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Effects of pH, Temperature, and Isotopically Labeled Substrates on Enzyme Activity

INTRODUCTION

In Chaps. 13 to 19 we examined various kinetic aspects of enzyme mechanisms. Several of the examples discussed throughout this book refer to particular amino acid side chains that may be involved in various aspects of a mechanism. Previously, the rate-limiting steps were discussed in terms of whether a particular bond-breaking event is involved. In both of these areas much useful information can be obtained from kinetic studies. In some instances this is preliminary to other studies, while in other cases the information can directly provide the answer to a question regarding the enzyme mechanism. In this chapter we examine the usefulness of pH studies, isotope effect studies, and temperature studies of various kinetic parameters.

pH-DEPENDENCE STUDIES

There are many ionizable side chains in a protein molecule. In addition, the α-amino and carboxyl terminal residues must be considered. These ionizable groups have a variety of potential roles in a protein's structure and function. Ionic interactions can clearly be important in secondary, tertiary, and quaternary structure. Ionizable groups also often play important roles in catalysis. In Chap. 7 we looked at a variety of ways to chemically modify amino acid side chains, with the intention of identifying which types of residues may be important in the function of the protein. A particularly useful guide to such chemical modification experiments can be pH studies of

various kinetic parameters for the enzyme; they can yield information about the pK_a values of groups involved in a variety of functional properties of the protein.

Basic Theory

The state of protonation of a group that can undergo a reversible protonation ($B + H \rightleftharpoons BH$) is given by the Henderson–Hasselbalch equation,

$$pH = pK_a + \log \frac{[B]}{[BH^+]} \tag{20-1}$$

where pK_a is given by

$$pK_a = -\log K_a \tag{20-2}$$

and K_a is the ionization constant, defined by

$$K_a = \frac{[B][H^+]}{[BH^+]} \tag{20-3}$$

Equation (20-1) is derived by rearranging Eq. (20-3) to the form

$$[H^+] = \frac{K_a[BH^+]}{[B]} \tag{20-4}$$

Taking the negative logarithm of Eq. (20-4) leads to Eq. (20-1). In terms of a protonatable group which upon association gives a neutral species ($HA \rightleftharpoons H^+ + A^-$), the Henderson–Hasselbalch equation becomes

$$pH = pK_a + \log \frac{[A^-]}{[HA]} \tag{20-5}$$

As discussed in Chap. 7, the pK_a of a particular side chain is characteristic of that group, with the reservation (stressed in Chap. 7) that the pK_a of a particular chemical group may, in the three-dimensional structure of the protein, be quite perturbed from its "normal" value.

pH studies of enzyme kinetics offer a means of obtaining information concerning pK_a values of groups involved in enzyme function. Before discussing possible interpretation of the pH parameters, we consider the way such data are usually presented.

Consider a parameter, K_{obs}, that is dependent on the state of ionization of a side chain in the protein. First, the side chain may need to be protonated or unprotonated (but not both for the value of K_{obs} to be maximal. Figure 20-1 illustrates two ways of plotting the dependence of K_{obs} on pH. K_{obs} may be plotted directly versus pH or log K_{obs} versus pH may be plotted. Figure 20-1 illustrates how the pK_a is obtained from either of these plots. In the example, K_{obs} is maximal when the ionizable group is in its unprotonated state. If K_{obs} required the ionizable group to be protonated, the two plots would be the inverse of those shown.

It is quite conceivable that K_{obs} depends on the state of ionization of two groups on the protein surface. As illustrated in Fig. 20-2, this results in a bell-shaped curve.

