Regulation of binding of initiator tRNA to eukaryotic initiation factor eIF-2

Effects of the haem-controlled repressor on the kinetics of ternary complex formation

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Ternary complex formation was studied in reticulocyte lysate supernatants and using rat liver eukaryotic initiation factor-2 (eIF-2) preparations. Haem-deficiency reduced the rate of formation of ternary (Met-$tRNA_f$ · GTP · eIF-2) complexes by the eIF-2 in reticulocyte supernatants, the reduction being more marked when complex formation was assayed in the absence of GTP-regenerating capacity. Pretreatment with the haem-controlled repressor (HCR) reduced the rate of ternary complex formation by crude (liver) eIF-2. In contrast, complex formation by an almost homogeneous eIF-2 preparation was unaffected by HCR: sensitivity to HCR was however restored by a factor which catalyses exchange of guanine nucleotides bound to eIF-2.

Protein synthesis *Reticulocyte lysate* *Eukaryotic initiation factor-2*

1. INTRODUCTION

The rate of protein synthesis in reticulocyte lysates is closely regulated by the concentration of haemin (review [1]). In the absence of haem protein synthesis is rapidly shut off due to the development of a block in peptide-chain initiation [2,3] associated with the activation of a cyclic nucleotide-independent protein kinase termed the haem-controlled repressor (HCR). HCR specifically phosphorylates the α-subunit of protein synthesis initiation factor-2 (eIF-2) [4,5]. eIF-2 normally promotes the binding of the initiator Met-$tRNA_f$ to 40 S ribosomal subunits in a two-stage process involving formation of a ternary complex between eIF-2, GTP and Met-$tRNA_f$, and the subsequent transfer of this complex to the 40 S subunit [6,7]. Levels of such 40 S initiation complexes are reduced in haem-deficient lysates [3].

It now seems clear that increased phosphorylation of the α-subunit of eIF-2 is associated not only with the inhibition of protein synthesis caused by haem-deficiency, but also with several other conditions which bring about the impairment of peptide-chain initiation in reticulocyte lysates [4,8]. However, the mechanism by which phosphorylation of eIF-2 impairs its ability to participate in initiation is not fully understood. This is largely because purified eIF-2 is equally active in a number of cell-free assay systems, irrespective of its phosphorylation state [4,9–13].

Regulation at the level of peptide-chain initiation also appears to be involved in the control of protein synthesis in other systems; e.g., regulation of protein synthesis by hormones (insulin [14], glucocorticoids [15]) in skeletal muscle or by amino acid starvation in Ehrlich ascites tumour cells [16]. To investigate how changes in the activity of eIF-2 may be involved in the control of protein synthesis in these less well characterised systems, it is necessary to devise suitable methods for assaying eIF-2 in extracts of these tissues or cells. Here we have

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Abbreviations: eIF-2, eukaryotic initiation factor-2; HCR, haem-controlled repressor; Hepes, 4-(2-hydroxyethyl)-1-piperazine sulphonic acid

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chosen to use reticulocytes as a model system for the development of such assays. In the course of these experiments, rates of ternary complex formation were determined in postribosomal supernatants derived from haem-deficient or haem-supplemented lysates. The results show that haem-deficiency reduces the rate of complex formation in these fractions. We have also observed that when crude eIF-2 from other sources was treated with HCR, a similar impairment in the rate of ternary complex formation was observed. In contrast, when more highly purified eIF-2 was used, HCR treatment was without effect on the rate of ternary complex formation by the initiation factor. Sensitivity of purified eIF-2 to HCR could be restored by addition of a factor which catalyses exchange of guanine nucleotides bound to eIF-2. The results show that phosphorylation inhibits the first step in initiation complex formation by eIF-2, and that another component is required for this inhibition to be expressed in vitro.

