Role of Reversing Factor in the Inhibition of Protein Synthesis
Initiation by Oxidized Glutathione*

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(Received for publication, February 17, 1988)

The inhibitions of protein synthesis initiation in heme-deficient reticulocyte lysates and in GSSG-treated heme-supplemented lysates are both characterized by the activation of heme-regulated eIF-2α kinase, which phosphorylates the α-subunit of eukaryotic initiation factor eIF-2 (1). In both inhibitions, the accumulation of eIF phosphorylated in α-subunit (eIF-2(αP)) leads to the sequestration of reversing factor (RF) in a phosphorylated 15 S complex, RF.eIF-2(αP), in which RF is nonfunctional. A sensitive assay for the detection of endogenous RF activity in protein-synthesizing lysates indicates that, in GSSG-inhibited (1 mM GSSG) lysates, RF is more profoundly inhibited than in heme-deficient lysates. RF inactivation in GSSG-induced inhibition appears to be due to two separate but additive effects: (i) the formation of the phosphorylated 15 S RF complex, RF.eIF-2(αP), and (ii) the formation of disulfide complexes which inhibit RF activity. Both inhibitory effects are overcome by catalytic levels of exogenous RF which permits the resumption of protein synthesis. RF activity and protein synthesis in GSSG-inhibited lysates are efficiently restored by the delayed addition of glucose-6-P or 2-deoxyglucose-6-P (1 mM). The rescue of protein synthesis by hexose phosphate (1 mM) is proportional to the extent of RF recovery and is due in part to NADPH generation; even at levels of hexose phosphate (50 μM) too low to support protein synthesis, partial restoration of RF activity occurs due to increased NADPH/NADP ratios. The ability of dithiothreitol (1 mM) to restore RF activity in GSSG-treated but not heme-deficient lysates also provides evidence for a reducing mechanism which functions at the level of RF. The results suggest that NADPH plays a role in the maintenance of sulfhydryl groups essential for RF activity.

The initiation of protein synthesis in rabbit reticulocytes is rapidly inhibited by the oxidation of intracellular glutathione (1, 2). A similar inhibition is achieved in reticulocyte lysates by the addition of oxidized glutathione (0.02 mM GSSG) (3–5). The inhibition by GSSG is due in part to the activation of the heme-regulated eIF-2α kinase (HRI) (3–5) that specifically phosphorylates the α-subunit (38 kDa) of the initiation factor eIF-2. In contrast to heme deficiency, the activation of heme-regulated eIF-2α kinase in GSSG-treated lysates takes place in the presence of 20 μM hemin. The two inhibitions display similar characteristics and are both reversed by the addition of high concentrations of eIF-2 or by high levels of cyclic AMP (8 mM). As shown in gel-filtered lysates, the two inhibitions are also prevented or reversed by physiological concentrations of glucose-6-P or 2-deoxyglucose-6-P (0.02–1 mM) (5–11). The sugar phosphate is involved in two independent functions in protein chain initiation: (i) the generation of NADPH, and (ii) an effector or cofactor function in some step in initiation (5, 8–11). Both functions are essential to prevent the inhibition caused by the activation of HRI in heme deficiency and in GSSG-treated lysates (5, 8–11). A recent study (11) suggests that NADPH is required in protein synthesis to maintain certain essential proteins (but not eIF-2) in the sulphydryl form, and that the formation of a disulfide bond in one or more of these proteins may be associated with the activation of HRI in heme deficiency.

Of particular interest in the GSSG-induced inhibition is the fate of reversing factor (RF). RF is a multipolypeptide initiation factor (12) involved in the recycling of eIF-2 (13–16). During the formation of the 80 S initiation complex in the last step of the protein chain initiation sequence, eIF-2 is presumed to be released as binary complex eIF-2.GDP. Before eIF-2 can take part in a new round of initiation, the GDP moiety must be replaced by GTP; this function is catalyzed by RF (13–16). In lysates inhibited by the action of heme-regulated eIF-2α kinase, RF interacts with phosphorylated binary complex eIF-2(αP).GDP to form a 15 S complex, RF.eIF-2(αP), in which RF is nonfunctional (17, 18). Reversal of inhibition in heme-deficient lysates is achieved by the addition of 20 μM hemin (20 μM) or MgGTP (2 mM); these components block HRI activity, enabling endogenous protein phosphatase(s) to dephosphorylate the phosphorylated 15 S complex and release functional RF (19).

