Origins of Enzyme Catalytic Power

6

Understanding enzyme function is an exciting research because diverse and often unpredictable solutions are developed to perform seemingly impossible tasks. Enzymes are such powerful catalysts as they lower the height of the activation energy barrier. How do enzymes bring about this decrease in $\Delta G^{\neq}$? Many excellent attempts to dissect this into discrete enthalpic and entropic factors have been made. Rate accelerations are favored when enzyme specifically binds and assembles substrates at the active site and then provides optimal arrangement of catalytic groups. This is clearly an entropic ($\Delta S^{\neq}$) contribution. Stabilization of transition state through enthalpic ($\Delta H^{\neq}$) factors like select hydrogen bonds, salt links, acid–base groups, covalent interactions, etc., is another feature. Various tricks that enzymes employ in achieving catalytic prowess are shown in Fig. 6.1. These components are best understood through case studies, and we will do this through representative examples for each.

6.1 Proximity and Orientation Effects

Entropic contributions in accelerating the enzymatic reaction rates are substantial. This is described by enzymologists variously as approximation, coming together, spatial relationship, pre-organization, propinquity effect, Circe effect (character from Odysseus of Homer!), orbital steering, restricted motion, loss of degrees of freedom, etc.

The reactants in solution have substantial degrees of rotational and translational freedom. This means a given molecule is an ensemble of many conformational states. Only one (or few) of these conformations is capable of reaching the transition state and beyond. By selectively binding to the enzyme active site such a reactant, conformation is frozen out of many. Clearly on binding to the enzyme active site, the substrate loses many degrees of freedom – becomes ordered. Recall that entropy is a measure of disorder or randomness. This entropy loss ($\Delta S^{\neq}$) is reflected in the

N. S. Punekar, *ENZYMES: Catalysis, Kinetics and Mechanisms*,
https://doi.org/10.1007/978-981-13-0785-0_6

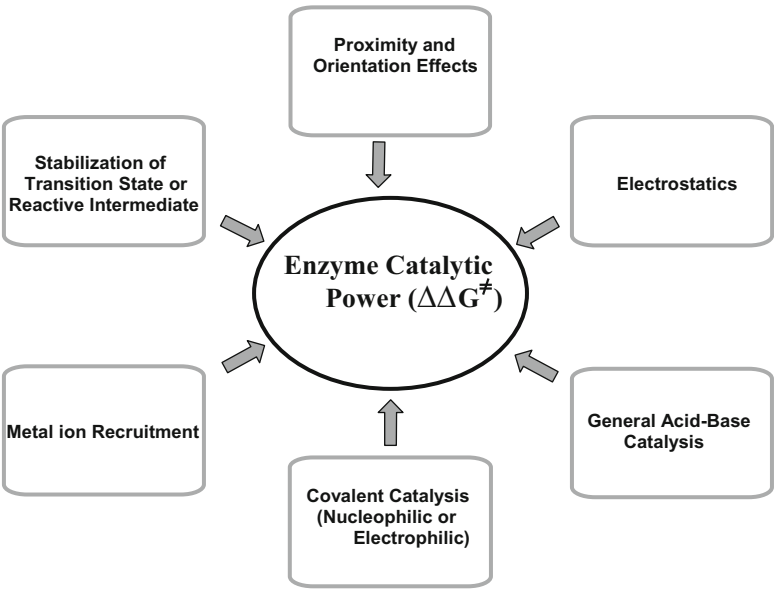

Fig. 6.1 **Various features that enzymes use to achieve reaction rate acceleration**

lowering of $\Delta G^{\neq}$. This entropic cost is paid in part by the substrate binding energy – the entropic cost of a chemical event is thus shifted to the binding (physical) event! Apart from *freezing the reactant conformation*, other consequences of substrate binding to enzyme active site are important. These are discussed below.

Every enzyme active site is endowed with functional groups (amino acid side chains or from the cofactor or both) that are involved in catalysis. The three-dimensional scaffold of the enzyme protein ensures that these groups create a well-defined *pre-organized* active site. Binding to such a site brings these catalytic groups in close proximity and proper orientation with the susceptible substrate bonds. Any enzyme catalytic group acting on the substrate will now be an *intramolecular* event. Intramolecular reactions generally proceed much more rapidly than their intermolecular counterparts. An excellent application of this concept is Cleland reagent (Cleland 1964). Dithiothreitol (HS-X-SH) is a better reducing agent than two equivalents of a monothiol (X-SH). Here the oxidation is made intramolecular, and formation of more number of product molecules ($n = 3$) confers entropic advantage (more degrees of freedom!) for the overall reaction.

$$\begin{array}{lll} \mathrm{R_1-S-S-R_2+X-SH+X-SH} & \rightarrow & \mathrm{R_1-SH+R_2-SH+X-S-S-X} \\ (n=3) & & (n=3) \\ \mathrm{R_1-S-S-R_2+HS-X-SH} & \rightarrow & \mathrm{R_1-SH+R_2-SH+(X-S-S)} \\ (n=2) & & (n=3) \end{array}$$

Substrate tethering (proximity) and proper orientation of catalytic group(s) for attack lead to large kinetic advantage. It appears as though the effective local

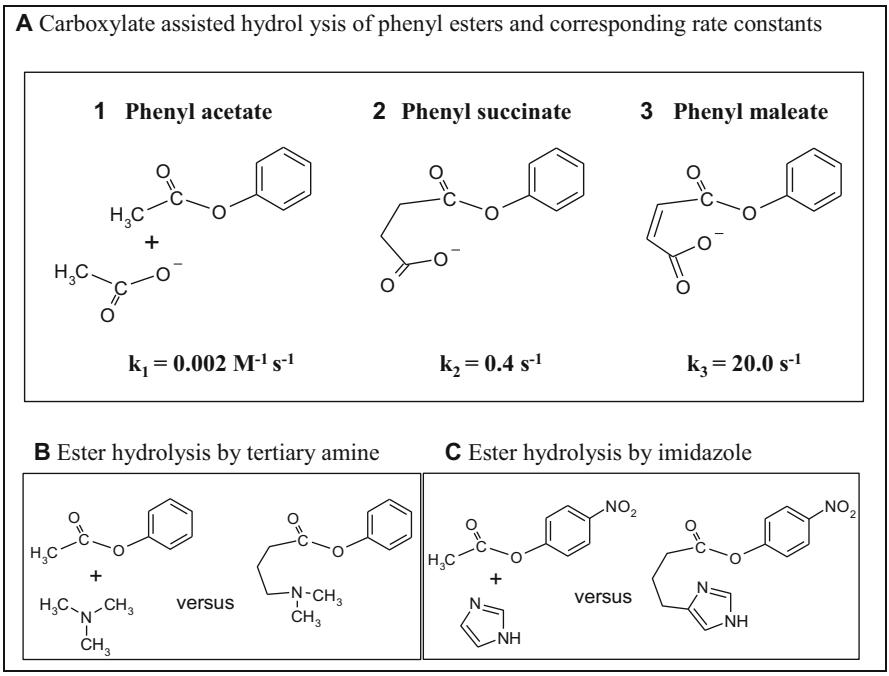

Fig. 6.2 Organic model reactions exemplifying the proximity and orientation effects that lead to rate accelerations. Hydrolysis of phenyl esters by carboxylate (**A**), tertiary amine (**B**), and imidazole (**C**) is shown

concentration of the catalytic group is raised enormously. Organic model reactions have helped to understand many of these features of enzyme catalysis. Examples illustrating these concepts are discussed below.

