THE ROLE OF GUANINE NUCLEOTIDES IN PROTEIN BIOSYNTHESIS

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ABSTRACT It is not possible to select one competing substrate molecule over another one when the selection system is at equilibrium. Therefore, it is convenient to view all substrate selections as transport phenomena.

The requirement for a displacement from equilibrium to effect a substrate selection can be met in different ways. One particularly convenient way is to drive a nonselected substrate, such as GTP in protein synthesis, far from equilibrium. This allows the flux of selected substrate to be relatively slow, but effectively irreversible. Accordingly, the conventional view that GTP hydrolysis drives protein synthesis is amended. It is suggested that the regeneration of GTP from GDP is the driving force for protein synthesis.

Several different selection mechanisms are described in the context of systems driven by displacements from equilibrium of the nonselected substrate. These are then evaluated in light of recent experimental results. The data argue against the relevance of proofreading mechanisms for aminoacyl-tRNA selection by the messenger RNA-programmed ribosome. Similarly, recent data suggesting that the translation of messenger RNA is not dependent on the presence of elongation factors and guanine nucleotides are reevaluated.

INTRODUCTION

The adapter hypothesis (1) postulates two substrate selections in which transfer RNA (tRNA) participates during protein synthesis. One of them is the matching of the amino acids with their cognate tRNA's by the aminoacyl(AA)-tRNA synthetases. The other is the matching of the aminoacyl-tRNA's with their cognate codons by the messenger RNA (mRNA)-programmed ribosome. Although these functions have been verified definitively, there is still considerable mystery surrounding the mechanism through which they are mediated. The principal difficulty at present is to understand how the accuracy of such substrate selections is obtained.

For example, the most recent analysis of model systems suggests that codon-anticodon interactions could provide a binding constant for cognate pairs that is only 10–100 times greater than that for pairs containing a single mismatched codon position (2,3). In contrast, observations of translation in vivo suggest that tRNA matching with codons is performed with an error frequency less than 10^{-4} (4,5). Since codon-

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anticodon interactions are the only possible source of sequence information for the elaboration of proteins, the discrepancy between the in vivo and in vitro data creates an engaging problem.

One way out of this dilemma is to recognize that measurements of the strength of the codon-anticodon interaction in model systems yield information only about the equilibrium constants for this interaction. If the matching of codon and anticodon during protein synthesis takes place far from equilibrium, it could be the rate constants of the process that determine the fidelity of this matching process and not the equilibrium constants (6). According to this interpretation there may be no real discrepancy between the in vitro and in vivo fidelity measurements because they refer to different processes.

Similarly, it has been argued that even if tRNA discrimination on the ribosome can be adequately described by a binding constant, it is possible that measurements of the stability of codon-anticodon interactions in the absence of ribosomes would be almost irrelevant to measurements in the presence of the ribosome. Thus, it has been suggested that tRNA molecules are bound by the codon-programmed ribosome in a unique conformation, the stability of which is affected by the nature of the codon-anticodon interaction (7): when the codon-anticodon interaction is the cognate one, the conformation favoring ribosome binding is stabilized. However, when the codon and anticodon are not a cognate pair, the conformation of the tRNA acceptable to the ribosome is disfavored. According to this idea, measurements of tRNA affinities for codon analogues in the absence of ribosomes could be insensitive to the different distributions of conformational states for the tRNA. Hence, the specificity of such interactions in the absence of the ribosome could be systematically underestimated.

Recently, Hopfield (8) and Ninio (9) have argued forcefully for a kinetic solution to the fidelity problem. They have taken up the idea, introduced by Baldwin and Berg (10), that the fidelity of an enzymatic process can be enhanced by introducing sequential checking or "proofreading" steps. What is important about Hopfield's and Ninio's studies is their emphasis on the need for thermodynamic forces to drive such "proofreading" systems far from equilibrium. In particular, they both stress the potential significance of the nonselected substrates, the nucleoside triphosphates (ATP and GTP), in driving the synthetases and ribosomes far from equilibrium during their respective substrate selections. Accordingly, both Hopfield (8) and Ninio (9) have shown that editing or, as they are called now, proofreading schemes can in principle yield greater fidelity of selection than expected from the relative affinities of cognate and noncognate substrates for their discrimination sites at equilibrium.

This paper is concerned with what is potentially a more general role for the guanine nucleotides in substrate selections. It is first demonstrated that no substrate selection can occur when a system is at equilibrium. Since displacements from equilibrium are required for a selection system to express its substrate specificity, it is convenient to view a substrate selection as a transport process. Accordingly, attention is redirected away from the binding constants of substrates for their discriminating sites, and focused instead on the kinetic parameters influencing the relative fluxes of cognate and

noncognate products. Furthermore, it turns out that displacement from equilibrium for a nonselected species, such as GDP in protein synthesis, provides a striking kinetic advantage for the flux of selected species, such AA-tRNA, through the selection system, in this case the ribosome. Therefore, it is suggested that the relationship between the GDP flux and the fidelity of protein biosynthesis may be general, and not limited to proofreading schemes.

A modest amount of formal development is required to illustrate these ideas. In the course of this development, three different kinds of substrate selections are described and their fundamental similarity to the process of messenger RNA movement is stressed. Finally, several important experimental results are reevaluated in the context of the present theory.

THE IMPOSSIBILITY OF ENZYMATIC SELECTION AT EQUILIBRIUM

Fundamental to all of the arguments to be presented here is the simple idea that at equilibrium a process such as protein biosynthesis is impossible. In particular, the selection of substrate molecules such as the AA-tRNA's and the directed movements of tRNA as well as mRNA will be impossible at equilibrium. This is such a general proposition that it can be illustrated by discussing a simple substrate selection by an enzyme.

First, we assume that two substrate species, A_1 and B_1, can react to form the product species A_2 and B_2. At equilibrium in the absence of an enzyme the concentrations of substrates and products are related by the equilibrium constant K_2. Now suppose that A_1 is the substrate species to be selected, and that it can be found in two forms: A_1^c and A_1^n, both of which react equally well with the nonselected substrate B_1, that is to say $K_2^c = K_2^n$. Clearly, at equilibrium in the absence of an enzyme, the ratio of product species A_2^n/A_2^c will be equivalent to the ratio of substrate species, A_1^n/A_1^c.

