Regulation of protein synthesis by phosphorylation of eukaryotic initiation factor 2α in intact reticulocytes and reticulocyte lysates

(heme deficiency/eukaryotic initiation factor 2α kinase/double-stranded RNA inhibition)

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ABSTRACT   Studies in intact rabbit reticulocytes and reticulocyte lysates provide further evidence of a functional role for the phosphorylation of eukaryotic initiation factor 2α (eIF-2α) in the regulation of initiation of protein synthesis in eukaryotic cells. In intact reticulocytes treated with isonicotinic acid hydrazide to inhibit heme synthesis, the phosphorylation of eIF-2α was significantly greater than in control cells. In heme-deficient reticulocyte lysates and in lysates treated with double-stranded RNA, significant phosphorylation of eIF-2α occurred prior to the onset of inhibition of protein synthesis; a large proportion, however, of the total eIF-2α remained unphosphorylated. These findings indicate that a modest concentration of phosphorylated eIF-2α can suffice to inhibit initiation, and they suggest that one of the factors with which eIF-2 must interact may be rate limiting, especially when eIF-2α is phosphorylated.

Protein synthesis in intact reticulocyte lysates is dependent upon the presence of heme (1–5). In heme deficiency, there is a decline in the rate of protein synthesis due to the activation of a heme-regulated cAMP-independent protein kinase (HRI) which specifically phosphorylates the small subunit (38,000 daltons) of eukaryotic initiation factor 2 (eIF-2α) (6–10). The heme-regulated eIF-2α kinase has been isolated and purified in its heme-reversible form (11, 12) and has been shown to bind heme with the formation of a heme–protein complex (12). The binding of hemin to the enzyme is associated with diminished kinase activity and diminished ability to inhibit the initiation of protein synthesis (12).

The addition of double-stranded RNA (ds RNA) in low concentrations (1–20 ng/ml) to hemin-supplemented lysates results in an inhibition of protein synthesis (13, 14) with characteristics similar to those of heme deficiency. The inhibitor induced by ds RNA (dsI) is also a cAMP-independent protein kinase that phosphorylates eIF-2α (6, 15). The ds RNA-induced eIF-2α kinase has been purified from reticulocyte lysates treated with ds RNA and has been shown to exist as a polypeptide doublet of 67,000 and 68,500 daltons on sodium dodecyl sulfate/polyacrylamide gel electrophoresis; the 68,500-dalton polypeptide represents a more phosphorylated and more active form of the 67,000-dalton polypeptide (16). Although the site(s) of phosphorylation of eIF-2α by HRI and dsI are very similar or identical (17–19), HRI and dsI are different and can be distinguished immunologically (20).

The mechanism by which the phosphorylation of eIF-2α results in inhibition of initiation is not yet fully elucidated. It has been shown that the phosphorylation of eIF-2α diminishes the interaction of eIF-2 with other initiation factors: with a factor variously called eIF-2-stimulating protein (ESP) (21), stabilization factor (SF) (22), or Co-eIF-2C (23) in the formation of the [Met-tRNA_f·eIF-2·GTP] ternary complex and with another fac-

tor called ternary complex dissociation factor (TDF) or Co-eIF-2B in the binding of the [Met-tRNA_f·eIF-2·GTP] complex to the 40S ribosomal subunit (24). In addition to eIF-2, there are factors that can prevent or reverse the inhibition of initiation by HRI or dsI. These reversing, rescue, or restoring factors (RF) are proteins or protein complexes found in the postribosomal supernatant, in the ribosomal salt wash, or in both (25–27). Recent studies indicate that the RF of Amesz et al. (27) and ESP are closely related: they form complexes with eIF-2 and have very similar polypeptide patterns on NaDodSO4/polyacrylamide gel electrophoresis (28). The mechanism by which RF prevents or reverses inhibition by HRI or dsI remains to be delineated.

