The biological significance of substrate inhibition: A mechanism with diverse functions

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Many enzymes are inhibited by their own substrates, leading to velocity curves that rise to a maximum and then descend as the substrate concentration increases. Substrate inhibition is often regarded as a biochemical oddity and experimental annoyance. We show, using several case studies, that substrate inhibition often has important biological functions. In each case we discuss, the biological significance is different. Substrate inhibition of tyrosine hydroxylase results in a steady synthesis of dopamine despite large fluctuations in tyrosine due to meals. Substrate inhibition of acetylcholinesterase enhances the neural signal and allows rapid signal termination. Substrate inhibition of phosphofructokinase ensures that resources are not devoted to manufacturing ATP when it is plentiful. In folate metabolism, substrate inhibition maintains reactions rates in the face of substantial folate deprivation. Substrate inhibition of DNA methyltransferase serves to faithfully copy DNA methylation patterns when cells divide while preventing *de novo* methylation of methyl-free promoter regions.

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The kinetics of an enzymatic reaction are typically studied by varying the concentration of substrate and plotting the rate of product formation as a function of substrate concentration. In the conventional case this yields a typical hyperbolic Michaelis-Menten curve, and a linear reciprocal Lineweaver-Burk plot, from which the kinetic constants of the enzyme can be calculated. A surprisingly large number of enzymes do not behave in this conventional way. Instead, their velocity curves rise to a maximum and then decline as the substrate concentration goes up. This phenomenon is referred to as substrate inhibition, and it is estimated that it occurs in some 20% of enzymes [1]. A partial list of enzymes that show substrate inhibition appears in Box 1.

Substrate inhibition is often interpreted as an abnormality that comes from using artificially high substrate concentration in a laboratory setting. In a review article on the mechanisms of substrate inhibition in 1994, Kuehl [2] commented that ''although recognized early on as an almost universal phenomenon, it has nevertheless met an almost universal disinterest. Probably the main reason for this neglect is that the majority of enzymologists and many authorities in the field regard substrate inhibition as being almost always a nonphysiological phenomenon.''

There are several reasons for suspecting that substrate inhibition is not a pathological phenomenon, but a biologically relevant regulatory mechanism. First, in many cases normal substrate concentrations are to the right of the velocity maximum, which indicates that these enzymes typically operate under substrate inhibition. Second, many enzymes have specialized sites where a second substrate molecule can bind and act as an allosteric inhibitor. For those enzymes, substrate inhibition is clearly a specially evolved property. Third, evidence is accumulating that substrate inhibition plays critical regulatory roles in a number of metabolic pathways.

Substrate inhibition means that the velocity curve of a reaction rises to a maximum as substrate concentration increases and then descends either to zero or to a non-zero asymptote. Many mechanisms are known that can result in such substrate-velocity curves [3, 4]. Here we discuss two simple mechanisms. Suppose that an enzyme, E, has two

Box 1

A short list of enzymes that are subject to substrate inhibition

4-hydoxyphenylpyruvate hydroxylase
acetylcholinesterase
adenosine 5′-pyrophosphate sulfurylase
adenosine kinase
adenylate cyclase
aldehyde dehydrogenase
alanine aminopeptidase
alcohol dehydrogenase
aldehyde dehydrogenase
aldose reductase
alkaline phosphatase
aminoacylase-I
aminoimidazolecarboximide ribotide transformylase
arylamidase
aspartate transcarbamylase
carboxypeptidase
cholinesterase
citrate synthase
cytochrome P450 (some)
diamine oxidase
diphospoglyceromutase
DNA-methyltransferase
enolase
esterase
formyltetrahydrofolate synthase
fructose-l,6-bisphosphatase
galactosyltransferase
gentamycin acetyltransferase
glutamate dehydrogenase
glutathione reductase
glycerol-3-phosphate dehydrogenase
HIV1-reverse transcriptase
isocitrate dehydrogenase
kynunrenine aminotransferase
lactate dehydrogenase
L-amino acid oxidase
lipoxygenase
malate dehydrogenase
N-methyl transferase
nucleotidediphosphate kinase
O-acetylserine sulfhydrolase
octopine dehydrogenase
PAPS synthetase
phenol sulfotransferase
prenyltransferase
purine nucleoside phosphorylase
pyrophosphatase
pyruvate decarboxylase
pyruvate kinase
ribonuclease A
ribonuclease T1
ribonuclease T2
ribonucleoside diphosphate reductase
serine hydroxymethyltransferase
sucrose-6-glycosyltransferase
sulfotransferases
trannsglucosyl-amylase
tRNA nucleotidyltransferase
trypsin
tryptophan hydroxylase
tyrosine hydroxylase
urease
uridine kinase
xanthine oxidase
α-D-galactosidase
α-glucosidase
β-fructofuranosidase
β-hydroxysteroid dehydrogenase

