Proc. Natl. Acad. Sci. USA
Vol. 76, No. 5, pp. 2118–2122, May 1979
Biochemistry

In situ phosphorylation of the α subunit of eukaryotic initiation factor 2 in reticulocyte lysates inhibited by heme deficiency, double-stranded RNA, oxidized glutathione, or the heme-regulated protein kinase

(protein synthesis regulation/Met-tRNA$_f^{Met}$ binding factor/inhibition of initiation/[^{32}P]phosphoprotein profiles)

VIVIAN ERNST*, DANIEL H. LEVIN*, AND IRVING M. LONDON*†

*Department of Biology, Massachusetts Institute of Technology; and †Department of Medicine, Harvard University, and Harvard–Massachusetts Institute of Technology, Division of Health Sciences and Technology, Cambridge, Massachusetts 02139

Contributed by Irving M. London, December 26, 1979

ABSTRACT **Protein synthesis initiation in reticulocyte lysates is inhibited by heme deficiency, low levels of double-stranded RNA (dsRNA), oxidized glutathione (GSSG), or the purified kinase (HRI) that acts on the α polypeptide of eukaryotic initiation factor 2 (eIF-2α). The phosphoprotein profiles produced in lysates in response to these various conditions have been monitored directly in lysates after labeling for brief periods with pulses of [γ-^{32}P]ATP. The [^{32}P]phosphoprotein profiles were analyzed by electrophoresis in sodium dodecyl sulfate/polyacrylamide slab gels under conditions in which the HRI and eIF-2α polypeptides were clearly distinguished. All four modes of inhibition produced a rapid phosphorylation of eIF-2α compared to control lysates, which displayed little or no phosphorylation of eIF-2α. In heme-deficient lysates, phosphorylation of eIF-2α occurred rapidly both before and after the shut-off of protein synthesis; the delayed addition of hemin to these lysates resulted in a decrease in the phosphorylation of eIF-2α and the subsequent restoration of protein synthesis. These data suggest that rapid turnover of phosphate occurs at the site(s) of eIF-2α phosphorylation. In lysates inhibited by heme deficiency, GSSG, or added HRI, the phosphorylation of eIF-2α was accompanied by the rapid *in situ* phosphorylation of HRI. The inhibition of initiation induced by dsRNA was accompanied by the phosphorylation of eIF-2α and a 67,000-dalton polypeptide but not HRI. These observations *in situ* indicate that (*i*) the phosphorylation of eIF-2α is the critical event in these inhibitions of protein chain initiation, and (*ii*) the phosphorylation of HRI is associated with its activation in heme deficiency.**

The rate of protein synthesis in reticulocyte lysates incubated (*i*) in the absence of hemin, or (*ii*) in the presence of hemin (20 μM) plus double-stranded RNA (dsRNA) (20 ng/ml), or (*iii*) with hemin (20 μM) plus 0.5 mM oxidized glutathione (GSSG), proceeds at control linear rates for several minutes and then declines abruptly (shut-off) (1–6). Under all three conditions, inhibition is accompanied by the activation of cyclic AMP-independent protein kinases that phosphorylate the α subunit (38,000 daltons) of the eukaryotic initiation factor eIF-2 (for review, see ref. 7), designated eIF-2α (8). Each of the three protein kinases has been isolated and purified to various degrees (9–15); all three activities appear to be specific for eIF-2α. When added to normal lysates, each of the kinase preparations produces biphasic kinetics of inhibition, and these three inhibitions are reversed by the addition of eIF-2.

Recent studies with reticulocyte lysates have confirmed that under conditions of heme deficiency there is a correlation between the inhibition of protein synthesis and the *in situ* phosphorylation of endogenous eIF-2α (16–19). The phosphorylation of endogenous eIF-2α in those studies was analyzed by two-dimensional polyacrylamide gel electrophoresis of the whole lysate (16, 17), or of isolated fractions enriched for endogenous eIF-2 (18, 19).

In this report, we analyze the phosphorylation *in situ* of endogenous eIF-2α by a simple procedure using one-dimensional sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis. Under various incubation conditions, normal or inhibited lysates are supplemented with a single pulse of high specific activity [γ-^{32}P]ATP. In general, 5-μl aliquots are removed at 1-min intervals and immediately heated at 90°C for 5 min in a $NaDodSO_4$/2-mercaptoethanol dissociation buffer (20). The [^{32}P]phosphoprotein profile in these lysates can be directly monitored in a $NaDodSO_4$/polyacrylamide gel electrophoresis system in which it is possible to distinguish the α polypeptide (38,000 daltons) of endogenous eIF-2. With this technique it can be shown that, under all conditions, there is a relatively rapid phosphorylation of several lysate polypeptides. Of particular interest is the finding that differences in the phosphorylation of eIF-2α are clearly apparent. In addition, in heme deficiency, there is a correlation between the phosphorylation of eIF-2α and the phosphorylation of a polypeptide component that comigrates with purified heme-regulated eIF-2α kinase (HRI).

