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Selective Neutrality and Enzyme Kinetics

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Abstract. This article appeals to a recent theory of enzyme evolution to show that the properties, neutral or adaptive, which characterize the observed allelic variation in natural populations can be inferred from the functional parameters, substrate specificity, and reaction rate. This study delineates the following relations between activity variables, and the forces—adaptive or neutral—determining allelic variation: (1) Enzymes with broad substrate specificity: The observed polymorphism is adaptive; mutations in this class of enzymes can result in increased fitness of the organism and hence be relevant for positive selection. (2) Enzymes with absolute substrate specificity and diffusion-controlled rates: Observed allelic variation will be absolutely neutral; mutations in this class of enzymes will be either deleterious or have no effect on fitness. (3) Enzymes with absolute or group specificity and nondiffusion-controlled rates: Observed variation will be partially neutral; mutants which are selectively neutral may become advantageous under an appropriate environmental condition or different genetic background. We illustrate each of the relations between kinetic properties and evolutionary states with examples drawn from enzymes whose evolutionary dynamics have been intensively studied.

Key words: Enzyme evolution — Allozyme variation — Substrate specificity — Diffusion-controlled rates — Absolute neutrality

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Introduction

The recognition more than two decades ago that a large degree of allelic variation exists in natural populations (Lewontin and Hubby 1966; Harris 1966) generated a controversy concerning evolutionary origins which remains unresolved. The issue concerns the relative importance of selection and random drift in determining allelic polymorphism.

Evolutionary genetics distinguishes between nonadaptive and adaptive mutations. The first refers to changes—the neutral mutations—which do not alter the fecundity and mortality of the organism. Adaptive mutations—namely, those changes which affect net reproductive rate and hence are relevant for selection—may be advantageous or deleterious. Assessing the relative importance of selection and drift in explaining the observed polymorphism in natural populations is essentially equivalent to determining the relative incidence of adaptive and nonadaptive evolutionary changes. The problem was put in sharp focus by the neutral hypothesis (Kimura 1968). This asserts that mutations which constitute the nonadaptive changes represent a very large class of the set of all observed changes.

The attempts to assess the validity of the neutral hypothesis have resulted in a large population-genetic literature concerning fixation of neutral mutants in a finite population (Kimura 1971; Kimura and Ohta 1971) and the fixation of mutants under random environmental conditions (Gillespie 1987), a statistical literature concerning tests to assess selective neutrality from electrophoretic data (Ewens 1972), and sequence data (Hudson et al. 1987), Sawyer (1994).

The neutral hypothesis concerns a relation between evolutionary processes at molecular and organismic lev-

Table 1. Selective neutrality and substrate specificity in enzymes

Specificity and reaction rate	Selective property	Examples
Broad specificity; intermediate rates	Adaptive	Most esterases, peptidases, nucleases
Absolute specificity; diffusion-controlled rates	Absolutely neutral—no potential for adaptation	Fumarase, catalase, superoxide dismutase
Absolute or group specificity; nondiffusion-controlled rates	Partially neutral—latent potential for adaptation	Kinases, synthetases, glycosidases, lyases

els, a synthesis which does not exist in the current models of evolutionary genetics. The hypothesis pertains to the putative weak or negligible effect of mutations at the DNA level on Darwinian fitness. In assessing the frequency of this family of mutations, the following question arises. Can we specify in structural or kinetic terms a class of genes in which random mutations have negligible effects on the life cycle of the organism?

The problem as stated is evidently intractable since it depends on an understanding of the relation between genes and organism which is beyond our current purview. However, the problems which the complexities of the developmental system generate can be obviated by considering enzymes rather than general proteins and addressing the following question. Can we characterize a class of enzymes in which nucleotide changes in the genes which encode them will be either deleterious or have negligible effects on the net reproductive rate of the organism?

This article proposes a characterization of this class of enzymes—which is equivalent to a categorization of selective neutrality—in terms of the functional property, *substrate specificity*.

