Irreversible Steps in the Ferritin Synthesis Induction Pathway*

Lissa S. Goessling, David P. Mascotti, Maitrayee Bhattacharyya-Pakrasi, Hu Gang, and Robert E. Thach†

From the Department of Biology, Washington University, St. Louis, Missouri 63130

The ability of cells to repress ferritin synthesis after removal of an inducing agent (iron or heme) was investigated. Re-repression, requiring approximately 4 (after iron removal) to 10 h (after heme removal) for completion. Desferrioxamine mesylate (Desferal) had only a slight effect on the rate of re-repression, whereas cycloheximide was strongly inhibitory, indicating that new protein synthesis is required for re-repression. Re-repression occurred at a slow but significant rate in the presence of both Desferal and cycloheximide. These results indicate that, in the absence of an iron chelator, the induction of ferritin synthesis is essentially irreversible.

The kinetics of the previously reported covalent modification of IRE-binding protein (IRE-BP) were then examined, to see whether this phenomenon might account (at least in part) for the irreversibility of induction. It was found that the heme- or iron-dependent disappearance of 98-kDa IRE-BP occurred rapidly (within 1 h), and was equally rapidly reversed upon removal of heme after a 1-h exposure. By contrast, after a 4-h exposure to heme, little 98-kDa IRE-BP could be regenerated after heme removal. These results suggest that the slow, irreversible step of the IRE-BP modification pathway closely over time with the induction of ferritin synthesis. The covalent modification of IRE-BP depends on cell growth rate, and is most readily detected in rapidly growing cells.

Synthesis of the iron storage protein, ferritin, is regulated at the translational level in higher eukaryotes (Eisenstein and Munro, 1990/1991; Klausner et al., 1993; Theil, 1990; Lin et al., 1990/1991; Kuhn, 1991; Rogers, 1992; Melefiors and Hentze, 1993). The regulatory mechanism includes a conserved stem/loop structure in the 5′-untranslated region of the ferritin mRNA (the iron responsive element (IRE)) and a 98-kDa protein that binds to the IRE in the absence of iron, thereby inhibiting translation. This protein is inactivated in the presence of iron or heme. It has been variously named the ferritin repressor protein, the IRE-binding protein (IRE-BP), and the iron regulatory factor. A related minor protein, IRE-BP II, has been identified by cloning and by band shift analysis (Rouault et al., 1989; Leibold and Munro, 1988). IRE-BP can bind iron to form a Fe₄S₄ cluster, the presence of which reduces IRE binding activity, but creates an aconitase active center (Kennedy et al., 1992; Haile et al., 1992a, 1992b; Constable et al., 1992).

The presence of iron can also trigger the covalent modification, and subsequent disappearance of IRE-BP (Goessling et al., 1992). This reaction is stimulated by porphyrin precursors (such as 5-aminolevulinic acid (ALA)) and is inhibited by antagonists of porphyrin synthesis (such as succinylacetone), which suggests the involvement of heme. This reaction was presumed to be irreversible, since it coincided with the appearance of small amounts of low molecular weight products. In contrast, the formation of the Fe₄S₄ center has been proposed to be readily and easily reversible (Constable et al., 1992; Tang et al., 1992; Klausner et al., 1993).

In order to determine whether either of these two iron-dependent reactions (iron-sulfur center formation or covalent modification) actually regulates ferritin synthesis in vivo, we have sought to determine whether induction of ferritin synthesis itself is reversible. To this end, we have studied the conditions under which repression is restored following a period of induction (i.e. "re-repression"). Both heme and iron (as ferric ammonium citrate (Fe³⁺) plus transferrin) were used as inducers. In both cases, it was found that cycloheximide inhibits re-repression in a dose-dependent manner, even in the presence of the iron chelator, desferrioxamine mesylate (Desferal). This result indicates that induction of ferritin synthesis is either not thermodynamically reversible, or is kinetically blocked under physiological conditions, and that de novo synthesis of some protein, probably IRE-BP itself, is required for re-repression.

