Fig. 1(c). Taking antilogarithms of the values in Fig. 1(c) gives the bell-shaped plots of Fig. 1(d), which gives the pH-dependence of the concentrations of the forms active in the two directions. It shows that the pK marked by an arrow characterizes the right-hand side of the bell representing the forward reaction (hydron uptake) and the left-hand side of the bell representing the reverse reaction (hydron output).

Fig. 2 shows that a similar picture is obtained if the pK of the participating group C is not between the pK values of the structural groups A and B. The product of the fractional concentrations of the forms of group required for the forward direction will be low, because there is no pH at which both $-CH$ and $-B^-$ are the predominant forms of groups C and B.

Comparison of Figs. 1 and 2 shows that the same curve types exist whether the pK of the participating group is between those of the structural groups or not. In each case the pK that characterizes the right-hand side of the bell for the forward reaction characterizes the left-hand side of the bell for the reverse reaction. But in one case it is the pK of the participating group, and in the other it is that of a structural group. This exemplifies the fact that a kinetic demonstration of the need for a hydron cannot determine the site where it is needed.

In section 1 it was mentioned that departure from typical bell shapes could be due to two deactivating hydronations close in pK . This is illustrated in Fig. 2(c), where it may be noted that the curve of $\log([A^-][BH][C^-])$ has a slope of 2 on the left. This hardly shows in Fig. 2(d), because it occurs at a pH at which there is very little of the active form, so it hardly distorts the bell shape (Brocklehurst *et al.*, 1983). What may be learnt from such curve shapes was explored by Brocklehurst & Dixon (1976, 1977), but the first hydronation or dehydronation to deactivate is usually the only one that need be considered.

Although the model presented in the last few paragraphs is for independent groups, and section 3 deals with interacting groups, section 4(b) shows that even the treatment used above is valid for interacting groups if the dissociation constants considered become 'titration constants' rather than the constants for independent groups.

If no participating group exists, and the hydron is taken up directly in the reaction, Fig. 3 shows that the same result is obtained. The addition of a line of slope -1 , representing the dependence of $\log[H^+]$ on pH, to one of slopes $+1$ and 0, representing the dependence of $\log[A^-]$ on pH, gives one of slopes 0 and -1 . So the left-hand side of the bell for the reverse reaction again becomes the right-hand side of the bell for the forward reaction. Presumably some further structural group will be hydronated as the pH is lowered, giving a fall of activity at low pH.

One particular case in which Fig. 3 applies may be noted, as follows. A reaction converts a reactant RH, an acid, into a product P, and RH is predominantly dissociated to R^- over the pH range studied. The enzyme

Fig. 2. Dependences of concentrations of particular enzyme forms on pH

This differs from Fig. 1 only in that the pK of the participating group C (5.0) has been assigned a value that is not between those of groups A and B (6.5 and 9.5). The arrow again marks the pK common to both bells; this time it is the pK of group A. The fractional concentration of the active form for the forward reaction is so low that it has had to be multiplied by a factor of 20 to show on the same graph as the other bell.

nevertheless binds the reactant in the form RH . When rates at negligible degrees of saturation of the enzyme with reactant are studied, i.e. specificity constants (section 1) are plotted, the reaction is of the form:

Fig. 3 therefore applies, because the concentration of the reactive form of the reactant, $[RH]$, is proportional to the product $[R^-][H^+]$, and the total reactant concentration $[RH] + [R^-]$ may be taken as $[R^-]$. When, however, rates at saturating concentrations of substrate are determined, i.e. catalytic constants (section 1), the reaction has become:

so no uptake of hydron is involved, since it had already occurred to achieve saturation.

It is thus seen that in this simple model, although nothing can be said about the widths of the bells, the pK

Fig. 3. Dependence on pH of a particular ionic form of an enzyme and of this multiplied by $[H^+]$

Graph (a) corresponds with graphs (a) and (b) of Figs. 1 and 2; (b) and (c) correspond with (c) and (d) of Figs. 1 and 2. The complete bell in (c) gives the active form of the enzyme. The pH-dependence of the forward reaction requires both this and H^+ , so follows the half-bell. Both bells share the pK marked with an arrow.

that characterizes the high-pH side of one of them also characterizes the low-pH side of the other.

3. SECOND MODEL: INTERACTING GROUPS

The second model allows the groups A, B and C to interact, so that the pK of each may have different values according to the ionization state of the others.