MATERIALS AND METHODS

Preparation of Reticulocyte Lysates. Reticulocytes were obtained from rabbits treated with 1-acetyl 2-phenylhydrazine (Sigma) and lysates were prepared as described (4, 21).

Phosphorylation *In Situ* in Reticulocyte Lysates and $NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Protein synthesis reaction mixtures (25 μl) containing 50% by volume of reticulocyte lysate (4); 10 mM Tris·HCl, pH 7.7; 1 mM $Mg(OAc)_2$; 76 mM KCl; 0.2 mM GTP; 60 μM leucine and the other 19 amino acids at 30 μM; 5 mM creatine phosphate and creatine kinase at 100 μg/ml were incubated at 30°C. Other additions to the reaction mixtures are described in the legends of the figures. [γ-^{32}P]ATP [25 μCi; 20–30 Ci/mmol (1 Ci = 3.7 × 10^{10} becquerels); New England Nuclear] was added at various times as indicated in the legends. Samples (5 μl) were removed at the times indicated and immediately mixed with $NaDodSO_4$ protein dissociation buffer (20) to give a final volume of 40 μl. These samples were analyzed on either 10% polyacrylamide slab gels (0.1% $NaDodSO_4$, bisacrylamide-to-acrylamide ratio 1:38.5) or 15% polyacrylamide gels (0.1%

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: eIF-2, eukaryotic initiation factor 2 [the initiation factor that forms a ternary complex with Met-tRNA$_f^{Met}$ and GTP, designation adopted by the 1976 Fogarty International Symposium on Protein Synthesis (31)]; eIF-2α, α polypeptide (38,000 daltons) of eIF-2 (8); $NaDodSO_4$, sodium dodecyl sulfate; HRI, heme-regulated eIF-2α kinase; GSSG, oxidized glutathione; dsRNA, double-stranded RNA.