The term substrate specificity in its widest sense refers to the capacity of the enzyme to discriminate between two competing substrates. We will use the term in a more restricted context to refer to the capacity of the enzyme to discriminate between two competing *natural* substrates under physiological conditions. In this context, substrate specificity characterizes the diversity of physiologically present substrates that produce appreciable reaction rates. Specificity can be categorized in terms of the decreasing diversity of the class of physiologically present substrates as follows: (1) *Broad specificity*. This describes the case when the activity of the substrates depends on the substrate containing a particular chemical bond, for example, the peptide bond in peptidases. (2) *Group specificity*. This defines the situation where the substrates are characterized by a particular group; for example, alcohol dehydrogenase will catalyze the oxidation of a variety of alcohol. (3) *Absolute specificity*. Enzymes are said to have this property when only a single substrate (or in the case of a bimolecular reaction, a single pair of substrates) produces a reaction at an appreciable rate; for example, triosephosphate isomerase is specific for the interconversion of D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.

This article appeals to a new theory of enzyme evolution (Demetrius 1992, 1995) in order to characterize selectively neutral and adaptive enzymes in terms of the functional properties, reaction rate, and substrate specificity. The characterization we derive is embodied in the following three propositions.

A(1). Mutations in enzymes with broad substrate specificity are adaptive. This class of mutations can result in a positive change in the fitness of the organism and hence will be relevant for selection.

A(2). Mutations in enzymes described by absolute substrate specificity and diffusion-controlled rates are absolutely neutral. This class of mutations will be either deleterious or will exert no effect on fitness.

A(3). Mutations in enzymes described by absolute or group specificity and nondiffusion-controlled rates are partially neutral. This family of mutations has a latent potential for selection; that is, mutants which are selectively neutral may become advantageous under an appropriate environmental condition or different genetic background.

This categorization of neutrality and adaptation of enzymes in terms of their degree of substrate specificity is summarized with examples in Table 1.

In Table 1, we have only included enzymes where all members of the group belong exclusively to one of the categories we have described. We note, for example, that among the dehydrogenases, phosphatases, and transferases, enzymes described by both broad and absolute specificity exist; consequently, these classes of enzymes do not fit neatly into the categories described.

Models to explain the incidence of neutral mutations in enzymes were developed in Hartl et al. (1985) and elaborated in later works; see, for example, Dean (1994). These studies were based on the metabolic control models of Kacser and Burns (1987) and the biochemical systems analysis of Savageau (1976). The main conclusion of the analysis in Hartl et al. (1985) is that conditions for the occurrence of neutral evolutionary states can be induced by the indirect result of the force of natural selection.

The theory advanced in this article aims to explain the incidence of both neutral *and* adaptive states of enzymes, and furthermore to relate this incidence to functional properties, such as substrate specificity and reaction rate.

The main conclusion of our analysis is expressed in the correspondence between evolutionary states (neutral or adaptive) and functional variables (substrate specificity, reaction rate), described by A(1), A(2), and A(3). This correspondence derives from a new theory of enzyme evolution which classifies enzymes in terms of their thermodynamic condition and studies directional trends in the rate constants of each of these categories under the dual forces of mutation and selection.

In this article we provide a summary of the basic elements of the evolutionary theory of enzymes developed in Demetrius (1995). We recall certain aspects of enzyme kinetics and transition state theory; an account of the directional trends in kinetic variables which derive from the synthesis of transition state theory and evolutionary dynamics is given. The application of this theory to address the problems of selective neutrality and adaptation is then developed. The text by Fersht (1985) provides a good source for the basic material in enzyme kinetics invoked in this article. The work of Jencks (1975) gives an account of the relations between thermodynamic parameters and catalytic mechanisms which inform our analysis.

Enzyme Dynamics: Thermodynamic and Kinetic Parameters

The scheme of reactions which define the catalysis of a single substrate S by an enzyme E to a product P can be described by the set of equations

$$E + S \rightleftharpoons ES \rightleftharpoons ES' \rightleftharpoons \ldots \rightleftharpoons EP \rightarrow E + P$$

where expressions ES, ES' ... represent the set of possible intermediates in the reaction.

In our study of this series of reactions, we consider the transition state involved in the rate-determining step as characterizing the transition state for the overall reaction. The steady-state kinetics of the reaction can be described in terms of both kinetic and thermodynamic variables.

