Regulation of Enzyme Activity

37

Most organisms, however evolutionarily distant, contain a set of common metabolites. But their intracellular concentrations are unique to each individual species. This metabolic identity of an organism is the consequence of quantitative differences in relevant enzyme properties and their associated regulation. Features of metabolic regulation are unique to each organism – often within a closely related group of organisms. It is becoming increasingly clear that the concept of unity in biochemistry does not always extend to the metabolic pathway control and enzyme regulation. Historically, metabolic regulation and control of enzyme activity have developed as closely related phenomena. What then is the justification to place regulation of enzyme activity here in "Frontiers in Enzymology"? Over the years, molecular developments in biology have outshone the progress made in physiological and system-level understanding of organisms. While the basic principles of enzyme/metabolic regulation may have been uncovered, novel modes of regulation continue to be discovered. Nature continues to surprise us with original ways of regulating enzyme activity. The novelty may be in the conceptual mechanism or the regulatory ligands involved. For instance, fructose-2,6-bisphosphate as a regulator of glycolysis (at the phosphofructokinase step) was discovered much later (in the early 1980s) – many decades after the complete description of glycolytic enzymes (Hers and Hue 1983). In this sense the topic of regulation of enzyme activity will always be at the frontiers of enzymology.

The multitude of biochemical changes in metabolism are brought about by the battery of different enzymes. *Regulation* (also termed *homeostasis*) is the ability to maintain metabolic constancy in the face of external perturbations. *Control* on the other hand is the ability to make changes to metabolism as and when necessary. Both these phenomena manifest through manipulation of enzyme activities. Without its regulation and control, the cell is essentially a bag of enzymes. However, *the cell is not just a bag of enzymes*. A remarkable degree of order is maintained inside because of the stringent and very efficient regulation of enzyme activities. Regulation of enzyme activity is desirable and is accomplished because (a) the catalytic rates

N. S. Punekar, *ENZYMES: Catalysis, Kinetics and Mechanisms*,
https://doi.org/10.1007/978-981-13-0785-0_37

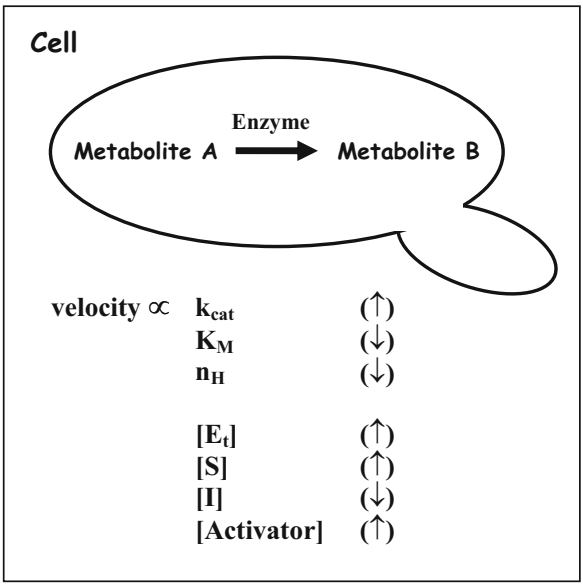

Fig. 37.1 Various factors that influence the overall rate of an enzyme-catalyzed reaction in cellular metabolism. Changes in the intensive or extensive properties listed may increase (↑) or decrease (↓) effective enzyme activity of that step

achieved, at times, may be too fast or too slow for the well-being of the cell and (b) more than one enzyme may share a metabolite as its substrate, thereby necessitating a logic for metabolic pathway flux distribution.

At any given time, the reaction rate taking metabolite A to metabolite B depends on the in vivo activity of the corresponding enzyme. Effective intracellular enzyme activity is a function of a number of intensive (K_M, k_{cat} and n_H) and extensive ([E_t], [S], [P], [I] and [*activator*]) properties (Fig. 37.1). The resultant rate is a consolidated response and an outcome of all these factors. Therefore, in principle, regulation of enzyme activity is possible by changing one or more of these properties.

Enzyme regulation can be achieved either by increasing/decreasing the number of enzyme molecules (through induction, repression, and turnover) or by modulating the activity of preexisting enzyme molecules (inhibition or activation). The former mechanisms respond relatively slowly to the changing external stimuli and provide for *long-term* control, while the latter respond rapidly to changing conditions and are *short-term* control mechanisms (Fig. 37.2). We will study representative examples for all these modes of enzyme regulation in this chapter. Looking at each and every variant of regulation would be arduous and would quickly grow into a textbook on metabolism. Instead of dwelling on the vast mechanistic permutations, case studies of well-established systems are highlighted. Furthermore, the emphasis will be on the examples of historical importance.

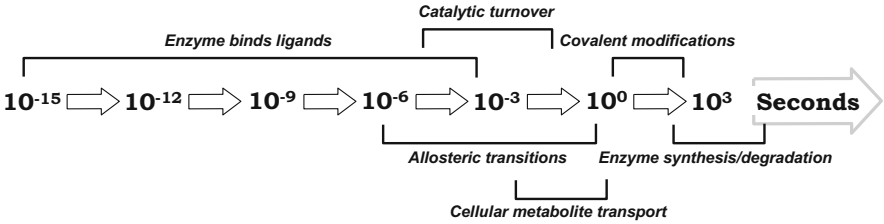

Fig. 37.2 Events relevant to regulation of enzyme activity. The timescales as indicated are approximate ranges

37.1 Control of Enzyme Concentration

We recall from Chap. 15 that maximal velocity (V_{max}) for an enzyme equals $k_{cat} \times [E_t]$. Of these, k_{cat} is an intensive property and is an intrinsic constant for a given enzyme. Enzyme concentration $[E_t]$ is an extensive property whose magnitude can be adjusted either by increasing or decreasing the number of enzyme molecules in the cell. This mode of regulation largely comes under genetic regulation but will be briefly mentioned here for the sake of completeness.

Induction and Repression Enzymes involved in catabolic routes are "induced" in the presence of compounds that are destined to be degraded through these routes. The *lac* operon of *E. coli* and its induction by lactose is an excellent example. The enzymes of the biosynthetic pathway are "repressed" by the end product of the pathway. In many bacteria histidine is known to repress the expression of enzymes from *his* operon. The genetic control of pathways is brought about by inducer or repressor ("effector" in general) molecules which may be small molecular weight metabolites. There is no uniformity with regard to the effector identity for a given pathway in different organisms. This is where *unity in biochemistry* concept faces its biggest challenge. In the biosynthetic pathways, repression by the end product and in the catabolic routes induction by the initial substrate affect all the enzymes of a given pathway. It is generally found that majority of the metabolic routes are nonlinear, i.e., there is considerable branching (for biosynthetic routes) and convergence (for catabolic routes). Compared to linear metabolic sequences, branching and convergence produces an added dimension of complexity in regulation both at the biochemical as well as the genetic level. The control of enzyme synthesis occurs by sequential induction and by multivalent repression, which are, in effect, variations of the same theme found in linear pathways.

Another prevalent genetic control mechanism, governing the number of enzyme molecules, is *catabolite repression*. The enzymes of catabolic routes may be repressed when glucose is abundant through *carbon catabolite repression*. This repression could be mediated through cAMP or some other effector. The *nitrogen metabolite control* is an adaptive mechanism which imparts hierarchy to nitrogen

sources – the ones most economically used generally being consumed first. *Nitrogen metabolite repression* is observed when ammonia is present as a nitrogen source. Ammonia acts by either inhibiting the uptake of other complex nitrogen sources by a phenomenon called "inducer exclusion" or serves as a direct or indirect (such as via L-glutamine) repressor of the genes involved in the catabolism of complex nitrogen sources. Both carbon catabolite repression and nitrogen metabolite repression are broad modes of control acting globally across pathways.

Posttranscriptional Regulation of mRNA The half-lives of the mRNA transcripts of key enzymes involved in metabolism are often determined by the cellular demands. The mRNA stability is known to increase during induction of a few structural genes in metabolic pathways. Stable, stored mRNAs of some microbial enzymes are translated in response to environmental cues. The mammalian ornithine decarboxylase antizyme is a protein regulator of the ornithine decarboxylase enzyme activity (see Sect. 37.6 below). This antizyme mRNA is significantly stable ($t_{1/2} = 12$ h). The synthesis of antizyme protein from its preformed mRNA is triggered by an increase in cellular polyamine levels – an example of translational control.

Regulation by Protein Degradation Cells continuously synthesize proteins from, and degrade them to, their component amino acids. This permits regulation of cellular metabolism by eliminating superfluous enzyme and other protein molecules. Remarkably, most rapidly turned over enzymes occupy important metabolic control points, whereas the relatively stable enzymes have nearly constant catalytic and allosteric properties so that cells can efficiently respond to environmental changes and metabolic needs. The half-lives of enzymes range from under an hour to more than 100 h. Enzyme protein turnover thus represents a regulatory mechanism belonging to longer timescales (Fig. 37.2). In general, the longer the life span of a cell (such as in eukaryotes), the more important is the process of enzyme turnover as a control mechanism.

The steady-state level of a given enzyme protein is a balanced outcome of its synthesis rate (which generally follows zero-order kinetics; $k_s \times [E]^0$ or simply k_s) and degradation rate (which normally obeys first-order kinetics; $k_d \times [E]$). Also, the degradation rate constant is related to half-life ($t_{1/2}$) by the following relation (see Chap. 9 for details):

$$k_d = \frac{\ln 2}{t_{1/2}} = \frac{0.693}{t_{1/2}}$$

The rate of change of enzyme level inside a cell is given by the following equation: $d[E]/dt = k_s - k_d \times [E]$. During the steady state, $d[E]/dt = 0$ and therefore we have the relation $k_s = k_d \times [E]$.

The first step to establish the occurrence of enzyme turnover is to show that the protein level of that enzyme is changing. It is also important to show that the change in enzyme activity is not due to any other reason such as covalent modification or a

conformational change. Enzyme turnover can be experimentally measured by using isotopically labeled amino acid precursors. The synthesis rate constant (k_s) may be evaluated by giving a single pulse of radiolabeled amino acid and subsequently following the incorporation (at short time intervals) of the label into the enzyme protein. For degradation rate constant (k_d) measurements, first the enzyme protein is labeled. Then the decay of specific radioactivity of this labeled protein is measured at fixed (often longer) time intervals. Among the liver proteins, ornithine decarboxylase is turned over much more rapidly ($t_{1/2} = 15$ min) than phosphofructokinase ($t_{1/2} = 168$ h). Many of the enzymes with short half-lives catalyze rate limiting steps in metabolic pathways.