Evidence on the role of phosphorylation of eIF-2α in the regulation of the initiation of protein synthesis was provided by studies that correlated the phosphorylation of endogenous eIF-2α in situ with the inhibition of protein synthesis in heme-deficient or ds RNA-treated reticulocyte lysates. When the addition of hemin to a heme-deficient lysate resulted in resumption of protein synthesis, there was concomitant dephosphorylation of eIF-2α (29).

The studies that we report in this paper were designed to determine (i) whether phosphorylation of eIF-2α occurs under quasi-physiologic conditions of heme deficiency in intact reticulocytes and in reticulocyte lysates and (ii) whether the kinetics of phosphorylation are consistent with the abrupt decline in the rate of protein synthesis observed in heme-deficient lysates—i.e., within 5 min after the start of incubation.

We found that in intact reticulocytes in which heme synthesis was inhibited by isonicotinic acid hydrazide (INH; isoniazid), the phosphorylation of eIF-2α was significantly greater than in control cells, a finding similar to that in our earlier preliminary study (30). In heme-deficient reticulocyte lysates, the phosphorylation of eIF-2α was rapid and reached a maximal level before the onset of inhibition of protein synthesis; in hemin-supplemented lysates, little or no phosphorylation of eIF-2α was observed.

These findings provide further evidence of a functional role for the phosphorylation of eIF-2α in the regulation of initiation of protein synthesis in eukaryotic cells.

MATERIALS AND METHODS

Protein Synthesis and *in Situ* Phosphorylation in Intact Reticulocytes and Reticulocyte Lysates. Reticulocytes were obtained from rabbits treated with 1-acetyl-2-phenylhydrazine

Abbreviations: eIF, eukaryotic initiation factor; HRI, heme-regulated cAMP-independent protein kinase; ds RNA, double-stranded RNA; dsI, inhibitor induced by ds RNA; RF, restoring factor; ESP, eIF-2-stimulating protein; INH, isonicotinic acid hydrazide (isoniazid); IEF, isoelectric focusing.
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and lysates were prepared as described (3).

(*i*) *Intact reticulocytes.* Intact reticulocytes were washed three times with cold saline and then resuspended in 1 vol of saline. Nutritional medium for reticulocytes was prepared according to Bitte and Kabat (31).

The cell suspension was gently rotated in a water bath at 37°C in air. Protein synthesis was assayed by the incorporation of [^{3}H]leucine (99.8 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) into protein. The intact reticulocytes were labeled with ^{32}P$_i$ by adding 1.2 mCi of carrier-free [^{32}P]orthophosphoric acid to 1 ml of incubation mixture. [^{3}H]Leucine (45.71 Ci/mmol) and carrier-free [^{32}P]orthophosphoric acid (50 mCi/ml) were obtained from New England Nuclear. Aliquots (50 μl) of the incubation mixture were applied directly to 2.3-cm filter paper discs (Whatman 3 MM), the filters were treated as previously described (32), and the amount of [^{3}H]leucine and [^{32}P]phosphate incorporated into protein was determined with an efficiency of 10% for ^{3}H and 70% for ^{32}P.

(*ii*) *Reticulocyte lysates.* Protein synthesis reaction mixtures (50 μl) were as described (32). [γ-^{32}P]ATP was generated at constant specific activity by the addition of 1 mM fructose 1,6-bisphosphate, 0.1 mM NAD$^+$, and 100 μCi of carrier-free [^{32}P]orthophosphoric acid (50 mCi/ml; New England Nuclear) (33).

Preparation of Samples for Two-Dimensional Gel Electrophoresis. (*i*) *Intact reticulocytes.* One volume (3–5 ml) of incubation mixture was transferred in conical graduated tubes containing 3 vol of ice-cold saline, and the cells were centrifuged at 800 × g at 4°C for 10 min. Aliquots of packed cells (15 μl) were removed from the pellet and immediately lysed in 55 μl of isoelectric focusing (IEF) buffer (lysis buffer) (34). Eight milligrams of solid urea (Schwarz/Mann, ultrapure) was then added to bring the final concentration of urea in the total mixture to 9.5 M. The samples were allowed to stand for 20 min at room temperature, and 20 μl of mixture containing 4.5 μl of cells was loaded on each IEF gel.

