Good Kinetic Practices

13

Generating reliable enzyme data requires clean experimental design and good kinetic practices. Certain practical considerations are important in this quest. This chapter will describe many such aspects of experimentation.

13.1 How to Assemble Enzyme Assay Mixtures

Stock Solutions and Dilutions Enzyme reaction rate, like any other chemical reaction rate, depends on the concentration of reactant(s), effector(s), pH, and ionic strength. Measurements therefore have to begin with precise definition of various concentrations involved. Solutions for enzyme assays must be prepared accurately. Analytical precision may be achieved by good experimental practices like (a) differential weighing of chemicals in a calibrated balance, (b) volumetric transfers using precision pipetting aids, and (c) use of reliable primary standards. A few standard solutions commonly employed in enzyme assays are listed in Table 13.1. Various buffers used to maintain reaction pH will be discussed a little later. Many assay components are required in very low concentrations; some of them may be hygroscopic. This necessitates the calibration of stock solutions before use. For instance, once prepared, the concentration of a stock NADH solution may be standardized by measuring its absorbance at 340 nm. From the knowledge of its molar extinction coefficient ($\boldsymbol{\varepsilon} = 6220\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$), the actual concentration can be ascertained.

Good quality water (double distilled or deionized) is always used to prepare solutions for enzyme assays. Most dilutions are also made in water. Few assay components are not readily soluble in water. They may have to be added to the assay as solutions in an organic solvent. In such cases, it is necessary to take suitable controls to check whether the solvent itself affects the enzyme activity or the assay method.

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Table 13.1 Standard solutions frequently used in enzyme assays

Component	Preparation/source[a]	Comments
NaOH	1.0 M, sodium hydroxide pellets (4.0 g) in 100 ml	Store in plastic (*not* glass!) bottle
Saline	0.9% NaCl (0.9 g in 100 ml)	Isosmotic with blood; commonly used as phosphate buffered saline – With 20 mM Na, K phosphate, pH 7.4
Ammonium sulfate	Saturated solution is 3.9 M (at 0°C)	Used to precipitate proteins; highest ionic strength of 23.4 in water
Potassium chromate	Potassium chromate (20 mg) and KOH (1.6 g) to make 500 ml solution	Spectroscopic standard with A_{375nm} of 0.991
Bovine serum albumin (BSA)	Crystalline BSA solution (1.0 mg per ml)	Protein standard with A_{280nm} of 0.66
Glycerol	5–50% solution (by volume)	Viscous, difficult to pipette; stabilizer, cryoprotectant
Ethylenediaminetetraacetic acid (EDTA)	100 mM stock; 373.2 mg of disodium salt in 10 ml	Chelating agent, typically used at 0.1–1.0 mM
2-Mercaptoethanol	Pure liquid is 14.3 M	Thiol protectant; typically used at 1–5 mM
Dithiothreitol (DTT)	100 mM stock; 154.3 mg in 10 ml	Thiol protectant; typically used at 1–5 mM
Phenylmethanesulfonyl fluoride (PMSF)	10 mM; 1.74 mg per ml isopropanol	Stock store at −20 °C; typically used at 0.1 mM
NAD^+	1.0 mM; 6.63 mg in 10 ml	
NADH	1.0 mM; 7.09 mg in 10 ml	On 1:10 dilution should give A_{340nm} of 0.622
ATP	10 mM; 60.5 mg of the disodium salt in 10 ml	Kinase/synthetase substrate; used along with excess of $MgCl_2$
Oxygen (O_2)	375 nM; solubility at 25°C, 12 mg in 1000 ml	Substrate for oxidation and oxygenases; solubility depends on temperature and ionic strength
p-Nitrophenol	10 mM; 13.9 mg in 10 ml	Formed as hydrolysis product of esterase and phosphatase; at pH 10.0 p-nitrophenol has $\boldsymbol{\varepsilon} = 18{,}700$ at 405 nm
5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB)	10 mM; 39.6 mg DTNB in 10 ml; prepare fresh, unstable in alkaline pH	Thiol estimations; 5-thio-2-nitrobenzoic acid formed with $\boldsymbol{\varepsilon} = 13{,}600$ at 412 nm

[a]All solutions are in water unless mentioned otherwise

It is generally desirable to prepare concentrated stock (from 5× to 200×) solutions. The idea is to provide sufficient space (volume) in the assay mixture to permit further additions. Concentrated stock solutions are also useful in minimizing significant changes (such as in terms of pH, temperature, buffer content, etc.) to the reaction mixture upon their addition. Too large a dilution of buffer affects pH since

ionization itself is concentration dependent. Component additions made should not be more than 5–10% of the total reaction volume. Otherwise, special care is required to ensure that they are well mixed and equilibrated for pH, temperature, etc. It is usual to start the reaction by the addition of 10–20 μl of the enzyme per assay. Such volumes can be easily pipetted and do not essentially change the total reaction volume. A 10 μl addition to 1.0 ml reaction corresponds to 1% increase in volume.