The first example in Fig. 6.2a is the typical hydrolysis of phenyl acetate by sodium acetate (CH_3COO^- ions). This is a bimolecular reaction where the ester and the acetate ion are independent entities. Note that accordingly the rate constant k_1 has units of $M^{-1}\ s^{-1}$. However, when the $-COO^-$ group is built into the same molecule (phenyl succinate), the reaction becomes intramolecular – and the absolute value of rate constant (k_2 with units of s^{-1}) increases by a factor of 200! A direct comparison of the two rate constants is difficult because they represent two different orders of reaction (see Chap. 9 for details). By fixing the acetate concentration (say $[CH_3COO^-] = 1.0$ M) and assuming pseudo-first-order condition in the first case, this can be work out. An effective $-COO^-$ concentration of 200 M may be calculated for phenyl succinate, while this would be an enormous number of 10,000 M for phenyl maleate! Achieving such high concentrations by acetate addition is practically impossible – concentration of glacial acetic acid itself is around 17 M!

We know from the above discussion that when a reaction is made intramolecular, rate accelerations take place as if the apparent reactive group concentration felt at the

site is enormously raised. Reactions in examples 2 and 3 above are intramolecular; then why is k_3 50 times larger than k_2? Because of two methylene groups (no double bond), phenyl succinate has many rotational degrees of freedom. The *cis* double bond in phenyl maleate restricts this motion, and the $-COO^-$ is always favorably oriented for attack – the reaction is entropically more favorable. However, if the double bond is *trans* (as with phenyl fumarate), no such rate acceleration occurs.

Two other examples of such rate accelerations due to intramolecular catalysis are ester hydrolysis by tertiary amine (b in Fig. 6.2) and imidazole (c in Fig. 6.2). The apparent concentrations felt, by making the reaction intramolecular in these two cases, are 1300 M and 24 M, respectively.

Enzyme active site generally occupies a small region compared to the total protein volume. With respect to reactions involving multiple substrates, their proper assembly on to enzyme active site takes place by a series of bimolecular collisions. Ultimately, when the reaction occurs at a fully occupied active site, it represents an intramolecular chemistry. This is an entropically favored situation as more degrees of freedom are lost! All the reactants (substrates) are bound very close to each other with their appropriate groups suitably aligned. This proximity and orientation of reactants effectively increase their local concentration, and profound rate accelerations are achieved (Jencks 1975; Page and Jencks 1971).

We note that proximity and orientation of reactive groups (both from the enzyme and the substrate) contribute substantially to rate enhancements. But what tools are employed by nature to gain this advantage? The formation of a non-covalent enzyme-substrate complex is the first step in enzymatic catalysis. Substrates are bound to enzymes by multiple weak interactions – often mediated by van der Waals forces, hydrophobic interactions, and hydrogen bonds. Hydrogen bonds are prominent among these as they are directional (Fersht 1987). In fact strength of a hydrogen bond is a function of its length, orientation, linearity, and the microenvironment. When multiple hydrogen bonds occur, the effect can be cooperative and dramatic! Apart from their cooperative effect observed between two strands of DNA, five well-directed hydrogen bonds define the strength of avidin–biotin complex. A special kind of hydrogen bond – the low-barrier hydrogen bond (LBHB) – has been implicated in some enzymic catalysis (Cleland et al. 1998). LBHBs are short, very strong hydrogen bonds formed when the partner electronegative atoms sharing the hydrogen have comparable pKa values. Evidence for LBHB formation exists for chymotrypsin catalysis, and this hydrogen is shared between His57(NH) and Asp102(COO^-). This is proposed to help stabilize the tetrahedral intermediate. Similarly, a LBHB between -OH group (of the substrate lactate) and His195(**N**, of the enzyme) is observed in lactate dehydrogenase catalysis. Whatever be the individual pKa of such hydrogen-bonding partners, the two pKas should transiently match (and the H is equally shared between the partners!) during the course of each catalytic event for LBHB to form. The transient formation of this LBHB may provide a convenient intermediate step for the large pKa change (from +15 for >CHOH of lactate to −5 for >C=O of pyruvate) during the catalytic trajectory of lactate dehydrogenase reaction.

6.2 Contribution by Electrostatics

Enzyme active sites are clefts, crevices, or pockets formed by the overall three-dimensional structure of the protein. Substrate binding to active site accompanies (a) de-solvation of substrate by replacing the water positions by the pre-organized polar frame work and (b) exclusion of water from the pocket unless it is a reactant. Active sites generally exclude bulk water and thus create unique local dielectric environment. This has profound consequences to the functional group reactivity. The pKa of a carboxylate may be elevated by low local dielectric constant (Glu35 of lysozyme has a pKa of 6.3!). Similarly, the pKa of an amino group may vary by several units from its normal value because of the proximity of charged groups in the neighborhood (Lys-NH_2 of acetoacetate decarboxylase displays an unusually low pKa of 5.9). Triosephosphate isomerase exploits one of its α-helix dipole to modulate the pKa of active site His95. Although not exhaustive, the following examples further illustrate the contribution of electrostatics in enzyme catalysis (Warshel et al. 2006).