Intracellular proteins destined for degradation are either tagged (ubiquitinated) and taken to proteasomes or processed through autophagy. Proteasome is a large multi-subunit protease found in all eukaryotes. This multicatalytic proteolytic complex degrades ubiquitin-tagged proteins in an ATP-dependent manner. A proteasome contains five different protease activities facing its lumen cavity – these are characterized as chymotrypsin-like, trypsin-like, postglutamyl hydrolase, branched chain amino acid protease, and small neutral amino acid protease. Lactacystin is a selective proteasome inhibitor. Autophagy is the other route of protein turnover and involves lysosomes. Proteins/enzymes that are generally not ubiquitinated take this nonselective process of autophagy. Lysosomes contain more than 50 different hydrolytic enzymes, all with an acidic pH optimum. These include nine different cathepsins and nine more exoproteases. Most lysosomal proteases are inhibited by leupeptin.

37.2 Control of Enzyme Activity: Inhibition

Another level of regulation of metabolic pathways is to control the activity of a strategically placed enzyme by sensing metabolite concentrations. Compared to the slow, limited means of control of enzyme concentration, there are a plethora of mechanisms to control the enzyme activity. This adds further diversity in regulation because one can modulate enzyme activity either positively (by activation) or negatively (by inhibition). Some such important control mechanisms are outlined below.

Inhibition of the activity of an enzyme by ligand binding is the quickest way of controlling its function. Being a non-covalent interaction, the binding equilibrium is concentration driven and reversible. In this timescale (see Fig. 37.2), the enzyme concentration remains effectively constant. While examples of inhibitory ligands are plentiful, we do find instances of enzyme activation by ligands. The concept of *enzyme activation* is analogous to the more common *enzyme inhibition*. It is just that the effects are opposite! We will elaborate on the various modes of enzyme inhibition (and leave examples of enzyme activation mostly to the imagination of the reader).

Inhibitors could be structurally similar to either the substrate or the product of an enzyme. By virtue of this similarity, they may bind at the active site and exhibit their

inhibitory effect. Such inhibitors are called *isosteric* inhibitors (see Chap. 20). Ligands that may or may not resemble the substrate (or the product) could bind to a site other than the enzyme active site. These inhibitors are termed as *allosteric* inhibitors. It is worth noting that a substrate itself could be an allosteric activator (see "cooperative/allosteric modulation" below).

Enzymes inhibited at higher substrate concentrations are rare. This phenomenon of *substrate inhibition* is known in enzyme kinetics (see Chap. 23). However, regulatory significance of substrate inhibition is not well understood. Enzymes acting on metabolites that are toxic when accumulated often display substrate inhibition behavior. An aldehyde dehydrogenase interacting with its substrate aldehyde is an example. It appears that such enzymes have evolved to catalyze forward reaction at low substrate concentrations. The evolutionary pressure on catalysis in the reverse direction may have been insignificant on them. In any case, the toxic metabolic intermediate is quickly cleared by such enzymes.

Inhibition by the reaction product is the most common yet frequently overlooked mode of enzyme regulation. A product can access and bind at the enzyme active site. Product can inhibit the reaction by titrating out the active enzyme available for catalysis. It can also drive the reaction backward by simple mass action. The extent to which a product inhibits an enzyme depends on its concentration and the binding constant. The lower the K_I, the greater is the inhibition at any given product concentration. As a thumb rule, biosynthetic enzymes are much more sensitive to *product inhibition* than the catabolic enzymes. This makes sense as catabolism should occur only when the corresponding substrate is in excess. Product inhibition ensures that a biosynthetic enzyme makes enough product (and is accumulated to satisfy the cellular needs) as is necessary, while wasteful metabolism is prevented.

End Product Inhibition is a common mode of metabolic regulation in biosynthetic pathways. Often a terminal metabolite, without any chemical analogy/reactivity to an earlier step, is a powerful inhibitor of its own synthesis. Since they have very little structural resemblance to the substrate(s) of the metabolic step they inhibit, these inhibitors invariably bind to an allosteric site of that enzyme (Pardee and Reddy 2003). In this mode of *feedback inhibition*, the end product (or a near end product) controls the metabolic flux by inhibiting the activity of one or more early enzymes of the pathway. Two examples are acetolactate synthase (in branched chain amino acid biosynthesis) feedback inhibited by L-valine and aspartate transcarbamoylase (in pyrimidine biosynthesis) inhibited by CTP (Umbarger 1956; Monod et al. 1963). In branched pathways, the maximum inhibition is often attained only by the combined action of multiple end products. This inhibition strategy circumvents the problem of completely shutting down a branched pathway by one end product, thereby ensuring availability of other end products to the organism. Figure 37.3 shows the schematic of a branched anabolic pathway to understand the various kinds of feedback inhibitions that may operate in biosynthetic pathways.

In branched pathways, often the end product inhibits the first (or an early) enzyme of the respective branch. The branch point intermediate preceding the branch in turn

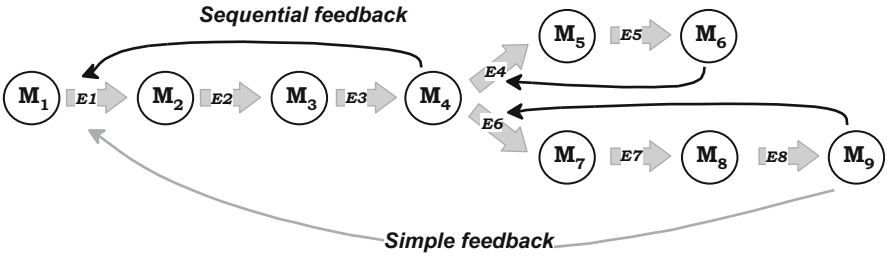

Fig. 37.3 Schematic of a branched anabolic pathway showing different modes of enzyme inhibition. Pathway metabolites are numbered M_1 through M_9 and enzymes are numbered *E1* through *E8*. A simple feedback inhibition of *E1* by end product M_9 (gray arrow) is depicted. Sequential inhibition involving inhibitions of *E4* by M_6 and *E6* by M_9 with the resultant accumulation of M_4 and subsequent inhibition of *E1* by M_4 (black arrows) is shown. Few other possible variations are described in the text

regulates the activity of the first enzyme common to all the end products, thus maintaining a balance of the products formed (Fig. 37.3; M_6 inhibits *E4*, M_9 inhibits *E6*, and consequent higher levels of M_4 inhibit *E1*). Examples of such *sequential feedback inhibition* may be found in aromatic amino acid biosynthesis (e.g., pathway control by Trp, Phe, and Tyr in *B. subtilis*) and the biosynthesis of aspartate family of amino acids. Variations of this sequential feedback control are also possible. For instance, inhibition of the first (or important) common enzyme of each branch by the product at the branch point along with a simultaneous activation of the first enzyme after the branch point by the same or other intermediate of the pathway (Fig. 37.3; M_4 inhibits *E2* and activates *E7*). Yet another possible mode involves *compensatory activation and deinhibition* – where the first common enzyme is inhibited by one product and activated by the other product (Fig. 37.3; M_6 inhibits *E4* and activates *E6*, and M_9 inhibits *E6* and activates *E4*), thus maintaining a balance in the products formed. Examples of this kind may be found in biosynthesis of purine nucleotides (AMP and GMP) and of dNTPs.

Examples of an enzyme inhibited by more than one ligand are known – the so-called multiple inhibition. In the case of *concerted or multivalent inhibition*, the products of a branched pathway do not singly inhibit the first common enzyme. However, the presence of two or more products is essential for significant inhibition (Fig. 37.3; either M_6 or M_9 alone do not inhibit *E1*, but when both are present, *E1* activity is markedly reduced). Threonine and lysine act in concert (but not individually) to inhibit *B. polymyxa* aspartokinase. A *synergistic inhibition* is observed when mixtures of M_6 and M_9 at low concentrations bring about more inhibition (of *E1*) than the same total specific concentration of M_6 or M_9 alone. Interaction with AMP, histidine, and glutamine as inhibitors of *B. licheniformis* glutamine synthetase is one such case (where synergistic inhibition of the enzyme by Gln + His pair and AMP + His pair is reported). The concerted inhibition is thus an extreme case of synergism for inhibition between the inhibitors. By contrast, in *cumulative feedback inhibition*, there is no cooperation or antagonism between several inhibitors of an

enzyme. Each end product is a partial inhibitor and brings about same percentage inhibition irrespective of whether other inhibitors are present or not. The *E. coli* glutamine synthetase provides an excellent case study of cumulative inhibition (also see later section) (Stadtman 2001). Suppose His alone at a given concentration yields 50% inhibition (enzyme retains 50% of the original activity) and Trp alone at a given concentration yields 30% inhibition (leaving 70% of the original activity). Then at the same concentrations of "His+Trp," the enzyme retains 35% of the original activity (50% + [30% of 50%] or 30% + [50% of 70%] = 65% inhibition). If the nature of inhibition was additive, then the final inhibition reached would have been 80% (i.e., 50% + 30% = 80%).

If an enzyme is inhibited by more than one ligand, then understanding their mutual interaction is of interest. *Multiple inhibition analysis* (or the interaction of more than one inhibitor with the enzyme) is meaningfully done through Dixon analysis (Chap. 22). For example, when *I* and *J* competitively inhibit the enzyme, the double reciprocal form of the rate equation is

$$\frac{1}{v} = \frac{K_M}{V_{max}}\left(1 + \frac{[I]}{K_I} + \frac{[J]}{K_J} + \frac{[I][J]}{\alpha K_I K_J}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$

Experimentally however, rates are measured at a fixed concentration of *S*, while [*I*] is varied. A $1/v \rightarrow [I]$ plot of this data is nothing but the Dixon plot. Now, in a similar setup, we can include different fixed concentrations of *J* and obtain a series of lines. The pattern of these lines is characteristic of the nature of interaction between the two inhibitors. The influence of one-bound inhibitor on the binding of the other is estimated by the interaction constant, α. This constant is conceptually similar to the interaction term α, we used in describing noncompetitive inhibition in Chap. 22. An α value of infinity indicates mutually exclusive binding of *I* and *J* (meaning only *EI* or *EJ* possible; *EIJ* does not form) and gives a parallel pattern in the Dixon analysis. A finite α value shows that both inhibitors can simultaneously bind the enzyme; values below unity are indicative of synergistic interaction between *I* and *J*.

37.3 Control of Enzyme Activity: Cooperativity and Allostery

In some enzymes the ligand binding and/or catalytic activity follows a non-Michaelian saturation pattern. This feature allows the enzyme to function as a ligand concentration-dependent switch. At higher concentrations ligand binding may become progressively easier (positive cooperativity) or more difficult (negative cooperativity). Enzyme conformational changes accompany ligand binding events in this mode of regulation.