Kinetic Parameters

We assume that the enzyme-substrate complex is in thermodynamic equilibrium with free enzyme and substrate. In this case, the system can be described by the Michaelis-Menten mechanism, namely:

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \overset{k_{\text{cat}}}{\rightarrow} E + P \qquad (1)$$

The parameter k_{cat} is the unimolecular rate constant for the conversion of the enzyme-substrate complex to an enzyme-product complex and K_m is the apparent dissociation constant. The quantity k_{cat}/K_m is the apparent second-order rate constant at very low substrate concentrations; under these conditions it is a useful measure of the overall efficiency of the catalyst.

Thermodynamic Variables

The reaction $E + S \rightleftharpoons ES^{\ddagger}$, describing the transition from the unbound enzyme and substrate to the transition state, can also be described in terms of thermodynamic variables—namely: activation free energy $\Delta G^{\ddagger}$, activation enthalpy $\Delta H^{\ddagger}$, the activation entropy $\Delta S^{\ddagger}$, and the heat capacity of activation $\Delta C^{\ddagger}$.

The function $\Delta G^{\ddagger}$ denotes the energy required to transform the system from the electronic and vibrational ground state to the transition state. This energy function is always nonnegative, $\Delta G^{\ddagger} \geq 0$. The activation enthalpy $\Delta H^{\ddagger}$ describes the energy difference between the unbound ground state and the bound state involving enzyme and substrate. The parameter $\Delta H^{\ddagger}$ can assume both positive and negative values. The activation entropy $\Delta S^{\ddagger}$ is a composite quantity which comprises (1) $\Delta S_1{}^{\ddagger}$, the conformational changes in enzyme and substrate induced by the interaction with the substrate in the active site and (2) $\Delta S_2{}^{\ddagger}$, the replacement of translational and orientational entropy by vibrational entropy. Activation entropy, $\Delta S^{\ddagger}$, can also assume positive and negative values. The heat capacity of activation $\Delta C^{\ddagger}$ is defined by $\Delta C^{\ddagger} = [\partial(\Delta H^{\ddagger})/\partial T]$. When equilibrium conditions obtain, $\Delta C^{\ddagger}$ is described by the variance in the enthalpy distribution, a nonnegative quantity. The quantities $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ are related by the identity

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \qquad (2)$$

Classification of Enzymes

The relation (2) plays a central role in our classification of enzymes in terms of the values assumed by their thermodynamic variables in their kinetically significant transition states.

When $\Delta C^{\ddagger} = 0$, a condition which implies that $\Delta H^{\ddagger}$ is independent of temperature, the state of the enzyme-substrate complex can be parameterized in terms of the two variables $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. Invoking the condition $\Delta G^{\ddagger} > 0$, we note that enzymes can be classified into three categories (Demetrius 1995) as follows:

Type 1: $\Delta H^{\ddagger} > 0$, $\Delta S^{\ddagger} < 0$

Type 2: $\Delta H^{\ddagger} < 0$, $\Delta S^{\ddagger} < 0$

Type 3: $\Delta H^{\ddagger} > 0$, $\Delta S^{\ddagger} > 0$

When $\Delta C^{\ddagger} > 0$, a state which corresponds to a dependence of $\Delta H^{\ddagger}$ on temperature, the parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ can each be divided into two groups: $\Delta H^{\ddagger} < T\Delta C^{\ddagger}$ and $\Delta H^{\ddagger} > T\Delta C^{\ddagger}$; and $\Delta S^{\ddagger} < \Delta C^{\ddagger}$, $\Delta S^{\ddagger} > \Delta C^{\ddagger}$. This

indicates that the type 3 model can be further described in terms of three subcategories.

Type 3(a): $\Delta H^{\ddagger} < T\Delta C^{\ddagger}$, $\Delta S^{\ddagger} < \Delta C^{\ddagger}$

Type 3(b): $\Delta H^{\ddagger} > T\Delta C^{\ddagger}$, $\Delta S^{\ddagger} < \Delta C^{\ddagger}$

Type 3(c): $\Delta H^{\ddagger} > T\Delta C^{\ddagger}$, $\Delta S^{\ddagger} > \Delta C^{\ddagger}$

Hence when $\Delta C^{\ddagger} > 0$, we now have five categories of enzyme-substrate reactions. The significance of this classification resides in the fact that the categories satisfy certain invariant and stability properties with respect to mutations which alter the free energy profile of the enzyme-substrate reaction (Demetrius 1995, 1997). These properties are as follows

Invariance. Each category is invariant under mutations which have no effect on $\Delta C^{\ddagger}$. Such mutations will induce very small changes in the activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ and consequently will cause no change in the mechanism of catalytic action.

