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REGULATION OF PROTEIN SYNTHESIS IN RABBIT RETICULOCYTE LYSATES: INHIBITION OF eIF-2 PHOSPHOPROTEIN PHOSPHATASE BY NaF, PYROPHOSPHATE AND CATIONS

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Summary: The nature and the role of eIF-2 phosphoprotein phosphatase in rabbit reticulocyte lysates have been examined. The eIF-2 phosphoprotein phosphatase is inhibited by a variety of divalent metal ions (Cd^{++} > Ag^{++} > Cu^{++} > Pb^{++} > Zn^{++} > Co^{++} > Sr^{++} > Mo^{++}) in lysates in situ. In addition, PPi, EDTA and NaF inhibit this enzyme. The eIF-2 phosphoprotein phosphatase is also inhibited by $NaHSO_3$ and $Na_2S_2O_5$. $Na_2S_2O_5$ is, however, more effective. $Na_2S_2O_5$ has been found to be a potent inhibitor of protein synthesis in lysates. This inhibition is associated with the phosphorylation of the 38,000-dalton subunit of initiation factor eIF-2. eIF-2 overcomes this inhibition. These findings suggest that under optimum conditions of protein synthesis the phosphorylation and dephosphorylation of eIF-2 are in a dynamic state of equilibrium in which dephosphorylation is favored. The inhibition of eIF-2 phosphoprotein phosphatase by $Na_2S_2O_5$ shifts this equilibrium in favor of eIF-2 phosphorylation, consequently, protein synthesis is inhibited. The sulfhydryl nature of eIF-2 phosphoprotein phosphatase has been established.

Protein synthesis in rabbit reticulocytes and their lysates is regulated by heme (reviewed in 1). In heme deficiency, a heme-regulated inhibitor (HRI) is activated that blocks protein chain initiation (1). HRI has been identified as an adenosine 3':5' cyclic monophosphate (cyclic-AMP) independent protein kinase that specifically phosphorylates the 38,000-dalton subunit of initiation factor eIF-2 (2-6) and, as a consequence, eIF-2 is inactivated (1,7,8).

There is little information available, however, on the proposed eIF-2 phosphoprotein phosphatase (4) that is present in the lysates (9,10). To gain some insight into the nature and the role of this enzyme in the regulation of protein synthesis we have examined the effect of NaF, pyrophosphate and metal ions on the activity of the enzyme in lysates in situ. The results show that NaF, pyrophosphate and various metal ions inhibit the activity of eIF-2 phosphoprotein phosphatase. The inhibition of eIF-2 phosphoprotein phosphatase by $Na_2S_2O_5$ is of particular interest

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because $Na_2S_2O_5$ also inhibits protein synthesis in lysates that is overcome by the addition of exogenous initiation factor eIF-2.

MATERIALS AND METHODS

The following procedures have been described: preparation of rabbit reticulocyte lysates, protein synthesis mixtures, assay of protein synthesis (11), preparation of purified eIF-2 (11,12), preparation of purified HRI (4), protein kinase assay, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the autoradiography of polyacrylamide gel (11).

Preparation of [^{32}P]eIF-2: Purified eIF-2 (30 µg) was incubated for 30 minutes with [γ^{32}P]ATP (specific activity 15,000 cpm/pmol) and purified HRI (2 µg) as described for the protein kinase assay (4). The free [γ^{32}P]ATP was removed by chromatography on a Biorex 70 column (0.5 x 4 cm). The bound eIF-2 was eluted with buffer containing 650 mM KCl (13). The [^{32}P]eIF-2 was dialyzed against buffer containing Tris-HCl (pH 7.8) 20 mM; KCl, 80 mM; DTT, 0.5 mM and 10 percent glycerol.

eIF-2 phosphoprotein phosphatase assay. eIF-2 phosphoprotein phosphatase activity was assayed in lysates. Reaction mixture (20 µl) contained 10 µl of lysates, appropriate concentration of various components and [^{32}P]eIF-2 (10,000-13,000 CPM). Samples were incubated at 30°. After 2 minutes of incubation, 5 µl aliquots were removed and transferred to 15 µl of denaturing solution (Tris-HCl, 50 mM [pH 7.0]; 2% SDS and 5% 2-mercapto-ethanol). The samples were heated at 100° and were then subjected to electrophoresis in SDS-polyacrylamide gel. After the gel had been autoradiographed, the 38,000-dalton polypeptide region of the gel was cut out and the radioactivity associated with the polypeptide was determined.