Decarboxylation of Hydroxyethyl Thiamine Pyrophosphate Adduct The decarboxylation of pyruvate is facilitated by the formation of initial hydroxyethyl thiamine pyrophosphate (HETPP) adduct, between thiamine pyrophosphate and pyruvate. This HETPP adduct is charged, but the charge is diffused due to delocalization of electrons (Fig. 6.3a). Since the active site of pyruvate decarboxylase is

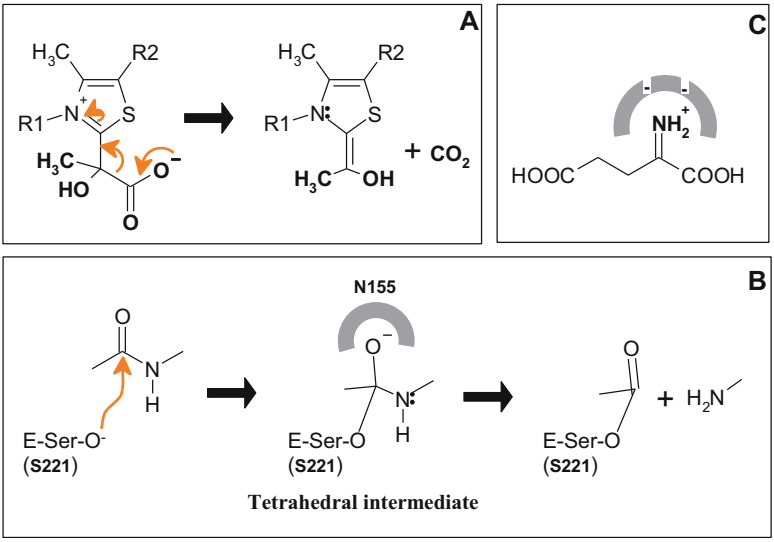

Fig. 6.3 Electrostatic effects at the enzyme active sites. (**A**) Hydrophobic active site of pyruvate decarboxylase facilitates expulsion of CO_2 from the HETPP adduct. (**B**) The oxyanion hole stabilizes the tetrahedral intermediate in subtilisin catalysis. (**C**) Negatively charged groups of glutamate dehydrogenase electrostatically stabilize the bound 2-iminoglutarate

nonpolar (hydrophobic), this adduct tends to lose charges by expelling CO_2. This electrostatic feature is supported by studies with an analog of HETPP adduct. The HETPP analog (Fig. 6.3a, where R1 = CH_3 and R2 = H) was prepared; its decarboxylation rate in ethanol is 10^5-fold faster than in water. This rate is even faster in other polar aprotic solvents. The model decarboxylation reaction points to a striking catalytic effect of de-solvation and subsequent charge destabilization at the enzyme active site.

Oxyanion Hole of Subtilisin During each catalytic cycle, subtilisin (and all other serine proteases like chymotrypsin, trypsin, etc.) goes through an attack of enzyme-Ser-OH on to the carbonyl carbon (sp^2 hybridized) of the scissile peptide bond. This leads to an initial "oxyanion" formation where the carbon is sp^3 hybridized (Fig. 6.3b). Stabilization of the "oxyanion" in a special pocket (oxyanion hole) is a strategy for rate acceleration. Site-directed mutagenesis was used to evaluate the contribution of this oxyanion stabilization at the active site. Mutant enzyme forms showed that even after a triple replacement (where the catalytically essential residues – D32, H64, and S211 – were replaced by Ala), the mutant subtilisin retained the ability to hydrolyze peptide bonds (at 10^3–10^4-fold above the uncatalyzed rates). This significant residual rate is attributed to the stabilization of oxyanion intermediate. An Asn residue (N155) contributes significantly to this oxyanion hole of subtilisin active site.

Selective Enrichment of 2-Iminoglutarate at the Glutamate Dehydrogenase Active Site There is strong evidence that the reductive amination of 2-oxoglutarate catalyzed by glutamate dehydrogenase proceeds through an enzyme-bound 2-iminoglutarate intermediate. However, the equilibrium (2-oxoglutarate + NH_3 ⇄ 2-iminoglutarate + H_2O) in solution is largely in favor of 2-oxoglutarate. It follows that the enzyme electrostatically stabilizes the bound 2-iminoglutarate complex by utilizing negatively charged groups and hydrogen-bonding basic groups at its active site (Fig. 6.3c). This results in substantially increased levels of 2-iminoglutarate ready for reduction. The same negatively charged enzyme groups while stabilizing the iminium ion (>C=NH_2^+) decrease the population of bound 2-oxoglutarate (>C=$O^{\delta-}$) due to charge repulsion. Better interaction with 2-methyleneglutarate (>C=CH_2, which is uncharged) at this pocket may account for its efficacy as a good inhibitor (Choudhury and Punekar 2007). Electrostatics permits glutamate dehydrogenase to discriminate between iminium and carbonyl groups and thus forms the chemical basis of ammonia recognition by the enzyme.

Guidance of Charged Substrates Charge distribution about the active site to stabilize transition states and/or intermediates is termed electrostatic catalysis. Besides this, in several enzymes the overall charge distribution of the protein matrix serves to guide polar substrates to their active sites. Superoxide dismutase (SOD) and acetyl cholinesterase are two well-studied examples of this "torch of charge guidance" effect (Getzoff et al. 1992; Silman and Sussman 2008).

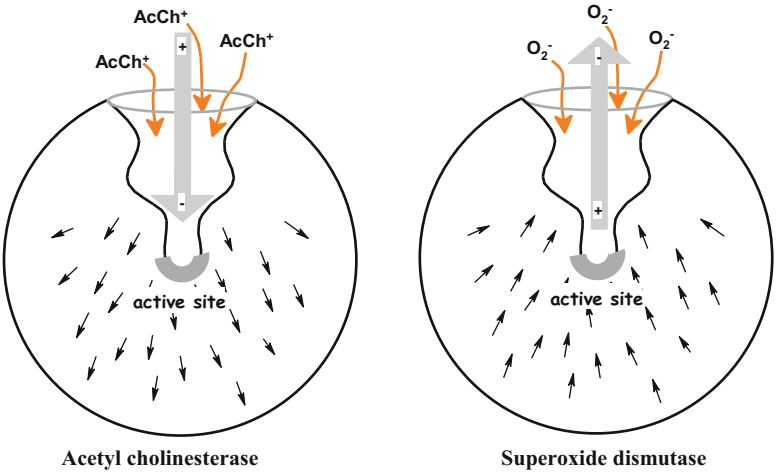

Fig. 6.4 Electrostatic torch of guidance effect in superoxide dismutase and acetyl cholinesterase. The gray block arrows indicate the overall enzyme electrostatic field vectors with respect to the active site present at the bottom of the channel/gorge. Small arrows represent various contributing dipoles to the overall field vector

The electrostatic contributions to SOD catalysis were analyzed through calculations of electrostatic potential and the electrostatic field vectors in the active site channel. Arrangement of global electrostatic charges in SOD promotes productive enzyme-substrate interaction through substrate guidance and charge complementarities. The extensive electrostatic field directs the negatively charged superoxide (O_2^-) substrate to the bottom of the active site channel (Fig. 6.4). Charge guidance is also suggested from studies of the enzyme's dipole and overall electrostatic potential. The maximal rate of SOD reaction is 2×10^9 $M^{-1}s^{-1}$, very close to diffusion-controlled limit. This is remarkable since the active site channel forms only about 10% of the enzyme surface! Electrostatic forces possibly guide the charged substrate and enhance oriented diffusion. The electrostatic field vectors indicate that attraction of O_2^- is a long-range process, occurring even beyond the active site channel!

