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Articles

Assembly and Breakdown of Mammalian Protein Synthesis Initiation Complexes: Regulation by Guanine Nucleotides and by Phosphorylation of Initiation Factor eIF-2†

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ABSTRACT: Eukaryotic cell polypeptide chain initiation factor eIF-2 forms ternary complexes with GTP and initiator Met-tRNA$_f$. These complexes can be destabilized in vitro by the addition of salt-washed 40S ribosomal subunits. Our evidence suggests that this destabilization is mediated by GDP generated by premature hydrolysis of the GTP molecule present in the ternary complex. With complexes formed by using a partially purified preparation of eIF-2 from Ehrlich ascites tumor cells, it is possible to reverse the 40S subunit induced inhibition by creating conditions which eliminate free GDP from the system. This reversal probably occurs due to exchange of GTP for the GDP bound to the initiation factor, in a reaction catalyzed by another factor present in the eIF-2 preparation. However, if the eIF-2 has previously been phosphorylated by the reticulocyte heme-controlled repressor, the 40S subunit induced inhibition cannot be reversed by elimination of free GDP. The instability of initiation complexes containing eIF-2, together with the impairment of guanine nucleotide exchange after phosphorylation of eIF-2 [Clemens, M. J., Pain, V. M., Wong, S.-T., & Henshaw, E. C. (1982) *Nature (London) 296*, 93-95], may be an important aspect of the mechanism of the inhibition of translation by the heme-controlled repressor.

Polypeptide chain initiation in eukaryotic cells involves a complicated series of events [reviewed in Thomas et al. (1982) and Clemens & Pain (1980)] in which initiator Met-tRNA$_f$ is first bound in a ternary complex with initiation factor eIF-2[1] and GTP; this complex then binds to a native 40S ribosomal subunit. In the presence of several other initiation factors, mRNA associates with the 40S complex, and finally a 60S subunit joins, with concomitant GTP hydrolysis (Trachsel & Staehelin, 1978; Merrick, 1979; Peterson et al., 1979), to give a functional 80S initiation complex. The factor eIF-2, as well as having a crucial role in this pathway, is subject to regulation by phosphorylation catalyzed by specific protein kinases such

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[1] Abbreviations: eIF, eukaryotic initiation factor; HCR, heme-controlled repressor; PEP, phosphoenolpyruvate; PK, pyruvate kinase (EC 2.7.1.40); HPLC, high-performance liquid chromatography; DEAE, diethylaminoethyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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as the heme-controlled repressor (HCR) of reticulocytes (Farrell et al., 1977; Clemens, 1980) and double-stranded RNA-dependent enzymes found in reticulocytes and induced by interferons in many other cell types (Sen et al., 1978; Kimchi et al., 1979; West & Baglioni, 1979; Levin et al., 1981).

There has been a problem in understanding the mechanism by which phosphorylation of eIF-2 inhibits activity of this factor, however, because highly purified eIF-2 is equally active in many in vitro assays, regardless of the extent of its modification by protein kinases (Trachsel & Staehelin, 1978; Safer et al., 1977). Furthermore, there is controversy in the literature over which precise stage in the initiation pathway is blocked when eIF-2 is phosphorylated. Several reports indicate impairment of formation of ternary complexes (de Haro & Ochoa, 1978) or 40S initiation complexes (Balkow et al., 1973a; Clemens et al., 1974; Pinphanichakarn et al., 1976; Clemens, 1976; Farrell et al., 1977; Kramer et al., 1977; de Haro & Ochoa, 1978) while others have suggested inhibition of the conversion of 40S to 80S initiation complexes (Balkow et al., 1973b; Gross, 1979). It has been suggested that eIF-2 can function stoichiometrically in all of these steps but, after its phosphorylation, may be unable to recycle and act catalytically in multiple rounds of protein synthesis (Clemens & Pain, 1980; Cherbas & London, 1976). Recently, we have examined the exchange of GTP for GDP which may remain associated with eIF-2 when the factor is released during 80S initiation complex formation and have reported that phosphorylation of partially purified eIF-2 inhibits the rate and extent of guanine nucleotide exchange on the factor (Clemens et al., 1982). This effect could explain the inhibition of eIF-2 recycling which has been proposed.

Much of the controversy over the identification of the stage in protein synthesis which is regulated by HCR and similar protein kinases would be resolved if it could be shown how GDP/GTP exchange reactions in vitro affect the ability of eIF-2 to form 40S initiation complexes. In this paper, we present evidence indicating that 40S subunit·eIF-2·GTP·Met-tRNA$_f$ complexes are unstable, apparently due to premature hydrolysis of the GTP bound to eIF-2. In a partially purified preparation of eIF-2, the GDP, which is believed to remain associated with the initiation factor, can be subsequently displaced by excess GTP. The effects of HCR on this displacement, and the role of a guanine nucleotide exchange factor which catalyzes the reaction, are described.

Materials and Methods

Materials. Radioactive compounds ([^{35}S]methionine, 700–1300 Ci/mmol, and ^{3}H-labeled guanine nucleotides, 10–15 Ci/mmol) were purchased from Amersham International Ltd., U.K. Unlabeled nucleotides were from Sigma Chemical Co., U.K. [^{35}S]Met-tRNA$_f$ was synthesized by using a preparation of *Escherichia coli* aminoacyl-tRNA synthetases and deacylated calf liver tRNA (Boehringer, U.K.) as described previously (Clemens et al., 1974).