The remaining labeled packed cells (150–250 μl) were lysed by adding 1 vol of cold water, and the hemolysate was stirred by Vortex mixing and was centrifuged at 10,000 × g at 4°C for 10 min, and the supernatant (100–150 μl) was obtained. Aliquots (50 μl) were then mixed with 100 μl of lysis buffer supplemented with urea to bring the final urea concentration to 9.5 M. After standing for 20 min at room temperature, 55 μl of the mixture, containing 9 μl of lysate, was loaded on first-dimension IEF gels. The remaining lysate was used for preparation of the pH 5 fraction as described below.

(*ii*) *Reticulocyte lysates.* Lysis buffer (100 μl) and 27 mg of urea were added to 50 μl of the protein synthesis reaction mixture described above. The samples (55 μl), containing 9 μl of lysate, were loaded on the first-dimension IEF gels.

(*iii*) *pH 5 fractions prepared from lysates.* Reticulocyte lysates (50 μl) prepared from labeled intact cells were diluted with 12 vol of cold water and immediately precipitated by adding 1.7 μl of 1 M acetic acid. After 15 min on ice, samples were centrifuged at 12,000 × g at 4°C for 10 min in a Brinkmann model 3200 centrifuge. The supernates were removed by aspiration and 50 μl of IEF buffer was added to the pH 5 precipitates. After 30 min at room temperature, the samples were stirred and clarified by a brief centrifugation in a Brinkmann model 3200 centrifuge; 45 μl of supernate was then loaded on the IEF gels.

The pH 5 fraction from the protein-synthesizing reticulocyte lysate was prepared by adding 0.8 ml of cold water to 100 μl of reaction mixture, and then 1.7 μl of 1 M acetic acid was immediately added to the solution. The pH 5 fraction was processed as described above.

Two-dimensional gel electrophoresis was performed according to O'Farrell (34). Ampholines (pH 3.5–10 and pH 5–7) were purchased from LKB. The final pH gradient in the IEF gels was linear between pH 4.5 and 6.6. In several experiments only the acidic sections (pH 4.5–5.8 or pH 4.9–5.9) of the first-dimension gels, which contained [^{32}P]eIF-2α, were run in the second dimension. Gels were stained with Coomassie brilliant blue and were dried, and autoradiograms were made, using Kodak XR-50 medical x-ray film (2- to 4-day exposure for lysate experiments, 7- to 14-day exposure for experiments with intact cells).

The initiation factor eIF-2 was prepared from rabbit reticulocytes (32).

Hemin chloride was obtained from Calbiochem. INH was purchased from Sigma. *Penicillium chrysogenum* ds RNA was the generous gift of Hugh D. Robertson (Rockefeller University, New York, NY). Purified heme-regulated eIF-2α kinase (step 7) (11) was kindly provided by Hans Trachsel.

RESULTS AND DISCUSSION

Location of eIF-2α in a Two-Dimensional Gel Electrophoresis System. The isoelectric point of the small α subunit (38,000 daltons) of the initiation factor eIF-2 was found to be pH 5.4 for the phosphorylated form. The position of eIF-2α was determined by adding eIF-2 phosphorylated in the α subunit to a lysate or to the pH 5 fraction previously mixed with denaturing sample buffer used for two-dimensional gels.

The technique of pH 5 precipitation permits the detection of more nonhemoglobin proteins and increases the sensitivity of detection of eIF-2α. The increased phosphorylation of eIF-2α causes a shift in the position of migration in the isoelectric focusing dimension and yields a slightly lower but clearly detectable isoelectric point (pH 5.4) as compared to unphosphorylated eIF-2α. On stained gels there are two spots, which correspond to unphosphorylated and phosphorylated forms of eIF-2α. These two spots can be observed after addition of labeled [^{32}P]eIF-2α to an unlabeled lysate or pH 5 fraction. They are also readily seen when the phosphorylation of endogenous eIF-2α in the lysate is accompanied by a significant shift of the stained spot to the position corresponding to phosphorylated eIF-2α (Fig. 1).

