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Catalysis of Guanine Nucleotide Exchange on eIF-2 by eIF-2B: Is it a Sequential or Substituted Enzyme Mechanism?

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The mechanism of action of the eukaryotic initiation factor eIF-2B in catalyzing the exchange of guanine nucleotides bound to eIF-2 is uncertain—evidence having been adduced for a sequential mechanism and for a substituted enzyme mechanism. Data purporting to support a substituted enzyme mechanism have been analysed and shown to be ambiguous and equally consistent with a sequential mechanism. Suitable rate constants for a sequential mechanism involving the transient formation of the quaternary complex eIF-2·eIF-2B·GDP·GTP are suggested.

The detailed mechanism for the exchange of guanine nucleotides bound to the eukaryotic initiation factor eIF-2 catalysed by eIF-2B remains uncertain. By analogy with results obtained for exchange of guanine nucleotides bound to the bacterial elongation factor EF-Tu catalysed by EF-Ts (1), a substituted enzyme mechanism has been generally assumed. Such a mechanism (reactions 1-4)

$$\text{eIF-2B} + \text{eIF-2}\cdot\text{GDP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{eIF-2B}\cdot\text{eIF-2}\cdot\text{GDP} \quad [1]$$

$$\text{eIf-2B}\cdot\text{eIF-2}\cdot\text{GDP} \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \text{eIF-2B}\cdot\text{eIF-2} + \text{GDP} \quad [2]$$

$$\text{eIF-2B}\cdot\text{eIF-2} + \text{GTP} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{eIF-2B}\cdot\text{eIF-2}\cdot\text{GTP} \quad [3]$$

$$\text{eIF-2B}\cdot\text{eIF-2}\cdot\text{GTP} \underset{k_4}{\overset{k_{-4}}{\rightleftharpoons}} \text{eIF-2B} + \text{eIF-2}\cdot\text{GTP} \quad [4]$$

has been substantiated most clearly in the work of Rowlands *et al.* (2). However, Wahba and colleagues (3,4) have championed the view that the mechanism of guanine nucleotide exchange catalyzed by eIF-2B is via a sequential (ternary complex) mechanism involving in this case formation of the quaternary complex of eIF-2·GDP·eIF-2B·GTP. This can be represented by reactions 5-8.

$$\text{eIF-2B} + \text{eIF-2}\cdot\text{GDP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{eIF-2B}\cdot\text{eIF-2pdGDP} \quad [5]$$

$$\text{eIF-2B}\cdot\text{eIF-2}\cdot\text{GDP} + \text{GTP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{eIF-2B}\cdot\text{eIF-2}\cdot\text{GDP}\cdot\text{GTP} \quad [6]$$

$$\text{eIF-2B}\cdot\text{eIF-2}\cdot\text{GDP}\cdot\text{GTP} \underset{k_3}{\overset{k_{-3}}{\rightleftharpoons}} \text{eIF-2B}\cdot\text{eIF-2}\cdot\text{GTP} + \text{GDP} \quad [7]$$

$$\text{eIF-2B}\cdot\text{eIF-2}\cdot\text{GTP} \underset{k_4}{\overset{k_{-4}}{\rightleftharpoons}} \text{eIF-2B} + \text{eIF-2}\cdot\text{GTP} \quad [8]$$

Dholakia and Wahba (4) observed that the presence of GTP is required for release of GDP from the eIF-2·GDP complex, a requirement not to be expected with a substituted enzyme mechanism, and this result was confirmed by Oldfield and Proud (5) and Gross *et al.* (6).

Rowlands *et al.* (2) studied the homologous displacement of [^{3}H]GDP by GDP. They provided (Fig. 6 of ref. 2) a Hanes plot of [GDP]/ν_0 versus [GDP] that is fully consistent with a substituted enzyme mechanism, ie. all the lines for the different [eIF-2·GDP] used intersected the *y* axis at the same point. Dholakia and Wahba (4) provided Lineweaver-Burk plots (Figs 5A and B of ref. 4) that appear equally convincing evidence of the sequential mechanism. How is this discrepancy in mechanism to be resolved?

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THEORY

The rate equation describing a substituted enzyme mechanism is

$$\nu_0 = V_{max}at/(at + K_at + K_ta) \quad [9]$$

and for a sequential mechanism

$$\nu_0 = V_{max}at/(at + K_at + K_ta + K'_aK_t) \quad [10]$$

where a and t are eIF-2·GDP and the displacing nucleotide, GDP or GTP, respectively, K_a and K_t the Michaelis constants for eIF-2·GDP and GDP or GTP, and K'_a an additional constant which from equation 11 below can be seen to be equal to k_{-1}/k_1.

