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Effects of skeletal muscle protein phosphatase inhibitor-2 on protein synthesis and protein phosphorylation in rabbit reticulocyte lysates*

(protein chain initiation/translational control/eukaryotic initiation factor 2α kinase/phosphorylation of eukaryotic initiation factor 2α)

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ABSTRACT **Reticulocyte lysates contain two major classes of protein phosphatase activities, designated type 1 and type 2. These designations are based on criteria derived from the analyses of protein phosphatase species in other tissues. The criteria include (*i*) chromatographic elution profiles on DEAE-cellulose; (*ii*) specificity of lysate phosphatases toward [^{32}P]phosphorylase *a* and [^{32}P]phosphorylase kinase; (*iii*) sensitivity of lysate phosphatases to Mg^{2+} ATP; and (*iv*) sensitivity to the heat-stable protein phosphatase inhibitor-2. The lysate phosphatase species are similar to those described in rabbit skeletal muscle and rabbit liver. Reticulocyte protein phosphatase type 1, but not type 2, is inhibited by heat-stable protein phosphatase inhibitor-1 and -2 which have been characterized from rabbit skeletal muscle. We have initiated a study on the function and specificity of lysate protein phosphatase activities involved in the regulation of protein synthesis by examining the effects of protein phosphatase inhibitor-2 on reticulocyte protein synthesis and protein phosphorylation. Our findings are as follows. (*a*) Protein phosphatase inhibitor-2 inhibits protein chain initiation in hemin-supplemented lysates. (*b*) Inhibition is characterized by biphasic kinetics and is reversed by the delayed addition of purified reticulocyte eukaryotic initiation factor 2 (eIF-2). (*c*) Inhibition of protein synthesis by inhibitor-2 is accompanied by the phosphorylation of the α-subunit (38,000 daltons) of eIF-2 (eIF-2α) and of two heat-stable polypeptides of 29,000 and 44,000 daltons. (*d*) The 29,000-dalton component is phosphorylated in lysates under conditions of protein synthesis and appears to be inhibitor-2, but the physiological significance of this modification of inhibitor-2 is not clear. (*e*) Inhibitor-2 has no effect on the activation *in vitro* of isolated heme-regulated or double-stranded RNA-dependent eIF-2α kinases. We propose that the inhibition of protein synthesis in hemin-supplemented lysates by added inhibitor-2 is due at least in part to the inhibition of a type 1 eIF-2α phosphatase activity, which permits a basal eIF-2α kinase activity to be expressed leading to the accumulation of phosphorylated eIF-2α and an inhibition of protein synthesis.**

Protein synthesis initiation in rabbit reticulocyte lysates is regulated by the reversible phosphorylation of the α-subunit (38,000 daltons) of eukaryotic initiation factor 2 (eIF-2; eIF-2α) (1–3). In the absence of added hemin, there is a rapid activation of the heme-regulated cAMP-independent eIF-2α kinase (HRI) from its latent inactive form (1, 4–8). Activation is accompanied by the phosphorylation of lysate HRI (80,000–85,000 daltons) (9), a rapid phosphorylation of endogenous eIF-2α, and the consequent inhibition of protein chain initiation. In protein-synthesizing hemin-supplemented lysates, HRI is presumably maintained in a latent state; however, a low but detectable level of phosphorylated eIF-2α is often observed (9–11). This is thought to be due to a basal eIF-2α kinase activity which is expressed even in the presence of hemin but at a level that is not inhibitory owing in part to eIF-2α phosphatase activity whose apparent function in normal lysates is to maintain eIF-2 in its active state. Evidence that phosphatase activity also is involved in the reversal of heme deficiency by hemin is provided by the observations that delayed addition of hemin to inhibited lysates produces (*i*) a partial dephosphorylation and inactivation of lysate HRI, (*ii*) a significant decrease in phosphorylated eIF-2α, and (*iii*) the consequent restoration of linear protein synthesis (1, 9).