Often, it may be required to add different amounts of a component (such as while performing substrate or inhibitor saturation studies). This can be done in different ways once a stock solution is prepared. (1) Directly add different required volumes by precision pipettes. This procedure should be discouraged because (a) different volume additions lead to volume changes, however small, and (b) pipettes come with volume ranges and are not uniformly accurate, particularly at the lower ranges. (2) Prepare a dilution series from the stock solution – such that a constant volume is added to the rest of the assay mixture. While making dilutions, it is desirable to independently pipette increasing amounts of stock solutions to each tube and make up the volumes. Serial dilution (stepwise from one dilution to the next!) should be avoided because a mistake in one tube is carried over to all the subsequent dilutions.

Use of Cocktails An enzyme assay mixture may consist of several components like substrate(s), cofactor(s), metal ion, buffer, and protective agent (thiol compounds such as DTT or 2-mercaptoethanol). It makes practical sense to prepare a bulk mixture of many (or all) of these and take suitable aliquots for individual assays. Such *assay cocktails* should contain all components with the exception of one (quite often this is enzyme) that is used to start the reaction. Assay methods employing cocktails are particularly useful (a) to avoid pipetting mistakes and related scatter and (b) if a number of assays are to be performed under identical conditions. They are valuable when monitoring enzyme fractions from a chromatography column.

While the use of cocktails to assay enzymes can be convenient, certain precautions are in order. Adequate controls are required to ensure that (a) there are no instabilities or incompatibilities of various assay components and (b) pre-incubation with and the sequence of addition of some components have no significant contributions to the outcome of the assay.

However mundane it may appear, accurate pipetting is a crucial determinant for good enzyme experiments. Largest contributions to measurement errors arise from pipetting mistakes.

Assay Dead Time and Mixing In a multicomponent reaction, it is essential to ensure that all the components are properly mixed at the start of the assay. Mixing in smaller volumes is not trivial. Too vigorous a shaking may lead to enzyme denaturation. When one of the components is more viscous (like a glycerol stock of an enzyme), mixing does require an effort. The contents can be satisfactorily mixed by covering the top with Parafilm and inverting the tube (or the cuvette) a few times. Some enzymes (like *Bam*H I) are supplied as 50% glycerol stocks – repeated pipetting/ejecting of such samples into the small volume of an assay mixture facilitates their quick mixing. Otherwise, the dense enzyme solution settles quickly

to the bottom of the tube. One other way to achieve gentle but complete mixing is to add the initiating component to the side of the reaction vessel – as droplet (of about 50 μl or less). At time zero, the reaction can now be initiated by simple repeated inversions of the closed vessel.

Regardless of how we mix the components to initiate the reaction, the process of mixing should be completed in a short period of time. With some practice, the fastest mixing time can be as short as 10 s. This is the *dead time* of any manual assay – we cannot make any meaningful measurements before this! One can however adjust temperature and enzyme concentration to ensure that the reaction rate is slow enough to allow convenient (longer) timescales for measurement. In a spectrophotometric continuous assay, the reaction is started by mixing the solution, placing the cuvette in the holder, and then starting the detector by pressing a button. The time lapse between mixing and actually starting the measurement can be up to 20 s. For reaction rates occurring below the seconds scale, fast reaction kinetic tools (see Chap. 11) may be employed.