Equation 10, by comparison with equation 9, involves an additional term in the denominator. If conditions were such that the term K'_aK_t were negligible in value in comparison with the other three terms, analysis of the sequential mechanism would give the same pattern of Lineweaver-Burk and Hanes plots as the substituted enzyme mechanism and vice versa. Such will tend to occur as a and t increase, and if K'_a and K_t are small.

Use of the King and Altman method (7), as described by Cornish-Bowden and Wharton (8), together with the rate constants described in equations 5-8, results in the following expression for $1/\nu_0$ for the sequential mechanism (in which eIF-2B does not appear and the quotients have the unit of sec).

$$\frac{(k_3 + k_4)}{k_{-3}k_{-4}} + \frac{1}{k_1a} + \frac{(k_2 + k_3)}{k_2k_{-3}t} + \frac{k_1(k_2 + k_3)}{k_1k_2k_{-3}at} \quad [11]$$

Equation 11 can be related to the reciprocal form of equation 10, namely

$$\frac{1}{V_{max}} + \frac{K_a}{V_{max}a} + \frac{K_t}{V_{max}t} + \frac{K'_aK_t}{V_{max}at} \quad [12]$$

from which it can be shown that $k_1 = V_{max}/K_a$, also that $K'_a = k_{-1}/k_1$ and that $V_{max} = k_{-3}k_{-4}/(k_{-3} + k_{-4})$.

RESULTS

Analysis of "Substituted Enzyme Mechanism" Results

Effect of [GDP]. Rowlands *et al.* (2) determined K_m^{GDP} for the results presented in their Fig. 6 to be about 2 μM. Thus all the [GDP] employed in the data of their Fig. 6, ranging from 4.5-72 μM, were substantially above the K_m^{GDP}. The consequence of this is most readily seen in the plot of ν_0, taken from Fig. 6, against [GDP] (Fig. 1). The range of [GDP] chosen seems to be ill advised. Those for eIF-2·[^{3}H]GDP, ranging from 12.6 to 37.9 nM, were more appropriately related to the $K_m^{eIF\text{-}2\cdot GDP}$ (34 nM), but unfortunately no Hanes plot of [eIF-2·[^{3}H]GDP]/ν_0 against [eIF-2·[^{3}H]GDP] was presented.[1]

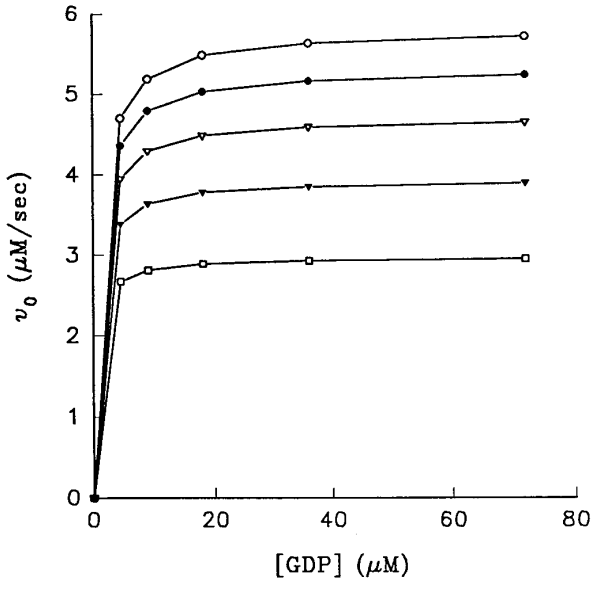

FIG. 1. Initial rates of displacement of [^{3}H]GDP bound to eIF-2 by GDP catalyzed by eIF-2B. The values of ν_0 employed in Fig. 6 of Rowlands *et al.* (2) can be obtained in two ways—either from equation 10 by employing the values they state for K_a and K_t of 34 nM and 2.0 μM respectively and a V_{max} of 0.011 pmol/sec, or by reading the values of [GDP]/ν_0 directly off Fig. 6. In Fig. 6 the y intercept appears to be about 0.5, which is inconsistent with it being K_t/V_{max} if K_t and V_{max} are 2.0 μM and 0.011 (x 1000) pmol/sec. Using a value for the intercept of 0.5 and comparing the slopes in Fig. 6 for the highest and lowest concentrations of eIF-2·GDP results in alternative values of K_a and K_t of 41 nM and 6.5 μM and V_{max} of 0.013 pmol/sec. The lines presented have been calculated using the kinetic parameters given by Rowlands *et al.* (2). [eIF-2·GDP] ○ 37.9 nM; ● 31.6 nM; △ 25.3 nM; ▲ 18.9 nM; □ 12.6 nM.