These findings and the rapid turnover of the phosphate moiety of eIF-2α observed in inhibited lysates (9) point to an important regulatory function for the protein phosphatase(s) that acts on phosphorylated eIF-2α. The identification and preliminary isolation of several protein phosphatase activities from reticulocyte lysates have been described (12). These activities fall into two classes which have been designated type 1 and type 2 based on several criteria, including (*i*) chromatographic elution profiles on DEAE-cellulose, (*ii*) specificity toward the phosphorylated substrates phosphorylase *a* and phosphorylase kinase, (*iii*) selective inhibition of type 1 (but not type 2) phosphatase by two heat-stable proteins from rabbit skeletal muscle, designated inhibitor-1 and inhibitor-2 (13–18), and (*iv*) a correspondence to similar species in rabbit skeletal muscle and rabbit liver (for review, see ref. 15).

In this study we examined the functions of protein phosphatases in the regulation of protein synthesis by using heat-stable protein phosphatase inhibitor-2 (I-2) as a probe in hemin-supplemented lysates undergoing protein synthesis. Our results show that the addition of I-2 to normal hemin-supplemented lysates produces an inhibition of protein synthesis which is accompanied by the phosphorylation of eIF-2α. The results suggest that I-2 blocks the action of a type 1 protein phosphatase with eIF-2α phosphatase activity, which permits expression of

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* We announce with sadness the death of our co-worker, Dr. Vivian G. Ernst, in Boston on Sept. 12, 1982. Her love of science and her pervasive enthusiasm in her work and in her person were always a source of inspiration. We feel privileged to have known her, and we take pride in our collaboration with her. Her death is a deeply felt loss to her husband, Dr. Daniel H. Levin, and to her professional colleagues.

Abbreviations: eIF-2, eukaryotic initiation factor 2; eIF-2α, α-subunit (38,000 daltons) of eIF-2; I-1 and I-2, heat-stable protein phosphatase inhibitor-1 and -2; HRI, heme-regulated eIF-2α kinase; ds RNA, double-stranded RNA; dsI, ds RNA-dependent eIF-2α kinase; $PP2A_1$, $PP2A_2$, PP2B, species of type 1 and type 2 protein phosphatases.

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a basal eIF-2α kinase activity. A preliminary report has appeared elsewhere (19).

METHODS AND MATERIALS

Protein Synthesis in Reticulocyte Lysates. Rabbit reticulocytes (20) and reticulocyte lysates (20, 21) were prepared as described. Protein synthesis reaction mixtures (25 μl) contained 12.5 μl (50%, vol/vol) of reticulocyte lysate (21), 10 mM Tris·HCl at pH 7.7, 1 mM $Mg(OAc)_2$, 76 mM KCl, 0.2 mM GTP, 60 μM [^{14}C]leucine (138 mCi/mmol; 280 cpm/pmol; 1 Ci = 3.7×10^{10} becquerels); 19 other amino acids at 30 μM, 5 mM creatine phosphate, and creatine kinase at 100 μg/ml. Incubation was at 30°C. Aliquots (3–5 μl) were removed at the indicated times and analyzed for [^{14}C]leucine incorporation into acid-precipitable protein as described (21).

***In Situ* Phosphorylation in Protein-Synthesizing Lysates.** Incubation mixtures were as described for protein synthesis, except that unlabeled leucine was utilized. Phosphoprotein profiles during protein synthesis were obtained in two ways. As described (9), phosphorylation was carried out with a brief pulse of [γ-^{32}P]ATP (25 μCi; 20–30 Ci/mmol). Alternatively, phosphorylation in lysates was performed under conditions of constant specific activity of [^{32}P]ATP and [^{32}P]GTP by supplementing lysate incubations with [^{32}P]orthophosphoric acid (100 μCi), 1 mM fructose 1,6-bisphosphate, and 100 μM NAD^+ as described (1, 22, 23). In these assays the standard energy-regenerating system of creatine phosphate and creatine phosphokinase was omitted. The pH 5 fractionation of lysate incubations was carried out as described (23, 24). Incubation aliquots (5 μl) or pH 5 fractions were directly analyzed for [^{32}P]phosphoprotein profiles by one-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography (1, 4, 9).

