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The energy relay: A proofreading scheme based on dynamic cooperativity and lacking all characteristic symptoms of kinetic proofreading in DNA replication and protein synthesis

(ribosomes/tRNA/accuracy/mutagenesis)

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ABSTRACT **A mechanism for proofreading biosynthetic processes requiring high accuracy is described. The previously understood "kinetic proofreading" mechanism of enhancing accuracy has distinguishing characteristics such as the nonstoichiometric use of substrate or cosubstrate that have allowed its identification in aspects of DNA and protein synthesis. The proofreading scheme developed here, though generically related, lacks all the previous identifying features. A DNA polymerase proofreading in this manner need neither generate dNMP nor have a 3′→5′ exonuclease activity. Protein synthesis could be proofread even with stoichiometric GTP consumption or without elongation factor Tu·GTP. The kinetic scheme that generates this proofreading makes use of an "energy relay" from previous substrate molecules and is a representative of a class of nonequilibrium processes displaying dynamic cooperativity. This proofreading mechanism has its own identifying characteristics, which are sufficiently subtle that they would have generally escaped notice or defied interpretation.**

The ability of an enzyme to discriminate between correct and incorrect substrates is based on energy differences between these substrates when bound to the enzyme in appropriate transition states. Simple Michaelis–Menten enzymes have a finite energy difference $\Delta G^\dagger$ between such states for similar substrates and thus must make errors at least a finite fraction $f_0 \equiv e^{-\Delta G^\dagger/RT}$ of the time when presented with equal concentrations of both substrates (1, 2). Errors can represent a serious limitation on the abiliity to process biochemical information, and cells make use of many error-reducing and error-correcting mechanisms to keep errors in DNA, RNA, and protein synthesis at low levels.

Kinetic proofreading is the simplest general mechanism of error prevention at the molecular level (2, 3) beyond a brute-force increase of $\Delta G^\dagger$ (and concomitant reduction of f_0) by precise stereochemical constraints. In cases in which the enzyme does not [or even in principle cannot (1, 4)] increase the simple discrimination energy $\Delta G^\dagger$, proofreading can nonetheless be used to reduce errors far below f_0 at the cost of an additional expenditure of energy (2, 5–7). Kinetic proofreading schemes have readily identified hallmarks (1–3). From these, it now appears that the "editing" of some prokaryotic DNA polymerases (8–11), the operation of several of the aminoacyl tRNA synthetases (12–14), and the use of elongation factor Tu·GTP in protein synthesis (15–17) and codon recognition all involve the same basic proofreading scheme and branched pathway.

Yet puzzles still abound in questions of accuracy. For example, if the easiest way to gain accuracy beyond f_0 is by kinetic proofreading, why do few eukaryotic DNA polymerases show the hallmarks of such a process? Are the requisite activities simply lost in purification, or are they for some reason not needed? Are the replicative assemblies and the variety of proteins used in DNA or RNA polymerization or protein synthesis increasing accuracy by improving the stereochemistry, or merely by producing kinetics that allow available stereochemistry to be expressed? Or in this welter of structural complication may new phenomena be going on? Such questions form the background of the present work.

The "energy relay" is a mode of enzyme operation in which the recent past can be "remembered" by an enzyme in a dissipative system. This memory can be used to obtain useful and novel results in enzyme properties. Its physical basis is the nonequilibrium populations generated in a driven kinetic system displaying multiple conformations. We will use this mechanism to produce a proofreading scheme that, though conceptually a form of kinetic proofreading, lacks all the conventional hallmarks of such proofreading. This proofreading through an energy relay and dynamic cooperativity may be taking place in polymerases, replicons, etc., where simple kinetic proofreading is believed not to occur because its identifying characteristics are absent. It may also occur in addition to simple kinetic proofreading.

The hallmarks of elementary kinetic proofreading

Kinetic proofreading is a general means of obtaining higher accuracy from a *given* discrimination energy $\Delta G^\dagger$ between correct and incorrect substrates (1–3). The topology of the reaction pathway is different from that of a nonproofreading enzyme in that a branched reaction pathway is essential. The simple

$$E + S \rightleftharpoons ES \rightleftharpoons (ES)^\dagger \ldots \rightleftharpoons E + P_S. \qquad [1]$$

Michaelis–Menten pathway is not branched—a substrate molecule bound to the enzyme is either released as product P_S or released as S with no change in it or any other cosubstrate. Such a pathway cannot proofread (18). The elementary kinetic proofreading process demands a branched pathway, as for example (2)

$$E + S \rightleftharpoons ES \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} (ES)^* \rightleftharpoons E + P_S \qquad [2]$$

$$E + S$$

or

$$E + S \rightleftharpoons ES \longrightarrow (ES')^* \rightleftharpoons E + P_S. \qquad [3]$$

$$E + S'$$

Abbreviation: Tu, elongation factor Tu.