Initiation Factors and HCR. The majority of the experiments described in this paper were performed by using partially purified initiation factor eIF-2 from Ehrlich ascites tumor cells, prepared in the laboratory of Dr. E. C. Henshaw (Cancer Center, University of Rochester, NY) as described in detail elsewhere (Clemens et al., 1982). Briefly, a ribosome salt wash was fractionated by ammonium sulfate precipitation (30–70% saturation), phosphocellulose chromatography, and DEAE-cellulose chromatography. The eIF-2 obtained was about 30–40% pure but still contained a guanine nucleotide exchange factor (see the text). Some experiments, where indicated, employed a partially purified reticulocyte ribosomal salt wash (the fraction eluting from DEAE-cellulose between 0.1 and 0.22 M KCl), and others used a highly purified preparation of rat liver eIF-2 obtained as described elsewhere (Proud et al., 1982). The latter material was 80–90% pure. Purified heme-controlled repressor (Hunt, 1979) was used in most of the experiments and was kindly provided by Dr. T. Hunt (University of Cambridge). In some earlier experiments, a crude preparation of HCR (Clemens et al., 1974) was used, as indicated in the figure legends.

Other Preparative Procedures. Salt-washed 40S ribosomal subunits were prepared from "run-off" rat liver or reticulocyte ribosomes by sucrose gradient centrifugation in a zonal rotor, as described by Schreier & Staehelin (1973). They were stored under liquid nitrogen at 30–45 A_{260} units/mL in a buffer containing 10 mM Hepes, pH 7.6, 100 mM KCl, 3 mM magnesium acetate, 1 mM dithiothreitol, and 10% (v/v) glycerol.

Rat liver GDPase preparations were obtained as described previously (Clemens et al., 1980), essentially as a byproduct of liver eIF-2 purification. The material from a DEAE-cellulose column which did not bind to a phosphocellulose column at 250 mM KCl contained the GDPase activity and was used in this study without further purification. The characterization of this GDPase as a stimulator of eIF-2 activity has been published (Clemens et al., 1980).

Assays of eIF-2 Activity. The formation of eIF-2·GTP·Met-tRNA$_f$ ternary complexes was assayed by their retention on cellulose nitrate filters (Safer et al., 1979). Incubations contained 20 mM Hepes (pH 7.6), 100 mM KCl, 2 mM magnesium acetate, 0.5 mM dithiothreitol, 0.1 mM EDTA, 0.2 mM ATP, 0.2 mM GTP, and [^{35}S]Met-tRNA$_f$ and eIF-2 in the amounts indicated in individual figures and tables. Other additions or omissions are described for the appropriate experiments in the legends. After incubation at 28 or 30 °C, 20-μL aliquots of the reaction mixture were diluted into 1 mL of cold wash buffer (20 mM triethanolamine hydrochloride, pH 7.6, 100 mM KCl, and 2 mM magnesium acetate) and immediately filtered. Each filter was then washed with 2 × 3 mL of wash buffer and dried, and the radioactivity was determined by liquid scintillation counting.

The binding of [^{35}S]Met-tRNA$_f$ to 40S ribosomal subunits was assayed under essentially the same conditions as for ternary complex formation, except that 40 mM triethanolamine hydrochloride (pH 7.6) replaced Hepes, the dithiothreitol concentration was 1 mM, and the Mg^{2+} concentration was 3 mM. Subunits were added at a concentration of 1 A_{260} unit per 100-μL incubation. After 5 min at 30 °C, the initiation complexes were fixed by addition of 1% formaldehyde (final concentration) (Henshaw, 1979) and analyzed on 5 mL of 20–50% sucrose gradients containing the same buffer, KCl, and magnesium acetate concentrations as the incubations. Centrifugation was at 189000*g* for 3.5 h in a Beckman SW50.1 rotor. Fractions were collected and passed through cellulose nitrate filters which were then washed, dried, and counted as for ternary complex assays (see above).

Binding of [^{3}H]GDP to eIF-2 was monitored by essentially the same procedures as for assays of ternary complex formation, except that GTP and Met-tRNA$_f$ were omitted. Details have been described elsewhere (Clemens et al., 1982).

Results and Discussion

Destabilization of eIF-2·Met-tRNA$_f$ Complexes by 40S Ribosomal Subunits. It is widely recognized that polypeptide chain initiation complexes consisting of 40S ribosomal subunits, eIF-2, GTP, and Met-tRNA$_f$ are higly unstable in vitro. Such

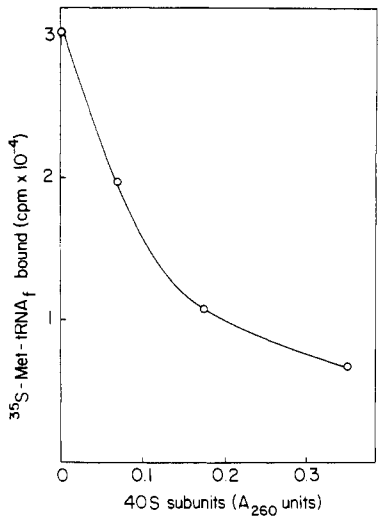

FIGURE 1: Inhibition of Met-tRNA$_f$ binding to eIF-2 by salt-washed 40S ribosomal subunits. Ehrlich cell eIF-2 (3 μg of protein) was preincubated for 5 min at 28 °C in a 100-μL reaction mixture containing 0.2 mM ATP and 0.2 mM GTP (free of GDP). The indicated amounts of 40S subunits were then added, together with [^{35}S]Met-tRNA$_f$ (4 × 10^5 cpm), and incubation was continued for a further 10 min. Complexes with eIF-2 were assayed by retention on cellulose nitrate filters and measurement of radioactivity as described under Materials and Methods.

