THE LIMITS FOR LIFE DEFINE THE LIMITS FOR ENZYMES

Summary

There are natural constraints that limit enzyme concentrations between 10 nM and 10 μM. For signaling switches k_{cat}'s are very low, at 10^{-2}–10^{-5} s^{-1}. For metabolic enzymes k_{cat}'s must be ≥ 1 s^{-1}, and are generally 10–3,000 s^{-1}. It then follows that for metabolic enzymes K_m values are generally limited to be between 1 μM and 1 mM. While increased K_m values would enable much faster k_{cat}'s, there is a clear need for enzymes to be sufficiently discriminating since so many have affinity constants below 100 μM.

2.1 Natural Constraints That are Limiting

The Michaelis–Menten equation expresses the rate of an enzymatic reaction, v, as a function of two other variables, the concentration of substrate, $[S]$, and the affinity of the enzyme for this particular substrate, K_m.

$$\frac{v}{v_{max}} = \frac{[S]}{K_m + [S]}.$$

(2.1)

But the maximum activity, V_{max},* itself is a function of the concentration of enzyme, $[E]$, so that we now have four variables that jointly define any catalytic rate:

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*Some writers object to the use of V_{max}, since this term does not represent a true maximum limit, but simply an upper limit that may vary according to the experimental conditions. If readers are aware of this caveat, then the use of this term will make it easier to be consistent with a large body of enzyme literature.

$$v = \frac{k_{cat}[E]_o[S]}{K_m + [S]}.$$

(2.2)

When doing reactions in a test tube, with an assay volume between 100 μl and 1 ml, scientists routinely vary each of these over a considerable range. Since both k_{cat} and K_m are intrinsic properties of enzymes that have been subject to modification by evolutionary selection, let us first consider the limits to the concentration of an enzyme within a cell.

2.1.1 The Possible Concentration of Enzymes is Most Likely to be Limiting

Bacteria such as *E. coli* have very small cellular volumes, with an average of about 1 μm³, and a range that goes below 0.5 μm³.[53] And only about 70% of this is the aqueous cytoplasmic volume wherein most enzymes will be located.[54] A simple calculation will show that for any enzyme to be present as only a single molecule in the smallest of these cells, this would equal a concentration of 0.5 nM.

Calculations:

Volume$_{cytoplasm}$ = $(0.7 \times 0.5 \ \mu m^3)(10^{-12} \ cc/\mu m^3)(10^{-2} \ L/cc) = 3.5 \times 10^{-15} \ L^†$

1 molecule = $1/(6.023 \times 10^{23} \ molecules/mol) = 1.66 \times 10^{-24} \ mol$

1 molecule/bacterial cell = $\dfrac{1.66 \times 10^{-24} \ mol}{3.5 \times 10^{-15} \ L} = 4.7 \times 10^{-10} \ mol/L$

This minimal quantity is clearly not likely, since whenever the single enzyme becomes damaged or inhibited, the cell would lose that activity completely. As few as 20 enzyme molecules would make this concentration equal to 10 nM, and therefore this value is a more realistic lower limit, and a value of 100 nM may be a more normal operational limit, since it still only stipulates about 200 enzyme molecules of a given type for an active bacterial cell. With the expanded cell volumes of mammalian cells, this same limited number of molecules will then equal a concentration in the low nanomolar range, consistent with the data in Table 1.6.

In Chap. 1, I described the average concentration of enzymes as being about 1 μM for mammalian cells. Table 1.4 shows that for glycolytic enzymes in mammalian cells, concentrations above 2 μM are standard. In a bacterial cell this would be about 4,000 molecules for each of these enzymes. And such a micromolar concentration range is also seen in Table 1.6 for the bacterial enzymes, ODCase and OPRTase, which are in pyrimidine biosynthesis. Since glycolytic enzymes should be at the high end of the concentration range, given their constant work load, then this may be the upper limit for

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†A volume of 3.2×10^{-15} L has been directly measured for *E. coli* cells.[55]

Table 2.1. Natural sources for chemical damage

Source	Intracellular agent	Formation rate (s^{-1})	Protective enzyme	k_{cat} (s^{-1})	Refs.
Oxygen	Oxygen radical ($O_2^{\cdot}$)	10^4	Superoxide dismutase	1×10^4	55, 56
	Hydrogen peroxide	$\geq 10^4$	Catalase	1×10^6	1
Metabolism	Acidity	$\geq 10^2$	Carbonic anhydrase	1×10^6	3, 57
UV light	High energy photon	0.1	Photolyase	0.4	58

concentrations of enzymes in general.[‡] This gives a very definite range limit for the number of each enzyme catalyst that may exist in a cell, and life is possible only when these enzymes function at an adequate rate at these limited concentrations.

2.1.2 The Rate for Enzymatic Steps Must Be Faster Than Natural, but Undesired and Harmful, Reactions

We have a natural sense for time frames that define human actions in the larger physical world. Due to a general enthusiasm for sports, many people have an idea of what the fastest rate is for running, or cycling, or swimming. We are not as interested in lower limits, though culturally we have some awareness of this with such expressions as "Rome was not built in a day." We will again see a range of rates, depending on the actual task to be performed, and guided by the principle that each enzyme must be good enough. A critical starting point for this question is the normal rate for insult to a living cell by the various, ever present sources of chemical and radiation damage, even though these are normally at low levels. Examples include damage from oxygen radicals that form spontaneously, from various types of chemical damage, and from radiation damage which is largely due to ultraviolet rays. Such damage may occur to almost any molecule in the cell, but has long term results mainly when it involves DNA. Therefore, at least a subset of enzymes, with the responsibility of preventing such damaging agents, or of reversing their effects, must have reaction times that are faster than the natural rates for damage, examples of which are in Table 2.1.

We intuitively expect that a damaging agent should not be allowed to exist even for a few seconds, since it may cause too much harm in that time. In addition, if the source appears to be at a high level for the cell, as in the example of the cell bathed by sunlight, then the effective exposure to the source of damage is constant for up to 16 h, or many cell lifetimes. While a single celled organism could clearly survive by remaining in environments that suffered no *UV* exposure, such as deep ocean bottoms, much of life has evolved by being directly dependent on solar energy, or by benefiting indirectly. And we currently have many examples of enzymes that negate oxygen radicals, and repair damage to DNA. The enzymes in Table 2.1 demonstrate appropriately high kinetic rates in this regard, as detailed below.

[‡]It is possible to insert special plasmids, containing a unique gene, into *E. coli* so that the protein coded by this plasmid is expressed at an excessively high concentration, approaching 5 mM. This is an unphysiological, aberrant state for these cells, and should not be seen as contradicting the discussion for normal concentration ranges.