Next, we introduce an enzyme that catalyzes the conversion of these substrates to products, but which has a higher affinity for A_1^c, (the cognate species) than for A_1^n (the noncognate species). What will the presence of catalytic amounts of the enzyme do to the ratio of noncognate to cognate product species? The answer is that at equilibrium the enzyme can do nothing to this ratio, and it is important for the rest of our discussion to understand why this is so.

The conversion of the above substrate species to product species in a closed system at equilibrium can be described by the following scheme:

$$\begin{array}{ccc} D + A_1 + B_1 & \overset{K_2}{\rightleftharpoons} & D + A_2 + B_2 \\ K_a \uparrow\downarrow & & \uparrow\downarrow K_b \\ D \cdot A_1 \cdot B_1 & \underset{K_1}{\rightleftharpoons} & D \cdot A_2 \cdot B_2 \end{array} \qquad (1)$$

Here, the association constants for substrates and products with the discrimination

site on the enzyme (D) are described by the equilibrium constants K_a and K_b, respectively. K_2 is, as above, the equilibrium constant describing the spontaneous reaction, while K_1 describes the catalytic conversion of substrates to products.

At equilibrium, the principle of detailed balance requires that:

$$K_a K_1 = K_2 K_b. \tag{2}$$

This relationship simply describes the condition under which the concentrations of substrates and products are constant in a closed system. It also explains why the enzyme cannot alter the equilibrium ratio between the competitive substrates and their product species.

Thus, at equilibrium the only thing that determines how the products and substrates are distributed is the free energy difference between these species, and this in turn is described by K_2. Since $K_2^n = K_2^c$, at equilibrium in the presence or absence of the enzyme, it follows from Eq. 2 that:

$$K_a^n K_1^n / K_b^n = K_a^c K_1^c / K_b^c. \tag{3}$$

This in turn means that the ratio of products A_2^n/A_2^c will be the same as the ratio of substrates A_1^n/A_1^c at equilibrium. This conclusion is a completely general one for any selection system at equilibrium. Indeed, it is a necessary consequence of what we mean by defining a catalyst as a substance that accelerates the approach to equilibrium but does not alter the free energy difference between substrates and products.

Nevertheless, we note that the two substrate species can be discriminated by the enzyme, i.e. bound with different affinities. Accordingly, it seems reasonable to distinguish between the discrimination capacity of the enzyme and the selection capacity of the system as a whole. This can be done formally in the following way:

Let us assume that there is an exit reaction for the product A_2 and that the rate constant for this reaction is k_e. When k_e is sufficiently small, the exit flux can be negligible and, therefore, it will not disturb the equilibria described in scheme 1. Now we can define an error function for the selection process (E_S) as the ratio of the flux of noncognate product out of the system to that of the cognate product ($k_e A_2^n / k_e A_2^c$) when the substrate concentrations for cognate and noncognate species are equal. For scheme 1 at equilibrium $E_S = 1$, that is to say there is no selection.

Next we can define an error function for the discrimination capacity of the enzyme (E_D) as the ratio for noncognate and cognate species bound at equilibrium to the site at which the enzymatic reaction is catalyzed when equal concentrations of the competitive species are present. For scheme 1 E_D will be given by the ratio K_a^n/K_a^c. Since we have already noted that these constants are different, it follows that E_D is less than one for scheme 1.

Now we expect there to be a functional relationship between E_S and E_D. E_S for scheme 1 is given by:

$$E_S = k_e A_2^n / k_e A_2^c = K_a^n K_1^n K_b^c / K_b^n K_a^c K_1^c = E_D \cdot (K_1^n K_b^c / K_1^c K_b^n). \tag{4}$$

Accordingly, we infer that the two error functions can be related to each other by a

third error function that we will refer to as E_k. Hence, we will define the third error function as:

$$E_k = E_S/E_D. \tag{5}$$

E_k describes the relative ease with which cognate and noncognate substrate are converted to products and released from the enzyme. Given the conservation law in Eq. 2, this error function at equilibrium is equivalent to the reciprocal of E_D. This is so because at equilibrium the binding energy of the substrates to enzyme, expressed in K_A, must be equivalent to the energy needed to convert the substrates to products and release the products from the enzyme, all normalized to the energy required to yield product spontaneously. Since the free energy change for cognate and noncognate reactions is the same in scheme 1, the more tightly bound cognate species will be processed and released from the enzyme less easily than the less tightly bound noncognate species. Consequently, at equilibrium there is no selection, i.e. $E_D \cdot E_k = E_S = 1$.

SELECTIONS LIMITED BY DISCRIMINATION

We consider next what sorts of displacements from equilibrium would permit a discrimination site to influence the outcome of a selection process. For the sale of simplicity we begin this inquiry by assuming that scheme 1 is operating with all of the rate constants in the system the same for cognate and noncognate species, except the dissociation rate constants of substrates and products from the enzyme. In particular, we assume that $k^n_{-a} \gg k^c_{-a}$, and $k^n_{-b} \gg k^c_{-b}$.
Hence,

$$\begin{array}{l} A_1 + B_1 + D \\ \quad k_a \uparrow\downarrow k_{-a} \\ A_1 \cdot B_1 \cdot D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A_2 \cdot B_2 \cdot D \underset{k_b}{\overset{k_{-b}}{\rightleftharpoons}} A_2 + B_2 + D \xrightarrow{k_e} . \end{array} \tag{6}$$

These boundary conditions yield a particularly simple interpretation for the effect of the principle of detailed balance (Eq. 2) on the scheme. This principle requires that the energy of interaction between substrate and enzyme be conserved so that it is also reflected in the energy of interaction between product and enzyme. As a consequence, it follows from Eq. 2 that for scheme 6 there is a fixed relationship for each species between the rate constants for the dissociation of substrate and product from the enzyme:

$$k^n_{-b}/k^n_{-a} = k^c_{-b}/k^c_{-a}. \tag{7}$$

Now, near equilibrium, i.e. with k_e negligibly small, the error functions for scheme 6 are $E_D = k^c_{-a}/k^n_{-a}$ and $E_k = k^n_{-b}/k^c_{-b}$, so that $E_S = 1$. Therefore, if we can drive this system away from equilibrium so that the influence of k^c_{-b} and k^n_{-b} on the fluxes through the enzyme is abolished, we can obtain a selection based solely on the relative values of k_{-a} for cognate and noncognate substrates.

