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Breakthroughs and Views

Determination of the kinetics of guanine nucleotide exchange on EF-Tu and EF-Ts: continuing uncertainties

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Abstract

An analysis is made of the rate constants for the reactions involving the interactions of EF-Tu, EF-Ts, GDP, and GTP recently derived by Gromadski et al. [Biochemistry 41 (2002) 162]. Though their measured values appear to allow a reasonable rate of nucleotide exchange sufficient to support rates of protein synthesis in vivo, their data underestimate the thermodynamic barrier involved in nucleotide exchange and therefore cannot be considered definitive. A kinetic scheme consistent with the thermodynamic barrier can be achieved by modification of various rate constants, particularly of those involving the release of EF-Ts from EF-Tu · GTP · EF-Ts, but such constants are markedly different from what are experimentally observed. It thus remains impossible at present satisfactorily to model guanine nucleotide exchange on EF-Tu, catalysed by EF-Ts by a double displacement mechanism, with experimentally derived rate constants. Metabolic control analysis has been applied to determine the degree of flux control of the different steps in the pathway.

Keywords: EF-Tu; EF-Ts; Guanine nucleotide exchange; Kinetic analysis; Metabolic control analysis

Each round of peptide bond formation in bacteria involves the release of one molecule of EF-Tu · GDP and in order for EF-Tu to continue functioning in protein synthesis the EF-Tu · GDP must release GDP and bind GTP. The very slow rate of release of GDP from EF-Tu · GDP is facilitated by the action of the factor EF-Ts which binds to the EF-Tu · GDP complex, causing a several thousand-fold decrease in the affinity of EF-Tu for GDP, largely as a consequence of a much enhanced 'off' rate constant for the nucleotide.

In order to resolve previously inconsistent data for some of the rate constants involved in the release of GDP and to determine how rapidly EF-Tu · GTP · aminoacyl-tRNA can be formed from EF-Tu · GDP under conditions in vivo (which might be found to limit the rate of peptide bond formation), Gromadski et al. [1] have recently re-evaluated the rate constants of all the association and dissociation reactions between EF-Tu, EF-Ts, GDP, and GTP. Interactions were studied by the stopped-flow technique, monitoring the fluorescence of tryptophan 184 in EF-Tu or of the mant group attached to the guanine nucleotide.

Their results offer a more coherent set of constants than have been obtained over the years using a rapid filtration technique [2,3], conventional filtration [4,5] or by stopped-flow and pressure jump techniques using a chromophoric GDP analogue, 2-amino-6-mercaptopurine riboside 5′-diphosphate (thioGDP) [6,7]. They conclude that at concentrations of nucleotides and factors found in *Escherichia coli*, the overall exchange rate, as calculated from the elemental rate constants, is $30\,s^{-1}$, a number which is compatible with the rate of peptide bond formation (estimated at about $10\,s^{-1}$ [8]) for the number of ribosomes in the cell. They further conclude that there is no single rate-limiting step in the mechanism of nucleotide exchange on EF-Tu, contrary to what can be concluded from earlier work [2,6] in which release of GDP from EF-Tu · GDP · EF-Ts was found to be extremely rapid, thus focussing attention on the release of EF-Ts from EF-Tu · GTP · EF-Ts as the limiting step [3,4]. Gromadski et al. [1] suggest that the three

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steps, EF-Ts binding to EF-Tu · GDP, dissociation of GDP from EF-Tu · GDP · EF-Ts, and dissociation of EF-Ts from EF-Tu · GTP · EF-Ts contribute about equally to the overall velocity of the nucleotide exchange.

In the present communication the sequence of reactions involving the participation of EF-Ts in nucleotide exchange has been modelled, and the principles of metabolic control analysis have been used to analyse the relative participation of each step in regulating the rate of functioning of the overall pathway. These results are compared with the findings of Gromadski et al. [1]; it is concluded that their data are only able to support likely rates of protein synthesis in vivo by use of a too favourable affinity of GTP relative to that of GDP for EF-Tu.

