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Kinetic Proofreading: A New Mechanism for Reducing Errors in Biosynthetic Processes Requiring High Specificity

(protein synthesis/DNA replication/amino-acid recognition)

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ABSTRACT **The specificity with which the genetic code is read in protein synthesis, and with which other highly specific biosynthetic reactions take place, can be increased above the level available from free energy differences in intermediates or kinetic barriers by a process defined here as kinetic proofreading. A simple kinetic pathway is described which results in this proofreading when the reaction is strongly but nonspecifically driven, e.g., by pliosphate hydrolysis. Protein synthesis, amino acid recognition, and DNA replication, all exhibit the features of this model. In each case, known reactions which otherwise appear to be useless or deleterious complications are seen to be essential to the proofreading function.**

Introduction

The proper functioning of protein synthesis depends on the ability to "read" the genetic code with few mistakes. In protein synthesis, the maximum frequency at which a wrong but similar amino acid is inserted has been estimated at 1 in 10^4 (1), so levels of discrimination superior to that must be maintained in the several recognition steps between amino-acid monomer and the product protein. Indeed, one of the fundamental general problems of biosynthesis is to understand how small error rates are achieved.

The customary view of the origin of such error rates can be described by an energy of discrimination. In typical biosynthetic processes in which "reading" is important, it is desired at some particular time to incorporate substrate C but not D, in spite of the fact that the final products of C or of D incorporated have essentially undistinguishable energies. Such incorporations are done through a recognition site c which is used somewhere along the reaction pathway, and which makes that region of the pathway energetically more favorable to C than to D. In simple reaction schemes, the frequency with which errors are made in site recognition is greater than or equal to $\exp - (\Delta G_{CD}/RT)$, where ΔG_{CD} is the largest difference between the free energy of D and of C moving along the reaction pathway containing the recognition site c for C.

It is often difficult to justify the 5.5 kcal (23 kJ) necessary to explain the known low error rates of 10^{-4} in protein synthesis, both in the case of codon-anticodon binding and in amino-acid recognition (2). The situation is much worse in the case of DNA replication, where the error-rate is about 10^{-9} (3, 4). Because the only simple discrimination mechanism is a ΔG_{CD} along the pathway, many descriptions of the energetics of recognition have an *ad hoc* character. One is, therefore, led to ask whether, with a *given* maximum ΔG_{CD} along the reaction path, it is possible to reduce the fraction of errors substantially below $\exp - (\Delta G_{CD}/RT)$. From a phenomenological point of view, if it were possible to *proofread* the product (or the site recognition during synthesis) once with the *same precision* as the conventional first identification, the fraction of errors would drop to $[\exp - (\Delta G_{CD}/RT)]^2$. While such proofreading is conceptually possible, there is no known mechanism for such proofreading in the recognition steps of protein synthesis. Proofreading or "editing" has been suggested in DNA replication (5, 6), but a detailed description of its chemical kinetic basis is lacking. The problem is thus to find a simple quantitative model containing the essential features of a proofreading scheme. Most highly-selective recognitions in biosynthesis are carried out enzymatically and are strongly driven by the hydrolysis of nucleoside triphosphates. These circumstances allow the construction of a simple mechanism of "kinetic proofreading." The known sequence of steps in several biosynthetic processes is precisely that necessary for the operation of this mechanism.

The kinetic proofreading model

The usual scheme for discrimination between substrates C and D by a recognition site c for substrate C is based on Michaelis kinetics. The reactions

$$C + c \underset{k_C}{\overset{k'_C}{\rightleftarrows}} Cc \xrightarrow{W} \text{correct product} \quad K_C = k'_C/k_C$$

$$D + c \underset{k_D}{\overset{k'_D}{\rightleftarrows}} Dc \xrightarrow{W} \text{error product} \quad K_D = k'_D/k_D \qquad [1]$$

are the starting point for a conventional description of reading errors (7, 8).

For clarity, we consider the simplest case expected to be biochemically relevant rather than the most general case. It is, therefore, presumed in [1] that incorporation take place from the Michaelis complex Cc or Dc at the same rate. Such indiscriminant incorporation is reasonable when the covalent bond formed upon incorporation is the same for either D or C, as in protein synthesis. Experiments have shown the independence of the maximum turnover rate on substrate species in some discrimination reactions (9, 10) in accord with a common W.

