Alternate Substrate (Product) Interactions 23

We have so far noted that a substrate molecule normally forms a productive complex at the enzyme active site. However there are cases where substrate also interacts with the enzyme (or the *ES* complex) in a nonproductive fashion. If this interaction is *kinetically silent*, it will not show up in routine steady-state kinetic analysis. Other methods (like equilibrium dialysis, fluorescence difference spectroscopy, or MALDI-TOF) may however be able to detect such binding phenomena. Most often nonproductive interactions of substrate are not considered at all – except when they also interact with the same enzyme either as activators or as inhibitors.

23.1 Substrate Inhibition

A decrease in enzyme activity as a consequence of high substrate concentration is termed substrate inhibition. Substrate inhibition may be observed due to one or more of the following reasons:

(a) The presence of a second set of low-affinity binding sites for S; and when so bound, can lead to nonproductive, inefficient enzyme forms.
(b) Unproductive binding of substrate by partial sub-site occupancy.
(c) Removal of an essential active site metal ion or cofactor by high [*S*].
(d) The presence of excess uncomplexed substrate such as ATP; note that in most cases Mg-ATP is the true substrate. It is therefore important to use proper concentration ratios of ATP and Mg^{2+} (Chap. 32, Phosphoryl Group Chemistry and Importance of ATP, in Part IV).

A simple binding equilibrium (where excess substrate acts as an uncompetitive inhibitor) and the corresponding rate equation for substrate inhibition are shown below:

N. S. Punekar, *ENZYMES: Catalysis, Kinetics and Mechanisms*,
https://doi.org/10.1007/978-981-13-0785-0_23

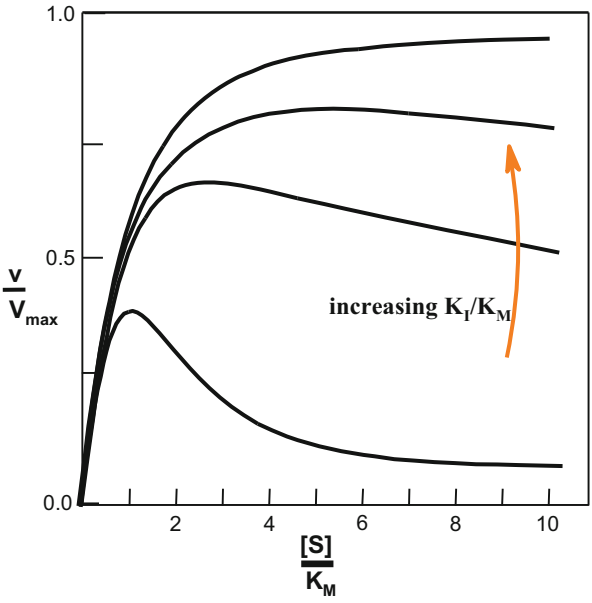


Fig. 23.1 Typical $v \rightarrow [S]$ plots for substrate inhibition of the enzyme. Enzyme activity (plotted as dimensionless v/V_{max}) as a function of substrate concentration (plotted as dimensionless $[S]/K_M$) at four different K_I/K_M values are shown

$$v = \frac{V_{max}[S]}{K_M + [S]\left(1 + \frac{[S]}{K_I}\right)} = \frac{V_{max}[S]}{K_M + [S] + \frac{[S]^2}{K_I}}$$

It is thus expected that an *SES* complex does form. Because of the $[S]^2$ term, the $v \rightarrow [S]$ relationship is nonlinear even when double reciprocals are taken. The nature of $v \rightarrow [S]$ plot (describing substrate inhibition) will depend on the relative magnitudes of K_M and K_I; an optimum is apparent in these plots. This is illustrated in Fig. 23.1.

23.1.1 Determination of Kinetic Constants and Their Significance

The double reciprocal form of the rate equation for substrate inhibition is as shown:

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

This equation may also be rearranged to

$$\frac{V_{max}}{v} = 1 + \frac{K_M}{[S]} + \frac{[S]}{K_I}$$

The $[S]/K_I$ term becomes significant at high $[S]$ values and a small K_I – leading substrate inhibition to set in. At low $[S]$ (and relatively large K_I values), this equation collapses to a regular Michaelis-Menten equation. An estimate of K_M can be made