Model developed

In the iterative model developed here [9], the following sequence of reactions are employed, using the rate constants derived by Gromadski et al. [1] and the concentrations of intracellular metabolites and components they describe:

$$\text{EF-Tu} \cdot \text{GDP} + \text{EF-Ts} \leftrightarrow \text{EF-Tu} \cdot \text{GDP} \cdot \text{EF-Ts} \quad (1)$$

$$\text{EF-Tu} \cdot \text{GDP} \cdot \text{EF-Ts} \leftrightarrow \text{EF-Tu} \cdot \text{EF-Ts} + \text{GDP} \quad (2)$$

$$\text{EF-Tu} \cdot \text{EF-Ts} + \text{GTP} \leftrightarrow \text{EF-Tu} \cdot \text{GTP} \cdot \text{EF-Ts} \quad (3)$$

$$\text{EF-Tu} \cdot \text{GTP} \cdot \text{EF-Ts} \leftrightarrow \text{EF-Tu} \cdot \text{GTP} + \text{EF-Ts} \quad (4)$$

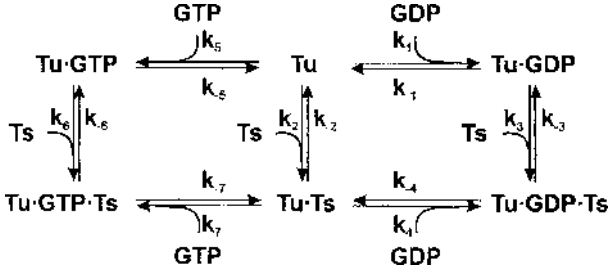
$$\text{EF-Tu} \cdot \text{GTP} + \text{aa-tRNA} \rightarrow \text{EF-Tu} \cdot \text{GTP} \cdot \text{aa-tRNA}. \quad (5)$$

The elemental rate constants resulting from their study are summarised in their Fig. 7 and are reproduced here in Table 1 which incorporates Fig. 1 of Gromadski et al. [1]. Assuming a constant availability of about 10 μM EF-Tu · GDP, a total cellular concentration of EF-Ts of about 10 μM and concentrations of GDP, GTP, and aminoacyl-tRNA of 100, 900, and 115 μM, respectively as used by Gromadski et al. [1] in their calculations, the model produces a steady conversion of EF-Tu · GDP to EF-Tu · GTP · aminoacyl-tRNA of 250 μM s^{-1}. Thus, the turnover rate constant for EF-Ts would be about 25 s^{-1}, in reasonable agreement with the 30 s^{-1} suggested by Gromadski et al. [1]. Since a rate of nucleotide exchange of 25–30 s^{-1} exceeds a rate of peptide bond formation of 10 s^{-1}, it is reasonable to assume as did Gromadski et al. [1] that the bulk of the EF-Tu in the cell (cf. 100 μM) will be in the form of EF-Tu · GTP · aminoacyl-tRNA.

Rate limiting steps

Gromadski et al. [1] suggested that the rate of binding of EF-Ts to EF-Tu · GDP would equal $k_3[\text{EF-Ts}]/(1+k_{-3}/k_{-4})$ and if the concentration of EF-Ts is about 10 μM would be 158 s^{-1}. The dissociation of GDP from the ternary complex equals $k_{-4}/[1 + (k_4[\text{GDP}])/(k_7\ [\text{GTP}])]$