Discrimination in [1] can be based on the kinetic "on" rates, the "off" rates, or on the equilibrium constants. To make it obvious where the energies of discrimination are (a choice also consistent with maximum proofreading) let

$$k'_C = k'_D;\ K_D/K_C = k_C/k_D \ldots \quad [2]$$

In this case there is no discrimination between C and D in the barrier to the formation of the Michaelis complex. The entire energy of discrimination then lies in the Michaelis complex itself and in the kinetic dissociation rates. This supposition is approximately true in the case of the binding of short complementary oligonucleotides, where the rate of binding of pairs does not change much with binding energy (8, 11).

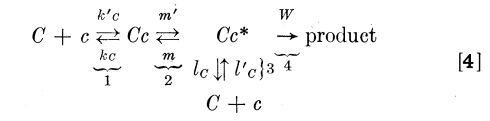
Define the error fraction f as the rate of incorrect product formation divided by the rate of correct product formation when substrate C and D are in equal concentrations. For the reactions [1] in steady state with simplifications [2],

$$f = (W + k_C)/(W + k_D) \quad [3]$$

The minimum error fraction attainable is

$$k_C/k_D = K_D/K_C \equiv f_0 = \exp - (\Delta G_{CD}/RT)$$

We next examine a two-stage kinetic model which *iterates* the same kind of discrimination. The reaction pathway for C (or for D, *mutatis mutandis*) is



[4]

$$K_C = k'_C/k_C;\ K = m'/m;\ L_C = l'_C/l_C$$

which adds an intermediate Cc^* (or Dc^*). Step **2** is assumed to be *totally insensitive to the difference between C and D*. Because step **2** is nonspecific, $K_D/K_C = L_D/L_C$. Offrates carry the specificity, so $l'_C = l'_D$ and $k'_C = k'_D$.

In the absence of side-reaction [3], the reaction path **1–2–4** provides no advantage over that of the simpler Eq. [1]. For $W \to 0$, the error fraction is f_0, and it increases as W increases. In the absence of reactions **1** and **2**, the side reaction pathway **3–4** is exactly Eq. [1] and also has a minimum error fraction of f_0.

The full reaction of Eq. [4] has the error fraction

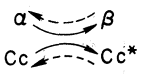
$$f = \frac{[l'_D(k_D + m') + m'k_D'][(k_C + m')(W + l_C) + k_C m]}{[(k_D + m')(W + l_D) + k_D m][l_C'(k_C + m') + m'k_C']} \cdots \quad [5]$$

(For reference purposes, no special suppositions about "on" rates are present in [5].) Reactions [4] as written, have an equilibrium constraint

$$(m'/m)_{\text{equilib.}} = (l_C'k_C/l_C k_C') = l_D'k_D/l_D k_D' \quad [6]$$

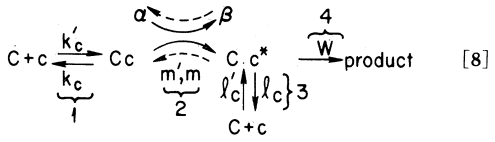
relating m and m'. Within this constraint, Eq. [5] never yields an error fraction less than f_0.

Increasing specificity in this system requires energy for reasons sketched in the following section. Let the intermediate step **2** be driven by enzymatic coupling to some other reaction $\alpha \leftrightarrow \beta$ which can be used as an energy source, as for example by



[7]

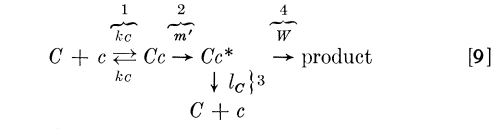
The rates m and m' are now coupled to an energy source, and need not be related by Eq. [6]. The total reaction pathway for the incorporation of C is



[8]

with an equivalent reaction for substrate D. The energy source might in a typical example use ATP for α, with AMP and pyrophosphate as the product β.