Subunit Cooperativity and Switch Behavior Most cooperative enzymes share a few features in common. These include:

1. Allosteric enzymes generally consist of multiple subunits (i.e., they are oligomeric).
2. The regulatory ligands (effectors) usually do not share any structural resemblance to the substrate(s) or product(s) of the enzyme reaction concerned.
3. Effectors may bind to an allosteric site distinct from the enzyme active site. It is thus possible to selectively destroy (by physicochemical or mutational methods) the allosteric site without affecting the catalytic site. Such a *desensitized* enzyme does not respond to allosteric effectors. For instance, upon limited heat treatment, *E. coli* aspartate transcarbamoylase loses its ability to bind CTP.
4. Allosteric enzymes do not show Michaelian substrate saturation kinetics. Their $v \rightarrow [S]$ plots are sigmoidal rather than being hyperbolic (see Fig. 15.4, Chap. 15). The sigmoid saturation curve indicates cooperative substrate binding – the binding of the first molecule facilitates the binding of subsequent molecules. The extent of cooperativity is measured by the value of **h** – the Hill coefficient (also denoted as $\mathbf{n_H}$; see Chap. 15). An enzyme with $\mathbf{h = 1}$ shows no cooperativity and is Michaelian. Negative cooperative enzymes have $\mathbf{h < 1,}$ whereas those with positive cooperativity will have $\mathbf{1 < h < n}$. If $\mathbf{h = n}$ for an enzyme with **n** binding sites (each monomer with an active site) then such an enzyme will be extremely cooperative. We note that $\mathbf{h} = 2.6$ for hemoglobin. The Hill coefficient for *E. coli* aspartate transcarbamoylase is 2.0; it decreases to 1.4 in the presence of an allosteric activator (ATP) and increases to 2.3 in the presence of an allosteric inhibitor (CTP).

There have been several attempts to capture the phenomenon of cooperativity into a theoretical model. These include mathematical descriptors of allosteric behavior (Hill equation and Adair equation, e.g., see box below) as well as physical models that incorporate enzyme structural information (see the two models by Monod, Wyman, and Changeux as well as by Koshland, Nemethy, and Filmer, briefly described later).

Oligomeric State, Subunit Cooperativity, and Metabolic Switch Behavior

We define "*Y*" (fractional saturation) using the rearranged form of Hill equation (see Chap. 15) as

$$Y = \frac{v}{V_{\max}} = \frac{K_{0.5}[S]}{1 + K_{0.5}[S]}, \text{and on rearranging we get } [S] = \frac{Y}{K_{0.5}(1 - Y)}$$

For dimeric ($n = 2$) enzymes, this will be.

$$Y = \frac{v}{V_{\max}} = \frac{K_{0.5}[S]^2}{1 + K_{0.5}[S]^2}, \text{and this gets rearranged to } [S]^2 = \frac{Y}{K_{0.5}(1 - Y)}$$

(continued)

In general, for an oligomeric enzyme consisting of "**n**" monomers, we arrive at a general form.

$$[S]^n = \frac{Y}{K_{0.5}(1-Y)}$$

Calculated $[S]_{0.9}/[S]_{0.1}$ ratios for oligomeric enzymes with various "***n***" values are shown below.

Oligomeric State (n monomers)	$[s]^1$ (n=1)	$[s]^2$ (n=2)	$[s]^3$ (n=3)	$[s]^4$ (n=4)
$[s]_{0.9}/[s]_{0.1}$ **Ratio**	81	9	4.33	3

This analysis is based purely on the mathematical assumption of **n** subunits interacting with each other in an oligomer. The larger the "**n**" value, the greater is the sensitivity to changing [*S*]; this is how typical *concentration-dependent switches* are expected to behave. However, as discussed above, only for extremely cooperative enzymes **n** is equal to **h**.

Enzymes exhibiting cooperative/allosteric regulation are often multi-subunit proteins. But it is not necessary that oligomeric state is always associated with cooperativity. Besides the subunit cooperativity described above, there may be other reasons why oligomeric proteins are selected by evolution. Multiple subunit structure of an oligomeric enzyme may confer the following possible advantages:

(a) Multimeric nature may bestow structural stability to an otherwise unstable structural fold of a monomer. Lactate dehydrogenase is one such example.
(b) Different subunit types may be dedicated to bind different ligands (one to bind substrates – the active site – while others to bind regulatory ligands, the allosteric site). Examples include aspartate transcarbamoylase and lactose synthase.

The earliest physical model to account for the behavior of allosteric proteins and enzymes was proposed by Monod, Wyman, and Changeux (Monod et al. 1965). According to this model, in an oligomeric allosteric enzyme, the subunits occupy equivalent positions within the oligomer. Each monomer can exist in one of the two conformational states: either the R (for *relaxed* – an active, high-affinity state with tighter binding to the ligand) or the T (for *tense* – an inactive, low-affinity state with weak/no binding to the ligand) state. Further, the monomers are conformationally coupled to each other – when one subunit takes the R conformation, all others also change to R state such that the symmetry of the oligomer is maintained (Fig. 37.4). Hence this model is known as the *symmetry model*. Allosteric ligands affect the $R \rightleftarrows T$ equilibrium, and the subunits change their conformation in a concerted

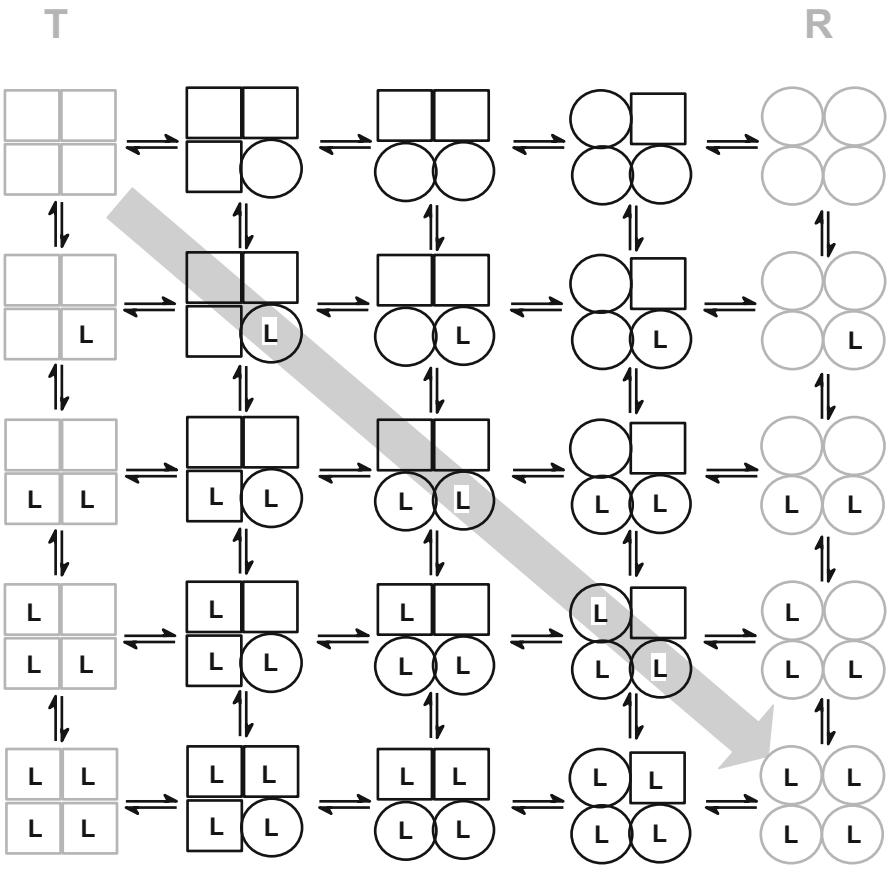

Fig. 37.4 Models of subunit cooperativity in a tetrameric enzyme. R represents a high affinity form (O) of the tetramer which is in equilibrium with T, the low affinity form (□) of the enzyme. The two vertical columns (in gray) show the species considered in the Monod, Wyman, and Changeux model. The species occurring along the diagonal (shown by the arrow) represent the forms considered by Koshland, Nemethy, and Filmer model. These two models are special cases of the more general Adair model (that includes all the enzyme species shown)

fashion. Therefore, it is also called the *concerted model*. Cooperative binding occurs when the ligand preferentially binds to the R state, thereby displacing the $R \rightleftarrows T$ equilibrium toward the *R* state. Sigmoidal oxygen binding to hemoglobin is a good example of this model. Nearly 100% of free hemoglobin occurs in *T* state, while O_2 binds 70 times more tightly to the *R* state.

Koshland, Nemethy, and Filmer proposed another physical model to describe allosteric phenomena – the so-called *sequential model* (Koshland et al. 1966). This model is based on the concept of ligand binding by "induced fit" (Chap. 8). In the absence of the ligand, the oligomer exists in one conformational state (and not as equilibrium of R and T states). The subunits change their conformation sequentially

as ligand molecules bind (Fig. 37.4). Conformational change in one subunit alters the interface of that subunit with its neighbors. This may result in more favorable (positive cooperativity) or less favorable (negative cooperativity) binding of the subsequent ligands. Unlike the symmetry model, this model can also account for and explain negative cooperativity.

More general models incorporating all possible conformational states for an allosteric enzyme have been proposed (Fig. 37.4), but with these the resulting kinetic treatment becomes extremely complex. The symmetry model (of Monod, Wyman, and Changeux) and the sequential model (of Koshland, Nemethy, and Filmer) have gained popularity over the years. The two models differ in the way ligand binding and conformational states are linked. Accordingly, they make specific predictions as to the allosteric behavior of an enzyme. The following experimental features are useful to distinguish between the two models:

(a) Observation of negative cooperativity in ligand binding points to a sequential model. For instance, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase binds NAD^+ in a negative cooperative manner.