Table 1
Kinetic mechanism of nucleotide exchange in EF-Tu



	k_+/k_- (μM^{-1} s^{-1}/s^{-1})	K_{eq} (μM^{-1})
1. EF-Tu + GDP ↔ EF-Tu · GDP (k_1/k_{-1})	2/0.002	1000
2. EF-Tu + GTP ↔ EF-Tu · GTP (k_5/k_{-5})	0.5/.03	16.7
3. EF-Tu + EF-Ts ↔ EF-Tu · EF-Ts (k_2/k_{-2})	10/0.03	333
4. EF-Tu · GDP + EF-Ts ↔ EF-Tu · GDP · EF-Ts (k_3/k_{-3})	60/350	0.171
5. EF-Tu · EF-Ts + GDP ↔ EF-Tu · GDP · EF-Ts (k_4/k_{-4})	14/125	0.112
6. EF-Tu · GTP + EF-Ts ↔ EF-Tu · GTP · EF-Ts (k_6/k_{-6})	30/60	0.5
7. EF-Tu · EF-Ts + GTP ↔ EF-Tu · GTP · EF-Ts (k_7/k_{-7})	6/85	0.070
8. EF-Tu · GTP + aa-tRNA → EF-Tu · GTP · aa-tRNA	50/	

The diagram in the upper part is from [1] and shows the various reactions for which they measured 'on' and 'off' rate constants, the values for which are taken from their Fig. 7 and are set out in the lower part of this table as reactions 1–8. In brackets the nomenclature used by Gromadski et al. [1] as in the diagram. For reaction 8 the equilibrium constant and 'on' rate constant appear to possess a similar numerical value [1,3] which leads to an 'off' rate constant of around 1 s^{-1}, a value which has little relevance in the present context.

and would be $100\,s^{-1}$. Together, these two reactions take about 16 ms (1/158 + 1/100 s), which corresponds to a rate of about $60\,s^{-1}$ for the displacement of GDP and formation of the binary complex EF-Tu · EF-Ts. The following step of GTP binding, due to the high cellular concentration of GTP, is likely to be extremely fast, whereas the dissociation of EF-Ts may be expected to be $57\,s^{-1}$ [rate $= k_{-6}/[1 + (k_6[\text{EF-Ts}])/(k_{\text{aa-tRNA-binding}}[\text{aa-tRNA}])]$, assuming that EF-Tu · GTP binds aminoacyl-tRNA with an association rate constant of $50\,\mu M^{-1}\,s^{-1}$ as indicated by Gromadski et al.]. Together, all steps of the nucleotide exchange in EF-Tu take about 34, 16 ms for the release of GDP and 18 ms for GTP binding and dissociation of EF-Ts, and the overall rate of the complete cycle of nucleotide exchange will be about $30\,s^{-1}$—no one particular step being particularly rate limiting. Use of metabolic control analysis enables this suggestion to be checked.

Metabolic control analysis

With the model we can calculate for each step a flux control coefficient, defined in this case as the percentage increase in flux for a 1% increase in the forward and back rate constants governing the reaction. These prove, for the particular conditions chosen above, to be 0.043 for formation of EF-Tu · GDP · EF-Ts in reaction 1, 0.319 for release of GDP in reaction 2, 0.088 for formation of EF-Tu · GTP · EF-Ts in reaction 3, 0.539 for release of EF-Ts in reaction 4 and 0.007 for formation of EF-Tu · GTP · aminoacyl-tRNA in reaction 5. These numbers add up to unity and suggest that under the particular conditions studied, the two dissociation reactions, the release of GDP from the EF-Tu · GDP · EF-Ts complex and of EF-Ts from the EF-Tu · GTP · EF-Ts complex are the principal rate determining steps. These results are in part similar to Gromadski et al.'s conclusions from estimates of the turnover times of reactions 1, 2, and 4, though addition of EF-Ts to EF-Tu · GDP, not of GTP to EF-Tu · EF-Ts, shows the lowest flux control coefficient. Both reactions 1 and 3 will exert relatively little influence on the flux, control of which appears to be more a function of reactions 2 and particularly 4.

From the model it is also possible to calculate elasticities, which are the ratio of the total forward reaction divided by the net forward reaction. These are 5.6 for EF-Tu · GDP · EF-Ts formation, 1.6 for release of GDP, 2.4 for formation of EF-Tu · GTP · EF-Ts, and 1.01 for release of EF-Ts—more or less mirroring the flux control coefficients. Calculation of metabolite responses shows that a 1% increase in [EF-Tu · GDP] increases flux by 0.23%, 1% increase of [GDP] inhibits flux by 0.20%, and 1% increase in [GTP] raises flux by 0.21%, whereas changing [aminoacyl-tRNA] has very little effect because of the limitation of rate of formation of EF-Tu · GTP in reaction 4.