2.1.2.1 Oxygen Radicals

The earliest life actually formed in an anaerobic environment, but with the advent of cyanobacteria a limited oxygen atmosphere was produced by 2.5 billion years ago. Although oxygen led to a dramatic increase in the diversity of microbes and then multicellular eukaryotes, it also provided a new source of toxicity, in the form of oxygen radicals. The formation of $O_2^{\cdot}$ in *E. coli* occurs at a rate of 5 μM s^{-1}.[55] For the actual volume of this cell, this concentration equals about 10,000 oxygen radicals per second. The observed k_{cat} for superoxide dismutase is also 10^4 s^{-1}. This enzyme cannot go much faster since it binds two molecules of the superoxide radical. However, to maximize the removal of superoxide, the enzyme exists at cellular concentrations of 10 μM.[59] This results in an overall very effective rate, so that the enzyme is able to reduce the steady-state concentration of the $O_2^{\cdot}$ to only 10^{-10} M. To assist superoxide dismutase in maintaining a maximum activity, an additional enzyme, catalase, removes the peroxide produced by the dismutase, so that there will never be any significant product inhibition. Catalase itself is also very fast, with a k_{cat} of 10^6 s^{-1}.[1] For rapidly growing cells such as bacteria, this low level of oxygen toxicity is no longer harmful. For very long lived organisms such as humans, this amount of toxicity is seen as a significant factor for cumulative damage leading to senescence.[55]

2.1.2.2 Metabolic Acidity

The normal metabolism of carbohydrates and fats produces carbon dioxide, which is hydrated to form carbonic acid, and the carbonic acid dissociates to produce bicarbonate and H$^+$. This is a potential source of acidity, but organisms have evolved proton pumps to excrete the acid protons, and retain the bicarbonate to act as a buffering agent against other sources of acidity. Additional acidity comes from the formation of lactate under anaerobic conditions, as well as the frequent formation of many organic acids from the sulfur containing amino acids and phosphates. For human metabolism about 80 mmol/day of acids are produced.[57] Approximating this standard rate to a bacterial cell leads to a production of about 110 protons per second for a bacterial volume. Carbonic anhydrase catalyzes the very rapid hydration of carbon dioxide, which dissociates to provide the bicarbonate used in buffering against acids. The activity of carbonic anhydrase in providing bicarbonate easily compensates for the metabolic rate of fixed acid production.

2.1.2.3 Ultraviolet Radiation

Ultraviolet exposure is constant during daylight hours. In vivo experiments with *E. coli* by Aziz Sancar and colleagues have demonstrated the formation of pyrimidine dimers in DNA at a rate of 0.1 s^{-1}.[58] These authors also measured the concentration of photolyase at about 17 molecules/cell (about 10 nM), and a repair rate of 0.4 pyrimidine dimers per second. While this damage rate is naturally a function of the intensity of the UV light, it was observed that over a range of light intensity, this number of photolyase molecules always maintained cell survival.

This low rate of 0.4 s^{-1} is a misleading assessment of this enzyme's activity. That is, the enzyme cannot repair more damaged nucleotides than exist. Unlike other enzymes that have access to a steady concentration of substrate molecules, photolyase must search for the infrequent damaged site. It binds DNA sufficiently well that it spends most of its time sliding along the DNA double helix, until it encounters a damaged site. Based on experiments where the enzyme could be excited by rapid laser pulse, the reaction time for the photochemical repair is on the order of 10^{-12} s, which is remarkably rapid.

We can now extend this concept regarding lower limits on enzyme rates to enzymes in general. Any required chemical reaction must occur faster than the lifetime of a cell. But any specific microbe cannot be too leisurely in its reproductive time, since then other species with faster rates will come to dominate the available resources. The natural driving force from competition will result in reproductive cycles that are fast enough for a species to maintain itself. Bacteria are the ancestral cells, and under optimal conditions of nutrients and temperature, they can undergo cell division to produce two cells in about 20 min. Since nutrients are at an optimum, this means that the concentration of the substrate is not a limiting variable. However, any necessary chemical reaction must normally occur many times within a cell's lifetime, since cell division is a cumulative process in which individual enzymatic reactions, such as the synthesis of the nucleotides required for the duplicate DNA strands, must be performed many times by each enzyme.

Since an *E. coli* genome consists of 4.6×10^6 base pairs, then 9.2×10^6 nucleotides must be produced in at most one half of the cell life time, 600 s, so that the many other steps required for cell division may also occur. If the number of each enzyme molecule is at 100 per cell (50 nM), then each must have a rate of 153 s^{-1} under cellular conditions, meaning that their k_{cat} must be somewhat higher. Since these bacteria are at the same time making almost an equal quantity of RNA (mRNA, rRNA, tRNA), then rates of nucleotides synthesis must actually be about twice as fast. While there are some approximations in this argument, it helps to set some lower limits on the concentration of enzymes, and therefore on their minimum catalytic rates.

There is clearly some flexibility in the final rate necessary as a function of the concentration of that enzyme. Similar to the calculations at the beginning of this chapter, one may readily demonstrate that for the smallest bacterial cell a concentration of 2,000 molecules equals 1 µM. For the calculation above to provide adequate nucleotides, at this higher concentration of 1 µM these same enzymes could satisfy their function with a k_{cat} 20-fold slower, at about 30 s^{-1}. Since enzyme concentrations are almost never above 10 µM, then at this upper limit these same enzymes could be slower, with a rate of about 3 s^{-1}, and still accomplish the needed production of nucleotides within the desired time limit. We again approach the lower rate barrier of 1 s^{-1}. But, since the total protein concentration is itself limited, then only some enzymes can reach such a high concentration, and the majority will clearly need to be faster. This helps to set some lower limits on the concentration of enzymes, and therefore on their minimum catalytic rates.

I have described here logical reasons to account for the observed concentration of enzymes in a bacterial cell, and these values correspond very nicely with the observed values for most enzymes. The not surprising conclusion is that living organisms, responding to the pressures of natural selection, have generally reached a state where
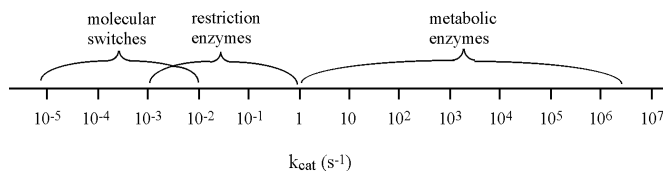
Fig. 2.1. The range for enzyme catalytic rates

their enzymes, as a total system, have reached an optimum balance between possible enzyme concentrations and the rates needed to maintain a dynamic and successfully reproducing organism. Since the majority of the estimated 20,000 enzymes in human cells have not yet been characterized, it is certainly possible that a few will emerge that do have k_{cat} values somewhat below $1\ s^{-1}$. A few such slower enzymes might be sustained by the system, if the greater majority remains consistent with the constraints that I have described.