Protein Kinase Assays. These were performed and the products were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography as described (4, 9).

Materials. Purified preparations of rabbit skeletal muscle heat-stable protein phosphatase inhibitor-1 (I-1) (16, 17) and I-2 (16, 18) were prepared as described (17, 18). Five different preparations of I-2 with specific activities ranging from 24,000 to 86,000 units/mg of protein (25–90% pure) yielded similar results in these studies. One unit of inhibitor activity is defined as the amount that produces 50% inhibition of phosphorylase phosphatase activity in the standard assay (12–15, 18).

Homogenous eIF-2 was kindly provided by William C. Merrick (Case Western Reserve University, Cleveland). *Penicillium chrysogenum* mycophage double-stranded RNA was a gift of Hugh Robertson (Rockefeller University).

[γ-^{32}P]ATP, [^{14}C]leucine, and inorganic $^{32}P_i$ were purchased from New England Nuclear. Fructose 1,6-bisphosphate and NAD^+ were obtained from Sigma; hemin Cl was purchased from Calbiochem.

RESULTS

Inhibition of Reticulocyte Protein Synthesis by I-2 from Skeletal Muscle and Reversal of Inhibition by eIF-2. Addition of I-2 to hemin-supplemented lysates resulted in a biphasic inhibition of protein synthesis similar to that observed in heme deficiency (Fig. 1); the degree of inhibition increased with increasing amounts of added I-2 (20–50 units). A similar inhibition was observed in response to phosphorylated I-1, but this was variable in different lysates and required higher concentrations of I-1 (100–300 units) to achieve comparable levels of inhibition (not shown). Moreover, we found that I-1 was dephosphorylated in lysates but that addition of cAMP (100 μM), which activates endogenous cAMP-dependent protein kinase (25), permitted

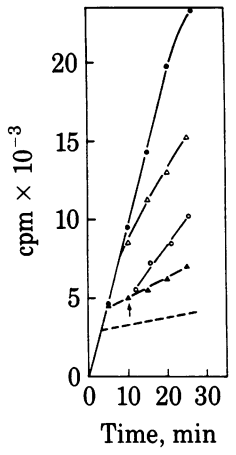

FIG. 1. Inhibition of reticulocyte protein synthesis by I-2 and reversal of inhibition by eIF-2. Protein synthesis reaction mixtures (25 μl) containing 20 μM hemin (unless stated otherwise) were incubated at 30°C under the following conditions: ●, no additions; △, plus 22.5 units of I-2; ▲, plus 45 units of I-2; ○, plus 45 units of I-2 (at 0 min) and 16 pmol of eIF-2 added at 10 min (arrow); ---, heme-deficient lysate. Incorporation of [^{14}C]leucine is given as cpm $\times 10^{-3}$ per 5-μl reaction mixture. One unit of I-2 is defined as the amount required to inhibit phosphorylase phosphatase by 50% under standard assay conditions (12, 18).

regeneration of phosphorylated I-1 (not shown). However, even with added cAMP, I-1 was less inhibitory than I-2. For these reasons, I-2 was utilized in the experiments described here. The extent of inhibition by added I-2 approached that observed in heme deficiency. In this regard we have found that lysates contain significant levels of I-2 but undetectable or very low levels of I-1 (ref. 12; unpublished data). The inhibition caused by I-2 was reversed by the delayed addition of purified eIF-2 (Fig. 1, arrow). This suggests that the inhibition is due to the inactivation of eIF-2.