(b) Conformational changes accompany ligand binding according to both the models. However, they predict different patterns for these conformational changes. The sequential model predicts a one to one correspondence between the number of sites occupied by the ligand ($\bar{Y}$, the fraction of sites saturated) and the extent of conformational change ($\bar{R}$, the fraction of enzyme in the R state) observed. A plot of $\bar{R}$ against $\bar{Y}$ should therefore be linear (Fig. 37.5). However, this $\bar{R} \rightarrow \bar{Y}$ plot will be nonlinear in the case of symmetry model – at every stage of ligand binding, there will be more than stoichiometric number of subunits in the R state. By this experimental criterion (the $\bar{R} \rightarrow \bar{Y}$ plot), the interaction of AMP with glycogen phosphorylase a conforms to the symmetry

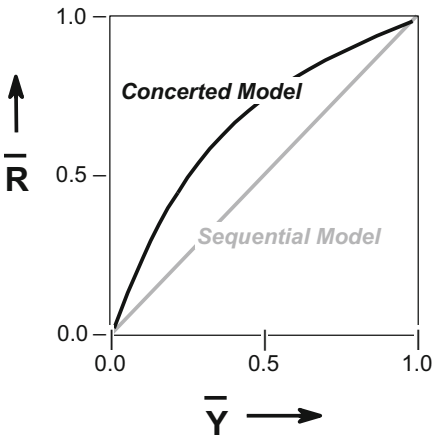

Fig. 37.5 Plot of $\bar{R}$ against $\bar{Y}$ is different for symmetry and sequential models of cooperativity. The linearity of this plot supports the sequential model of Koshland, Nemethy, and Filmer, while the nonlinear plot favors Monod, Wyman, and Changeux model ($\bar{R}$, the fraction of enzyme in the R state; $\bar{Y}$, the fraction of sites saturated)

model, while that of NAD^+ with glyceraldehyde-3-phosphate dehydrogenase follows the sequential model.

Structural information on allosteric enzymes and their various conformational states is necessary to understand the phenomenon of cooperativity. Ligand-induced conformational changes are the most valuable indicators. These changes could be assessed indirectly by spectroscopic probes or directly by X-ray crystallographic data. Best studied examples of distinct R and T states for allosteric proteins are hemoglobin (the honorary enzyme!), *E. coli* aspartate transcarbamoylase, and phosphofructokinase.

The two original physical models account for cooperativity through ligand binding features. Direct ligand-binding data (obtained by equilibrium dialysis, gel filtration, and/or ultracentrifugation) allows Scatchard analysis of the cooperative behavior. If the binding of a ligand to one subunit affects the affinity of another subunit for the same ligand, then such a cooperative interaction is termed *homotropic*. The sigmoid saturation of *E. coli* aspartate transcarbamoylase by L-aspartate is an example. On the other hand, *heterotropic* allosteric effects are observed between substrates and effectors (other than that substrate). Allosteric inhibition by CTP observed on the L-aspartate saturation of aspartate transcarbamoylase illustrates this point. The enzyme kinetic data (as opposed to direct ligand binding data) often provides evidence of cooperativity. Since the enzyme activity is a manifestation of binding as well as catalysis, one or both of these may account for the cooperative enzyme behavior. If an effector modifies the affinity of the enzyme for its substrate (i.e., it affects the $K_{0.5}$), then it is a *K system*. In this sense, CTP allosterically inhibits *E. coli* aspartate transcarbamoylase by shifting the aspartate saturation curve to the right (the $K_{0.5}$ is increased without affecting the V_{max}). In a *V system*, the effector influences the V_{max} and not the $K_{0.5}$.

The structural biology today has offered strong evidence for multiple conformations in preexisting equilibrium for many enzyme proteins. This more dynamic view of enzyme structure needs to be addressed in describing allosteric phenomena. Accordingly, allosteric control may manifest by a *population shift* in the statistical ensembles of many states. This new outlook is discussed in a later section (Chap. 39 Future of enzymology – An appraisal).

Other Origins of Sigmoid Enzyme Kinetics Enzymes displaying cooperative/allosteric regulation are often multi-subunit proteins. However, besides true subunit cooperativity described above, there may be other reasons why an enzyme displays sigmoid enzyme kinetics. Nature has evolved a varied set of mechanisms for generating sigmoidal effects for enzyme regulation.

(a) The enzyme activity may be a function of its state of aggregation (Lynch et al. 2017; Traut 1994). Enzyme monomers may be less active than the oligomeric aggregates. Further, this association–dissociation equilibrium may be influenced by substrate and/or regulatory ligands. Sigmoid kinetic behavior could arise from such association–dissociation. For instance, chicken liver

acetyl CoA carboxylase monomers polymerize extensively to more active filamentous, polymeric aggregates; and this is helped by citrate (an activator) while palmitoyl CoA facilitates depolymerization (an inhibitor). Systems undergoing association–dissociation are amenable to experimental verification because protein concentration effects can be easily observed.

(b) Multiple kinetic paths exist for an enzyme exhibiting random mechanism. The reaction path followed at lower substrate concentrations may differ from the one favored at higher [*S*]. If the net rates of the two paths are different (and the one at higher [*S*] is faster), a sigmoid kinetic curve ensues because of the substrate concentration-dependent reaction path switching!

(c) Although rare, there are examples of single subunit enzymes showing sigmoid kinetics. This kinetic behavior arises due to slow conformational change as a part of the enzyme catalytic cycle. Such systems with conformational memory are also called as *hysteretic* enzymes or *mnemonic* enzymes. For instance, glucokinase is monomeric but shows sigmoid kinetics with respect to its substrate glucose. The monomer undergoes a slow, glucose concentration-dependent conformational transition. An **h** value of 1.7 for glucose is reported.

(d) Complex non-Michaelian kinetics ensues when a mixture of isoenzyme forms with differing kinetic constants is analyzed. One isoform may saturate earlier than the other, and the resultant $v \rightarrow [S]$ curve (overlap of the two!) could be sigmoid. We will look at isoenzyme regulation in some detail in a subsequent section.

How to Study Regulatory Enzymes First and the foremost, one should ensure that the so-called regulatory kinetic behavior is not due to some artifacts of the assay. Many such caveats are discussed in an earlier chapter (Chap. 12 Principles of enzyme assays). A misleading sigmoid kinetics may be recorded when oxidation of enzyme or substrate, depletion of substrate due to complex formation, etc. occur during the enzyme assay. Before ascribing sophisticated regulatory mechanisms to unusual enzyme kinetic behavior, such artifacts must be discounted.

In order to assess cooperative behavior, it is necessary to examine enzyme activity over a wide range of substrate concentration. The kinetic data may then be analyzed through suitable plots (like the Hill plot) to measure the degree of cooperativity. An idea about the enzyme architecture is required to ascertain the possibility of subunit interactions leading to cooperativity. Typical protein quaternary structure determination techniques such as study of molecular weight in the absence and presence of denaturing agents, subunit composition, and cross-linking studies are useful. The kinetic cooperativity may correspond to ligand binding in an oligomeric protein. This can be tested through direct binding methods such as equilibrium dialysis, ultracentrifugation, etc. Once again, the ligand-binding cooperativity may be evaluated through Scatchard plot or Hill plot of the binding

data (see Chap. 17). Conformational changes often accompany ligand binding. These can be scored directly by X-ray crystallography and NMR spectra. Indirect approaches with chromophores and fluorophores as reporters or accessibility of amino acid residues to chemical modification reagents are also useful. Finally, presence of allosteric sites (distinct from the active site) to bind regulatory ligands may be ascertained by desensitizing the enzyme through physicochemical and mutational tricks.

37.4 Isozymes and Regulation

Isozymes are multiple molecular forms of an enzyme catalyzing the same chemical reaction. They differ from each other in their primary sequence but often are of comparable size and are unique translational products of distinct genes. The covalent modification states (like the phosphorylated forms, etc.) of the same enzyme are not isoenzymes by this definition. Isozymes play critical roles in cellular and metabolic regulation. They may be found in the same cell but in different (a) metabolic states (such as NADP-glutamate dehydrogenase versus NAD-glutamate dehydrogenase), (b) organelles to integrate cellular metabolism (malate shuttle; mitochondrial and cytosolic malate dehydrogenase isoforms in heart muscle), (c) tissues to facilitate inter-organ metabolism (lactate dehydrogenase in the skeletal muscle versus the liver), and (d) stages of development (e.g., laccases and trehalases during sporulation). Isoenzymes may be catalogued according to their distinguishing features and perceived metabolic significance (Table 37.1).

For the same chemical reaction, enzymes can be evolved that are more effective catalysts for one direction than the other. This is possible despite the fact that Haldane relationship (see Chap. 15) places certain constraints on the kinetic parameters of the enzyme. Recall that

Table 37.1 Isoezymes grouped according to their metabolically significant features

Differing feature	Examples[a]
Michaelis constant	Hexokinase (m), aldolase (m)
Substrate and cofactor specificity	Glutamate dehydrogenase (f), isocitrate dehydrogenase (p)
Allosteric properties	Hexokinase (m), aspartate kinase (b)
Subcellular localization	Carbamyl phosphate synthetase (f), malate dehydrogenase (m)
Tissue/organ localization	Arginase (m), lactate dehydrogenase (m)
Catabolic or biosynthetic (inducibility)	Alcohol dehydrogenase (f), threonine deaminase (b)

[a]Isoenzymes observed in bacteria (b), fungi (f), mammals (m) and plants (p)

$$\frac{V_{\text{maxf}} \times K_{\text{MP}}}{V_{\text{maxr}} \times K_{\text{MS}}} = \frac{[P]_{\text{eq}}}{[S]_{\text{eq}}} = K_{\text{eq}}$$

and K_{eq} is an immutable thermodynamic parameter that the catalyst cannot tinker with. It is however perfectly possible to have more than one numerical solution to satisfy the above equation. For example, two isozymes may have the same V_{maxf} values but different K_{MS}s. This is compensated by appropriate V_{maxr} and K_{MP} values in the two cases thereby resulting in an identical K_{eq} value. The isozyme with a lower $V_{\text{maxf}}/K_{\text{MS}}$ could either have a suitably lowered V_{maxr}, an elevated K_{MP} or both. The forward reaction rate (the first order rate with respect to [S]) is given by

$$v_f = \frac{V_{\text{maxf}}}{K_{\text{MS}}}[S] = \frac{k_{\text{catf}}[E_{\text{t}}]}{K_{\text{MS}}}[S]$$

On comparing the v_f for the two isozymes at a given [*S*], the enzyme form with a lower K_{MS} performs better in this direction. Two general observations can now be made:

(a) As many enzymes never need to catalyze a reaction in the reverse direction in vivo, there is no evolutionary pressure to achieve catalytic perfection in that direction. If the active site is strictly complementary to the transition state, then the enzyme will be an optimized catalyst for both directions. Efficiency in one direction could however be preferentially improved by evolving an active site that binds either *S* or *P* better than it binds the transition state. Indeed, methionine adenosyltransferase is one such *one-way enzyme* (with its limiting forward rate about 10^5 times greater than the reverse one).