The reactions [8] have expression [5] for the error fraction, but *without* the constraint [6]. The elimination of the constraint allows far better error fractions. Suppose Cc^* is a high energy intermediate, so that $l_C' = l_D'$ is negligible. The population of Cc^* comes from the driven reaction [2] coupled to phosphate hydrolysis. The back reaction m can be made negligible by keeping the PP_i concentration low. Under these circumstances the effective reaction scheme is



[9]

If $m' \lesssim k_C$, the first intermediate Cc or Dc will achieve a near equilibrium ratio between D and C when equal quantities of the two substrate are present. Thus $[Dc]/[Cc] \approx f_0$. The reaction sequence **2–3–4** behaves in a fashion analogous to the Michaelis scheme of Eq. [1], with "on" rates and incorporation rates independent of substrate but off-rates different in the ratio f_0. However, the entrance to the second intermediate is from the first intermediate, which is biased by a factor of f_0 against D. Equation [9] is thus equivalent to the use of Eq. [1] in a situation where the *source* population is already weighted against D by a factor f_0. The same off-rate "reading" mechanism is used in each of the two consecutive discriminations. When $W \lesssim l_c$, the net result is an error fraction $f \approx f_0^2$ expected for a double discrimination. *This driven kinetic pathway using a high energy intermediate achieves an error fraction equal to one achievable by doubling the differences in binding energy between C and D for a simple process like Eq.* [1], or to proofreading once.

To achieve an f-value approaching f_0^2 several conditions must be met. First, the wrong substrate arriving at Dc^* must come typically through step **2** rather than step **3**, so $m'k_D'/(m' + k_D) \geq l_D'$. Second, the rate of loss of molecules Dc^* must be dominantly by path **3**, so m and $W \lesssim l_D$. Third, just as for Eq. [1], $m' \lesssim k_C$. The first two of these inequalities together require

$$m'/m \gtrsim (1/f_0)(m'/m)_{\text{equilib.}} \quad [10]$$

Thus, to obtain an error fraction comparable to f_0^2, reaction **2** must be driven hard enough to the right so that the effective equilibrium constant (Eq. [6]) for the two intermediates of a given substrate is increased by a factor of at least $(1/f_0)$. Driven less hard, f will still be enhanced, but not to the level of f_0^2. The hydrolysis reactions of nucleoside triphosphates are out of equilibrium by factors up to 10^8, so large driving "forces" are available *in vivo*.

Further enhancement of selectivity can be achieved by stacking in such driven stages of the reaction. Several different driving steps, or a single very high energy intermediate followed by n downhill steps to lower intermediates (each of

which can break up) could be used to reach a discrimination level of f_0^{n+1}.

From the representation [**9**], the flexibility available in details becomes clearer. *Any* arrangement of kinetic constants such that m' when driven is substrate independent and small, can discriminate against D by the factor $f_0 = K_D/K_C$ in populating Dc^*. Dc^* can be dissociated in step **3** more than Cc^* by $W_D/W_C = f_0$, $L_C = L_D$, $l_C = l_D$ just as well as by the particular kinetic combinations emphasized. Care must be taken in constructing such a scheme not in fact to increase the maximum difference ΔG_{CD} somewhere along the reaction pathway, but properly done, such modifications also proofread.

Proofreading in a Michaelis viewpoint

The following representation of the basis of proofreading may be useful in examining other related schemes. The reader interested in applications of the given scheme can omit this section.

It is possible to "collapse" the complicated kinetics generated by the Eq. [**9**] approximation to Eq. [**4**] into a modification of the Michaelis scheme Eq. [**1**] with only one intermediate, Cc. Since both intermediates in [**9**] can break up into $c + C$ with discrimination between C and D in the off-rates, the single intermediate in a "collapsed" scheme will have this property, and acts as if $l_C = k_C$, $l_D = k_D$. The problem in collapsing [**4**] onto [**1**] is the representation of W. In [**1**], as soon as cC is formed, it immediately starts to make product at a rate W. In [**4**], the formation of Cc does not begin product formation, but instead begins the generation of Cc^*. The rate of generation of product in [**9**] starts at *zero* when Cc is formed. Thus, [**9**] will be equivalent to a scheme like [**1**], but with an effective time-dependent $W(t)$ (the measuring time from the time of formation of a Cc complex) starting at zero and rising later, rather than a constant time-independent W for the incorporation rate. It is as though there were an effective "delay" in the turning on of W after the formation of Cc in an otherwise normal Michaelis discrimination.