After Kaiser (1980)

binding sites for its substrate S, a catalytic site for binding that can produce the product, P, and a non-catalytic (or allosteric) site that can produce the product at a reduced rate (see Fig. 1). We denote by E·S the substrate bound to the catalytic site, by S·E·S two substrate molecules bound to both the catalytic and the non-catalytic site. Haldane [5] considered the simple case when a substrate molecule binds first to the catalytic site, followed by a substrate binding to the non-catalytic site, (as shown in Fig. 1), and assumed $k_4 = 0$. Then, using the rapid equilibrium assumption, one can derive the kinetic formula

$$\frac{V}{E_0} = \frac{k_2[S]}{K_m + [S] + \frac{[S]^2}{K_i}} \quad 1$$

where E_o is the total amount of enzyme present, K_m the Michaelis constant, and K_i is the dissociation equilibrium

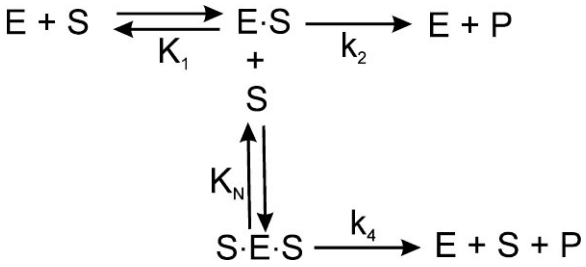

Figure 1. A reaction diagram for substrate inhibition.

constant ($1/K_N$) for the reaction $S \cdot E \cdot S \leftrightarrow S + E \cdot S$. This is Haldane's formula for substrate inhibition [5]. As there are two powers of [S] in the denominator, the velocity goes to zero as [S] becomes large. Intuitively, this is because more and more of the enzyme is tied up in the unproductive ternary complex.

If we relax Haldane's assumption and allow random-order binding of the substrates to the catalytically active and inactive sites, and allow $0 < k_4 < k_2$, so the ternary complex can produce the product, but at a reduced rate, then one can derive the velocity formula

$$\frac{V}{E_0} = \frac{k_2[S] + k_4\frac{[S]^2}{K_i}}{K_m + [S] + \frac{[S]}{k_1 K_i} + \frac{[S]^2}{K_i}} \quad 2$$

In this case, the velocity curve rises to a maximum and then descends to $E_o k_4$ as [S] gets large. The value of K_i affects the shape of the velocity curves described by Equations (1) and (2). As K_i gets larger the peak moves to the right and the curve descends more slowly. Figure 2 shows the substrate inhibition curves for tyrosine hydroxylase and tryptophan hydroxylase. The K_m values are the same but tryptophan hydroxylase has a much higher K_i value. As $K_i \to \infty$, one regains the hyperbolic Michaelis-Menten curve. This makes sense because when K_i is large there is very little enzyme tied up in the ternary complex.

Our purpose here is not to describe the biochemical origins of substrate inhibition beyond this brief introduction. Rather, we want to discuss the biological functions of substrate inhibition. We use as our exemplars five enzymes that show substrate inhibition: tyrosine hydroxylase, acetylcholinesterase, phosphofructokinase, folate cycle enzymes, and DNA methyltransferase. In each of these cases, substrate inhibition plays a distinctly different regulatory role. The collection of examples illustrates the broad diversity of physiological functions of substrate inhibition.