In an earlier analysis of the results of Rowlands *et al.* (2), the present author (9), assuming, as had Rowlands *et al.* (2), that reactions 1-4 constituted the mechanism for the displacement of [^{3}H]GDP by GDP, concluded that the most plausible values of the rate constants k_1 and k_{-1} were 2.60×10^8 $M^{-1}sec^{-1}$ and 11.5 sec^{-1} and for k_2 and k_{-2} were 1.6×10^7 $M^{-1}sec^{-1}$ and 32 sec^{-1}. For a homologous displacement k_3 and k_{-3} are equal to k_2 and k_{-2} and k_4 and k_{-4} are equal to k_1 and

[1] It is not possible meaningfully to use the values of ν_0 read off Fig. 6 of Rowlands *et al.* (2) to make a Hanes plot of [eIF-2·GDP]/ν_0 versus [eIF-2·GDP] because reading off the data from Fig. 6 involves measuring the slopes of the lines that have a common point of intersection on the y axis and therefore demonstrate, even if misleadingly, a substituted enzyme mechanism.

k_{-1}. As for equation 10, the fourth term of equation 11 will contribute progressively less to the value of $1/\nu_0$ as the value of a or t increases.[2]

When a Hanes plot of $[GDP]/\nu_0$ versus [GDP] is made with the values ν_0 calculated according to equation 11 using the above rate constants, mathematically the lines for the different concentrations of the second substrate eIF-2·[^{3}H]GDP do not intersect the y axis at the same point, but intersect at a point with x, y coordinates of -1.44, -0.046. This point, however, is very close to the origin, since the y axis rises to 40 and the x to 72. In practice, given normal scatter of data, it would be quite impossible to establish that the lines did intersect at such a common point, consistent with a sequential mechanism, or at a common point on the y axis, consistent with the substituted enzyme mechanism.

In the case of the Lineweaver-Burk plot of $1/\nu_0$ against 1/[GDP] (not shown), the line for the lowest fixed concentration of eIF-2·GDP in the simulation is visibly not parallel to the lines for the other concentrations, but it is difficult to see any convergence in the other four lines because it is so slight (though mathematically it is there). Given the problems of the accuracy of the nucleotide binding assay, it would be virtually impossible in practice to say for certain that any of the lines were or were not parallel to each other.

For the Lineweaver-Burk and Hanes plots with GDP as fixed substrate, the data points calculated according to equation 11 do show lack of parallelism of the curves for the former plot and lack of convergence to a single point on the y axis for the latter, indicating clearly that the data do not derive from a substituted enzyme mechanism. The plots, however, are not so striking that a scatter in the real data would make it very difficult to draw any firm conclusions.

If lower [GDP] had been used (eg 10x less and spanning the range above and below K_t), although the coordinates for the intersection in the Hanes plot remain the same, the scale of the axes is reduced and the point of intersection appears further from the origin. Under these conditions the Hanes plot could provide more clear cut evidence for or against either mechanism. It is the use of excessively high [GDP] that prejudiced the validity of the results obtained by Rowlands *et al.* (2). A better spread of [eIF-2·GDP], giving a larger range of slopes for the different lines, would also have been advantageous.

[2] The rate constants quoted above actually refer to results of Rowlands *et al.* (2) obtained at 30°C, whereas the data for their Fig. 6 was obtained at 15°C. Possible rate constants consistent with the data of Rowlands *et al.* (2) at 15°C (their Fig. 4B) are k_1 and k_2 of 2.94×10^8 and 2.9×10^5 $M^{-1}sec^{-1}$ and k_{-1} and k_{-2} of 10 and 0.58 sec^{-1}. The 30°C rate constants become more relevant when considering the data of Dholakia and Wahba (4).