Kinetics of *in Situ* Phosphorylation of eIF-2α in Reticulocyte Lysates Inhibited by Heme Deficiency or by ds RNA. Protein-synthesizing reticulocyte lysates (50 μl) were incubated in the presence and absence of hemin (20 μM), and the phosphorylation of eIF-2α was carried out by labeling lysate proteins with [γ-^{32}P]ATP generated at constant specific activity (33). In the autoradiograms the intensity of the two-dimensional gel spots corresponding to the phosphorylated form of eIF-2α was markedly increased in heme-deficient lysates compared to hemin-supplemented lysates (Fig. 2). This finding is in agreement with results obtained by Farrell et al. (35), who used an iodo[1-^{14}C]acetate labeling procedure and two-dimensional gel analysis, and with results obtained in our laboratory by Ernst et al. (29), who used a brief pulse of high specific activity [γ-^{32}P]ATP followed by analysis in one-dimensional NaDodSO$_4$/polyacrylamide gel electrophoresis.

In the heme-deficient lysate the rate of phosphorylation of eIF-2α is very rapid. At 2 min of incubation, a significant increase of intensity of [^{32}P]eIF-2α was observed, and maximal intensity was reached at 4 min (Fig. 2), about 1 min prior to the onset of inhibition of protein synthesis (Fig. 3). No further increase in phosphorylation seemed to occur after 4 min, as judged by the autoradiogram of phosphorylated eIF-2α at 10 and 15 min in heme-deficient lysates (Figs. 2 and 4B). On the stained gel, the spot corresponding to phosphorylated eIF-2α is barely visible at 2 min of incubation, a finding consistent with

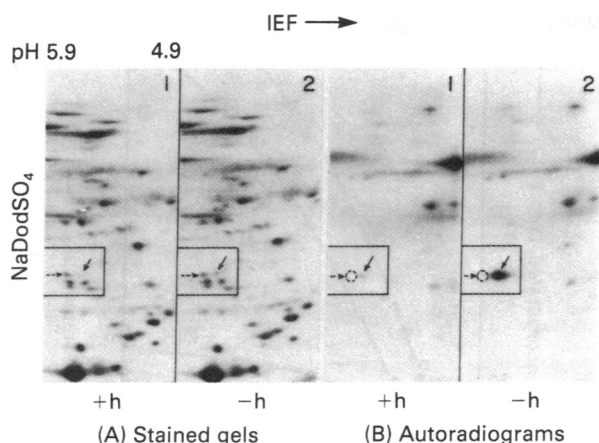Biochemistry: Leroux and London

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FIG. 1. Phosphorylation of eIF-2α in hemin-supplemented (+h) and heme-deficient (−h) lysates. Lysate protein synthesis incubation (50 μl) and labeling of lysate proteins with $^{32}P_i$ (100 μCi), two-dimensional gel electrophoresis, and autoradiography were as described in *Materials and Methods*. Aliquots of lysate stored in liquid nitrogen were thawed on ice. For hemin-supplemented lysates, 40 μM hemin was added during thawing. The final concentration of hemin in the lysate protein synthesis incubation was 20 μM. At 4 min of incubation the reaction was stopped by adding 100 μl of IEF buffer and 27 mg of urea to the reaction mixture. The 55 μl of mixture, containing 9 μl of lysate, was loaded on the IEF gels. Only the sections of IEF gels between pH 5.9 and 4.9 were run in the second dimension. The figure shows stained gels (*A*) and autoradiograms (*B*) of proteins in hemin-supplemented (1) and heme-deficient (2) lysates. Only the areas of the two-dimensional gels contained within the rectangles are shown in Figs. 2 and 4. In this and subsequent figures, the horizontal dashed arrows indicate the unphosphorylated form of eIF-2α, and solid diagonal arrows indicate the phosphorylated form. Dashed circles outline the position of the unphosphorylated form of eIF-2α on the autoradiograms.

the observed relatively low level of phosphorylation of eIF-2α. However, at 4 min of incubation, when the maximal level of phosphorylation of eIF-2α is observed, there is a clear increase in the size of the spot that corresponds to phosphorylated eIF-2α (Fig. 2). It should be noted, however, that 60–70% of the eIF-2α is in unphosphorylated form in the heme-deficient lysates (Fig. 2), an observation similar to that of Farrell *et al.* (35).