RESULTS

The results presented in Fig 1, A to G show that metal ions Ag^{++}, Cd^{++}, Co^{++}, Cu^{++}, Mo^{++}, Pb^{++}, Sr^{++}, and Zn^{++} inhibit eIF-2 phosphoprotein phosphatase. The order of effectiveness being: $Cd^{++}>Ag^{++}>Cu^{++}>Pb^{++}$ $Zn^{++}>Co^{++}>Sr^{++}>Mo^{++}$. The divalent metals Cd^{++} and Ag^{++} in 1 mM concentration inhibit eIF-2 phosphoprotein phosphatase activity by 65-75 percent. The Mn^{++} and Ca^{++}, on the other hand, neither stimulate nor inhibit eIF-2 phosphoprotein phosphatase (results not shown). The eIF-2 phosphoprotein phosphatase is also inhibited by pyrophosphate (1-5 mM), EDTA (0.4-8 mM) and by 40-50 percent by NaF (50 mM). Whereas GDP (0·1 to 1 mM) inhibits the enzyme poorly.

Previous studies have shown that $NaHSO_3$ is a potent inhibitor of phospho-protein phosphatase(s) that dephosphorylate histones in situ (14). These findings prompted us to examine the effect of $NaHSO_3$ and of $Na_2S_2O_5$ (the effect of $Na_2S_2O_5$ on phosphoprotein phosphatases is not known) on the activity of eIF-2 phosphoprotein phosphatase. The results in Fig 1, H

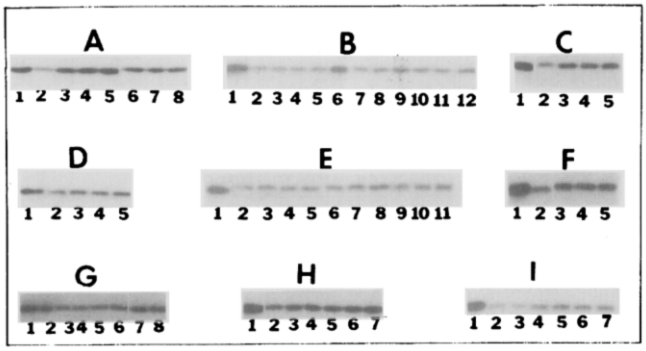


Fig 1. Inhibition of eIF-2 phosphoprotein phosphatase by metal ions, PPi, NaF, EDTA, $Na_2S_2O_5$ and $NaHSO_3$. Lysate (10 µl) was incubated in a total volume of 20 µl with indicated reagents and [^{32}P]eIF-2 for two minutes at 30° as described in "Materials and Methods" section. Aliquots (5 µl) were removed and transferred to 15 µl of denaturing solution. The samples were subjected to electrophoresis in SDS-polyacrylamide gel and autoradiograms were prepared (11).The sections of the autoradiograms containing 38,000-dalton subunit of eIF-2 are presented.

A: Lane 1, unincubated [^{32}P]eIF-2 control; lane 2, incubated control lysate; lanes 3 to 5, samples contained 1, 2 and 3 mM cadmium chloride, respectively; and lanes 6 to 8, samples contained 1, 5 and 10 mM silver acetate, respectively.

B: Lane 1, unincubated [^{32}P]eIF-2 control; lane 2, incubated control lysate; lanes 3 to 6, samples contained 0·25, 0·5, 1 and 5 mM lead chloride, respectively; lanes 7 to 9, samples contained 1, 5, and 10 mM sodium molybdate, respectively; and lanes 10 to 12, samples contained 1, 5 and 10 mM cobaltus chloride, respectively.

C: Lane 1, unincubated [^{32}P]eIF-2 control; lane 2, incubated control lysate; lanes 3 to 5, samples contained 1, 2 and 3·5 mM lead chloride, respectively.

D: Lane 1, unincubated [^{32}P]eIF-2 control; lane 2, control incubated lysate; and lanes 3 to 5, samples contained 1, 2 and 3 mM cupric sulfate, respectively.