Table I: Requirement for GTP Hydrolysis for Inhibition of Initiation Complex Formation with eIF-2 on Addition of 40S Ribosomal Subunits[a]

guanine nucleotide added	concn (mM)	[^{35}S]Met-tRNA$_f$ bound to eIF-2 (cpm × 10^{-3}) control	+40S subunits	% inhibition by 40S subunits
GTP	0.2	7.6	2.0	74
GDPNP	0.2	14.8	19.6	none

[a] The experiment was carried out under conditions similar to those described in Figure 1, except that the temperature was 30 °C, the reaction volume was 50 μL, [^{35}S]Met-tRNA$_f$ (1.05 × 10^5 cpm) was present from zero time, and the incubation time was 20 min after addition of the 40S subunits (0.07 A_{260} unit). GDPNP replaced GTP where indicated.

complexes must either be fixed with formaldehyde or glutaraldehyde (Henshaw, 1979; Clemens et al., 1974) or require the addition of other components of the protein synthesis machinery (Trachsel & Staehelin, 1979) in order to stabilize them for analysis by sucrose density gradient centrifugation or other means. This intrinsic instability of 40S complexes can be demonstrated when salt-washed 40S subunits are added to assays containing all the components required for eIF-2·GTP·Met-tRNA$_f$ ternary complex formation (Figure 1). As little as 0.1 A_{260} unit of subunits per 100-μL incubation inhibits Met-tRNA$_f$ binding to eIF-2 by 50%. We have investigated the cause of this 40S-induced inhibition, and we have established that it is not an artifact arising from competition between subunits and eIF-2 for binding to cellulose nitrate filters, since subunits previously passed through the filters do not inhibit the subsequent binding of ternary complexes. Futhermore, no ternary complexes are recoverable from the material which passes through the filters (data not shown). The possibility was then considered of involvement of guanine nucleotides in the inhibition. Replacement of GTP with a nonhydrolyzable analogue, 5′-guanylyl imidodiphosphate (GDPNP), abolishes the inhibition by 40S subunits (Table I). The inhibition can also be overcome by adding either an energy-regenerating system (phosphoenolpyruvate and pyruvate kinase) or a guanosine diphosphatase preparation previously described by us (Clemens et al., 1980). These effects are illustrated in Figure 2. When nonphosphorylated eIF-2 preparations are used, the 40S-induced dissociation of ternary complexes is completely reversed on addition of PEP + pyruvate kinase or GDPase. These results all indicate that the inhibition of Met-tRNA$_f$ binding to eIF-2 by 40S subunits is due to the presence of GDP. This nucleotide could be generated by premature hydrolysis of the GTP in a 40 S·eIF-2·GTP·Met-tRNA$_f$ complex, and there is evidence in the literature for such a reaction (Mazumder, 1975; Odom et al., 1978; Anderson et al., 1978). An alternative, but unlikely, explanation is that GDP present as a trace contaminant of the added GTP somehow binds to eIF-2 with increased affinity, in the presence of 40S subunits, displacing the bound GTP and Met-tRNA$_f$.

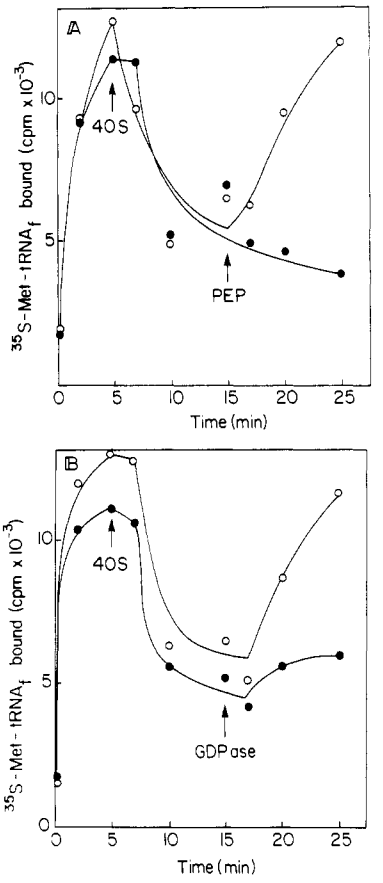

FIGURE 2: Reversal of 40S subunit induced inhibition of Met-tRNA$_f$ binding by an energy-regenerating system or by treatment with guanosine diphosphatase. Incubation conditions were similar to those in Table I except that highly purified HCR (8 units/mL) was present where indicated. The reaction volume was 200 μL, and 20-μL aliquots were removed for analysis at the times indicated. 40S subunits (0.25 A_{260} unit) were added at 5 min, and either (A) 3 mM phosphoenolpyruvate plus 8 units/mL pyruvate kinase or (B) 130 μg/mL partially purified GDPase was added at 15 min. (○) Control eIF-2; (●) HCR-treated eIF-2.

Effects of HCR-Catalyzed Phosphorylation. The above results show that agents which eliminate free GDP from assays of initiation complex formation reverse the inhibitory effect of 40S subunits in vitro. This has allowed us to study the effect of prior phosphorylation of eIF-2 on its response to conditions which should favor exchange of bound GDP for free GTP. When such assays are performed, quite dramatic differences are observed between HCR-treated and control eIF-2 (Figure 2). In contrast to the latter, eIF-2 which has been incubated