Instability. The categories are unstable under mutations which cause changes in $\Delta C^{\ddagger}$. This class of mutations will induce relatively large changes in $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ and may therefore transform one category of enzyme into another.

The invariance property implies that each of the five categories define a particular catalytic mechanism and hence can be considered as representing an enzyme superfamily. Typical examples of each category are described as follows: type 1—the serine proteases, most esterases, phosphatases; type 2; type 3(a)—enzymes with diffusion-controlled rates (triosephosphate isomerase); type 3(b)—synthetases; type 3(c)—kinases.

Evolutionary Catalysis

The evolutionary dynamics of enzymes is a two-level process involving (1) mutation, which acts on the gene that codes for the enzyme, and (2) selection, which acts on the organism that carries the enzyme. The models developed in Demetrius (1995) exploited the classification of the enzyme-substrate complex to study the evolutionary changes in the kinetic parameters, reaction rate, k_{cat}/K_m, and binding affinity, K_m. The analysis showed that the evolutionary trends in the kinetic parameters are constrained by the thermodynamic conditions that define the enzyme categories.

When mutations do not alter the mechanisms of catalysis, the evolutionary trends in the kinetic variable assume the following pattern.

Type 1: $\Delta H^{\ddagger} > 0$, $\Delta S^{\ddagger} < 0$. Random, nondirectional changes in reaction rate and binding affinity.

The random nondirectional trajectory of the kinetic variables requires that enzymes in the evolutionary limit will be characterized by a large variability in reaction rate and a broad substrate specificity.

Type 2: $\Delta H^{\ddagger} < 0$, $\Delta S^{\ddagger} < 0$; **type 3(a):** $\Delta H^{\ddagger} < T\Delta C^{\ddagger}$, $\Delta S^{\ddagger} < \Delta C^{\ddagger}$. A unidirectional increase in reaction rate and binding affinity.

The thermodynamic constraints that define the type 2 and type 3(a) models indicate that in the evolutionary limit, maximal reaction rates and maximal binding affinity will be achieved. Hence, modern type 2 and type 3(a) enzymes will be described by diffusion-controlled rates and absolute substrate specificity.

Type 3(b): $\Delta H^{\ddagger} > T\Delta C^{\ddagger}$, $\Delta S^{\ddagger} < \Delta C^{\ddagger}$; **type 3(c):** $\Delta H^{\ddagger} > T\Delta C^{\ddagger}$, $\Delta S^{\ddagger} > \Delta C^{\ddagger}$. A unidirectional increase in reaction rate and binding affinity.

The thermodynamic constraints which define the type 3(b) and type 3(c) models impose different bounds on the reaction rate and binding affinity in the evolutionary limit. In the type 3(b) model, enzymes in the evolutionary limit will be described by a local maximum of k_{cat}/K_m and a global maximum of K_m. Molecules in the limiting condition will therefore be described by nondiffusion-controlled rates, absolute specificity. In the type 3(c) model, the evolutionary limit will now be described by a local maximum of k_{cat}/K_m and a local maximum of K_m. Molecules in the evolutionary limit will now be characterized by nondiffusion-controlled rates and group specificity.

The relation between the thermodynamic condition of the enzymes, the properties reaction rate, and substrate specificity (which is derived from the evolutionary model) is summarized in Table 2.

Table 2.

Thermodynamic condition	Reaction rate k_{cat}/K_m	Substrate specificity
$\Delta H^{\ddagger} > 0$, $\Delta S^{\ddagger} < 0$	Highly variable	Broad
$\Delta H^{\ddagger} < 0$, $\Delta S^{\ddagger} < 0$ $\Delta H^{\ddagger} < T\Delta C^{\ddagger}$, $\Delta S^{\ddagger} < \Delta C$	Diffusion-controlled rates	Absolute
$\Delta H^{\ddagger} > T\Delta C^{\ddagger}$, $\Delta S^{\ddagger} < \Delta C^{\ddagger}$	Nondiffusion-controlled rates	Absolute
$\Delta H^{\ddagger} > T\Delta C^{\ddagger}$, $\Delta S^{\ddagger} > \Delta C^{\ddagger}$	Nondiffusion-controlled rates	Group

One of the main predictions that has emerged from this new synthesis of transition state theory and evolutionary dynamics asserts that enzymes with diffusion-controlled rates have absolute substrate specificity. Those enzymes whose activity is consistent with this prediction are triosephosphate isomerase, fumarase, catalase, and carbonic anhydrase. These enzymes have k_{cat}/K_m values in range 10^7–10^8 $M^{-1}s^{-1}$ (which approximates the diffusion limit) for reactions with their respective natural substrates and significantly inferior rates for related substrates under physiological conditions.

