Biol Metals (1991) 4:48–55

BIOLOGY OF METALS

Characteristics of the interaction of the ferritin repressor protein with the iron-responsive element

Greg R. Swenson, Maria M. Patino, Mannie M. Beck, Livia Gaffield and William E. Walden

Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, IL 60612, USA

Summary. The iron-responsive regulation of ferritin mRNA translation is mediated by the specific interaction of the ferritin repressor protein (FRP) with the iron-responsive element (IRE), a highly conserved 28-nucleotide sequence located in the 5′ untranslated region of ferritin mRNAs. The IRE alone is necessary and sufficient to confer repression of translation by FRP upon a heterologous message, chloramphenicol acetyltransferase, in an in vitro translation system. The activity of FRP is sensitive to iron in vivo. Cytoplasmic extracts of rabbit kidney cells show reduction of FRP activity when grown in the presence of iron, as detected by RNA band shift assay. Using a nitrocellulose filter binding assay to examine the interaction of FRP with the IRE in more detail, we find that purified FRP has a single high-affinity binding site for the IRE with a K_d of 20–50 pM. Hemin pretreatment decreases the total amount of FRP which can bind to the IRE. This effect is dependent on hemin concentration. Interestingly, the FRP which remains active at a given hemin concentration binds to the IRE with the same high affinity as untreated FRP. A variety of hemin concentrations were examined for their effect on preformed FRP/IRE complexes. All hemin concentrations tested resulted in rapid complex breakdown. The final amount of complex breakdown corresponds to the concentration of hemin present in the reaction. The effect of hemin on FRP activity suggests that a specific hemin binding site exists on FRP.

Key words: Ferritin – Repressor – Iron-responsive element – Translation – Iron – Hemin

Offprint requests to: W. E. Walden

Abbreviations. IRE, iron-responsive element; FRP, ferritin repressor protein; CAT, chloramphenicol acetyltransferase; ORF, open reading frame

Introduction

Ferritin is the major intracellular iron-storage protein in eukaryotes. Its synthesis is regulated at the level of translation in coordination with the iron status of the cell (for review see Theil 1987). When iron is low, the majority of ferritin mRNA in the cell is in the form of free, untranslated mRNP (Zahringer et al. 1976; Walden and Thach 1986; Aziz and Munro 1986). An increase in iron stimulates ferritin mRNA translation, resulting in a shift of ferritin message into polysomes (Zahringer et al. 1976; Aziz and Munro 1986). Induction of translation of pre-existing ferritin mRNA can result in as much as a 50-fold increase in ferritin synthesis (Shull and Theil 1982).

At least two components facilitate the effect of iron on translation of ferritin mRNA. The first is a highly conserved sequence of approximately 28 nucleotides found in the 5' untranslated region of all ferritin mRNAs for which sequence is known (Murray et al. 1987; Hentze et al. 1987; Aziz and Munro 1987; Theil 1990). This sequence has been named the iron-responsive element (IRE) by virtue of its ability to confer iron-regulated translation upon a mRNA in vivo (Hentze et al. 1987; Aziz and Munro 1987). Furthermore, deletion of this element from ferritin mRNA renders it unresponsive to iron (Hentze et al. 1987; Aziz and Munro 1987). The second component of this regulatory system is a translational repressor which specifically represses ferritin mRNA translation in an iron-responsive manner (Zahringer et al. 1976). Such a repressor has been identified in rabbit reticulocytes (Walden et al. 1988), and recently purified from rabbit liver (Walden et al. 1989). This repressor is a 90-kDa protein which has been named the ferritin repressor protein (FRP; Walden et al. 1989). Its properties are that it is a highly specific inhibitor of ferritin mRNA translation in vitro, and it binds specifically to transcripts containing the IRE sequence (Brown et al. 1989; Walden et al. 1989). FRP does not inhibit translation of ferritin mRNAs from which the IRE sequence has been deleted, nor does it have an effect on the translation of other

mRNAs (Walden et al. 1988; Brown et al. 1989; Walden et al. 1989). Proteins with similar characteristics and activity have been purified from human liver (IRE binding protein; Rouault et al. 1989) and from human placenta (iron responsive factor; Neupert et al. 1990). Both of these have been shown to bind specifically to the IRE sequence, but have not yet been reported to inhibit translation of ferritin mRNA in vitro.