Similarly, acetyl cholinesterase also has a strong electrostatic dipole (but of opposite orientation to that of SOD!). This dipole is aligned with the deep gorge leading to its active site, so that the positively charged acetylcholine is drawn to the active site by electrostatic field. By comparison, a structurally related lipase (from *Geotrichum candidum*) has a poor and markedly different dipole orientation. Charge guidance and electrostatics may not operate in the case of lipase because the lipase substrate is neutral.

Electrostatic forces that are felt at the enzyme active site are the effects of the extended environment of that protein – including dipoles from the second shell and much beyond. Mutations at locations remote to active site, yet significantly affect enzyme activity, implicate a role for electrostatics in catalysis.

6.3 Metal Ions in Catalysis

A third of all the known enzymes require metal ions for their function. Such metal ions may function as determinants of protein structure, but more importantly they could directly participate in the catalytic process. Metal ions may bind to substrate and as a consequence enhance its interaction with the enzyme – by stabilizing and presenting one specific substrate conformation for catalysis. Aspects of metal chemistry and various roles played by metal ions in redox biochemistry are found in Part IV (Chaps. 32 and 33). In this section, however, the emphasis will be on their role in rate accelerations. Metal ions may be viewed as "super acids" because they can (a) have charge density greater than +1, (b) be present at concentrations higher than those achieved by protons (H^+ ions) around neutral pH, and (c) coordinate with several groups to act as templates. They are recruited in enzyme active sites as tools for electrostatic catalysis (see above). In terms of enzyme catalysis, metal ions contribute to reaction rate accelerations in the following ways.

Charge Shielding A number of anionic (negatively charged) compounds act as substrates only when present in their divalent metal ion complexes. Enzymes acting on citrate, ATP, etc. are some examples of this kind. Requirement of Mg^{2+} for most ATP reactions (nucleotide triphosphate reactions in general!) led to the recognition that Mg-ATP complex is the true substrate for such enzymes (also see Chap. 32). Apart from orienting the highly flexible oligomeric phosphates of ATP, the divalent metal ion partly neutralizes (and shields!) the negative charges on the polyphosphate chain. Charge shielding becomes particularly important when an anionic nucleophile has to attack the substrate during catalysis. Charge repulsion is expected to reduce the efficacy of negatively charged attacking groups, while neutral nucleophiles are not always feasible (especially due to pH constraints). For example, a kinase active site nucleophile (anionic) can easily approach the negatively charged polyphosphate of ATP (or any other NTP) – when it is complexed with Mg^{2+} ions (Fig. 6.5).

Charge Stabilization An important role for metal ions in catalysis is to serve as an electrophilic catalyst (Lewis acid), by stabilizing a negative charge on the reaction intermediate. This is better achieved by multivalent metal cations than mere protons.

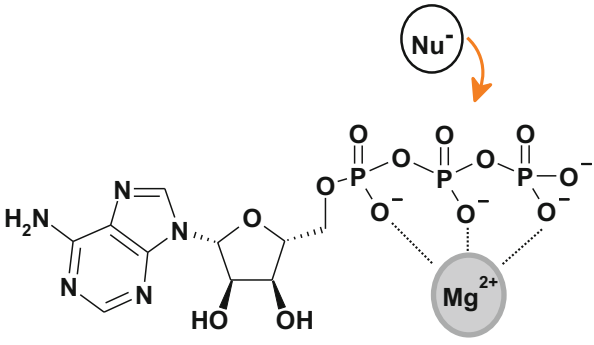

Fig. 6.5 The structure of ATP with Mg^{2+} shielding the polyphosphate negative charges. An anionic nucleophile can favorably approach such a polyphosphate

Three different enzyme chemistries exemplify this aspect of metal catalysis: (a) decarboxylation of oxaloacetate is catalyzed by divalent metal ions. For instance, Mn^{2+} chelated by oxaloacetate electrostatically stabilizes the developing enolate ion during reaction (Fig. 6.6a). Indeed most oxaloacetate-decarboxylating enzymes do require a divalent metal ion for activity. Decarboxylation of oxalosuccinate by isocitrate dehydrogenase similarly requires Mn^{2+}. (b) Increased electron delocalization to stabilize the enolate intermediate also occurs during aldol cleavage. Class II aldolases (from fungi) usually contain Zn^{2+} to polarize the carbonyl oxygen of the substrate (Fig. 6.6b). (c) Zn^{2+} ion of carboxypeptidase A is coordinated to the carbonyl oxygen of scissile peptide bond (Fig. 6.6c). This facilitates the polarization

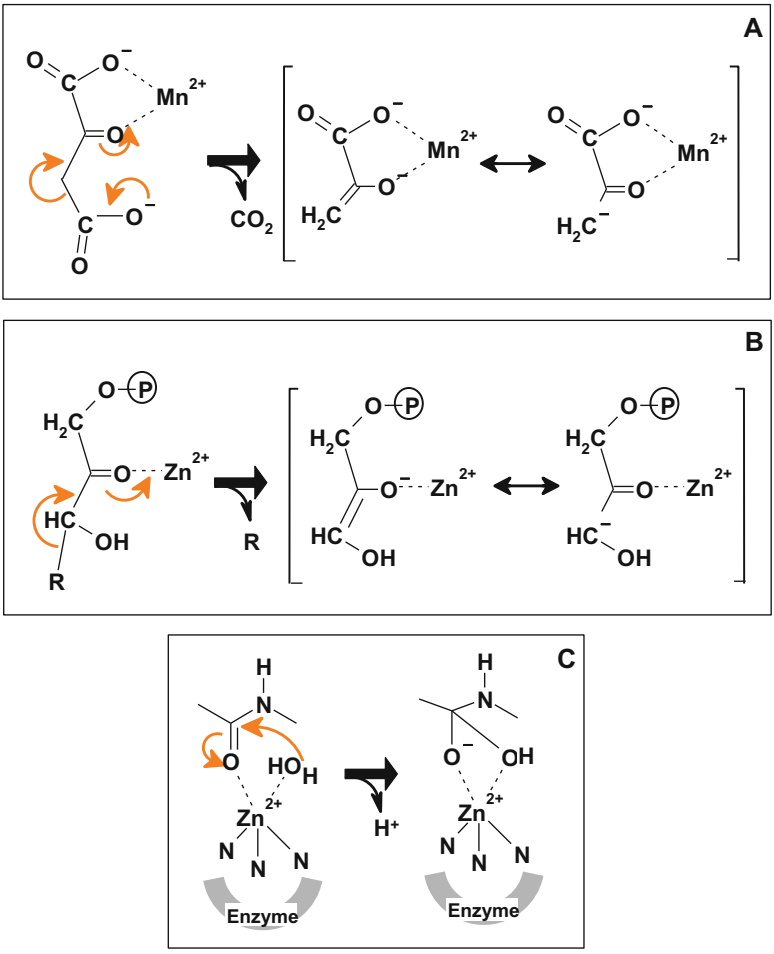

Fig. 6.6 Metal ions involved in charge stabilization during catalysis. (**A**) Oxaloacetate decarboxylase – Mn^{2+}; (**B**) class II aldolase – Zn^{2+}; (**C**) carboxypeptidase A – Zn^{2+}

