# pH Studies with Enzymes

# 24

Enzyme-catalyzed reactions involve one or more proton transfers. Acid–base chemistry permeates most of enzyme chemical mechanisms. Ionizable amino acid side chains of the enzyme protein are typically involved in such catalysis. Each ionizable group can be viewed as an acid and also a conjugate base. A general exposure to acid–base chemistry and catalysis may be found in Chap. 30 of the book. The reader is well advised to read that background material to better appreciate the subject covered in this chapter.

Interactions of enzyme with its substrate (or any ligand for that matter) involve at least one or few groups whose correct ionization is necessary for its optimal function. The ionization state of acid–base groups on the enzyme, substrate, and inhibitor directly affects catalytic activity. These ionizable groups may affect the enzyme activity by influencing the binding or catalysis or both. Almost all protonation–deprotonation events occurring in an enzyme reaction are catalyzed. The relevant acid–base groups on the enzyme may be identified with the help of techniques such as:

(a) Group specific chemical modifications (see Chap. 21, Irreversible Inhibitions),
(b) pH dependence of kinetic parameters,
(c) Structure–activity correlations through physical techniques like X-ray, nuclear magnetic resonance (NMR) spectroscopy, etc.
(d) Site-directed mutation analysis (discussed later in Part V; Chap. 39, Future of Enzymology: An Appraisal).

All the four approaches give useful complementary information; however, pH-dependence of enzyme kinetics gives the best insight as it reports on the reaction being catalyzed while it occurs. The pH dependence of kinetic parameters provides information on kinetic as well as chemical mechanism. The kinetic aspects will be the main focus here, while the other approaches pertaining to pH are dealt with in later sections (see Part IV; Chap. 30, Acid–Base Chemistry and Catalysis).



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## 24.1 Enzyme pH Optimum

Determination of activity as a function of pH is the first and simplest experiment one conducts to determine the effect of $H^+$ ions on the enzyme. In all enzymatic studies, maintaining a well-defined pH ($H^+$ concentration) of the system is very crucial. Since a range of pH values (between 0 and 14 in an aqueous environment) are to be used, more than one kind of buffer ion may be required in the experiment. Effects on the enzyme due to switching of buffer species and change in ionic strength, if any, become important and have to be eliminated. This is achieved by the judicious use of suitable buffers (refer to Part II; Chap. 13, Good Kinetic Practices). Most enzymes display a bell-shaped pH-activity curve with maximal activity around neutral pH. However there are enzymes with a pH optimum in the acidic (such as pepsin) and alkaline (such as arginase) range as well (Fig. 24.1).

The decrease of activity on either side of pH optimum may result from (a) instability of the enzyme and/or (b) changes in the kinetic parameters of the enzyme due to pH. Enzyme stability over broad pH ranges is a desirable feature for industrial application. Information about the stability of the enzyme over the pH range studied is also necessary in designing correct kinetic studies.

In order to establish the pH stability profile, the enzyme is preincubated at different pH values. For each pH of preincubation, aliquots are withdrawn as a function of time, and activity remaining is assayed. In this process it must be ensured that the active enzyme remaining is stable after taking the preincubation sample into assay mixture. A plot of percent activity remaining versus pH (after a fixed time of preincubation) gives a fair idea about enzyme's pH stability. Meaningful pH dependence of enzyme activity is then sought within this range of pH defined for stability.

Experimental determination of pH optimum (plot of pH versus activity; Fig. 24.1) serves two purposes. It is of practical importance in enzyme assay optimization. Secondly, the ascending and descending limbs of such profiles give some idea about the range of pKas and hence possible ionizable groups involved.

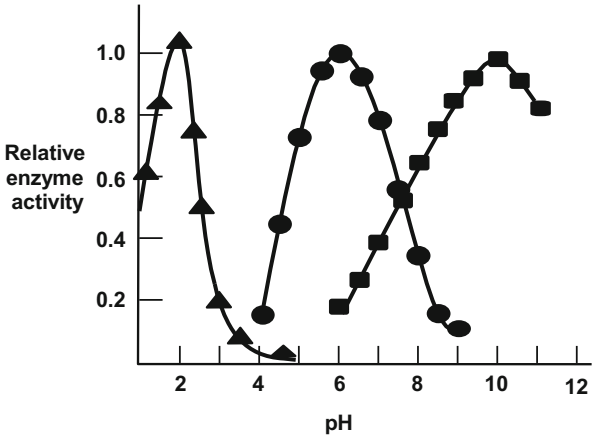


**Fig. 24.1 pH-activity profile of an enzyme**. Three representative enzyme examples namely pepsin (▲), amylase (●), and arginase (■) are shown