Numerical problems

While in principle the data of Gromadski et al. [1] are an advance on previous work, there are some specific problems. From the top line of their Fig. 7 employing the rate constants reproduced in the lower part of Table 1, it can be concluded that in the absence of EF-Ts the affinity of EF-Tu for GDP is 60-fold greater than for GTP, as found by Romero et al. [3]. This is consistent with other estimates of about 100 [10,11]. However, in the presence of EF-Ts the apparent affinity of EF-Tu for GDP, obtained by multiplying out the rate constants and hence affinities for each step in their Fig. 7 (i.e., reactions 4–7 in the lower part of Table 1), is seen to be only 4.6-fold greater than for GTP, i.e., 13-fold less than in the absence of EF-Ts. Such a situation for the interactions displayed in Fig. 1 is not permissible.

It is obvious that discrepancies will arise given inevitable experimental error in the measurement of the rate constants determined for EF-Tu · GDP formation from EF-Tu by either direct addition of the nucleotide or during intermediate participation of EF-Ts, and in a like manner to the formation of EF-Tu · GTP. It is not meaningful, however, to calculate rates of nucleotide turnover unless the relative affinity of EF-Tu for GDP and GTP is the same in the presence of EF-Ts as in its absence, as Gromadski et al. [1] themselves point out. The equilibrium constant for EF-Tu · GDP formation from EF-Tu and GDP in the absence of EF-Ts (line 1 of the lower part of Table 1) is $1000\,\mu M^{-1}$ but with the involvement of EF-Ts, i.e., combining lines 3, 4, and 5, the value is seen to be $218\,\mu M^{-1}$—a 4.6-fold difference. The equilibrium constant for EF-Tu · GTP formation from EF-Tu and GTP in the absence of EF-Ts (line 2 of Table 1) is $16.7\,\mu M^{-1}$ but with the involvement of EF-Ts, i.e., combining lines 3, 6, and 7, the value is $47\,\mu M^{-1}$—a discrepancy of 2.8-fold. From these numbers, if it is assumed that the ratio of the relative affinities in the absence of EF-Ts is not likely to be less than 60, it can be concluded that the discrepancy in the relative affinities of EF-Tu for GDP versus GTP determined in the presence of EF-Ts is distributable in some manner between reactions 4 and 7. It is not possible from these data to attribute the inappropriate ratio to one particular step, nor is it dependent on the values, measured or postulated, for interaction of EF-Tu and EF-Ts (reaction 3), though, if the affinity of EF-Ts for EF-Tu were greater than that in line 3 of Table 1, the affinity of EF-Tu · GDP formed via reactions 3–5 would more nearly approach that found in the absence of EF-Ts. Conversely, however, the difference between the affinities for EF-Tu · GTP formation in the absence and presence of EF-Ts would increase, and vice versa.

Table 2
Changing rates of EF-Tu · GTP · aminoacyl-tRNA formation from EF-Tu · GDP with change of rate constants

	Rates (s^{-1})				
	As calculated from formulae in Section 3				Iterative procedure
Using data of Gromadski et al.	58	99	57	= 29.5	25.2
$4 \times k_{-3}$ $3 \times k_6$	49	99	52	= 20	15.6
$4 \times k_{-3}$ $3 \times k_{-7}$					12.0
$4 \times k_4$ $3 \times k_6$	158	61	52	= 24	15.4
$4 \times k_4$ $3 \times k_{-7}$					9.8
$0.25 \times k_3$ $0.33 \times k_{-6}$	39	99	19	= 11	8.2
$0.25 \times k_3$ $0.33 \times k_7$	39	70	57	= 18	9.7
$0.25 \times k_{-4}$ $0.33 \times k_{-6}$	49	25	19	= 8.8	5.6
$0.25 \times k_{-4}$ $0.33 \times k_7$	49	18	57	= 11	5.8

The first three columns are figures derived by the procedure of the authors as described in the text to give the overall rate as indicated in the fourth column, which can be compared with the rate obtained using the iterative procedure in the final column.