In the present study we have monitored the activity of reversing factor (RF) directly in GSSG-inhibited lysates in order to determine the role of RF in the mechanism of inhibition. A previous study (18) indicated that, as in heme-deficient dsRNA-inhibited lysates, the loss of RF activity in GSSG-treated lysates was correlated with the shut off of protein synthesis. We show here that (i) GSSG treatment produces a more profound inactivation of RF than heme deficiency and that (ii) the recovery of RF activity and protein synthesis in GSSG-treated lysates may depend upon the restoration of sulfhydryl groups of RF as well as the dephosphorylation of the 15 S RF.eIF-2(αP) complex. The data suggest that NADPH generation plays a role in the maintenance of RF activity. In this regard, a recent study (20) has
determined that isolated RF contains NADPH and that RF function is inhibited by NAD⁺ and NADP⁺.

**EXPERIMENTAL PROCEDURES**

**Materials**—Purified preparations (80–90% pure) of RF (14) and eIF-2 (14, 21) were prepared from reticulocyte lysates as described. 1-[^3H]GDP (304 mCi/mmol), [8-^3H]GDP (9 Ci/mmol), and [γ-^32P]ATP (3000 Ci/mmol) were obtained from Du Pont-New England Nuclear. Glucose 6-phosphate, 2-deoxyglucose 6-phosphate, ATP, GDP, GSSG, GTP, and dithiothreitol were purchased from Sigma.

**Protein Synthesis in Reticulocyte Lysates**—Rabbit reticulocyte lysates were prepared as described (5, 22). Protein synthesis in reticulocyte lysates was carried out in 20–30-μl reaction mixtures as described (5, 22). In most experiments, protein synthesis was carried out under three conditions: (i) plus 20 μM hemin, (ii) minus hemin, and (iii) plus 20 μM hemin plus 1 mM GSSG. Other conditions and additions are detailed in the legends. The extent of protein synthesis was measured by the incorporation of [14C]leucine into protein as described (5, 22).

**Assay for Endogenous RF in Protein-synthesizing Lysates**—RF activity in the lysate was measured by the ability of lysates to stimulate the dissociation of added labeled binary complex eIF-2-[^3H]GDP in the presence of excess unlabeled GDP as described (18, 19). Lysate RF activity was determined in a two-step procedure. In step 1, protein synthesis was allowed to proceed in the presence of unlabeled leucine under various conditions as indicated in the legends. In step 2, lysate RF activity was determined as follows. At various time intervals (5–15 min) an aliquot (15 μl) of the protein synthesis reaction mixture was supplemented with an equal volume of labeled binary complex, and incubation was allowed to proceed for a further 15 min at 30 °C. RF activity was measured by the amount of labeled binary complex dissociated as described previously (18, 19) and detailed in the summary to Table I. Other modifications are described in the legends.

**In Vitro Assay for RF**—RF activity of purified RF was measured by the dissociation of the labeled binary complex eIF-2-[^3H]GDP as described previously (14).

**In Situ [^32P]Phosphoprotein Profiles of Normal and Inhibited Protein-synthesizing Lysates**—Protein synthesis was carried out in the presence of unlabeled leucine as described above and in the summary to Table I. In the course of incubation, reaction mixtures (30 μl) were pulsed with 10 μCi of [γ-^32P]ATP (3000 Ci/mmol) at 5–10 and 10–15 min, and [γ-^32P]labeled phosphoprotein profiles were analyzed as described previously (19, 23). Details are noted in the legend to Fig. 2.

**Sucrose Gradient Fractionation of Lysates**—For the data in Table III, protein synthesis was allowed to proceed at 30 °C for 15 min under three conditions (+h, −h, and +h + GSSG), where h indicates hemin, in 100-μl incubation volumes containing unlabeled leucine. After incubation, protein synthesis mixtures were chilled in ice, brought to 25 μM NaF, and diluted with an equal volume of a dilution buffer containing 40 mM Tris-HCl (pH 7.6), 40 mM KCl, 3 mM Mg(OAc)₂. The mixtures were each layered over 5 ml of a 15–30% linear sucrose gradient containing 25 mM Tris-HCl (pH 7.6), 25 mM KCl, 2 mM Mg(OAc)₂. Centrifugation was at 107,000 × g at 2 °C for 90 min in a Beckman SW50.1 Ti rotor (17). Gradients were collected in 20–0.25-ml fractions by upward displacement with continuous monitoring at 280 nm in an ISCO fractionator. The 15 S RF complex was largely contained in fractions 3 and 4; aliquots were used directly in the experiments in Table III.