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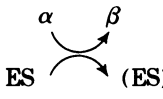
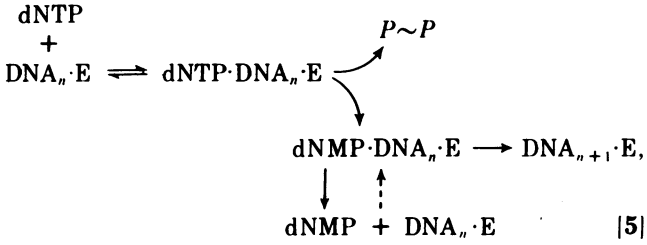
Other equivalent branched diagrams (3) produce the same result for the same reason. In scheme 2, (ES)* is a high-energy intermediate, so high in energy that the reaction indicated by the dashed arrow is negligible. This intermediate is reached by coupling ES → (ES)* reaction with the degradation of a high-energy molecule α into a lower energy state β. This step



[4]

should be highly irreversible (2) for the system to proofread well. This scheme seems to be used in acylating tRNA (12–14). Scheme 3 is used in protein synthesis, in which S is the ternary complex Tu·GTP·aatRNA, and S′ is aatRNA (15–17). Scheme 3 also reflects the way in which prokaryotic DNA polymerases can proofread (8–10). In this case, S is a deoxynucleoside triphosphate (dNTP) and S′ is dNMP. Pyrophosphate is cleaved from dNTP in the ES → (ES′)* step. The detailed reaction becomes



[5]

in which DNA_n is a growing DNA 3′ terminus having n base pairs on the double-stranded side, and E represents the enzyme or replicon. As in case 2, the branching ratio for going forward to DNA_{n+1}·E or for aborting the reaction (going to dNMP) from dNMP·DNA_n·E will be substrate dependent, allowing the rejection path dNMP·DNA_n·E → DNA_n·E + dNMP to be used for erroneous substrates (i.e., a mispaired terminus) while dNMP·DNA_n·E → DNA_{n+1}·E chiefly occurs for the correct substrate. Again, the reaction is set up in such a way that dNMP·DNA_n·E is dominantly reached from DNA_n·E + dNTP (and not directly via the dashed arrow pathway) because dNMP·DNA_n·E is a high-energy intermediate compared to dNMP + DNA_n·E.

Let the error fraction of the system in making the first irreversible product (ES)* or (ES′)* be f_o when equal amounts of correct and incorrect substrates are present. Let r_A be the stoichiometry ratio of the number of times the energy reaction ($\alpha \rightarrow \beta$) is used to the number of P_A form when only A is present. The observed error fraction f_{obs} for the entire reaction is thus

$$f_{obs} = \left(f_o \frac{r_A}{r_B}\right) \frac{[B]}{[A]} \equiv f \frac{[B]}{[A]} \qquad [6]$$

when B and A are present at arbitrary concentrations. f_o is the intrinsic error fraction of the enzyme with the trivial factor of substrate availability removed. The proofreading enhancement of accuracy comes solely from the stoichiometry product r_A/r_B, which must be $\ll 1$ if effective proofreading is present. In reaction scheme 3 an analogous statement can be made. These considerations do not include the effect of "peelback" in DNA replications, which can readily be included when relevant (19, 20).

Simple kinetic proofreading has identifying characteristics because of the branched pathway and the position of the branch after the energy-use step. For definiteness, these will be stated for a DNA polymerase, with parenthetic remarks about protein synthesis. Correct nucleoside triphosphates will be called dATP; incorrect triphosphates will be called dBTP.

(*i*) In the presence of a *large* excess of incorrect dBTP compared to correct (dATP) ones, there *must* be turnover of dBTP → dBMP. *The entire scheme depends on such turnover.* (The analog in the case of protein synthesis and codon reading is the hydrolysis of GTP from the ternary complex. Tu·GTP·$aatRNA^{aa}$·ribosome·mRNA without amino acid incorporation when the codon match is wrong.)