E: Lane 1, unincubated [^{32}P]eIF-2 control; lane 2, incubated control lysate; lanes 3 to 5, samples contained 0·1, 0·5 and 1 mM GDP, respectively; lanes 6 to 8, samples contained 0·1, 1 and 5 mM pyrophosphate, respectively; and lanes 9 to 11 contained 1, 5 and 50 mM NaF, respectively.

F: Lane 1, unincubated [^{32}P]eIF-2 control; lane 2, control incubated lysate; and lanes 3 to 5, samples contained 1, 5 and 10 mM strontium chloride, respectively.

G: Lane 1, unincubated [^{32}P]eIF-2 control; lane 2, incubated control lysate; and lanes 3 to 8, samples contained 0·4, 0·8, 1·6, 2·4, 4 and 8 mM EDTA, respectively.

H: Lane 1, unincubated [^{32}P]eIF-2 control; lane 2, incubated control lysate; and lanes 3 to 7, samples contained 2·5, 5, 10, 25 and 50 mM $Na_2S_2O_5$, respectively.

I: Lane 1, unincubated [^{32}P]eIF-2 control; lane 2, incubated control lysate; and lanes 3 to 7, samples contained 2·5, 5, 10, 25 and 50 mM $NaHSO_3$, respectively.

and I, show that both $NaHSO_3$ and $Na_2S_2O_5$ inhibit eIF-2 phosphoprotein phosphatase. $Na_2S_2O_5$ is, however, more effective.

The experimental finding that $Na_2S_2O_5$ inhibits eIF-2 phosphoprotein phosphatase at low concentrations (2.5 mM) suggested that this reagent

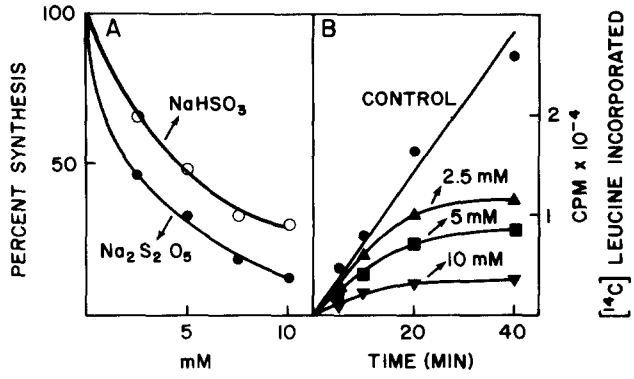


Fig 2. Inhibition of protein synthesis by $Na_2S_2O_5$. Protein synthesis reaction mixtures (20 µl) containing 10 µM hemin were incubated at 30° with indicated concentrations of $Na_2S_2O_5$ or $NaHSO_3$. After 40 minutes of incubation (A) or after indicated time intervals (B), aliquots (5 µl) were removed and protein synthesis was assayed.

could provide a valuable tool in the investigation of the role of eIF-2 phosphoprotein phosphatase in the maintenance of protein synthesis in lysates. The results in Fig 2, A show that both $Na_2S_2O_5$ and $NaHSO_3$ are potent inhibitors of protein synthesis. Once again $Na_2S_2O_5$ is more effective than $NaHSO_3$. The observed biphasic kinetics of inhibition (Fig 2, B) are reminiscent of the inhibition of protein synthesis that is observed by the phosphorylation of the 38,000-dalton subunit of eIF-2 in heme-deficiency or by the addition of HRI to hemin-supplemented lysates (1). Similar kinetics of inhibition are observed with $NaHSO_3$ (results not shown). The suggestion that the phosphorylation of eIF-2 may be involved in protein synthesis inhibition by $Na_2S_2O_5$ is supported by the *in situ* phosphorylation of lysate polypeptides (Fig 3). These results show that in the presence of $Na_2S_2O_5$ there is a 2-3 fold increase in the phosphorylation of the 38,000-dalton subunit of eIF-2 over the control. It should be pointed out that in addition to enhanced phosphorylation of the 38,000-dalton subunit of eIF-2, in the presence of $Na_2S_2O_5$, there is a marked increase in the phosphorylation of a polypeptide(s) of 90,000-95,000-daltons. The conclusion that increased phosphorylation of the 38,000-dalton subunit of eIF-2 may be associated with the inhibition of protein synthesis in the presence of $Na_2S_2O_5$ is supported further by the finding that initiation factor eIF-2 overcomes this inhibition (Table 1). It should be noted that DTT is also effective in partially preventing inhibition of protein synthesis by $Na_2S_2O_5$.