Tyrosine hydroxylase

In the terminals of dopaminergic neurons in the central nervous system, dopamine is synthesized in a two step process from tyrosine → L-Dopa → dopamine. The enzyme that catalyzes the first step, tyrosine hydroxylase (TH), shows strong substrate inhibition by tyrosine [6–8]. The velocity curve for TH as a function of cytosolic tyrosine concentration is shown in Fig. 2, and it has the typical substrate inhibition form. The

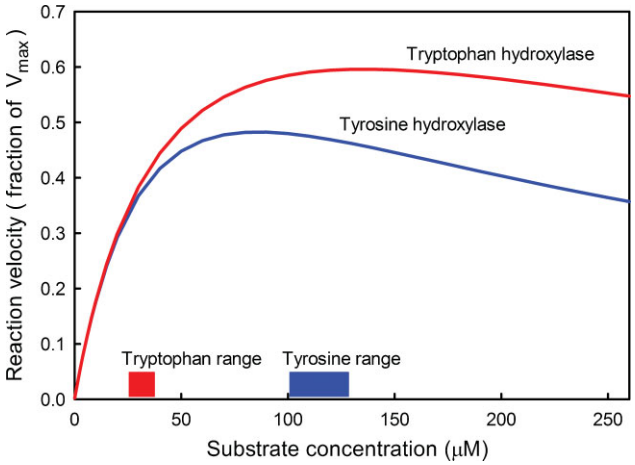

Figure 2. Substrate inhibition of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH). The velocity curve for TH as a function of tyrosine concentration is shown using parameters ($K_m = 46$, $K_i = 160$) obtained from fitting data [8]. The velocity curve for THP as a function of tryptophan concentration uses parameters ($K_m = 46$, $K_i = 400$) obtained by fitting experimental curves [12, 13]. For both tyrosine and tryptophan, the range of normal daily average concentration is indicated on the *X*-axis. For TH the average concentration is near the point where the velocity curve has its maximum, but for TPH the average concentration is in a region where the velocity curve rises sharply.

normal concentration of cellular tyrosine in the brain [9] is in the range 100–125 μM; the midpoint of this range is close to the maximum of the velocity curve. However, this "normal concentration" is, in fact, a daily average because the tyrosine concentration in the brain varies by as much as a factor of two before and after meals [10]. What effect does substrate inhibition have on the synthesis of dopamine? We have investigated this question using a mathematical model of dopamine synthesis, release, and reuptake [11], and the answer can be seen in Fig. 3. Without substrate inhibition, the velocity of the TH reaction varies quite a lot with meals, but the reaction rate is very stable in the presence of substrate inhibition. Similarly (simulations not shown) the vesicular stores of dopamine are very stable in the presence of substrate inhibition and vary quite a bit in its absence.

It is interesting to contrast TH with tryptophan hydoxylase (TPH), a closely related enzyme that catalyzes the first step in the synthesis of serotonin out of tryptophan. TPH shows substrate inhibition for tryptophan [12, 13]; however, as indicated in Fig. 2, the normal concentration of tryptophan is in the range 25–35 μM [14], below the K_m of TPH for tryptophan, which is about 46 μM [15]. Therefore, the normal velocity of the TPH reaction is on the steeply rising part of the velocity curve, which suggests that the synthesis of tryptophan should be sensitive to brain tryptophan level alterations caused by meals. Indeed, this is the case. Brain dopamine is not very affected by meals but brain serotonin varies a lot [14, 16]. Since increased serotonin has an inhibitory affect on appetite [17, 18], it makes sense that serotonin synthesis should be sensitive to meals. This leaves open, however, the question of the biological significance of substrate inhibition of TPH. Since the genes for TH and TPH are paralogs that diverged relatively