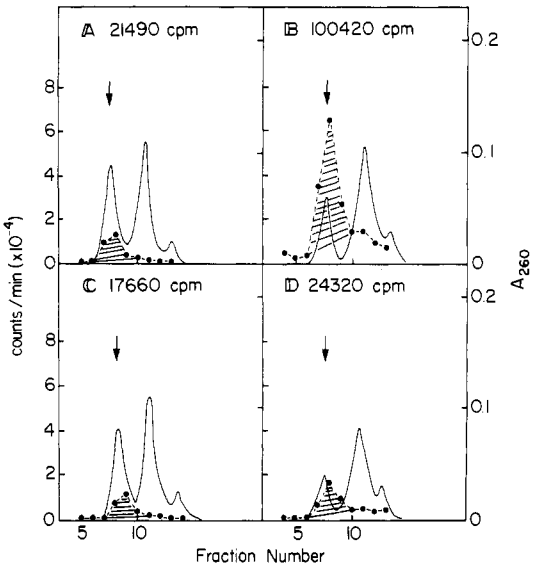

FIGURE 3: Stimulation of 40S initiation complex formation by phosphoenolpyruvate and pyruvate kinase is inhibited by HCR. A partially purified reticulocyte ribosomal salt wash was incubated with washed 40S ribosomal subunits (1 A_{260} unit) and [^{35}S]Met-tRNA$_f$ for 5 min at 28 °C, in the presence or absence of crude HCR and 3 mM phosphoenolpyruvate and 8 units/mL pyruvate kinase. Each 100-μL incubation was immediately fixed with 1% formaldehyde and subjected to centrifugation, fractionation, and analysis as described under Materials and Methods. Sedimentation was from left to right. The 40S subunit monomer peak is indicated on each gradient by an arrow; the faster sedimenting peak is 40S dimers, which do not bind Met-tRNA$_f$. (—) A_{260}; (●) ^{35}S radioactivity. The total [^{35}S]-Met-tRNA$_f$ bound to 40S subunits (hatched area) is given by the figures in each panel. (A) Control incubation; (B) +PEP/pyruvate kinase; (C) HCR treated; (D) HCR treated + PEP/pyruvate kinase.

with HCR and ATP remains inhibited by the 40S subunits when PEP + pyruvate kinase or GDPase is subsequently added. These results are consistent with the conclusion that phosphorylated eIF-2 to which GDP is bound cannot readily exchange this nucleotide for free GTP, even when the latter is in large excess in the incubation (Clemens et al., 1982; see also below).

This interpretation of how HCR-catalyzed phosphorylation inhibits eIF-2 activity may explain the observation that HCR not only affects the formation of 40S initiation complexes but also apparently destabilizes them and inhibits their conversion to 80S complexes (Balkow et al., 1973b; Gross, 1979). Such behavior could simply reflect the dynamic nature of 40S complexes (Darnbrough et al., 1973) in which a significant proportion of them may undergo GDP-induced breakdown rather than successful conversion to 80S complexes. Reutilization of the eIF-2 released during breakdown of 40S complexes may require exchange of GTP for GDP bound to the factor, in a reaction which is inhibited by phosphorylation. This is discussed in more detail later. The highly unstable nature of 40S initiation complexes is emphasized by the observation that fixation with formaldehyde or glutaraldehyde, in our hands, permits recovery of up to a 20-fold greater yield than with unfixed material.

Similar conclusions to those drawn from Figure 2 are reached when the effects of HCR on formation of 40S initiation complexes, isolated by sucrose density centrifugation, are assayed in the presence and absence of phosphoenolpyruvate and pyruvate kinase. The energy-regenerating system eliminates traces of free GDP and stimulates initiation complex formation by a factor of 4.7-fold. However, in the presence of HCR, this stimulation is almost abolished (Figure 3). As in the above experiments, this result can be explained by the inability of phosphorylated eIF-2 to exchange bound GDP for free GTP.

Guanine Nucleotides and eIF-2 Activity. We have analyzed the proportion of total guanine nucleotide present as GDP under various conditions and the fate of eIF-2·GDP complexes upon removal of free GDP by treatment with phosphoenolpyruvate plus pyruvate kinase or with GDPase. The results of thin-layer chromatography and high-performance liquid chromatography (HPLC) indicate that the presence of neither 40S subunits nor HCR enhances the hydrolysis of total free GTP (data not shown). Very little GDP or GMP was detectable under any conditions. The small amount of free GDP present due to spontaneous GTP hydrolysis was totally eliminated by PEP + pyruvate kinase. In spite of the efficiency of the latter process, however, neither PEP + pyruvate kinase nor GDPase appears able to act on GDP bound to eIF-2. Evidence for the former conclusion comes from studies of the kinetics of ternary complex formation by partially purified eIF-2 in the presence and absence of PEP + pyruvate kinase (Proud et al., 1982). The lack of effect of GDPase on eIF-2·GDP can be deduced from more direct measurements of [^{3}H]GDP binding. When the initiation factor is preincubated with the labeled nucleotide, and then sufficient GDPase is added to hydrolyze all free GDP to GMP and P_i, there is no detectable loss of radioactivity bound to eIF-2 during the subsequent incubation (data not shown). As we have shown previously (Clemens et al., 1982), guanine nucleotides remain firmly bound to eIF-2 unless they can be displaced by free nucleotides, in a process which may be catalyzed by an accessory factor (see below).

GTP and GDP can displace each other from partially purified eIF-2, to an extent which depends on their relative concentrations, and phosphorylation of the initiation factor by HCR inhibits the rate of displacement in either direction. This is clearly illustrated by the experiments described in Figures 4 and 5. Figure 4 shows the kinetics of displacement of [^{3}H]GDP from complexes with eIF-2 during subsequent incubation with either unlabeled GDP or GTP. In incubations containing partially purified eIF-2, the rate of [^{3}H]GDP displacement by unlabeled GDP is inhibited if the initiation factor is phosphorylated by HCR and ATP (Figure 4A), in confirmation of our earlier observations (Clemens et al., 1982). In contrast, the slower kinetics of [^{3}H]GDP displacement from highly purified eIF-2 by unlabeled GDP are identical whether or not the factor is exposed to HCR and ATP (Figure 4B). The ability of GTP to displace [^{3}H]GDP from eIF-2 is particularly sensitive to the state of purity of the initiation factor. Thus, when partially purified eIF-2 is used, GTP can displace [^{3}H]GDP relatively rapidly, and this process is strongly inhibited by HCR treatment (Figure 4C). However, complexes between highly purified eIF-2 and [^{3}H]GDP are not dissociated at all by the subsequent addition of 2 μM GTP (Figure 4D). Indeed, an apparent stimulation is observed, which in reality probably reflects the continued formation of a small number of additional eIF-2·[^{3}H]GDP complexes. HCR prevents this small extent of additional binding, although the significance of this effect is not certain. The converse experiment to that shown in Figure 4, to examine displacement of labeled GTP, cannot readily be performed because of the difficulty of binding sufficient radioactivity from a ligand for which the affinity of eIF-2 is comparatively low (Walton & Gill, 1976). However, an indirect approach is to measure GTP-dependent Met-tRNA$_f$ binding in ternary complex assays. When this is done, a result analogous to that in Figure 4 is obtained. In Figure 5, the kinetics of dissociation of