These observations prompted a further investigation of the IRE-BP covalent modification pathway. In particular, it was of interest to determine which of the two steps previously described (Goessling et al., 1992) is irreversible. Thus the initial formation of a high molecular weight species (HMS), as well as its subsequent disappearance, were examined for reversibility. We also wished to determine whether either of these steps correlates in time with the induction of ferritin synthesis. The results of these experiments indicate that the formation of HMS, which is very fast, is reversible, whereas the slower disappearance of HMS is not. Moreover, it is evident that the induction of ferritin synthesis correlates closely in time with the second, irreversible, step.

Finally, we have sought to determine why others have reported difficulties in observing the IRE-BP covalent modification phenomena (Tang et al., 1992). A possible explanation arises from our observation that the degree of IRE-BP modification is dependent on cell growth rate. Thus while IRE-BP disappearance triggered by either heme or iron is readily apparent in growing cells, this phenomenon is reduced in non-growing cells. These and other results suggest that the variation in results reported by different laboratories may be explained by differences in cell culture conditions. The implications of these observations for the biochemical mechanism of ferritin induction are discussed.

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† To whom correspondence should be addressed. Tel.: 314-935-6775; Fax: 314-935-4432.

The abbreviations used are: IRE, iron responsive element; ALA, 5-aminolevulinic acid, IRE-BP; IRE-binding protein; PAGE, polyacrylamide gel electrophoresis; Desferal, desferrioxamine mesylate; HMS, high molecular weight species.
Irreversibility of Ferritin Induction

EXPERIMENTAL PROCEDURES

Cell Culture—A normal rabbit skin fibroblast line, RAB-9, was used for all experiments reported. These cells were seeded at low density in Earle’s minimal essential medium supplemented with 2% fetal calf serum and nonessential amino acids (alanine, aspartate, glutamate, glycine, proline, and serine, all at 0.1 mM) and 1 mM sodium pyruvate. Cells were allowed to grow for 2 days, by which time they had reached from two-thirds to three-fourths confluency, or for 7 days by which time all growth had ceased, prior to conducting the experiments described.

Analysis of Radiolabeled IRE-BP by Immunoprecipitation—RAB-9 cells were labeled with [35S]methionine plus [35S]cysteine (Trans33-S-label; ICN) for 2 h in serum-free medium and then transferred to spent media that contained 1% unlabeled methionine and, where indicated, 100 μM ferric ammonium citrate (Fe+++ ) plus 0.2 mg/ml transferrin, 200 μg desferroxamine mesylate (Desferal), 2 mM 5-amino-2-norleucine (AL-5), 7.5 mM succinylacetone, or heparin at the indicated concentrations and times. Cells were lysed by addition of immune buffer (phosphate-buffered saline solution, supplemented with 1% deoxycholate, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA) or band shift lysis buffer (25 mM Tris-HCl, pH 7.4, 40 mM KCl, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.7 μg/ml pepstatin A, and 7 μM β-mercaptoethanol). Residual radiolabeled IRE-BP was then analyzed by immune precipitation of equal counts per min of labeled lysate (pre-treated with 1% SDS at 100 °C for 5 min, then diluted 5-fold with immune buffer that contained bovine serum albumin (15 mg/ml) and no SDS) with rat anti-rabbit IRE-BP serum, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and fluorography, all as previously described (Goessling et al., 1992).

Analysis of Ferritin Synthesis Rate by Immune Precipitation—Ferritin synthesis was induced by treatment of cells with heme or Fe+++ plus transferrin. In some experiments, the inducing medium was replaced with fresh media containing Desferal, cycloheximide, or no supplement, and incubation was continued for the times indicated. Newly synthesized proteins were then labeled for equal quantities of protein (usually 5 μg) by the technique of Leibold and Munro (1992) as modified by Walden et al. (1992). At various times thereafter, samples of cells were labeled with [35S]methionine plus [35S]cysteine for 1 h. Labeled ferritin was then analyzed by cell lysis, immunoprecipitation with anti-ferritin antibody and alkaline phosphatase-conjugated to goat anti-rat IgG antibody, as described by Blake et al. (1984).