In hemin-supplemented lysates undergoing maximal protein synthesis, there is a low level of eIF-2α phosphorylation, which is probably due to some basal activity of eIF-2α kinase. However, the phosphorylation of eIF-2α in heme-deficient lysates is significantly greater than in hemin-supplemented lysates as

FIG. 3. Inhibition of protein synthesis in reticulocyte lysates by heme deficiency or by ds RNA. Protein synthesis reaction mixtures (50 μl) were incubated at 30°C with 20 μM hemin (●) and without hemin (○) and, in a separate experiment, with 20 μM hemin plus *P. chrysogenum* ds RNA at 20 ng/ml (△). At intervals, aliquots (5 μl) were removed and assayed for protein synthesis.

judged by the autoradiograms and the stained two-dimensional gels (Fig. 2).

The kinetics of phosphorylation of eIF-2α were found to be the same in the pH 5 fractionated lysates (Fig. 4) and in unfractionated lysates. In the heme-deficient lysate, there is rapid and extensive phosphorylation of eIF-2α, which reaches a maximum at 4 min of incubation; no further increase in phosphorylation at 15 min of incubation is observed. As noted above, a considerable proportion of eIF-2α remains unphosphorylated. In lysates inhibited by ds RNA (20 ng/ml) the rate of phosphorylation is slower. At 2 min of incubation there is no increase in phosphorylation of eIF-2α as compared to control hemin-supplemented lysates (data not shown). A modest increase in phosphorylation of eIF-2α is observed at 4 min of incubation, and