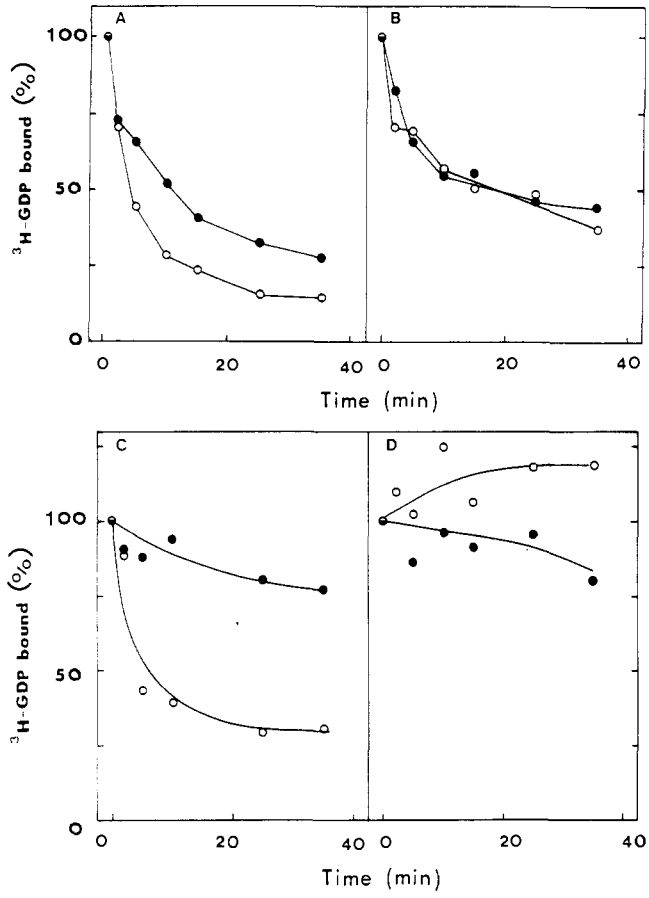

FIGURE 4: Displacement of [^{3}H]GDP from complexes with eIF-2. Partially purified eIF-2 (panels A and C) or highly purified eIF-2 (panels B and D) was preincubated with [^{3}H]GDP for 10 min and then with (●) or without (○) HCR for 5 min. Unlabeled GDP (panels A and B) or GTP (panels C and D) was then added (final concentration 2 μM), and the subsequent displacement of bound [^{3}H]GDP was followed by the retention of the remaining eIF-2·[^{3}H]GDP complexes on cellulose nitrate filters. Results are expressed as a percentage of the initial level of complexes (present immediately before addition of unlabeled nucleotide). These values were as follows: (A) control (2890 cpm), HCR treated (3670 cpm); (B) control (880 cpm), HCR treated (830 cpm); (C) control (4180 cpm), HCR treated (3140 cpm); (D) control (1220 cpm), HCR treated (1090 cpm).

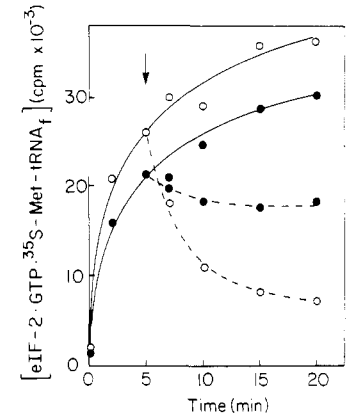

FIGURE 5: Dissociation of ternary complexes by GDP and protection from this effect by phosphorylation of eIF-2. Ternary complexes between Ehrlich cell eIF-2 (30 μg of protein/mL), GTP (0.2 mM), and [^{35}S]Met-tRNA$_f$ were formed at 30 °C and assayed as described under Materials and Methods. Twenty-microliter aliquots were removed at the times indicated. (○) Control incubations; (●) HCR (8 units/mL) present throughout. No energy-regenerating system was present. At 5 min, 0.2 mM GDP was added (arrow) to half of each incubation (- - -). The other half was further incubated without added GDP (—) to complete the time course.

ternary complexes in response to the addition of an unphysiologically high concentration of GDP (0.2 mM) show that HCR actually *protects* eIF-2·Met-tRNA$_f$ complexes, once formed, from the inhibitory effect of the diphosphate. (Note that no such protection is evident when 40S subunits are used rather than GDP itself, as in Figure 2, since in this case GDP is apparently generated in situ rather than exchanging with GTP in the complexes.) The effect of HCR on GDP-induced dissociation of ternary complexes is not due to a decrease in free GDP concentration since HCR does not eliminate this nucleotide from incubations (data not shown).