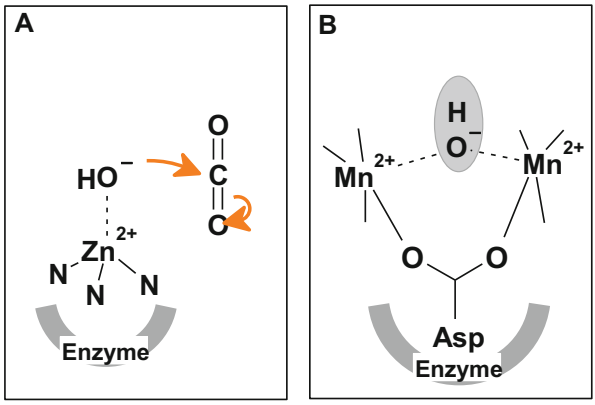

Fig. 6.7 Metal ions enhance the nucleophilicity of water during catalysis. (**A**) Carbonic anhydrase, Zn^{2+}; (**B**) arginase, Mn^{2+} bimetallic cluster

of carbonyl group, and stabilizes negative charge on the oxygen, during reaction. Zinc ion in carboxypeptidase A also enhances the nucleophilicity of coordinately bound water for attack (see below).

Enhance Nucleophilicity of Water Metal ions may generate a nucleophile by increasing the acidity of a nearby molecule. When this molecule happens to be water, a reactive hydroxide is generated. Water molecule bound to a transition metal, in its inner coordination sphere, is a better source of OH^- than bulk water. Metallo-hydrolases exploit this feature for catalysis and impart better nucleophilic nature to water. Carbonic anhydrase provides a good example of this effect. When bound to the positively charged Zn^{2+} ion, the pKa of water molecule is reduced from 15.7 to 7.0. Thus a substantial concentration of hydroxide ion (metal bound!) is generated at near-neutral pH (Fig. 6.7a). Carbonic anhydrase utilizes the reactivity intrinsic to a zinc-bound hydroxide ion in its catalysis.

Arginase employs a bimetallic (Mn^{2+}) cluster to hydrolyze arginine to form ornithine and urea. It appears that two metal ions are better than one in generating the hydroxide. A water molecule is sandwiched between two Mn^{2+} ions (Fig. 6.7b) and is made sufficiently nucleophilic to attack the guanidinium group. A variation on this theme is the *H. pylori* enzyme with a bimetallic Co^{2+} cluster. In accordance with differences in the chemical reactivity of Mn^{2+} and Co^{2+}, this cobalt arginase has a different pH optimum. Two other examples of bimetallic centers are jack-bean urease with two Ni^{2+} ions and the *E. coli* alkaline phosphatase with two Zn^{2+} ions.

6.4 General Acid–Base Catalysis

Majority of the enzyme-catalyzed reactions involve one or more proton transfers and hence general acid–base chemistry permeates most of enzymology. Almost all these proton transfers are catalyzed. Regardless of what other tools an enzyme catalyst

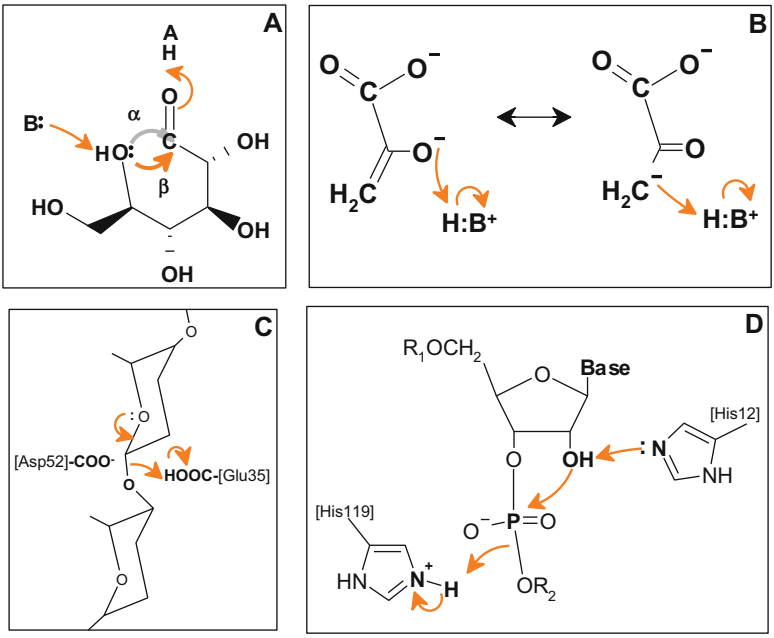

Fig. 6.8 General acid–base catalysis in rate accelerations. (**A**) Mutarotation of glucose is both acid and base catalyzed; (**B**) keto-enol tautomerization, as shown for pyruvate here, could involve a single acid–base group in the 1,3-prototropic shift; (**C**) schematic of the lysozyme chemistry involving the two active site carboxylates; (**D**) RNase A catalysis is initiated by His12 abstracting the proton from 2′ OH. His119 acts as a general acid to donate a proton to the leaving group. The cyclic intermediate generated is subsequently attacked by water where the roles of two His residues are reversed

exploits, general acid–base catalysis is a common ploy. Rate accelerations effected by this mode of catalysis may involve abstraction, donation, or movement of H^+ ions. These proton transfers – *the prototropic shifts* – may involve (i) a single general base or (ii) multiple ionizable groups that relay the proton from one atom to another. Among numerous examples of general acid–base catalysis include glucose isomerization, enolization of pyruvate, lactam–lactim interconversion of purine–pyrimidine bases, pyridoxal phosphate chemistry with amino acids. Etc. While almost every enzyme recruits acid–base catalysis, the concept is best illustrated through a few case studies.