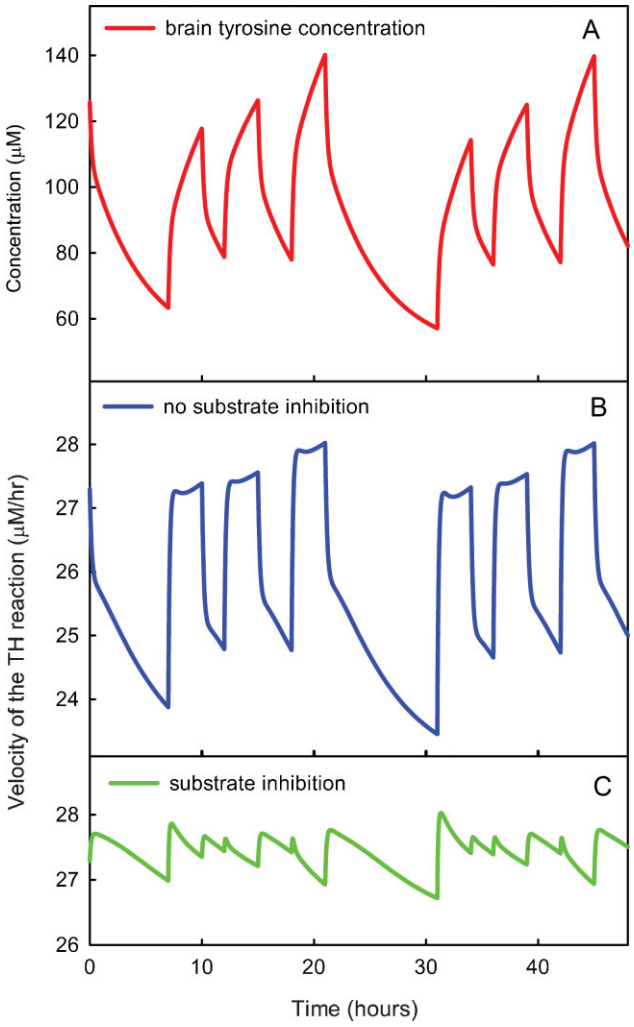

Figure 3. Stabilization of dopamine synthesis. **A:** The variation of tyrosine concentration in dopaminergic neurons due to meals over a 48-h period computed using the mathematical model [11]. It is known [10] that the brain concentration of tyrosine varies by as much as a factor of two before and after meals. **B:** The variation in the rate of the TH reaction over the 48-h period if the substrate inhibition of TH by tyrosine is removed. **C:** The variation in the rate of the TH reaction over the 48-h period if substrate inhibition of TH by tyrosine is included. Substrate inhibition stabilizes the synthesis of dopamine in the face of large variations in tyrosine availability [11].

recently [19, 20], the substrate inhibition of TPH may be an ancestral trait.

Acetylcholinesterase

Unlike dopamine and serotonin, which are rapidly removed from the synaptic cleft and re-enter the synaptic terminals via specialized transporters, acetylcholine (ACH) is degraded in the cleft by the enzyme acetylcholinesterase (AChe) [18]. The choline moiety is transported into the presynaptic terminal and ACH is resynthesized there. Because of the importance of ACH as a neurotransmitter, and also because the inhibition of AChe is the mechanism of action of certain nerve gasses [21]

and insecticides [22], the synthesis and structural properties of ACHe have received considerable attention [23–25]. Salpeter and coworkers [26, 27] and Rosenberry and coworkers [28, 29] have carried out detailed studies of the kinetics of ACH release, binding to post-synaptic receptors, and degradation by ACHe.

Degradation as a method for clearing the synaptic cleft poses some obvious problems. In order to terminate the signal before the next action potential arrives, ACHe should be a very efficient enzyme or have a very high concentration. Indeed, it is known that ACHe is exceptionally efficient [30]. But then much of the released ACH might be degraded before reaching receptors at the post-synaptic membrane. A solution for this problem would be to have ACHe be inhibited by its substrate ACH so that when ACH is at high concentrations the degradation proceeds relatively slowly and then accelerates as the concentration drops. Substrate inhibition of ACHe by ACH was noticed as long ago as 1969 [31], but its functional importance has been emphasized only recently [28, 32]. For this scenario to work, ACH should be released very rapidly into the cleft so that the concentration of ACH rises quickly into the inhibitory range. This is exactly what occurs. The peak of the ACHe velocity curve occurs at about 1 mM [28]. The rise time of ACH release is less than 100 μs [26, 33], and the concentration of ACH is quickly driven past 1 mM and rises to 10 mM and perhaps higher [32]. This is possible because of the very high concentration of ACH in presynaptic vesicles, approximately 1 M [34].