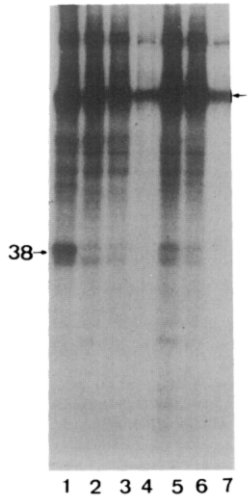


Fig 3. Phosphorylation of lysate polypeptides in situ in the presence and absence of $Na_2S_2O_5$. Lysates (10 µl) containing 10 µM hemin and ATP generating system (11) were incubated with (lanes 5 to 7) 2·5 mM $Na_2S_2O_5$ or without $Na_2S_2O_5$ (lines 2-4) and 3·75 µC of [^{32}P]ATP. At intervals of 0.2, 2 and 4 minutes, aliquots (3 µl) were removed and transferred to 15 µl of denaturing solution. They were subjected to electrophoresis in SDS-polyacrylamide gel and autoradiograms were prepared (11). Lane 1, samples were treated as in the control sample except that eIF-2 (0·2 µg) and HRI (0·05 µg) were added to the sample as an internal control for the phosphorylation of eIF-2; lanes 2 to 4, control samples incubated for 0.2, 2 and 4 minutes; and lanes 5 to 7, samples incubated with $Na_2S_2O_5$ for 0.2, 2 and 4 minutes. The position of the 38,000-dalton subunit of eIF-2 is indicated. The arrow alone (right side) indicates the position of 90,000-95,000-dalton phosphopolypeptide(s).

The findings that heavy metals inhibit eIF-2 phosphoprotein phosphatase activity and the fact that DTT overcomes the $Na_2S_2O_5$ induced inhibition of protein synthesis strongly suggested that the eIF-2 phosphoprotein phosphatase may be a sulfhydryl enzyme. This prediction is confirmed by the experimental results (Fig 4). These results show that N-ethylmaleimide (0·5-10 mM) strongly inhibits eIF-2 phosphoprotein phosphatase.

DISCUSSION

The nature of eIF-2 phosphoprotein phosphatase was investigated *in situ* in lysates. Under these conditions the specificity of the phosphatase is likely to be preserved. This is supported by the distinctive inactivation pattern of eIF-2 phosphoprotein phosphatase by various metals. Unlike other phosphatases (15-20), the activity of eIF-2 phosphoprotein phosphatase is not enhanced by Mn^{++} and unlike skeletal muscle phosphoprotein phosphatase (20) the eIF-2 phosphoprotein phosphatase is not

Table I. Effect of DTT and eIF-2 on $Na_2S_2O_5$ induced inhibition of protein synthesis.

Additions	Protein synthesis CPM x 10^3	Percentage Inhibition
I. None	33	-
$Na_2S_2O_5$(3·8 mM)	9	73
$Na_2S_2O_5$(3·8 mM) + eIF-2	16	52
II. None	29	-
DTT(2 mM)	26	10
$Na_2S_2O_5$(3 mM)	14	52
$Na_2S_2O_5$(3 mM) + DTT(2 mM)	22	24

Protein synthesis reaction mixtures (25 µl) containing 10 µM hemin were incubated at 30° with indicated concentrations of $Na_2S_2O_5$, DTT or eIF-2 (1 µg). After 40 minutes of incubation 5 µl aliquots were removed and protein synthesis was determined (11).

inactivated by ATP. This observation is significant in view of the fact that 1 mM ATP concentration is maintained during protein synthesis in lysates (11). Similarly, unlike eIF-2 phosphoprotein phosphatase, Co^{++} and Zn^{++} do not inactivate the skeletal muscle phosphoprotein phosphatase (20), in fact, many substitute for Mn^{++} ions, albeit less efficiently. The inactivation of eIF-2 phosphoprotein phosphatase by the addition of EDTA suggests an apparent requirement of a metal ion for enzyme activity. The identity of this metal has not been defined. The distinctive inactivation profile of eIF-2 phosphoprotein phosphatase by metal ions suggests that this enzyme, unlike other phosphatases, may be substrate specific. This conclusion is supported by our finding that the skeletal muscle phosphoprotein phosphatase (20) and the rabbit liver phosphatase (17) are ineffective in the rescue of protein synthesis in