It can be shown (Adams, 1916; Edsall & Wyman, 1958) that the molecule containing these groups has molecular pK values for its first, second and third hydrons to dissociate. The molecular pK values are functions of the values for the contributing groups and of the interactions between them. The first molecular dissociation constant, K_1 , is defined as $[H^+][EH_2^-]/[EH_3]$, where $[EH_2^-]$ represents the sum of the concentrations of all forms that have lost one hydron, i.e. those with (1) $-A^-$, $-BH$ and $-CH$ groups; (2) $-AH$, $-B^-$ and $-CH$ groups; and (3) $-AH$, $-BH$, and $-C^-$ groups. Since the concentration of a particular form required for activity, say with the ionic forms $-A^-$, $-BH$ and $-CH$, is a fixed fraction of $[EH_2^-]$, the dependence of $[EH_2^-]$ on pH is all that is needed to find how activity varies with pH. This is fortunate, because there is no method of measuring (as opposed to estimating) the values of group dissociation

Fig. 4. The pH-dependences of the four forms of a tribasic acid

Molecular pK values have arbitrarily been set at 3.7, 6.0 and 6.9.

constants (Dixon, 1976), such as the four for group B: $K_B = [H^+][AH, B^-, CH]/[AH, BH, CH]$, $K_B' = [H^+][A^-, B^-, CH]/[A^-, BH, CH]$, $K_B'' = [H^+][AH, B^-, C^-]/[AH, BH, C^-]$, and $K_B''' = [H^+][A^-, B^-, C^-]/[A^-, BH, C^-]$. In contrast, molecular pK values are measurable quantities.

The forward reaction requires the ionization form A^- , BH , CH , and its concentration depends on pH in the same way that $[EH_2^-]$, the sum of the concentrations of it and its tautomers, does. Similarly, the reverse reaction requires the form A^- , BH , C^- , and its concentration varies with pH in the same way that $[EH^{2-}]$ does. Hence the bell-shaped curves representing the two activities share a molecular pK , pK_2 , that characterizes the high-pH side of the bell for the forward reaction, and the low-pH side for the back reaction. Fig. 4 gives an illustration of this behaviour.

4. BREADTH OF APPLICATION OF MODELS

(a) Positively co-operative hydronations

The closest that the two molecular pK values of a dibasic acid can get is 0.6 (i.e. $\log 4$) without positively co-operative hydronation. It reaches this value with identical and independent groups. The constants for consecutive hydronations spread among more groups can approach each other more closely.

Nevertheless it is perfectly conceivable that hydronations can be positively co-operative, so that loss of one hydron would facilitate the loss of the next, as appears to occur with reactions of the papain catalytic site (Brocklehurst & Malthouse, 1980). In that case the molecular pK values could approach more closely still, and even cross over. The conclusions of section 3 are still valid if this should occur. Fig. 5 shows the consequences of letting pK_3 be lower than pK_2 .

It is far from obvious from Fig. 5 that the pK characterizing the right-hand side of the bell of $[EH_2^-]$ is that characterizing the left hand side of that for $[EH^{2-}]$. Nevertheless, the narrowness of the peak of $[EH^{2-}]$ shows that the pH at which it is half its maximal value on its left-hand side is quite far from the relevant pK . This may be found from the graph given by Tipton & Dixon (1979), as their Fig. 4, which gives the pK difference, here $pK_3 - pK_2$, as a function of the width of the bell at half its maximal height. Their graph is based on the relation that if q is defined by letting the pH values at which the bell is half-maximal in height differ from the central pH

Fig. 5. The pH-dependences of the four forms of a tribasic acid that shows positively co-operative hydronation

The pK for the loss of the third hydron (7.5) has been set below that for the loss of the second (8.0). Hence there is never much EH^{2-} , and it is not obvious that 8 is the pK that characterizes the dehydronation that forms it. Note that the alkaline side of the bell for $[EH_2^-]$ and the acid side of the curve for $[E^{3-}]$ are steeper than the one-site titration curves shown for the $EH_3 \rightleftharpoons EH_2^-$ interconversion.

by $\log q$, then pK_3 and pK_2 are $\log[q - 4 + (1/q)]$ (which may be negative) above and below the central pH respectively (Dixon, 1979). For a bell as narrow as that shown, $pK_3 - pK_2$ is negative, i.e. there is positively co-operative hydronation.

Fig. 5 also shows that the bell representing $[EH^{2-}]$ is very low, since dissociation of the second hydron facilitates that of the third, and that the slope on the right-hand side of the bell for $[EH_2^-]$, like the curve for $[E^{3-}]$, is much steeper than one characteristic of a one-site hydronation, seen on the left of this bell. Thus the two characteristics, of the narrowness of one bell and the steepness of one side of another, can serve as a warning not to expect the simple visual identification of the pK characterizing the alkaline side of the bell for the forward reaction with that characterizing the acid side of the bell for the reverse reaction. Nevertheless, in fact, the two curves share the same pK value.