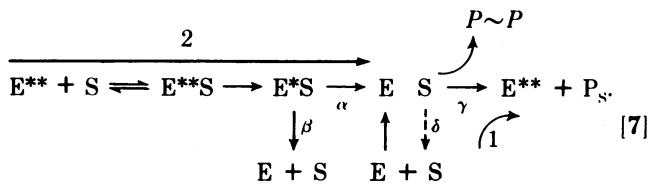
(*ii*) The scheme requires the stoichiometry ratio for the *correct* substrate alone to be greater than 1.0, so there *may* be measurable turnover of dATP → dAMP. In principle, it can be reduced to a very low level by multiple state schemes, so this stoichiometry ratio can be very close to 1.0. Examining this stoichiometry is sensible when searching for proofreading, but a failure to find dAMP formation does not rule out simple kinetic proofreading. (The same statement can be made of Tu·GTP usage in protein synthesis.)

(*iii*) Because the reaction (ES′)* ⇄ E + P_S may be reversible under some circumstances, those circumstances can lead to the reaction $P_S \rightarrow P + S'$ for either the correct or the incorrect substrate. If this reaction is reversible, the 5′→3′ polymerase will also be a 3′→5′ exonuclease. Under some circumstances this exonuclease activity could be experimentally missed—for example, its activity might require bound pyrophosphate on the polymerase, and the activity might then be absent in a naive assay. The activity might be present on a separate protein in the replicon and absent in a purified polymerase. But the presence of a possible exit path for dNMP means that at least in some conditions, a 3′→5′ exonuclease activity must be present. (A tRNA-dependent ribosomal degradation of growing polypeptides has never been identified.)

These three hallmarks of conventional kinetic proofreading by DNA polymerases all exist by virtue of the fact that the exit path for errors in proof is as dNMP. [A similar scheme can obviously be generated with dNDP as the exit path, but does not seem to occur (21).] It has generally been presumed that in the absence of *i*, *ii*, and *iii* a simple DNA polymerase reaction (without additional energy couplings, as to an additional external ATP cleavage) cannot proofread. The next section is a counter example to this presumption.

An energy relay in kinetic proofreading

Let E** be a high-energy metastable state of enzyme E, and E* be a less high-energy state.



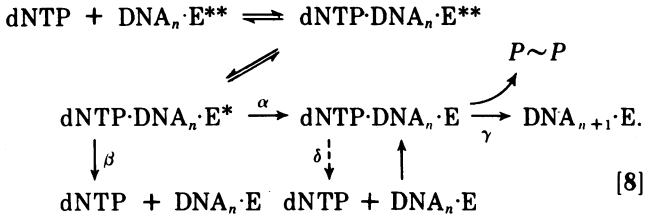
[7]

The qualitative description of the enzyme reaction is as follows. If the enzyme is in state E, the substrate binds, and product forms with normal Michaelis accuracy f_o by pathway 1. This reaction, however, does *not* regenerate E, but leads to E**, using some of the available energy of the phosphate cleavage to alter the state of the enzyme. The formation of (E**S) is then done with the usual Michaelis accuracy, followed by an "irreversible" change to E*S. The forward rate $\vec{\alpha}$ to ES and the abortion rate $\downarrow\beta$ to E + S can be arranged so that the material reaching the state ES via pathway 2 has been effectively proofread by exit path $\downarrow\beta$. E*S can be such a high-energy intermediate that the reverse reaction to $\downarrow\beta$ is immaterial. Reaction $\vec{\gamma}$ is now made rapid compared to $\delta\downarrow$ for *all* substrates, so that essentially all material that arrives at ES from pathway 2 (and has thus been proofread) is converted to product. (γ

could be followed by other slow steps before P_S was formed with no alteration of accuracy considerations.)

In this process, some substrates E*S have followed path $\downarrow\beta$, yielding E + S. These enzyme molecules must be recycled via pathway 1, and the substrate processed in this recycling step will not be proofread. In addition, because $\overrightarrow{\gamma}$ is fast, the reaction δ must exhibit its discrimination in the "on" kinetic constants, contrary to reaction $\downarrow\beta$, for which discrimination in the "off" kinetic constants is possible. This is why it is necessary in this scheme to have two side branches rather than one. In general, it seems that the "exit path for errors" cannot be the restarting pathway and still obtain a net proofreading.