(b) Since $V_{\text{maxf}} = k_{\text{catf}} \times [E_{\text{t}}]$, any unfavorable k_{catf} changes during catalyst design/evolution (arising out of thermodynamic constraints – such as Haldane relationship) can be compensated by the system. Despite having a lower k_{catf}, one can maintain the desired V_{maxf} by increasing $[E_{\text{t}}]$. In reality, this implies that the cellular concentrations (abundance!) of various isozymes need not necessarily be maintained at the same level.

Isozyme Dedicated to a Pathway Nature employs isozymes as a means to compartmentalize and regulate metabolism. These may be wired into metabolism to perform specific roles. Isozymes with dedicated function may be differently regulated at the genetic level. Bacteria elaborate two distinct isoforms of threonine deaminase: one for biosynthesis (with higher affinity for Thr; low K_{M}) and the other for catabolism (with lower affinity for Thr; high K_{M}). Similarly, the two carbamyl phosphate synthetases serve to feed the biosyntheses of arginine and pyrimidine, respectively. Multiple ω-amino acid transaminases are expressed in response to the availability of respective ω-amino acid inducers in the medium. They may be specific (such as GABA transaminase) or generic in their substrate specificity. There are two glutamate dehydrogenases in fungi to satisfy the cellular needs.

They catalyze the same chemical reaction but in opposite directions! The NAD-glutamate dehydrogenase (catabolic enzyme) is induced upon nitrogen starvation and/or when glutamate is the sole nitrogen source available to the cell. The NADP-dependent enzyme is biosynthetic and is responsible for the synthesis of cellular glutamate. Interestingly, yeast displays yet another isoform (of NADP-glutamate dehydrogenase) during diauxic growth on ethanol – dedicated to make glutamate when the carbon source is switched to ethanol (and it is not glucose).

Isozyme to Suit a Metabolic Demand Isozymes may have evolved distinct kinetic virtues to suit the metabolic demands of an organism. The two mammalian lactate dehydrogenase isoforms are well suited for the requirement of converting lactate to pyruvate (H_4 form in the heart) or pyruvate to lactate (M_4 form in the skeletal muscle). In addition, pyruvate inhibits the muscle form of lactate dehydrogenase. On the whole, pyruvate is completely oxidized by the heart (glycolysis + Krebs cycle), while in the skeletal muscles, it is reduced to lactate and sent into the blood stream. The liver displays both isoforms and is able to reconvert lactate to pyruvate so that gluconeogenesis can occur. While the Cori cycle depends on their functionality, the proposed metabolic role of lactate dehydrogenase isozymes is far from conclusive.

Kinetic differences between isozymes may favor forward reaction in one tissue and the back reaction in another. Despite the immutability of the equilibrium constant, one can envision various absolute values for V_{maxf} and V_{maxr} as well as different V_{maxf}/V_{maxr} ratios. This is possible within the constraints of Haldane relationship (discussed above). Aldolase isozymes are good examples where such kinetic comparisons have been made. The liver isozyme (aldolase B) is clearly more effective in fructose-1,6-bisphosphate synthesis (and hence gluconeogenesis!). Table 37.2 summarizes the relevant kinetic data for the two aldolase isozymes.

Glucokinase occurs only in the liver, while hexokinases are found both in the muscle and the liver. Further, hexokinase, but not glucokinase, is inhibited by the product glucose 6-phosphate. These features, in combination with their respective Michaelis constants, are in accordance with their role in glucose homeostasis in the

Table 37.2 Kinetic features of rabbit aldolase isozymes

	Isozyme form	
Kinetic property	Aldolase A (muscle)	Aldolase B (liver)
V_{maxf} (Fructose 1,6-bisphosphate cleavage)	5300 min^{-1}	250 min^{-1}
V_{maxr} (Fructose 1,6-bisphosphate synthesis)	10,000 min^{-1}	2600 min^{-1}
K_M (Fructose 1,6-bisphosphate)	60 μM	1 μM
K_M (Dihydroxyacetone phosphate)	2000 μM	400 μM
K_M (Glyceraldehyde 3-phosphate)	1000 μM	300 μM
(Fructose 1,6-bisphosphate)/(Fructose 1-phosphate) activity ratio[a]	50	1

[a]The ratio favors the liver isozyme (aldolase B) for fructose metabolism via fructose 1-phosphate

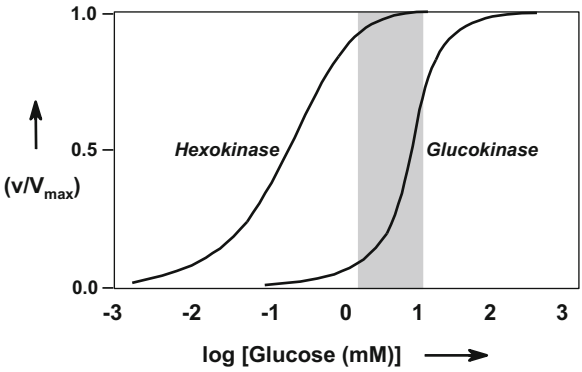

Fig. 37.6 Glucose saturation of hexokinase and glucokinase of mammalian liver. As the two isoenzymes show significantly different K_M for glucose (summary table above), a semi-log plot of log [Glucose] with fractional velocity (v/V_{max}) on Y-axis is shown (see Chap. 17)

body. The glucose concentration response of the two isoenzymes is shown in Fig. 37.6. It is obvious that liver (with its glucokinase) is able to respond and process high glucose presented to it in the blood.

Yet another example of isozymes tailored for catalysis in opposing directions is alcohol dehydrogenase (ADH) in yeast. Yeast growing in the absence of oxygen displays the constitutive ADH-I that is designed for aldehyde reduction. The ADH-II is induced upon aerobic growth and is well suited to oxidize alcohol to aldehyde for its further entry into Krebs cycle.

Isoenzymatic Regulation and Additive Inhibition A single enzyme reaction leading to several end products is a potential problem for regulation. Feedback inhibition by one of these products would not only affect its own formation but also interferes with that of the other products. Therefore, in some branched biosynthetic pathways, a number of discrete isoenzymes exist for the first committed step. Each one of them responds specifically and differently to inhibition by the various end products. The aspartokinases from *E. coli* were first examples described by Earl Stadtman's group at NIH. These isoforms are involved in the biosynthesis of L-aspartate family of amino acids (Lys, Thr, Met, and Ile). The multiplicity of aspartokinases is feedback inhibited by different end products of the branched pathway. One isozyme is Thr-sensitive, while the other is Lys-sensitive. Because of this aspartokinase from *E. coli* extracts exhibits *additive inhibition* by Thr and Lys. Further, the Lys-sensitive isozyme is also under Lys repression. The Thr-sensitive isozyme is subjected to multivalent inhibition by Thr and Ile. A second interesting example of enzyme multiplicity is 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase from *E. coli*. Formation of DAHP from erythrose-4-phosphate and PEP is the first step in the aromatic amino acid biosynthesis. Of the three isozymes of *E. coli* DAHP synthase, one is inhibited by Phe, while the second by Tyr. The third minor isoform is inhibited by Trp. Although there are many sequence differences between the DAHP synthase (Phe) and the DAHP synthase (Tyr) of yeast, a single residue determines the sensitivity to feedback inhibition; the

isozyme with Gly-226 is Tyr-sensitive, whereas that with Ser-226 responds to Phe inhibition.

37.5 Covalent Modifications and Control

Many enzymes (and hence pathways) are controlled by mechanisms that involve posttranslational covalent modification of proteins (Walsh et al. 2005). Here the actual enzyme protein concentration (i.e., $[E_t]$) remains unaffected, but the activity of existing enzyme molecules is altered. We are interested in covalent modifications that lead to altered enzyme activity/specificity. The modification may result in a less active or more active form of the unmodified enzyme. Examples of covalent modification types amenable to regulation of enzyme activity are listed in Table 37.3. As we shall see shortly, such covalent modification may be reversible or irreversible.

Zymogen Activation by Limited Proteolysis Is Irreversible Proteolytic cleavage of polypeptides (and enzymes) is an essentially irreversible event. More often, we think of this process in terms of protein degradation. However, in some cases,

Table 37.3 Well-characterized covalent modifications in enzymes

Type of modification	Target in protein	Examples
Irreversible (once only)	**Peptide bond**	
Limited (specific) proteolysis	-Asp-Lys – Ile-Val-, -Ala-Arg – Ile-Val-, -Ser-Arg – Ile-Val-	Pancreatic zymogens (trypsinogen, chymotrypsinogen, proelastase, procarboxypeptidases A and B)
	Arg – Ile, Arg – Gly	Blood clotting factors (factors XIIa, XIa, IXa, VIIa, Xa and prothrombin)
	Single site?	Complement system (C1r, C1s, C2)
Reversible (back and forth)	**Amino acid**	
Phosphorylation–dephosphorylation	Ser, Thr, Tyr, Lys	Glycogen synthase, glycogen phosphorylase, phenylalanine hydroxylase, triglyceride lipase, acetyl CoA carboxylase, cdc kinase, NAD-glutamate dehydrogenase
Nucleotidylation–denucleotidylation	Tyr, Ser	Glutamine synthetase and P_{II} protein (Gram-negative bacteria)
Acetylation–deacetylation	Cys?	Citrate lyase (anaerobic bacteria)
Thiol-disulfide interchange	Cys	Plants (light activation)
ADP-ribosylation	Arg, Glu, Lys	EF-2 (diphtheria toxin), G protein (cholera toxin), glutamine synthetase (mammalian)
Methylation	Asp, Glu, Lys, His, Gln	Bacterial chemotaxis, histones