2.1.3 DNA Modifying Enzymes: Accuracy is More Important Than Speed

I have shown various data to support a lower limit for enzymatic rates of about $1\ s^{-1}$ for enzymes with normal metabolic functions (Fig. 2.1). There is a special group of enzymes whose function is to alter genomic DNA. They may methylate certain bases along the host's genomic DNA to transiently make such genes less available for transcription. They may also cut foreign DNA, belonging to invading viruses or other pathogens, into fragments to make it inactive. Such enzymes must be accurate as to where they modify the host DNA, and also be specific in recognizing restriction site sequences that are unique to the foreign DNA. This accuracy is achieved by greater slowness. Type III restriction enzymes have a k_{cat} of about $1\ s^{-1}$,[60] while type II and type I enzymes have rates of $0.1–0.05\ s^{-1}$,[61, 62] and $0.001\ s^{-1}$.[63]

2.1.4 Signaling Systems: Why Very Slow Rates Can Be Good

There is a special group of enzymes where a much slower activity is necessary, since it defines a limited time for a signal to exist. These signals occur in processes where it is necessary to switch between states of activity, and to maintain the altered state for a transient, but defined period. Depending on the process, this transient time may last for only tens of seconds, for many hours, or even for many years. The defining limit for this transient period is the slow rate at which a key regulatory enzyme makes or cleaves a phosphate bond. Currently known examples include the various G proteins, enzymes that control circadian clocks, and enzymes involved in memory storage. These enzymes are also referred to as *molecular switches* (Table 2.2).

G proteins are themselves regulatory, having two different conformations when they are binding GTP or GDP. Since GTP acts as a regulatory signal, it stabilizes a new conformation in the GTP binding domain, which in turn influences the activity of an adjoining domain in the same multifunctional protein, or a separate target enzyme. The GTP binding domain has a very slow rate for the hydrolysis of GTP, which permits the

Table 2.2. Molecular switches – the slowest enzymes known

Enzyme	k_{cat} (s^{-1})	Reaction	Refs.
GTPase	1×10^{-2}	GTP + H$_2$O $\rightarrow$ GDP + P$_i$	64
KaiC	10^{-3}–10^{-4}	KaiC + ATP $\rightarrow$ KaiC-P$_{1-3}$+ ADP	65
KaiC phosphatase	10^{-3}–10^{-4}	KaiC-P$_{1-3}$ + H$_2$O $\rightarrow$ KaiC + 1–3 P$_i$	65
CaMKII	8×10^{-6}	CaMKII + ATP $\rightarrow$ CaMKII-P + ADP	66

active conformation of this domain to maintain the regulatory stimulus on its neighbor for as long as the GTP remains intact. Upon hydrolysis of the GTP to form GDP, which occurs very slowly at a rate of about 10^{-2} s^{-1},[64] a new conformation occurs, which now has little influence on the neighboring catalytic function that it is intended to control. This slow rate of hydrolysis therefore serves as a built in clock that limits the duration of the regulatory signal to about 100 s. In addition, the GTP binding domain has tighter binding for GDP, so that this product is released slowly, so that the less active/inactive form of the enzyme is now stable for many minutes.

Cyanobacteria have a circadian clock that depends on the phosphorylation state of the protein KaiC.[65] KaiC acts to regulate gene expression in a circadian pattern. It has autophosphorylation and autodephosphorylation activities, and these two activities are regulated by the additional proteins KaiA and KaiB. KaiA stimulates the auto-phosphorylation, while KaiB attenuates this function. Both the phosphorylation and the dephosphorylation rates are remarkably slow (Table 2.2), so that it takes many hours to phosphorylate the protein, and a similar length of time to dephosphorylate. The alterations between these two very slow rates set the circadian pattern as the KaiC protein is converted to the phospho-enzyme state, and then to the native state.

A similar switch pattern is observed for CaMKII, a calcium/calmodulin dependent protein kinase that is involved in memory storage.[66] A memory impulse activates this enzyme by the release of calcium/calmodulin which bind to the hexameric enzyme, and induce it to begin autophosphorylation of that subunit, until the hexamer is completely phosphorylated and activated. The phosphorylated CaMKII can in turn be dephos-phorylated by a specific protein phosphatase. The duration of the signal is enhanced by the fact that the postsynaptic density contains only about 30 enzyme molecules.[67] The postsynaptic density is the visible structural region on the postsynaptic membrane that contains a highly structured complex of molecules.

2.1.5 What is the Meaning of the Many Metabolic Enzymes for Which Slow Rates Have Been Published?

In the literature over the past 50 years there are many published values for metabolic enzyme activities that are well below 1 s^{-1}. This is easily observed with a general data base, such as BRENDA.[33] Inspection of the published values for many enzymes often shows a range in the specific activity for the same enzyme of 100-fold or greater. I tend to trust the higher values. Unless one makes a significant error in recording the activity rate, or in its calculation, one cannot make an enzyme go faster than is normal for it. However, enzymes are often sensitive, and kinetics are done with enzymes that are not in

their normal milieu. It is therefore not unusual that researchers observe low rates, since the enzyme may have become partly denatured during the purification procedure, or some aspect of the assay conditions are not optimal.

Among the most common problems are that intracellular enzymes function in a reducing environment, and those that have surface cysteines may form unwanted intra- or inter-subunit disulfide bonds in an oxidizing storage or assay buffer. Adding reducing reagents, such as dithiothreitol is now normally tested early in a purification. A better choice of buffer is sometimes needed. Phosphate makes an excellent buffer and is very economic. But, when assaying enzymes that bind nucleotides, the phosphate of the buffer will always be a background inhibitor that prevents measurement of the true V_{max}. Cells have many types of proteases that are often constrained in a special organelle (Golgi and endoplasmic reticulum). Disruption of cells to obtain the desired enzyme normally breaks these organelles, so that their proteases now have contact with the desired enzyme. Inhibitors of such proteases are now routinely employed in the early stages of enzyme purification. Further problems emerge with enzyme storage, or loss of a cofactor during dialysis, and so forth. The list of potential problems that are generally preventable can be daunting to new researchers. Because of the ease with which enzyme activity may be unwittingly decreased by the experimenter, caution and judgment are necessary in accepting some of the published rates for enzymes.

2.2 Parameters for Binding Constants

A few simple examples will help to clarify binding constants. To emphasize the general nature of this discussion, let us consider the binding of a proton by acetate, as shown in a normal titration curve (Fig. 2.2). Although the affinity of acetate for binding a proton is poor, since the pK_a is 4.8, it serves as a useful model. This binding constant, the pK_a, defines the concentration of the ligand to be bound, H^+ that is needed for 50% binding. For an approximately tenfold change in concentration above this pK_a, at pH = 3.8, the curve continues to be almost linear before reaching a plateau at 100% saturation. In the same way, down to a proton concentration tenfold lower than the pK_a, at a pH of 5.8, the curve continues to be almost linear before reaching a plateau where there is no binding. Since titration curves are always shown on log plots, it is then a simple mnemonic to remember that the effective range for binding is over almost 2 logs of the concentration of the ligand. This will be true for any binding interaction which occurs at a *constant affinity* by the receptor for the ligand being bound. What is demonstrated in Fig. 2.2 for the binding of a very small ligand, H^+, to a very small receptor, acetate, will also hold true for the binding of much larger ligands to normal enzymes.