Order of Component Addition and Pre-incubations Assays are routinely initiated by the addition of enzyme – as the last component – to the rest of the reaction mixture. However, this may not always be the best option. The reaction can also be initiated by adding (at time zero) other components like substrate(s) or cofactor(s). Actual choice of how to start the reaction will depend on one or many of the following factors:

(a) If the enzyme is unstable in the assay mixture, it should be the last component to be added.
(b) Due to compatibility issues, a component may not be suitable for inclusion in the assay cocktail. The assay format then would require that such a component be added last and made the initiating component.
(c) There may be significant blank rates – in one or more combinations of the incomplete reaction mixtures. Such combinations offer the best controls and should be used to measure blank rates.
(d) An enzyme may need enough time to establish a binding (and/or conformational) equilibrium with one or other substrate, cofactor, or inhibitor. This is best achieved by pre-incubating the enzyme with appropriate ligand(s) before starting the reaction with the missing component. Many enzymes bind their cofactors loosely. Therefore significant proportion of it upon purification is present as apoenzyme. Interaction of pyridoxal phosphate with serine hydroxymethyltransferase is one such example. Pre-incubation with the requisite cofactor is necessary to convert the apoenzyme fraction into fully active holoenzyme. The NADP-glutamate dehydrogenase from *A. niger* offers a different example. Upon pre-incubated with NADPH plus 2-ketoglutarate (and not individually!) and when the reaction started with ammonia, enhanced initial rates are observed. Clearly, ligand-dependent conversion to a more active form occurs during the pre-incubation step.

Table 13.2 Design of a typical 1.0 ml enzyme assay with controls

	Addition in µl		
Component	Enzyme blank	Substrate blank	Test
Substrate (20×)	50	0	50
Buffer (10×)	100	100	100
Water	850	890	840
Enzyme	0	10	10
Rate measured	$(\Delta A/t)_{-E}$	$(\Delta A/t)_{-S}$	$(\Delta A/t)_{Test}$

About Blanks and Controls We have earlier mentioned that a common practice is to start the reaction by the addition of the enzyme. This however assumes that all controls and blanks have been taken into account. An assay system where all the components are present except the enzyme – whose volume is made up for with buffer – allows the measure of nonenzymatic rates, for example, the CO_2 hydration rate in the absence of carbonic anhydrase in the assay. Any nonenzymatic rate has to be corrected for – actually subtracted from the rate recorded for the complete assay. Thus, *enzyme minus* blank is an important and useful control. If blank rates occur in the absence of added enzyme, failure to subtract this blank rate results in an enzyme concentration curve that intersects Y-axis above zero – implying finite enzyme activity when no enzyme is present!

A *substrate minus* control should always be included. The substrate blank rate allows us to detect any time-dependent changes in the assay that are independent of substrate conversion step (oxidation of NADH in the absence of pyruvate, in lactate dehydrogenase assay, for instance). In principle, for multi-substrate reactions, "substrate minus" controls (and blanks) may be measured for each one of them. It is possible that a blank rate will only occur with certain components of an incomplete assay mixture. It is thus necessary to test for all such rates using different possible combinations.

A typical experimental design to meaningfully measure enzyme activity is shown in Table 13.2. This includes the two important controls mentioned above. While measuring change in absorbance (ΔA) as a function of time, $\Delta A/t$ would represent the rate. Experimentally we obtain the three rates, namely, $(\Delta A/t)_{Test}$, $(\Delta A/t)_{-S}$, and $(\Delta A/t)_{-E}$. The true enzymatic rate would then be given by

$$\left(\frac{\Delta A}{t}\right)_{\text{enzymatic}} = \left(\frac{\Delta A}{t}\right)_{\text{Test}} - \left(\frac{\Delta A}{t}\right)_{-\text{S}} - \left(\frac{\Delta A}{t}\right)_{-\text{E}}$$

The two types of controls and blanks (substrate blank and enzyme blank) are essential so that their contributions can be corrected for.

Enzyme Stability During Storage, Pre-incubation, and Assay Enzymes are prone to inactivation like any other protein. They are optimally stable under specific conditions of temperature, pH, ionic strength, and presence or absence of ligands. These parameters differ from enzyme to enzyme. Furthermore, conditions are not

necessarily the same for optimal stability when compared to optimal enzyme activity. Hence enzyme stocks should be maintained under conditions of their maximal stability. But enzyme assays should be conducted under the conditions of optimal activity. Enzyme stocks are best stored at low temperatures but without repeated freezing and thawing. Their stability is greatly enhanced by additives like sucrose or glycerol (at 20–50% level). It is also useful to maintain suitable aliquots so that just enough enzyme is taken out to thaw before use.