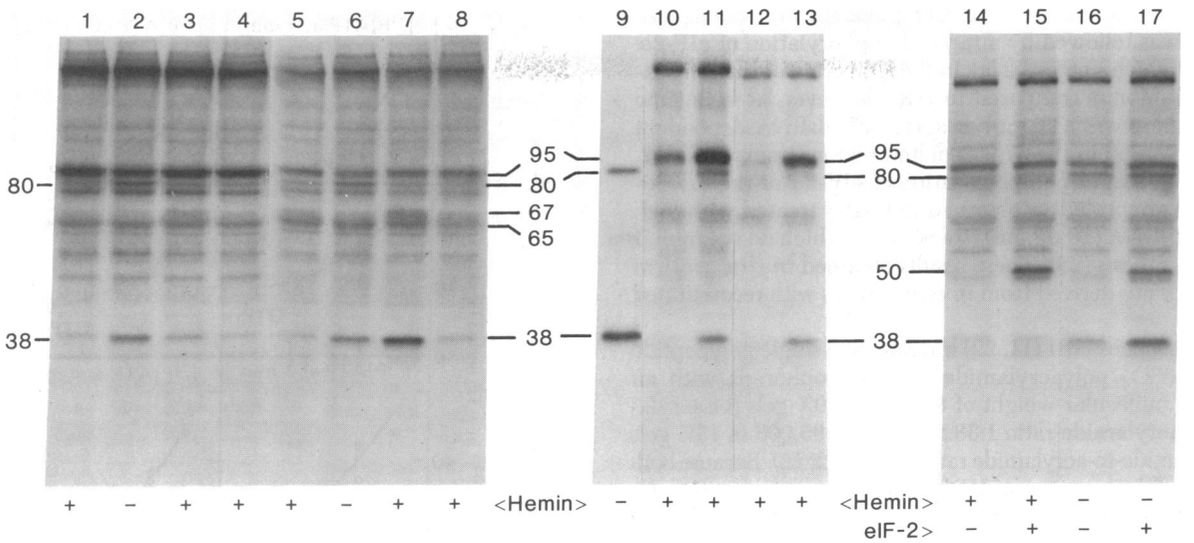

FIG. 1. Phosphorylation of eIF-2α in reticulocyte lysates inhibited by heme deficiency, dsRNA, or GSSG. Protein synthesis reaction mixtures (25 μl) were prepared and [γ-^{32}P]ATP (25 μCi) was added at the times indicated below. Samples (5 μl) of the incubations were removed 2 min after the addition of [^{32}P]ATP and analyzed by 10% $NaDodSO_4$/polyacrylamide gel electrophoresis. The figure shows the autoradiograms of selected tracks from three gels. [γ-^{32}P]ATP was added at 0 min (tracks 14–17), 1 min (tracks 1–4, 10, and 11), or 4 min (tracks 5–8, 12, and 13). Other additions to lysate incubations at 0 min were: tracks 1, 3–5, 7, 8, and 10–15, 20 μM hemin; tracks 2, 6, 16, and 17 contain no added hemin. Tracks 3 and 7, 20 μM hemin plus *P. chrysogenum* dsRNA at 20 ng/ml; tracks 4 and 8, 20 μM hemin plus poly(I·C) at 20 μg/ml; tracks 11 and 13, 20 μM hemin plus 0.5 mM GSSG; track 15, 20 μM hemin plus eIF-2 at 20 μg/ml; track 17, minus hemin plus eIF-2 at 20 μg/ml. Track 9 is a protein kinase assay (20 μl) containing 1 μg of eIF-2, 0.5 μl of purified HRI (step 7) (22), 62.5 μM [γ-^{32}P]ATP (160 mCi/mmol), 60 mM KCl, 2 mM $Mg(OAc)_2$, 10 mM Hepes at pH 7.3 and incubated at 37°C for 10 min (9). The reaction was stopped by the addition of protein dissociation buffer containing 5 μl of nonincubated lysate reaction mixture. In this gel system eIF-2 migrates as three polypeptide components with molecular weights of 52,000 (γ-subunit); 50,000 (β subunit), and 38,000 (α subunit) (8); HRI migrates as an 80,000-dalton polypeptide (23). Molecular weight markers used to calculate the molecular weights shown ($\times 10^{-3}$) were phosphorylase *a* (92,500), bovine serum albumin (67,000), ovalbumin (45,000), aldolase (40,000), and chymotrypsinogen (25,000). All samples were loaded in wide wells (>5 mm).

$NaDodSO_4$ bisacrylamide-to-acrylamide ratio 1:172) (9, 20). Electrophoresis was at 25 mA (60–120 V) for 4–5 hr. When 10% polyacrylamide gels were used electrophoresis was continued to permit the dye front and hemoglobin to migrate off the gels. Both 10% and 15% gels were stained for 1 hr at 37°C in 0.2% Coomassie brilliant blue R-250 (Bio-Rad), dissolved in 7% acetic acid/50% methanol (vol/vol). Gels were destained by repeated washing in fresh 7% acetic acid/50% methanol for a total time of 2 hr, at 37°C, transferred to 10% acetic acid, and left overnight at room temperature. Autoradiograms of stained dried gels were made with Kodak RP-5 X-Omat x-ray film, (36- to 72-hr exposure). By using these procedures, the background of gel autoradiograms was reduced to a minimum.

Protein synthesis was assayed by the incorporation of [^{14}C]leucine (140 mCi/mmol) into protein as described (4, 21). [^{14}C]Leucine (350 mCi/mmol) was obtained from New England Nuclear. Aliquots (5 μl) of reaction mixtures were assayed for [^{14}C]leucine incorporation on filter paper discs as described (21). Hemin chloride was obtained from Calbiochem. Oxidized glutathione (GSSG) and poly(I·C) were purchased from Sigma. *Penicillium chrysogenum* dsRNA and purified reticulocyte eIF-2 were gifts of Hugh D. Robertson (Rockefeller University, New York, NY) and William C. Merrick (Case Western Reserve, Cleveland, OH), respectively. Purified heme-regulated eIF-2α kinase (step 7, ref. 22) was kindly donated by Hans Trachsel (Massachusetts Institute of Technology, Cambridge, MA).

RESULTS AND DISCUSSION

Phosphorylation of eIF-2α during the inhibition of protein synthesis by heme deficiency, dsRNA, GSSG, or purified HRI

Reticulocyte lysates were incubated under several conditions: (*i*) with hemin (20 μM), (*ii*) without hemin, (*iii*) with hemin plus dsRNA (20 ng/ml), or (*iv*) with hemin plus GSSG (0.5 mM). The [^{32}P]phosphoprotein profiles in these lysates, which were incubated for brief periods after a single pulse of [γ-^{32}P]ATP, were monitored by one-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1). Lysates incubated in the absence of hemin (tracks 2, 6, 16) or with hemin plus dsRNA (tracks 3, 7) or with hemin plus GSSG (tracks 11, 13) showed an increased phosphorylation of a 38,000-dalton polypeptide compared to hemin-supplemented control lysates (tracks 1, 5, 10, 12, 14). This polypeptide comigrated with ^{32}P-labeled eIF-2α that was phosphorylated *in vitro* by HRI (track 9). The identity of the 38,000-dalton lysate polypeptide as eIF-2α is further supported by the observation that the addition of low levels of purified eIF-2 to heme-deficient lysates resulted in a considerable increase in the phosphorylation of this same polypeptide (track 17); this phosphorylation was not observed in control hemin-supplemented lysates containing the same level of added eIF-2 (track 15). It should be noted that the β subunit (50,000 daltons) of eIF-2 was significantly phosphorylated in lysates only when eIF-2 was added in excess (tracks 15, 17); this phosphorylation occurs in the presence or absence of hemin, and is not related to inhibition of protein synthesis (8, 13, 15, 24). This is in agreement with Farrell *et al.* (12), who observed that the β subunit of eIF-2 is not phosphorylated on crude ribosomes unless eIF-2 is present in excess.