**RESULTS**

**Effect of GSSG Addition on Protein Synthesis and RF Activity**—In previous studies (17–19), we described a highly sensitive assay for the detection of endogenous RF activity directly in protein-synthesizing lysates. Based on an estimate of 2–4 nM RF in our lysates, the assay can detect 0.01–0.02 pmol of endogenous RF contained in 5–10 μl of lysate. The assay for lysate RF are carried out in a two-step procedure in which lysates are allowed to undergo protein synthesis under various conditions in step 1; at intervals, aliquots are transferred to RF reaction mixtures containing labeled binary complex eIF-2-[^3H]GDP in step 2. The amount of RF activity in the lysate is measured by the extent or rate of dissociation of labeled binary complex in the presence of excess unlabeled GDP as described under “Experimental Procedures” and in the legend to Table I. As shown in Table I, the addition of increasing concentrations of GSSG (0.1–1.5 mM) normal heme-supplemented lysates (+h + GSSG) resulted in increasing inhibition of protein synthesis and a parallel decrease in RF activity. At high GSSG concentrations (1–1.5 mM), lysate RF activity is often not detectable after protein synthesis shuts off. In contrast, in heme-deficient lysates (−h), a basal level of RF activity (15–20%) is generally observed. The addition of 1 mM dithiothreitol to lysates treated with 1 mM GSSG largely prevented or reversed inhibition (Table I, Experiment II). When dithiothreitol was added at the start of incubation, GSSG was chemically reduced, and normal protein synthesis and RF activity were observed. Addition of dithiothreitol at 5 or 10 min after the start of incubation produced significant restoration of both protein synthesis and RF activity. In contrast, addition of dithiothreitol to heme-deficient lysates did not restore protein synthesis or RF activity (Table I, Experiment II).

**The Effects of Exogenous RF and of MgGTP in GSSG-treated Lysates**—The delayed addition of purified RF, hemin (20 μM), or MgGTP (2 mM) is effective in reversing the inhibition of protein synthesis in heme-deficient lysates (14,

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**Table I**

| Protein synthesis conditions | Protein synthesis: [14C]leucine incorporated (step 1) | Protein synthesis: [14C]leucine in lysates | RF activity: [^3H]GDP released (step 2) |
|-----------------------------|----------------------------------------------------------|---------------------------------------------|----------------------------------------|
|                             | cpm | % | pmol | cpm | % | pmol |
| **Experiment I**            |     |   |      |     |   |      |
| +h                          | 43,095 | 100 | 1.91 | 100 |
| +h + GSSG (0.10 mM)         | 36,112 | 84  | 1.77 | 93  |
| +h + GSSG (0.25 mM)         | 25,716 | 60  | 0.85 | 45  |
| +h + GSSG (0.50 mM)         | 17,201 | 40  | 0.49 | 26  |
| +h + GSSG (1.00 mM)         | 14,716 | 34  | 0    | 0    |
| +h + GSSG (1.50 mM)         | 8,544  | 20  | 0    | 0    |
| −h                          | 10,668 | 25  | 0.29 | 15  |
| **Experiment II**           |     |   |      |     |   |      |
| +h                          | 34,196 | 100 | 2.26 | 100 |
| +h + GSSG (1.00 mM)         | 31,460 | 92  | 2.32 | 103 |
| +h + GSSG (2.50 mM)         | 21,178 | 67  | 0.80 | 40  |
| +h + GSSG (5.00 mM)         | 12,808 | 36  | 0.49 | 26  |
| +h + GSSG (10.00 mM)        | 11,666 | 34  | 0    | 0    |

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18, 19). Hemin and MgGTP exert their effects by blocking lysate HRI activity (9). We examined the effects of the addition of MgGTP or RF in hemin-supplemented lysates inhibited by 0.5 mM GSSG. Catalytic levels of exogenous RF cause a rapid restoration of protein synthesis (Fig. 1B) similar to that observed in heme-deficient lysates (14, 18). MgGTP is considerably less efficient in reversing inhibition in GSSG-treated lysates than in heme-deficient lysates (Fig. 1A). This difference in response to MgGTP is also apparent in the kinetics of restoration of RF activity in the two lysates. MgGTP efficiently restores RF activity in hemin-deficient lysates, but is inefficient in GSSG-treated lysates (Fig. 1C). These data suggest that GSSG acts in two ways to diminish these data suggest that GSSG acts in two ways to diminish RF activity including 

(i) the activation of HRI leading to the formation of the 15 S complex, RF: eIF-2 (eP), and (ii) the formation of mixed disulfides, perhaps of RF itself. This hypothesis would explain why both effects are overcome by added RF, but not by MgGTP. In vitro, we find that purified RF and the labeled binary complex are rendered inactive in the presence of 1 mM GSSG; even pretreatment of purified RF with GSSG under controlled conditions decreases its activity (data not shown).