At high enzyme concentrations, one may observe the "Zulu" effect – where inactivation of significant proportion of the total enzyme may apparently go unnoticed; but below a critical concentration, there may be a sudden decrease. Immobilized enzymes may behave in this manner due to limits on substrate diffusion rates.

There are several reasons why an enzyme loses activity during the assay. The irreversible loss of enzyme may be due to specific adverse conditions of assay like pH, temperature, ionic strength, ligands, etc. Apart from these, possible inactivation due to proteolysis (by contaminating protease activity, however minor!) of the enzyme protein may occur. Enzymes, especially the intracellular kind, are found naturally at high concentrations. On dilution to much lower concentrations in an assay mixture, many of them lose activity rapidly. In dilute protein solutions, normally found in an assay with pure enzyme preparations, concentration effects come into play. Proteins bind avidly to glass or polystyrene (plastic) surfaces. Adsorption onto surfaces of containers, assay tubes, and pipette tips is a serious concern – particularly when the assay contains a dilute solution of the enzyme protein. Adsorbed enzyme may reflect as loss of enzyme activity – with an enzyme concentration curve intersecting the X-axis rather than passing through the origin. Therefore, it is prudent to use assay tubes and pipette tips made of low protein binding material. Silicone-coated glassware may also be used. Another option is to add an inert carrier protein to pure enzyme samples. This ensures that potential protein binding surfaces are coated/saturated by the carrier protein. Non-interfering carrier proteins like bovine serum albumin (BSA) or gelatin (lacks aromatic amino acid residues!) are preferred as additives to enzyme stock solutions and/or assay mixtures. Many restriction enzymes are best used in a buffer fortified with BSA. For example, *Bam*H I is supplied as a 50% glycerol solution with 0.01% BSA.

Inactivation of the enzyme during assay could be a likely cause for nonlinearity. Addition of fresh enzyme aliquot, to an ongoing assay, should proportionately enhance the rate before it again falls off with time (Fig. 13.1). Enzyme inactivation makes estimation of the initial rate difficult and inaccurate. The possibility of enzyme inactivation can be ruled out by a simple test described by Selwyn (1965). It is based on the fact that the value of $[P]$ formed in an enzyme assay is determined solely by the product of time and enzyme concentration (that is: $[P] \propto [E] \times t$). A set of progress curves may be generated in which all the parameters are kept constant – except the amount of enzyme. These time courses when plotted with normalized $[E]$ should be superimposable. The data points from all progress curves (at different $[E]$), when plotted as $[P]$ versus $[E] \times t$, should fall on a single curve (*Selwyn plot*, right panel in Fig. 13.1). This implies in order to form certain $[P]$ in a standard assay,

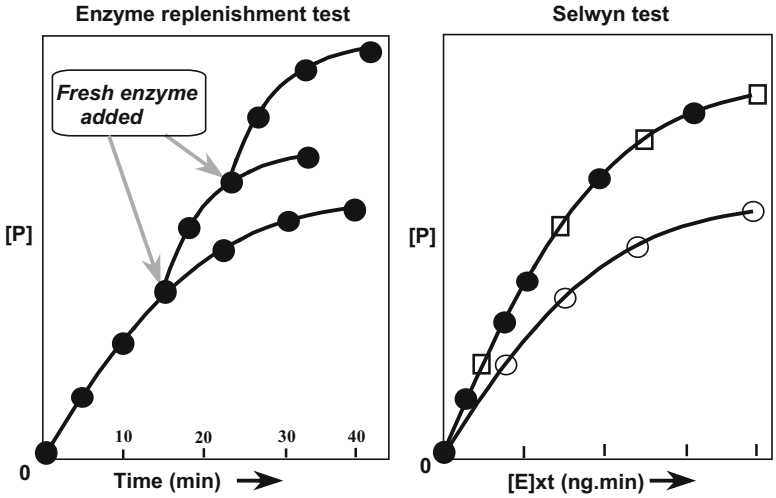

Fig. 13.1 Enzyme inactivation during assay. This may be tested by fresh addition of enzyme (left panel) and by Selwyn test (right panel). The curves for two different enzyme concentrations (● and □) superimpose when there is no inactivation. With inactivation however, the curve for lower enzyme concentration (○) is distinct and falls off much faster

twice the amount of enzyme (i.e., 2 × [*E*]) should take half the time (i.e., *t*/2). If the enzyme is getting inactivated during the course of the assay, then the value of [*E*] itself decreases with time. Therefore the data for different [*E*] should fall on different curves.