In assessing the empirical validity of the prediction

relating catalytic efficiency with substrate specificity, it is important to emphasize that the term *absolute specificity,* as used in this article, pertains to the capacity of the enzyme to discriminate between different natural substrates—that is, substrates that are physiologically present in the cell. A discussion of aspects of the catalytic activity of alkaline phosphatase and β-lactamase will help to illustrate this point.

Alkaline phosphatase is a broadly specific enzyme that is found in both prokaryotes and eukaryotes. The natural substrate for this enzyme consists of the monoesters of orthophosphoric acid. We would therefore infer from the theory we have described that alkaline phosphatase will catalyze its natural substrates at rates inferior to the diffusion limit. Simopoulos and Jencks (1994) recently reported that alkaline phosphatase catalyzes 4-nitrophenyl phosphate at diffusion-controlled rates. This observation, however, is not inconsistent with our general prediction, since 4-nitrophenyl phosphate is an artificial substrate—that is, it is not physiologically present in cells. The behavior of this artificial substrate can be understood in a more general context. We have observed in Demetrius (1995) that the type 1 condition is not evolutionarily stable; hence, evolution by mutation and selection can transform a type 1 enzyme defined by nondiffusion-controlled rates and broad specificity into a type 2 model described by diffusion-controlled rates and absolute specificity. The perturbation arguments used to establish this property can be invoked to show that the natural substrate of the type 1 enzyme can be structurally perturbed to react with the type 1 enzyme at diffusion-controlled rates. This property indicates that, in the case of type 1 enzymes, there do exist artificial substrates, structural variants of the natural substrate, which will react with the type 1 model at diffusion-controlled rates. Artificial substrates with this property, however, do not exist in the case of type 3 and type 4 enzymes. This situation is a consequence of the fact that mutation and selection are unable to transform either the type 3 or type 4 model into a type 1 enzyme (Demetrius 1995). This constraint on the evolutionary dynamics of the type 3 and type 4 models implies that under no circumstances can diffusion controlled rates be achieved for this class of enzymes.

The enzyme β-lactamase is known to react with the substrate benzylpenicillin at diffusion-controlled rates (Hardy and Kirsch 1984). We would therefore predict that β-lactamase would be characterized by absolute specificity. It is well established however, that β-lactamase has broad specificity—appreciable reaction rates being achieved for a wide variety of penicillins and cephalosporins. This anomaly, in the context of our theory, derives from the fact that antibiotics do not constitute the natural substrates of the enzyme. Penicillins and the related β-lactam antibiotics are exogenous substances. Their effects are uniquely bactericidal—a property which is expressed by inhibiting the activity of the D-alanyl-D-alanine peptidase involved in cell wall synthesis (Tipper and Strominger 1965). Hence the evolutionary dynamics of β-lactamase is determined by competition between β-lactamase and the cell wall enzymes for the β-lactam antibiotics. For the large majority of enzymes, however, the evolutionary dynamics is determined uniquely by competition between physiologically present substrates for the active site of the enzyme.

We will now appeal to the correspondence expressed in Table 2 in order to determine relations between enzyme activity and properties such as selective neutrality and adaptation.

Selectively Neutral and Adaptive Enzymes

The neutralist and selectionist hypotheses constitute the two main arguments proposed to account for the observed allelic variation in natural population. The neutral hypothesis asserts that the observed variations at a locus represent alleles that are selectively neutral. These variations involve substitutions at noncritical sites of the protein and thus have negligible effects on protein function. According to the selectionist hypothesis, the various observed allelic proteins have a significant effect on fitness. It is furthermore claimed that at each polymorphic locus, the polymorphism is maintained by heterozygote superiority. In the context of these two hypotheses, the following three modes of nucleotide substitutions may be distinguished.