(A) Stained gels

(B) Autoradiograms

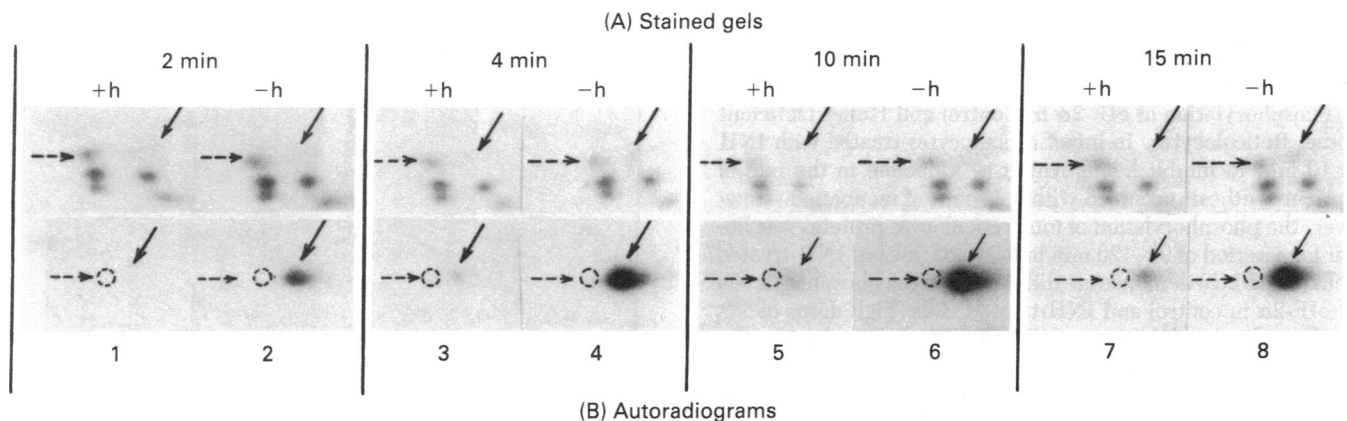FIG. 2. Kinetics of phosphorylation of eIF-2α in hemin-supplemented (+h) and heme-deficient (−h) complete lysate. Stained gels (*A*) and autoradiograms (*B*) of segments of two-dimensional gels at 2 min (segments 1 and 2), 4 min (segments 3 and 4), 10 min (segments 5 and 6), and 15 min (segments 7 and 8) of lysates prepared as in Fig. 1.
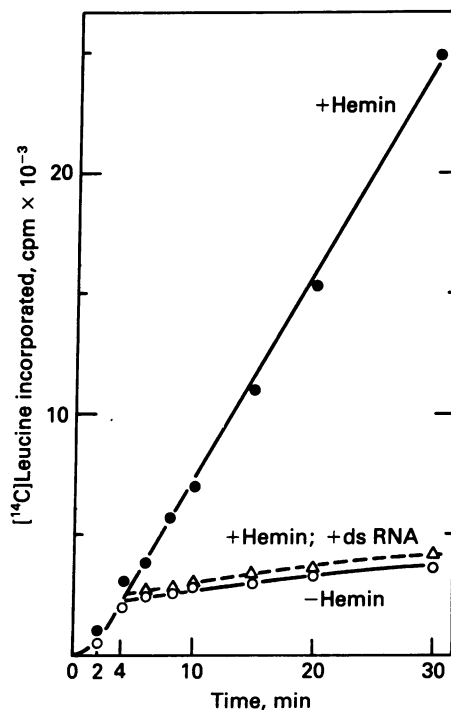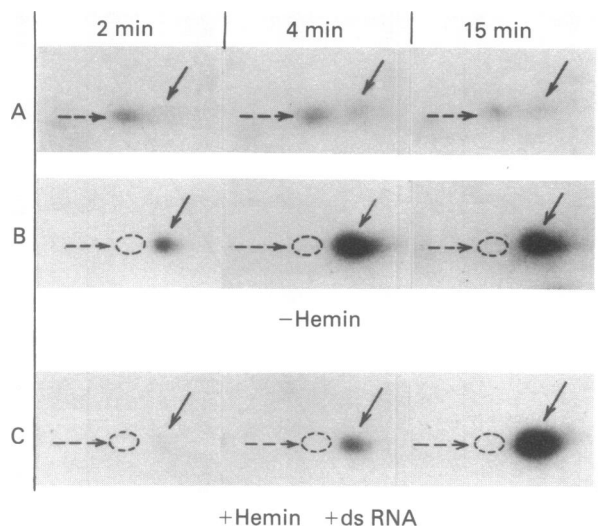
FIG. 4. Kinetics of phosphorylation of eIF-2α in lysates inhibited by heme deficiency or by ds RNA. Lysates were incubated at 30°C; (*i*) with 20 μM hemin, (*ii*) without hemin, (*iii*) with 20 μM hemin plus *P. chrysogenum* ds RNA (20 ng/ml) as described in Fig. 3 but under conditions that permit labeling of lysate proteins with $^{32}P_i$. Lysate protein synthesis incubation (100 μl), labeling of lysate proteins with $^{32}P_i$ (200 μCi), two-dimensional gel electrophoresis, and autoradiography were as described in *Materials and Methods*. The figure shows the segments of two-dimensional gels of pH 5 fractions prepared from lysates inhibited by heme deficiency (*A*, stained gel; *B*, autoradiogram) or by *P. chrysogenum* ds RNA at 20 ng/ml (*C*, autoradiogram). At 2, 4, and 15 min of incubation the reaction was stopped by the dilution of the protein synthesis mixture with 8 vol of ice-cold water followed by immediate addition of 1.7 μl of 1 M acetic acid. The pH 5 fractions were processed as described in the text.

at 15 min there is very strong phosphorylation of eIF-2α (Fig. 4*C*), which exceeds that observed in heme-deficiency. The marked phosphorylation of eIF-2α seen at 15 min of incubation in ds RNA-treated lysates is accompanied on the stained gel by a greater shift from the unphosphorylated to the phosphorylated form than is observed at 15 min incubation in heme-deficient lysates (data not shown).