13.2 pH and Ionic Strength Considerations

Enzyme activity is profoundly affected by pH, buffer species used, ionic strength, and the dielectric constant of the solution. Enzyme-catalyzed reactions almost always involve ionizable groups on the enzyme and/or on the substrate. As proton transfers are crucial, maintaining a well-defined pH (H^+ concentration) for an enzyme assay is important. Since a range of pH values (between 0 and 14 in an aqueous environment) are in use, more than one kind of buffer ion may be required in an experiment. This is achieved by the judicious use of suitable buffers; some useful buffer components and their characteristics are listed in Table 13.3. Apart from the desired pH, and hence its pKa, choice of a buffer depends on many other factors. Some buffer components may have additional effects. For instance, phosphate may be an enzyme substrate/inhibitor or may chelate metal ions. Tris is known to inhibit some enzymes like succinic semialdehyde dehydrogenase. Amine buffers may form unwanted Schiff bases with substrate/product carbonyl groups. Counterions (like SO_4^{2-}, Cl^-, K^+, Na^+, NH_4^+, etc.) may influence the enzyme directly or through ionic strength effects.

Table 13.3 Buffers frequently used in enzyme studies

Component (and its full name)	pKa (at 25 °C)	ΔpKa/ Δ °C
Acetate (with its Na or K salt)	4.76	+0.0002
MES[a]; 2-(N-morpholino)ethanesulfonic acid	6.15	−0.0110
Maleate (with its Na or K salt)	6.26 (pKa2)	–
PIPES[a]; piperazine-N,N′-bis(2-ethanesulfonic acid)	6.80	−0.0085
Imidazole (with HCl)	6.95	−0.0200
Phosphate (with its Na or K salt)	7.21 (pKa2)	−0.0028
HEPES[a]; N-(2-hydroxyethyl)piperazine-N′-(ethanesulfonic acid) (with NaOH)	7.55	−0.0140
Tris, tris(hydroxymethyl)aminomethane (with HCl)	8.06	−0.0310
Glycine (with NaOH)	9.78 (pKa2)	−0.0250
Carbonate (with its Na or K salt)	10.33 (pKa2)	−0.0090

[a]These pKa values are reported at 20 °C

Buffering of metal ion concentration is also a consideration in design of enzyme assays. Besides substrates like ATP, many buffer species (for instance, phosphate and citrate) are significantly chelate divalent metal ions. This has to be accounted for, and suitable buffers that do not bind metal ions (like Mg^{2+}) should be used.

Several buffer components were designed and synthesized (like HEPES) to minimize interference effects and provide a range of pH values (Good et al. 1966).

Effective buffering capacity is limited to ±1.0 pH unit about the pKa value of a given buffer species. The strength and choice of buffer used should take into account the required pH range and the nature of the reaction. Strong buffering is necessary to maintain pH with reactions that generate or consume H^+ ions (like urease, glucose oxidase, etc.). Buffers are usually prepared by dissolving the buffering component in a small volume, adjusting the pH, and then making up the desired volume – the pH of this solution is confirmed finally. Two considerations are very important in the use of stock buffer solutions: (a) ionization is affected upon dilution; it is therefore prudent to measure the final assay mixture pH and ensure that the required pH is reached, and (b) pKa of a buffer species is temperature dependent; Tris is notorious for this (Table 13.3). It is thus necessary to measure/adjust the buffer pH at the same temperature at which it will be used. Finally, pKas of ionizable species are subject to perturbation by ionic strength and dielectric constant changes. Extra caution is needed to measure/report pH values in aqueous-organic solvent mixtures (see Chap. 24).

Because of pKa limitations, a single buffer system cannot be used over a wide range of pH values. Further, buffer-specific effects (see above) may also exist. Effects on the enzyme due to switching of buffer species, if any, have to be eliminated. This is achieved by using either *single buffers with overlap* or *mixed buffer systems*. Two different buffers with overlapping pH ranges may be used to make measurements at the same pH. This informs us about the effects of buffer components other than those due to pH alone. Since different buffers contribute

Table 13.4 Mixed buffer systems that span a broad pH range

pH range	Components of the mixed buffer		
5.2–8.5	MES (50 mM)	PIPES (50 mM)	HEPES (50 mM)
5.2–8.6	Maleate (50 mM)	Tris (50 mM)	–
6.0–10.0	Acetate (25 mM)	Tris (25 mM)	Ethanolamine (50 mM)
5.0–9.0	Acetate (25 mM)	MES (25 mM)	Tris (50 mM)

different ionic strengths, it is desirable to use mixed buffer systems (Table 13.4) that also provide for constant ionic strength.