Phosphoprotein Profiles in Lysates Inhibited by I-2: Phosphorylation of eIF-2. The observation that reticulocyte protein synthesis becomes inhibited by the addition of I-2 led us to examine the phosphoprotein profiles of inhibited lysates. To obtain these profiles, the creatine phosphate/creatine phosphokinase energy-regenerating system normally used in protein synthesizing lysates (20, 21) was replaced by a system that generated [^{32}P]ATP and [^{32}P]GTP at constant specific activity. This was accomplished by utilizing 1 mM fructose 1,6-bisphosphate, 0.1 mM NAD^+, and [^{32}P]orthophosphoric acid, a system that generates ATP of constant specific activity through glycolysis at a rate which efficiently supports linear protein synthesis in lysates (1, 22, 23). This method has the advantage of permitting comparative analysis of lysate phosphoprotein profiles at any interval during protein synthesis. Labeled lysate phosphoproteins then were directly analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography (Fig. 2, lanes 1–6). Under these conditions, specific changes in the phosphorylation of endogenous heme-regulated eIF-2α kinase (HRI, 80,000 daltons) and eIF-2α (38,000 daltons) are readily distinguished (9). A further improvement in resolution was achieved by utilizing the pH 5 fractions (Fig. 2, lanes 7–12) of the lysate samples; this enhanced the resolution in gel electrophoresis and also permitted larger samples to be analyzed.

As shown in Fig. 2, the inhibition of protein synthesis in hemin-supplemented lysates treated with I-2 was associated with increased phosphorylation of eIF-2 (lanes 5 and 11) compared to uninhibited control lysates (lanes 1 and 7). In I-2-inhibited lysates, two additional phosphoproteins (29,000 and 44,000 daltons) appeared within 5 min of the start of incubation

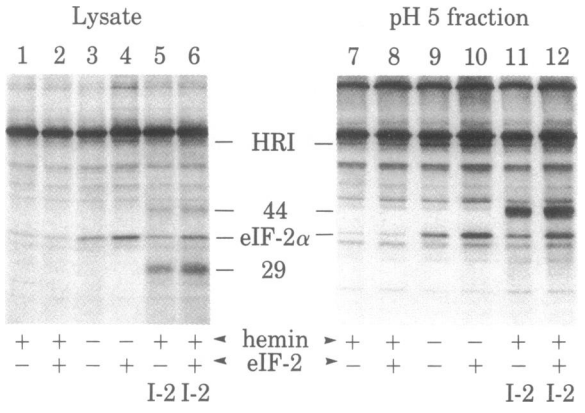

FIG. 2. Phosphoprotein profiles in lysates inhibited by heme deficiency and by I-2: Use of glycolytic ATP-generating system. Protein synthesis reaction mixtures (25 μl) were incubated with 1 mM Fru-1,6-P_2, 100 μM NAD^+, and 50 μCi of [^{32}P]orthophosphoric acid (1, 23, 24). After 5 min at 30°C, aliquots (5 μl) were added directly to sample dissociation buffer for direct analysis in $NaDodSO_4$/polyacrylamide gels (0.1% $NaDodSO_4$/10% acrylamide/0.26% bisacrylamide) (9). At the same time, aliquots (10 μl) were added to a solution containing 50 mM NAF and 4 mM EDTA and the mixture immediately was brought to pH 5 by addition of 0.5 M acetic acid (23, 24). The pH 5 precipitates were recovered by centrifugation, dissolved in sample buffer, and analyzed on the same gel as the lysate samples; lanes 7–12 represent the pH 5 fractions of the lysate samples in lanes 1–6. Changes in the reaction mixtures were: lanes 1, 2, 7, 8, 20 μM hemin; lanes 3, 4, 9, 10, without hemin; lanes 5, 6, 11, 12, 20 μM hemin plus 45 units of I-2; lanes 2, 4, 6, 8, 10, 12, also supplemented with 1.5 pmol of purified eIF-2 at the start of incubation (this concentration of eIF-2 does not restore synthesis in inhibited lysates). Autoradiograms of stained gels are shown. HRI, heme-regulated eIF-2α kinase; 44 and 29, positions corresponding to 44,000 and 29,000 daltons.