Mutarotation in glucose is facilitated by an acid or a base. The interconversion of α-D-glucose and β-D-glucose (via the linear form) is subject to acid–base catalysis as shown in Fig. 6.8a. The ring closure to form the hemiacetal may occur through the attack of oxygen on either face of the C1 carbonyl. Similarly a *keto-enol tautomerization* is facilitated by general base, general acid, or both. For instance, the developing negative charge on carbon (the carbanion-like transition state) can be stabilized by the protonation of the oxygen (Fig. 6.8b). In both these examples proton donation (acid catalysis) or proton abstraction (base catalysis) may occur

independently. However, the two events could occur in concert. It is also possible that the same acid–base group is involved in the prototropic shift – a net proton transfer from one atom to the other. *1,3-prototropic shifts* (where a proton is moved from the first atom to the third) are quite common during enzyme catalysis. Enolization of pyruvate (as represented in Fig. 6.8b) is one such case where proton shifts between C3 of pyruvate and the carbonyl oxygen.

That biological catalysis has primarily/predominantly originated in an aqueous environment is consistent with this. 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. Therefore, the pH dependence of enzyme activity is a reflection of its ionizable groups involved in binding and/or catalysis (see Chap. 24 for a detailed treatment). Ionizable amino acid side chains of the enzyme protein are typically involved in such catalysis. These could be through concerted action of acid–base groups on the enzyme. Most hydrolytic enzymes rely on general acid–base catalysis as a tool. Acid proteases (such as pepsin) and glycosidases (like lysozyme, cellobiohydrolase, and amylase) are some well-documented examples. In accord with two carboxylate groups as catalytic residues, these enzymes exhibit acidic pH optima. The Glu35 (-COOH) and Asp52 ($-COO^-$) form the catalytic residues of lysozyme active site (Fig. 6.8c). Glu35 (acting as general acid) facilitates bond cleavage by protonating the glycosidic oxygen. Ribonuclease A (RNase A) active site however displays two His residues – His12 as general base and His119 as the general acid (Fig. 6.8d). Their roles are reversed during 2′,3′-cyclic intermediate hydrolysis, and the enzyme regains its original ionization state. RNA is alkali labile – hydrolyzed by general base catalysis. However, the rate accelerations achieved by the combined action of two His residues (at the RNase A active site) are many orders of magnitude higher.

The nature and chemical reactivity of important acid–base groups in enzyme catalysis are extensively covered in a later section (see Chap. 30).

6.5 Covalent Catalysis

In many reactions, at some stage during catalysis, an enzyme-substrate covalent intermediate may occur. This catalytic trick may even follow a different, more facile reaction path than the uncatalyzed one. An enzyme could break down a complex reaction into two or more simple ones – each step with its own activation energy barrier. However these new barriers are lower than that of the uncatalyzed reaction. This is the crux of covalent enzyme catalysis.

A covalent bond could be established between the enzyme and the substrate in one of the two ways – [a] an enzyme nucleophile may attack an electron-deficient center on the substrate or [b] an enzyme-bound electrophile could be attacked by the electron-rich center of the substrate. Side chains of many amino acid residues are known to participate in nucleophilic catalysis by forming covalent enzyme-substrate

Table 6.1 Catalysis involving covalent enzyme-substrate intermediates

Amino acid [side chain]	Intermediate	Examples
Ser [–CH_2OH]	Acyl-enzyme	Chymotrypsin, lipase, acetylcholinesterase
	Phospho-enzyme	Phosphoglucomutase
Thr [–$CH(CH_3)OH$]	Phospho-enzyme	Phosphotransferases
Tyr [–PhOH]	Phospho-enzyme	DNA integrase, topoisomerase
Cys [–SH]	Acyl-enzyme	Glyceraldehyde 3-phosphate dehydrogenase, acyltransferases, papain
Asp/Glu [–COOH]	Enzyme-ester	Epoxide hydrolase, haloalkane dehalogenase
	Glycosyl-enzyme	Lysozyme
His [-imidazole-NH]	Phospho-enzyme	Glucose 6-phosphatase, succinyl-CoA synthetase
Lys [–NH_2]	Schiff's base	Acetoacetate decarboxylase, aldolase (type I), transaldolase

intermediates (Table 6.1). It has been possible to trap and directly demonstrate the existence of few of these intermediates. The role of nucleophilic catalysis by Ser195 (-OH) of chymotrypsin is very well-established (Carter and Wells 1988). Schiff base formation in fructose 1,6-bisphosphate aldolase is another classic example. More recently, the "Phillips mechanism" for lysozyme was revised based on the electrospray ionization mass spectrometry (ESI-MS) evidence in combination with a E35Q mutant of lysozyme (Kirby 2001). Active site residue Asp52 acts as a nucleophile and forms a covalent bond to the C1 carbon of the substrate glycoside.

Enzymes (being proteins) have a choice of many nucleophilic groups (side chains of amino acid residues) but have little to offer in terms of good electrophiles. This is one of the reasons why a number of small molecules (cofactors and prosthetic groups) are recruited by nature to complement an apoenzyme – resulting in a functional holoenzyme. These molecules act as temporary electron sinks during catalysis. Electrophilic recruitment may involve (a) cofactor molecules like pyridoxal phosphate, thiamine pyrophosphate, etc. or (b) simple apparatus like Schiff base, protein-bound pyruvate, or dehydroalanine. The nature and chemical reactivity of important nucleophiles and electrophiles are discussed in detail, in a later section (Chaps. 31 and 35).

6.6 Transition State Binding and Stabilization

The substrate is held in a unique active site environment by enzyme groups through proximity orientation and electrostatics. It may be argued on similar grounds that the active site discriminates between the substrate and the transition state. Catalysis is

Table 6.2 Differential binding of enzyme to substrate and transition state

Equilibrium	Dissociation constant	ΔG°	Rate constant
$E + S \rightleftharpoons E.S$	K_S	$\Delta G^\circ{}_S$	k_{uncat}
$E + TS \rightleftharpoons E.TS$	K_{TS}	$\Delta G^\circ{}_{TS}$	k_{cat}

thus a consequence of the preferential binding (and therefore stabilization) of the transition state. Linus Pauling lucidly stated it first: "I think that enzymes are molecules that are complementary in structure to the activated complexes of the reactions that they catalyze,. . ." in his 1948 discourse (Pauling 1948).

Enzyme may be viewed as a device that preferentially binds/stabilizes the transition state (*TS*) rather than the ground state of the reactant (substrate). Although the *TS* is of highest free energy and is extremely unstable, we can visualize the consequences of its preferential binding to the enzyme. The two binding equilibria and relevant kinetic and thermodynamic parameters are shown in Table 6.2.