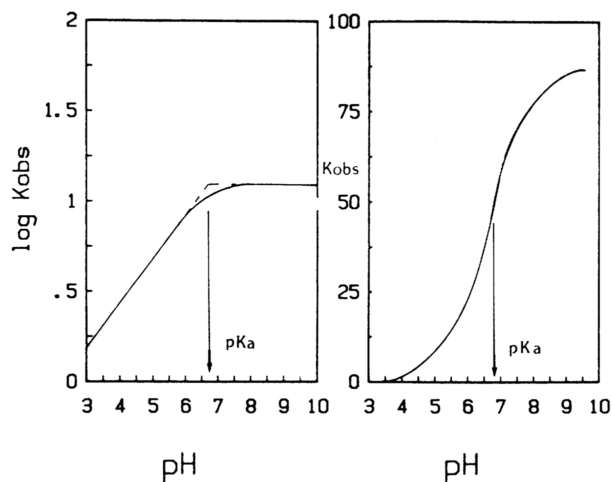


Figure 20-1 Methods of plotting the pH dependence of a parameter, K_{obs}, and the determination of pK_a.

Effects on K_m

As discussed extensively in Chaps. 13 to 15, the K_m of an enzyme-catalyzed reaction can, depending on the formal kinetic mechanism, have a variety of physical significances. In an equilibrium mechanism the Michaelis constant for a particular substrate can be equated with the dissociation constant of the substrate from the ternary or quaternary enzyme–substrate complexes for a two or three-substrate

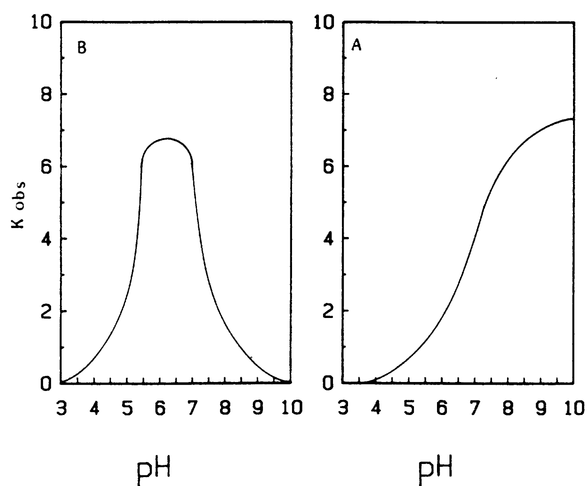


Figure 20-2 Plots of K_{obs} versus pH for a system dependent on the ionization of a single side chain (A), or one dependent on two side chains (B).

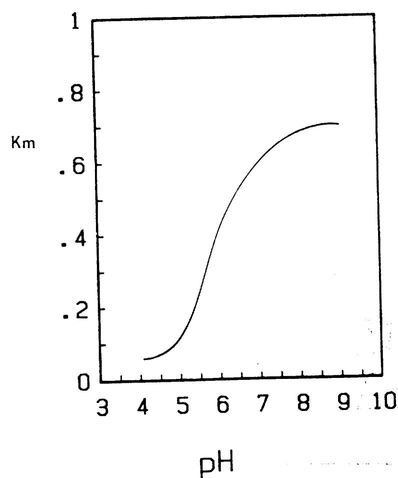


Figure 20-3 Typical plot of K_m versus pH. From the plot shown, a pK_a of approximately 6 is obtained.

reaction, respectively. Clearly, in such an instance a pK_a from a plot of K_m versus pH (as in Fig. 20-3) gives information concerning a group in the enzyme–substrate complex involved in the stability of the complex. This group can be on either the substrate or the enzyme. If the pK_a values (if any) of ionizable groups on the substrate are known (or independently determined), it is possible that the pK (or pK's) obtained from a plot of K_m versus pH can be assigned to ionizable groups on the enzyme.

Where a steady-state mechanism is involved, the K_m for a substrate has no simple physical significance. Any pK values obtained in such a case may reflect groups on the enzyme (assuming that substrate pK values have been eliminated from consideration) that are involved with any of the rate constants in the K_m term.