1. *Adaptive:* The allelic substitutions may be advantageous or deleterious and thus constitute the material basis for current adaptive evolution.
2. *Absolutely neutral:* The mutations are either deleterious or have no effect on the net reproductive activity.
3. *Partially neutral:* The mutants are selectively neutral under certain environmental conditions but may become advantageous under an appropriate environmental state. Hence *partially* neutral mutants have a *latent* potential for selection and can be the raw material for future adaptive evolution.

We will now invoke the evolutionary theory of enzymes we have outlined to show that the properties, reaction rate, and substrate specificity provide a complete categorization of the three states: adaptation, absolute neutrality, partial neutrality.

Our analysis predicts the following patterns.

1. The condition of adaptation is common: It is described by enzymes with broad substrate specificity.
2. The condition of absolute neutrality is rare: It exists only in enzymes with diffusion-controlled rates—a small subset of the class of all enzymes.

3. The condition of partial neutrality is common: It is characterized by enzymes with absolute or group substrate specificity and nondiffusion-controlled rates.

Adaptive Enzymes

These molecules are the evolutionary descendants of type 1 enzymes. The directionality theory for enzyme evolution shows that mutation and selection acting on type 1 enzymes will result in random, nondirectional changes in k_{cat}/K_m and K_m. Present-day type 1 enzymes will therefore be characterized by a large variability in the parameters k_{cat}/K_m and K_m and also by a broad substrate specificity.

The kinetic parameters of present-day type 1 molecules will be described by nonextremal values of the rate constants. This implies that allelic substitutions in this class of enzymes can lead to both increases and decreases in the kinetic variables and hence induce variations in fitness. Type 1 enzymes will therefore have a potential for selection, which will be retained under different environmental conditions and different genetic background.

Examples: Most esterases, peptidases, and phosphatases are characterized by broad substrate specificity. Our model predicts that this class of enzymes will be subject to adaptive evolution. The strongest support for our general thesis is provided by alcohol dehydrogenase from *Drosophila melanogaster*. This enzyme has a broad specificity for a variety of alcohols. Analysis of the observed geographic frequency distribution with the distribution expected from a purely stochastic process has provided evidence that the patterns of geographic variation are maintained by selective environmental gradients (Oakeshott et al. 1982).

Two other intensively studied enzymes with broad substrate specificity and selective properties consistent with the predictions of our model can be noted.

1. Leucine aminopeptidase (LAP) in *Mytilus edulis*. The adaptive significance of the allelic variation of the enzyme was investigated by Hilbish and Koehn (1985), who showed that two allozyme variants at the Lap locus in *Mytilus edulis* differ in their catalytic efficiencies. The biochemical properties are known to influence the fitness of the organism through their effect on viability.
2. Esterase-6 (EST-6) in *Drosophila melanogaster*. The est-6 locus in *D. melanogaster* codes for two allozyme variants, which differ in their kinetic activity. The specific activity of the variant phenotypes determines the fitness of the organism through its effect on reproduction (Richmond et al. 1980).

Absolute Neutrality

Enzymes characterized by this state are the evolutionary descendants of the type 2 molecules. Our theory predicts that evolution will result in a unidirectional increase in both k_{cat}/K_m and K_m. Also, the increase in k_{cat}/K_m will continue until diffusion-controlled rates are attained. Present-day type 2 enzymes are operating at maximal catalytic effectiveness; consequently, random point mutations which result in amino acid substitutions will generate either a reduction in catalytic activity or have no effect on the kinetic variables. We predict that enzymes in this class will therefore be characterized by absolute neutrality.

Table 3. Enzymes with diffusion controlled rates

Enzyme	Substrate	k_{cat}/K_m (s^{-1} M^{-1})
Acetylcholinesterase	Acetylcholine	1.6×10^8
Carbonic anhydrase	CO_2	8.3×10^7
Catalase	H_2O_2	4×10^7
Crotonase	Crotonyl-CoA	2.8×10^8
Fumarase	Fumarate	1.6×10^8
Triosephosphate isomerase	Glyceraldehyde-3-phosphate	2.4×10^8

Examples: Table 3 gives a list of enzymes with diffusion-controlled rates together with their natural substrates. The enzymes in this group have absolute specificity—they exhibit appreciable reaction rates for a unique substrate (or in the case of bimolecular reactions, for a unique pair of substrates).