RNA Band Shift Assays—Cells were lysed in band shift buffer, and equal quantities of protein were analyzed by SDS-PAGE, and then transferred by electroblotting for 36 h at 100 mA onto nitrocellulose (Schleicher & Schuell) membranes. IRE-BP was then detected using anti-IRE-BP antibody and alkaline phosphatase-conjugated to goat anti-rabbit IgG antibody, as described by Blake et al. (1984).

RESULTS

Ferritin synthesis in rabbit fibroblasts (RAB-9) was induced by addition of 100 μM Fe+++ plus 0.2 mg/ml transferrin to the culture media as previously described (Goessling et al., 1992). At various times thereafter, samples of cells were labeled with [35S]methionine plus [35S]cysteine for 1 h. Labeled ferritin was then analyzed by cell lysis, immunoprecipitation with anti-ferritin antibody, SDS-PAGE, and fluorography. The results of this experiment (Fig. 1A) show that induction of ferritin synthesis is relatively slow, peaking at 4–5 h after iron addition. Interestingly, the induction process did not always stop immediately upon removal of iron, especially at early times. Thus after 2 h of iron treatment, ferritin synthesis continued to rise for at least 1 h after iron removal, prior to declining. This behavior is consistent with the interpretation that iron does not immediately inactivate IRE-BP, but rather potentiates a much slower inactivation process. Re-repression of ferritin synthesis was then studied by removing excess iron and returning cells to the original media. In some cases, Desferal (at 200 μM) or cycloheximide (at 3 μg/ml) was included in this re-repression media (cycloheximide was removed from the media prior to pulse labeling; see “Experimental Procedures”). In Fig. 1B it is evident that the resumption of the repressed state after iron removal is slow, requiring over 4 h to complete. This process is only slightly affected by the presence of Desferal. By contrast, inclusion of cycloheximide strongly inhibits re-repression (Fig. 1B), either in the presence or absence of Desferal. In most experiments a very slow re-repression could be observed in the presence of both cycloheximide and Desferal (compare lanes 14 and 18 in Fig. 1B). This re-repression does not appear to occur to a significant extent in the absence of Desferal, since even overnight treatment with cycloheximide prevents full re-re-

![Image of Fig. 1](image-url)
persion to be achieved (Fig. 1C). Similar results were obtained when ferritin synthesis was induced with heme instead of iron (Fig. 2). However, in this case re-repression is slower than after induction by iron. This observation may reflect the fact that heme is a considerably more effective inducer of ferritin synthesis than iron in growing cells, and causes a more extensive loss of IRE-BP; moreover, the effects of heme are substantially more resistant to Desferal treatment than those of iron (Goessling et al., 1992).

Prolonged treatment of cells with cycloheximide alone sometimes produced detectable induction of ferritin synthesis (Fig. 2B, lanes 5 and 6). A similar effect was observed with actinomycin D (Daniels-McQueen et al., 1992). Both effects may be a manifestation of the slow turnover of IRE-BP that is due to slow levels of iron present in normal growth media.

These results suggest that protein synthesis is ordinarily required for the re-repression of ferritin synthesis following induction. This conclusion is consistent with previous studies which suggested that IRE-BP may be degraded in the presence of iron during induction (Goessling et al., 1992). We next sought to obtain more direct evidence that this apparent loss of IRE-BP is in fact irreversible. (If it were not, then our current results might be explained by a requirement for de novo synthesis of some protein other than IRE-BP, which is necessary for re-repression.) It was also of interest to determine whether either of the two steps previously described, the rapid conversion of IRE-BP to HMS, and the slow disappearance of HMS, might be reversible. (The presence of only one irreversible step in a pathway could be sufficient to render the overall process irreversible.) Indeed, the results in Fig. 3 show that approximately one-half of the prelabeled IRE-BP that is apparently lost during a 1-h exposure to heme can be recovered by subsequent incubation in the absence of heme.