The phosphorylation of eIF-2α in inhibited lysates was rapid and was observed well before the onset of inhibition, as early as 30 seconds after the start of incubation in some experiments. Rapid phosphorylation of eIF-2α was observed in heme-deficient (Fig. 1., tracks 2, 6) or GSSG-treated lysates (tracks 11, 13), whether the [γ-^{32}P]ATP pulse was added at 1 min or 4 min after the start of incubation. On the other hand, in lysates treated with dsRNA (20 ng/ml), there was no significant la-

beling of eIF-2α after a [γ-^{32}P]ATP pulse at 1 min, but a pulse at 4 min was followed by a rapid phosphorylation of eIF-2α (track 7), which suggests a lag in the activation of the dsRNA-induced inhibitor compared to HRI; however, at both time periods, there was a slight but detectable dsRNA-dependent phosphorylation in the 67,000-dalton region (tracks 3, 7) (12, 14). No significant phosphorylation of eIF-2α or the 67,000-dalton polypeptide was observed in lysates treated with high levels of dsRNA (20 μg/ml) (tracks 4, 8), which do not inhibit protein synthesis (25). These results obtained *in situ* confirm the conclusions derived from *in vitro* studies with reconstituted systems.

Homogeneous HRI (11, 22) migrates as a single polypeptide in NaDodSO$_4$/polyacrylamide gel electrophoresis with an apparent molecular weight of 80,000 in 10% gels (bisacrylamide-to-acrylamide ratio 1:38.5) (23) and 95,000 in 15% gels (bisacrylamide-to-acrylamide ratio 1:172) (12, 22). Because both crude and homogeneous HRI preparations undergo autophosphorylation (12, 22, 23, 26), this criterion has been used to determine if phosphorylation of endogenous HRI occurs *in situ* (Fig. 1). In the [^{32}P]phosphoprotein profiles of heme-deficient lysates (tracks 2, 6) and GSSG-treated lysates (tracks 11, 13), as monitored in 10% gels, there was increased phosphorylation in an 80,000-dalton polypeptide that comigrated with purified HRI (track 9). In lysates incubated with hemin alone (tracks 1, 5, 14), or with hemin plus dsRNA (tracks 3, 4, 7, 8), this band was not significantly phosphorylated. A third [^{32}P]phosphoprotein, which migrated at 95,000 daltons in 10% gels, was strongly phosphorylated in GSSG-treated lysates (tracks 11, 13). However, its identity and role are not known at present.

Analysis of ^{32}P-labeled ATP and GTP pools in lysates

In all of the experiments described here, the endogenous ATP and GTP lysate pools were labeled at selected times during incubation by a single pulse of high specific activity [γ-^{32}P]-ATP. At intervals of 1–5 min, aliquots were removed for [^{32}P]phosphoprotein analysis by NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 3). At the same time, additional samples were analyzed for radiolabeled ATP and GTP to determine the extent of label associated with the endogenous pools of ATP and GTP (Fig. 2). By using two systems of thin-layer chromatography, it was found that, within 1 min after [γ-^{32}P]ATP addition, 30–40% of the added label was present in the ATP pool and 20% of the label was present in the GTP pool, indicating a rapid labeling of GTP (Fig. 2). As expected, continued incubation was accompanied by a gradual decrease of the radioactivity in each pool, so that by 5 min 20% of the added radioactivity was in the ATP pool and 10% in the GTP pool (Fig. 2). The relative rates of decline in both pools were the same in hemin-supplemented and in heme-deficient lysates (Fig. 2). Because all incubations contained an ATP/GTP-regenerating system (creatine ~P and creatine kinase), the decrease in label reflects decreased specific radioactivities and not pool concentrations. Hence the regenerating system acts as a pulse chase due to the *in situ* generation of unlabeled ATP and GTP. Therefore, the phosphoprotein profiles obtained in the studies tend to be selective for those phosphate sites that turn over rapidly during the course of the ATP pulse. Consequently, experimental samples were generally monitored during the first 3 min after the pulse.

Kinetics of phosphorylation of eIF-2α in heme-deficient lysates

The relative rates of phosphorylation of eIF-2α were examined in heme-deficient lysates after single [γ-^{32}P]ATP pulses at 1 min or 4 min (before shut-off) or at 9 min (after shut-off) (Fig. 3). Aliquots were removed at 1-min intervals after each pulse for