When "off" kinetic constants are different between two substrates, a time delay can greatly enhance the discrimination between them. Let W be always small but time-dependent. If the complex Cc forms at time zero, the probability of the incorporation of C before the complex breaks up is approximately

$$\int_0^\infty e^{-k_C t} W(t) dt \qquad [11]$$

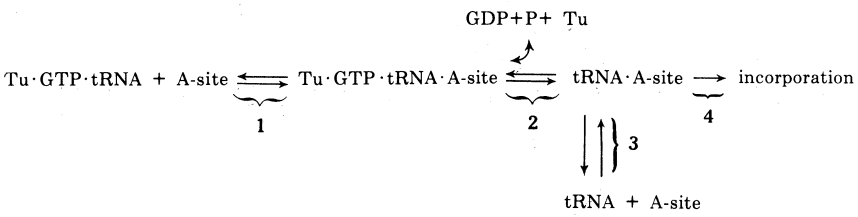
with a similar expression for D. If $W(t)$ is very small near $t = 0$ and then grows, the exponential in Eq. [**11**] can decay considerably for substrate D (which escapes rapidly) before $W(t)$ grows, while it will not do so for the smaller decay rate of substrate C. A simple example related to the previous section uses $W(t)$ equals a constant times t in Eq. [**11**]. This time-dependence yields the error fraction $f = (k_C/k_D)^2 = f_0^2$ found for the kinetic proofreading kinetics under optimal conditions.

The model of Eq. [**8**] works by an effective delay in turning on of W fully when viewed as a Michaelis-like scheme. It is impossible to have a delay in equilibrium, where there is no sense of the direction of flow of time. The mechanism which is to generate the delay must consume free energy in order not to be a Maxwell demon, which gives a general explanation of why [**4**] only functions when it is driven. Equally clear is the need for off-rate discrimination when W is nonselective. We were in fact led to consider schemes like Eq. [**4**] by an understanding of the use of time delay to enhance specificity.

The reading of the genetic code in protein synthesis

The elongation of a protein polymer growing on a ribosome involves the binding of a specific charged tRNA molecule at the empty A-site of the mRNA-ribosome complex (12, 13). The protein polymer is attached to the amino acid of a tRNA bound to the adjacent ribosomal P-site. Both enzymatically directed and nonenzymatic binding of tRNA at the A-site can occur. Nonenzymatic binding at a 20 mM Mg concentration, is sufficiently specific that it was used (14, 15) in deciphering the genetic code.

The enzymatic binding process involves the prior formation of a ternary complex of tRNA, elongation factor Tu, and a molecule of GTP. During binding, the GTP is hydrolyzed and the Tu factor is released. Thus, the total reaction scheme for the specific binding of charged tRNA to the ribosome includes



The inclusion of **3** as a possible *in vivo* side reaction at 7 mM Mg seems reasonable since the dissociation rate is faster in the absence of Mg (11). *This reaction scheme parallels that of Eq.* [**8**] except for irrelevant details of how the energetic coupling takes place.

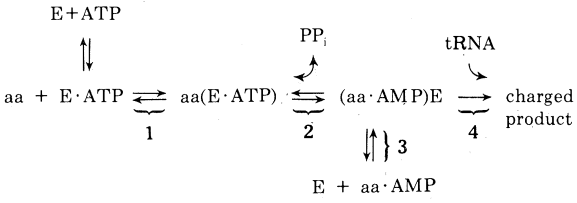
In this case, an argument can be made for the occurrence of kinetic proofreading. Kinetic studies of base pairing in double helical fragments show that the on-rates are relatively independent of pairing, and the off-rates presumably dominate the discriminatory ability (8, 11). *In vivo*, the enzymatic pathway **1–2–4** dominates in the incorporation of protein. In nonenzymatic binding at higher Mg concentration, W is slow enough to allow Eq. [**3**] to operate fairly effectively, so W must be much slower than the off-rate l_D of improper tRNA in step **3**. The l_D, l_C, and W thus have the correct kind of relation for kinetic proofreading. If this relation is not radically altered on going to 7 mM Mg, (11) all qualitative conditions for kinetic proofreading are met *in vivo*.

The side-pathway of the reaction to produce nonenzymatic release (or binding) appears harmful in normal protein synthesis. It acquires use in this reaction scheme as a rejection pathway after proofreading.