Effects on V_{max}

As with the interpretation of the pH dependence of K_m, the significance of any pK_a values determined in plots of V_{max} versus pH (Fig. 20-4) depends on knowledge of the formal kinetic mechanism. In equilibrium mechanisms the V_{max} is directly proportional to the rate constant for the interconversion of central ternary or quaternary complexes. In such an instance the pK (or pK's) obtained reflect a group (or groups) involved in the catalytic mechanism of the enzyme. The nature of such involvement can range from a group's participating in acid–base catalysis or some other aspect of the chemical mechanism to one whose ionization is important in maintaining the correct conformation of the enzyme's active site, thus allowing efficient catalysis to occur. In addition, the interconversion of central complexes may involve a rate-limiting conformational change that is dependent on the ionization state of one or more amino acid side chains.

When the formal kinetic analysis of the reaction indicates a steady-state mechanism, the V_{max} reflects the rate-limiting step. In steady-state mechanisms this can

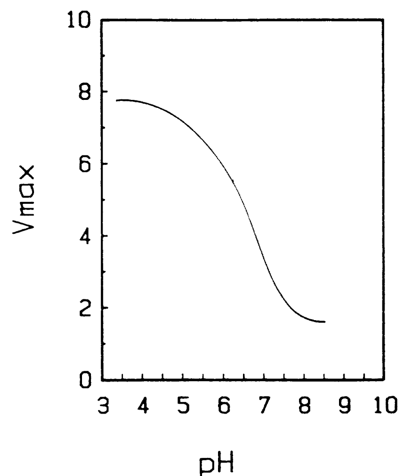


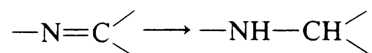
Figure 20-4 Typical plot of V_{max} versus pH. From the plot shown, a pK_a of approximately 5.8 is estimated.

be a substrate binding step, a product release step, a component of the catalytic reaction, or a combination of several of these stages. If the overall rate-limiting step in the reaction is known from other experiments, the interpretation of the pH dependence of V_{max} is straightforward. In the absence of this it is sometimes possible to obtain information by examining the pH dependence of K_m for substrates in the reverse reaction. Such substrates are of course the products of the reaction whose V_{max} is being studied. In the case where product release is rate limiting, any pK associated with a group involved in product release is also associated with the reverse reaction. A pK reflected in V_{max} in this case also appears in K_m (for the product as a substrate in the reverse reaction).

Clearly, knowledge of the formal kinetic mechanism is of considerable help in the interpretation of the pH dependence of an enzyme reaction. In place of plotting K_m versus pH it is often advantageous to plot a particular θ parameter, or ratio of θ parameters versus pH, as in Fig. 20-5.

As discussed in Chaps. 14 and 15, depending on the formal kinetic mechanism, these parameters (or ratios) can be related uniquely to particular steps in the overall mechanism. For example, in compulsory-order steady-state mechanisms $\theta_1 = 1/k_1$, where k_1 is the rate constant for the first substrate binding. Other such identities were given in Tables 14-1, 14-3, and 15-1.

A particularly interesting example that may be considered is the enzyme dihydrofolate reductase. This enzyme uses NADPH to reduce dihydrofolate in an overall reaction that includes the hydride transfer from the reduced nicotinamide ring of NADPH and a protonation (using a solvent proton) of an imine bond in dihydrofolate. The reaction, which in terms of the involved atoms is shown by



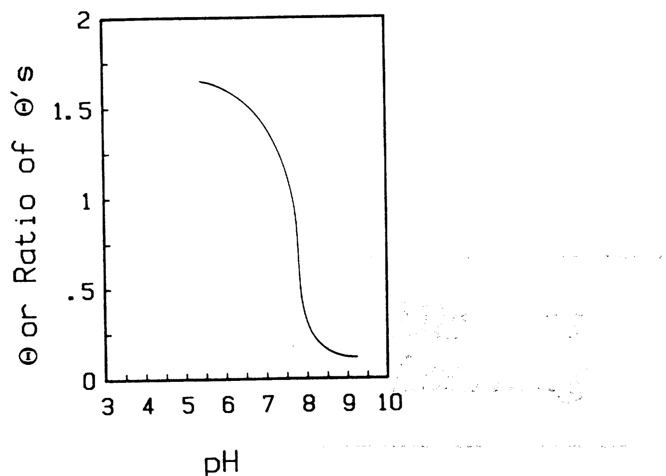


Figure 20-5 Plot illustrating the dependence of either an individual rate parameter or some ratio of rate parameters on pH. From the curve shown, a pK_a of approximately 7.5 is estimated.

is presumed to involve the protonation of the nitrogen prior to the hydride transfer to the carbon since this would enhance the carbonium-ion character of the carbon. Two important questions that can be raised involve the protonation pathway and stabilization of the protonated transition state, which will be positively charged.