If an enzyme binds (and/or stabilizes) it's *TS* better than S, then one expects that $\Delta G^\circ{}_{TS}$ is more negative than $\Delta G^\circ{}_S$. The two ΔG° values are related to their corresponding $\Delta G^{\neq}$ values, and the ratio of the rates of catalyzed versus the uncatalyzed reaction (k_{cat}/k_{uncat}) is related to K_S/K_{TS}. More tightly an enzyme binds its reaction *TS* relative to the substrate (i.e., the smaller the K_{TS} compared to K_S) the greater is the rate acceleration. The magnitude of $\Delta G^\circ{}_{TS} - \Delta G^\circ{}_S$ therefore significantly contributes to the overall decrease in activation energy ($-\Delta\Delta G^{\neq}$) during catalysis. Recalling (from Chap. 5) that $\frac{k_{cat}}{k_{uncat}} = 10^{\frac{\Delta\Delta G^{\neq}}{2.303RT}}$, a rate enhancement factor of $\sim 10^6$ may be estimated for an enzyme that binds its *TS* complex with 8.0 kcal.mol^{-1} greater affinity at 25°C, than its substrate. This is worth two hydrogen bonds that can form only in "*E.TS*" but not in the "*ES*" complex! LBHBs may be one such tool for the *TS* to make better contacts with the enzyme. There are at least two consequences of the tighter binding of an enzyme to its *TS*. Devices that are designed to appreciably bind *TS* should catalyze the corresponding reaction! The concept of antibodies against *TS* mimics – called *abzymes* or *catalytic antibodies* – arose from the seminal idea of Linus Pauling. Secondly, *TS analogs* (stable molecules that resemble the *TS*) should be potent competitive inhibitors of the enzyme. Such *TS* analogs, as a corollary, provide insights into catalytic mechanism (Mader and Bartlett 1997; Radzicka and Wolfenden 1995).

It is not necessary for a good substrate to have high affinity for the enzyme as long as the corresponding *TS* form does so. The concept "underestimation of binding energy (in substrate binding) is utilized for catalytic rate acceleration" has also been variously recognized in the enzyme literature. These include (a) destabilization of the ground state by the enzyme, (b) rack mechanism leading to strain and distortion in the substrate molecule, (c) induced fit versus nonproductive binding, and (d) sequestration of the *TS* (intermediate) at the active site. The following select examples illustrate the concept of transition state binding and stabilization by the enzyme.

Strain and Distortion Ferrochelatase catalyzes the formation of heme by inserting Fe^{2+} into protoporphyrin IX. The iron entry requires that the planar porphyrin be bent. Indeed the enzyme binds the porphyrin substrate in a distorted form to facilitate iron entry. Lysozyme is another example of this kind. The substrate glycosidic sugar (the N-acetyl muramic acid, ring D) is bound by the enzyme in a distorted/strained half-chair conformation (Fig. 6.9a). It is worth noting that the sugar residues at the other five subsites of lysozyme are in the normal, chair conformation. Introducing strain in the substrate glycosidic residue appears to be a feature of many glycohydrolases.

Preferential TS Binding According to Richard Wolfenden, enzymatic reaction transition state presents a "moving target." The *TS* develops and disappears very rapidly along the reaction coordinate. One could however build stable structures that resemble the *TS* – enzyme may bind them in preference to either the substrate or the product of that reaction (Radzicka and Wolfenden 1995). Seeking *TS* mimics as powerful enzyme inhibitors is an active enterprise in drug discovery. Some of them like the α-glucosidase inhibitor acarbose (Fig. 6.9b) have matured into useful drugs. Pepstatin inhibits the aspartyl proteases (such as pepsin and rennin, in nM range) because of the unusual amino acid statine in its structure. It is thought that the statine structure mimics the tetrahedral intermediate (*TS*) formed during catalysis (Fig. 6.9c). Pyrrole-2-carboxylate resembles planar *TS* of proline racemase and is a competitive inhibitor (Fig. 6.9d); it binds the enzyme with 160-fold better affinity than proline itself. Similarly, 2′-carboxy-D-arabinitol 1,5-bisphosphate is a powerful *TS* inhibitor of ribulose bisphosphate carboxylase (RubisCO) as it is the analog of carboxyketone intermediate (Fig. 6.9e). As a last example, we consider adenosine deaminase that catalyzes the irreversible deamination of adenosine to inosine. The enzyme is strongly inhibited by analog of an unstable tetrahedral intermediate – formed with a change of hybridization from sp^2 to sp^3 at C-6 of adenosine. Nebularine 1,6-hydrate (Fig. 6.9f) binds the enzyme with a K_I of 3×10^{-13} M, whereas the K_M for adenosine is 3×10^{-5} M. In comparison, 1,6-dihydronebularin is a poor inhibitor with a K_I of 5.4×10^{-6} M. The lone –OH on the tetrahedral C-6 of the purine contributes approximately 10 kcal/mol (at 25°C) in enzyme binding!

Protection of TS (Intermediate) Intermediates in some chemical reaction paths are either reactive or unstable. Enzymes protect/stabilize such species by embedding them in their active sites. One such reactive intermediate occurs during the interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. The *cis*-enediol formed in the triose phosphate isomerase reaction has a tendency to eliminate phosphate and form methyl glyoxal (Fig. 6.10). A short loop of polypeptide closes the active site and protects the reactive intermediate from bulk solvent – closing of this lid prevents methyl glyoxal formation. A mutant enzyme without this loop is a poor catalyst and indeed produces significant quantities of methyl glyoxal.