2.2.1 The Importance of Being Good Enough

We know that enzymes should evolve to have a binding constant appropriate for optimizing their normal activity. But what defines normal activity for different enzymes? The two obvious constraints are speed and accuracy. If we consider three professions, neurosurgeon, barber, and candy vendor, we intuitively appreciate that we cannot expect
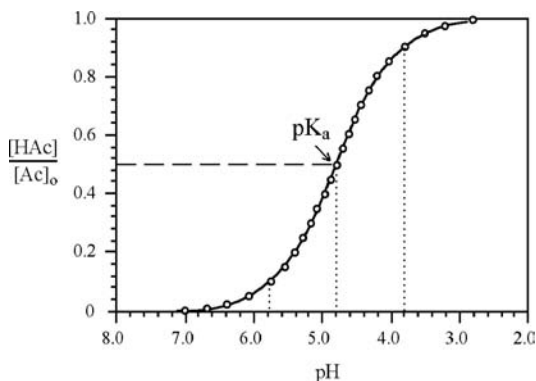
Fig. 2.2. Proton binding by acetate

of each one an equal number of transactions with patients/customers per day. Surgeons need to be very discriminating in what/where they cut. Their speed should be no faster than that speed at which they will make no error. The art of cutting hair is not quite as exact, and barbers can proceed at a moderate speed. Vendors may clearly proceed at faster rates, since they may safely correct occasional errors with no harm to the customer. In the spirit of this metaphor, we expect enzymes involved in DNA synthesis to be more stringent in binding the correct nucleotide to avoid mutations. The main requirement is that their error rate should be low enough so that a sufficient majority of organisms succeed in producing offspring without many mutations. Since the degree of fidelity in mammalian DNA synthesis has an error rate of $<10^{-8}$, it would not be effective to have an enzyme bind with such stringent affinity so as to accomplish this, for the catalytic rate would then be far too slow. An ingenious proof reading function has evolved, which divides the recognition of the correct nucleotide into two steps, so that neither has to be too stringent, and thereby limit the rate of DNA synthesis.

We have already seen the demand for speed in enzymes such as catalase and superoxide dismutase (Table 2.1). These enzymes work in sequential steps to neutralize oxygen radicals. These compounds are formed spontaneously in an oxygen environment, and are very damaging to DNA and therefore mutagenic. It is then not surprising that speed has been selected in enzymes that prevent oxidative damage (superoxide dismutase, catalase) or constantly replenish our buffering capacity (carbonic anhydrase). These enzymes have rates of about 10^4–10^6 s^{-1}. When comparing enzymes, such as the glycolytic enzymes shown in Table 1.4, we see that triose phosphate isomerase is 30 times faster than enolase, and we may be suitably impressed by this very fast enzyme. But, it is also evident in Fig. 1.2 that not all chemical reactions are equally difficult. Therefore, although carbonic anhydrase is a thousand times faster than staphylococcal nuclease, it is the rate enhancement performed by staphylococcal nuclease that is truly astounding. While we will see a spectrum of values for both K_m and k_{cat}, as a general rule each enzyme has been selected to be at least good enough for its specific function.

Table 2.3. Estimated values of K_d consistent with a normal k_{cat}

k_{off} (s^{-1})	K_d (M)	k_{cat} (s^{-1})	
10^8	10^{-1}	10^7	
10^7	10^{-2}	10^6	↑
10^6	10^{-3}	10^5	normal
10^5	10^{-4}	10^4	limits
10^4	10^{-5}	10^3	normal
10^3	10^{-6}	10^2	range
10^2	10^{-7}	10^1	
10^1	10^{-8}	1	↓
1	10^{-9}	0.1	

2.2.2 The Range of Binding Constants

It has been observed that glycolytic enzymes have a K_m for their normal substrate that is equal, or at least comparable to the normal concentration of that substrate.[68] This feature permits some variation in the enzymatic rate as the concentration of its substrate varies under cellular conditions. Under normal conditions the enzyme would be 50% active if $K_m = [S]_{cell}$, and the enzyme would still have an almost linear response to the substrate, even if it declined or increased by about tenfold. But, would it be inefficient to have a binding constant significantly different from the normal $[S]$? Table 1.3 lists a few normal metabolites and their cellular concentrations. We might expect enzymes using such metabolites to have affinities comparable to these concentrations. However, it would be equally correct to say that cells arrange to maintain their metabolites at concentrations that are consistent with the K_ms of the respective enzyme. This may be the more meaningful constraint, since enzymes under selective pressure may evolve to have a binding constant that is good enough. To keep this from being a circular argument, let us first consider the limits for enzyme binding constants.

The on/off binding of the substrate is shown in (2.1). The on rate is assumed to be fairly standard for the encounter of enzyme and substrate, and the actual rate has been calculated to be as high as 7×10^9 s^{-1} M^{-1} for two molecules of equal size in water. In a more physiological medium of appropriate ionic strength, rates of 10^9 s^{-1} M^{-1} have been observed. Also, for the majority of enzymes k_{cat} is slower than k_{off}. Since k_{off} then determines the binding affinity, we may easily approximate the limits for both k_{off} and thence K_d using (2.3).

$$K_d = \frac{k_{off}}{k_{on}}. \tag{2.3}$$

Table 2.3 shows the calculated results for assuming $k_{on} = 10^9$ s^{-1} M^{-1}, when k_{off} varies between 1 and 10^8 s^{-1}. The values for K_d in this table are calculated with (2.4).

$$K_{d} = \frac{k_{off}\,(s^{-1})}{10^{9}\,M^{-1}\,s^{-1}}. \tag{2.4}$$

To estimate values of k_{cat}, also assume that k_{cat} will be tenfold lower than k_{off}, though the true difference is frequently much greater. From these calculations we then see that in order to have the minimal activity of 1 s^{-1}, K_{d} should be no lower than 10^{-8} M. To have the highest activities so far measured, K_{d} can be as high as 10^{-2} M. This range of K_{d} values has been observed for different enzymes, and is close to the limit of what appears to be possible. These values define the boundaries for normal enzymes, although for most enzymes k_{cat} has values of 10–3,000 s^{-1}. Though we normally have a sense that faster enzymes should be better, only about 20 different enzymes have been shown to have kinetic rates greater than 10,000 s^{-1}.[33] Even for the enzymes of glycolysis, the highest flux pathway in the cell, no enzyme has a rate greater than 3,000 s^{-1} (Table 1.4).

A note of caution is necessary, since the assumptions used to calculate Table 2.3 do not absolutely apply to every enzyme. But, they are a useful guide for the majority of enzymes, and the values so calculated are very consistent with measured values that are currently known. These calculations then tell us that for enzymes to have normal rates, with a k_{cat} of 10–3,000 s^{-1}, they should have affinity constants of 10^{-7}–10^{-4} M. And these affinity values are quite comparable to the normal concentrations of the respective substrates.