This sort of displacement is accomplished in the steady state for biochemical schemes by postulating that the products of the reaction are removed from the system so fast that they can not equilibrate with the enzyme. Then, with the rate-limiting step in the process being the nonspecific rate constant k_1, the effect of k_{-b} on the rate of product evolution is abolished. The formal requirements for scheme 6 to function independently of k_{-b} are that the steady state concentrations of product as well as the intermediate $D \cdot A_2 \cdot B_2$ are negligibly small. This in turn can be accomplished if k_e is much greater than both k_{-b} and k_b, and if k_{-b} is much greater than both k_1 and k_{-1}. Given these boundary conditions, scheme 6 in the steady state is adequately described by:

$$A_1 + B_1 + D \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} A_1 \cdot B_1 \cdot D \xrightarrow{k_1}. \tag{8}$$

The flux of product in the steady state is now given by:

$$J = k_1 k_a (A_1)(B_1)(D)/(k_1 + k_{-a}). \tag{9}$$

Accordingly, we have the desired result that the only parameters distinguishing the fluxes of cognate and noncognate species are k^c_{-a} and k^n_{-a}, respectively.

If we return now to the error functions defined in the previous section, we see that the particular constraints applied to scheme 1 in order to obtain Eq. 9 cannot influence the discrimination capacity of the enzyme, E_D. In contrast, the error function E_k, which describes the kinetic properties of the system, is altered radically: thus, from Eq. 9 we obtain:

$$E_S = (K^n_a/K^c_a)(1 + k_1/K^c_{-a})/(1 + k_1/K^n_{-a}), \tag{10}$$

with

$$E_k = (1 + k_1/k^c_{-a})/(1 + k_1/k^n_{-a}), \tag{11}$$

and

$$E_D = K^n_a/K^c_a. \tag{12}$$

Clearly, the relative magnitudes of k_1, k^c_{-a} and k^n_{-a} will determine how effective the selection can be. Thus, any substrate molecule reaching the intermediate $D \cdot A_1 \cdot B_1$ can either dissociate from the complex at a rate determined by k_{-a} or it can be converted to product at a rate determined by k_1. If k_1 is much greater than k^n_{-a}, which is greater than k^c_{-a}, virtually any substrate that binds to the enzyme will become product and no selection is possible i.e. $E_S = 1$ with $E_D^{-1} = E_k$. In contrast, with $k^n_{-a} \gg k^c_{-a} \gg k_1$, the cognate and noncognate species can equilibrate at the enzymatic site and the selection will reflect the full discrimination capacity of the enzyme, i.e. $E_S = E_D$ with $E_k = 1$.

The error function E_k describes the effects of displacements from equilibrium on the selection process, and it can vary as the magnitude of the displacement increases. In

contrast, the error function E_D describes the relative affinities of competing substrate species for the discriminating site, and it is an invariant for any given system. This difference between the two error functions suggests how inadequate substrate affinity measurements can be for the description of a selection system. Indeed, selection systems will be described below in which the error of selection is significantly smaller than the error of discrimination, and, here, it will be shown that E_k is much less than 1. First, however, we will attend to a more fundamental issue.

FLUX COUPLING OF THE NONSELECTED SUBSTRATE

The point of the foregoing is to demonstrate that there can be no selection unless the substrates and products of a reaction are displaced from equilibrium. We have illustrated one way of obtaining the required displacement, namely, by consuming the selected product as fast as it is produced. However, reducing the selected product to what is effectively zero concentration may be inconvenient, particularly if the selected product can only be slowly processed in the next step within a chain of reactions (.e.g. the ribosome-mediated manipulations of tRNA or the resynthesis of AA-tRNA). It is, however, possible to operate on the concentration terms for the nonselected substrate and product so that these are displaced from equilibrium while the concentrations of selected substrate and product are free to range over a broad spectrum of values.

In order to illustrate this idea we can rearrange scheme 6 so that:

$$A_1 + B_1 + D \overset{K_a}{\rightleftharpoons} D \cdot A_1 \cdot B_1 \overset{K_1}{\rightleftharpoons} D \cdot A_2 \cdot B_2 \overset{K_b^{-1}}{\rightleftharpoons} D + \underset{\downarrow k_e}{A_2} + B_2 \overset{C/K_n}{\rightleftharpoons} B_1. \tag{13}$$

Here, we have done two things: first, we have separated the exit reaction for the selected product (A_2) from that for the nonselected product (B_2). Second, we have introduced a nonselected substrate regeneration system that holds the steady-state concentrations of nonselected species at fixed but controllable levels: $(B_2)/(B_1) = K_n/C$. Here K_n is the equilibrium constant describing the hydrolysis of B_1 and the parameter C describes the displacement from equilibrium of the ratio of the nonselected product to substrate. Clearly at equilibrium $C = 1$, and when $C > 1$ the nonselected product is being "pumped" back to its substrate form by the regenerating system as fast as it is being produced.

Now, if the reaction is run in such a way that there is no exit reaction ($k_e = 0$) the ratio of (A_2/A_1) will be displaced from its equilibrium value to an extent proportional to C. Similarly, the consequence of large values of C in the steady state, with $k_e > 0$, is to make the transition described by C/K_n effectively irreversible by reducing the effective concentration of B_2 to zero. Furthermore, it becomes possible to generate selections of the sort described by Eq. 8 with large positive values of C and with virtually any value for the rate of removal of A_2. In other words, by displacing the nonselected substrate and product from equilibrium, the rate of selected product removal is no longer the sole determinant of the fidelity of the selection process.