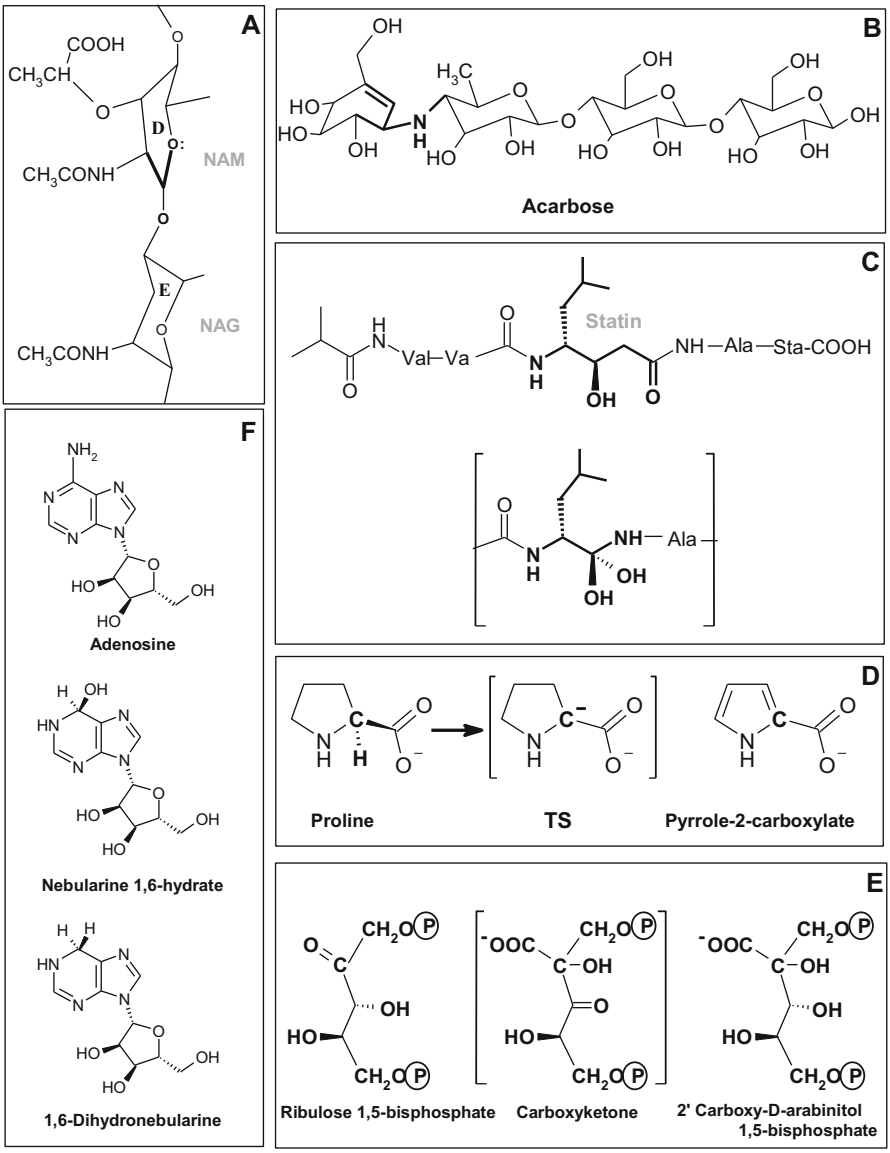

Fig. 6.9 Transition state binding and stabilization. (**A**) Half-chair conformation of the NAM sugar bound to lysozyme active site; (**B**) acarbose, α-glucosidase inhibitor; (**C**) structure of statine found in pepstatin and the corresponding enzyme-bound tetrahedral intermediate; (**D**) the planar TS of proline racemase and pyrrole 2-carboxylate; (**E**) the carboxyketone intermediate of RubisCO and its inhibitor analog 2′-carboxy-D-arabinitol 1,5-bisphosphate; (**F**) nebularine 1,6-hydrate is a potent inhibitor of adenosine deaminase because of the –OH group on the tetrahedral C-6 of purine

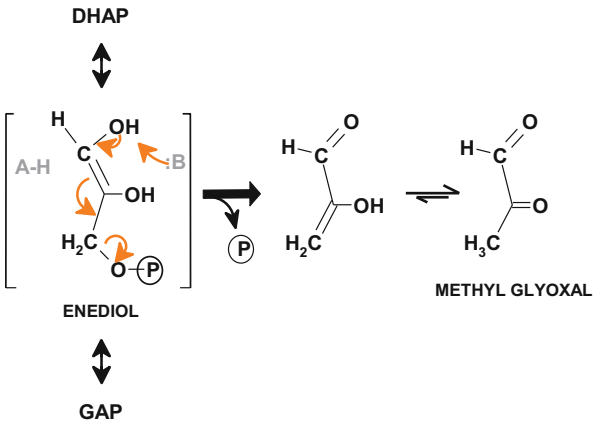

Fig. 6.10 Triosephosphate isomerase protects the *cis*-enediol intermediate. In the absence of this feature, methyl glyoxal is the undesired product

Enzymes bring about rate accelerations by recruiting a number of tricks highlighted in this chapter. They include features that bring down the activation energy of the reaction by a combination of enthalpic and entropic factors; these factors contribute in different measure for each enzyme. Two common examples are used to illustrate this concept in the next chapter.

References

Carter P, Wells JA (1988) Dissecting the catalytic triad of a serine protease. Nature 332:564–568

Cleland WW (1964) Dithiothreitol, a new protective reagent for SH groups. Biochemistry 3:480–482

Cleland WW, Frey P, Gerlt JA (1998) The low barrier hydrogen bond in enzymatic catalysis. J Biol Chem 273:25529–25532

Choudhury R, Punekar NS (2007) Competitive inhibition of glutamate dehydrogenase reaction. FEBS Lett 581:2733–2736

Fersht AR (1987) The hydrogen bond in molecular recognition. Trends Biochem Sci 12:301–304

Getzoff ED, Cabelli DE, Fisher CL, Parge HE, Viezzoli MS, Banci L, Hallewell RA (1992) Faster superoxide dismutase mutants designed by enhancing electrostatic guidance. Nature 358:347–351

Jencks WP (1975) Binding energy, specificity, and enzymic catalysis: the Circe effect. Adv Enzymol Relat Areas Mol Biol 43:219–410

Kirby AJ (2001) The lysozyme mechanism sorted – after 50 years. Nat Struct Biol 8:737–739

Mader MM, Bartlett PA (1997) Binding energy and catalysis: the implications for transition-state analogs and catalytic antibodies. Chem Rev 97:1281–1301

Page MI, Jencks WP (1971) Entropic contributions to rate accelerations in enzymic and intramolecular reactions and the chelate effect. Proc Nat Acad Sci USA 68:1678–1683

Pauling L (1948) Nature of forces between large molecules of biological interest. Nature 161:707–709

Radzicka A, Wolfenden R (1995) Transition state and multisubstrate analog inhibitors. Meth Enzymol 249:284–312

Silman I, Sussman JL (2008) Acetylcholinesterase: how is structure related to function? Chem Biol Interact 175:3–10

Warshel A, Sharma PK, Kato M, Xiang Y, Liu H, Olsson MHM (2006) Electrostatic basis for enzyme catalysis. Chem Rev 106:3210–3235

Suggested Reading

Gutteridge A, Thornton JM (2005) Understanding nature's catalytic toolkit. Trends Biochem Sci 30:622–629
Hansen DE, Raines RT (1990) Binding energy and enzymatic catalysis. J Chem Edu 67:483–489
Kirby AJ, Hollfelder F (2008) Biochemistry: enzymes under the nanoscope. Nature 456:45–47
Knowles JR (1991) Enzyme catalysis: not different, just better. Nature 350:121–124
Kraut J (1988) How do enzymes work? Science 242:533–540
Scott LT (2014) Enzyme catalysis, Basic principles in one easy lesson. Nat Chem 6:177–178