Most enzymes display a bell-shaped pH-activity curve with maximal activity around neutral pH. However, there are enzymes with an extreme pH optimum in the acidic (such as pepsin) and alkaline (such as arginase) range as well. 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. It is important to know whether the effects of pH on enzyme activity are reversible or they result from irreversible changes leading to inactivation. Activity data in a pH range where the enzyme is rapidly losing activity is difficult to interpret – often meaningless. Enzyme pH stability can be evaluated by incubating it at different pH values (with and without substrate, effector, etc.) before readjusting to a pH where it is known to be stable. Subsequently, the activity remaining in these samples can be determined in a standard assay. Information about the stability of the enzyme over the pH range studied is necessary in designing correct kinetic studies (more on this in Chap. 24). Meaningful pH dependence of enzyme activity may be sought within a range of pH defined for stability.

Experimental determination of pH optimum (plots of pH versus activity) serves two purposes: firstly, it is of practical importance in enzyme assay optimization, and secondly, the ascending and descending limbs of such profiles give some idea about the range of pKas and hence possible ionizable groups involved. Different enzymes have different pH optima – this pH optimum may be different from the physiological pH in which the enzyme functions. If in vitro data are to be related to in vivo situation, then it is relevant to assay the enzyme at physiological pH values.

13.3 Temperature Effects

Rate of chemical reaction is directly affected by temperature. Normally the rate doubles for every 10 °C rise in temperature (Chap. 9). While this is also true for enzymatic reaction rates, there is one major difference. Like any other protein, an enzyme undergoes thermal denaturation at higher temperatures. Beyond a particular temperature, enzyme-catalyzed rate starts to decline – due to inactivation of the catalyst. Hence temperature optimum is a practically convenient expression with no absolute significance. It suggests something about the heat stability of the enzyme preparation but is not a definite characteristic of the given enzyme sample, much less of the enzyme itself. At the temperature optimum, enzyme activity and enzyme

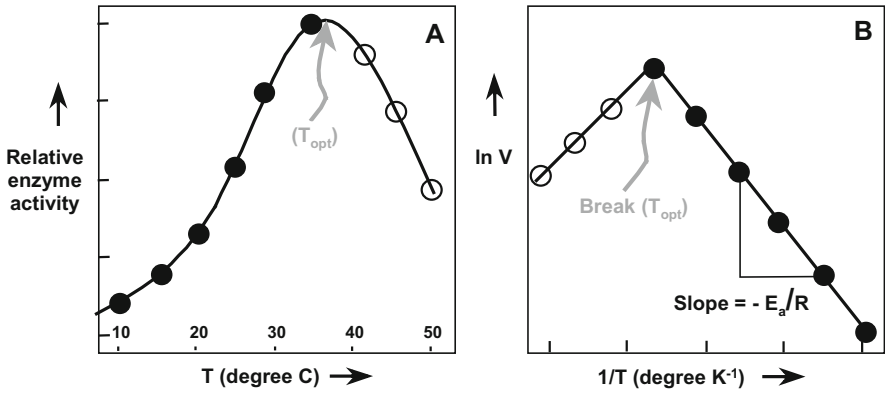

Fig. 13.2 Temperature dependence of enzyme activity. (**A**) Temperature optimum of an enzyme. Inactivation rate predominates in the descending limb (open symbols). (**B**) Arrhenius plot. The velocity data (*V* is maximal enzyme activity at a fixed enzyme concentration) is plotted against absolute temperature (°K) in ln *V* versus 1/T format. The gas constant R = 8.31 J × $mol^{-1} \times K^{-1}$ (or 1.98 cal × $mol^{-1} \times K^{-1}$)

inactivation rates compete – leading to a maximum in the curve. *Temperature optimum* (T_{opt}) is a consequence of these two competing processes. Optimal temperature for the same enzyme may vary depending on the presence of stabilizers, pH, etc.