Behavior of eIF-2 in Different States of Purification. We have shown above that the state of purity of initiation factor eIF-2 influences its response to HCR treatment. This is not the result of any significant difference in the degree of phosphorylation of eIF-2 in its different states of purity. In an experiment in which partially purified and highly purified factor preparations were incubated with [γ-^{32}P]ATP and HCR, in the presence of 0.5 μM GDP, 11 250 cpm of radioactivity was incorporated into the α subunit of the cruder eIF-2, and 10 990 cpm was incorporated into an equivalent amount (by biological activity) of the purified factor. These figures were obtained by excising the appropriate bands from parallel tracks of a sodium dodecyl sulfate–polyacrylamide gel for measurement of radioactivity by scintillation counting. In addition, the displacement of GDP by excess GTP is not accompanied by any major change in the extent of ^{32}P labeling of eIF-2 (data not shown).

Both partially and highly purified eIF-2 preparations show inhibition of Met-tRNA$_f$ binding by 40S subunits (implying that this effect is an intrinsic property of eIF-2 and the subunits, in the absence of other factors), but whereas subsequent elimination of GDP by PEP/pyruvate kinase virtually abolishes the inhibition of the less pure eIF-2, this treatment still leaves an approximately 50% impairment of the more purified initiation factor (data not shown). This difference in behavior is consistent with the fact that the kinetics of displacement of GDP by GTP are much faster with the impure eIF-2 (Figure 4A,C) than with the pure eIF-2 (Figure 4B,D). This difference is due to the presence of a guanine nucleotide exchange factor, associated with the former (L. R. V. Panniers and E. C. Henshaw, unpublished results). It seems likely that GTP, generated and maintained in the presence of PEP + pyruvate kinase, can only slowly displace from highly purified eIF-2 the GDP which is produced when the initiation factor becomes abortively associated with 40S subunits. In this respect, pure eIF-2 behaves like phosphorylated eIF-2.

Conclusions

In this study, we have produced data which shed further light on the problem of whether HCR-catalyzed phosphorylation of eIF-2 inhibits initiation at a step before or after 40S initiation complex formation (or at both stages). Resolution of this problem comes from the observation that 40S complexes are unstable and must therefore undergo rapid turnover. This instability does not appear to be an artifact of the in vitro system used here (due, for example, to the absence of other initiation factors) since Darnbrough et al. (1973) have presented clear evidence for Met-tRNA$_f$ exchange within 40S complexes in the reticulocyte lysate system, in the complete absence of protein synthesis. Their observation can only be explained by continuous dissociation and reassociation of 40S complexes independently of the rate of translation. We propose that the basis of the instability of 40S complexes is the premature hydrolysis of eIF-2-bound GTP in these complexes. Such a reaction has been described previously (Mazumder,

1975; Odom et al., 1978; Anderson et al., 1978), and requirements for an eIF-(4 + 5) fraction (Odom et al., 1978) and AUG trinucleotide (Anderson et al., 1978) were implicated in those reports. [Our own results suggest, however, that AUG as well as initiation factor eIF-3 partially *stabilizes* eIF-2·Met-tRNA$_f$ complexes in the presence of 40S subunits (data not shown).] The exact nature of the protein(s) responsible for premature GTP hydrolysis is not clear. A ribosomal salt wash from *Artemia* contained such an activity which was inhibited by fusidic acid (Mazumder, 1975). Anderson et al. (1978) described a 37000-dalton acidic protein which was distinct from eIF-2, -3, or -5. Our results with highly purified eIF-2 suggest that GTPase activity is a property either of eIF-2 or salt-washed 40S subunits or of a minor contaminant of either of these preparations (which would have to be very active indeed relative to its concentration in these incubations). Whatever the mechanism, the generation of GDP in situ in these experiments has proved a useful tool in the analysis of the effects of phosphorylation on eIF-2, as well as suggesting an explanation for apparent inhibition of both formation and utilization of 40S complexes by HCR (Gross, 1979). Our conclusions concerning the turnover of 40S complexes and the point at which phosphorylation of eIF-2 may act to prevent their re-formation are summarized in Figure 6A.

Several investigators have reported the failure of phosphorylation to affect the abilities of pure eIF-2 to form ternary complexes with GTP and Met-tRNA$_f$, to bind to 40S initiation complexes, or to participate in polypeptide synthesis in fractionated cell-free systems (Trachsel & Staehelin, 1978; Safer et al., 1977; Benne et al., 1980). Furthermore, phosphorylated and nonphosphorylated eIF-2 are equally capable of restoring protein synthesis in heme-deficient reticulocyte lysates (Safer et al., 1977; Benne et al., 1980). A feature common to all these assays, however, is that the eIF-2 molecules function stoichiometrically rather than catalytically and large amounts of the factor are required for activity. This has led to the suggestion that phosphorylation only impairs the ability of eIF-2 to recycle between successive rounds of initiation rather than affecting its participation in initiation complex formation as such. Some kinetic evidence for the failure of eIF-2 recycling in heme-deficient lysates has been published (Cherbas & London, 1976) although others have claimed that phosphorylation does not inhibit this process (Benne et al., 1980).

The GTP that is required for eIF-2-dependent binding of Met-tRNA$_f$ to 40S initiation complexes has been shown to be hydrolyzed to GDP and phosphate on joining of the 60S subunit to give an 80S initiation complex (Trachsel & Staehelin, 1978; Merrick, 1979; Peterson et al., 1979). This step is brought about by eIF-5 and is accompanied by release of eIF-2 and other factors from the ribosome (Trachsel & Staehelin, 1978; Peterson et al., 1979). It is also well-known that GDP binds tightly to eIF-2, with a dissociation constant 100-fold lower than that for GTP (Walton & Gill, 1976). It therefore seems probable, although not yet proven, that the eIF-2 is released as a complex with GDP. Such a complex would be inactive until the GDP is removed because this nucleotide is a very strong inhibitor of eIF-2 (Walton & Gill, 1976). In vitro, several enzyme/substrate systems such as phosphoenolpyruvate and pyruvate kinase, nucleosidediphosphate kinase + ATP, and guanosine diphosphatase stimulate eIF-2 activity (Clemens et al., 1980; Walton & Gill, 1976). All of these share the property of eliminating GDP from the incubations. In the intact cell, GDP must be removed from its association with eIF-2 by being exchanged with GTP.