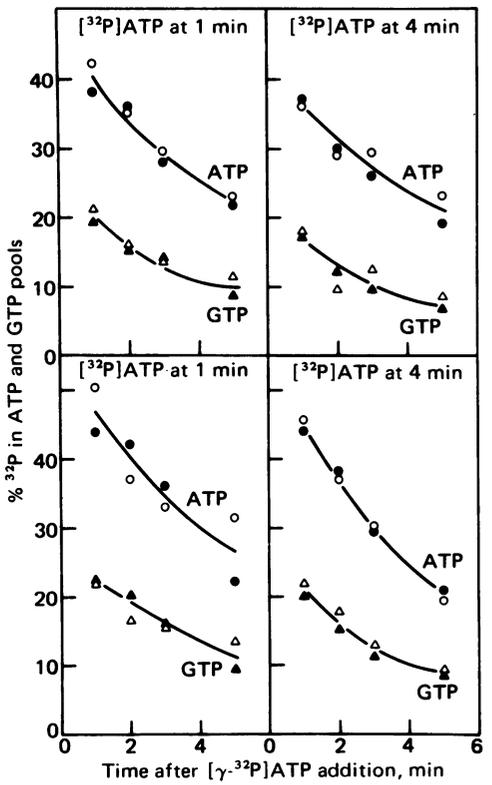

FIG. 2. Analysis of ^{32}P-labeled lysate ATP and GTP pools by thin-layer chromatography. Protein synthesis reaction mixtures were incubated in the presence (●, ▲) or absence (○, △) of 20 μM hemin as described for Fig. 1. [γ-^{32}P]ATP was added at 1 min or 4 min and aliquots (1 μl) were removed at the indicated times and immediately diluted in an equimolar mixture of carrier ATP and GTP (25 mM) in 1-ml Eppendorf plastic tubes. These diluted samples were immediately frozen and stored in liquid nitrogen. For analysis, samples were thawed in ice, mixed, and centrifuged at 4°C in an Eppendorf bench centrifuge (12,000 × *g*) to remove precipitated protein. The clarified supernatants were removed and aliquots (1 μl, approximately 60,000 cpm) were loaded onto 20 × 20 cm polyethyleneimine-cellulose thin-layer plates (Polygram, CEL 300 PEI, Macherey-Nagel, West Germany). Ascending chromatography was carried out for 24 hr at room temperature in either 0.8 M acetic acid/0.8 M LiCl (*Upper*) or in 0.75 M KH_2PO_4, pH 3.4 (*Lower*), using a 20 × 15 cm Whatman 3 MM paper wick. Under these conditions, inorganic phosphate migrates with the solvent front into the 3 MM wick (not shown). Carrier ATP and GTP were identified under a UV lamp and autoradiograms of the dried plates were made (exposure time 2–4 hr). The ^{32}P-labeled ATP and GTP spots were excised and assayed for radioactivity directly in liquid scintillation fluid (Liquifluor, New England Nuclear). The figure shows the ^{32}P radioactivity associated with the ATP and GTP markers from experiments in which [^{32}P]ATP was added at either 1 min (*Left*) or 4 min (*Right*). The data are corrected for dilution of the lysate incubation in the carrier and the radioactivity associated with each nucleotide is plotted as a percent of the total radioactivity added to the protein synthesis incubation; i.e.,

$$\text{Percent } ^{32}\text{P in ATP (or GTP)} = \frac{\text{cpm in extracted ATP (or GTP)}}{\text{cpm in [}\gamma\text{-}^{32}\text{P]ATP added}} \times 100.$$

3 min and monitored for phosphorylation of eIF-2α in the NaDodSO$_4$/polyacrylamide gel electrophoresis system in 15% gels (Fig. 3). There was a rapid phosphorylation of eIF-2α after each pulse in heme-deficient lysates, but not in hemin-supplemented lysates. The incorporation of ^{32}P into eIF-2α in heme deficiency appeared to increase for about 3 min after the 1-min pulse (tracks 2, 4, 6), the 4-min pulse (tracks 8, 10, 12), and the 9-min pulse (tracks 14, 16, 18). Within 4 min after each pulse the label in eIF-2α began to decline as the radioactivity