How, then, can we realistically imagine generating large values of C in normal biochemical reactions? Let's begin by assuming that the nonselected substrate in our scheme is GTP, and that the nonselected products are GDP + P. At equilibrium the concentrations of these reactants are fixed by the constant K_G, which describes the hydrolytic reaction for GTP. However, the living cell does not permit these nucleotides to reach equilibrium with each other. Instead, there is, apparently, a rapid flux of GDP and P back to GTP at the expense of whatever free energy sources are available to the cell. The constant C is therefore a convenient way of describing the coupling between the flux of GTP through the selection system and the flux of GDP through the selection system and the flux of GDP through the GTP-generating systems. Furthermore, C is a parameter easily measured in the cell or in vitro.

The equilibrium constant K_G is of the order of 10^6 M, which means that at equilibrium the ratio of (GTP)/(GDP) (P) should be close to $10^{-6}\,M^{-1}$. However, in *E. coli* the steady state ratio (GTP)/(GDP) is close to 10 (11) and the concentration of (P) is of the order of 10^{-2} M.[1] In other words, the guanosine nucleotides, as well as the adenosine nucleotides, are driven quite far from equilibrium with $C \sim 10^9$. Since C is much greater than K_n in scheme 12, this selection will operate far from equilibrium under physiological conditions, and with the appropriate kinetic characteristics will yield the overall error fraction described in Eq. 10. What then are the appropriate kinetic characteristics?

First, we can assume, as we did earlier for the development of scheme 8, that k_{-b} is much greater than both k_1 and k_{-1}. Then our problem is reduced to determining the minimum value of k_e at which the steady-state formation of product is effectively irreversible; this constraint is summarized by:

$$k_e(A_2) \gg k_b(A_2)(B_2)(D). \tag{14}$$

We can now analyze the effect of a regeneration system for the nonselected substrate by calculating the effect of C on the critical value of k_e in scheme 14 for an appropriate conservative system. Here, the sum of (B_1) and (B_2) is fixed at a steady-state level given by (B_0). Since (B_2) is now given by $(B_0)K_n/(K_n + C)$, the inequality in 14 becomes:

$$k_e(1 + C/K_n) \gg k_b(B_0)(D). \tag{15}$$

Accordingly, the larger the steady-state value of C, the smaller the critical value for k_e need be. Thus, with sufficiently large C, k_e can limit the flux of selected substrate through the system, but it will not limit the fidelity of the selection of this substrate, and we obtain a scheme which functions as in scheme 8, with the error functions described in schemes 10, 11, and 12. In addition, the flux of substrate can be described by Eq. 9. This in turn means that with B_1 given by $(B_0) \cdot C/(C + K_n)$ the steady-

[1] H. Rosenberg. 1977. Personal communication.

state concentration of B_1 increases with C. Therefore, the rate of the selection as well as the fidelity of the selection are increasing functions of C.

Before going on to apply the foregoing idea to more complicated schemes, it would be worthwhile anticipating some potential misunderstandings. First, the nonselected substrate per se does not drive the selection. Furthermore, it matters very little how great or small the energy of hydrolysis is for nonselected substrate (K_n). It is the parameter C, in some sense analogous to an equilibrium constant, that is the measure of the free energy driving the system away from equilibrium. The nonselected substrate merely provides a convenient way of coupling the flux of the selected substrate to this free energy source. Accordingly, the nonselected substrate need not participate directly in the transformations of the selected substrate. Hence, what appears to be a gratuitous component in a selection system may turn out to be the molecular link that couples the selection system to the metabolic pathways of the cell, which in the final analysis drive all of the substrate selections.

KINETIC ENHANCEMENT OF SELECTION

The discussion so far has been restricted to selection systems limited by their discrimination capacity as expressed in E_D. Now we will consider systems in which $E_k \ll 1$, which is the condition that permits $E_S \ll E_D$.

The idea that the ribosome can bind tRNA molecules in restricted conformational states, the occupancy of which is influenced by the character of the codon-anticodon interaction, has been discussed in detail previously in the context of a selection system only minimally displaced from equilibrium (7). It is, however, also possible to imagine a selection system that exploits conformational differences between cognate and noncognate tRNA even when the system is displaced far from equilibrium.

Thus, it has been suggested that AA-tRNA molecules could in principle be selected by the codon-programmed ribosome on the basis of the rate constants that characterize the transition from an initial binding state to a second more stable one (6). Such a transition might be more rapid when the conformation of tRNA is the one favored by cognate codon-anticodon interactions than when the tRNA is in the conformation favored by noncognate interactions. Here, the equilibrium constant for the transition could be the same for cognate and noncognate species, but the rate constants in both directions could be larger for cognate than for noncogante species. If we identify such a transition with that described by K_1 in scheme 8, then

$$K_1^c = K_1^n, k_1^c > k_1^n, \quad \text{and} \quad k_{-1}^c > k_{-1}^n.$$

Given these assumptions, the rates of cognate and noncognate substrate selection for scheme 8 would be as described in Eq. 9. The error function of such a selection would be:

$$E_S = (k_1^n K_a^n / k_1^c K_a^c) \frac{(1 + k_1^c/k_{-a}^c)}{(1 + k_1^n/k_{-a}^n)}. \tag{16}$$

Here, the error function describing the departure from equilibrium would be:

$$E_k = k_1^n/k_1^c \frac{(1 + k_1^c/k_{-a}^c)}{(1 + k_1^n/k_{-a}^n)}. \tag{17}$$

Accordingly with $k_a^n \gg k_1^n$ and $k_{-a}^c \gg k_1^c$, $E_k = k_1^n/k_1^c$ and the system will select substrate more accurately when it is displaced from equilibrium than would be expected from its discriminatory capacity at equilibrium (i.e. $E_S < E_D$).

PROOFREADING

A far less trivial way than that described in the previous section has been suggested by Hopfield (8) and Ninio (9) to obtain kinetic enhancement of the fidelity of selection. They motivate their idea by recognizing that there must be limits to the discrimination obtainable between closely related substrate species. However, they suggest that a selection system could circumvent these limitations if the elementary discrimination step could be repeated one or more times before the product is selected. Such sequential repetitions of the substrate discrimination have come to be called proofreading.