Figure 4A shows the results of some simple model calculations. The black curve is the rate of input of ACH into the cleft, consistent with known rapid input [33, 35]. Typical apparent K_m values are in the range 50–100 μM [28, 36, 37]; we used the value 58 μM in the model [28]. The blue curve in Fig. 4A shows the ACH concentration in the cleft as a function of time if we assume simple Michaelis-Menten kinetics. The green curve shows the concentration of ACH in the cleft if substrate inhibition is added with $K_i = 17\,900$ as determined experimentally [28]. In each case, the V_{max} was the same and its value was chosen so that the concentrations in the cleft go over 10 mM. The effect of substrate inhibition is clear. The peak of the ACH concentration is higher and the ACH pulse lasts about 1 msec longer. We note that it takes the binding of two ACH molecules to the receptor to open the channel on the post-synaptic membrane [38], so the real effect on neural transmission is proportional to the squares of the blue and green curves where the difference is even greater. If one squares the curves in Fig. 4A, one obtains the curves in Fig. 4B. The green curve is very similar to experimental curves [26] for the number of open channels as a function of time at frog and lizard neuromuscular junctions.

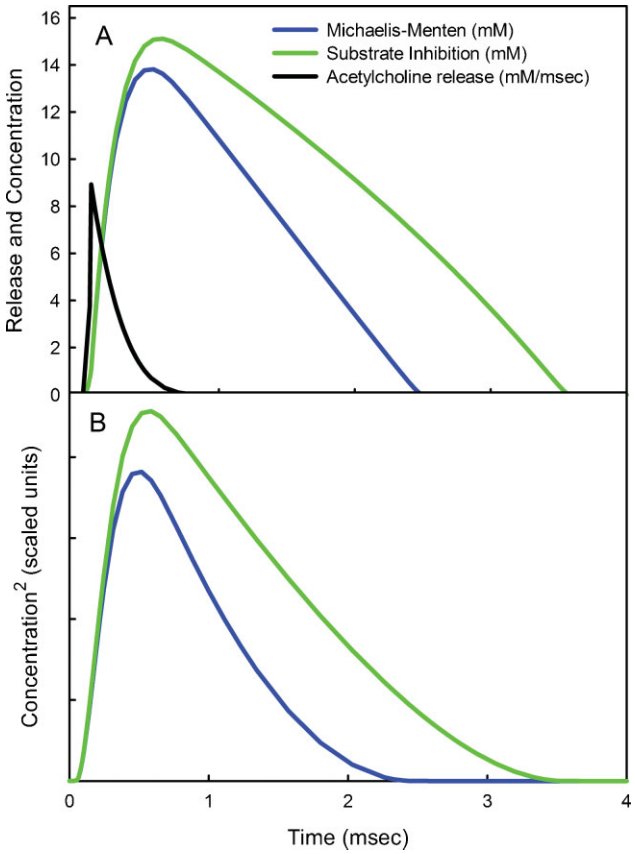

Figure 4. Effect of substrate inhibition of ACHe. **A:** The blue curve (Michaelis-Menten kinetics) and the green curve (substrate inhibition kinetics) show that the effect of substrate inhibition is to raise the peak concentration of ACH in the cleft and to prolong the length of the effect; the black curve indicates the rate at which ACH was released into the cleft. **B:** The squares of the curves in (**A**) are proportional to the number of open channels on the post-synaptic membrane. Calculations were done using a simple mathematical model.

Phosphofructokinase

Phospofructokinase (PFK) is the third enzyme in glycolysis and uses ATP to phosphorylate fructose-6-phospahate to fructose-1-6-biphosphate. The activity of PFK is under complex regulation: it is activated by fructose-2,6-biphosphate, AMP and ADP, inhibited by citrate, and is under strong substrate inhibition by ATP [39–41]. At physiological concentrations (0.5–3 mM), ATP strongly inhibits the activity of PFK (Fig. 5). This inhibition makes sense, because the main function of glycolysis is the production of ATP, so when ATP is present in abundance, glycolysis is inhibited. Glycolysis both uses ATP and is a net producer of a small amount of ATP, but the majority of ATP is produced after the products of glycolysis are further metabolized in the citric acid cycle and the energy released is used to power chemiosmotic ATP synthesis in the mitochondria. The inhibition of PFK by ATP makes the rate of glycolysis sensitive to the rate at which ATP is being used, and therefore to the demand for ATP.