2. MATERIALS AND METHODS

2.1. *Materials*

All biochemicals and radiochemicals were obtained as in [17]. Reticulocyte lysates (1:1) and [^{35}S]methionyl-tRNA$_f$ were prepared as in [18]. The haem-controlled repressor was a generous gift from Dr Tim Hunt (University of Cambridge). The guanine nucleotide exchange factor [19] was provided by Dr Richard Panniers (University of Rochester, New York).

2.2. *Preparation of rat liver eIF-2*

The procedure used was based on those described in [20] for pig liver and in [21] for Ehrlich ascites tumour cells. Briefly, rat livers were homogenised in 0.25 M sucrose containing 35 mM Tris–HCl (pH 7.6), 30 mM KCl, 5 mM Mg-acetate and 10 mM β-mercaptoethanol. A postmitochondrial supernatant was prepared and the microsomes were sedimented from it by centrifugation at 253 000 × g for 30 min. After resuspension in homogenisation buffer and adjustment of [KCl] 0.5 M, the preparation was recentrifuged as above. The resulting supernatant (microsomal salt wash) was subjected to ammonium sulphate fractionation. The material precipitating between 40–50% saturation was collected and dissolved in buffer A (20 mM morpholinopropanesulphonic acid (pH 7.6), 0.1 mM EDTA, 0.5 mM dithiothreitol, 10% (v/v) glycerol) containing 0.35 M KCl, and dialysed extensively against this buffer. (Samples of this material were used in section 3.2.) The dialysed preparation was applied to a column (1.6 × 4.5 cm) of phosphocellulose in the same buffer. The eIF-2 bound to the column and was eluted with a gradient (total vol. 50 ml) of KCl (0.35–0.8 M) in buffer A. The fractions containing the most activity were pooled, dialysed (1 h) against buffer A containing 0.05 M KCl and then diluted with an equal volume of buffer A, prior to application to a DEAE-cellulose column (3.5 ml) equilibrated in buffer A containing 0.09 M KCl. To elute the eIF-2 a gradient (25 ml) of KCl (0.09–0.4 M) in buffer A was applied. The eIF-2 was eluted between 0.15 and 0.20 M KCl. The eIF-2 preparation was concentrated by repeating the DEAE-cellulose step using a smaller column (1 ml) and eluting the eIF-2 stepwise with buffer containing 0.35 M KCl. The final eIF-2 preparation was stored at −70°C.

2.3. *Incubation and fractionation of reticulocyte lysates*

Protein synthesis in reticulocyte lysates was assayed essentially as in [22]. Where present, haemin was 20 μM. The postribosomal supernatant samples were prepared as follows: after 14 min incubation, at which time protein synthesis was still proceeding linearly in the haem-containing lysate but had almost completely shut off in the haem-deficient one, lysates were diluted 2-fold with ice-cold buffer (20 mM Tris–HCl (pH 7.8), 80 mM KCl, 2 mM Mg-acetate and 2 mM GTP). The GTP was added to prevent pressure-activation of HCR during centrifugation [23]. The diluted lysate was centrifuged in a Beckman 50Ti rotor at 140 000 × g for 2.5 h at 2°C. The resulting postribosomal supernatant was dialysed for 2 h against 20 mM Tris–HCl (pH 7.8), 0.2 mM EDTA, 10 mM β-mercaptoethanol, prior to assay of ternary complex formation.

2.4. *Assay of ternary complex formation*

Formation of [eIF-2 · GTP · Met-tRNA$_f$] complexes was assayed as in [17] with the following modifications: the incubation volume was 100 μl, 15 μl samples were taken at the times indicated, and phosphoenolpyruvate plus pyruvate kinase

was omitted, where indicated, from some incubations. Amounts of eIF-2 present were always kept well within the linear range of the assay.