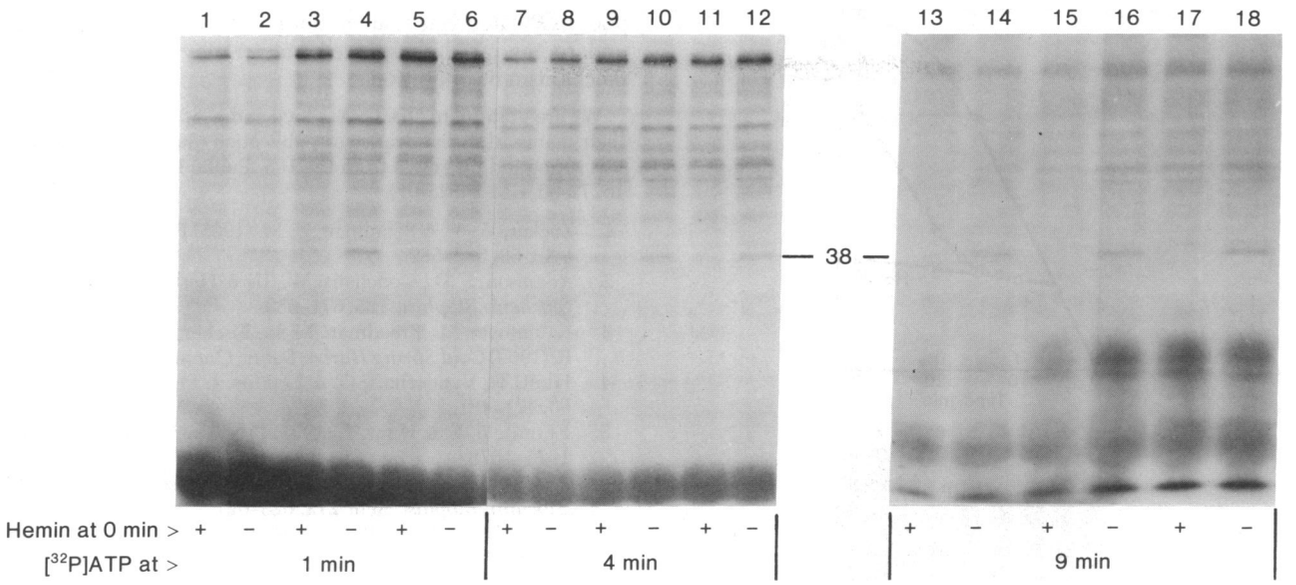

FIG. 3. Kinetics of phosphorylation of eIF-2α in hemin-supplemented and in heme-deficient lysates. Lysate protein synthesis incubations, electrophoresis in 15% $NaDodSO_4$/polyacrylamide gels, and autoradiography were as described in *Materials and Methods*. [γ-^{32}P]ATP (25 μCi) was added at 1 min (tracks 1–6); 4 min (tracks 7–12); and 9 min (tracks 13–18). Aliquots (5 μl) were removed at 1 min (tracks 1, 2, 7, 8, 13, and 14), 2 min (tracks 3, 4, 9, 10, 15, 16), and 3 min (tracks 5, 6, 11, 12, 17, 18) after the addition of [γ-^{32}P]ATP. Incubation mixtures contained 20 μM hemin where indicated.

in the endogenous nucleotide pool was correspondingly diminished (unpublished observations), which supports a mechanism involving a rapid phosphate turnover. This was also examined by adding purified HRI to hemin-supplemented lysates.

Phosphorylation of eIF-2α in hemin-supplemented lysates inhibited by purified HRI

Incubation of hemin-supplemented lysates with HRI results in an inhibition of protein synthesis similar to that observed in heme deficiency (3, 4, 11). The phosphorylation profile of endogenous eIF-2 in lysates inhibited with either partially or highly purified HRI and pulsed with [γ-^{32}P]ATP indicates that a rapid phosphorylation of eIF-2α occurs that is similar to that found in heme deficiency (Fig. 4). When [γ-^{32}P]ATP was added 1 min after the start of incubation there was a significant increase in eIF-2α phosphorylation at 3 min and 5 min in heme-deficient lysates (tracks 3, 4), and in hemin-supplemented lysates treated with either highly purified HRI (tracks 5, 6) or with crude HRI (tracks 7, 8). Similar profiles were observed when the [γ-^{32}P]ATP pulse was added at 4 min (not shown). The phosphorylation of HRI itself was masked in these 15% gels (tracks 1–8) by a 95,000-dalton polypeptide that comigrated with HRI and was extensively phosphorylated under essentially all conditions. As shown in Fig. 1, in 10% gels HRI migrated as an 80,000-dalton polypeptide. The phosphorylation of HRI in hemin-supplemented lysates inhibited by highly purified HRI was clearly detected in 10% gels (Fig. 4, tracks 9 and 10).