It turns out that the treatment of the nonselected substrate displacement from equilibrium developed here provides an attractive way to reformulate the ideas originally described by Hopfield and Ninio. As a consequence, the proofreading scheme to be described here will differ in some details from their schemes, but it yields the same error functions.

We begin by rearranging scheme 13 so that the initial discrimination step is repeated in the new scheme:

$$\begin{array}{ccccccccc}
A_1 + B_1 + D & \overset{K_n/C}{\rightleftarrows} & A_1 + B_2 + D & & & & & & \\
k_a \uparrow\downarrow k_{-a} & & k_{-c} \uparrow\downarrow k_c & & & & & & \\
A_1 \cdot B_1 \cdot D & \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} & A_1 \cdot B_2 \cdot D & \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} & A_2 \cdot B_2 \cdot D & \underset{k_b}{\overset{k_{-b}}{\rightleftarrows}} & A_2 + D + B_2 & \overset{C/K_n}{\rightleftarrows} & B_1 . \\
 & & & & & & \downarrow k_e & & \\
 & & & & & & P & &
\end{array} \tag{18}$$

Here, the only rate constants different for cognate and noncognate species are k_{-a}, k_{-b}, and k_{-c}. We recall that from the principle of detailed balance this means that:

$$k_{-a}^n/k_{-a}^c = k_{-b}^n/k_{-b}^c = k_{-c}^n/k_{-c}^c. \tag{19}$$

Therefore, the dissociation step characterized by k_{-c} will be in effect a repetition of that characterized by k_{-a}.

An additional step characterized by $K_2^n = K_2^c$ with $k_2^n = k_2^c$ has been introduced and for scheme 18 to function optimally we require that $(k_{-c}^c + k_2) \gg k_{-1}$ and that $k_{-b}^n \gg k_{-b}^c \ll k_{-2}$. This step is motivated by the need to break the detailed balance constraint at the branch point corresponding to $(D \cdot A_1 \cdot B_2)$ so that $k_{-c}^n/k_2 \gg k_{-c}^c/k_2$. Had we ignored this problem, the fluxes of product would otherwise have been characterized by: $k_{-b}^n/(k_{-b}^n + k_{-c}^n)$ and $k_{-b}^c/(k_{-c}^c + k_{-c}^c)$, respectively. In

light of Eq. 19 this would mean that without the step characterized by k_2, the system could not proofread.

Now, assuming that $k^n_{-a} \gg k^c_{-a} \gg k_1$, and for values of $C \gg K_n$, scheme 18 is reduced to the form:

$$\begin{array}{ccccc} A_1 + B_1 + D & \overset{K_n/C}{\rightleftharpoons} & A_1 + B_2 + D & & \\ k_a \downarrow \uparrow k_{-a} & & & \uparrow K_{-c} & \\ A_1 \cdot B_1 \cdot D & \xrightarrow{k_1} & A_1 \cdot \quad B_2 \cdot D & \xrightarrow{k_2} & . \end{array} \tag{20}$$

Hence, the error function for the selection at large values of C is:

$$E_S = (K^n_a/K^c_a)(k_2 + k^c_{-c})/(k_2 + k^n_{-c}), \tag{21}$$

and the proofreading effect is found in the kinetic error function:

$$E_k = (k_2 + k^c_{-c})/(k_2 + k^n_{-c}) \tag{22}$$

Clearly, the displacement from equilibrium of the nonselected substrate is essential to the enhanced selectivity of the system. Thus, we require the flux due to k_c to be negligible for the proofreading effect of k^n_{-c} and k^c_{-c} to be fully expressed. This in turn depends on values of C sufficiently large to reduce the steady-state concentration of B_2 to negligible levels. Inspection of the schemes of Hopfield (8) and Ninio (9) lead to the same conclusion: a displacement from equilibrium for the nonselected substrate appears to be required for proofreading schemes.

This is an important conclusion because it provides one analytical property with which to distinguish a proofreading system from the other types of selections described above. These, as shown above, can in principle be driven by a displacement from equilibrium of the selected product. Therefore, any selection system that does not display a significant increase of the error function when operating without a displacement from equilibrium for the nonselected substrate cannot be a proofreading system of the sort described so far.

Finally, it is worth emphasizing that the error function in Eq. 22 does not approach its minimum bound of k^c_c/k^n_c unless $k^n_c \gg k^c_c \gg k_2$. However, this limit is characterized by a system that yields a negligible flux of selected product compared to the flux of nonselected product. The excess production of nonselected product compared to selected product is therefore another analytic property of proofreading systems.

DIRECTIONAL MOVEMENTS

Two novelties have been introduced for the description of nonproofreading schemes. One is the treatment of all substrate selections as transport processes. The other is the use of the displacement from equilibrium of the nonselected substrate, as summarized in the coupling parameter C, to facilitate the transport of the cognate species preferentially. The motivation for this approach has been a very general one; namely, recog-

nition of the constraint imposed on any selection process by the principle of detailed balance, which has not figured very prominently in previous treatments of this problem. However, we will now turn to more specific issues concerning the ribosome and its particular functions. In so doing, it should become evident that all of the steps in protein biosynthesis are most conveniently viewed as transport phenomena.

We have explored three types of substrate selections that might be employed by the ribosome to select AA-tRNA's. There is, in addition, a related problem in protein synthesis, namely, the movements of mRNA attending each cycle of peptide bond formation. Here too a displacement from equilibrium is required to effect the process. To illustrate this conclusion, we can consider a very simple model for the movements of ribosome relative to mRNA; this is a unidirectional process beginning at the 5′ end of the mRNA and proceeding one codon at a time to the 3′ end of the mRNA (see ref. 12).