The pH behavior of an enzyme is a complex outcome of all the ionizable groups present on the enzyme and the substrate. A simplistic treatment of bell-shaped activity curve is to compare it to the ionization of a dibasic acid. Examples of enzymes in which at least two active site ionizable groups have been assigned for function are ribonuclease A (H12 and H119) and lysozyme (E35 and D52). Of many ionizable groups present on the enzyme and the substrate, two may be considered kinetically significant with the enzyme represented as *HEH*. The ascending limb defined by the ionization of the first proton ($HEH \rightleftarrows EH^- + H^+$) and the descending limb by the other ($EH^- \rightleftarrows E^{2-} + H^+$). The active enzyme species is the singly deprotonated form $EH^-$. In such a simplistic model representation, the pH behavior of $V_{max}/K_M$ (the first-order rate constant) is given by the following equation:

$$\frac{V_{max}}{K_A} = \frac{\left(\frac{V_{max}}{K_A}\right)^0}{\frac{[H^+]}{K_{H1}}} + 1 + \frac{K_{H2}}{[H^+]}$$

where $K_{H1}$ and $K_{H2}$ are the acid dissociation constants for the two groups and $(V_{max}/K_M)^0$ is the pH-independent value when the two groups are correctly protonated. At lower pH values (i.e., high $[H^+]$) the $K_{H2}/[H^+]$ term in the denominator is insignificant, while at higher pH values (i.e., low $[H^+]$), the $[H^+]/K_{H1}$ term becomes insignificant. Although much oversimplified, this equation does capture the essence of pH dependence of enzyme kinetic parameter ($V_{max}/K_M$ in this case).

## 24.2 pH Kinetic Profiles

Best mechanistic information can be obtained by performing substrate saturation at different pH values. It must be borne in mind, however, that the kinetic mechanism may itself change with change in pH of the reaction. For instance, the normal random mechanism exhibited by creatine kinase changes to an equilibrium ordered one (Chap. 19, Analysis of Initial Velocity Patterns; Mg-ATP binding first) as the pH is decreased from 8.0 to 7.0. Such effects have to be checked up beforehand. The pH-kinetic experiments are so conducted that effects of ionic strength and changing buffer species are properly controlled. With this data one can simultaneously determine the effects of pH on the kinetic constants such as $V_{max}$, $V_{max}/K_M$, and also $K_M$. Similarly, one can determine the pH dependence of (a) $K_I$ for a competitive inhibitor and (b) $K_{activator}$ for an activator. The variation of kinetic parameters with pH is best plotted as log–log plots; this makes sense as pH is a logarithmic scale ($-\log[H^+]$). While the horizontal axis is always pH, the Y-axis could be $\log V_{max}$, $\log V_{max}/K_M$, $pK_I$ ($-\log K_I$), $pK_{activator}$ ($-\log K_{activator}$), $pK_{metal\ ion}$ ($-\log K_{metal\ ion}$), etc. Profiles typically schematized to indicate such log–log plots are shown in Fig. 24.2.

**Interpretation of pH Profiles** Any singly ionizable group can access either a protonated or a deprotonated state. As fractional proton cannot be transferred, one encounters line segments with zero or unit slopes. Rarely, ionization of two protons