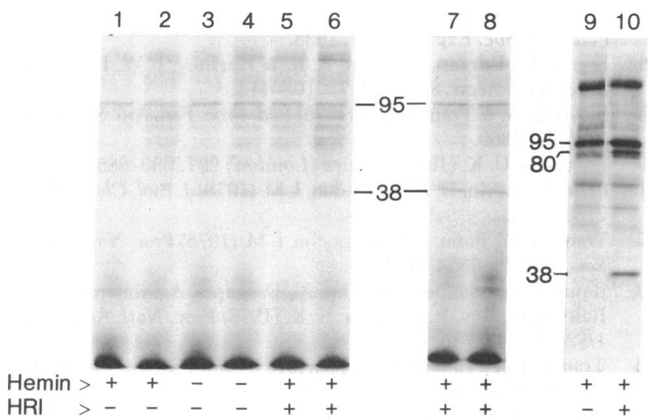

FIG. 4. Phosphorylation of eIF-2α in hemin-supplemented lysates inhibited by purified HRI. Protein synthesis reaction conditions and autoradiography were as described in the text. [γ-^{32}P]ATP (25 μCi) was added at 1 min and aliquots (5 μl) were removed at 3 min (tracks 1, 3, 5, 7) or 5 min (tracks 2, 4, 6, 8) for analysis on 15% gels (tracks 1–8). In another experiment, [γ-^{32}P]ATP was added at 0 min and samples (5 μl) were removed at 2 min for analysis on 10% gels (tracks 9, 10). The figure shows the [^{32}P]phosphoprotein profile of lysates incubated with 20 μM hemin only (tracks 1, 2, 9) or with 20 μM hemin plus 0.5 μl of highly purified HRI (step 7, ref. 23) (tracks 5, 6, 10) or with 20 μM hemin plus a crude CM-Sephadex preparation of *N*-ethylmaleimide-activated HRI (13, 14) (3.6 μg) (tracks 7, 8). Tracks 3 and 4 show profiles of control lysates incubated in the absence of hemin.

It is of interest that crude preparations of HRI and highly purified HRI produce similar [^{32}P]phosphoprotein profiles in lysates despite the fact that crude HRI contains several kinase activities, including casein kinase activities, that phosphorylated *in vitro* the β subunit of eIF-2 (8, 13, 15) and three polypeptides of eIF-3 (18, 27, 28).

Effect on the phosphorylation of eIF-2 after the delayed addition of hemin to heme-deficient lysates

The inhibition of protein synthesis in heme-deficient lysates can be reversed by the delayed addition of hemin (4, 29). In a typical heme-deficient lysate, protein synthesis declined abruptly at 5 min after the start of incubation (shut-off) (Fig. 5 *upper*). The addition of hemin at 9 min resulted in a restoration of synthesis in this lysate after a lag period of about 6–8 min. We examined the effect of this phenomenon on the phosphorylation of lysate eIF-2α by using the one-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis system. [γ-^{32}P]ATP was added at 9 min to lysate incubation mixtures that either contained hemin from the start of incubation or contained no added hemin. [γ-^{32}P]ATP and 20 μM hemin were added simultaneously at 9 min to a third, heme-deficient, lysate incubation mixture. Aliquots were removed at 1-min intervals from each incubation and monitored for eIF-2α phosphorylation (Fig. 5 *lower*). Within 1–3 min after the delayed addition

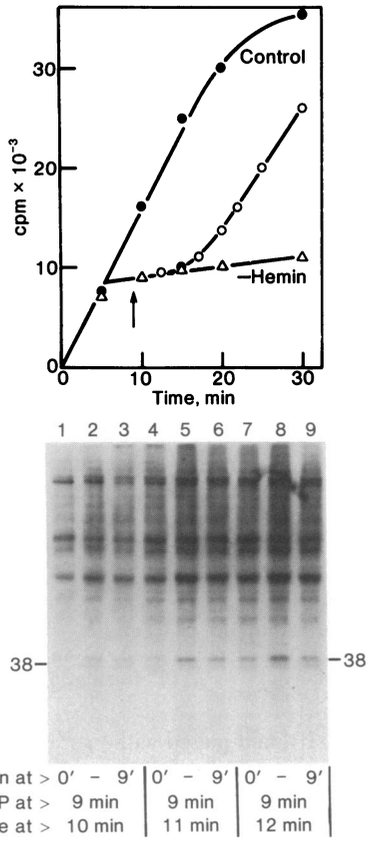

FIG. 5. Effect of the delayed addition of hemin on the phosphorylation of eIF-2 in heme-deficient lysates. Protein synthesis and the phosphorylation of the α subunit of eIF-2 were monitored in lysates incubated under the three following conditions: (*i*) with hemin added at 0 min, (*ii*) with no added hemin (heme deficiency), and (*iii*) with hemin added after the onset of inhibition (9 min). (*Upper*) Kinetics of protein synthesis as measured by the incorporation of [^{14}C]leucine into 5-μl aliquots as described (21). ●, Control lysate, 20 μM hemin at 0 min; △, no added hemin; ○, 20 μM hemin added at 9 min (arrow). (*Lower*) Phosphoprotein profile of the lysate incubations shown in *Upper*. [γ-^{32}P]ATP (25 μCi) was added at 9 min to each incubation mixture and aliquots (5 μl) were removed at the indicated times for analysis in 10% $NaDodSO_4$/polyacrylamide gels as described for Fig. 1. Tracks 1, 4, and 7: lysate incubated with hemin (added at 0 min); tracks 2, 5, and 8: lysate incubated without added hemin; tracks 3, 6, and 9: lysate with hemin added at 9 min.