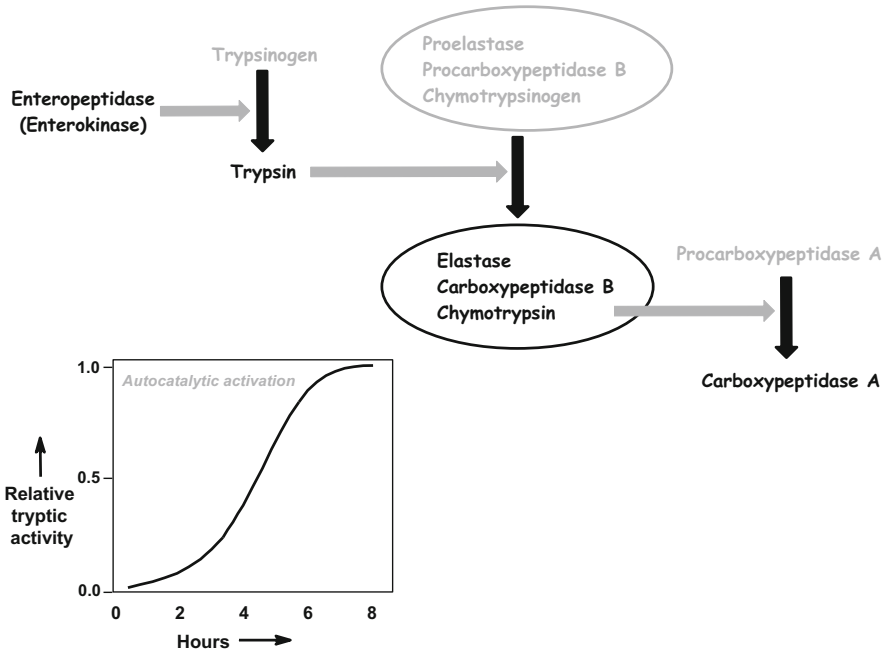

Fig. 37.7 Sequence of protease activation events associated with pancreatic zymogens. Activated enzyme forms are in black and zymogens are in gray. Black arrows indicate activation events catalyzed by the relevant enzyme (gray arrows). Autocatalytic activation of trypsinogen by trypsin is shown in the inset

limited and selective peptide bond cleavage can lead to activation of an inactive precursor – the so-called zymogen activation. The well-known example of zymogen activation in the small intestine is a regulatory trick (safety mechanism) to guard against premature activation of pancreatic proteases. Enteropeptidase (earlier known as enterokinase) of the small intestine initiates the zymogen activation cascade (Fig. 37.7) by converting trypsinogen to trypsin. This is achieved by the cleavage of a single peptide bond of the zymogen. Once small amount of trypsin is generated, the further conversion of trypsinogen to trypsin becomes autocatalytic (see inset, Fig. 37.7). A significant feature of zymogen activation is amplification of the initial signal. The irreversible cascades of zymogen activation also occur in blood clotting and activation of the complement system. In these two examples, each factor upon activation proteolytically cleaves the next zymogen in the sequence (for details see standard text books of biochemistry). These cascade systems are amenable to control and exhibit great signal amplification by using a catalyst to create more catalysts.

Enzyme Regulation by Reversible Covalent Modification Many key enzymes of metabolism interconvert between two forms that differ in catalytic properties. While more than 100 different covalent modifications of amino acid residues are known, in terms of enzyme regulation, phosphorylation, nucleotidylation, and

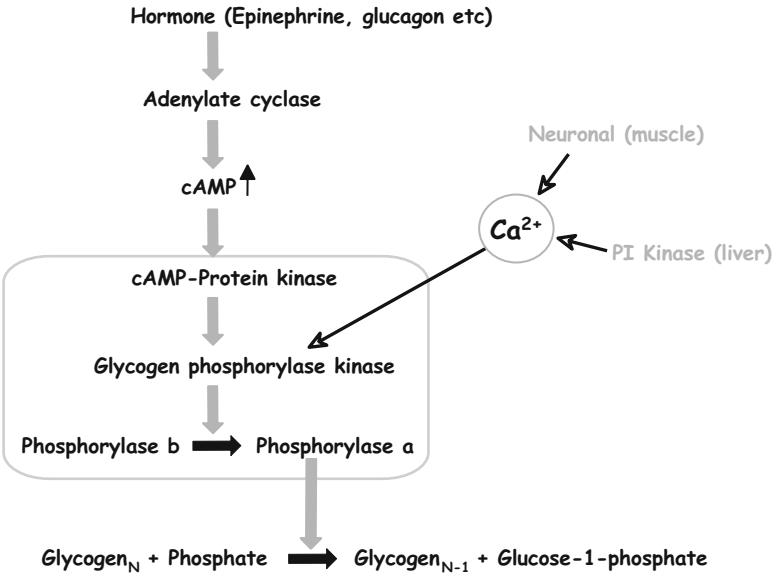

Fig. 37.8 Protein phosphorylation cascade of glycogen breakdown. Glycogenolysis in the liver and muscle is triggered by hormone action (also by neuronal stimulation in the muscle). Multiple protein phosphorylation steps allow exquisite control of glycogen phosphorylation (gray box). The phosphorylation cascade is further complicated by that a) the phosphorylation state itself may also be determined by associated protein phosphatases and b) at every level, both phosphorylation and dephosphorylation may be differently (reciprocally) affected by allosteric modulators like AMP and glucose-1-phosphate. This regulation also receives inputs from multiple second messengers like cAMP, Ca^{2+}, inositol-1,4,5-trisphosphate (IP_3), and diacylglycerol (DAG). The tissue-specific differences are not explicitly shown in this figure, but the reader is directed to excellent texts on this subject. Finally, a similar mechanism reciprocally controls glycogen synthase activity – phosphorylations leading to net decrease in its activity

ADP-ribosylation are important and frequent. Regulation by thiol-disulfide interchange, well demonstrated in plants, may also be significant in other organisms. The best documented example of adenylylation control is that of *E. coli* glutamine synthetase (see below). An extremely versatile mechanism of reversible covalent modification is via protein phosphorylation and dephosphorylation (Krebs and Beavo 1979). This is a predominant mode of control in eukaryotes. Reversible phosphorylation–dephosphorylation event occurs at the –OH of a Ser, Thr, or Tyr on the enzyme protein. The enzymes accomplishing phosphorylation and dephosphorylation are known as *protein kinase*s and *protein phosphatase*s, respectively. The modification and its reversal are catalyzed by separate (converter) enzymes – a catalyst acts upon another – to create a *cascade* system. A well-studied example of an interconvertible enzyme is glycogen phosphorylase (Fig. 37.8). Glycogen phosphorylase along with its converter enzymes (the kinase and the phosphatase) defines a *monocyclic cascade*. In such a system allosteric effectors can bind to either or both converter enzymes or directly to the interconvertible enzyme. One or more effectors

can thus lead to an adjustment in the steady-state level of phosphorylation and the activity of glycogen phosphorylase. Monocyclic cascades can thus sense changes in the concentrations of many different metabolites and act as metabolic integration systems. When a converter enzyme (glycogen phosphorylase kinase) itself is subject to phosphorylation/dephosphorylation (a kinase kinase!), then a *bicyclic cascade* is defined. Glycogenolysis in the liver provided the first example of this kind (Fig. 37.8). Nucleotidylation control of *E. coli* glutamine synthetase (see below) provides yet another case of a bicyclic cascade (although a closed one!) (Stadtman and Chock 2014). Reader may refer to literature on many other examples of excellent metabolic control by enzyme covalent modifications.

The phosphorylation of enzyme targets is a well-recognized mode of regulating enzyme activity (and hence metabolism) in eukaryotes. Typically protein kinases recognize -Arg-Arg-X-Ser- or -Arg-Lys-X-Ser- sequences of the target enzyme and phosphorylate at the Ser-OH. Experimental analysis and interpretation of phosphorylation/dephosphorylation events is tricky because of the following: (a) A single protein kinase may be able to phosphorylate many different targets in vitro. Elucidating their in vivo relevance can be daunting. (b) Many target enzymes are subject to multiple phosphorylations at different sites. Not all of these phosphorylations may be directly involved in the control of activity. (c) The issues discussed for protein kinases (in the above two points) are equally relevant to the functioning of phosphoprotein phosphatases. Nevertheless, some early discoveries like the phosphorylation/dephosphorylation cascade control of glycogen metabolism (Fig. 37.8) have been resolved to significant details.

Metabolic Significance of Covalent Modifications The reversible covalent modification and cyclic cascade systems are of value in the regulation and integration of cellular metabolism. This mode of control has certain obvious advantages.

- The system develops a capacity for *signal amplification*. A small amount of signal (such as a hormone acting via cAMP; Fig. 37.9) can act on a much larger amount of target enzyme (via the converter enzyme(s)). For example, in the muscle tissue, the molar concentrations of cAMP protein kinase (0.2 μM), phosphorylase kinase (2.5 μM), and phosphorylase (80 μM) are widely different, whereas the

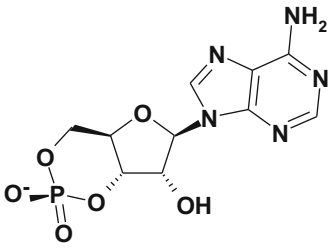

Fig. 37.9 Structure of cyclic AMP – the second messenger

intracellular cAMP levels do not exceed 2–3 μM. At these concentrations, cAMP obviously cannot activate phosphorylase directly! (Ferrell Jr 1996)

- They can act as *catalytic amplifiers* as a small amount of converter enzyme can act on a much larger amount of interconvertible enzyme target. For instance, the initial signal of enteropeptidase is quickly amplified by converting trypsinogen to trypsin.
- With multi-cyclic cascades, besides the capacity for signal and rate amplification, the *flexibility of response* of the system increases exponentially. Individual enzyme proteins, on an average, are not really big molecules. Their surface area is not unlimited and may accommodate a few additional sites for regulatory ligands besides the active site. Cyclic modification systems involve more enzymes and therefore can harbor more sites for effector binding. For example, *E. coli* glutamine synthetase responds directly to about 15 ligands but indirectly (through the other players of the bicyclic cascade) to another 20 more regulators. Such systems have the capacity to respond to a range of signals thereby achieving *integration of metabolism* (Chock et al. 1980).

Role of Signal Molecules and Energy Charge Protein phosphorylation is the most common (and prevalent) covalent modification observed in eukaryotic enzyme regulation. Protein kinases (that phosphorylate target enzymes) themselves respond to control signals. The major signal molecules include cyclic nucleotides (cAMP and cGMP), Ca^{2+}, Ca^{2+}/calmodulin, and diacylglycerol. Each one of these activates a unique class of protein kinases. The cAMP-dependent protein kinase is an important member of the hormonal activation pathway of glycogen phosphorylase (Fig. 37.8). Phosphorylation of phosphofructokinase-2 by cAMP-dependent protein kinase leads to its inhibition; this leads to decrease in fructose-2,6-bisphosphate levels and lowered phosphofructokinase and of glycolysis.

Similarly, diacylglycerol activates protein kinase C, which in turn catalyzes the phosphorylation of a range of proteins involved in cellular processes. The function of molecules like cAMP (Fig. 37.9) appears to be solely to act as signal in controlling enzyme activities. Sutherland called cAMP the *second messenger* for hormone action (Blumenthal 2012). Many hormones act on adenylate cyclase through G-proteins. Activation of adenylate cyclase leads to the synthesis and hence a rise in the intracellular levels of cAMP. Regulation by various second messengers and hormonal/neural control of cellular activities is beyond the scope of this book. The reader may refer to excellent and detailed account of this area in advanced texts on biochemistry.