3. RESULTS AND DISCUSSION

3.1. *Ternary complex formation by eIF-2 in postribosomal supernatants from reticulocyte lysates.*

Postribosomal supernatants prepared as in section 2 contained > 90% of the total Met-tRNA$_f$-binding activity present in the reticulocyte lysate. When postribosomal supernatants were prepared in buffers containing lower (60 mM) concentrations of KCl, however, more Met-tRNA$_f$-binding activity was associated with the ribosomal pellet and under these conditions only 60–70% of the total was recovered in the ribosome-free supernatant (C.G. Proud, unpublished).

Two lines of evidence indicate that the Met-tRNA$_f$-binding activity in the postribosomal supernatant represents eIF-2 and only eIF-2:

(1) Met-tRNA$_f$ binding in the ternary complex assay was dependent on the presence of GTP (not shown) and was strongly stimulated by phosphoenolpyruvate plus pyruvate kinase (a GTP-regenerating system) (fig.1). This behaviour is characteristic of eIF-2 [24,25].

(2) When postribosomal supernatants were subjected to ion-exchange chromatography on phosphocellulose in buffer containing 0.25 M KCl, almost all the protein passed through the column, whilst all the Met-tRNA$_f$-binding activity bound to the column and could be quantitatively recovered by elution with buffer containing 0.7 M KCl (data not shown). This step has frequently been used in the isolation of eIF-2 from reticulocytes [26,27].

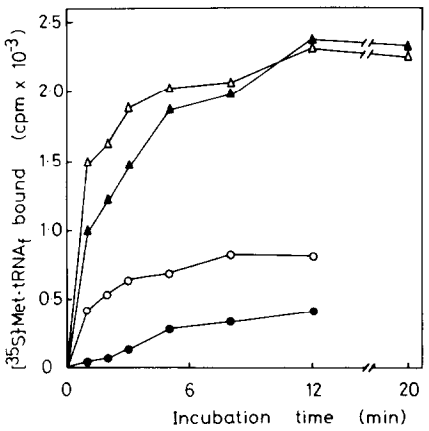

Fig.1. Formation of ternary complexes by eIF-2 in postribosomal supernatants derived from reticulocyte lysates incubated in the presence (○,△) or absence (●,▲) of haemin. Time courses of [eIF-2 • GTP • [^{35}S]Met-tRNA$_f$] complex formation were followed, as in section 2 in incubations containing GTP and the GTP-regenerating system (△,▲) or GTP alone (○,●).

Fig.1 shows that formation of [eIF-2 • GTP • Met-tRNA$_f$] complexes occurred at a faster initial rate in the supernatants derived from haemin-supplemented lysates than in those from lysates which had been incubated without haemin, when assayed in the presence of GTP and the GTP-regenerating system. This difference in rates was even more marked when complex formation was assayed in the absence of the regenerating system (fig.1). Here, complex formation did not attain the same plateau level as in the 'complete' assay (during the time period studied), but both the rate and extent of complex formation were considerably greater in the supernatant from the haem-supplemented lysate than in the corresponding fraction from the haem-deprived one. Therefore, GTP alone appeared to be less efficient in supporting complex formation in the latter supernatant than in the former one. These differences in rates of ternary complex formation seem to reflect the state of activity of the endogenous eIF-2 at the end of the incubations of the lysates with or without haemin. They are not due to the effects of activators or inhibitors which might have been present in the assays of Met-tRNA$_f$ binding. The evidence for this is:

(1) When protein synthesis was assayed in reticulocyte lysates to which aliquots of postribosomal supernatants had been added, no inhibition was observed (not shown). This was perhaps surprising since the haem-deficient supernatant might be expected to contain HCR. The probable explanation is that since the lysates from which the supernatants were derived had been incubated for only 14 min without haem, only 'reversible HCR' was formed and this was subsequently inactivated, perhaps during the centrifugation in the presence of GTP [28].