Why are not affinity constants much lower than $[S]_{cell}$? Tight binding might be better, since it will give the best discrimination for the specific substrate. In accord with this hypothesis is the fact that there is a distinct, unique enzyme for almost every single reaction. As a simple example, purine and pyrimidine nucleosides need to be phosphorylated three times to produce the nucleoside-triphosphates that are essential metabolites. It might be possible to have a single kinase able to perform each of these reactions, if it had a nondiscriminating catalytic site at which any of the intermediates could bind. We find that there is almost a separate kinase for each nucleoside, and nucleoside monophosphate, with only a few enzymes serving two substrates. Only nucleoside-diphosphate kinase is able to bind and phosphorylate all of the nucleoside diphosphates. This demonstrates that the ability to specifically control each of the pathways leading to ATP, GTP, CTP, and UTP is sufficiently necessary that almost all organisms make the appropriately distinct enzymes for each step. Clearly, for an enzyme to be distinct, it must bind its specific substrate well, while binding close analogs fairly poorly. This means the binding constant for the normal substrate must be below 1 mM, and preferably below 100 μM. In Fig. 2.3, we see that three fourths of the K_{m} values are below 100 μM. But again, binding should only be as stringent as necessary, while not impeding the required catalytic rate. Therefore, only one K_{m} value is below 1 μM.

Why should not affinity constants be sufficiently higher than $[S]_{cell}$? A high K_{m} means poor binding, and that in turn means the rate can be much faster. If the enzyme does not bind the substrate tightly, it will not bind the product tightly, since most of the
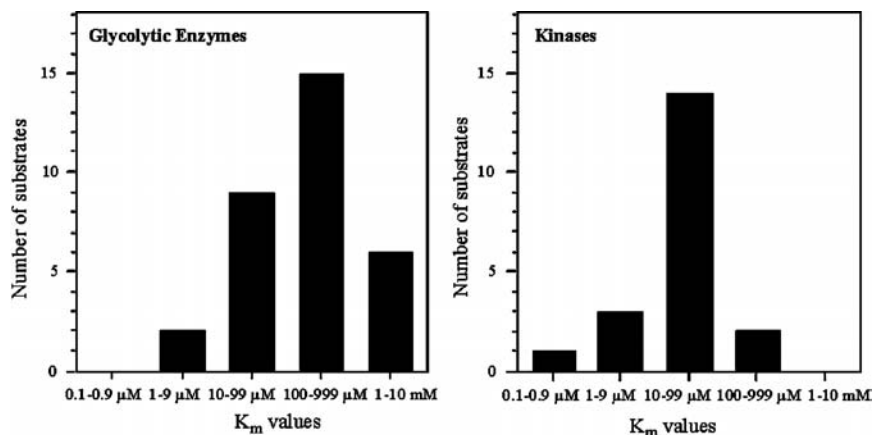
Fig. 2.3. K_m values for substrates of the glycolytic enzymes, adapted from Fersht,[68] and of nucleoside/nucleotide kinases. For the kinases the K_m values are for the acceptor substrate[69–86]

same binding determinants are in both of these molecules. If the product is not bound tightly, then it will normally dissociate very rapidly from the enzyme, so that the enzyme is again free to bind another substrate molecule and continue to be productive. But poor binding means that the substrate-binding site is not well defined, and similar molecules that resemble the substrate may also bind there. This means that the enzyme is no longer very discriminating. Sometimes this feature is desirable, as with general proteases which function in the catabolism of proteins to recover the amino acids.

More generally this poor discrimination may not be useful, and the need to control the synthesis or catabolism of most molecules has led to enzymes somewhat more specific for a preferred substrate, as suggested by the normal range and limits indicated in Table 2.3. If speed were sufficiently desirable, then by now we should see that need expressed in a lot of weak binding constants. Figure 2.3 shows such values for the ten glycolytic enzymes, as well as for a group of nucleoside and nucleotide kinases. For the glycolytic enzymes about three-fourths of the K_m values are below 500 μM, and over one-third are below 100 μM. On average, K_m values for the kinases are more than tenfold lower than for the glycolytic enzymes, since most of the kinases have K_m values that are below 100 μM. K_m values above millimolar would be more consistent for an emphasis on the speed of the reaction.

An important contrast emerges for the two groups in Fig. 2.3. Glycolytic enzymes generally have higher K_ms. Glycolysis is the highest flux pathway. Therefore, we see that K_m values are higher than average for these enzymes, to enable the turnover rates required. With these higher K_ms, the enzymes may frequently bind an incorrect metabolite, but they will not bind it tightly and will therefore release it almost instantly. And should they react chemically with it, the new compound produced may still have a use, since the cell has a variety of six carbon sugars, and their three carbon derivatives.
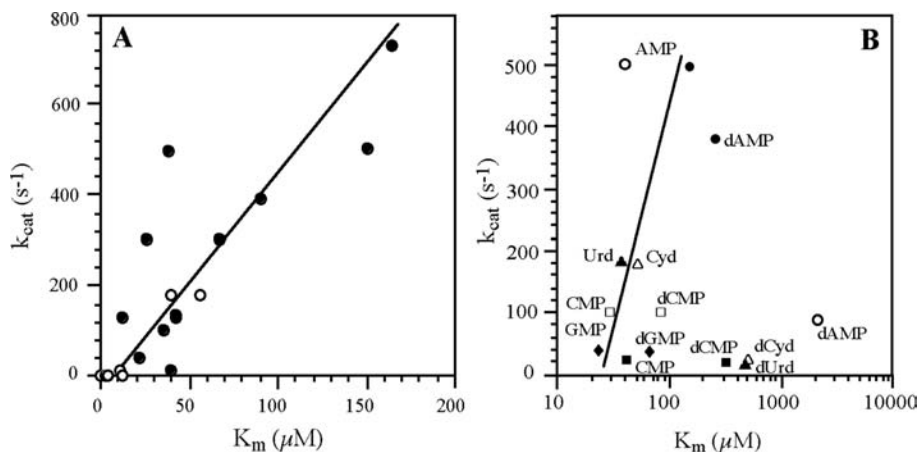
Fig. 2.4. Specificity of nucleoside and nucleotide kinases. (**A**) Nucleoside (*open circle*) and nucleotide (*filled circle*) kinases with values for their principal acceptor substrate. The best fit defines the specificity constant as 3.9×10^6 s^{-1} M^{-1}. (**B**) For some enzymes in (**B**), the data point for the principal substrate, a ribonucleoside or ribonucleotide is reproduced, along with the line denoting the specificity constant, as well as data for the deoxy analogs tested with these enzymes. The same symbol is used for data points for the same enzyme. Note that the scale for the abscissa has changed

With the kinases we see a spectrum of affinity values (Figs. 2.3 and 2.4). The highest K_ms for these are at 170 μM, which is below the average for the glycolytic enzymes, but many show much tighter binding. These latter are deoxynucleoside kinases, whose activity is not constantly needed, since most cells do not need a steady supply of deoxynucleotides at all times. Overall, we see a balance between the need for speed vs. the need for specificity and control. A guiding restraint is the need for an appropriate, or at least a minimal rate for each specific enzyme. Because tighter binding leads to a slower overall k_{cat} there is a limit to how discriminating an enzyme can be.