Many genetic varients of dihydrofolate reductase have been described and a particular residue, Asp-27, appears from the crystal structure to be in a position to play a role in either the protonation or the transition-state stabilization or both.

Elegant studies using site-directed mutagenesis have provided an answer to the role of Asp-27 in the mechanism and shed further insight into the delicate balance of factors contributing to the overall catalytic efficiency of dihydrofolate reductase. Oligonucleotide-directed mutagenesis (see Chap. 6) was used to generate a mutant gene with asparagine in position 27. In addition, a primary site revertant of the Asn-27 gene, where the spontaneous transition AAC (Asn) to AGC (Ser) had occurred, was obtained. Native enzyme, together with both mutants, gave binary complexes with the inhibitor methotrexate, which gave isomorphous crystal structures. The major difference between the three structures (obtained to 1.9-Å resolution) involved a water molecule which in the wild-type enzyme is hydrogen bonded to the carboxyl of Asp-27.

The native enzyme shows initial rate kinetics consistent with a rapid-equilibrium, random-order mechanism. In such a mechanism either hydride transfer or proton transfer could be rate limiting, although the lack of an effect on V_{max} of deuterated coenzyme suggests that the hydride transfer step is not rate limiting. Detailed kinetic analysis of all three enzymes indicates that in both mutants k_{cat} is decreased while K_m is increased, indicating a role for Asp-27 in both binding and catalysis. The pH

dependence of these parameters, however, gives a clue to the detailed role of Asp-27. With both mutant enzymes k_{cat} increases as the pH decreases, unlike the wild-type enzyme, which shows pK values of approximately 5 and 8. This suggests that when the substrate is pre-protonated (as it will be increasingly at low pH), it can function independently of the presence of Asp-27, indicating that the primary role of this carboxyl group involves substrate protonation, presumably via a concerted mechanism involving the hydrogen-bonded water molecule rather than being involved in the hydride transfer step in any way.

Interestingly, with the mutant enzymes at low pH (where, as indicated above, the substrate is pre-protonated) a kinetic isotope effect with deuterated NADPH is now observed, showing that in the mutant enzymes hydride transfer is now rate limiting.

Before leaving the topic of pH dependence of kinetic parameters, it must be reiterated that any pK assigned to a group on the enzyme, on whatever basis, need not necessarily reflect the pK of a group involved directly with the substrate or the catalytic reaction. It is possible that such a group does reflect the pK of a group involved directly with the substrate or the catalytic reaction; it is also possible that such a group can affect the overall stability of the protein (and hence have an effect on V_{max} by decreasing the effective enzyme concentration) or the K_m of a substrate via a conformational change triggered by the ionization of a group located spatially far from the binding site.

Effects on Protein Structure

The protonatable groups of a protein can interact to form noncovalent ion pairs that act to stabilize the protein's structure. At neutral pH there are a number of both positive and negative charges in a protein. The positive charges come from the side chains of lysine, arginine, and histidine (which, depending on pK_a, are positive at pH 7). The N-terminal amino acid, in addition, has an α-amino group. Negative charges arise from glutamate and aspartate carboxyl side chains as well as the C-terminal residue. The crystal structures of a variety of proteins have shown the existence of ion pairs involving various partners. In hemoglobin (deoxy) they appear to be involved in both intra-subunit and inter-subunit interactions, and both side chain moieties and terminal amino acid residues are involved. (The making and breaking of these ion pairs in terms of the allosteric properties of hemoglobin are discussed in more detail in Chap. 21). Such ion-pair formation is an entropy-driven process since it results in the disordering of the solvating water molecules of the individual partners. The disruption of ion–dipole interactions with the solvating water molecules results in a positive ΔH on complex formation.