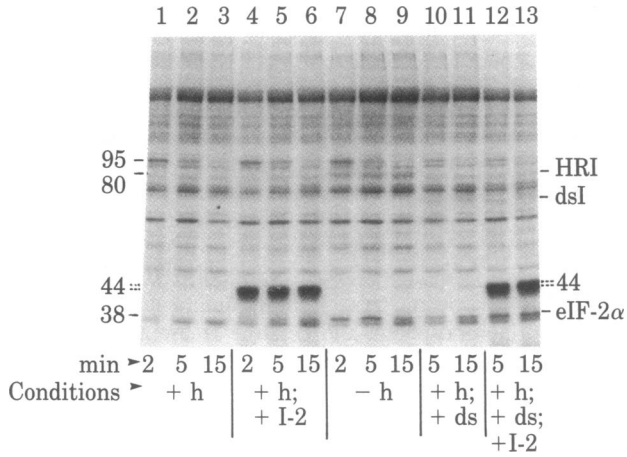

FIG. 3. Effect of I-2 on the turnover of [^{32}P]eIF-2α in inhibited lysates. Protein synthesis reaction mixtures (25 μl) were incubated at 30°C with [γ-^{32}P]ATP (25 μCi). At 2, 5, or 15 min, as indicated, 10-μl samples were removed, concentrated by pH 5 fractionation (see legend to Fig. 2), and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography (9). Additions were as follows: lanes 1–3, 20 μM hemin (+ h); lanes 4–6, 20 μM hemin plus 50 units of I-2; lanes 7–9, without hemin; lanes 10, 11, 20 μM hemin plus double-stranded RNA (ds) at 20 ng/ml; lanes 12, 13, 20 μM hemin plus 20 ng/ml of ds RNA plus 50 units of I-2.

(lane 5). The 29,000-dalton polypeptide appears to be I-2 which is phosphorylated by a cAMP-independent protein kinase in the lysate. The physiological significance, if any, of this I-2 phosphorylation is not yet known. The 44,000-dalton phosphoprotein appears to be a protein contaminant of the I-2 preparations based on titration studies in the lysate and on $NaDodSO_4$ gel electrophoresis of the I-2 preparations (not shown). The phosphorylation of the 44,000-dalton component in I-2-inhibited lysates was rapid, cAMP-independent, and relatively stable. In lysates, both the 29,000- and 44,000-dalton proteins remained soluble after heating at 95°C for 2 min. However, only the 44,000-dalton polypeptide was precipitated by pH 5 fractionation (Fig. 2, lanes 11 and 12).

In the same experiment (Fig. 2), when duplicate assays of control and of inhibited lysates were supplemented with low levels of eIF-2, both endogenous and exogenous eIF-2α were phosphorylated (compare lanes 5 and 6), confirming the presence of active eIF-2α kinase in heme-deficient (lanes 4 and 10) and I-2-inhibited lysates (lanes 6 and 12).

Hemin-supplemented lysates undergoing optimal linear protein synthesis often displayed low but detectable steady-state levels of phosphorylated eIF-2α (Fig. 2, lanes 1 and 7) which are not inhibitory (1, 9, 24, 25). By comparison, heme-deficient lysates displayed a rapid phosphorylation of both HRI and eIF-2α (lanes 3 and 9). In these lysates, protein synthesis stopped at 5 min and the phosphorylation of eIF-2α approached a maximal steady-state level (Fig. 2, lanes 3 and 9); at this time, about 30–40% of lysate eIF-2α is phosphorylated (1, 9, 10, 24). The turnover of phosphate on eIF-2α in inhibited lysates is rapid and is due to the presence of a highly active phosphatase (1, 9, 26). We estimate that, in heme-deficient lysates, the *α-P* moiety of endogenous eIF-2 has a half-life of approximately 2–3 min; hence, the restoration of protein synthesis produced by the delayed addition of hemin may be explained by decreased eIF-2α kinase activity and the subsequent dephosphorylation of eIF-2α by the endogenous phosphatase activity (1, 9).