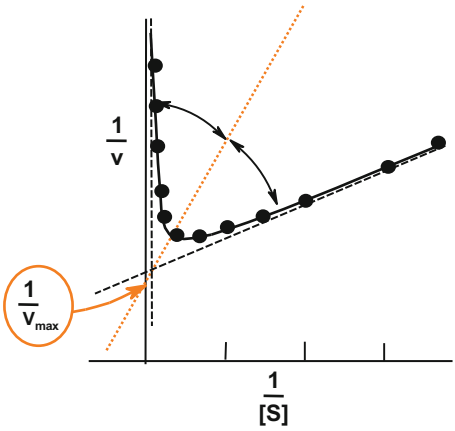


Fig. 23.2 Double reciprocal plot of $v \rightarrow [S]$ data for an enzyme showing substrate inhibition. Graphical estimates of V_{max} and K_M are indicated as extrapolations through broken lines

from $v \rightarrow [S]$ data at lower substrate concentrations by extrapolating the apparent linear region of a normal double reciprocal plot (dashed line tangent in Fig. 23.2). Graphically the value of $1/V_{max}$ may be obtained by drawing a line bisecting the angle made by the two limbs of the $1/v \rightarrow 1/[S]$ curve (dotted line bisector; Fig. 23.2). The estimated V_{max} and K_M values may be subsequently substituted in the rate equation to obtain K_I. Best way to extract all the kinetic parameters (V_{max}, K_M and K_I) is to fit the data to the equation.

Substrate inhibitions are not usually important if $[S]$ is kept relatively low – below its physiological levels. The evolutionary process normally eliminates dead-end combination of substrate that leads to substrate inhibition. However, if physiologically $[S]$ does not reach inhibitory levels (for instance, aldehyde substrates that can be toxic to the cell!), then dead-end combinations do persist. This often manifests as substrate inhibition in kinetic studies, particularly in the nonphysiological direction.

Substrate inhibition may be induced by an inhibitor in an ordered sequential mechanism. In most cases such induced substrate inhibitions are partial (and not complete) because the inhibitor does escape from the *EIB* complex at a reduced but finite rate. Inhibition of hexokinase by Mg-ATP is an example of this kind. Lyxose (an inhibitor resembling glucose, the first substrate) induces a competitive substrate inhibition by Mg-ATP. Such induced substrate inhibition occurs because much of the enzyme is trapped as E.Lyxose.Mg-ATP complex, from which lyxose cannot dissociate.

23.2 Use of Alternate Substrates in Enzyme Studies

Alternate products or substrates compete with the normal substrates for the same enzyme form(s). They may also be viewed as inhibitors of the normal reaction. Such an analysis does enrich our kinetic understanding of enzyme action. Glucose and

galactose compete for the same form of hexokinase enzyme (Fig. 23.3). Therefore, galactose may be seen as a competitive inhibitor of glucose reaction with hexokinase and *vice versa*.

Two useful areas of application of alternate substrates (and alternate products – because they are substrates in the reverse direction) in the kinetic study of enzymes are enumerated below.

23.2.1 Information About the Active Site Shape, Geometry, and Interactions

Enzymes are specific catalysts and hence can accommodate a narrow range of substrate structures in their active sites. Conversely, testing different structural variants of the natural substrate for catalysis by the enzyme defines the active site. This was an attractive but simple option to probe active sites much before the use of X-ray structural data. Excellent insight into mammalian glutamine synthetase active site was adduced by Meister and his colleagues by this approach (Table 23.1).

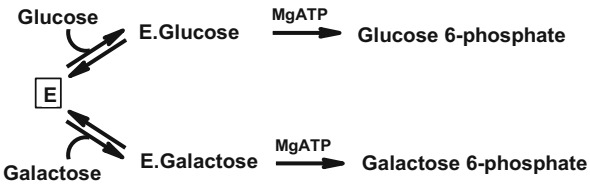


Fig. 23.3 Glucose and galactose compete for the same enzyme. Enzyme hexokinase is represented as *E*

Table 23.1 Interaction of different structural variants of L-glutamate with glutamine synthetase

Compound	Substrate activity (%)[a]	Competitive inhibition[a]
α-Aminomalonate	00	No
L-Aspartate	00	No
D-Aspartate	00	No
L-Glutamate	100	Yes
D-Glutamate	54	Yes
β-Glutamate	46	Yes
α-Methyl-L-glutamate	67	Yes
β-Methyl-D-glutamate (*threo*)	46	Yes
γ-Methyl-L-glutamate (*threo*)[b]	63	Yes
γ-Hydroxy-L-glutamate (*threo*)	89	Yes
γ-Hydroxy-D-glutamate (*threo*)	02	–
L-α-Aminoadipate	22	–
D-α-Aminoadipate	11	–

Adapted with permission from A. Meister, Adv Enzymol, 31:183–218. Copyright (Meister 1968) John Wiley & Sons Inc

[a]Both substrate activity and inhibition are scored against L-glutamate, the natural substrate

[b]Of the four possible isomers only this is a substrate

While some molecules may not be substrates, they may interact at the active site and inhibit the enzyme. A quantitative structure–activity analysis for different substrates and non-substrates taken together succinctly defines the active site perimeters (Fig. 23.4). Molecular models were used to map the enzyme active site space occupied by L-glutamate. Arguments are based on steric hindrance by various substitutions on the glutamate structure, and the active site occupancy of this substrate was elegantly worked out. Accordingly, ovine brain glutamine synthetase binds L-glutamate in a fully extended conformation in which the carboxyl groups are as far apart as possible. The intercarboxyl carbon distance of about 5 Å is required, and the molecule is anchored at the active site through two carboxyl groups and an amino group.

