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# Binding Constants in the Formation of Mammalian Protein Synthesis Initiation Complexes and the Role of mRNA

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**The findings of Parkhurst *et al.* (Biochemistry 33, 15168-15177:1994) that a 10-mer oligoribonucleotide containing the AUG triplet enhances the binding of the eIF-2·Met-tRNA$_i$ complex to the 40S ribosomal subunit are questioned on the basis of a re-evaluation of their calculations. It is not possible to conclude, as they did, that addition of the AUG-containing oligonucleotide produces an exceptionally large increase (as judged by the magnitude of the coupling free energy) in the binding of the eIF-2·Met-tRNA$_i$ complex to the 40S subunit, or that their results are more consistent with internal initiation than with the scanning initiation model.** 

The first step in mammalian protein synthesis is the formation of a ternary complex between eukaryotic initiation factor 2 (eIF-2), GTP and Met-tRNA$_i$ to form eIF-2·GTP·Met-tRNA$_i$. The ternary complex is then generally considered to interact with the 40S ribosomal subunit, which complex then binds mRNA. In prokaryotes by contrast mRNA is necessary for the interaction of 30S subunit and ternary complex (1). Roy *et al.* (2) have demonstrated in eukaryotes it can also be the case that mRNA is required for significant binding of ternary complex and 40S subunit to occur. Recently Parkhurst *et al.* (3) have analysed the binding constants for the various interactions, using an AUG-containing 10-mer oligoribonucleotide as their mRNA. They found an association constant ($K_a$) of 0.94 $\mu M^{-1}$ for interaction of 40S subunit (R) with ternary complex (which in their studies is actually a quaternary complex (Q) containing, in addition to eIF-2, GTP and Met-tRNA$_i$, the factor Co-eIF-2C). For binding of their oligoribonucleotide (M) to the Q·R complex $K_a$ was 110 $\mu M^{-1}$. R was also found to bind M ($K_a$ 1.78 $\mu M^{-1}$) and possibly M also bound to Q with an upper limit for $K_a$ of 0.14 $\mu M^{-1}$. From the different measured $K_a$'s they calculated that $K_a$ for binding of Q to R·M was about 58 $\mu M^{-1}$. Their major conclusion was that the addition of messenger RNA produced a 60-fold enhancement of the binding of Met-tRNA$_i$ to 40S-an increase which they considered to be unusually large. The purpose of the present communication is to re-evaluate their data and final conclusions which it appears may not be unique.

## METHODS

A program for analysis of multiequilibria, previously donated to the author by Dr Athel Cornish-Bowden of the Laboratoire de Chimie Bactérienne, Centre National de la Recherche Scientifique, in Marseilles, France, has been used to determine concentrations of components present in equilibrium with each other. Though not shown, the validity of the calculations can be verified by manual recalculation of association constants used from the concentrations of components arising from the calculations, to ensure that there is agreement.

## RESULTS AND DISCUSSION

Table 1 shows the calculated concentrations of the Q·R·M complex to be expected from the association constants that Parkhurst *et al.* (3) derived, and the comparisons of these values with those actually found. The procedure used for experimentally determining the actual values cannot distinguish Q·R·M from Q·R, i.e. they are actually (Q·R·M + Q·R). From the calculations, however, it is apparent that Q·R is always less than 1% of Q·R·M. Thus there is very little error if (Q·R·M + Q·R) is assumed to equal Q·R·M. Table 1 shows that with a $K_a$ of 110 $\mu M^{-1}$ for interaction between Q·R and M, there is better agreement between "found" and "calculated" values when, in addition to formation of Q·R and Q·R·M (column a), the interaction of R and M with the association constant of 1.78 $\mu M^{-1}$, which the authors measured independently, is also assumed (column b). Assumption of an additional interaction between Q and M (column c) at what Parkhurst *et al.* (3) considered the upper limit of the possible association required increase of interation



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**TABLE 1**

Comparison of Found and Calculated Concentrations of Q·R·M Complex

| 40S Ribosomal subunits added | Q·R·M | | | |
|---|---|---|---|---|
| | | Calculated | | |
| | Found | (a) | (b) | (c) |
| 6.8 | 3.6 | 5.9 | 4.4 | 4.2 |
| 20.4 | 15.3 | 16.9 | 12.4 | 11.6 |
| 68 | 30.7 | 39.9 | 30.2 | 28.5 |
| 204 | 42 | 47 | 43 | 42 |
| 612 | 45 | 49 | 47 | 47 |

*Note.* All concentrations are nM. That of Q initially was 49.7 mM and of M 1.5 $\mu$M. The concentrations of Q·R·M headed "found" are those of Parkhurst *et al.* (3) and are more correctly (Q·R·M + Q·R), whereas the calculated values are for the Q·R·M complex alone. In the presence of M the amount of Q·R was always less than 1% of the total. For column (a) no binding between R and M, or between Q and M, was assumed; in (b) the formation of R·M ($K_a$ 1.78 $\mu M^{-1}$) was assumed, in addition to formation of Q·R ($K_a$ 0.94 $\mu M^{-1}$) and Q·R·M from Q·R and M ($K_a$ 110 $\mu M^{-1}$); in (c) an additional formation of Q·M ($K_a$ 0.14 $\mu M^{-1}$) was included in the calculations.

between Q·R and M, as they found, to about 133 $\mu M^{-1}$ for a comparable fit of the data for Q·R·M formation.

Columns (b) and (c) do in fact show quite reasonable agreement between calculated and experimentally derived concentrations. Given that $K_a$ for Q·R formation multiplied by the $K_a$ for the interaction of Q·R with M must equal the $K_a$ for R·M formation multiplied by the $K_a$ for its interaction with Q, Parkhurst *et al.* (3) were able to calculate this last association constant from the other three constants as 58 $\mu M^{-1}$. The figure for addition of Q to R·M is thus 62-fold greater than for addition of Q to R in the absence of M, and suggests that the presence of M greatly enhances the binding of Q to R. The data of Table 1 therefore support this conclusion.