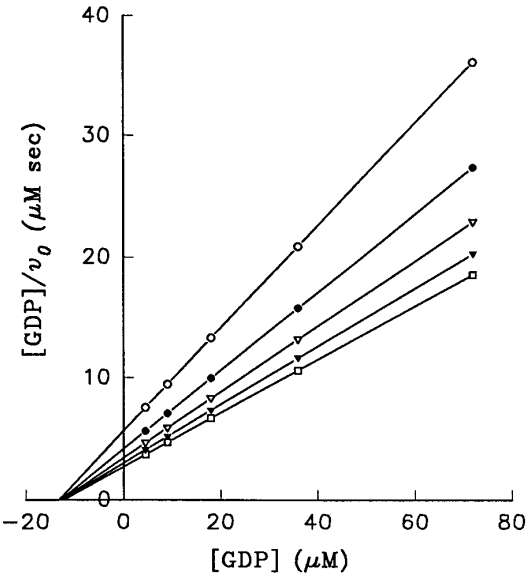

FIG. 2. Hanes plot for a heterologous displacement of GDP from eIF-2·GDP according to the sequential mechanism. Values of ν_0 were calculated from equation 11 using [GDP] and [eIF-2·GDP] of Rowlands *et al.* (2), assuming now that free GDP is GTP and rate constants for k_1 and k_4 of 2.6×10^8 $M^{-1}sec^{-1}$, for k_2 of 1.6×10^6 $M^{-1}sec^{-1}$, for k_{-1} and k_{-4} of 11.5 sec^{-1} and for k_{-2} and k_{-3} of 32 sec^{-1}. Intersection is at x, $y = -14.4$, -0.46. If rate constants of k_1 and k_4 of 2.6×10^8 $M^{-1}sec^{-1}$ and k_2 of 1.6×10^7 $M^{-1}sec^1$ and k_{-1} and k_{-4} of 11.5 sec_{-1} and k_{-2} and k_{-3} of 320 and 32 sec^{-1} are used, the point of intersection has the x, y coordinates -7.9, 0.25. [eIF-2·GDP] ○ 12.6 nM; ● 18.9 nM; △ 25.3 nM; ▲ 31.6 nM; □ 37.9 nM.

Heterologous displacement. Rowlands *et al.* (2) did not present data for a heterologous displacement of GDP bound to eIF-2 by GTP. In such a case there is no reason to expect k_2 and k_{-2} to equal k_3 and k_{-3} or possibly k_4 and k_{-4} to equal k_1 and k_{-1}. It is well established that GTP binds to eIF-2 less tightly than does GDP. Panniers *et al.* (10) estimated that the affinity of GTP for eIF-2·eIF-2B was about 10-fold less than of GDP. If, for sake of example, the 'on' rate constant k_2 is reduced from 16 to 1.6×10^6 $M^{-1}sec^{-1}$ or k_{-2} raised to 320 sec^{-1}, then the Hanes plot for data calculated with the equation 11 shows quite clearly (Fig. 2) the stigmata expected for a sequential mechanism, even with the displacing nucleotide concentrations used by Rowlands *et al.* (2).

A further possible reason for the opposite conclusions arrived at by Rowlands *et al.* (2) and Dholakia and Wahba (4) seems, therefore, to lie in the fact that the former group chose to study only the homologous exchange, the data from which, though possibly resulting from a sequential mechanism, are too similar to those assuming a substituted enzyme mechanism for it to be possible easily to distinguish between the two unless conditions are carefully chosen.

Analysis of "Sequential Mechanism" Results

Estimation of V_{max}. The results of Dholakia and Wahba (4) are less easy to analyse in that the two reciprocal plots they show in their Figs 5A and B, the first of $1/\nu_0$ versus 1/[GTP] and the second of $1/\nu_0$ against 1/[eIF-2·GDP], whilst clearly supporting a sequential mechanism, are also clearly separate experiments that cannot be precisely integrated in mathematical terms.

In the text of their paper Dholakia and Wahba (4) talk of a V_{max} of 2,610 pmol of GDP released/min/μg eIF-2B, but from the secondary plot derived from their Fig. 5A V_{max} can be anything between 3,000 and 11,000 pmol of GDP released/min/μg eIF-2B, whereas from the secondary plot from Fig. 5B V_{max} falls fairly close to 2,900 pmol of GDP released/min/μg eIF-2B. A rate of 2,610 pmol of GDP released/min/μg eIF-2B translates to a turnover number (T_N) of 11 sec^{-1}, measured probably at 30°C or 37°C, to be compared with about 8 sec^{-1} at 30°C calculated from Rowlands *et al.* (2), which extrapolates to about 22 sec^{-1} at 37°C (not 38 sec^{-1} as they suggest).

From the secondary plots of $1/\nu_0$ against 1/[eIF-2·GDP] for different set [GTP] (Fig. 5B), V_{max} in the present analysis is about 2,900 pmol/min/μg eIF-2B, K_a is 37 nM and K_t 2.9 μM. K'_a (the negative reciprocal of the value on the x axis for the point of intersection of the various lines (11) and equal to k_{-1}/k_1) can be estimated to be about 19 nM. Unfortunately, the secondary plots from $1/\nu_0$ against 1/[GTP] for different set [eIF-2·GDP] (Fig. 5A) are too erratic from which to draw conclusions, but were probably the source of Dholakia and Wahba's estimates of K_a and K_t of 100 nM and 3.6 μM respectively.