A similar result was observed when total IRE-BP was detected by immunoblotting (Fig. 4). Thus IRE-BP that had apparently been lost can be partially regenerated. The source of this recoverable IRE-BP may be the HMS. (This material is difficult to remove from the SDS-PAGE gel, so it frequently is not detected by immunoblotting. Since the technique employed allows electrophoretic transfer of a 200-kDa marker protein, it seems likely that HMS may possess a branched-chain structure.) In any event, these results indicate that a covalent modification of IRE-BP can be partially reversed if the exposure to heme is sufficiently brief. This reversal process is quite rapid, being virtually complete in 1 h (Fig. 4).

By contrast, when the period of heme treatment was extended to 4 h, little of the lost IRE-BP could be recovered in the 98-kDa region (Fig. 5, A and B). Similar results were obtained when the samples were analyzed by immunoprecipitation (Fig. 5C). This confirms the ultimate irreversibility of the heme-dependent loss process.

Quantitative comparison of immunoblot with immunoprecipitation data sometimes revealed a component of the IRE-BP population that is resistant to heme-dependent degradation. This "refractory component" represents up to 25% of the total IRE-BP (Fig. 6). This iron-stimulated loss of IRE-BP is also detectable by immunoprecipitation (see "Experimental Procedures"). The arrow indicates IRE-BP, at ~100 kDa, and HMS, at ~200 kDa.

These data confirm that the loss of IRE-BP is at least a two-step process, the first being fast and reversible, the second being slower and irreversible. Comparison of the data in Figs. 3–7 with the data shown in Figs. 1 and 2 indicates that the rapid, reversible step occurs well before any ferritin synthesis

**Fig. 2. Induction of ferritin synthesis by heme and re-repression after heme removal.** The experimental protocols were similar to those described in the legend to Fig. 1, except that ferritin synthesis was induced by 20 μM heme, instead of iron. Where indicated, 400 μM Desferal was present.

**Fig. 3. Reversibility of heme effect on IRE-BP after 1 h of treatment.** Rabbit RAB-9 cells were labeled with [35S]methionine plus [35S]cysteine for 2 h (first incubation), and then chased with excess cold methionine (1 ms) in the presence of 50 μM heme (H), or 200 μM Desferal (D), or 20 μg/ml cycloheximide (C) for the indicated times (second incubation). Heme was then removed, and incubation was continued for the indicated times and conditions (third incubation). Labeled IRE-BP was then analyzed by immunoprecipitation with anti-IRE-BP antibody, SDS-PAGE, and fluorography. The arrows indicate IRE-BP, at ~100 kDa, and HMS, at ~200 kDa.

**Fig. 4. Kinetics of reversal of heme effect as indicated by immunoblotting.** Experimental conditions and procedures were as described in the legend to Fig. 3, except that 20 μM heme was used, and IRE-BP was analyzed by immunoblotting (see "Experimental Procedures"). The arrow indicates IRE-BP, at ~100 kDa.