Table 1

Effect of reticulocyte postribosomal supernatants on ternary complex formation by purified eIF-2

Source of postribosomal supernatant	Muscle eIF-2 (ng)	Ternary complexes formed (cpm [^{35}S]Met-tRNA$_f$ bound)					
		After 3 min incubation			After 10 min incubation		
		Actual	Predicted	Diff.	Actual	Predicted	Diff.
None	8.4	3033			4744		
None	16.8	6484			9690		
Haemin-supplemented lysate	None	3108			6252		
	8.4	5787	6141	− 354	9746	10996	−1250
	16.8	7480	9593	−2113	13717	15942	−2225
Haemin-deficient lysate	None	2456			5465		
	8.4	4052	5289	−1237	9237	10209	− 972
	16.8	6462	8940	−2478	12958	15155	−2197

The formation of ternary complexes by purified skeletal muscle eIF-2 (prepared as in [17]) was assayed in the presence or absence of supernatant samples (10 μl) from haemin-supplemented or haemin-deficient lysates. Incubations (100 μl) were carried out as in section 2 and contained GTP, phosphoenolpyruvate and pyruvate kinase. 'Predicted values' are the sums of the activities of the two components (purified eIF-2 and postribosomal supernatant) obtained when each was assayed separately.

(2) Measurement of ternary complex formation in incubations containing mixtures of supernatants from haem-deprived and haem-containing lysates indicated that neither fraction contained dominant activators or inhibitors of complex formation, since complex formation was strictly additive (not shown). In a further experiment designed to test whether fractions from haem-deficient lysates contained a specific inhibitor of [eIF-2 · GTP · Met-tRNA$_f$] formation, aliquots of postribosomal supernatants were added to assays containing purified eIF-2 (from rabbit muscle). The results (table 1) showed that, although formation of complexes by purified eIF-2 was partially inhibited by the added supernatant, the degree of inhibition was the same whether the supernatant was from a haem-deprived or a haem-supplemented lysate. This inhibition, which may be due to the presence in these fractions of unlabelled Met-tRNA$_f$, is therefore not the cause of the differences described above.

Whilst it is well established that haem-deficiency results in a fall in the level of 40 S-initiation complexes [3], this is the first time, to our knowledge, that an effect of haem-deficiency on peptide-chain initiation in reticulocyte lysates at the level of ternary complex formation has been reported. Qualitatively similar results to those in fig.1 were observed in 18 separate experiments utilising 5 different lysate preparations.

3.2. *Effect of HCR on ternary complex formation by eIF-2 preparations from rat liver*

Two distinct preparations of rat liver eIF-2 were used in these experiments: (A) material from the 40–50% ammonium sulphate fractionation step of the purification procedure (section 2); (B) highly purified eIF-2 from the final DEAE-cellulose column. On SDS–polyacrylamide gel electrophoresis (fig.2), preparation (B) showed the 3 major components characteristic of mammalian eIF-2 (α-, β- and γ-subunits; M_r 36 000, 49 000 and 52 000, respectively) [17,19,29]. Preparation (A) was very impure, showing many bands on SDS–polyacrylamide gels (fig.2).

Fig.3 shows the effect of preincubation with HCR on the formation of ternary complexes by