Effect of I-2 in Lysates Pulsed with [γ-^{32}P]ATP. Normal and inhibited lysates were supplemented with brief pulses of high-specific-activity [γ-^{32}P]ATP (9) and the phosphoprotein profiles were analyzed by $NaDodSO_4$ gel electrophoresis (Fig. 3). In this experiment, polypeptides smaller than ≈32,000 daltons were run off the gels to enhance resolution. Under normal conditions, the label from [γ-^{32}P]ATP added at the start of incubation is initially incorporated into lysate proteins and then is gradually diluted at a rate depending both upon the rate of phosphate turnover and the rate of dilution of the label in the endogenous ATP pool (9). When both rates are rapid, then label incorporated initially is likely to be lost rapidly—for example, as in the 95,000-dalton component in Fig. 3 (lanes 1–3). More stable phosphates with slower rates of turnover would tend to show little or no loss of label. When I-2 was added to lysates under various conditions, the level of labeled phosphate on eIF-2α tended to remain stable or increase (lanes 4–6, 12, 13) for up to 15 min of incubation compared to the level observed in corresponding inhibited lysates with no added I-2 (lanes 7–11). The data indicate that I-2 prevents the dephosphorylation of eIF-2α in the inhibited lysate by blocking a type 1 phosphatase. At the same time, the inability of I-2 to prevent the loss of label on the 95,000-dalton component (Fig. 3, lane 6) suggests that its phosphate turnover is probably mediated by a type 2 phosphatase.

One explanation for the effect of I-2 is that the inhibition of eIF-2α phosphatase by I-2 permits phosphorylated eIF-2α to accumulate due to a basal eIF-2α kinase activity. This model also predicts that delayed $^{32}P_i$ pulses in I-2 treated lysates would produce less ^{32}P-labeled eIF-2α than earlier pulses would. Evidence in support of this is found in the phosphoprotein profiles in Fig. 4. Lysates were inhibited by three different modes including heme-deficiency, double-stranded (ds) RNA, and I-2, all of which gave rise to increased phosphorylation of eIF-2α. All of the assays were supplemented with 1 mM fructose 1,6-bisphosphate and 0.1 mM NAD^+. In four assays (lanes 1–4), $^{32}P_i$ was present at 0–10 min; in duplicate assays it was present at

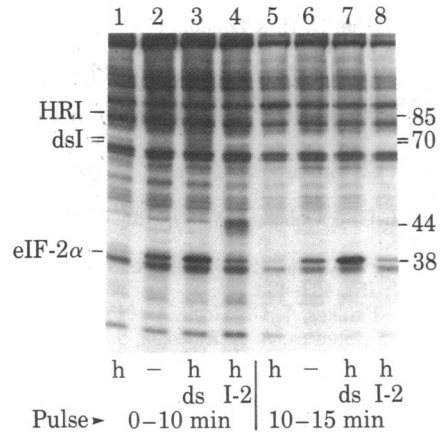

FIG. 4. Comparison of early and delayed $^{32}P_i$ pulses in inhibited lysates. Protein synthesis reaction mixtures (25 μl) were incubated with 1 mM Fru-1,6-P_2, 100 μM NAD^+, and [^{32}P]orthophosphoric acid (25 μCi) as in Fig. 2. In four assays (lanes 1–4), $^{32}P_i$ was added at 0 min and samples (10 μl) were taken at 10 min. In the remainder (lanes 5–8), $^{32}P_i$ was added at 10 min and samples (10 μl) were taken at 15 min. All samples were concentrated by pH 5 fractionation and analyzed by $NaDodSO_4$ gel electrophoresis (see Figs. 2 and 3). Conditions were as follows: lanes 1 and 5, 20 μM hemin (h); lanes 2 and 6, without hemin; lanes 3 and 7, 20 μM hemin plus 20 ng/ml of double-stranded RNA (ds) at 20 ng/ml; lanes 4 and 8, 20 μM hemin plus 50 units of I-2. The figure is an autoradiogram.