With these two opposing constraints, enzymes have evolved to have just the right affinity for their substrate. Due to the type of selection constraints described here, the concentration of the substrate is normally comparable to the affinity constants of enzymes that bind it. And the concentration of different substrates may vary over a range from micromolar to perhaps 10 mM, but again each substrate exists at a concentration appropriate for the rate at which it is consumed in however many pathways that require it.

2.3 Enzyme Specificity: k_{cat}/K_m

We can simplify (2.2) for the situation where the normal substrate concentration is low, since this would be the condition when an enzyme might more easily bind an available analog. At low [S] this term drops out of the denominator of (2.2) to give:

$$v = \frac{k_{cat}}{K_m}[E]_o[S]. \tag{2.5}$$

The preceding discussion on defining the limits for rates and affinities of enzymes has established that these two features are related, and therefore an enzyme may achieve an ideal balance by optimizing the ratio of k_{cat}/K_m, which describes the enzyme's efficiency for any substrate or metabolite. This ratio is also known as the *specificity constant*. Since within a cell any enzyme may encounter a variety of molecules that are analogs of its normal substrate, either of these terms in the specificity constant may vary depending on how well the enzyme and metabolite interact. When both k_{cat} and K_m change correspondingly, the specificity constant is not varied. But, in vitro one might demonstrate, that some analog binds with a K_m 100-fold higher than for the normal substrate, but with the same k_{cat}. Then the specificity constant for the analog is 100-fold lower. The specificity constant then permits a meaningful quantitative comparison for an enzyme's ability to chemically react with a substrate.

2.3.1 A Constant k_{cat}/K_m may Permit Appropriate Changes for Enzymes with the Same Enzyme Mechanism

To see the effects of the constraints discussed above, it is helpful to examine a single group of enzymes that all have the function of phosphorylating either a nucleoside or a nucleotide. These enzymes appear to be descended from a common ancestor,[87] but have diversified so that most of them are fairly specific for a single substrate, or sometimes for two similar substrates. As shown in Fig. 2.4a, they have also diverged in the affinity for their principal substrate, and concomitantly in their maximum rates. The separate data points in this figure are based on values from the literature for the different enzymes. The best fit to these data defines a line with a slope equal to k_{cat}/K_m, which has a value of 3.9×10^6 s^{-1} M^{-1}. This specificity defines this set of enzymes as completely normal or average for these values. A few of the sample data points in Fig. 2.4A deviate noticeably from the average values. These may be true outliers for which some special explanation may yet be obtained, but they may also reflect some variation in how the data were obtained by different laboratories and with changing technologies.

   Although they are descended from a common precursor, we also see that the individual enzymes have in fact altered their affinities and therefore their rates, while maintaining the same specificity constant. The most discriminating enzymes are deoxythymidine kinase and deoxycytidine kinase. They have a K_m at 1–2 μM, and consequently are also very slow with a k_{cat} of just above 1 s^{-1}. At the other end are UMP kinase from pig, and AMP kinase from chicken. These enzymes have very poor affinities, with high K_ms at 150–170 μM, but are therefore significantly faster with maximum rates at 500–700 s^{-1}.

   In Fig. 2.4A we again see the specificity that many of these enzymes have for their normal ribonucleoside or ribonucleotide substrates. These enzymes will also phosphorylate the deoxy versions of the normal substrates, but normally at a lower k_{cat}, despite a very much greater K_m. Only two enzymes, GMP kinase and one of the CMP kinases, have almost as good a rate with the deoxy substrate. The difference in specificity is normally greater for enzymes that already have a low K_m for the normal substrate.

There is a definite trend between the measured K_ms of the enzymes for their acceptor substrates, and the measured concentration of these substrates in different cells: AMP and UMP normally exist at >100 μM, while deoxynucleosides are below 1 μM.[31] If an enzyme such as deoxycytidine kinase has a substrate normally present at 1 μM or below, then it must have an appropriately lower K_m in order to discriminate for this uncommon substrate. Enzymes such as UMP kinase can afford higher K_ms, since their normal substrate is sufficiently abundant at a concentration above 100 μM. While this has not generally been measured, it would be logical for the high K_m, high k_{cat} enzymes to be present at lower concentrations, as long as their actual rate of catalysis is adequate for the conditions of the cell in which they function. Then, even though UMP kinase may also bind some of the other pyrimidine substrates in the cell, such as deoxythymidine or deoxycytidine, it will bind them more poorly, and because UMP kinase is itself at a lower concentration, it will not contribute much to the normal synthesis of dCMP or dTMP. Therefore, the varied kinetic properties for the enzymes in Fig. 2.4 are consistent with the cell being able to have enough control for the formation of each nucleotide.

2.3.2 The Specificity Constant may Apply to Only One of the Two Substrates for a Group of Enzymes with the Same Mechanism

The kinases in Fig. 2.4 are named for the acceptor substrate, to which the phosphate group will be transferred. And as we see in Fig. 2.4, there exists the same specificity constant for all the acceptor substrates of this set of related kinase enzymes. Since all these enzymes use the same phosphate donor substrate, ATP, it is interesting to note that they have no constant specificity for ATP, as shown in Fig. 2.5. This figure shows no common feature for the use of ATP, though K_m values are mostly below 200 μM. While it is logical for these kinases to show discrimination for their preferred acceptor substrate,
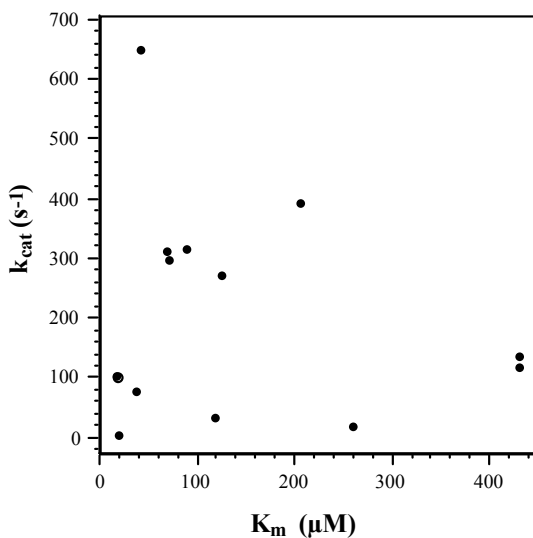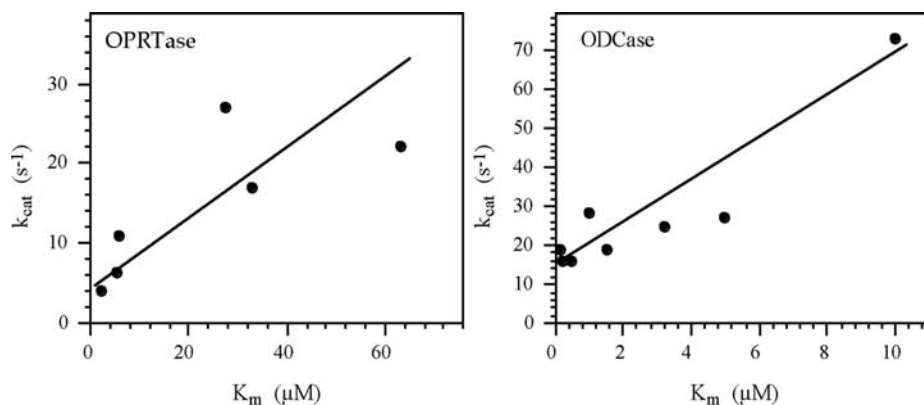

Fig. 2.5. Specificity of nucleoside and nucleotide kinases for ATP

Fig. 2.6. Specificity constants for the same enzyme activity, for enzymes from different organisms. OPRTase, orotate phosphoribosyltransferase; ODCase, OMP decarboxylase

it is not necessary for them to show a comparable affinity for ATP, though this might be expected given that these enzymes are related to the same ancestor.