of hemin to the heme-deficient lysate, the rate of labeling of endogenous eIF-2α (tracks 3, 6, 9) was significantly less than in the heme-deficient control to which [γ-^{32}P]ATP was added but not hemin (tracks 2, 5, 8); this reduced level of phosphorylation of eIF-2α precedes the reversal of protein synthesis produced by the delayed addition of hemin, which presumably acts by diminishing or blocking endogenous HRI activity. However, it should be noted that some labeling of eIF-2α persisted even after the addition of hemin. Recently, Safer *et al.* (17) reported that the restoration of protein synthesis in heme-deficient lysates by delayed hemin addition was not accompanied by a decrease in the phosphorylation of eIF-2α; their incubation mixtures contained $^{32}P_i$ and an energy-regenerating system that maintained the lysate [^{32}P]ATP and [^{32}P]GTP pools at constant specific activities (30). The differences between the two observations may in part reflect the different methods used for labeling the ATP and GTP pools. Our pulse-labeling method tends to monitor for rapid phosphate turnover in a necessarily limited time period, whereas the method used by Safer *et al.* (17) monitors for all kinase activities during the course of incubation. These combined observations suggest the possibility that more than one site of eIF-2α can be phosphorylated but that in heme deficiency only the site(s) undergoing rapid phosphate turnover may be involved in the mechanism of inhibition.

We are grateful to Drs. G. A. Floyd and J. A. Traugh for copies of their manuscripts in advance of publication (30). This investigation was supported by grants from the U.S. Public Health Service (AM-16272-06, GM-24825-01).

1. Zucker, W. V. & Schulman, H. M. (1968) *Proc. Natl. Acad. Sci. USA* **59**, 582–589.
2. Adamson, S. D., Godchaux, W., III & Herbert, E. (1968) *Arch. Biochem. Biophys.* **125**, 671–683.
3. Rabinovitz, M., Freedman, M. L., Fischer, J. M. & Maxwell, C. R. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34**, 567–578.
4. Hunt, T., Vanderhoff, G. & London, I. M. (1972) *J. Mol. Biol.* **66**, 471–481.
5. Ehrenfeld, E. & Hunt, T. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 1075–1078.
6. Kosower, N. S., Vanderhoff, G. A. & Kosower, E. M. (1972) *Biochim. Biophys. Acta* **272**, 623–627.
7. Levin, D. H., Ranu, R. S., Ernst, V., Trachsel, H. & London, I. M. (1977) in *Proceedings of Eleventh Meeting of FEBS*, eds. Clark, B. F. C., Klenow, H. & Zeuthen, J. (Pergamon, Oxford), Vol. 43, pp. 27–36.
8. Tahara, S. M., Traugh, J. A., Sharp, S. B., Lundak, T. S., Safer, B. & Merrick, W. C. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 789–793.
9. Levin, D. H., Ranu, R. S., Ernst, V. & London, I. M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3112–3116.
10. Kramer, G., Cimadivella, M. & Hardesty, B. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3078–3082.
11. Ranu, R. S. & London, I. M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4349–4353.
12. Farrell, P., Balkow, J., Hunt, T., Jackson, R. J. & Trachsel, H. (1977) *Cell* **11**, 187–200.
13. Gross, M. & Mendelewski, J. (1977) *Biochem. Biophys. Res. Commun.* **74**, 559–569.
14. Levin, D. H. & London, I. M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1121–1125.
15. Ernst, V., Levin, D. H. & London, I. M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4110–4114.
16. Farrell, P. J., Hunt, T. & Jackson, R. J. (1978) *Eur. J. Biochem.* **89**, 517–521.
17. Safer, B., Lloyd, M., Jagus, R. & Kemper, W. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 1938.
18. Benne, R., Edman, J., Traut, R. R. & Hershey, J. W. B. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 108–112.
19. Floyd, G. A. & Traugh, J. A. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 2869.
20. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
21. Ernst, V., Levin, D. H. & London, I. M. (1978) *J. Biol. Chem.* **253**, 7163–7172.
22. Trachsel, H., Ranu, R. S. & London, I. M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3654–3658.
23. Ranu, R. S., London, I. M., Das, A., Dasgupta, A., Majumder, A., Ralston, R., Roy, R. & Gupta, N. K. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 745–749.
24. Traugh, J. A., Tahara, S. M., Sharp, S. B., Safer, B. & Merrick, W. C. (1976) *Nature (London)* **263**, 163–165.
25. Hunter, T., Hunt, T. & Jackson, R. J. (1975) *J. Biol. Chem.* **250**, 409–417.
26. Gross, M. & Mendelewski, J. (1978) *Biochim. Biophys. Acta* **520**, 650–663.
27. Issinger, O.-G., Benne, R., Hershey, J. W. & Traut, R. R. (1976) *J. Biol. Chem.* **251**, 6471–6474.
28. Traugh, J. A. & Lundak, T. S. (1978) *Biochem. Biophys. Res. Commun.* **83**, 379–384.
29. Adamson, S. D., Herbert, E. & Kemp, S. F. (1969) *J. Mol. Biol.* **42**, 247–258.
30. Floyd, G. A. & Traugh, J. A. (1978) *Methods Enzymol.* **60**, 511–521.
31. Anderson, W. F., Bosch, L., Cohn, W. E., Lodish, H., Merrick, W. C., Weissbach, H., Wittman, H. G. & Wool, I. G. (1977) *FEBS Lett.* **76**, 1–10.