We recall that enzymes are said to be operating at diffusion-controlled rates when the reaction rate is determined by the encounter frequency of the enzyme and substrate. For enzyme-substrate systems, the encounter frequency is of the order 10^8–10^9 M^{-1} s^{-1}; consequently, values of k_{cat}/K_m in the range 10^7–10^9 M^{-1} s^{-1} are considered to have attained the diffusion limit.

An interesting example of an enzyme which is diffusion controlled and selectively neutral is superoxide dismutase (SOD). Superoxide dismutase is a copper metalloenzyme which catalyzes the removal of the highly reactive O_2^- to molecular oxygen and hydrogen peroxide. This enzyme has a k_{cat}/K_m of the order 10^9 M^{-1} s^{-1}, which represents one of the fastest reaction rates observed. Getzoff et al. (1992) has shown that exchange of a lysine for a glutamine residue resulted in increased reaction rate. This change, however, caused an increased inhibition by phosphate anions—a property which is presumed to confer a selective disadvantage to any organism that carries the mutant enzyme. This example illustrates the general claim that in the case of enzymes functioning at the diffusion limit, amino acid substitutions will be either neutral or result in a reduced selective advantage (even though the kinetic variable k_{cat}/K_m is increased).

This characterization of the properties of superoxide dismutase provides a new perspective on the studies by Hudson et al. (1994). The analysis of SOD locus in *Drosophila melanogaster* has revealed a pattern of variation which suggests that a rare variant has risen rapidly in

frequency. Hudson et al. (1994) proposes that the high frequency of the haplotypes is due to natural selection at the SOD locus or at some tightly linked locus. Our observation that the SOD enzyme functions at diffusion-controlled rates and is therefore absolutely neutral would tend to exclude natural selection at the SOD locus as the determining mechanism underlying the variation. The argument developed in this paper predicts natural selection at some tightly linked locus as the driving force behind the polymorphism.

Partial Neutrality

These molecules are the evolutionary descendants of type 3 and type 4 enzymes. Our theory shows that mutation and selection acting on type 3 and type 4 enzymes will result in a unidirectional increase in both k_{cat}/K_m and K_m. However, owing to the constraints on the thermodynamic parameters, the diffusion limit will not be attained; present-day type 3 enzymes will be described by a local minima of k_{cat}/K_m and a global maximum of K_m; present-day type 4 enzymes will be characterized by a local minima of k_{cat}/K_m and a local minima of K_m. Changes in the amino acid sequence in both classes of enzymes will result in a shift of k_{cat}/K_m from one local minima to another. This shift will induce either an increase, a decrease, or no change in the reaction rate—the nature of these transitions being highly dependent on the genetic background and the environmental conditions. We therefore predict that mutants of type 3 and type 4 enzymes will display a latent potential for selection—enzymes may be selectively neutral in one environment and become subject to selection in another environment.

Example: The enzyme phosphoglucose isomerase (PGI) is a type 3(b) enzyme: it has absolute substrate specificity—it converts glucose-6-phosphate (G6P) to fructose-6-phosphate. It also acts at nondiffusion-controlled rates. We predict that this enzyme will be described by partial neutrality—selectively neutral in one environment but subject to balancing selection in another environment. The empirical evidence accords with our prediction. Dykhuizen and Hartl (1983), working with the bacterium *E. coli,* studied several alleles of PGI in competition in chemostats. The studies revealed complete neutrality: There was little functional difference among allelic isozymes. Watt (1977, 1983), working with PGI in *Colias* butterflies, observed a multiallelic polymorphism under balancing selection. We thus observe for PGI selective neutrality under one environmental condition (the bacterial model) and balancing selection under another environmental condition (the *Colias* model).

Absolute Neutrality—Relative Incidence

Our theory of enzyme evolution enables one to predict the relative incidence of the different states: absolute neutrality, partial neutrality, and adaptation.