Again, it is also worth noting that the affinity for ATP is almost as strong as it is for the acceptor substrates. There presumably is no stringent need for these enzymes to show a preference for the phosphate donor. In terms of the chemical reaction for a kinase, any nucleoside triphosphate (NTP) would be an energetically equivalent donor substrate. And studies with uridine kinase have shown that this enzyme does not discriminate at the catalytic site between ribo-NTPs and deoxyribo-NTPs, and also accepts purine and pyrimidine NTPs.[74] Such results are consistent with the phosphate donor site of this enzyme being mostly occupied by the three phosphate groups, since little binding discrimination is evident for the ribose or the base.[88] One might then expect a very high, nondiscriminating K_m for ATP, and uridine kinase does have the highest K_m for ATP in Fig. 2.5. Most of the other enzymes show a better affinity, suggesting again that some degree of discrimination is normally needed even for the phosphate donor.

ATP is one of the most abundant metabolites in cells, normally having a total concentration of 2.5 mM or higher.[31] One might then expect that kinases could have quite a high K_m for ATP, since they would always be able to bind it well enough. However, an average cell has at least several thousand kinases, for a total concentration of these ATP-binding enzymes of perhaps 2 mM. Then, the actual free concentration of ATP is perhaps only 0.5 mM, or even lower. If most kinases should be more active only when the cell ATP pool is abundant, then their affinities for ATP should be consistent with such available ATP concentrations. This hypothesis is consistent with the otherwise surprising data that kinases generally have low K_ms for ATP.

However, if discrimination for a phosphate donor is not in fact necessary, then the variation that is observed may simply be a concomitant result as these enzymes have evolved their separate specificity for the primary acceptor substrate. That is, a mutation leading to a desired change in affinity at the acceptor site, may have a modest influence

on the adjacent ATP binding site, leading to a variety of affinities for ATP that are still good enough for normal phosphotransfer reactions.

2.3.3 The Same Enzyme Can Maintain Constant Specificity While Adapting to Changes

We saw in Fig. 2.4 that a group of enzymes with the same type of reaction can have a constant specificity for their acceptor substrate, while still varying in their specific rates and affinities. The exact same flexibility is also evident if one examines a single specific enzyme reaction. Figure 2.6 shows such results separately for the enzymes orotate phosphoribosyltransferase (OPRTase),[36, 89–94] and OMP decarboxylase (ODCase).[36, 89, 94–99] Based on sequence alignments the OPRTases come from a common ancestor, as do the ODCases.[100]

For both of the enzymes in Fig. 2.6 there is a greater than tenfold range in the affinities for the principal substrate when enzymes from different organisms are compared. These variations in affinity may then reflect some differences in the need for how discriminating the enzyme needs to be in whatever cell it serves. For both enzymes in Fig. 2.6, those examples with the lowest values for k_{cat} and K_m are from mammals. If one interprets this sample set from microbes to humans as an evolutionary continuum, these results would support the interpretation that discrimination is more important than speed for these two enzymes. This is then an interesting evolutionary choice, since these two enzymes have activity rates at the low end of the range for such values.

2.3.4 The Limits to k_{cat}/K_m

The formulation of the specificity constant allows this value to be highest when either k_{cat} is maximized or when K_m is lowest. As a simple illustration of this, let us use the extreme limits of k_{cat} and K_m for calculating a specificity constant of 10^7 s^{-1} M^{-1}:

$$\frac{k_{cat}}{K_m} = 10^7 \, \text{s}^{-1} \, \text{M}^{-1} = \frac{10^7 \, \text{s}^{-1}}{1 \, \text{M}} = \frac{1 \text{s}^{-1}}{10^{-7} \, \text{M}}.$$

This is intended to illustrate the range for either of the two variables in this relation. For most enzymes, a balance between these two extreme positions is observed. It does emphasize the point that high specificity not only is provided by the obvious high affinity of a low K_m, but may also be produced by a very poor K_m when that leads to an exceptional k_{cat}. Examples of this diversity are shown in Table 2.4.

For natural enzymes, the efficiency is normally $\geq 10^5$ s^{-1} M^{-1}. But for artificial enzymes, such as DNAzymes and abzymes, the specificity constant is normally at 10^3 s^{-1} M^{-1} or much lower. The efficiency for the DNAzyme in Table 2.4 approaches the lower range for normal enzymes. Although it is still very slow, it is quite an achievement for the scientists who constructed it. The abzyme shown is also one of the most efficient artificial enzymes developed, but since it only has to increase the activity by 10^6 over k_{non}, this is not that difficult a chemical reaction.

Table 2.4. The range of observed specificity constants

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m (s^{-1} M^{-1})	Refs.
4-Oxalocrotonase tautomerase	2-Hydroxymuconate	2.9×10^6	1.9×10^{-4}	1.5×10^{10}	37
Superoxide dismutase	$O_2^{\cdot-}$	1×10^4	1.3×10^{-3}	8×10^8	56
Carbonic anhydrase	CO_2	1×10^6	1.2×10^{-2}	8×10^7	3
Catalase	H_2O_2	4×10^7	1.1	4×10^7	1
Uridine kinase	Uridine	180	4×10^{-5}	4×10^6	74
Orotate phosphoribosyltransferase	Orotate	4	2×10^{-5}	2×10^5	94
β-Alanine synthase	NCβA[a]	0.6	9×10^{-6}	7×10^4	101
Abzyme	Nitrobenzisoxazole	0.66	1.2×10^{-4}	5×10^3	102
DNAzyme	ODC RNA[b]	2×10^{-4}	6×10^{-7}	3×10^3	103

[a] N-carbamoyl-β-alanine
[b] Ornithine decarboxylase mRNA

Since the specificity constant, as a second order constant, cannot exceed the rate of diffusion that governs the encounter of two molecules, values $\geq 10^8$ s^{-1} M^{-1} are normally interpreted as indicating near perfection for such enzymes. In a general sense, we might assume that only a little mutational fine tuning is needed to adjust any enzyme to have somewhat better k_{cat} or K_m, and thus to approach this plateau of perfection. It is quite likely that for many enzymes this will remain an unattainable limit. A limiting feature that is frequently unappreciated is the actual difficulty of the chemistry for some reactions. Evidence for this is in Fig. 1.2, where we see that for some reactions, the un-catalyzed chemistry is incredibly slow, because it is so difficult. Considering the architecture of most catalytic sites, it is almost standard for two or three amino acid residues to participate in the actual chemistry, as opposed to the binding of the substrate. Frequently a metal cofactor or an organic cofactor may also be involved when they provide an appropriate benefit.