We take the movement of the ribosome from codon (n) to codon ($n + 1$) as our paradigm:

$$R(n) + B_1 \overset{K_a}{\rightleftharpoons} R(n)\cdot B_1 \overset{K_1}{\rightleftharpoons} R(n+1)\cdot B_2 \overset{K_b^{-1}}{\rightleftharpoons} R(n+1) + B_2 \overset{C/K_n}{\rightleftharpoons} B_1. \qquad (23)$$
$$\downarrow k_e$$

Scheme 23 is formally the same as scheme 6, and, indeed, large values of C will have the same effects here as in the selection of an AA-tRNA. Thus, by reducing the steady-state concentration of B_2 to negligible levels, the rate constant k_e, which describes the initiation of the next cycle of peptide bond formation, can be relatively small and the scheme will still operate effectively and irreversibly. This is important because once the mRNA has been advanced so that the next codon is in the reading frame, any significant tendency for slippage of the mRNA backwards would represent an error-catastrophe for the system.

The value of the displacement from equilibrium represented by C can now be appreciated in its broadest aspect. During protein synthesis we can employ schemes such as 23 to represent alternately the selection of the AA-tRNA and the subsequent movements of mRNA as well as tRNA on the ribosome. The displacement from equilibrium represented by large values of C "punctuates" each of these steps in such a way that the rate of one step does not limit the fidelity of the previous step. Thus, the elaboration of a protein, or for that matter any polymer, can be driven in sharp, distinct steps and yet each such step is provided a significant degree of kinetic freedom, by virtue of the displacement from equilibrium being applied not to selected species (e.g. AA-tRNA) but to the nonselected product (e.g. GDP).

THE ROLE OF ELONGATION FACTORS

A cycle of peptide bond formation has the following elements: AA-tRNA bound by the codon-programmed ribosome is accompanied by elongation factor EFT_u as well as GTP at the so-called A site (12). After the hydrolysis of GTP, EFT_u and GDP are

released from this site, and a peptide bond is formed. This sequence can be summarized as follows:

$$\begin{array}{l} \text{AA-tRNA} + \text{EFT}_u + \text{GTP} + \text{R}_1(n) \\ \quad k_1 \downarrow\uparrow k_{-1} \\ \text{Complex I} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Complex II} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{R}_2(n) + \text{EFT}_u + \text{GDP} + \text{P}. \end{array} \quad (24)$$

Here $R_1(n)$ represents the ribosome with the n^{th} codon in position to choose an AA-tRNA, and $R_2(n)$ represents the ribosome with the peptide bond corresponding to the n^{th} amino acid formed and attached to the tRNA in the A site.

The next part of the cycle, referred to as the translocation step, is mediated by elongation factor EFG as well as GTP:

$$\begin{array}{l} \text{R}_2(n) + \text{EFG} + \text{GTP} \\ k_4 \downarrow\uparrow k_{-4} \\ \text{Complex III} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \text{Complex IV} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} \text{R}_1(n+1) + \text{EFG} + \text{GDP} + \text{P} + \text{tRNA}. \end{array} \quad (25)$$

Here, $R_1(n + 1)$ is the ribosome carrying the growing polypeptide chain in the P site and the mRNA has been advanced so that the $(n + 1)$ codon is now sitting in the A site, calling for the next AA-tRNA.

Now, if we assume that there is a strong displacement from equilibrium for the GDP, and if the rate constants for the successive reactions are arranged as for the paradigm in scheme 6, then in the steady state, schemes 24 and 25 will be reduced to the same form as scheme 8:

$$\begin{array}{l} \text{AA-tRNA} + \text{EFT}_u + \text{GTP} + \text{R}_1(n) \\ \quad k_1 \downarrow\uparrow k_{-1} \\ \text{Complex I} \xrightarrow{k_2} \end{array} \quad (26)$$

$$\begin{array}{l} R_2(n) + \text{EFG} + \text{GTP} \\ \quad k_4 \downarrow\uparrow k_{-4} \\ \text{Complex III} \xrightarrow{k_5}. \end{array} \quad (27)$$

These two kinetic schemes lead to a modest reevaluation of the functions of the elongation factors and GTP in a cycle of peptide bond formation.

First, it would appear that the factors EFT_u and EFG can influence the steady-state rate of protein synthesis simply by catalyzing the reactions corresponding to k_2 and k_5, respectively. These two reactions involve, among other things, the hydrolysis of GTP before the formation of the peptide bond (k_2) and that attending translocation (k_5). What then is the functional relationship between GTP and the elongation factors?

Studies on the protein factors required for bacterial protein synthesis as well as the involvement of GTP hydrolysis in their functions all lead to the same general view (13–19). The functional association of the factors with the ribosome is as efficient with a noncleavable analogue of GTP as it is with GTP itself. The reason that the noncleavable analogues are competitive inhibitors of protein synthesis is that the hydrolysis of GTP is required for the release of the respective factors. In effect, GTP complexed with factor allows the interaction with the ribosome, while GDP complexed with factor destabilizes that interaction. Hence, the GTP-GDP interconversion can be conveniently viewed as a functional switching signal, with the respective nucleotides functioning as allosteric effectors in the system (20). In other words, the GTP can be viewed as a passive form of GDP, and its hydrolysis may simply provide the activation energy for the release of the respective factors from the ribosome.

We have argued that the role of GDP is first to provide a coupling mechanism to drive protein synthesis and second to trigger the detachment of the factors from their ribosomal binding sites. What then would be the point of having detachable protein factors, particularly, if it is conceivable that the hydrolysis of GTP might be performed by a ribosomal enzymatic center? It seems reasonable to suggest that the protein factors facilitate the otherwise difficult conformational rearrangements of tRNA and mRNA that represent the different movements on the ribosome (see for example refs. 7 and 22).

In summary, the dependence of the molecular movements of tRNA and mRNA on protein factors which kinetically facilitate these movements and which dissociate from the ribosome in response to the hydrolysis of the nonselected substrate, GTP, is one way to solve the problem of the directionality of protein synthesis. At equilibrium, the occupancy of a molecular state (e.g. AA-tRNA in the ribosomal A site) will be determined strictly by the energy of that state compared to other accessible states. In contrast, an irreversible process can in principle fix the state of a molecule, independently of the relative energy of that state, by kinetically preventing access to other energetically favorable states. Thus, by coupling the transitions of the tRNA and mRNA on the ribosome to the presence or absence of the elongation factors and by controlling the functional state of the factors through the guanine nucleotide flux, the cycle of protein synthesis can be driven unidirectionally and very precisely.