Transferrin receptor synthesis is also regulated by iron (Rao et al. 1985; Rao et al. 1986). In contrast to ferritin, however, this synthesis is depressed in cells exposed to elevated levels of iron. It is regulated by iron primarily at the level of mRNA stability (Mullner and Kuhn 1988; Mullner et al. 1989). Transferrin receptor mRNA is degraded much more rapidly in iron-replete cells than in iron-poor cells. It has been shown that sequences contained within the 3′ untranslated region of the transferin receptor mRNA are required for regulation of stability of this mRNA in response to iron (Owen and Kuhn 1987; Casey et al. 1989). Interestingly, five IRE-like sequences have been identified within the regulatory region of this mRNA and recent results have shown that these IREs are involved in its iron-regulated instability (Casey et al. 1988; Casey et al. 1989). This implicates FRP in the regulation of transferrin receptor synthesis as well (Klausner and Harford 1989). Additional evidence supporting this notion is that the IRE binding protein isolated from human tissues binds specifically to the transferrin receptor IREs in RNA binding asays (Casey et al. 1988; Neupert et al. 1990).

The simplest model explaining the mechanism through which iron regulates the synthesis of ferritin and tansferrin receptor is that iron interacts directly with FRP, thus preventing its binding to the IRE. As a result, translation of ferritin mRNA is derepressed, while a putative nuclease-sensitive site is exposed on the transferrin receptor mRNA causing rapid degradation. However, free iron salts do not inhibit FRP activity in vitro (Lin et al. 1990a). It is highly possible that, in vivo, iron has its effect through another more complicated mechanism, or that another form of iron is the 'regulator'. In vitro repression of ferritin mRNA translation by FRP can be relieved by hemin (Lin et al. 1990a). Thus, it has been proposed that hemin is a form of iron which regulates FRP activity in vivo (Lin et al. 1990a). Evidence from other studies suggest that exposure of cells to elevated levels of iron leads to inactivation of FRP through an oxidation/reduction pathway involving critical sulfhydryls (Hentze et al. 1989). The regulation of FRP activity through oxidation and reduction has been called the sulfhydryl switch (Hentze et al. 1989). It is an interesting notion that FRP activity could be regulated through multiple pathways.

In this communication we present results from a study of the interaction of the FRP with the IRE. Our findings show that this interaction is very tight, having a K_d of the order of 40 pM. Binding of FRP to the IRE is sufficient for translational repression in vitro since a heterologous mRNA carrying the IRE at its 5′ end is repressed by FRP in vitro. Interestingly, hemin reduces the amount of active FRP but has no effect on the affinity of the FRP/IRE interaction. The relevance of these findings to translational repression and iron induction in vivo is discussed.

Materials and methods

Purification of FRP. FRP was purified as previously described (Walden et al. 1989).

Constructs and in vitro transcriptions. All constructs used for in vitro transcriptions were cloned into pTZ18R (United States Biochemical Corp.) adjacent to the promoter for T7 polymerase. Construction of pTZM1 and pTZNS2 were described previously (Walden et al. 1988). The chloramphenicol acetyltransferase (CAT) gene was excised from pCM1 (Pharmacia) as a 780-base-pair *Sal*I fragment. This fragment was cloned into the *Sal*I site of pTZ18R, placing its transcription under the direction of the T7 promoter. The CAT gene fragment was similarly cloned into the plasmid pTZM1, placing it under the direction of the T7 promoter and 3′ of a 92-base-pair sequence from the human ferritin L-chain cDNA which contains the IRE (Walden et al. 1988). Orientation of cloned fragments was determined by restriction mapping and sequencing.