Type 2 and type 3(a) enzymes, the absolutely neutral class, constitute a small subset of the class of all enzymes. This follows from the property observed in Demetrius (1995)—that evolution from the primitive type 1 (large positive $\Delta H^{\ddagger}$, large negative $\Delta S^{\ddagger}$) to the type 2 (small negative $\Delta H^{\ddagger}$, small negative $\Delta S^{\ddagger}$) and type 3(a) models (small positive $\Delta H^{\ddagger}$, small positive $\Delta S^{\ddagger}$) involves surmounting a large energy barrier, a highly rare event. It is also important to note that the type 2 and type 3(a) conditions (small $\Delta H^{\ddagger}$) impose constraints on the chemistry of the enzyme-substrate reaction (Eyring et al. 1985) which for structural reasons are rarely satisfied.

Type 1 enzymes, the adaptive class, constitute a large subset of the class of all enzymes. The type 1 molecule, as we observed, includes the serine proteases, esterases, phosphatases—enzymes with broad substrate specificity. Type 3(b) and Type 3(c) enzymes, the partially neutral, partially adaptive class, also constitute a large subset. This superfamily includes kinases and synthetases and is described by nondiffusion-controlled rates with absolute or group specificity.

The relative incidence of the different categories can be understood from another perspective, one based on a classification of enzyme-substrate reactions in terms of the structural properties of molecular rigidity and flexibility (Demetrius 1997). These properties pertain to the conformational changes in enzyme and substrate which occur when the substrate binds to the active site at the transition state. Rigidity pertains to the absence of significant change in the average position of residues on binding of ligand; the enzyme in the presence of bound ligand is congruent to the enzyme alone; flexibility pertains to significant structural changes induced by binding (Koshland 1976).

In Demetrius (1997), we appealed to a thermodynamic theory of molecular deformation to determine a relation between the thermodynamic condition of the enzyme-substrate complex and molecular flexibility. This correspondence is described as follows:

$\Delta H^{\ddagger} > 0, \Delta S^{\ddagger} < 0$ (type 1):	Rigid enzyme—flexible substrate
$\Delta H^{\ddagger} < 0, \Delta S^{\ddagger} < 0$ (type 2):	Rigid enzyme—rigid substrate
$\Delta H^{\ddagger} < T\Delta C^{\ddagger}, \Delta S^{\ddagger} < \Delta C^{\ddagger}$ (type 3a):}	
$\Delta H^{\ddagger} > T\Delta C^{\ddagger}, \Delta S^{\ddagger} < \Delta C^{\ddagger}$ (type 3b):	Flexible enzyme—rigid substrate
$\Delta H^{\ddagger} > T\Delta C^{\ddagger}, \Delta S^{\ddagger} > \Delta C^{\ddagger}$ (type 3c):	Flexible enzyme—flexible substrate

This correspondence enables us to infer a characterization of selectively neutral and adaptive enzymes in terms of the structural criteria, rigidity and flexibility. In particular, we observe that flexible enzymes, characterized by type 3(b) and type 3(c) conditions, will be described by partial neutrality.

Flexible enzymes, that is, molecules which are subject to large conformational changes on binding of substrate, include the allosteric enzymes as a subgroup. We can therefore postulate the following general principle: Allosteric enzymes are characterized by partial neutrality.

Allosteric control refers to the regulation of enzyme

activity via cooperative protein–ligand interactions and via effector ligands which switch on or off individual enzymes. Allosteric control mechanisms are involved in all regulatory processes in cells and are therefore quite common. Accordingly, the condition of partial neutrality which characterizes allosteric enzymes is a common evolutionary property.

Conclusion

What proportion of the accepted mutations in the evolution of enzymes are adaptive or selectively advantageous for the organism and what proportion are selectively neutral and accumulate during evolutionary divergence as a result of drift? This article has exploited an analytical theory of enzyme evolution to address this question. Our analysis has delineated three classes of enzymes:

1. *Adaptive:* Accepted mutations in this class can be selectively advantageous for the organism.
2. *Absolutely neutral:* Mutations in this class are deleterious or have no effect on Darwinian fitness and hence possess no potential for selection.
3. *Partially neutral:* Mutations in this group have a latent potential for selection and may be selectively neutral in one environment and selectively advantageous in another.

We completely characterize, in terms of catalytic power and substrate specificity, each of these classes, and we show that adaptive enzymes (common) are characterized by intermediate reaction rates and broad substrate specificity; absolutely neutral enzymes (a rare class) are described by diffusion-controlled rates and absolute substrate specificity; and partially neutral enzymes (common) are described by nondiffusion-controlled rates and absolute or group substrate specificity.

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