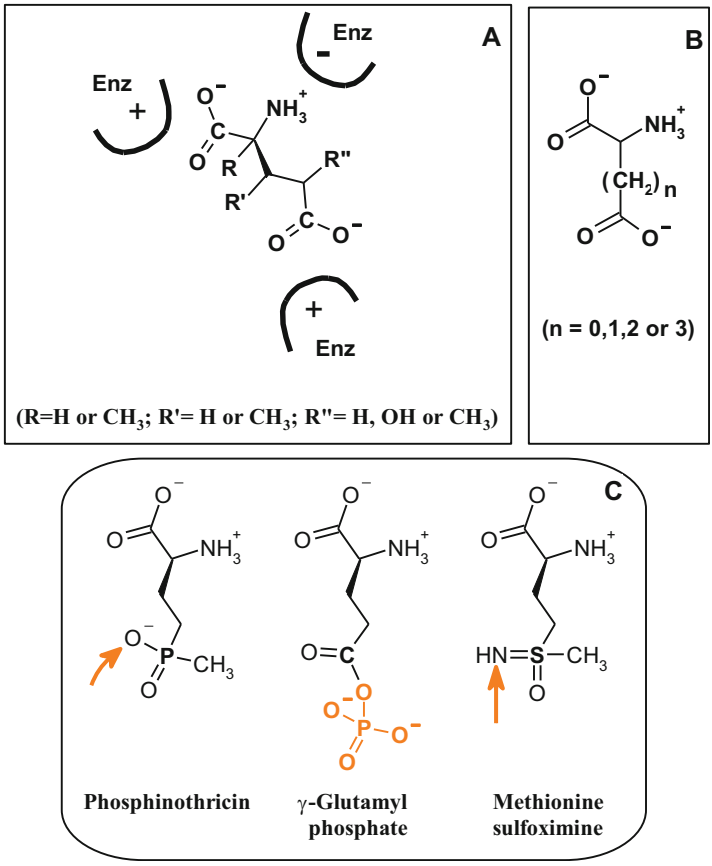


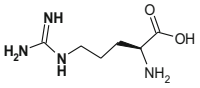
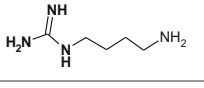
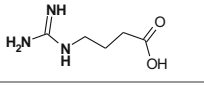
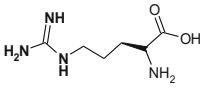
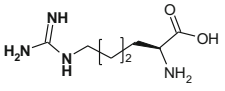
Fig. 23.4 Different substrate structural variants used to define glutamine synthetase active site geometry. (**A**) Systematic variations in glutamate structure at α, β, or γ carbon (corresponding to data in Table 23.1). (**B**) Variation of chain length separating the two carboxylates. (**C**) The γ-glutamyl phosphate enzyme-bound intermediate. Arrows indicate the potential phosphorylation position on the two tight-binding inhibitors, namely, L-phosphinothricin and L-methionine-DL-sulfoximine

It must be noted that glutamine synthetases from other sources possess much higher degree of substrate specificity; many of the structures described above are not accepted, while only L-glutamate serves as a substrate. On the other hand, L-phosphinothricin and L-methionine-DL-sulfoximine (Figure above) markedly inhibit all glutamine synthetases. The two potent tight-binding inhibitors may be phosphorylated and resemble the γ-glutamyl phosphate intermediate of the enzyme reaction.

A number of biologically relevant guanidinium group-containing compounds occur in nature. Life has solved the chemistry of guanidinium group hydrolysis by recruiting metal ions for activating water attack. Majority of these ureohydrolases exhibit an alkaline pH optimum (greater than pH 9.0) and contain a bimetallic Mn^{2+} cluster at the active site. While the apparatus to hydrolyze guanidinium group (to release urea) is more or less conserved, members of ureohydrolase superfamily display exquisite substrate specificity (Table 23.2). Indeed they are classified based on this feature.