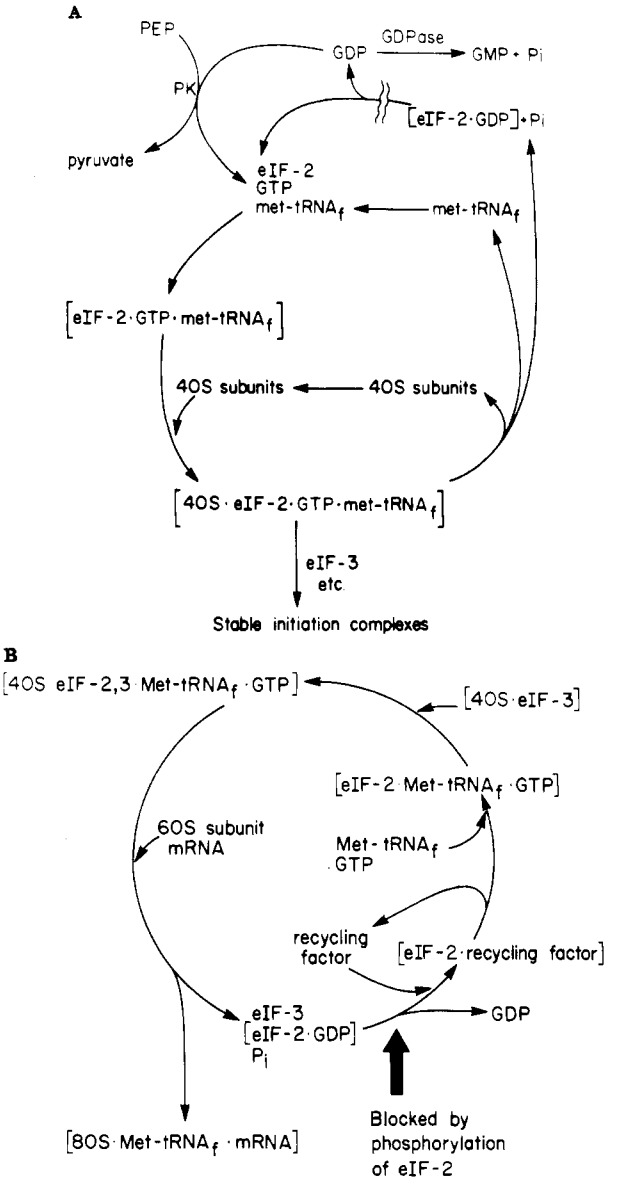

FIGURE 6: Pathways involving eIF-2 during polypeptide chain initiation. (A) Proposed mechanism for regulation of eIF-2 activity by 40S subunits and guanine nucleotides in vitro. The initiation factor is shown as capable of ternary complex formation with GTP and Met-tRNA$_f$. These ternary complexes can bind to 40S ribosomal subunits to form unstable complexes. In the presence of other components required for polypeptide chain initiation, these 40S complexes are stabilized, but in the absence of such components, it is proposed that premature GTP hydrolysis occurs with dissociation of the eIF-2 probably still bound to the resulting GDP. The latter must be exchanged for GTP before the eIF-2 can become active again; this is favored by conditions which continuously remove free GDP (PEP + pyruvate kinase or GDPase treatment). It is further suggested that phosphorylation of eIF-2 by HCR and ATP inhibits this nucleotide exchange reaction (as indicated in the figure by the parallel wavy lines), thus rendering the factor more susceptible to the 40S subunit induced inhibition and refractory to the effects of PEP + PK or GDPase. (B) The "eIF-2 cycle" and its control by phosphorylation. It is proposed that during polypeptide chain initiation in vivo eIF-2 is released when GTP hydrolysis occurs on formation of the 80 S·Met-tRNA$_f$·mRNA complex. The eIF-2 remains bound to the GDP which is generated and then exchanges this GDP for GTP in a reaction catalyzed by a recycling or exchange factor. As in panel A, phosphorylation of eIF-2 inhibits this exchange and thus impairs the rate of initiation. It is possible that the pathways shown in panel A may be superimposed upon this cycle in vivo.

It is this reaction which appears to be the target for inhibition by HCR.

The sensitivity of partially purified eIF-2 and the resistance

of the pure factor to inhibition by phosphorylation suggest the presence of one or more additional components which are involved in guanine nucleotide exchange. Such a factor would be functionally analogous to EF-T_S, which acts during polypeptide elongation (Kaziro, 1978). In collaboration with L. R. V. Panniers and E. C. Henshaw (University of Rochester Cancer Center, Rochester, NY), we have recently characterized such an activity from the ribosomal salt wash of Ehrlich ascites cells (unpublished results). This material can be separated from eIF-2 and, when added back to the purified factor, stimulates the rate of ternary complex formation and of GDP/GTP exchange and restores sensitivity to HCR-catalyzed phosphorylation (Proud et al., 1982). These observations, in combination with the fact that HCR treatment of partially purified eIF-2 inhibits both the binding and the displacement of GDP (Clemens et al., 1982; this paper, Figures 2–5), suggest that the interaction between eIF-2 and the nucleotide exchange factor is altered by phosphorylation. These observations are analogous to the reported failure of phosphorylated eIF-2 to interact with various preparations of stimulatory proteins in vitro. Thus, one or more of the activities designated as ESP (de Haro & Ochoa, 1978), anti-inhibitor (Amesz et al., 1979), SF (Ranu & London, 1979), RF (Siekierka et al., 1981), or Co-eIF-2B and Co-eIF-2C (Das et al., 1979), which behave in this way, may have a role in guanine nucleotide exchange during initiation. ESP has indeed been reported to stimulate GTP binding to eIF-2 (de Haro & Ochoa, 1979) and has recently been characterized as a guanine nucleotide exchange catalyst, the effects of which are blocked when eIF-2 becomes phosphorylated (Siekierka et al., 1982).