(b) Titration constants

Simms (1926) pointed out that the titration curve of a polybasic acid can be duplicated by a mixture of independent monobasic acids. The pK values of the acids of such an equivalent mixture are called titration constants. The titration constants approach the molecular constants if these are well separated. (Dixon, 1973, 1974, mistakenly identified 'titration' constants with 'molecular' or 'macroscopic' constants.)

These titration constants characterize the one-site titration curves that are the factors of the Michaelis (1914) functions (the functions that give the dependence on $[H^+]$ of the various ionic forms of a polybasic acid) (Dixon, 1975). They are only real if the molecular constants do not approach more closely than is allowed without co-operative hydronation (Dixon, 1975).

Since such one-site titration curves multiply together to give the actual concentrations of intermediate species (e.g. those of Fig. 4), the treatment of section 2 applies to molecules with interacting groups if the pK values, such as those of Figs. 1(a) and (b), are interpreted as titration constants rather than as group constants.

(c) Change of rate-limiting step with pH

The need for groups in particular ionic forms is not the only reason why the dependence of a rate of reaction on pH may follow titration curves. Exactly the same curve:

$$y = a/(a + \cosh x) \quad (\text{Dixon, 1974, 1979})$$

is followed if the rate-limiting step changes with pH (Dixon, 1973). It might do this if a substrate needed to be dehydrated to form an intermediate, and this intermediate needed to be hydronated to react to give products. Then the first step could be rate-limiting at low pH, and the second step at high pH. In the simplest, non-enzymic, example of such a reaction, in which there is one intermediate whose concentration is negligible in comparison with that of the reactant, the pH-dependence is of the normal bell shape; one of the pK values that characterize this dependence is that of the reactant, and the other is this same pK added to a function of various rate constants (Dixon, 1973). Hence if an enzyme-catalysed reaction were also to change rate-limiting step with pH, there may be no simple relation between the bells in the two directions. Hence the conclusion that the bells share a common pK may not extend to such reactions.

5. BASIS OF PHENOMENON

Although the common pK of the curves for the forward and reverse reactions does not seem to have been pointed out previously, it amounts to a reflection of the well-known fact that the pH-dependence of the concentration of a hydronated species, HA, is the same as that of [H⁺][A⁻]. It also corresponds to the fact that, for enzyme-catalysed reactions under rapid equilibrium conditions, the substrate constant (K_s) is equal to the inhibitor constant (K_i) for that compound acting as an inhibitor in the reverse reaction (see Segel, 1975). Under steady-state conditions this simple relationship will not necessarily hold, just as full steady-state conditions for enzyme reactions involving hydronation can also be complex if hydronations are not at equilibrium (Laidler, 1955; Ottolenghi, 1971).

6. COMPARISON WITH EXPERIMENT

Fig. 6 reproduces a figure from Yoshida & Freese (1965), which gives their results on alanine dehydrogenase (EC 1.4.1.1), which catalyses the reaction:

It is the best published study we know of the pH-dependences of such a reaction, although it is not ideal in three respects. First, it may not involve the release and uptake of exactly 1 mol of hydrons/mol of substrate over the whole pH range studied, because substrate and reactant titrate over this range. Nevertheless the pK values for dissociation of H⁺ from NH₄⁺ and from the ammonio group of alanine are close, so the reaction is fairly suitable in this respect. Secondly, the bells are not those of simple three-hydronic state curves, so that successive hydronations may be accompanied by partial deactivations, and the models would not apply. Thirdly, since the observations are at fixed substrate concentrations, the rates reflect neither catalytic nor specificity constants, since the degree of saturation may be changing.

Fig. 6. Effect of pH on alanine dehydrogenase activity

This Figure is redrawn, with permission, from the paper by Yoshida & Freese (1965). The reaction studied is:

where the concentrations shown on the left were used for the forward reaction (—) and those shown on the right for the back reaction (----).

Hence, although the predictions might have been expected to be realized only approximately in this example, they appear to hold remarkably well.

7. CONCLUSION

For an enzyme-catalysed reaction studied in both directions by determining the reversible dependence on pH of either the catalytic constant or the specificity constant, the following conclusion applies: the pK value characterizing the alkaline limb of the pH-dependence in one direction will be identical with that characterizing the acid limb in the other if the hydron is a reactant in the first direction and a product in the second. This conclusion may not hold if there are pH-dependent changes in the rate-limiting step of the reaction.

Note added in proof (received 13 October 1987)

We are grateful to Dr. W. P. Jencks for pointing out that, although the conclusions of this paper are valid for the specificity constant, circumstances could arise when they would not hold for the catalytic constant. This constant is determined by the rates of the steps that follow saturation of the enzyme with substrate. Since they exclude saturation, those for the forward direction may exclude a step that is rate-limiting in the reverse direction. Thus the rate-limiting steps for this constant may differ in the two directions and so may require different ionic forms of the enzyme; this would allow them to have unrelated pH-dependences.

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