FACTOR-FREE PEPTIDE BOND FORMATION

Gavrilova and Spirin along with their associates have developed a system for studying polypeptide elongation in the absence of elongation factors and GTP (22–27). Such a factor-free system, provides a way of exploring the effects of removing GTP and factors on the various steps in protein biosynthesis. The most obvious gross difference between the factor-free and the factor-dependent systems is that the latter functions at a much faster rate than the factor-free system. The factor-free and factor-dependent systems are both inhibited by the same antibiotics, which suggests that the mechanism of peptide bond synthesis is the same for both (27). Nevertheless, only a small number

of synthetic messenger RNA's have been translated in the factor-free system. Accordingly, it is not certain that the mechanisms of peptide elongation are identical in the presence and absence of factors plus GTP. On the other hand, the results with this system are so striking that we are obliged to consider them in our discussion of the role of GTP in protein synthesis.

Gavrilova et al. (26) have been concerned with showing that the mechanism of polypeptide chain elongation is the same in their factor-free system as that of the normal factor-dependent systems. To this end, they have studied the relationship between the chain length of the oligopeptides synthesized in the factor-free system and the chain length of the oligonucleotides used to prime the system. If the movement of tRNA and mRNA is normal for the factor-free system, the chain length of the oligopeptide products must be limited to the largest number of consecutive triplets in the oligonucleotides used as mRNA. Hence, the size distribution of the oligopeptides will correlate with the size of the oligonucleotide primers straightforwardly if translation is normal. If it is abnormal, say for example that there can be slippage, then the oligopeptides might be larger than predicted from the sizes of the oligonucleotide primers.

Qualitatively speaking, the results are in line with what might have been predicted if the system were translating normally. Thus, Gavrilova et al. (26) show that at least 98% of the products are equal to or less than the size predicted from the largest number of consecutive codons in each of the tested oligonucleotides. On the surface, such results contradict our argument for the essential contributions of factors and GDP to drive the system unidirectionally.

However, we note an important difference between the binding of AA-tRNA and the subsequent movements of tRNA as well as mRNA in this system. If an excess of AA-tRNA is presented to the mRNA-programmed ribosome, AA-tRNA will certainly be bound. Furthermore, there is a chance that two AA-tRNA's can be bound to a ribosome in the A and P sites simultaneously. Since the formation of the peptide bond is an energetically favored reaction (see for example ref. 20), this means that the occasional peptide bond can be made and dipeptide can accumulate to a limited extent. But our theory requires that the rates and fidelity of the subsequent steps in the elongation process, e.g. the orderly advance of mRNA, should be quite dependent on the presence of factors as well as GTP. Accordingly, we would expect that the formation of products longer than dipeptide should be very much restricted in the factor-free system.

While Gavrilova et al. (26) have stressed the fact that the oligopeptides synthesized in their system are not too large, our opinion is that they are too small. If we assume that a ribosome can begin to translate an oligonucleotide at any point along its length, it is possible to predict the fraction of Phe incorporated into the different sizes of oligo-Phe with each oligo-U mRNA. For example, $(Up)_9$ should mediate the incorporation of 67% Phe into dimer and 33% into trimer. In contrast, $(Up)_{12}$ should yield 31% Phe in dimer, 47% in trimer, and 22% in tetramer. However, Gavrilova et al. (26) find that all of the oligo-U primers with chain length between 9 and 12 yield more than 80% dimer. This is precisely the sort of result that we would predict for such a system

operating without GTP; it should have difficulty producing peptides with chain lengths greater than two.

This interpretation of the data suggests that the failure of Gavrilova et al. (26) to observe the accumulation of oligopeptides longer than predicted from the sizes of the oligonucleotides used to prime the system may have been a statistical accident. Thus, with a limited capability to translate even the predicted oligopeptides in significant amounts, the accumulation of even longer slippage products may have been impossible, i.e. the slippage may have been masked.

THE ROLE OF GTP IN AA-tRNA SELECTION

We have previously discussed two classes of selection mechanisms: conventional selections in which displacements from equilibrium of either the selected or nonselected species can drive the selection, and proofreading schemes that depend strictly on displacements from equilibrium of a nonselected substrate. Clearly, the two classes of selection mechanisms should behave differently in the GTP-factor-free system, because proofreading should not be possible in this system.

Contrary to what might have been expected, Gavrilova et al. (26) have found that the error function of peptide synthesis decreases when the factors and GTP are removed from the system. Thus, the factor-dependent system can incorporate up to 17% Leu in place of Phe with poly U as messenger, while the factor-free system incorporates less than 1% Leu in place of Phe. Similarly, streptomycin, which normally enhances the error frequency, will inhibit the factor-free system; however, the error rate for Leu is 32% for the factor-dependent system in the presence of streptomycin and 0.4% in the factor-free system. On the surface, these results suggest that the hydrolysis of GTP enhances the rate of translation dramatically, and in parallel increases the error fraction, as might be expected for systems dependent solely on discrimination (see scheme 10). However, we must be cautious with such results. The error frequencies in all in vitro systems are orders of magnitude higher than those in vivo, as indicated by the above figures. This means that the errors measured in vitro may originate from sources different from those expressed in vivo. For example, the present theory implies that high concentrations of GDP and inorganic phosphate, never seen in vivo, could enhance the error frequency significantly. Accordingly, the errors seen by Gavrilova et al. (27) as well as all others in factor-dependent systems may result from a rapid hydrolysis of GTP, independent of protein synthesis, which results in artificially high concentrations of GDP and inorganic phosphate. We have already observed this phenomenon in another protein-synthesizing system (Wagner, Pettersson, and Kurland. Unpublished results). In summary, the results of Gavrilova et al. (27) show a GTP-dependent enhancement of the rate of AA-tRNA selection, but they do not reveal proofreading enhancement of the fidelity of the selection.