Our observations suggest that the exchange factor (and phosphorylation of eIF-2) has effects on both the rate and extent of GDP exchange. At saturating concentrations of GDP, HCR treatment of the Ehrlich cell eIF-2 we have used here lowers the apparent total number of binding sites by 50–65% (data not shown). The latter observation is, however, complicated by the possibility that eIF-2 may be isolated partially or completely in the form of eIF-2·GDP and eIF-2·GTP complexes, which then have to exchange their nucleotides for the labeled one in vitro. The mechanism of the "eIF-2 cycle", which is summarized in Figure 6B, predicts that the factor should exist in vivo predominantly as ribosome-bound or soluble complexes containing GTP or GDP, and with the ratio between the latter determined by the state of eIF-2 phosphorylation. We are currently testing this prediction in the reticulocyte lysate system.

Finally, we emphasize that the conclusions drawn here concerning the effects of HCR-catalyzed phosphorylation on the behavior of eIF-2 apply equally in situations where the initiation factor is modified by other protein kinases, such as those activated by double-stranded RNA in reticulocytes (Levin et al., 1981) or induced by interferon treatment of eukaryotic cells (Sen et al., 1978; Kimchi et al., 1979; West & Baglioni, 1979). Such a conclusion is valid because these enzymes all phosphorylate an identical site on the 36 000-dalton subunit of this initiation factor (Samuel, 1979; Levin et al., 1980).

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Registry No. GDP, 146-91-8; 5′-guanylyl imidodiphosphate, 34273-04-6.

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Solid-Phase Synthesis of Thymosin β_4: Chemical and Biological Characterization of the Synthetic Peptide†

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ABSTRACT: The chemical synthesis of thymosin β_4 using a solid-phase procedure has been accomplished. The synthetic product was found to be homogeneous on paper electrophoresis at pH 6.5, high-performance liquid chromatography on a reversed-phase column, and isoelectric focusing using polyacrylamide gels. The synthetic material was also shown to be identical with the natural thymosin β_4 by tryptic peptide mapping, amino acid compositional analyses, and polyacrylamide gel isoelectric focusing. Biologically, synthetic thymosin β_4 was found to be as active as the natural compound in a terminal deoxynucleotidyltransferase induction assay and in a macrophage migration inhibition assay. The proposed structure of the peptide hormone was thus confirmed by a chemical synthesis.

It is now well established that the endocrine thymus produces a family of hormonal-like peptides which controls development of the thymic-dependent lymphoid system and participates in the process of immune regulation (Low & Goldstein, 1978; Low et al., 1979a). Our previous studies demonstrated that a partially purified thymic preparation termed thymosin fraction 5 is effective in partially or fully reconstituting immune functions in thymic-deprived or immunodeprived animals (Thurman et al., 1975; Bach et al., 1971; Dauphinee et al., 1974), as well as in humans with immunodeficiency diseases (Wara et al., 1975; Wara & Ammann, 1976) and cancer (Cohen et al., 1979; Chretien et al., 1978). We have previously reported the isolation and biological properties of several components of thymosin fraction 5, including thymosin α_1 (Low & Goldstein, 1979; Low et al., 1979b), α_7 (Goldstein et al., 1981; Ahmed et al., 1979), β_3 (Pazmino et al., 1978; Hu et al., 1981), β_4 (Hu et al., 1981; Low et al., 1981; Low & Goldstein, 1982), and polypeptide β_1 (Low & Goldstein, 1979; Low et al., 1979b). The biological studies of these purified peptides indicate that they act on various pre-T and T-cell[1] subpopulations to maintain normal immunological reactivity.

The amino acid sequence has been determined for thymosin α_1 (Low & Goldstein, 1979), β_4 (Low & Goldstein, 1982), and polypeptide β_1 (Low & Goldstein, 1979). Thymosin α_1 is a polypeptide consisting of 28 amino acid residues. It is highly active in amplifying T-cell immunity (Low et al., 1979b; Ahmed et al., 1979) and is capable of modulating the expression of terminal deoxynucleotidyltransferase (TdT) (Hu et al., 1981). Thymosin β_4 consists of 43 amino acid residues with a molecular weight of 4963 and an isoelectric point of 5.1 (Low & Goldstein, 1982). The amino acid sequence of this peptide is shown in Figure 1. This peptide exhibits important activities in the regulation and differentiation of thymus-dependent lymphocytes (Pazmino et al., 1978; Low et al., 1981; Thurman et al., 1981). It induces expression of TdT activity in TdT-negative thymocytes both in vivo (Low et al., 1981) and in vitro (Pazmino et al., 1978). It also inhibits the migration of macrophages (Thurman et al., 1981). Most recently, it has been found that β_4 exerts biological effects on the hypothalamus and pituitary (Rebar et al., 1981; Hall et al., 1982).

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[1] Abbreviations: T-cell, thymus-dependent lymphocytes; TdT, terminal deoxynucleotidyltransferase; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; PAM-resin, hydroxymethylphenylacetamidomethyl resin; HF, hydrogen fluoride; HPLC, high-performance liquid chromatography; MMI, macrophage migration inhibition; LRF, luteinizing hormone-releasing factor; LH, luteinizing hormone; 2-ClZ, 2-chlorobenzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; DEAE, diethylaminoethyl; TCA, trichloroacetic acid; PPD, preservative-free tuberculin-purified protein derivative; PBL, peripheral blood lymphocyte, PEC, peritoneal exudate cell; MIF, macrophage migration inhibitory factor.

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