Effect of Glucose-6-P on Protein Synthesis and RF Activity in GSSG-treated Lysates—Table II demonstrates that both Glc-6-P (1 mM) and 2-deoxy-Glc-6-P (1 mM) prevent or reverse GSSG-induced inhibition of protein synthesis. The delayed addition of 1 mM Glc-6-P at 5 or 10 min to GSSG-treated lysates produces a partial recovery of protein synthesis (Table II, Experiment II). More significantly, both Glc-6-P and 2-deoxy-Glc-6-P are highly effective in restoring RF activity in GSSG-inhibited lysates. This ability is best demonstrated in the reversal of inhibition by the delayed addition (5 or 10 min) of 1 mM Glc-6-P, where RF activity is nearly completely restored (90%) (Table II, Experiment II). Even at low sugar phosphate levels (10–50 μM), where protein synthesis is not restored, RF activity shows recovery from 0% activity to a level (~20%), which is normally observed in heme-deficient lysates. We have previously shown (5) that low sugar phosphate levels do not satisfy the cofactor requirements in initiation but can generate high NADPH/NADP ratios in lysates. The data suggest that RF activity may be dependent upon the availability of NADPH. The efficient recovery of RF activity induced by glucose-6-P or dithiothreitol argues in favor of a reducing action on RF.

Phosphoprotein Profile of GSSG-treated Lysates—The activation of heme-regulated eIF-2α kinase (HRI) in hemin-supplemented GSSG-treated lysates is demonstrated in the phosphoprotein profiles shown in Fig. 2. A pulse of [γ-32P]ATP at 5–10 or 10–15 min after the start of protein synthesis labels those polypeptides whose phosphates are turning over. Fig. 2 shows that in both pulse periods eIF-2α is phosphorylated in heme-deficient lysates (tracks 2 and 5) and in GSSG-inhibited lysates (tracks 3 and 6) but not in normal lysates (tracks 1 and 4). There is also some indication that HRI (85 kDa) is phosphorylated in both pulse periods in heme-deficient lysates (tracks 2 and 5). There does not appear to be a

Fig. 1. Protein synthesis and endogenous RF activity in normal and inhibited reticulocyte lysates. A, protein synthesis mixtures (30 μl) were incubated as described in Table I and under "Experimental Procedures." Where indicated, incubations were supplemented with 2 mM MgGTP or 20 μM hemin at 5 min (arrows). The extent of protein synthesis was determined with time by the incorporation of [3H]leucine into protein in 1 μl of incubation mixture. ●, plus 20 μM hemin (+h); ○, minus hemin (−h); □, plus 20 μM hemin plus 1 mM GSSG (+h + GSSG); ▲, minus hemin plus hemin added at 5 min (−h + h); △, minus hemin plus 2 mM MgGTP added at 5 min (−h + MgGTP); ■, plus hemin plus GSSG with 2 mM MgGTP added at 5 min (h + GSSG + MgGTP). B, protein synthesis mixtures were incubated as in A. Where indicated, incubations were supplemented with 0.1 pmol of RF at 10 min (arrows) and protein synthesis was measured with time as in A. ●, plus hemin; ○, minus hemin; □, plus hemin plus GSSG; ▲, minus hemin plus 0.1 pmol of RF added at 10 min; ■, plus hemin plus GSSG plus 0.1 pmol of RF added at 10 min. C, protein synthesis mixtures were incubated as in A. Where indicated, protein synthesis (step 1) incubations were supplemented with 2 mM MgGTP at 5 min (arrows). RF activity (step 2) was measured at the indicated intervals in 15-μl aliquots of parallel unlabeled protein synthesis incubation mixtures as described in Table I. Additions to step 1 assays: ●, plus hemin; ○, minus hemin; □, plus hemin plus GSSG; ▲, minus hemin plus 2 mM MgGTP added at 5 min; ■, plus hemin plus GSSG plus 2 mM MgGTP added at 5 min.
**TABLE II**  
Effect of Glu-6-P and 2-deoxy-Glu-6-P on protein synthesis and RF activity in normal and inhibited lysates