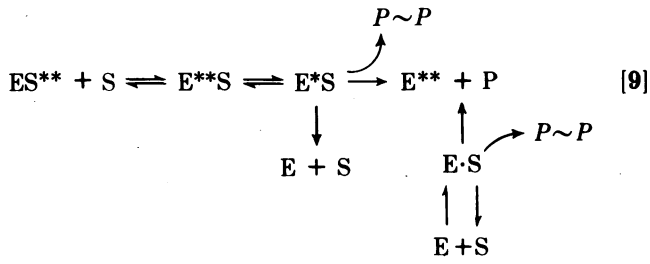
Note that the only way the substrate leaves the enzyme is as S or P_S. Applied to a DNA polymerase, such a scheme becomes



[8]

Path β, the exit for errors in proof, rejects errors as dNTP. *No release of dNMP is ever possible in this scheme.* Each pyrophosphate released increases the DNA length by one base. *All the hallmarks of elementary kinetic proofreading are lacking,* and yet the enzyme manages to proofread, by relaying forward energy stored from the chemical reaction involving a previous substrate molecule.

The scheme for the energy relay requires specificity to be expressed in the "on" constants, a not particularly common event in the discriminations for which proofreading is most necessary. This limitation is not intrinsic to the energy relay scheme. The minor variant



[9]

has the same proofreading features and hallmarks (next section), but could have all its specificities expressed in "off" constants.

The one critical set of kinetic parameters is the relative rate off in step $\downarrow\beta$ and forward in step $\overrightarrow{\alpha}$. Let the off kinetic constants for process β be denoted by k_A for correct substrate and k_B for incorrect substrate. k_o is the rate for process α, the same for both substrates. If there is a given $\Delta G \rightarrow f_o$, and the incidental steps such as the first enzymatic recognition display the full f_o, the overall accuracy of this system is

$$f_{obs} = f_o \frac{[B]}{[A]} \times \left\{ \frac{\frac{[A]}{[A]+[B]f_o}\cdot\frac{k_A}{k_o+k_A} + \frac{[B]f_o}{[A]+[B]f_o}\cdot\frac{k_B}{k_o+k_B} + \frac{k_o}{k_B+k_o}}{\frac{[A]}{[A]+[B]f_o}\cdot\frac{k_A}{k_o+k_A} + \frac{[B]f_o}{[A]+[B]f_o}\cdot\frac{k_B}{k_o+k_B} + \frac{k_o}{k_A+k_o}} \right\}. \quad [10]$$

When k_o is very large or very small, the expression in brackets goes to 1.0 and the system does not proofread. For [B]/[A] ≈ 1, if $k_o \approx \sqrt{k_A k_B}$ the system shows best proofreading. For example, if $k_A = 1$, $k_B = 1000$ (corresponding to $f_o = 1000$), and $k_o = 31.6$, then

$$f_{obs} = \left(\frac{1}{1000}\right)\frac{[B]}{[A]} \times \left\{ \frac{\frac{0.031}{1+0.001[B]/[A]} + \frac{0.000969\cdot[B]/[A]}{1+0.001[B]/[A]} + 0.031}{\frac{0.031}{1+0.001[B]/[A]} + \frac{0.000969\cdot[B]/[A]}{1+0.001\cdot[B]/[A]} + 0.0969} \right\}.$$

For [B]/[A] = 1, the error fraction is reduced by a factor of 0.064 through proofreading (i.e., the expression in brackets is 0.062). For [B]/[A] = 70 the proofreading (the expression in brackets) is only half as good (i.e., the expression in brackets now has the value 0.125). For very large [B]/[A], a situation not normally faced by a cell, the proofreading disappears.

The above proofreading enhancement of accuracy only reached a level of 0.064 rather than the level of 0.001 possible in the simplest ordinary kinetic proofreading scheme. The difference has little importance. Both schemes are capable of using the one-shot energy boost to achieve multiple proofreading through further pathway branches (5, 12) and so are not limited by the numbers above. And in the simplest of kinetic proofreading schemes, so much substrate is wasted in trying to approach the ultimate in accuracy that as a practical matter the system would in such a mode probably run nearer 0.010 than 0.001 for its proofreading enhancement (5, 22).

In spite of the lack of usual symptoms, the energy relay scheme for proofreading has conceptually much in common with simple kinetic proofreading. Yet the chemical physics of the enzyme molecule is fundamentally different—it now must "remember" its recent history. Most enzyme reaction schemes, including that of simple kinetic proofreading, use no such memory of previous substrate molecules.