These results are consistent with the suggestion that newly synthesized heme is necessary for this process. By contrast, in the absence of cycloheximide, de novo synthesis of IRE-BP occurred after iron removal, which ultimately restored IRE-BP to its initial level (Fig. 7B).
Fig. 5. Irreversibility of heme effect on IRE-BP after 5 h of treatment. Rabbit fibroblasts (RAB-9) were labeled with [35S]Met plus [35S]Cys for 2 h (first incubation), and then chased with excess cold methionine (1 mM) in the presence of 50 μM heme (H), or 200 μM Desferal (D), or 20 μg/ml cycloheximide (C) for 4 or 7 h (second incubation). Where indicated, heme was removed and the incubation was continued for an additional 3 or 6 h (third incubation). At the end of each incubation series, the residual prelabeled IRE-BP was then analyzed by immunoprecipitation with anti-IRE-BP antibody, SDS-PAGE, and fluorography, and quantitated by densitometry. Densitometric quantitation of residual 98-kDa repressor as detected by immune techniques by using the band shift assay to monitor RNA binding activity. These experiments were done with and without pretreatment with 2% β-mercaptoethanol (Leibold and Munro, 1988; Haile et al., 1992a). The results shown in Fig. 8 indicate that this loss of activity is specific to IRE-BP, as little activity ascribable to IRE-BP II could be detected. Similar results have recently been obtained by others.2

In contrast to the results described here, others have reported no loss of IRE-BP activity or protein in response to iron or heme treatment (Tang et al., 1992). A comparison of these authors’ results with ours suggested that the difference observed may be due to differences in cell growth conditions. (In our system, pretreatment of cells for 16 h with 50 μM heme or 100 μM Desferal, as described by Tang et al. (1992), would inhibit cell growth.) To test this hypothesis, we compared the heme response of growing cells to non-growing cells (Figs. 8 and 9). The differences are striking: while a loss of IRE-BP is clearly evident in growing (two-thirds confluent) cells, this loss is difficult to detect after 5 additional days in culture without feeding. Similarly, labeling experiments followed by immunoprecipitation reveal only a slight loss of newly synthesized

2 E. Leibold, personal communication.
Additions: D H

IRE-BP (not shown). Thus the refractory component of IRE-BP previously noted in Fig. 5 appears to have increased in the absence of cell growth.

These observations prompted us to compare the induction of ferritin synthesis in growing versus non-growing cells. Results of such an experiment are shown in Fig. 10. Two differences are evident: (i) growing cells tend to be more responsive to heme than non-growing cells; (ii) induction of ferritin synthesis in non-growing cells is a slower process than in growing cells. These observations not only may account for variability in published results, but they also suggest the existence of biochemically distinct iron- and heme-dependent pathways which...
may act in variable proportions to regulate iron metabolism as a function of cell growth.

DISCUSSION

The results presented here indicate that the induction of ferritin synthesis is ordinarily an irreversible process in growing cells: synthesis of new protein is required for full re-repression. While addition of Desferal after iron removal can cause a partial restoration of the repressed state, this process is much slower than the normal re-repression mechanism. This Desferal-dependent re-repression may be due to the removal of iron from cytoplasmic aconitase, thereby regenerating the apo form of IRE-BP, which is an active repressor, as shown by others (Walden et al., 1989; Haile et al., 1992).

Results presented here also demonstrate that IRE-BP is irreversibly lost during the induction process, and that the time course of this phenomenon inversely parallels that of ferritin synthesis. Results obtained with this preparation were indistinguishable from cytoplasmic aconitase, thereby rendering it invisible to our antibody preparation and, thus, consistent with the hypothesis that IRE-BP loss can be a direct cause of induction, which would explain why new IRE-BP ordinarily must be synthesized in order to restore the repressed state.

Previous experiments have suggested that the irreversible loss of IRE-BP is due to its proteolytic degradation. However, more detailed information about this process has been difficult to obtain, due to the small amounts and short half-lives of putative degradation intermediates. We have considered the possibility that some of the apparently "lost" IRE-BP protein is reversibly lost during the induction process, and that the time course of this phenomenon inversely parallels that of ferritin synthesis. In any case, both reactions must be irreversible under physiological conditions, as deduced by the fact that induction of ferritin synthesis itself is irreversible. The relative impact of these two reactions on the induction of ferritin synthesis may depend on the cellular growth rate, as well as on cell type (Eisenstein and Munro, 1990/1991).

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