The inhibition of PFK by ATP is particularly strong, and is not relieved unless ATP falls to an extremely low level, or unless an allosteric activator is present. ADP acts as an activator, which makes sense, because its accumulation will be associated with the depletion of ATP. Another important regulator is fructose-2,6-biphosphate. This metabolite is the product of PFK2, an enzyme whose activity is stimulated, among others, by epinephrine and insulin, and this is a

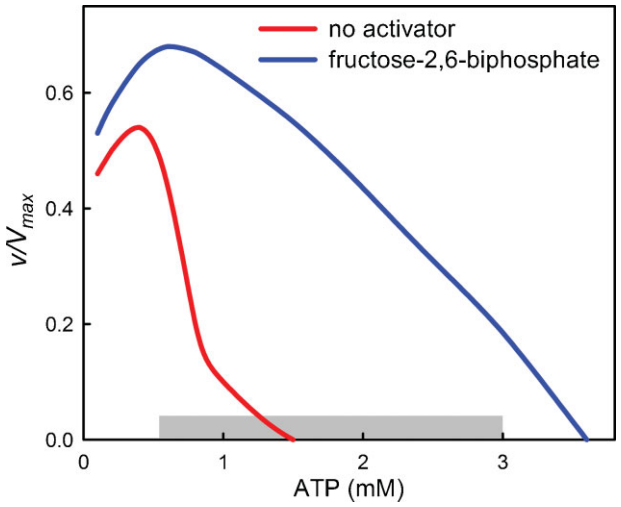

Figure 5. Substrate inhibition of PFK by ATP. The curves show the velocity of the reaction for PFK isolated from rat muscle in the presence (blue) and absence (red) of the activator fructose-2,6-biphosphate. In both cases the curves are monotone decreasing in the range of normal ATP concentrations indicated by the gray bar. The curves are based on experimental data [40].

mechanism by which these hormones override the inhibition of PFK by ATP and stimulate glycolysis and the further production of ATP.

The inhibition of PFK by ATP could, of course, also be viewed as an example of end-product inhibition. End-product inhibition is a well-known and extensively studied regulatory mechanism in metabolism. In the case of PFK, the end-product inhibition acts *via* substrate inhibition. Thus, the inhibition of PFK by ATP is not just substrate inhibition or just product inhibition, but is a system property of glycolysis.

The folate cycle

The folate cycle plays an important role in cell metabolism. It uses one-carbon units for the synthesis of purines and pyrimidines and sends one-carbon units to the methionine cycle for use in methylation reactions. Folate is a B vitamin and deficiency is known to be associated with neural tube defects [42], a decrease in DNA methylation [43], and the higher homocysteine levels that are biomarkers for cardio-vascular disease [44]. Folate deficiency and folate excess are associated with a variety of cancers [45, 46].

Each reaction of the folate cycle has a form of folate (oxidized or reduced, with or without an extra carbon unit) as a cofactor. Most of these folate substrates bind allosterically to one or more of the enzymes that catalyze reactions in the folate cycle [47–50]. In fact, each folate enzyme is inhibited by at least one of the folate substrates and several are inhibited by many of them [51]. Note that this is ''substrate inhibition'' at the level of a whole system rather than an individual reaction.

The traditional view of the allosteric binding of folates to folate enzymes is that it is a mechanism for storing folate units, which are released by the reversible allosteric binding when total folate gets low. This view is correct and the consequences are quite dramatic. When total folate drops, not only are folates released but free enzyme is also released, and this tends to keep up the velocities in the folate cycle despite the loss of total folate. We have shown that this is true in a mathematical model of the folate cycle [51]. Panel A of Fig. 6 shows the velocities of several important reactions in the folate cycle as total folate varies from normal (20 μM in liver cells) to zero without substrate inhibition. The velocities descend approximately linearly to zero. However, in the presence of substrate inhibition (Panel B) the velocities have long plateau regions and do not drop precipitously toward zero until total folate is below 5 μM.