Protein synthesis reaction mixtures were incubated under three conditions (+h, −h, and +h + GSSG) as described in Table I. Protein synthesis and lysate RF activity were measured as described in Table I and under "Experimental Procedures." As in Table I, the extent of protein synthesis (step 1) was determined after 30 min at 30 °C. Lysate RF (step 2) was determined in parallel unlabeled lysate incubations after 20 min of protein synthesis. In experiment I, Glu-6-P and 2-deoxy-Glu-6-P were added in increasing concentrations at 5 min after the start of incubation. In experiment II, 1 mM Glu-6-P was added at 0, 5, or 10 min as indicated.

| Protein synthesis conditions | Protein synthesis: [14C]Leu incorporated (step 1) | RF activity: [3H]GDP released (step 2) |
|-----------------------------|-----------------------------------------------|----------------------------------|
|                             | cpm                                          | pmol               | %     |
| Experiment I                |                                              |                    |
| +h                          | 32,736                                       | 1.90               | 100   |
| +h + GSSG                   | 10,125                                       | 0                  | 0     |
| +h + GSSG + Glu-6-P (10 μM) | 9,203                                        | 0.25               | 13    |
| +h + GSSG + Glu-6-P (50 μM)| 9,836                                        | 0.50               | 26    |
| +h + GSSG + Glu-6-P (1 mM)  | 23,436                                       | 1.76               | 93    |
| +h + GSSG + 2-deoxy-Glu-6-P (10 μM) | 8,889 | 0.37       | 19    |
| +h + GSSG + 2-deoxy-Glu-6-P (50 μM) | 9,382 | 0.43       | 23    |
| +h + GSSG + 2-deoxy-Glu-6-P (1 mM) | 17,120 | 1.41     | 74    |
| Experiment II               |                                              |                    |
| +h                          | 34,485                                       | 2.13               | 100   |
| +h + GSSG                   | 7,242                                        | 0.23               | 11    |
| +h + GSSG + Glu-6-P (0 min) | 18,058                                       | 2.09               | 98    |
| +h + GSSG + Glu-6-P (5 min) | 15,219                                       | 1.98               | 93    |
| +h + GSSG + Glu-6-P (10 min)| 11,696                                       | 1.85               | 87    |
| −h                          | 9,660                                        | 0.30               | 13    |
| −h + Glu-6-P (0 min)        | 9,057                                        | 0.23               | 11    |

**Table III**  
RF activity in the 15 S RF complexes obtained from the gradient fractions of three lysates

Protein synthesis was carried out under three conditions as in Table I: (i) plus hemin, (ii) minus heme, and (iii) plus hemin plus GSSG. Incubation was carried out for 15 min at 30 °C. Reaction mixtures were treated with NaF at a final concentration of 25 μM and then were separated in sucrose density gradients as described under "Experimental Procedures." The peak fractions containing the nonribosomal 15 S RF complexes were collected, pooled, and assayed. RF activity was measured in a 75-μl aliquot of the pooled 15 S fraction in a final assay volume of 150 μl containing 2.4 pmol of binary complex as described under "Experimental Procedures."

| 15 S RF complex: protein synthesis conditions (step 1) | RF activity: [3H]GDP released (step 2) | pmol | %    |
|------------------------------------------------------|---------------------------------------|------|------|
| +Hemin                                               | 0.85                                  | 100  |
| −Hemin                                               | 0.11                                  | 13   |
| +Hemin + GSSG                                        | 0.05                                  | 6    |

**RF Activity in the 15 S RF Complexes of Normal and Inhibited Lysates—A previous study (17) has shown that most of the lysate RF sediments in a broad 15 S peak during density gradient centrifugation. In heme-deficient lysates, the RF in the 15 S fraction is sequestered in a nonfunctional phosphorylated complex [RF-eIF-2(aP)]. Treatment of the phosphorylated 15 S complex with alkaline phosphatase releases RF activity (17). We examined the RF activity of the isolated 15 S complex obtained from gradient fractions of three lysates (+h, +h + GSSG, and −h). As shown in Table III, we found that the residual RF activity in the 15 S complex obtained from GSSG-treated lysates (+h + GSSG) was lower (6%) than the residual RF activity (13%) in the 15 S complex of heme-deficient lysates (−h). These data correlate with the levels of RF activity found in the intact protein-synthesizing lysates and confirm the finding that RF activity is more inhibited in GSSG-treated lysates than in heme-deficient lysates. Some of these findings are described in preliminary reports (24, 25).
DISCUSSION