Derivation of rate constants. To try to simulate the data of Figs 5A and B, a V_{max} (actually T_N) of 11 sec^{-1}, K_a of 37 nM, K_t of 2.9 μM and K'_a of 19 nM have been assumed. Values for rate constants have been evaluated with the restraint these data impose. Although in principle 8 rate constants govern the behaviour of reactions 5-8, in equation 11 the rate constants k_3 and k_4 are not present; moreover, k_1 is equal to V_{max}/K_a and therefore equal to 297 $\mu M^{-1}sec^{-1}$, and k_1 and k_{-1} are interrelated through K'_a, i.e. $k_{-1} = 0.019k_1$. The constants k_{-3} and k_{-4} are interrelated in V_{max} (first term of equation 11). From equation 11 it can also be seen that $K_t/V_{max} = (k_{-2} + k_{-3})/k_2k_{-3}$. Dholakia and Wahba (3) found a K_d of 4 μM for the binding of GTP to eIF-2B. If this value is assumed to apply to the ratio k_{-2}/k_2, then these two rate constants are related and it follows that $k_{-2}k_{-3} = 15.4(k_{-2} + k_{-3})$. Thus, setting either k_{-2} or k_{-3} at some predetermined value has the effect of defining the other rate constant of this pair and hence of k_2 and k_{-4}. Apart from k_3 and k_4, therefore, all the other rate constants can be fixed. If k_{-2} is arbitrarily set at 32 sec^{-1}, k_{-3} and k_{-4} are about 30 and 17.5 sec^{-1} respectively. If k_{-2} is made smaller, k_{-3} becomes negative. As k_{-2} increases k_{-3} falls and k_{-4} rises to limiting values of 15 and 40 sec^{-1} respectively. Thus k_{-3} appears to be in the range 15-30 sec^{-1} and k_{-4} 17.5-40 sec^{-1} at 30°C.

As is to be expected, data derived from use of these rate constants reproduce Fig. 5B very well, and Fig. 5A reasonably well (the intercept of the lines to the left of the y axis (K_a/K'_aK_t) is at $x = -0.67$, as opposed to -0.83 in Fig. 5A).

DISCUSSION

The substituted enzyme mechanism was initially favoured by analogy with the action of EF-Tu and EF-Ts (1), but fails to explain why eIF-2B cannot be displaced from binding to eIF-2 by GDP (3-6) and why a second nucleotide is required for displacement of the existing bound nucleotide. The present results do not prove the sequential mechanism, but do suggest that the evidence supposedly favouring the substituted enzyme mechanism may be suspect. The problems of interpretation emphasise once again that when working with the Michaelis-Menten equation and its transformations, wherever possible substrate concentrations used should span the range in which K_m is to be found.

The observation (4) that GTP can bind to eIF-2B suggests the possibility that an alternative mechanism of exchange to that outlined in equations 5-8 could be the interaction of eIF-2·GDP and eIF-2B·GTP. However, there is good evidence (3) that eIF-2B binds firmly to eIF-2 and eIF-2·GDP, but that the presence of GTP leads to dissociation of the eIF-2B and presumably GDP from the eIF-2 in favour of GTP. A molecular mechanism for the displacement is still required.

Another approach to distinguishing between a sequential and a substituted enzyme mechanism could be a comparison of progress curves for GDP displacement. For this purpose the reciprocal of equation 11 for ν_0 has been expanded to cover the reverse reaction and compared with the comparable equation (12) derived for the substituted enzyme mechanism. Although for only the substituted enzyme mechanism for homologous displacement is the progress first order, the difference in rates between the two mechanisms are sufficiently small such as to make experimental detection virtually impossible. Given the range of concentrations and rate constants discussed so far, reduction in k_1 slows the progress of the displacement and decrease of k_4 increases the rate of displacement. Only change of k_{-3} produces a markedly different response to the two progress curves - its 10-fold decrease, though decreasing the initial velocity, results in a marked progressive

increase in the fractional exchange rate as time proceeds for the sequential mechanism, whereas the fractional exchange rate for the substituted enzyme mechanism does not change with declining [eIF-2·GDP]. Unfortunately there are no published experiments which can be analysed in terms of this prediction.

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