The half-life of folate in the body is about 100 days and the first symptoms of folate deficiency (typically megaloblastic anemia) do not appear until after 3 months [52] of folate deprivation. This is consistent with the velocity profiles in Panel B because in the presence of substrate inhibition velocities have declined very little when half the folate has been depleted. Our ancestors had diets that likely varied seasonally in their content of folate and other B vitamins. Thus, substrate inhibition in the folate cycle is probably an evolutionary mechanism to protect us against large seasonal swings in folate availability [51].

DNA methyltransferase

DNA methylation plays an important role in the epigenetic regulation of gene expression. In vertebrates, DNA methylation occurs primarily on cytosines that are adjacent to guanines. Regions that are rich in CpG dinucleotides are called CpG islands and these are particularly common in the promoter regions of mammalian genes. Methylation of CpG islands is believed to block transcription and is the most common epigenetic mechanism for gene inactivation. Not all CpG sites are methylated. The pattern of methylation is tissue specific and this is believed to contribute to tissue-specific patterns of gene expression. Inappropriate methylation patterns as well as general hyper- or hypomethylation of the genome are associated with a diversity of diseases such as Fragile X syndrome, Prader-Willi syndrome and various cancers [53].

DNA methylation is accomplished by a family of DNA methyltransferases (DNMTs). DNMT3a and DNMT3b control *de novo* methylation during embryonic development, and are responsible for the initial establishment of the tissue-specific pattern of DNA methylation. DNMT1 is a maintenance methyltransferase that copies the existing methylation pattern onto the newly synthesized DNA strand when DNA is replicated during cell division.

DNMT1 is subject to strong allosteric substrate inhibition by regions of unmethylated DNA [54, 55]. Interestingly, DNMT1 is also activated by nearby methylated cytosines on the complementary DNA strand [56–60]. The exact molecular mechanisms by which the inhibition and activation of DNMT1 take place are an area of active investigation. Nevertheless, one can see the probable biological reasons for these effects. The inhibition of methylation by strands of unmethylated DNA guarantees that unmethylated regions normally remain unmethylated. By contrast, the activation of DNMT1 by methylated