The direct experimental approach of measuring all the rate constants at the same Mg concentration for correct and incorrect tRNA at pathway **3** and **4** is strongly desirable. Proofreading could also be directly checked by measuring the rate of GTP hydrolysis per amino acid added when the mRNA is coding for a single amino acid. If step **3** is not used as an exit step, one GTP will be hydrolyzed per added amino acid regardless of whether the tRNA added is correct or incorrect. If **3** is used for proofreading, more GTP will be hydrolyzed when incorrect tRNAs are used, for much of it should be rejected in step **3** after GTP hydrolysis but before incorporation.

The charging of tRNA with amino acids

In the reaction, synthetase + amino acid + ATP + tRNA → synthetase + aminoacyl·tRNA + AMP + pyrophosphate (in abbreviations E + aa + ATP + tRNA → E + AMP + PP_i + aa·tRNA) the synthetase must recognize with high specificity both the amino acid and the tRNA which is to be charged. We deal only with the former recognition problem. In the simplest cases, e.g., methionine synthetase (16), the amino-acid recognition and the tRNA recognition are independent steps. The initial stages of charging are



The last step is a composite which could be expanded. The reaction scheme **1**–**2**–**3**–**4** is isomorphic with Eq. [**8**] (except that the methionyl AMP rather than the substrate methionine itself is released in step **3**) and is therefore ideally set up for kinetic proofreading.

The intermediate, aa(E·ATP), and reactions **1** and **2** have been studied by phosphate exchange between ATP and pyrophosphate (but for valine and isoleucine synthetases). The phosphate exchange is characterized (9, 10) by a turnover number which for a given synthetase is essentially the same for valine and isoleucine, and by Michaelis constants for the two amino acids in the ratio 1/10 to 1/1000, depending on the synthetase and assay method. Thus reaction **1** is specific and reaction **2** is nonspecific, like those of Eq. [**8**]. Reaction **2** will be strongly driven by the low level of pyrophosphate *in vivo*.

The pathway **1**–**2** leads to a 1/10 to 1/1000 discrimination against the incorrect amino acid in the intermediate complex (aa·AMP)E. Methionyl AMP has a binding constant of $2 \times 10^6\ M^{-1}$ to its synthetase, and an off rate of $2\ sec^{-1}$ (16). The synthetase has no strong binding for other aminoacyl AMP compounds. For methionine, and also for tyrosine (17), step **3** is well verified and is specific. Kinetic proofreading can operate to reduce f if this discrimination between correct and incorrect aminoacyl AMP is at least partly in off-rates l_C and l_D. In addition, the rate of W of reaction **4** must be slower than the rate l_D of step **3**. What little is known about these rates is compatible with kinetic proofreading.

There is in this view a functional purpose to the side reaction which produces free aminoacyl AMP, a reaction which otherwise would appear biologically harmful. Kinetic proofreading would not occur without a reaction which provides a channel for the rejection of incorrect amino acids in the proofreading step. Only a small flux of correct amino acids need be rejected in this channel.

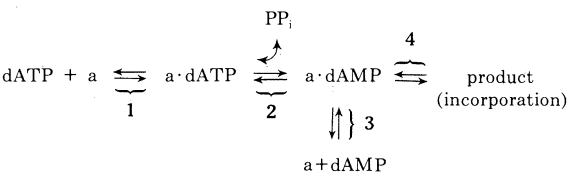
One way to prove the existence of kinetic proofreading is to measure all of the relevant rates for correct and incorrect aminoacyl AMP in reaction **3** and the rate of reaction **4**. Such an approach though is certainly most fruitful in the long run. In the case at hand, however, since the proofreading errors are rejected as aaAMP rather than as substrate amino acid, the removals in proofreading are specifically identified. One could, therefore, directly gain evidence for proofreading in this reaction by studying aminoacyl AMP production for correct and incorrect substrates. Synthetases which reject the incorrect amino acid itself after tRNA binding (18), releasing amino acid + AMP, also have a reaction scheme appropriate for kinetic proofreading, but are more difficult to examine for that effect. The expected increase in the ratio (phosphate hydrolysis)/(charged tRNA) for *mis*charging when proofreading is being used, can assay for proofreading in this case.