In pH 5 fractions prepared from hemin-supplemented lysates, only a low level of phosphorylation was found at any time of incubation, and no spot corresponding to phosphorylated eIF-2α was detected on stained gels (data not shown).

Differences in the kinetics of *in situ* phosphorylation of eIF-2α in lysates inhibited by heme-deficiency or by addition of ds RNA to hemin-supplemented lysates are consistent with the findings that the heme-regulated and ds RNA-dependent eIF-2α kinases are distinct even though they appear to phosphorylate the same or very similar site(s) on eIF-2α.

Phosphorylation of eIF-2α in Control and Heme-Deficient Intact Reticulocytes. In intact reticulocytes treated with INH at 10 mM to inhibit heme synthesis, a decline in the rate of protein synthesis occurred within 15 min of incubation. However, the phosphorylation of total reticulocyte proteins was linear for a period of 90–120 min in both control and INH-treated cells (Fig. 5). To demonstrate differences in the phosphorylation of eIF-2α in control and INH-treated cells, high doses of $^{32}P_i$ were used to label the intact cells (1 mCi/ml of incubation mixture containing 0.1 ml of cells). Increased phosphorylation of eIF-2α was observed at 60 min of incubation in INH-treated cells as compared to control cells (data not shown), but the differences in phosphorylation of eIF-2α were more pronounced when pH 5 fractions prepared from control and INH-treated cells were used for analysis (Fig. 6). At earlier times of incu-
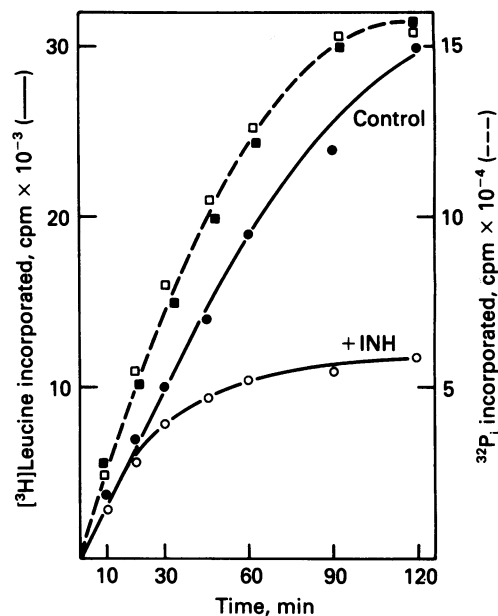

FIG. 5. Inhibition of protein synthesis in intact reticulocytes by heme deficiency. Intact reticulocytes were incubated in nutritional medium at 37°C. After preincubation for 5 min, 10 mM INH in saline was added to one incubation mixture of cells. The identical volume of saline was added at the same time to control cells, and incubation of both was continued for 120 min. At indicated times, aliquots (50 μl) were removed and assayed for protein synthesis in control (●) and INH-treated (○) cells. The phosphorylation of proteins was measured by the incorporation of $^{32}P_i$ into total proteins in control (■) and INH-treated (□) cells. The values represent cpm per 50 μl of incubation mixture.

bation (10 and 30 min), the difference in phosphorylation of eIF-2α in control and INH-treated cells was observed only in samples concentrated by pH 5 precipitation of lysates. At 10 min of incubation, when the rates of protein synthesis were similar in control and INH-treated cells (Fig. 5), there was a slight increase in phosphorylation of eIF-2α in INH-treated cells as compared to control cells (data not shown). At 30 min of incubation, there was a marked increase in phosphorylation of eIF-2α in INH-treated cells that persisted at 60 min of incubation (Fig. 6). It should be noted that control cells, actively engaged in protein synthesis, had a low level of phosphorylated eIF-2α (Fig. 5).