Possible solutions

It is possible to bring closer the relative affinity of EF-Tu for GDP and GTP in the absence and in the presence of EF-Ts by raising k_{-3} 4-fold and k_6 3-fold, or equally by increasing k_4 4-fold and k_{-7} 3-fold, or other similar changes, or by decreasing the value of the forward steps in a similar fashion, but the inevitable result of such changes is to decrease the rate of guanine nucleotide exchange. This is more consistently seen with the iterative model than with the formulae used by Gromadski et al. [1]. In Table 2 of the present paper a comparison is made of the effect of change of rate constants on the fluxes anticipated for the three steps postulated by Gromadski et al. [1] and described above, and on the flux determined by the iterative procedure. Since k_{-7} does not appear in Gromadski et al.'s formulae its variation does not influence apparent turnover rates, whereas all the constants play a role in the iterative procedure.

Although Chau et al. [2] found substantially higher 'on' and 'off' rate constants than did Gromadski et al. [1] for the addition of EF-Ts to EF-Tu · GDP and the release of EF-Ts from EF-Tu · GDP · EF-Ts, their equilibrium constants (K_{eq}) were very similar to those of Gromadski et al. [1]. Conversely, whereas the 'on' and 'off' rate constants determined by Romero et al. [3] for binding of GTP to EF-Tu · EF-Ts and the release of EF-Ts from EF-Tu · GTP · EF-Ts are numerically in the same range as those found by Gromadski et al. [1], their equilibrium constants are appreciably different, particularly for K_6 where its values range from $70\,\mu M^{-1}$ [3], through 9.3 as previously estimated by myself [12] to 0.5 found by Gromadski et al. [1].

Unfortunately my suggestions [12] for revised rate constants for the pairs of reactions 4–5 and 6–7 of Table 1 suffer from the same limitation as those of Gromadski et al. [1], namely that they lead to an apparent relative affinity of EF-Tu for GDP versus GTP in the presence of EF-Ts fivefold less than found in its absence. (This error arises as a result of using the equilibrium constants set out by Romero et al. [3].) Both sets of data clearly are in some manner inadequate to explain the functioning of EF-Ts. In the iterative model, using the data of Gromadski et al. [1], the release of EF-Ts from EF-Tu · GTP · EF-Ts has a low elasticity coefficient, indicating little flux in the reverse direction. If k_6 is raised 13-fold, then the ratio of the affinity of EF-Tu for GDP and GTP becomes the same, at about 60, irrespective of whether EF-Ts is involved or not. If both k_6 and k_{-6} are now raised 20% to $468\,\mu M^{-1}\,s^{-1}$ and $72\,s^{-1}$ (K_{eq}, $6.5\,\mu M^{-1}$) the flux in the model is $255\,\mu M\,s^{-1}$, that is, as initially found with Gromadski et al.'s rate constants. While their experimental results provide no evidence for this revision of k_6 and k_{-6}, it seems as likely that it is in this step that the problems arise in providing kinetic constants consistent with the presumed sequence of reactions for the facilitation of replacement of GDP by GTP on EF-Tu by EF-Ts.

Similar problems have been encountered previously [13] in trying to determine appropriate rate constants for the displacement of GDP from eIF2 · GDP and its replacement by GTP under the influence of eIF2B. It was suggested that Met-tRNA may bind to eIF2 · GTP · eIF2B and that only subsequently is the eIF2B released. Unequivocal evidence, however, for this step has yet to be observed. Finally, it must be stated that all attempts to compare the competence of kinetic data determined in vitro to explain intracellular events are compromised by the difficulty of knowing the influence in vivo of macromolecular crowding [14].

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