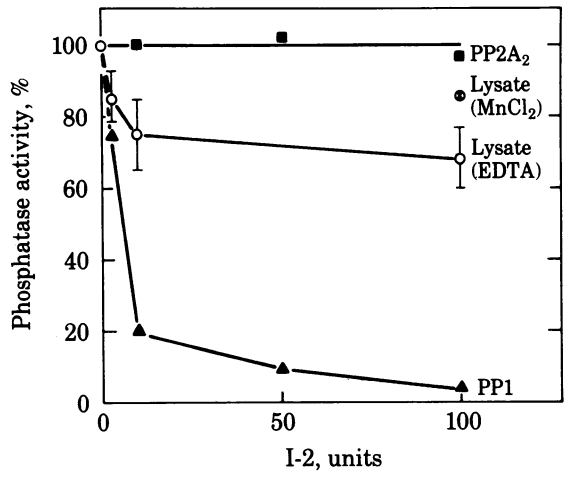

FIG. 5. Effect of skeletal muscle I-2 on type 1 and type 2 lysate phosphatases and on total lysate phosphatase activity. Protein phosphatase assays (60 μl) were carried out using [^{32}P]phosphorylase *a* (1 mg/ml) as substrate as described (12). Assay mixtures contained increasing concentrations of I-2 and were carried out with 1 mM $MnCl_2$ except where 1 mM EDTA was utilized as indicated. Other additions were partially purified reticulocyte type 1 phosphatase (PP1) (0.01 unit/ml), partially purified reticulocyte type $2A_2$ phosphatase ($PP2A_2$) (0.01 unit/ml), and total lysate diluted as a source of phosphorylase phosphatase to a final concentration of 0.01 unit/ml. Each point represents the percentage of total ^{32}P released at 30 min (30°C).

10–15 min (lanes 5–8). The delayed pulses in heme deficiency (lane 6) and ds RNA-inhibited lysates (lane 7) produced levels of [^{32}P]eIF-2α similar to those in the 0–10 min pulses (lanes 2 and 3), reflecting the rapid turnover of phosphate on eIF-2α and the potent eIF-2α phosphatase activity in these lysates. In I-2-inhibited lysates, there was less [^{32}P]eIF-2α in the later pulse (lane 8) than in the early pulse (lane 4), as would be expected if less phosphate turnover were taking place due to the inhibition of phosphatase activity.

One related observation of interest is a 24,000-dalton phosphoprotein that was phosphorylated in both normal and inhibited lysates during early linear synthesis (lanes 1–4) but was labeled only in normal lysates in response to a delayed ^{32}P pulse (lanes 5–8). One explanation for this is that the phosphate on the 24,000-dalton component is turning over during linear synthesis but is not turning over in shut-off lysates. We previously noted that this phenomenon is associated with all of the inhibitions induced by eIF-2α kinase activity (1). However, the nature of this association is unknown.