A more recent set of experiments reported by Thompson and Stone (28) is explicitly directed at the kinetic proofreading theory. They studied the relative extents of AA-tRNA and GTP hydrolysis when a poly U-programmed ribosome is presented

with either cognate (Phe) or noncognate AA-tRNA in the presence of EFT_u and GTP. The results showed that while there is relatively less binding of the noncognate AA-tRNA than Phe-tRNA, there is a relatively greater extent of GTP hydrolysis with two of the noncognate species, namely Leu and Ile-tRNA. Accordingly, Thompson and Stone (28) conclude that proofreading by the ribosome has been established. However, a closer look at their data contradicts this conclusion.

An example of what Thompson and Stone observed is a nearly equivalent stoichiometry of GTP hydrolysis associated with Phe-tRNA binding, as opposed to roughly four times more GTP hydrolysis than Leu-tRNA binding when the latter is presented to the ribosome. Now, from the point of view developed in earlier sections, the fourfold excess GTP hydrolysis is not really very much upon which to build a proofreading function. Therefore, we have taken the data reported by Thompson and Stone (29) and calculated the relevant error functions using the assumptions expressed in scheme 20 and Eq. 21.

Using the average values obtained from the 30-s and 6-min incubations to average out the sampling errors, we obtain the following pairwise error fractions: for Leu/Phe, $E_k = 0.46$ and $E_D = 0.10$, and for Ile/Phe, $E_k = 0.78$ and $E_D = 0.18$. Clearly, there is something wrong with the Thompson and Stone interpretation. Thus, the values for E_k indicate a proofreading effect of at most a factor of 2, a far cry from the factor of 100 that they explicitly anticipate in the introduction of their paper.

There are other problems with this experiment. For example, it is assumed that peptide bond formation is an irreversible reaction, which is not only unacceptable a priori but contradicted by the analysis presented by Spirin (29). Similarly, the absence of elongation factor G from the incubation mixtures will increase the probability that AA-tRNA can dissociate from the ribosome and, thereby, artifactually increase the apparent extent of the discard reaction. Therefore, the figures for GTP hydrolysis presented by Thompson and Stone (28) appear to be upper limits.

In summary, two experiments have been performed in which a proofreading function should have been evident if the kinetic proofreading reaction were relevant to the ribosome. In neither case was any evidence for such a proofreading function forthcoming. Nevertheless, the limitations of these experiments must be taken seriously. No one has succeeded in reproducing in vitro the fidelity of translation observed in vivo. Until that is done, no definitive conclusions concerning the role of GTP in AA-tRNA selection can be drawn from in vitro experiments.

SUMMING UP

The present study is an attempt to relate the rate and fidelity of protein biosynthesis to the flux of GTP that drives the functions of the ribosome. Our emphasis is on the flux of GTP, summarized in a phenomenological parameter C, and not, as is traditional, on the equilibrium between GTP and its hydrolytic products GDP and P. Thus, our starting point is that, even in the presence of GTP, at equilibrium the selection of AA-tRNA's and the sequential translation of the mRNA would be impossible.

That demonstration is merely an illustration of the obvious, namely, that protein biosynthesis is an irreversible process. Accordingly, it must be the fluxes of substrates that determine the efficacy of the process.

On the other hand, because we are primarily concerned with the behavior of this system in the steady state, it turns out to be convenient to describe the fluxes of guanine nucleotides in the form of a steady-state displacement of GTP, GDP, and P from their equilibrium concentrations. This displacement corresponds to the parameter C and, as we have indicated, it is a dimensionless parameter that reaches the level of 10^8–10^9 in bacteria. That is to say, the displacement of the guanine nucleotides from their equilibrium concentrations in vivo is immense, and corresponds to an impressive thermodynamic potential with which to drive processes that can be coupled to the fluxes of the nucleotides.

The advantage of driving a process such as protein synthesis through the displacement from equilibrium of a nonselected substrate, such as GTP, is straightforward. By effectively reducing the "back reactions" of the process through the flux coupling with the nonselected substrate, the rate of the subsequent processing of selected products is no longer a critical parameter, as it would be if the system were being driven by the displacement from equilibrium of selected substrates. This sort of system is aided by protein factors that can help mediate the transitions of tRNA and mRNA to and from particular sites on the ribosome, and then after the hydrolysis of GTP, be ejected from the system.

This then is the general framework in which we have attempted to assess the relevant data. Three different AA-tRNA selection mechanisms have been briefly discussed: simple discrimination based on the relative binding constants of cognate and noncognate species; kinetic enhancement of selection based on a combination of discrimination and selection on forward rate constants; and finally, kinetic proofreading, restated here in a way somewhat different from its earlier forms described by Hopfield (8) and Ninio (9).

So far, all of the relevant data argue against the proofreading mechanism for AA-tRNA selection, but the data are consistent with simple discrimination models. Nevertheless, the experimental systems currently available are really not sufficiently accurate to be relied upon. Thus, with error fractions orders of magnitude greater than those characteristic of protein synthesis in vivo, the value of the data concerning the fidelity of translation in vitro seems at best doubtful.

The formal arguments developed in the present study are not meant as exercises in theory, but rather as a guide to the execution and interpretation of experiments. If our arguments are correct and relevant, recognition of the potential significance of the GTP/GDP ratios in in vitro systems may hep in providing better ones for the analysis of translation. Similarly, the value of the approach developed here is, we hope, documented by the present analysis of the experiments of Gavrilova et al. (27) and those of Thompson and Stone (29). In both cases the important conclusions of these authors required modification in light of a quantitative analysis of their data.

Finally, the mechanism suggested here for effecting a substrate selection by the dis-

placement from equilibrium of a nonselected substrate may be a very general one. The relevance of this mechanism to specific selection processes must now be decided experimentally. It is hoped that at the very least this study has shown the impossibility of deciding such experimental issues by measuring only the binding constants of competing substrates for a discriminating site on an enzyme or ribosome. The major point of this study is to demonstrate that any substrate selection is an irreversible process, and, therefore, the description of such a process must entail the kinetic characteristics of the system.

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