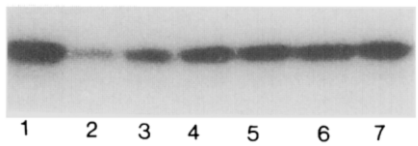
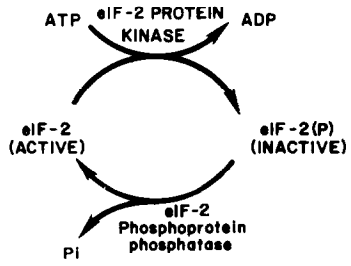


Fig 4. Inactivation of eIF-2 phosphoprotein phosphatase by N-ethylmaleimide (NEM). Lysates (8 µl) were incubated in a final volume of 10 µl with NEM at 30° for 10 min. DTT (20 mM) was then added to each sample. After 10 min in an ice bath, [^{32}P]eIF-2 (13,000 CPM) was added to each sample to a final volume of 16 µl. The samples were incubated at 30° for 2 min. Aliquots (5 µl) were removed and transferred to 15 µl of denaturing solution. The samples were subjected to electrophoresis in SDS-polyacrylamide gel and an autoradiogram was prepared. Lane 1, control without incubation; lane 2, control with incubation; and lanes 3 to 7, samples incubated with, 1, 2, 4, 6 and 10 mM NEM, respectively.

lysates and also do not increase the rate of dephosphorylation of eIF-2 when added to lysates (results not shown).

The finding that $Na_2S_2O_5$ inhibits eIF-2 phosphoprotein phosphatase at low concentrations (2·5-10 mM) provided us with an important tool to study the role of eIF-2 phosphoprotein phosphatase in the regulation of protein synthesis. It should be emphasized that this inhibition of the phosphatase by $Na_2S_2O_5$ is not due to Na^+ ions because addition of an equivalent concentration of NaCl neither inhibits eIF-2 phosphoprotein phosphatase nor protein synthesis in lysates. The inhibition of eIF-2 phosphoprotein phosphatase activity by 2·5-10 mM $Na_2S_2O_5$, the inhibition of protein synthesis in lysates by the same $Na_2S_2O_5$ concentrations and the increased phosphorylation of eIF-2 in situ in lysates in the presence of $Na_2S_2O_5$, strongly suggests that the eIF-2 phosphoprotein phosphatase plays an important regulatory role in protein synthesis by maintaining eIF-2 in a dephosphorylated state. These findings also suggest that low level of the active form of the inhibitory protein kinase (HRI) is present in lysates even in the presence of optimal hemin concentrations. A contention, that is supported by the detectable level of eIF-2 phosphorylation observed in the control samples (Fig 3) and by our earlier findings in intact reticulocytes (21). The presence of a highly active eIF-2 phosphoprotein phosphatase maintains the bulk of eIF-2 in a dephosphorylated state. As a result, protein synthesis rate is maintained at optimal level. This finding supports the view that phosphorylation-dephosphorylation of eIF-2 under conditions of optimal hemin concentrations are in a dynamic state of equilibrium which favors dephosphorylation. When either eIF-2 phosphoprotein phosphatase is inhibited or level of inhibitory protein kinase is increased, disequilibrium would result. There would be an increased phosphorylation of eIF-2, consequently protein synthesis would be inhibited. This is shown schematically:



Protein synthesis, therefore, may be regulated by the modulation of the activity of the eIF-2 protein kinase (HRI) or the phosphoprotein phosphatase. The results presented here provide the first evidence of the inhibition of lysate protein synthesis that is associated with the inhibition of eIF-2 phosphoprotein phosphatase.

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Note added in proof: In a recent report Wang, S. T., Mastropaola, W. and Henshaw, E. (1981), Fed. Proc. 40, 1550 (abstract) show that NaF inhibits eIF-2 phosphoprotein phosphatase in Ehrlich ascites cell extracts.

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