Effect of Purified I-2 on Total Lysate Phosphatase and on Purified Protein Phosphatase Types 1 and 2. To confirm the ability of I-2 to act on lysate phosphatases, we compared the phosphorylase phosphatase activity in the lysate with that of the partially purified lysate protein phosphatases type 1 and type 2 (12), in the presence of increasing I-2 (Fig. 5). In this assay, [^{32}P]phosphorylase *a* was used as the substrate and assays were carried out in the presence of 1 mM $MnCl_2$ for maximal phosphatase activity (12). It had been shown that both type 1 and type 2 phosphatases act on [^{32}P]phosphorylase *a* (for a review, see ref. 15), but only type 1 phosphatase is inhibited by I-2 (12, 18). In the presence of 100 units of I-2, the capacity of protein phosphatase 1 to dephosphorylate [^{32}P]phosphorylase *a* was inhibited >95%, whereas the activity of protein phosphatase $2A_2$ was not affected. When unfractionated lysate was used as the source of phosphatase activity, about 15% of total lysate phosphatase activity was inhibited by 100 units of I-2. This indicates that 85% of lysate phosphorylase phosphatase activity is insensitive to I-2 and therefore represents type 2 phosphatase. This ratio of I-2 sensitive (15%) to I-2 resistant (85%) phosphatase is in good agreement with the type 1/type 2 phosphatase ratio isolated from reticulocyte lysates (12).

This correlation was also reflected in lysate assays in which $MnCl_2$ was replaced by 1 mM EDTA (Fig. 5), a condition that diminishes type 2 phosphatase activity about 50% but has little or no effect on type 1 phosphatase (12). In the presence of 1 mM EDTA and 100 units of I-2, total lysate phosphorylase phosphatase activity was inhibited about 30%, which is in good agreement with values determined by other procedures (12). When $MnCl_2$ was replaced in the lysate phosphatase assays by 1 mM $MgCl_2$/0.5 mM ATP (MgATP), conditions that approximate physiological concentrations, type 2 phosphatase activity decreased about 80% and type 1 activity decreased approximately 15% (12). Under these conditions, the relative percentage of type 1 phosphatase activity increased from 15% assayed with $MnCl_2$ to 40% assayed with MgATP, a value that may reflect physiological lysate activities more accurately.

DISCUSSION

Based on the data reported here and elsewhere (12), we conclude that I-2 promotes the phosphorylation of eIF-2α in hemin-supplemented lysates, presumably by the inhibition of an eIF-2α phosphatase with type 1 properties. Crouch and Safer (27) isolated a highly purified protein phosphatase from reticulocyte lysates which dephosphorylates eIF-2α and displays properties of a type 2 phosphatase (28). Stewart *et al.* (28) demonstrated that a type 1 protein phosphatase from rabbit skeletal muscle dephosphorylates eIF-2α *in vitro* at a rate comparable to that of a type 2 phosphatase. The model suggested by our data is that in the lysate a type 1 phosphatase acts on phosphorylated eIF-2α.

We have considered the alternative that the observed effects of I-2 are due to an eIF-2α kinase activator rather than an eIF-2α phosphatase inhibitor. In this regard, Henderson *et al.* (29) recently described a heat-stable protein from lysates which functions as an eIF-2α kinase activator. Similarly, de Haro *et*

al. (30) purified, from preparations of bovine heart muscle cAMP-dependent protein kinase, a heat-stable protein that inhibits protein synthesis and elicits phosphorylation of eIF-2α in lysates. To clarify this issue we examined the effect of our I-2 preparations *in vitro* on the activation of purified heme-regulated eIF-2α kinase and ds RNA-dependent eIF-2α kinase (31) and found little or no activator effect by I-2. On the other hand, we recognize that our I-2 preparations may contain eIF-2α kinase activator properties when added to lysates, which are not elicited with purified components; however, this remains to be demonstrated. The results of the present study are consistent with the conclusion that the effect of added I-2 on protein synthesis in lysates is mediated by its inhibition of type 1 phosphatase. In support of this, we find that added I-2 decreases the rate of eIF-2α phosphate turnover in I-2-inhibited lysates. In addition, five separate preparations of skeletal muscle I-2 of varying degrees of purity, from 25% to 90%, produced similar inhibitions of protein synthesis and similar phosphoprotein profiles in lysates, including the phosphorylation of eIF-2α.

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