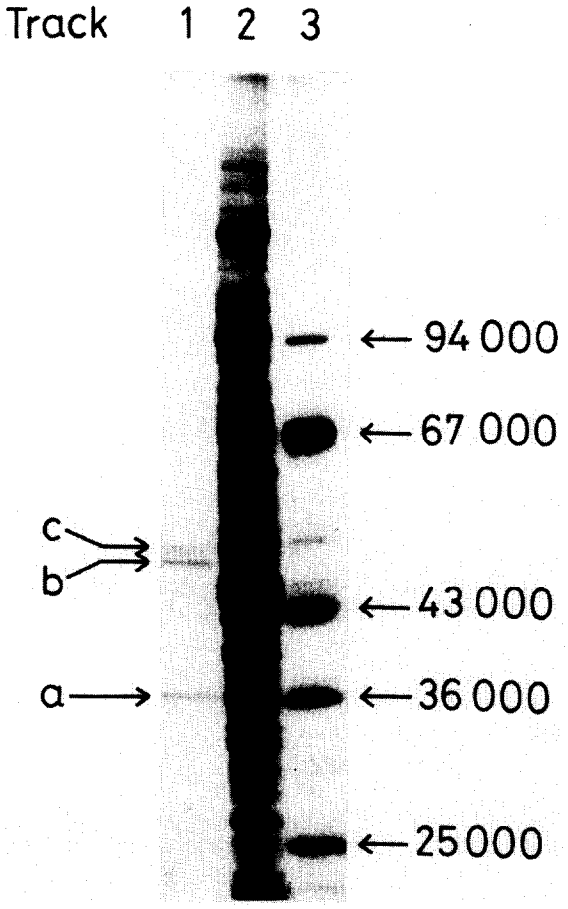

Fig.2. SDS–Polyacrylamide gel electrophoresis of rat liver eIF-2 preparations. Preparations (A) (40–50% ammonium sulphate fraction, ~ 150 μg, track 2) and (B) (final, purified eIF-2 fraction, 5 μg, track 1) were subjected to electrophoresis in 12.5% polyacrylamide gels [30]. Numbered arrows indicate the positions and M_r-values of marker proteins (track 3). The arrows labelled a,b and c show the positions of the α-, β- and γ-subunits of eIF-2, respectively.

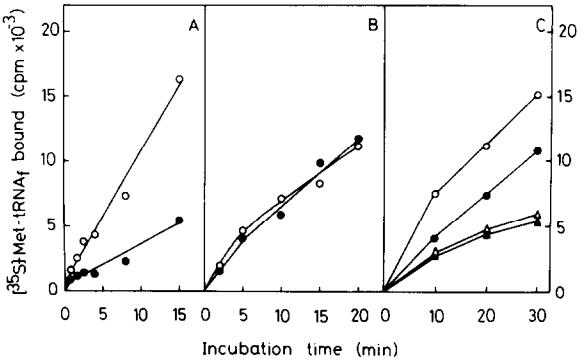

Fig.3. Effect of HCR on formation of ternary complexes by rat liver eIF-2 preparations. Relatively crude (preparation (A), panel A) or highly purified (preparation (B), panel B) eIF-2 was preincubated (5 min, 30°C) with (•—•) or without (○—○) HCR (8 U/ml; [34]) in 150 μl incubations containing 20 mM Hepes (pH 7.6), 2 mM Mg-acetate, 0.5 mM dithiothreitol, 100 mM KCl, 0.2 mM ATP, 0.05 mM EDTA, and ~ 5 μg of purified eIF-2 or an equivalent amount of the crude preparation. After the preincubation, GTP (to give 0.33 mM final conc.), phosphoenolpyruvate (to give 3 mM), pyruvate kinase (0.5 U) and ~ 0.2 μCi [^{35}S]Met-tRNA$_f$ were added (in that order) and the incubation was continued. Samples, taken at the times indicated, were assayed for ternary complex formation by retention on cellulose nitrate filters [17]. Panel C: purified eIF-2 (preparation (B)) (5 μg) was preincubated with (•,▲) or without (○,△) HCR as above. After 5 min, GTP (to give 0.2 mM) and 0.2 μCi labelled Met-tRNA$_f$ were added to each incubation. Guanine nucleotide exchange factor ([19]; 1.6 μg) was also added to some assays and ternary complex formation was then followed in the presence (○,•) or absence (△,▲) of this factor.

each of these preparations of eIF-2. It is clear that HCR-pretreatment substantially reduced the rate of ternary complex formation by preparation (A). In contrast, HCR-pretreatment had little or no effect on the formation of ternary complexes by preparation (B), in agreement with previous reports that HCR-treatment does not affect the activity of highly purified eIF-2 [4,9–13].