The replication of DNA

The completed part of a growing DNA strand is hydrogen-bonded to its complementary template. The next base to be incorporated is matched as the nucleoside triphosphate to the template, pyrophosphate is released, and the monophosphate is incorporated into the growing strand in a reaction catalyzed by DNA polymerase. *In vivo*, the pyrophosphate concentration is kept low by a pyrophosphatase, but *in vitro*, the reaction can be driven backward to produce triphosphate monomers by the addition of pyrophosphate (5, 6, 19).

DNA polymerase also exhibits an exonuclease activity, releasing monophosphates by hydrolysis of the end of the chain nominally growing. Others (5,6) have suggested that this exonuclease activity serves an editing role by causing an incorrect monophosphate base to be removed after it has been erroneously incorporated. How this editing takes place has not been described in physical chemical terms. Statements (5, 6, 19) that DNA polymerase cannot go on to incorporate a second base if the first base is not a correct match, imply a large ΔG_{CD} and a large specificity to mismatch in W. Existing data does not, however, demand this brute-force interpretation.

Denote the template-DNA polymerase complex ready to incorporate dAMP (for example) by a. The incorporation scheme is



The step **4** might include the DNA polymerase moving on one unit to be prepared for the addition of the next base. The reaction scheme again fits that of Eq. [**8**], except that the substrate itself is hydrolyzed in the driving reaction **2**, and step **4** is not irreversible. The reaction step **3** producing the exonuclease activity is essential to allow the escape of error products at the proofreading step. The off-rates for correct and

incorrect substrates in step **3** are not directly known, but the hydrolysis rate of an incorrectly matched terminal base is at least 40 times that of a correctly matched terminus (20).

The particular case of DNA replication is different from and more complicated than cases (a) and (b), for the growing polymer remains paired and can continue to be influenced by prior misincorporations. It is rather more likely in such a case that step **4** is also specific for base-paired termini, but such an additional specificity could be in addition to the use of kinetic proofreading.

One way of demonstrating that kinetic proofreading is used would be to attempt to copy a template via path **3** using base monophosphates as substrate material. If **3** is normally used to proofread the result of **1**–**2**, the use of **3** alone should greatly increase the error rate.

In an antimutator strain of bacteriophage T4 with an error rate less than 10^{-3} times that of the wild type, most of the base triphosphate is hydrolyzed to free monophosphate instead of adding to the growing polymer (20). (The interpretation is not definitive-see also ref. 21.) This must mean that the off-rate for step **3** with a correct match is comparable to the forward rate W for such a match. If the off-rate for an incorrect match in **3** is much faster than for a correct base (20) this antimutator strain of pathologically low error rate must make use of kinetic proofreading.

Discussion

Errors in identification in strongly driven systems can be reduced far below the level expected from simple ratios of binding constants or kinetic rate constants for simple reaction schemes. In strongly driven reactions, the new kinetic scheme of Eq. [**8**] results in error rates as low as "proofreading" once would produce. The amount of intrinsic free energy difference necessary to discriminate between two species at a given level of accuracy is cut in half by a single proofreading, and further reductions are similarly possible for more complicated reaction schemes. The error reduction mechanism is equivalent to the introduction of a lag or time delay between the formation of the activated complex and the formation of product, in an otherwise normal Michaelis scheme. In three cases we have examined, known steps and likely intermediates provide a reaction scheme which in essence is isomorphic to the new kinetic model. Sufficient details and numbers are known to suggest the use of kinetic proofreading in each of the three systems.

Circumstantial evidence on the use of proofreading in biosynthesis can be taken from the following questions. Why is DNA polymerase an exonuclease? Why is tRNA binding (and unbinding) to the messenger-ribosome complex permitted nonenzymatically as well as enzymatically? Why is the side product amino-acid AMP (or aa + AMP) a possible product in the charging of amino acids? All these three processes are at first sight wasteful side reactions which should have been eliminated if possible. Each is, however, given the same functional meaning as an essential side reaction for kinetic proofreading, since each is located at a point in the pathway where a second chance is possible for the rejection of incorrect associations.

The basis of good reading discrimination may often lie in proofreading and the kinetic complexity of biosynthetic pathways, and not in the existence of some particular intermediate with an extremely large free energy difference between correct and incorrect substrates. Understanding the meaning of biosynthetic pathways in such cases will involve the nuances of minor pathways, competitive rates, and side reactions. The dominant direct reaction pathway need not by itself contain the explanation of large specificity.

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