As the pH of the protein solution is varied, groups involved in such ion-pair formation are titrated and the stabilization energy provided by their interaction is lost. On a local scale, such effects might be expected to produce conformational changes affecting the activity by influencing K_m or V_{max}. On a larger scale, if enough ion pairs are involved in the overall structure and stability of the protein, it will

TABLE 20-1 *H* ion for titratable groups in proteins

Group	ΔH *ion* (kcal/mol)
Carboxyl	± 1.5
Imidazole	6.9–7.5
Amino	10–13
Sulfhydryl	6.5–7.0
Guanidine	12–13
Tyrosyl hydroxyl	6

unfold at extremes of pH. In general, proteins do denature at either low or high pH, and disruption of ion pairs contributes to this instability.

Temperature Dependence of pK_a Values

As indicated, local environmental effects can have significant influence on the pK_a of a group, which can complicate the interpretation of an experimentally determined pK_a. The temperature dependence of pK_a, however, can give a more reliable indication of which particular side chain is being titrated. As shown in Table 20-1, each type of side chain has a characteristic ΔH ion.

Since the standard free change ($\Delta G°$) of a reaction is given by

$$\Delta G° = -2.3RT \log K = \Delta H° - T\,\Delta S° \tag{20-6}$$

where K is the equilibrium constant, $\Delta H°$ the standard enthalpy change, and $\Delta S°$ the standard entropy change, one can write

$$-\log K = \frac{H°}{2.3R}\frac{1}{T} - \frac{\Delta S°}{2.3R} \tag{20-7}$$

The effects of temperature (T) on K are given by the van't Hoff equation,

$$\frac{d \ln K}{dt} = \frac{\Delta H°}{RT} \tag{20-8}$$

which can be rewritten as

$$\frac{d \ln K}{d(1/T)} = -\frac{\Delta H°}{R} \tag{20-9}$$

Equation (20-9) can be integrated between the limits of K_1 and K_2 at T_1 and T_2 to give

$$\log \frac{K_2}{K_1} = \frac{\Delta H°}{2.3R}\frac{T_2 - T_1}{T_2 T_1} \tag{20-10}$$

and $\Delta H°$ can be estimated by determining the equilibrium constant at two different temperatures. More reliably, $\Delta H°$ is determined using Eq. (20-7), which indicates

that a plot of $-\log K$ versus $1/T$ is linear, with a slope $= \Delta H°/2.3R$. In the context of pK_a values of groups, $-\log K$ is of course pK_a and all that is necessary is to measure the temperature dependence of pK_a and $\Delta H°$ ion is readily determined.

ISOTOPE EFFECTS

Although isotopes have been discussed in many chapters, their use in determining potential rate-limiting steps has not been formally examined. If we consider an example, the hydride transfer step involved in many dehydrogenase reactions, possible uses of isotopically labeled substrates in establishing a reaction's rate-limiting step become obvious. If a substrate is deuterium labeled in the position to be transferred, various experimental observations are possible. The deuterated substrate may show essentially identical steady-state kinetics to the normal substrate: Since an isotope effect is not observed, the inescapable conclusion is that the hydride transfer step is not the rate-limiting step. In such a case, an effect of the deuterated substrate on the pre-steady-state phase of the reaction may well be seen. As detailed in Chap. 18, if the reaction does not involve a rapid-equilibrium mechanism, it is likely that a pre-steady-state "burst" of product accumulation occurs. If the rate-limiting step of this pre-steady-state phase is the hydride transfer step, an isotope effect is expected. As illustrated in Fig. 20-6, an effect on this pre-steady-state phase has a minimal effect on the steady-state rate.

In a rapid equilibrium mechanism no pre-steady-state phase is observed since k_{cat} is the overall rate-limiting step in the reaction. Under these circumstances an isotope effect is seen but only in steady-state kinetic studies.