In accounting for the differing sensitivities of these two eIF-2 preparations to HCR, the most obvious difference between them is their degree of purity. This suggests that the crude preparation (A) may contain a factor which sensitises the eIF-2 to the effects of phosphorylation by HCR, but which is removed during the purification and is absent from the almost homogeneous preparation (B). A number of factors which stimulate the activity of eIF-2 have been reported (review [8]) and the stimulatory effects of several of them are abolished after eIF-2 has been phosphorylated by

HCR [8,13,31–32]. The best characterised of these is the reticulocyte factor in [31,33], which stimulates eIF-2 by promoting the exchange of guanine nucleotides bound to eIF-2 [33]. We have provided evidence that HCR treatment of partially purified eIF-2 inhibits such an exchange reaction [34]. During initiation GTP bound to eIF-2 in initiation complexes is hydrolysed to GDP [35]. As GDP has a high affinity for eIF-2 [36] and inhibits it [37], it is likely that eIF-2 is released from the ribosome upon 80 S initiation complex formation as an inactive [eIF-2 · GDP] complex. Participation of the eIF-2 in further rounds of initiation would then require displacement of GDP by GTP to form the active [eIF-2 · GTP] complex. The factor in [31,33] does promote this guanine nucleotide exchange reaction, and hence initiation complex formation, with unphosphorylated, but not with phosphorylated, eIF-2. If this is so in haem-deficient lysates, eIF-2 will accumulate as inactive [eIF-2 . GDP] complexes and initiation will then be impaired.

An analogous factor to the one from reticulocytes [31,33] discussed above has been isolated from Ehrlich ascites cells [19]. This factor similarly promotes exchange of guanine nucleotides bound to eIF-2. When added to purified eIF-2 (which is normally insensitive to the effects of phosphorylation by HCR, fig.3B), ternary complex formation was stimulated and rendered sensitive to inhibition by pretreatment of the eIF-2 by HCR (fig.3C). These results support the idea that crude eIF-2 is sensitive to HCR because it contains a factor of this type, and that phosphorylation inhibits ternary complex formation because it alters the interaction of eIF-2 with the guanine nucleotide exchange factor, thus imparing formation of the productive [eIF-2 . GTP] complex in the above manner.

The likely importance of the relationship between the guanine nucleotide exchange reactions and initiation complex formation is further supported by the following findings. HCR-catalysed phosphorylation inhibits both formation of ternary complexes by crude eIF-2 (fig.3A) and displacement of GDP by GTP from crude eIF-2 preparations (from Ehrlich ascites cells [34] or rat liver (M.J.C., unpublished)) whilst with pure initiation factor, HCR has no effect on either complex formation (fig.3B) or nucleotide exchange (M.J.C. unpublished).

In reticulocyte supernatants, GTP alone is less able to support ternary complex formation in fractions from haemin-deficient lysates than in fractions from control lysates (fig.1). This would be predicted from the above model, since the eIF-2 in supernatants from haem-deficient lysates would be more highly phosphorylated, and displacement of GDP by GTP would be impaired, relative to control supernatants. Increasing the GTP:GDP ratio (by providing a GTP-regenerating system) partially overcomes this, but the intitial rate of ternary complex formation is slower than in control fractions, as would be expected, since GDP displacement would still be impaired.

Results from both reticulocytes and liver eIF-2 preparations are therefore consistent with the idea that HCR inhibits eIF-2 by interfering with its interaction with a stimulatory factor, which is important in promoting exchange of bound guanine nucleotides. Experiments are now in progress to identify this factor in rat liver.

4. CONCLUSIONS

Effects of phosphorylation (by added HCR or during haem-deficiency) on eIF-2 activity are apparent in unfractionated reticulocyte supernatants and in crude eIF-2 preparations, but not in highly purified samples of the initiation factor. This suggests that measurement of kinetics of ternary complex formation in tissue extracts may be a valuable approach in assessing the importance of changes in eIF-2 activity in the regulation of peptide-chain initiation in other, less well characterised (or more technically difficult) systems; e.g., muscle [14,15] or tumour cells [16].

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