It is well accepted that key glycolytic enzymes are responsive to adenylate compounds namely ATP, ADP and AMP. Atkinson introduced the term *energy charge* to denote the relative concentrations of these three adenine nucleotides:

$$\text{Energy Charge} = \frac{[\text{ATP}] + 1/2[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

According to this definition, the energy charge of a cell can take values from 0 (when all the adenine nucleotide pool is present as AMP) to 1 (when all the adenine nucleotide pool is present as ATP). Typically, the energy charge of cells is maintained at or above 0.95. At least in the short term, the cellular concentration of adenine nucleotide pool (the sum [ATP] + [ADP] + [AMP]) remains constant, while their relative concentrations are [ATP] >> [ADP],[AMP] (for instance, a 5 mM total of adenine nucleotides may be present in the ratio ATP:ADP:AMP as 4.48:0.50:0.02). It appears that relative concentration change in the adenine nucleotides is a key determinant in sensing the state of metabolism, with AMP as the regulatory signal. This is because [AMP] is related to [ATP] through the equilibrium reaction catalyzed by adenylate kinase (also known as myokinase):

$$2\text{ADP} \rightleftarrows \text{ATP} + \text{AMP} \quad \left(K_{\text{eq}} = 0.44\right)$$

Due to this equilibrium, small changes in [ATP] are quickly amplified many fold in terms of [AMP]. Again, it is the fractional change in [ATP], and [AMP] is critical (and not their absolute concentrations) for the regulation of target enzyme activity. AMP is an intracellular allosteric signal sensed by many enzymes. A specific protein kinase is activated by AMP in two ways: (a) AMP is an allosteric activator of this enzyme, and (b) AMP makes this protein more susceptible to phosphorylation by a kinase kinase.

37.6 Protein-Protein Interactions and Enzyme Control

Regulation of enzyme activity by reversible interactions with small molecular weight metabolites (as inhibitors or activators) is a common theme in metabolism. Many of them are described earlier in this chapter (Sect. 37.2 Control of enzyme activity: Inhibition). Enzyme-enzyme interactions may also play significant role in the regulation of metabolic reaction pathways (Srivastava and Bernhard 1986). What about another protein binding to enzymes and regulating their activity? Although very few in number, there are indeed well-defined examples of this mode of enzyme control as well. A distinct regulatory protein may reversibly (or irreversibly) associate with an enzyme thereby increasing or decreasing its overall activity (Table 37.4). In one sense these protein modulators could be viewed as *regulatory subunits* of the target enzyme. However, the concept is different from regulatory (R) subunits of allosteric enzymes, permanently associated with the catalytic (C) subunits of an enzyme (e.g., *E. coli* aspartate transcarbamoylase with its $3R_2$-$2C_3$ architecture).

Protein modulators may regulate enzyme activity in different possible ways. This mode of regulation is often unique to an enzyme system or group of organisms. Examples include modifying the substrate specificity of an enzyme (lactalbumin), mediating the activation/inhibition of an enzyme upon receiving environmental cues

Table 37.4 Well-characterized protein modulators of enzyme activity

Regulator	Target enzyme	Comments
Lactalbumin	Lactose synthase (α subunit)	Lactalbumin is devoid of any catalytic activity; but it alters the specificity of the α subunit to synthesize lactose instead of N-acetyllactosamine
Calmodulin	Protein kinases	Many protein kinases (e.g., phosphorylase kinase) are activated by Ca^{2+}/calmodulin
G-Proteins	Adenylate cyclase	Upon interacting with hormone receptor complex, α subunit exchanges GTP for GDP and detaches from the βγ dimer of the G-protein; adenylate cyclase is either stimulated by the α_s or inhibited by α_i
Arginase	Ornithine transcarbamoylase	Yeast arginase and ornithine transcarbamoylase form a complex in the presence of ornithine/arginine. Ornithine carbamyltransferase activity is thereby inhibited (termed *epiarginasic control*)
Serpins	Serine proteases	Serpins (*ser*ine *p*roteinase *in*hibitor*s*) inhibit serine proteases by an irreversible suicide substrate mechanism. Well-known examples include antithrombin (clotting), C1-inhibitor (complement activation), and antiplasmin (fibrinolysis)
Antizyme	Ornithine decarboxylase	Antizyme levels increase in response to elevated cellular polyamines. Upon antizyme binding, proteasome-mediated degradation of mammalian ornithine decarboxylase ensues

(Ca^{2+}/calmodulin and α_s or α_i subunits of G-proteins), coupling a biosynthetic enzyme activity to end product availability (epiarginasic control in *S. cerevisiae*), titrating out the active enzyme by presenting themselves as suicide substrates (serpins) (Silverman et al. 2001), or marking the enzyme for proteolysis (antizyme) (Small and Traut 1984). We have already noted the role of ubiquitination in targeting proteins for degradation.

37.7 Compartmental Regulation and Membrane Transport

We have seen early in this chapter that access to the substrate (as well as inhibitors and activators) is a major factor that determines the overall rate of an enzyme-catalyzed reaction in vivo. Controlling this access itself can be used as a regulatory feature. One way to go about this is to keep the substrate (or inhibitor or activator) and the enzyme in distinct compartments so that they do not see each other unless required. This separation is achieved in three principle ways.

Many enzymes are selectively expressed during specific phases of growth and/or development. However, they may not encounter corresponding substrates until much later. For instance, glutamate decarboxylase and trehalase are stored in the fungal spores in latent forms. They get to see their substrates (glutamate and trehalose, respectively) only at the time when conditions are right for spore germination. Such *temporal compartmentalization* (separation of enzyme and its substrate

in time) is also common in secondary metabolism. Fatty acid synthase and polyketide synthases share a few common substrates but rarely compete with each other. Polyketide synthases are generally expressed after the cessation of growth phase. Similarly, the sugar precursors are directed to aminoglycoside antibiotic synthesis as secondary metabolism is associated with post-growth phase.

Essentially similar metabolites may serve as substrates for two distinct enzymes located in the same subcellular compartment. One way to dedicate their use by one or the enzyme is to differentially tag the metabolite and exploit enzyme specificity. Best example of this is selective use of NAD for catabolic purposes and NADP for biosynthetic reactions. For instance, despite the availability of NADH in the same compartment, the biosynthetic glutamate dehydrogenase uses NADPH. The two cofactors differ in a phosphate group but have essentially same redox potential. Other instances of *chemical compartmentalization* include selective use of ATP or GTP as phosphate donor (and ADP or GDP as phosphate acceptor), UDP glucose for glycogen synthesis and glucose-1-phosphate from glycogen breakdown, CDP derivatives in phospholipid metabolism, etc. Fructose-1,6-bisphosphatase acts on the α anomer of fructose-1,6-bisphosphate, whereas phosphofructokinase generates the β anomer of fructose-1,6-bisphosphate. Many steps of fatty acid biosynthesis and catabolism involve chemically similar intermediates. They are segregated in the same physical compartment of a prokaryotic cell by distinct chemical tags. All the intermediates of fatty acid biosynthesis are tethered to acyl carrier protein, while the similar intermediates of catabolism are free as CoA derivatives; the stereochemistry of the β-hydroxy intermediates are also opposite. An interesting example of similar set of reactions but distinct chemical compartments is L-proline and L-ornithine biosynthesis in fungi. Glutamate is the precursor for proline biosynthesis, whereas N-acetylation of glutamate dedicates it for the biosynthesis of L-ornithine. The acetyl tag is removed from N-acetyl L-ornithine subsequent to its synthesis.

A prominent feature of eukaryotic cells is their reliance on achieving enzyme/metabolic regulation through *physical compartmentalization*. The presence of distinct membrane-enclosed organelles allows them to spatially separate otherwise competing reactions. *N. crassa* arginine metabolism is an excellent case in point. The L-ornithine biosynthesis occurs in mitochondria. Of the two carbamyl phosphate synthetase isozymes, the mitochondrial form is dedicated for ornithine/arginine biosynthesis. Excess cellular arginine is actively transported and sequestered into fungal vacuoles. The ornithine arising out of cytosolic arginase reaction cannot access entry into mitochondria – relevant membrane permease is sensitive to competitive inhibition by cytosolic arginine. Since participation of ornithine in the biosynthesis as well as catabolism of arginine is obligatory, wasteful cycling of ornithine is a distinct possibility. The overall compartmental organization of arginine metabolism in *N. crassa* ensures that futile cycling of ornithine does not occur. Besides regulation of enzyme activity, this is achieved by recruiting at least two specific transporters located on vacuolar and mitochondrial membranes. Yet another example of physical compartmentalization is that of nitrogen fixation in filamentous cyanobacteria. The oxygen generated by photosynthetic cells is toxic to nitrogenase. The nitrogenase and the nitrogen-fixing apparatus is quarantined to specialized

Table 37.5 Different modes of transport across biological membranes

Nature of transport	Transporter mediated	ΔG°_{tr} (= − RT ln ($[A]_{in}$/$[A]_{out}$))	Examples
Passive transport			
Simple diffusion	No	Negative (driven by concentration gradient)	Urea, water
Facilitated diffusion	Yes	Negative (driven by concentration gradient)	Glucose carrier of erythrocytes
Active transport			
	Yes	Positive (coupled to ATP hydrolysis, ion/pH gradient, etc.)	(Na^+K^+)-ATPase on plasma membrane, maltose transporter from *E. coli*

non-photosynthetic cells called heterocysts, which are fortified with defense enzymes like catalase, peroxidase, superoxide dismutase, etc.

Mediated transport versus enzyme kinetics Cellular physical compartments most often are separated by phospholipid bilayers. Transport across these biological membranes is an important phenomenon. With the exception of a few molecules, most nutrients, metabolites, and ions are transported across such biological membranes through protein mediators. Various modes of transport and their characteristics are listed in Table 37.5. Rate of transport due to simple diffusion increases proportional to the existing concentration gradient – and no saturation is observed. On the other hand, like enzyme catalysis, mediated transport processes show saturation kinetics.

Mediated transport may be passive (facilitated diffusion) or active (input of energy to drive the transport against the prevailing concentration gradient). Both these forms of mediated transport are also amenable to general kinetic analysis used to analyze enzyme catalysis. Rate of transport may be saturated with the substance (denoted as A) transported – plots of initial rates of transport (transport flux, J_{tr}) versus substance concentration show a hyperbolic saturation (similar to the Michaelis–Menten kinetics for an enzyme; see Chap. 15).

$$J_{tr} = \frac{J_{trmax}[A]}{K_{tr} + [A]}$$

Other important features of mediated transport that resemble enzyme kinetics are specificity toward the ligand (compound or ion) being transported, competitive inhibition, pH dependence of transport, and the ability to be modulated by inhibitory substances. For example, the glucose carrier of erythrocyte membranes facilitates the transport of glucose down the concentration gradient. This carrier (a) transports some other sugars like mannose and fructose, albeit less effectively, and (b) is competitively inhibited by 2,4,6-trihydroxyacetophenone. The erythrocyte membrane also has a system for the facilitated transport of glycerol of which ethylene glycol is a competitive inhibitor.