The differences in phosphorylation of eIF-2α in control and heme-deficient lysates are significantly greater than the differences observed in control and INH-treated cells. The more
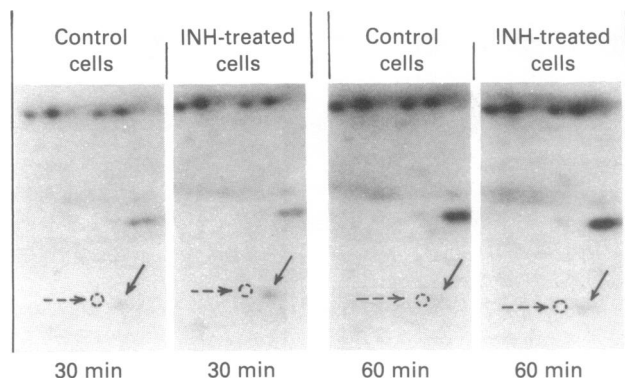

FIG. 6. Phosphorylation of eIF-2α in control and INH-treated intact reticulocytes. Autoradiograms of segments of two-dimensional gels of the pH 5 fractions prepared from control and INH-treated cells after 30 and 60 min of incubation performed as described for Fig. 5.

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striking effects in lysates may be explained in terms of the degree of heme deficiency achieved in lysates and in intact cells. In the lysate, mitochondria have been removed so that heme synthesis cannot occur; accordingly, heme deficiency is marked and there is clear dependence on exogenous hemin. In intact reticulocytes with functioning mitochondria, endogenous heme synthesis may be inhibited but not eliminated by INH, so that less marked heme deficiency would be expected.

In heme-deficient lysates, the equilibrium between phosphorylation of eIF-2α by the heme-regulated kinase (HRI) and dephosphorylation by phosphoprotein phosphatase activity is shifted, with a net increase in phosphorylation. On delayed addition of hemin, when HRI activity is diminished or blocked, a decrease in phosphorylation of eIF-2α and concomitant restoration of protein synthesis occur. In our previous studies (29), pulse labeling with [γ-^{32}P]ATP and analysis by one-dimensional NaDodSO$_4$/polyacrylamide gel electrophoresis were used. In the present study, using a system generating [^{32}P]ATP of constant specific activity and analysis by two-dimensional gel electrophoresis, we have obtained similar results (data not shown).

The observation that the inhibition of protein synthesis is associated with phosphorylation of as little as 30% of the total eIF-2α raises several questions. Does the relatively large amount of unphosphorylated eIF-2α reflect inhomogeneous pools of eIF-2? Is the ratio of phosphorylated to unphosphorylated eIF-2α different in the postribosomal supernatant fraction from the ratio in eIF-2 bound to the ribosomes? Is the unphosphorylated eIF-2 that is observed in a heme-deficient inhibited lysate functionally unavailable for participation in initiation? In intact control reticulocytes there is a low basal level of phosphorylation of eIF-2α (about 10%); in INH-treated reticulocytes, in which heme synthesis is inhibited with concomitant inhibition of protein synthesis, there is increased phosphorylation of eIF-2α (30–40%), but still a large portion of the total eIF-2α remains unphosphorylated. The narrow range between phosphorylated eIF-2α observed in control reticulocytes and in INH-treated heme-deficient reticulocytes suggests that a relatively modest concentration of phosphorylated eIF-2α can suffice to inhibit the cycle of initiation. This suggestion appears reasonable if one of the factors with which eIF-2 must interact is rate limiting and if this rate limitation is most marked when eIF-2α is phosphorylated. Because RF forms a complex with eIF-2, it is a likely candidate to serve as a rate-limiting factor. Our current hypothesis is that phosphorylation of eIF-2α renders RF rate limiting in the formation of the [Met·tRNA$_f$·eIF-2·GTP] and [40S·Met·tRNA$_f$] complexes and, accordingly, in the recycling of eIF-2 (36).

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