**TABLE 2**

Comparison of Found and Calculated Concentrations of Q·R Complex

| 40S Ribosomal subunits added | Q·R | |
|---|---|---|
| | Found | Calculated |
| 6.8 | 1.04 | 0.30 |
| 20.4 | 1.60 | 0.89 |
| 68 | 3.27 | 2.87 |
| 204 | 7.70 | 7.74 |
| 612 | 7.12 | 17.8 |

*Note.* All concentrations are nM. That of Q initially was 49.7 nM. The concentrations of Q·R headed "found" are those of Parkhurst *et al.* (3). $K_a$ for binding of Q and R used in the calculation was 0.94 $\mu M^{-1}$ as claimed by Parkhurst *et al.* (3).

**TABLE 3**

Comparison of Q·R and Q·R·M Formation

| 40S Ribosomal subunits added | Q binding complexes | |
|---|---|---|
| | | Calculated |
| | Found | (a) | (b) |
| 6.8 | 3.6 | 4.4 | 4.4 |
| 20.4 | 15.3 | 12.2 | 12.4 |
| 68 | 30.7 | 30.0 | 30.2 |
| 204 | 42 | 43 | 43 |
| 612 | 45 | 48 | 47 |

*Note.* All concentrations are nM. That of Q was initially 49.7 nM and of M 1.5 $\mu$M. The concentrations under the heading "found" are the experimental results of Parkhurst *et al.* (3) and are assumed to represent the complex Q·R·M as in Table 1. The concentrations under (b) are the calculated values of Q·R·M as determined in Table 1. The concentrations under (a) are the Q·R complex if the $K_a$ for the interaction of Q and R is 40 $\mu M^{-1}$. In this case any role of M in the formation of the Q·R complex or any interaction between R and M is ignored.

However, a problem over the acceptance of the findings of Parkhurst *et al.* (3) is that it is questionable whether their value of the association constant for Q·R formation is correct. Table 2 shows the amounts of binary complex found and to be expected for a $K_a$ of 0.94 $\mu M^{-1}$. Clearly, there is very poor agreement between calculation and what was observed, nor is it possible to vary the value of $K_a$ to provide a more satisfactory fit. A possible explanation of the experimental findings is that, contrary to the belief of Parkhurst *et al.* (3), Q·R in the absence of M is not stable under the conditions of the assay, and that the values observed represent a higher association constant than calculated, coupled with dissociation taking place when the [$Mg^{2+}$] is raised in the final stage of the assay.[1] In fact a $K_a$ of 40 $\mu M^{-1}$ for Q·R formation accurately reproduces the data of Table 1, column (b) and the "found" data (Table 3).

Whether this explanation is correct or not, an invalid estimate of $K_a$ for interaction between Q and R, such as appears to be the case from the data presented by the authors, prejudices any deductions that can be made dependent on its numerical value. The authors point out that the coupling free energy of −2.4 kcal/mol that they calculate for the effect of the presence of M in their experiments is greater

[1] The controls for the assay used by Parkhurst *et al.* (3) are contained in their Figure 2. Unfortunately this Figure is incomplete because the concentration of R used is not stated, and, since R in the subsequent experiments was the principal variable, it is not possible to guess what it may have been. It is also not clear whether a constant B, used in the equation for the evaluation of the data of Figure 2, whose numerical value is stated, itself varied as R varied, as it must.

than any of those listed by Weber (4) for various biochemical interactions. However, an incorrectly low value of $K_a$ for Q·R formation results in an incorrectly high value for the estimates of $K_a$ for Q·R·M formation from Q·R and M. This in turn results in an incorrectly large value for $K_a$ for Q·R·M formation from R·M and Q, then to a falsely high ratio of this $K_a$ to that for Q·R formation, and hence an incorrect estimate of the coupling free energy.

Credence is lent to this possibility by Table 2 of Parkhurst *et al.* (3) in which the enhancement of binding of Q to R produced by M is shown to be four fold. With the stated concentrations of Q, R and M such would be achieved with a $K_a$ of about 1 $\mu M^{-1}$, that is 100-fold less than their formal estimate. In this instance there is no evidence of cooperativity between binding of Q and M.

The thermodynamic considerations that Parkhurst *et al.* (3) employed to calculate the value of $K_a$ for binding of Q to R·M, and from which they estimated that addition of M increased the $K_a$ for binding of Q to R 60-fold, also suggest, *mutatis mutandis* that addition of Q, by first forming a Q·R complex, facilitates binding of M to R by an equivalent factor. Since the $K_a$ found by Parkhurst *et al.* (3) for binding of M to R is only about 2-fold greater than of R to Q and it is questionable whether the latter is underestimated, it is not possible to predict which might be the preferred route for formation of the Q·R·M complex. It is therefore not possible to determine whether the results favour an internal initiation model or the scanning initiation model of Kozak (5).

## REFERENCES

1. Maitra, U., Stringer, E. A., and Chaudhuri, A. (1982) *Ann. Rev. Biochem.* **51,** 869–900.
2. Roy, A. L., Chakrabarti, D., Datta, B., Hileman, R. E., and Gupta, N. K. (1988) *Biochemistry* **27,** 8203–8209.
3. Parkhurst, K. M., Hileman, R. E., Saha, D., Gupta, N. K., and Parkhurst, L. J. (1994) *Biochemistry* **33,** 15168–15177.
4. Weber, G. (1975) *Adv. Protein Chem.* **29,** 1–83.
5. Kozak, M. (1986) *Cell* **44,** 283–292.