While the ΔG° for a chemical reaction is related to equilibrium constant (K_{eq}), ΔG° for transport (ΔG°_{tr}) is related to concentration ratio of the substance ($[A]_{in}/[A]_{out}$). Just as some enzymes catalyze endergonic reactions at the expense of ATP hydrolysis, in active transport a transporter pumps molecules against a concentration gradient when it is coupled to energy supply. This input of energy may be in the form of ATP hydrolysis or dissipation of a preexisting ion/pH gradient. In a nutshell, mediated transport may be analyzed similar to enzyme catalysis – the former measuring rates of transport from one compartment to the other (across the biological membrane) while the latter deals with reaction rates. Examples of various types of mediated transport may be found in the relevant literature dealing with biochemistry and bioenergetics.

37.8 Glutamine Synthetase: An Anthology of Control Mechanisms

Glutamine is an essential metabolite and serves as a nitrogen donor in many crucial biosynthetic reactions. The enzyme that is responsible for glutamine synthesis is therefore central to cellular nitrogen metabolism. Glutamine synthetase is the sole de novo biosynthetic path to satisfy the glutamine needs of a cell. Along with glutamate synthase, glutamine synthetase offers an ATP-driven route to form glutamate from limiting concentrations of ammonia. In most organisms therefore, glutamine synthetase occupies a pivotal role in the regulation of nitrogen metabolism. The enzyme is particularly well regulated in organisms that have to mobilize ammonia into organic nitrogen. According to their specific needs, the importance of glutamine synthetase and its modes of regulation vary (Table 37.6). In general, the enzyme levels are inversely related to the availability of favorable nitrogen source.

Table 37.6 Glutamine synthetase from different organisms and its regulation

Organism	Oligomeric state	Regulation
Prokaryotes	Dodecamers	Genetic level (induction, repression, etc.); product and feedback inhibition; covalent modification cascade (such as in gram-negative bacteria); divalent metal ion-specific modulation
Yeasts and filamentous fungi	Tetramers and octamers	Genetic level (induction, repression, etc.); different monomeric subunits; aggregation–disaggregation equilibria; substrate (ammonia) availability; product and feedback inhibition
Plants	Dodecamers?	Product and feedback inhibition; substrate (ammonia) availability; covalent modification (symbiotic bacteria associated enzyme)
Mammals	Tetramers and octamers	Availability of substrates (glutamate and ammonia); energy charge; divalent metal ion specific modulation; inhibition by certain metabolites

Glutamine synthetase from *E. coli* is an excellent example of enzyme regulation. It is regulated by the following mechanisms:

- The enzyme protein levels are subject to transcriptional control depending upon the availability of carbon and nitrogen compounds in the growth medium. Glutamine synthetase levels are induced under nitrogen-limiting conditions. Favorable nitrogen sources (like ammonia and glutamine) normally repress its expression.
- Regulation occurs through cumulative feedback inhibition by the multiple end products of glutamine metabolism. Eight different metabolites are known to inhibit the enzyme with separate binding sites for each one.
- Divalent cations (Mg^{2+} and Mn^{2+} in particular) are known to bind and cause kinetically meaningful conformational changes. The native enzyme contains tightly bound Mn^{2+}.
- Through the work of Earl Stadtman's group, *E. coli* glutamine synthetase provides the best characterized example of reversible covalent modification. The biosynthetic ability of the enzyme is controlled by adenylylation and deadenylylation. The addition and removal of AMP moiety (on a surface-exposed Tyr-OH group) modulates catalytic potential, susceptibility to feedback inhibition, and divalent cation specificity. The adenylylated glutamine synthetase ($GS_{(AMP)n}$ in Fig. 37.10) is much less active, has a lower pH optimum, requires Mn^{2+} for activity, and is more susceptible to feedback inhibition. The physiological significance of adenylylation–deadenylylation is apparent from the high levels of covalent modification and low biosynthetic activity on favorable/sufficient nitrogen availability; and the converse was found when the growth medium contained a limiting nitrogen source.

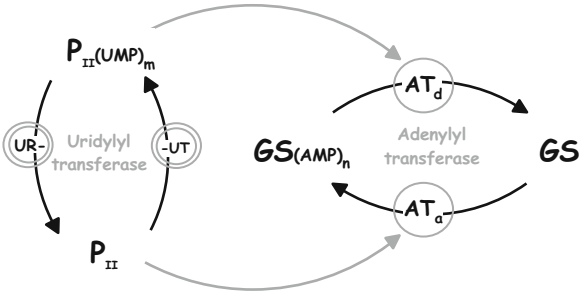

Fig. 37.10 The covalent regulation of *E. coli* glutamine synthetase. The uridylylation cycle and adenylylation cycle are linked through the regulatory protein P_{II}. Glutamine is an activator of UR activity, while 2-oxoglutarate activates UT. The P_{II} protein activates AT_a, while $P_{II\text{-}UMP}$ activates AT_d. Ultimately, AT_a and AT_d are, respectively, responsible for the adenylylation and the deadenylylation of glutamine synthetase. AT_a transfers AMP from ATP to form one adenylyl-O-tyrosyl bond per each subunit of the dodecameric glutamine synthetase (where $n = 1$–12). Covalent modification by UT similarly involves the transfer of UMP from UTP to P_{II} (where $n = 1$–3)

The covalent regulation of *E. coli* glutamine synthetase actually is a closed bicyclic cascade as shown in Fig. 37.10. A single adenylyltransferase catalyzes the adenylylation and deadenylylation reactions at separate non-interacting sites (AT_a and AT_d). This in turn is coupled to another nucleotidylation cycle in which P_{II} protein undergoes reversible uridylylation–deuridylylation. The uridylylation and deuridylylation of P_{II} protein is achieved by a bifunctional enzyme with separate catalytic centers (UT and UR, respectively). The unmodified form of P_{II} protein activates adenylylation (AT_a) of glutamine synthetase while the modified form ($P_{II\text{-}UMP}$) activates deadenylylation (AT_d). In this closed bicyclic cascade, all the covalent modification–demodification steps are dynamic processes and steady states get established rapidly. Glutamine synthetase is a dodecamer and each subunit can be covalently modified; therefore, average state of adenylylation can take values between 0 and 12, depending on the nutritional status of *E. coli*. Between UT, UR, P_{II}, $P_{II\text{-}UMP}$, AT_a, and AT_d, the cellular levels of 2-oxoglutarate and glutamine are sensed. In fact, more than 40 different metabolites are known to affect the activities of one or more of the enzymes of this bicyclic cascade. Thus *E. coli* glutamine synthetase is a very finely tuned enzyme for regulation of its activity and to sense the cellular nitrogen demands.

- Maintaining covalently modified glutamine synthetase for long periods is a poor investment for the cell. Such modified enzyme protein form ($GS_{(AMP)n}$ in Fig. 37.10) is oxidatively modified and leads to inactivation. This eventually renders the protein susceptible to proteolytic turnover.

37.9 Summing Up

Reining in a runaway chemical reaction is just as important as accelerating a sluggish one. While metabolism cannot do without enzyme catalysts, it can be catastrophic not to control reaction rates. Enzymes themselves provide the means for this modulation. Actual intracellular enzyme activity is determined by a combination of parameters like K_M, k_{cat}, n_H, $[E_t]$, $[S]$, $[P]$, $[I]$, and activators.

Steady-state cellular enzyme concentration is an outcome of gene expression at the level of induction, repression, mRNA stability, and translation and by protein degradation. These long-term modes of control often predominate in many prokaryotic microorganisms. On the other hand, preponderance of metabolic regulation via the control of enzyme activity is a common feature of eukaryotes. Control of preexisting enzyme activity is advantageous in terms of rapidity of the system response. This is typically achieved by ligand interaction phenomena such as enzyme inhibition, allostery, and cooperativity. Noncompetitive inhibition is important for the regulation of cell metabolism as the enzyme activity can be affected without a direct substrate analogy.

Table 37.7 Citrate synthase from different organisms: Function and its regulation

Organism	Function	Regulation
Mammalian cells (mitochondria; aerobic)	ATP production	Feedback inhibition by ATP
E. coli (mainly anaerobic)	Production of NADH and biosynthetic precursors	End product inhibition by NADH
Germinating seeds (glyoxysomes)	Glyoxylate cycle; conversion of fatty acid to sucrose	Not regulated by either (as above)

Sigmoid substrate saturation (the so-called homotropic allosteric interactions) can offer means to achieve significant changes in enzyme activity (and response) with just three- to fourfold increase in [*S*]. With increasing n_H values smaller fold increases in [*S*] are required to give the same relative increase in enzyme activity. Since [*S*] in vivo is often held constant (does not vary much), homotropic allostery alone is often unimportant. Its real value is in responding to relevant metabolite activator(s) through reversible shifts between hyperbolic and sigmoid substrate saturation kinetics.

Organisms solve similar metabolic problems in distinctly different ways. The same enzyme in two different organisms may be feedback inhibited by a different set of end products. Aspartate transcarbamoylase is inhibited by CTP in *E. coli*, whereas UTP is the regulatory ligand in plants. Depending upon the organism, inhibition by the same group of end products may be mechanistically distinct (cumulative, additive, concerted, or synergistic). The enzyme and corresponding substrate (or inhibitor or activator) may be sequestered in distinct compartments in different organisms – access itself may be a control feature. Interestingly, the same enzyme may perform different metabolic roles in different organisms; its regulation will accordingly be different in those organisms. Citrate synthase is a good case in point (Table 37.7).

Posttranslational covalent modification of enzymes, either reversible or irreversible, offers unique opportunities for metabolic regulation. Phosphorylation and proteolysis are more common. Besides the capacity for signal and rate amplification, covalent modification of preexisting enzymes allows a system-level mechanism to integrate a range of metabolic signals.

Nature is replete with examples of subtle variety in the mechanisms of control in different organisms – the "unity of biochemistry" concept does not always extend to the metabolic pathway control. Most organisms, however evolutionarily distant, produce/utilize a set of common metabolites; but their concentrations are unique to each individual. This metabolic identity is the consequence of quantitative differences in relevant enzyme properties and their associated regulation. The patterns of enzyme regulation outlined in this chapter are common themes but are not exhaustive by any standard. It would be a surprise if we do not find novel variations of enzyme regulation in the future.

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