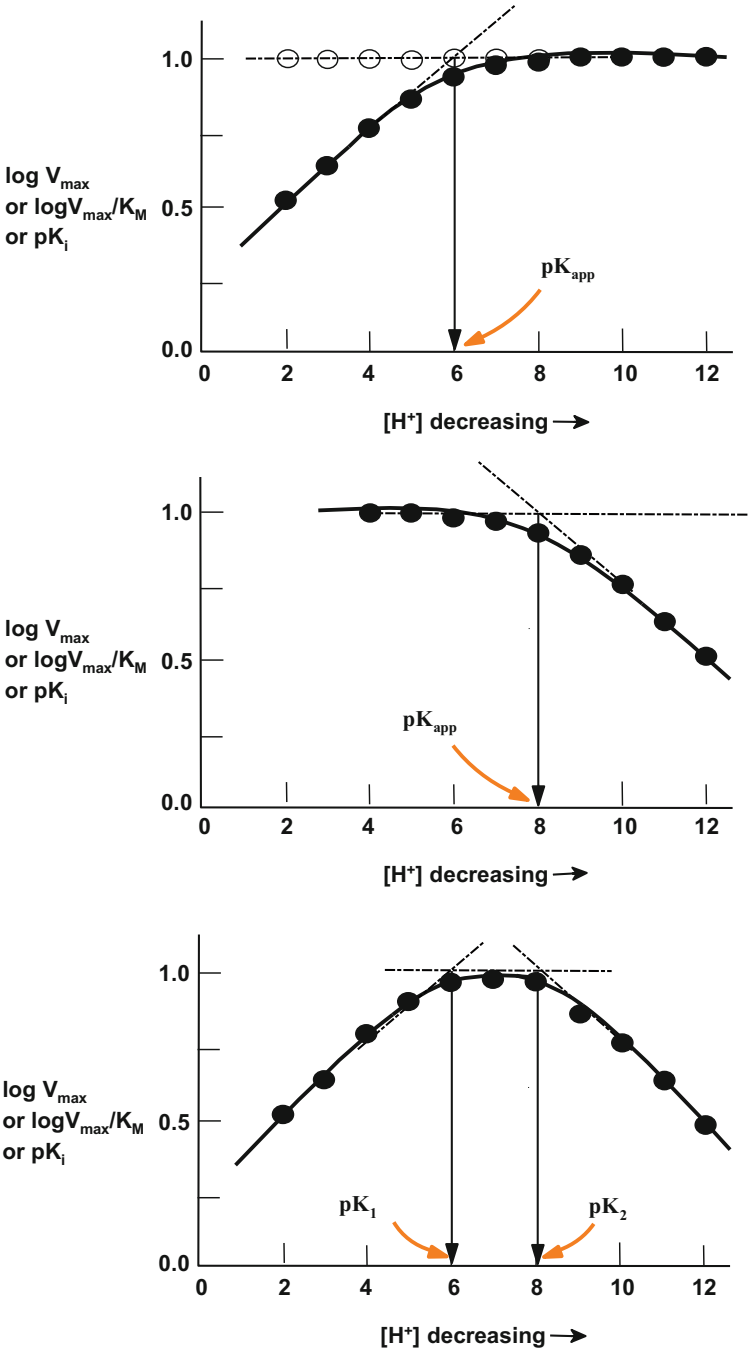


**Fig. 24.2 Possible pH versus kinetic constant profiles encountered for an enzyme**. Protonation of the group results in decreased activity/binding (top panel), deprotonation of the group results in decreased activity/binding (middle panel) and combination of the two – indicating titration of two ionizable groups (bottom panel). In each case, the kinetic constant ($V_{max}$, $V_{max}/K_M$, or $K_I$) is plotted on the Y-axis as its log value. Open circles (top panel) indicate the profile when that group does not titrate

at a single pH may however appear as lines with a slope of 2. A curve with a unit positive slope followed by a plateau (i.e., zero slope; Fig. 24.2 top panel) indicates that the protonation of that group results in decreased activity/binding. The point of intersection (extrapolated to pH axis) gives the pKa of the group involved. At higher pH values, a group may lose a proton, and this may lead to decreased activity/binding (line segment with zero slope followed by one with slope − 1; Fig. 24.2 middle panel). The presence of both an ascending and a descending limb with a plateau in the middle (Fig. 24.2 bottom panel) represents an enzyme example with two kinetically significant group protonations. Going from the left to right, deprotonation of the first group increases activity/binding, while the deprotonation of the second group decreases activity/binding. We have already come across an equation describing such a pH behavior of $V_{max}/K_M$ above. The observed pKa values of the two groups give a glimpse of their possible identity.

If one encounters two ionizable groups with their pKa values not sufficiently apart (more than two pH units), then it is difficult to distinguish the two and identify which one is protonated. We can further imagine an unusual situation: going up the pH scale, a group behaving like that in Fig. 24.2 middle panel may occur before a second group with a profile like that in Fig. 24.2 top panel. This is an example of a *reverse protonation* state, and the plateau in Fig. 24.2 bottom panel is suppressed (to much less than the true value). For an active enzyme, therefore, the group with lower pKa is required in the protonated state, while the group with higher pKa has to be in the deprotonated form. For example, fumarase catalysis requires an active site carboxylate in the deprotonated state and a protonated imidazole. Such reverse protonation cases are not uncommon. It is however conceivable that the catalyst may be locked into an inactive state – possibly because of the proximity of two oppositely charged groups (they may form a salt bridge). Mother nature may have therefore selected suitable pairs of ionizable groups to avoid such thermodynamic wells; lysozyme active site recruits two carboxylates – alternately acting as a general base and general acid for catalysis.