Most enzymes have three amino acids that participate in the reaction chemistry.[111] While two amino acids, or even one, might be enough for some types of chemistry, with

Table 2.5. Kinetic rates for ribozymes[a]

Enzymatic reaction	k_{cat} (s^{-1})	K_m	k_{cat}/K_m (s^{-1} M^{-1})	Refs.
Natural ribozymes				
RNA cleavage	0.2	20 nM	10^7	104
RNA cleavage	0.0017	1 nM	1.5×10^6	5
Self-splicing intron	0.001	1 nM	9×10^5	105
RNA cleavage	0.004	43 nM	9×10^4	106
Peptide bond formation	5	5 mM	10^3	107
Engineered ribozymes				
RNA self-ligation	1.1	9 μM	1.2×10^5	108
Aminoacyl esterase	0.1	450	2.2×10^5	109
RNA self-cleavage	0.023	49	4.8×10^5	110

[a] When more than one RNA was studied, only the most active is listed

three amino acids the active site will always assure that the substrate binding has the correct chirality. However, with three or more amino acids, a limited number of special arrangements are possible for these catalytic agents, and the perfect three-dimensional organization may not be available for all chemical reactions. And even when it is achievable, the process of natural selection appears generally to have been satisfied with enzymes that have not attained this ideal of perfection. In this sense, we may appreciate those enzymes with the highest specificity constants, without expecting this to be a standard that most enzymes will achieve.

2.3.5 Ribozymes and the RNA World?

We have increasing reports of RNAs that have a catalytic function.[112] Since such RNAS are found in so many different species, their existence is frequently used to support a model for an "RNA world,"[113, 114] to signify a time before proteins had appeared, and when RNAs were the principal molecules for both catalytic functions and information storage. This model proposes that proteins came later since they require ribosomes to be synthesized, and ribosomes contain many RNAs. DNA also appeared later, and being much more stable, it then assumed the storage of information. Since proteins are more complex and versatile they emerged to take on almost all catalytic functions. Current ribozymes are seen as the vestiges of an early more complex RNA world.

One serious difficulty with the RNA world hypothesis is that natural ribozymes have very limited catalytic functions, with the majority only able to cleave or ligate phosphodiester bonds. They are also very slow catalysts (Table 2.5), seldom having k_{cat} values greater than 0.1 s^{-1}. These features may be explained by assuming that the RNA world was slower, and that other catalytic functions for RNAs had also existed, but have disappeared as protein enzymes replaced them. But, since we have many examples of very efficient protein nucleases, why have the currently existing ribozymes also not been replaced by the more efficient protein enzymes?

An attractive answer is that currently existing ribozymes generally function as riboswitches to control transcription of a gene, or the processing of its transcript.[114] Since such functions are not directly involved in maintaining a steady state level of some metabolite, speed is not as critical as discrimination for the correct bond to cleave. When compared to protein regulatory switches (Fig. 2.1), these riboswitches have similar kinetic qualities, and there would be no benefit to a cell for replacing them with proteins that could not do the job any better. Base pairing provides the most direct means for binding to a specific site on a nucleic acid, and we see that the ribozymes/riboswitches mostly have binding constants in the low nanomolar range (Table 2.2), much tighter than some of the protein transcription factors. There is no surprise that many RNAs have been employed for such a function.

The literature promoting an RNA world is too extensive for a proper discussion here. The second serious difficulty with this hypothesis is: how was RNA produced without enzymes? While this question also applies to enzymes or proteins in general, it is worth noting that there is sound experimental evidence for the formation of amino acids and polypeptides in an abiotic system, with only ammonia, carbon monoxide, and metal catalysts (Fe, Ni, and Na$_2$S).[115, 116] This provides evidence for the spontaneous formation of polypeptides, requiring only the simplest of starting compounds and conditions, that

are comparable to what should have been available in the earliest abiotic seas. We have no such demonstration for an abiotic synthesis of polynucleotides.

Many metals would have been available in the abiotic seas, and they would have influenced the emergence and diversification of protein enzymes in two phases. Those polypetides or small proteins that were able to fold and bind to an available metal would have been stabilized by such binding, making them more abundant. Therefore, simply due to the stabilizing benefit of binding a metal, metalloproteins would have become more widely established in this initial phase. Since metals contribute to the chemistry of so many reactions, some of these new metalloproteins would have had an enzymatic function, and this would then have led to the natural formation of a diverse mixture of simple metalloenzymes. This suggestion is supported by the fact that the majority of currently characterized enzymes are metalloenzymes.

Once such simple catalysts are present, more complex molecules including RNAs can then be produced in a steady manner. While RNA clearly preceded DNA in early life forms, the available research data suggest that the earliest biological world must have included an abundant mixture of simple enzyme catalysts. While some ribozymes should have existed at this time, they were not the unique species for catalysis. Initially RNA would have become important for storing genomic information, while proteins continued to evolve into better catalysts.

As life forms continued to evolve, better methods for the regulation of metabolism would have enhanced the survival of such cells. Such improvements in the control of metabolism would have largely involved proteins to produce ever more complex allosteric regulatory enzymes. However, the process of life is opportunistic. The appearance and continued use of riboswitches as transcription factors provides a natural and logical benefit to their cells.

The one clear exception among the ribozymes is the ribosome which has a rate for peptide bond formation of 5 s^{-1} (Table 2.5). Since the atomic structure of the large ribosomal subunit shows only RNAs at the catalytic center, then the ribosome is also a ribozyme.[117] A distinctive feature for the ribosome is that it has a very modest affinity for the peptide, which permits more rapid turnover, although the k_{cat} is still near the lower limit for a metabolic enzyme (Fig. 2.1). Note that the value of k_{cat}/K_m is $10^3 \text{ M}^{-1} \text{ s}^{-1}$, making the ribosome one of the least efficient enzymes.

The need for a certain level of accuracy in the process of translation precludes that this mechanism should go very rapidly. Since the other two components of the translational complex are mRNA and tRNA, then the possibility of specific alignments is a direct benefit in having rRNAs as the central catalytic reactants of the ribosome. As an example, the 23S rRNA can bind to the conserved CCA terminus of any tRNA.

It must also be noted that ribosomes contain more than 50 proteins, and these had to exist before the ribosomal translation process had become standard. It is therefore plausible that the ribosome emerged in an early "protein world" where the above benefits of using RNAs made the RNA–protein complex of current ribosomes a successful catalyst to mediate the translation of messenger RNAs.