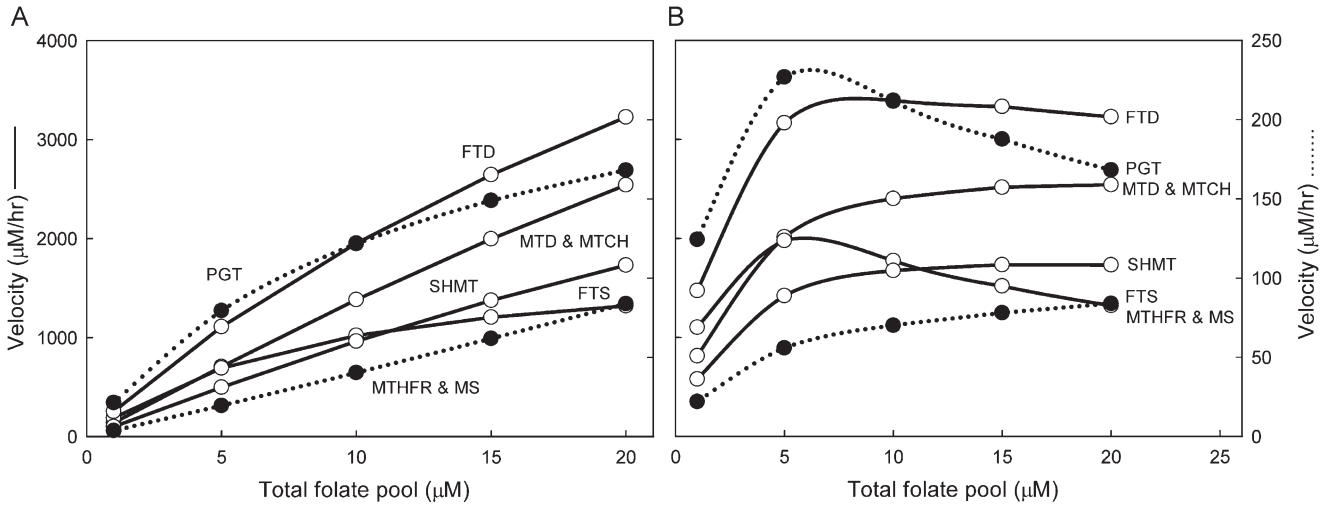

Figure 6. Model reaction velocities in the folate cycle as a function of total folate. Reaction velocities are identified by the acronyms of the enzymes that catalyze them: FTS, 10-formyltetrahydrofolate synthase; FTD, 10-formyltetrahydrofolate dehydrogenase; MTCH 5,10-methenyltetrahydrofolate cyclohydrolase; MTD, 5,10-methylenetetrahydrofolate dehydrogenase; MTHFR, 5,10-methylenetetrahydrofolate reductase; MS, methionine synthase; PGT, phosphoribosyl glycinamidetransformylase; SHMT, serinehydroxymethyltransferase. **A:** The velocities of the reactions descend linearly toward zero as total folate goes from 20 μM to 0 if substrate inhibition is not present. **B:** In the presence of substrate inhibition the velocities show long plateau regions and do not descend rapidly toward zero until total folate is below 5 μM. Calculations were done with a mathematical model [51]. Left and right *Y*-axes labels are the same for each panel.

cytosines guarantees that methylation is stimulated wherever the complementary strand is methylated. Substrate inhibition in this case is part of a mechanism that provides a bistable switch that ensures both the faithful reproduction of methylation patterns and the maintenance of methylation-free regions of the DNA.

Conclusions

Substrate inhibition is an extremely widespread phenomenon in enzyme kinetics. We have shown five examples here that illustrate different biological functions that substrate inhibition can create in different contexts. Indeed, one should not expect that any particular biochemical motif will have one unique function, because the function of a mechanism will depend on the larger system in which it is embedded. These systems have been molded by natural selection and therefore one should expect diversity in both the biological functions of the systems and the roles of substrate inhibition in creating the functions.

We selected our examples to display this diversity. In the case of TH, substrate inhibition plays the rather straightforward role of stabilizing dopamine synthesis against large swings in substrate availability due to meals. The effect here is to stabilize the amount of the end-product, dopamine, in synaptic vesicles. By contrast, the substrate inhibition of AChe prevents the excessively rapid degradation of ACH, ensuring that a sufficient amount reaches the post-synaptic receptors. PFK is a particularly interesting case. It is strongly inhibited by one of its substrates, ATP. However, the glycolytic chain, the citric acid cycle, and chemiosmosis produce a large number of ATPs so the effect of ATP on PFK is also an example of end-product inhibition. In this case, end-product inhibition acts *via* substrate inhibition.

Substrate inhibition in the folate cycle presents yet a different scenario. Enzymes are not only inhibited by their own substrates but also by other folate substrates in the cycle. Also, some folate substrates inhibit distant enzymes in the network but not necessarily enzymes that use them as substrates. The net result is that large amounts of folate and large amounts of enzyme are bound together unproductively. When total folate declines, these complexes dissociate releasing free folate and free enzyme that keep the reactions from slowing under moderate folate deficiency. Here substrate inhibition is a system homeostatic mechanism.

The mechanism by which the tissue-specific pattern of cytosine methylation on DNA is established is not well understood. But once this pattern is established, the allosteric regulation of DNA methyltransferase acts to maintain the pattern. The allosteric properties suggest that activation of the enzyme by nearby methylated cytosines allows the new complementary strand to be methylated at those locations during cell division. Substrate inhibition by unmethylated DNA protects unmethylated regions from inappropriate methylation.

The enzymes we have discussed are but a small fraction of those listed in Box 1. The biological functions of substrate inhibition of most of the others are waiting to be discovered. We expect that these functions will be diverse and that their investigation will give novel insights into the ways that biological systems are regulated. These systems are complex and their investigation will therefore require both biological experimentation and mathematical analysis.

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