The hallmarks of proofreading by an energy relay

The energy relay system for proofreading rejects some substrates, both correct and incorrect, by pathway β. Immediately after doing so, the enzyme is in state E, and the next substrate molecule to be turned into product (by pathway 1) will not be proofread. Such a restarting pathway is essential to the simple system. (It could be avoided by a separate energy-supplying external restarting system that converted E to E** by using an energy source such as ATP. Such an energy relay system would normally consume much less than stoichiometric ATP, because restarting is not a common event. It would lack *both* the characteristics of simple kinetic proofreading *and* the following characteristics *i* and *ii* and have instead more subtle symptoms.)

The use of this essential restarting pathway 1 produces most of the behaviors by which the occurrence of such a proofreading might be recognized.

These characteristics include:

(*i*) The error fraction f_{obs} rises more rapidly than linearly as a function of [B]/[A] (see Eq. **10** and compare with Eq. **6**), unlike the Michaelis–Menten or simple kinetic proofreading cases, which are linear.

(*ii*) The addition of a second incorrect substrate B′ raises the error fraction for substrate B compared to correct substrate A. (For polymerases copying heteropolymers, the effects can appear more complicated due to the changing definition of "correct" and "incorrect." For totally processive polymerases, the foregoing statement is adequate, but for less processive

polymerases, an increased probability of using the proofreading pathway while attempting to read base X will increase the error fraction for *other* base pairings.)

(*iii*) The start-up round from E is less accurate than the typical turnover and is characterized by different kinetics.

(*iv*) The system may exhibit phenomena such as pyrophosphate exchange without pyrophosphorolysis in DNA replication. Such a possibility depends on many details, but arises because in reaction 7 "reversal" by pyrophosphate may generate E rather than E**.

The existence of three structural and chemical states, E, E*, and E**, with appropriate kinetic constants is fundamental to the operation of the enzyme in this proofreading mode. E** and E may be relatively easy to study either structurally or kinetically. The existence of E* will be much harder to demonstrate in detail. In view of this difficulty, a search for the identifying characteristics seems the easiest way to look for examples of this phenomenon.

Biological questions and circumstances related to energy relay proofreading

A simple polymerase with a proofreading exonuclease activity such as *Escherichia coli* DNA polymerase I or III (8, 9) or T4 DNA polymerase (10) has features that are not ideal in a DNA replicating system. First, in the absence of substrate they degrade their product. Second, under conditions of low substrate concentrations or to maximize accuracy much of the precious substrate is lost, turned into dNMP rather than product. Third, when there is a mutilated base or damage in the DNA sequence being copied, these polymerases uselessly turn over substrates, dNTP → dNMP, searching in vain for a correct match when none is possible. Energy relay proofreading does not degrade DNA (except trivially, by pyrophosphorolysis), and never turns over dNTP except to add a base to a growing strand. There may thus be a biological circumstance that favors this solution to the accuracy problem.

In many systems involving accuracy there are systematic discrepancies or incompletenesses of understanding which leave room for the occurrence of energy relay proofreading. The following examples are *not evidence for* such proofreading, but merely indications of systems whose characteristics suggest an energy relay *might* be operating.

The accuracy of codon reading on the ribosome depends on the energization of the system in ways beyond that expected from simple kinetic proofreading (23) alone. It is also clear that tRNA being recognized by a codon triplet at the ribosomal A (aminoacyl) site has *at least* two states on the ribosome (24–27), one weakly bound and one more tightly bound. Kurland *et al.* (28) described an "accuracy enhancement" scheme in which the multiple configurations of tRNA bound to a ribosome were used to enhance accuracy. While close inspection and thermodynamics indicates that this system is merely postulated to have a large $\Delta G^{\dagger}$ in a particular configuration reached after several steps, a true enhancement function of the multiple configurations can be correct if the system uses energy relay proofreading. Energy relay proofreading could occur even for protein synthesis without Tu, using either the peptide bond formation energy or the translocation GTP to drive the reaction.

The aminoacyl-tRNA synthetases, which select amino acids and their tRNA, also have peculiarities. While some of them do seem to exhibit proofreading, others with perhaps similar fidelity problems (12, 13) do not. For the *E. coli* protein activating isoleucine, complications were of such a nature that Loftfield (29) was led to surmise that the normal enzymatic pathway was not the same as that followed by kinetic attempts to examine intermediates through artificially arresting the reaction. And while this enzyme seems to show proofreading, there is a discrepancy in the kinetic parameters associated with enzymatic deacylation. The apparent rate seems to depend on whether it is measured in the acylation reaction or in the reverse direction, and in addition (30) it is unexpectedly biphasic when charged tRNA is used as a substrate. An enzyme displaying unsuspected states such as E** and E* could readily show this kind of discrepancy.