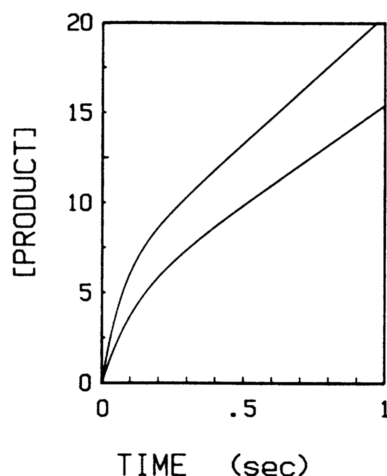


Figure 20-6 Simulated pre-steady-state kinetic results for a normal and an isotopically labeled substrate where the isotope effect is seen in the pre-steady-state phase of the reaction.

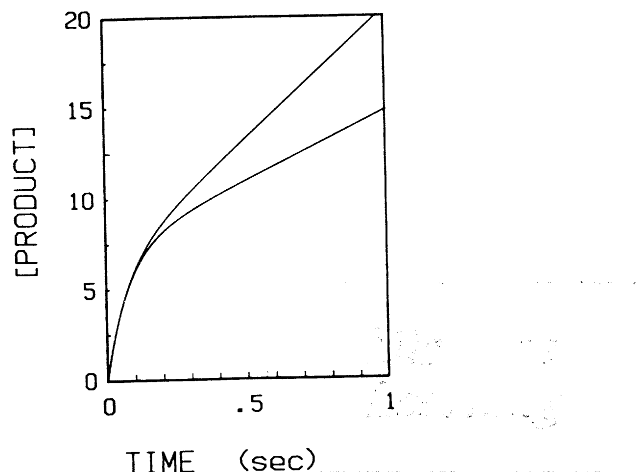


Figure 20-7 Simulated pre-steady-state kinetic results where the isotope effect is seen only in the steady-state rate.

In both cases we have assumed that the rate-limiting step contributing to k_{cat} or the pre-steady-state burst is the hydride transfer itself. It is, of course, possible that a rate-limiting conformational change in the central complex governs the rate of hydride transfer; in such an instance no isotope effect is observed on any phase of the reaction.

In cases where an isotope effect is seen in a step involving bond making or breaking, the magnitude of the effect depends on the atom involved. With hydrogen–deuterium or hydrogen–tritium substitutions, isotope effects up to two- or threefold are often observed.

In some circumstances, as examined in Chap. 15, the formal kinetic mechanism may be random order but not obey the rapid-equilibrium conditions. Under such conditions the hydride transfer step may not be rate limiting and a burst is seen. If the steady-state rate is affected but not the burst rate, as illustrated in Fig. 20-7, it indicates that the isotopically labeled product is released more slowly than the normal product, and that this is the rate-limiting step of the overall mechanism.

Although a large isotope effect in such an instance is unlikely unless the isotopically labeled product is a small molecule, small isotope effects may well be observed if the isotope atom is involved directly in some interaction with the protein.

TEMPERATURE EFFECTS

The effects of temperature on an enzyme-catalyzed reaction are diverse. As with the pH studies already examined, a detailed knowledge of the formal kinetic mechanism of the reaction considerably increases the merit of such experiments. Before

considering some of the enzyme parameters whose temperature dependence might be examined, it should be noted that the stability of the protein itself has a temperature dependence that must be taken into account when V_{max} effects are studied.