We make a few general observations on the pKas and acid–base groups obtained from pH kinetics studies. Groups that titrate in the $\log V_{max} \rightarrow$ pH profiles are mainly implicated in the catalytic step, and their ionization occurs in the *ES* complex (saturating concentration of all reactants). Since $V_{max}/K_M$ signifies first-order rate constant and interaction of *S* with *E*, $\log V_{max}/K_M \rightarrow$ pH data reflects on essential ionizable groups on the free substrate and the enzyme form with which it combines. Such groups therefore are important for substrate binding as well as catalysis. This interpretation is further simplified if ionization of acid–base group(s) on the substrate molecule is accounted for or they do not exist (like in glucose). An example of this is glucose isomerase (actually a xylose isomerase from *Actinoplanes* spp.) and its site-directed mutant (H54Q) form. This imidazole group (pKa around pH 6.0) of His54 is titrated in the $\log V_{max} \rightarrow$ pH profile of the native glucose isomerase but not in the H54Q mutant (the profile with open circles, Fig. 24.2 top panel).

The Michaelis constant – $K_M$ for the substrate – is not an independent kinetic parameter but may be viewed as a ratio of $V_{max}$ and $V_{max}/K_M$. Therefore its pH

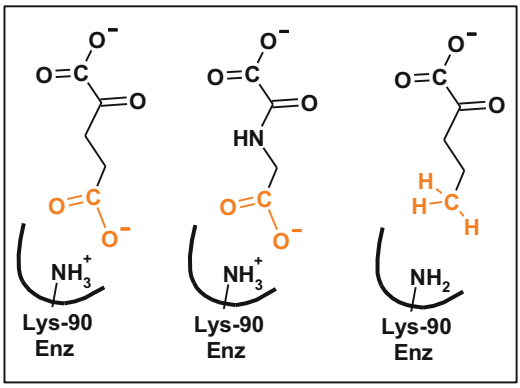


**Fig. 24.3 Interaction of glutamate dehydrogenase with the γ-COOH of 2-oxoglutarate through its active site Lys amino group**. Analogous active site interactions with oxalylglycine (competitive inhibitor, middle) and 2-oxovalerate (alternate substrate, right) are also shown

dependence is a composite of $\log V_{max} \rightarrow$ pH and $\log V_{max}/K_M \rightarrow$ pH plots. The $pK_M \rightarrow$ pH profile is rarely plotted. Such profiles are useful indicators of acid–base groups involved in binding (found either on the enzyme or on the substrate) provided $K_M$ approximates $K_S$ (see Chap. 15, Henry-Michaelis-Menten Equation, in Part II). The simplest pH profile to interpret however is that for the pH dependence of $K_I$ for a competitive inhibitor. In these $pK_I \rightarrow$ pH profiles, ionizable groups responsible for binding alone are titrated. The $K_I$ being equilibrium dissociation constant, the pKa values obtained from such plots are actual pKa values. They can be meaningfully compared with the group pKas obtained from $\log V_{max} \rightarrow$ pH and $\log V_{max}/K_M \rightarrow$ pH profiles. pH studies on bovine glutamate dehydrogenase provide an excellent example for such analysis. Glutamate dehydrogenase titrates an ionizable group around pH 8.0 – both in the $pK_M$ profile for 2-oxoglutarate (substrate) and $pK_I$ profile for oxalylglycine (competitive inhibitor). However, this group is not seen in the $pK_M$ profile for 2-oxovalerate – an alternate substrate but missing the γ-COOH (Fig. 24.3).

Since –COOH group is fully deprotonated at this alkaline pH, a positively charged group on the enzyme is clearly implicated. Deprotonation of this enzyme Lys side chain adversely affects 2-oxoglutarate binding but not that of 2-oxovalerate; indeed this Lys-$NH_2$ must stay unprotonated for 2-oxovalerate binding. The involvement of this group (K90) anchoring the γ-COOH of 2-oxoglutarate was clearly borne out much later, by X-ray structural data on this enzyme.

Lastly, $pK_I$ and $pK_{activator}$ profiles provide information about the ligands provided by the enzyme that bind the metal ion. The pKa for the ionization of metal ion-bound water may also be seen in such profiles.

## 24.3 Identifying Groups Seen in pH Profiles

X-Ray, NMR, and chemical modification information on active site functional groups are useful but are limited by the fact that (1) they represent one of the many protein conformations and (2) even when active site pictures with a substrate

or inhibitor bound are available, one may be looking at nonproductive complexes. An analog/inhibitor may bind differently from the way a productive substrate interacts at the active site. Site-directed mutations are useful in ruling out important role for a particular group, but mere loss of activity may result from structural changes and not necessarily due to specific role in catalysis.