The substrate specificity of human arginase was extensively probed by site directed mutations. Arginase specificity could be changed (in the N130D variant), to recognize and accommodate agmatine in place of arginine, thereby converting it into an agmatinase. Exploring the arginase active site with substrates, substrate

Table 23.2 Substrate structural requirements for three ureohydrolases[a]

Compound	Structure	Used as substrate by		
		Arginase	Agmatinase	4-Guanidinobutyrase
L-Arginine		**Yes**	No	No
Agmatine		No	**Yes**	No
4-Guanidinobutyrate		No	No	**Yes**
D-Arginine		No	No	No
L-Homoarginine		No	No	No

[a]Enzyme sources: arginase from *Aspergillus niger*, agmatinase from *Escherichia coli*, and 4-guanidinobutyrase from *Pseudomonas putida*

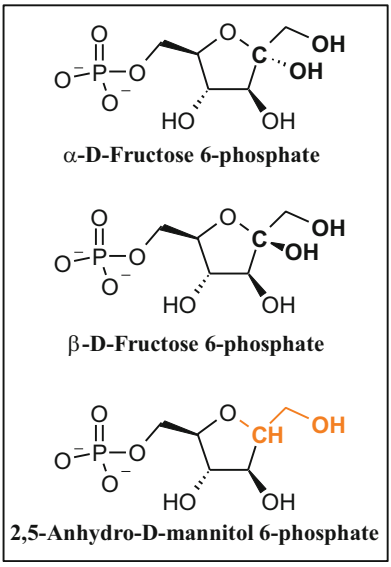


Fig. 23.5 Structural similarity between α-D-fructose 6-phosphate, β-D-fructose 6-phosphate, and 2,5-anhydro-D-mannitol-6-phosphate. Note that 2,5-anhydro-D-mannitol is an ether; the –OH on C2 carbon is missing and hence cannot mutarotate

analogues, and non-substrates has gained importance. Potent, selective arginase inhibitors (like NOHA) have found therapeutic application in channeling arginine pools into NO synthesis.

Most enzymes bind an otherwise conformationally flexible substrate, at their active sites, in a fixed geometry. As seen above, an extended L-glutamate conformation is frozen out at their active sites by glutamine synthetase and NADP-glutamate dehydrogenase. Alternate substrate structures with rigid geometry are useful in extracting such information. Phosphofructokinase phosphorylates the 1-position of D-fructose 6-phosphate in its furanose form. Because its two anomers (α-D-fructose 6-phosphate and β-D-fructose 6-phosphate) equilibrate very rapidly in water, it is hard to tell which anomer is the substrate. The β-anomeric structure may be frozen out as its corresponding 2,5-anhydro-D-mannitol-6-phosphate (Fig. 23.5). It is an ether (-OH missing on C-2!) and cannot undergo mutarotation; this is accepted as an alternate substrate by phosphofructokinase and not the α-isomer. The conclusion is inescapable that β-D-fructose 6-phosphate is the natural substrate for this enzyme. Similar studies with fructokinase showed that the β-furanose form of D-fructose is its substrate.

Substrate structures with fixed geometry may also be used to determine (a) which conformational isomer of ATP is the substrate for a given enzyme and (b) whether the α-phosphate of ATP is ever coordinated to the divalent metal ion during reaction. Further treatment on the biochemistry of ATP and its reactivity may be found in a later section (Chap. 32; Phosphoryl Group Chemistry and Importance of ATP).

Finally, the vast diversity of semisynthetic β-lactam structures (Chap. 3, Exploiting Enzymes: Technology and Applications, in Part I) provides practical

examples of how the knowledge of their binding to the active sites of D-Ala-D-Ala carboxypeptidase (the target) and β-lactamase (that confers resistance) has been successfully exploited in new antibiotic discovery.

23.2.2 Understanding Kinetic Mechanism

Alternate substrates and substrate analogues provide useful kinetic information. We will discuss two of their possible applications.

(a) Dead-end inhibition by an analogue of substrate B can be used as diagnostic of an ordered bi-reactant mechanism. This inhibition is uncompetitive with A as the varied substrate for the ordered case.
(b) A sticky substrate (high commitment to catalysis upon binding to E – see Chap. 25 for details) often leads to complex rate equations in the analysis of pH kinetics and isotope effects. A slow substrate with a K_M in the mM range will usually be not sticky. The binding of such slow alternate substrates will be in rapid equilibrium. Fructose 6-sulfate, being a mono-anion, binds 6-phosphofructokinase poorly. It therefore is a non-sticky, slow substrate when compared to fructose 6-phosphate (a di-anion!). Substrate affinity may also be lost by changing the reaction pH due to incorrect protonation states. For instance, creatine kinase loses affinity for creatine as the pH is decreased from 8 to 7; and it becomes non-sticky. In both these kinases, the loss of substrate affinity also leads to a change in the kinetic mechanism. A normal random mechanism changes to equilibrium ordered (Chap. 19; see 19.3 Few Unique Variations) with Mg-ATP binding before the other substrate.

Reference

Meister A (1968) The specificity of glutamine synthetase and its relationship to substrate conformation at the active site. Adv Enzymol 31:183–218