A variety of thermodynamic parameters can be obtained from temperature studies. As discussed in Chap. 13, the Arrhenius equation [Eq. (20-11)] relates the activation energy, E_a, to the rate constant, k, of a process;

$$k = Ae^{-E_a/RT} \qquad (20\text{-}11)$$

where A is a constant related to the probability of the reaction occurring. As with the van't Hoff equation [Eq. (20-8)], Eq. (20-11) can be written in a linear form [Eq. (20-12)] or an integrated form [Eq. (20-13)].

$$\log k = -\frac{E_a}{2.3R}\frac{1}{T} + \log A \qquad (20\text{-}12)$$

$$\log\frac{k_2}{k_1} = \frac{E_a}{2.3R}\frac{T_2 - T_1}{T_2T_1} \qquad (20\text{-}13)$$

The activation energy can be obtained by determining values for k at several temperatures. Enthalpies can be calculated from the temperature dependence of equilibrium constants in much the same way as described earlier for the temperature dependence of pK_a. Initial rate kinetic studies can yield much information concerning the rates of substrate binding or product release (in certain steady-state mechanisms), allowing the calculation of activation energies for these steps. The formal kinetic mechanism must be established, but once this is done the temperature dependence of the various θ parameters or ratios of θ parameters can yield a wealth of thermodynamic information.

In equilibrium mechanisms k_{cat} is the rate-limiting step and its temperature dependence allows the calculation of the activation energy for the chemical reaction (under conditions where it can be established that k_{cat} is a direct measure of the chemical reaction rather than a rate-limiting conformational change). Various relationships between initial rate parameters, as detailed in Chaps. 14 and 15, give a variety of equilibrium constants for substrate binding in various enzyme–substrate complexes. The temperature dependence of these equilibria permit the calculation of thermodynamic parameters.

Although the discussion so far has involved initial rate studies, similar experiments can be accomplished using rapid kinetic approaches (described in Chap. 18) where direct estimates of rate constants can be made. Similarly, the temperature dependence of conformational parameters can be employed to give thermodynamic information concerning interactions that may be involved in conformational changes. The temperature dependence of hydrophobic interactions or ion-pair formation are readily distinguishable from other types of interactions since they each have positive ΔH values.

The major problem with temperature studies of the types examined here is that it is possible that the formal kinetic mechanism may change in different steps in the

reaction contributing to particular kinetic parameters, which complicates the interpretation. Such changes may result from the temperature dependence of the various steps in the mechanism themselves; as the temperature is changed, new steps may become rate limiting. Alternatively, protein conformational effects may alter the formal kinetic mechanism: These are usually manifested as nonlinear van't Hoff of Arrhenius plots, emphasizing the need to use the linearized forms of these equations rather than calculating parameters from the integrated forms and experimental data at only two temperatures.

CRYOENZYMOLOGY

In the preceding section of this chapter we discussed the temperature dependence of kinetic parameters. Such effects are taken to extremes in the concept of cryoenzymology, the study of enzyme reactions at low temperatures. Under such conditions it should be (in theory) possible to measure steps in a reaction pathway which, at room temperature, are too rapid for techniques such as stopped flow. Since temperatures below 0° are often used, it is necessary to use antifreeze solvent systems, and this presents the first problem—it is essential to demonstrate that the solvent system does not affect the process being studied. The major problem, however, is the fact that very different rate-limiting steps may exist at low temperatures, meaning that simply determining kinetic parameters and interpreting their temperature dependence is no easy task. Despite these problems, cryoenzymology is being used with some systems.

Each of the protocols in this chapter (i.e., the study of pH effects, isotope effects, or temperature effects, etc.) has been examined from the standpoint of kinetic studies. The detailed interpretation of such studies usually requires a knowledge of the formal kinetic mechanism of the enzyme. In some instances similar information can be obtained from direct ligand binding studies. It is often easier, however, to use initial rate studies.

Although it might seem that the topics covered in this chapter are rather diverse, the *combination* of approaches is essential in the detailed interpretation of pH or temperature dependence of kinetic parameters. Each technique offers different insights into the makeup of the various kinetic constants that constitute the initial rate equation. Their application, in conjunction with thorough initial rate or stopped-flow studies, allows the enzymologist to dissect conformational effects from chemical steps and ligand binding or release steps in a kinetic mechanism. This in turn permits a far more detailed interpretation of the pH dependence of kinetic parameters in terms of which amino acid side chains may be involved in the actual chemical catalysis steps of the reaction.