The pKa values of functionally important acid–base groups are usually significantly shifted from corresponding pKa for groups on free amino acids. Such perturbation of normal pKas is due to unique active site environment – created by the protein – and shielding of these groups from the bulk aqueous phase. Nevertheless, the comparison of experimentally determined enzyme pKa values with reported pKas of amino acid side-chain functional groups can help in identifying their chemical nature. Regardless of our knowledge on exact nature of the acid–base group, its role in catalysis and/or binding can often be assigned from pH-kinetic profiles. Two different techniques may be useful in identifying the functional group whose pKa is observed in enzyme pH profiles.

**Inspection of Their Enthalpy of Ionization ($\Delta H_{ion}$)** The temperature dependence of equilibrium ionization constant is different for different acid–base groups (Table 24.1). These may be broadly categorized into three groups: groups with low $\Delta H_{ion}$ (carboxylate and phosphate), groups with moderate $\Delta H_{ion}$ (imidazole, thiol, and phenolic OH), and groups with high $\Delta H_{ion}$ (amino, metal-bound water, and guanidinium). In practice, pH profiles are generated at different temperatures, and the pKa values so obtained are plotted against respective 1/T values. The slope of such a plot gives $\Delta H_{ion}/2.303R$. Typical $\Delta H_{ion}$ values of 6 kcal/mol and 12 kcal/mol correspond to a ΔpKa (between 0 °C and 25 °C) of 0.4 and 0.8 pH units, respectively. A potential identification of groups from their characteristic pKa and $\Delta H_{ion}$ is thus feasible. A word of caution – unusual $\Delta H_{ion}$ values – may be obtained when the ionization of the group is coupled with protein conformational change (Knowles and Jencks 1976). In such cases experimentally observed $\Delta H_{ion}$ may include a component for that as well.

**Through Solvent Perturbation** According to the Bronsted definition of acids and bases, any species of a functional group that has a tendency to lose a proton is an acid. This definition eminently suits our understanding of the role of acid–base groups at the enzyme active site. We can classify all ionizable functionalities on the enzyme (including side chains of amino acid residues) into two groups – cationic acids and neutral acids (Table 24.2).

**Table 24.1 Range of $\Delta H_{ion}$ values for ionizable groups on the enzyme**

| Group | pKa range (at 298 K) | $\Delta H_{ion}$ (kcal×mol$^{-1}$) |
|---|---|---|
| Carboxylate, phosphate | 2–5 | 0 ± 1.5 |
| Imidazolium, thiol, phenolic OH | 5–10 | 6 ± 1.5 |
| Amine, metal-bound water, guanidinium | 8–12 | 12 ± 1.5 |

**Table 24.2 Different Bronsted acid groups on the enzyme**

| Acid type | Functional group |
|---|---|
| Neutral acids ($X\text{-}H \rightleftarrows X^- + H^+$) | Carboxylate, phosphate, thiol, phenolic OH, metal-bound water |
| Cationic acids ($X\text{-}H^+ \rightleftarrows X: + H^+$) | Imidazolium, amine, guanidinium |

A clear difference between the two Bronsted acid classes is in the net charge generated upon dissociation. Two charges are generated (one proton and a –ve ion) on dissociation of a neutral acid, while there is one +ve charged species on either side of the dissociation equilibrium for a cationic acid (see Table 24.2 above). This has implications to their differential behavior upon solvent perturbation (altered dielectric constant, etc.). The pKa values of neutral acids are elevated by adding water miscible organic solvents like different alcohols, formamide, dioxane, dimethyl sulfoxide, or N,N-dimethylformamide. Cationic acid pKas are largely unaffected by such solvent addition. In practice, pH profiles are generated with and without the solvent, and the relevant group pKas are compared. The groups whose pKas are elevated in the presence of an added solvent are all neutral acids. The two histidine residues (H12 and H119) at the active site of ribonuclease A were identified by such a study. There are two limitations of solvent perturbation approach. Groups that are not exposed to solvent will not be affected or accounted for. Secondly, since 30–50% of the solvent may have to be used, it becomes important to ensure that the enzyme is not inactivated by such addition.

## Reference

Knowles JR, Jencks WP (1976) The intrinsic $pK_A$-values of functional groups in enzymes: improper deductions from the pH-dependence of steady-state parameter. CRC Crit Rev Biochem 4:165–173