The inhibitions of protein chain initiation in heme-deficient lysates and in GSSG-treated lysates are both characterized by the activation of HRI and the inactivation of RF (3–5, 8–11, 18). However, in GSSG-inhibited lysates, RF inactivation appears to be more profound than that observed in heme deficiency. We conclude that the inhibition of protein chain initiation in GSSG-treated reticulocyte lysates results mainly from two separate but additive effects which diminish RF activity. These are (i) the sequestration of RF in a 15 S phosphorylated complex, RF-eIF-2(aP), resulting from the activation of HRI; and (ii) the oxidation of sulfhydryl groups required for functional RF activity. We have utilized a sensitive RF assay which enabled us to examine the fate of endogenous RF directly in protein-synthesizing lysates. Previous studies (5, 8–11) have demonstrated a requirement for NADPH generation at some step in initiation. Our present findings support the hypothesis that NADPH generation plays a role in the maintenance of RF activity by preventing or reversing the formation of RF disulfide complexes. A recent study by Dholakia et al. (20) indicates that RF function is controlled by an oxidation-reduction equilibrium; they demonstrated that isolated RF contains bound NADPH, and under appropriate conditions, both NADPH and NADP+ inhibit RF activity in vitro.

As in heme-deficient lysates, the rescue of protein synthesis in GSSG-treated lysates is dependent upon and is proportional to the extent of RF recovery. Efficient restoration of RF activity in GSSG-treated lysates is achieved by the delayed addition of glucose-6-P. Previous studies have shown that glucose-6-P has two functions in protein chain initiation: (i) the generation of NADPH, and (ii) a putative effector function in some step in initiation. Both functions are also fulfilled by 2-deoxyglucose-6-P which generates NADPH, owing to its requirement for NADPH in the conversion of GSSG to GSH by glutathione reductase (5).

GSSG produces a rapid depletion of lysate NADPH and glucose-6-P, due to the requirement for NADPH in the conversion of GSSG to GSH by glutathione reductase (5). The delayed addition of Glc-6-P restores NADPH levels, giving rise to high steady state NADPH/NADP+ ratios (5) in which >80% of the nucleotide is in the reduced state. Even at Glc-6-P concentrations too limiting (10–50 μM) to rescue protein synthesis, NADPH/NADP+ ratios are increased sufficiently to yield some recovery of RF activity.

Further evidence which emphasizes the importance of reduced sulfhydryls in RF activity is suggested by the results obtained with dithiothreitol, which has no effect in heme-deficient lysates, but promotes rapid RF recovery in GSSG-inhibited lysates. Although GSSG is rapidly metabolized in lysates (5), its inhibitory effects are expressed within a few minutes of the start of incubation. The rapid restoration of RF activity by the delayed addition of dithiothreitol (at 10 min) argues in favor of a direct reduction of disulfide complexes of RF. However, the molecular composition of RF disulfide complexes is not clear; possible structures include intra- or intermolecular disulfides, as well as mixed disulfides with GSSG. An alternative possibility is that GSSG oxidizes sulfhydryl group(s) of an RF-associated activity such as casein kinase II, which appears to increase RF activity in vitro by phosphorylation of the largest RF subunit (82 kDa) (26). In similar studies we have found that both casein kinase I and II phosphorylate the 82-kDa subunit of RF and that these activities are largely blocked by 1 mM GSSG (data not shown). At present, there is no evidence that GSSG has any other deleterious effect on protein synthesis. The conclusion that RF is susceptible to two modes of inactivation and is the principal site of inhibition is supported by the observation that exogenous RF alone efficiently restores protein synthesis.

The mechanism of HRI activation induced in lysates by GSSG remains unclear. In GSSG-treated lysates, HRI activation takes place in the presence of sufficient hemin (20 mM) to sustain optimal protein synthesis in normal lysates. In addition, many experiments have shown that GSSG (0.1–1 mM) has little or no direct effect on the activation of purified HRI in vitro. These phenomena suggest an indirect activation mechanism in the lysate.

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