A temperature-dependence curve (Fig. 13.2a) is more of practical value in designing enzyme assays. Enzymes should be assayed at temperature that is convenient, supports high activity, and does not significantly denature it. Most enzyme activities are measured at a standard temperature of 25 °C or 30 °C. However in some cases it may be desirable to use an appropriate physiological temperature. It is usually 37 °C for mammalian enzymes and is upward of 72 °C for *Thermus aquaticus* DNA polymerase used in polymerase chain reaction (PCR), for example.

Assay temperatures are normally maintained by keeping the reaction mixture in a water bath. Temperature equilibration takes time and depends on the starting temperature of the mixture and also the nature of the reaction vessel used. Frequently, for reasons of stability, enzyme (or some other labile component of the assay) is stored on ice. Adding such a cold component can lead to a drop in the effective temperature of the assay and therefore a slower reaction rate. Adequate time should be given in such cases for the assay mixture to reach the required temperature. Poor temperature equilibration can cause nonlinear reaction rates, and spurious lags may be observed.

Temperature dependence of enzyme activity data can also be analyzed according to Arrhenius equation (Chap. 9). In a temperature range over which inactivation is insignificant – a plot of ln *V* against 1/T gives a straight line (Fig. 13.2b). From its slope we obtain the value of activation energy ($E_a = -R \times$ slope). A *break in the Arrhenius plot* is observed when data at higher temperatures, with loss of catalytic activity, is also included (open symbols, Fig. 13.2b). It should be possible to compare activation energies (Ea) for enzyme-catalyzed reaction with the

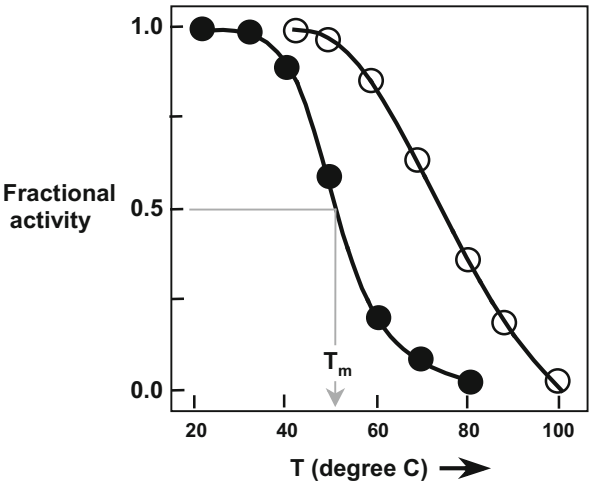

Fig. 13.3 Decrease in the fraction of enzyme activity as a function of increase in temperature. The temperature at the midpoint of inactivation is shown as T_m. For an enzyme with higher thermal stability (curve with open symbols) the T_m is higher

corresponding uncatalyzed reaction. As expected, the catalyzed reactions have much lower Ea (also see Chap. 5).

Temperature stability of an enzyme can be characterized kinetically (rate of inactivation) or thermodynamically (inactivation treated as a reversible process with an equilibrium). For kinetic characterization of enzyme stability, enzyme solutions are incubated at different temperatures and aliquots removed at suitable time intervals. The enzyme activity in these samples is then measured (usually immediately), in a standard assay at its optimal temperature. A plot of relative activity versus temperature can be informative (Fig. 13.3). The temperature at the midpoint of inactivation (T_m) corresponds to the temperature at which half the enzyme has lost its activity. A high T_m implies a more thermostable enzyme form. Thermal stability could arise due to (a) protection by a ligand or a stabilizing agent or (b) an inherently more stable enzyme (mutant form).

13.4 Summing Up

Many factors and a variety of artifacts influence the accuracy of enzyme activity measurements. Properly defined assay conditions of pH, temperature, and ionic strength go a long way in collecting reliable primary data on the enzyme of interest. Enzyme data without due attention to quantitative aspects is meaningless. Quality of this data begins with good kinetic practices and forms the foundation of further sophisticated analysis – with or without the use of computational support. Finally, the quality and completeness of enzyme data depend on reporting the essential metadata details (such as temperature, pH, ionic strength, concentrations of *E* and *S*, presence and concentrations of inhibitors/activators, etc.), conditions under which the kinetic parameters were obtained. Standards for reporting enzymology

data (STRENDA; available at http://www.strenda-db.org) is an effort to prescribe and follow best approaches for reporting data in enzyme research (Tipton et al. 2014).

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