In DNA polymerases, there are anomolous results involving pyrophosphate. While *E. coli* DNA polymerase I exhibits pyrophosphorolysis and pyrophosphate exchange, calf thymus polymerase α does not, and it is inhibited both competitively and noncompetitively by pyrophosphate (31). Such an unnecessarily tightly bound pyrophosphate could have a function in defining E**. The avian myeloblastosis virus reverse transcriptase exhibits much lower fidelity in pyrophosphate exchange (32) than in polymerization, a result perhaps associable with the distinction between E and E**. Unfortunately, both these results are also compatible with more mundane explanation.

Alberts and coworkers (ref. 33; W. Miller and B. Alberts, personal communication) found in their study of the T4 polymerase replicon reason to pursue the idea of a "pre-reading" of a base at the next site, not yet at the pyrophosphate cleavage site on the polymerase. To use this hypothesized event as a kinetic proofreading process necessitated coupling it to an additional ATPase. Energy relay proofreading provides a mechanism for such a process even without an ATP coupling. The general nature of the accuracy enhancement from the addition of single-strand binding protein to polymerase reactions (34) causes one to wonder whether something other than brute force accuracy is involved.

In DNA replication, there is evidence that an undecipherable lesion at one point in the DNA reduces the accuracy of replication at distant sites. These SOS "inducible" widespread errors (35, 36) are normally thought of in terms of a new (or modified) error-prone polymerase. Energy relay proofreading coupled with somewhat nonprocessive (at least at lesions) DNA synthesis would in itself produce fidelity reduction throughout the genome in the presence of DNA damage. Substances or conditions that slow DNA synthesis would generally be expected to be mutagens in this scheme, particularly if spontaneous relaxation from E** to E takes place. (This effect is in the opposite direction from that to be expected of simple kinetic proofreading, in which a slowing-down often gives the exonuclease proofreading path a better chance to operate.)

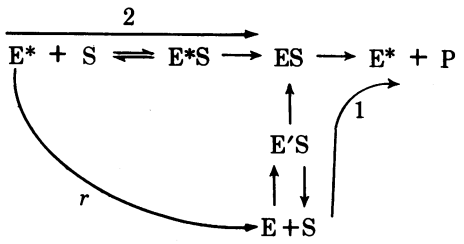
The relationship between observations and biological functions is very subtle in this problem. If nature has chosen to use this mechanism, only a fanatic enzymologist would have been so complete that he might have directly constructed the mechanism from experimental results. The foregoing "evidences" indicate only that there is room, in what is known and speculated, for energy relay proofreading to be operative.

Appendix: The energy relay—an example of dynamic cooperativity

"Cooperativity," as normally used to describe enzyme properties, refers to a system with several distinct binding sites that interact when they are occupied (37–39). In the most common usage, there are at least two binding sites for a given substrate, with the binding or enzymatic activity or both of one site dependent on the occupancy of the other site at the same time. This kind of cooperativity might be termed "static," because its properties arise from the occupancy at one point in time of two spatially separated sites.

An enzyme with a single binding site for a given substrate, but with multiple internal states, can have properties (for interacting with its present bound substrate molecule) that depend on what has happened to a substrate molecule that recently left that same site. This kind of cooperativity between binding events at a *single spatial site at different times* might be termed "dynamic" (or temporal). The energy relay uses dynamic cooperativity.

Dynamic cooperativity can mimic some properties of static cooperativity. For example, a sigmoid dependence of the rate of product generation on free



substrate concentration can be produced by this kinetic scheme, a minor variant of 7, if pathway 1 is rapid and pathway 2 is slow. r represents a relaxation process. Theoretical descriptions (40, 41) have been given for such behavior, using the binding of cosubstrates to produce states equivalent to multiple enzyme conformations. Hysteretic behavior (42) is another aspect of the same kinetics.

In general, dynamic cooperativity has been hard to document because secondary binding sites and static cooperativity are hard to distinguish from dynamic cooperativity. For most biological functions, static cooperativity can generate the important effects that dynamic cooperativity could. In the case of the energy relay, however, dynamic cooperativity provides a mechanism of proofreading that static cooperativity cannot.

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