Transcripts made for binding studies were synthesized in vitro in the presence of 120 μCi [α-^{32}P]UTP (Amersham Corp.; 800 Ci/mmol), 300 μM each of ATP, CTP, GTP, 200 μM unlabeled UTP, transcription buffer (40 mM Tris/HCl pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine), 64 U RNasin and 50 U T7 RNA polymerase in a 40 μl reaction volume. The resulting transcripts were treated with RNase-free DNase, phenol/chloroform extracted, and passed through Sephadex G-50. The transcripts were purified on a 10% polyacrylamide/8 M urea gel and located by autoradiography. RNA was eluted from the gel in a buffer containing 0.1% SDS, 1 mM EDTA and 5 mM ammonium acetate, run over a Sephadex G-50 column to remove SDS, and collected by ethanol precipitation.

CAT transcripts to be used in in vitro translations were synthesized using the same reaction conditions except that ATP, UTP, and CTP were used at 1 mM, GTP was used at 0.5 mM and 1 mM $m^7G(5')ppp(5')G$ cap analog was added; 5 μCi [α-^{32}P]UTP was also added to allow quantification and the gel purification step was omitted.

In vitro translations. In vitro translations in wheat germ extracts (Promega Biotech) and analysis of translation products by SDS/PAGE were performed as described previously (Walden et al. 1988). In all cases [^{35}S]methionine (Amersham Corp.; ≥600 Ci/mmol) was used as label. Each reaction contained 10 fmol transcript, 5 μl wheat germ extract and the indicated amounts of FRP. Reactions were performed at 25° C for 1.5 h.

In vivo iron effects. Rabbit kidney cells (RK-1; kindly provided by Dr W. Carey Hanly) were grown to approximately 90% confluence, after which cells were incubated for the indicated times in growth media containing 100 μM ferric ammonium citrate. Control cells were incubated in normal growth media for the same periods. Extracts were prepared by freeze thawing cells in 5 ml of buffer containing (10 mM HEPES Kolt pH 7.5, 10 mM KCl, 1 mM dithiothreitol). The cell extracts were centrifuged at 100000 × *g* for 1 h and the supernatant was removed and concentrated by ultrafiltration.

Binding assays were performed using ^{32}P-labeled transcripts made from the pTZM1 vector. Reactions were performed at 4° C by incubating this transcript (10000 cpm; 0.008 pmol) with 25 μg extract and analyzed following published procedures (Leibold and Munro 1988).

Nitrocellulose filter binding assay. Protein saturation experiments were performed by adding increasing amounts of FRP over a range of 1 pM to 20 nM to a constant amount of ^{32}P-labeled RNA

transcript (19.2 pM). Standard binding conditions consisted of 50 mM Tris/HCl pH 7.5, 40 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 20 μg/ml bovine serum albumin, and 66 μg/ml *Escherichia coli* tRNA. Reactions were performed at 37° C for 20 min at which time samples were passed through 24-mm nitrocellulose filters (Millipore-HAWP) which had been soaked in binding buffer for a minimum of 30 min prior to use. Sample filtration was followed by a wash with 2 vol. binding buffer. Filtration time of samples was approximately 2 s. Aliquots of labeled RNA were spotted on filters to determine the amounts of input RNA. Filters were dried at 80° C for 30 min in a vacuum oven prior to counting.

Experiments for Scatchard analysis were performed in triplicate in a similar manner except that protein was held constant while varying the amount of ^{32}P-labeled transcript. Curve fitting was performed with the aid of the computer program Ligand (Munson and Rodbard 1980).

FRP was pretreated with hemin at 37° C for 10 min in the presence of a glutathione redox buffer as previously described (Lin et al. 1990a). (Indistinguishable results were obtained in the absence of the glutathione buffer.) To analyze the effects of hemin on preformed FRP/IRE complexes, FRP was incubated with ^{32}P-labeled M1 transcript for 20 min at 37° C at which time hemin was added at the concentrations indicated. Aliquots were removed at timed intervals after hemin addition and filtered through nitrocellulose as described above.

Results

Analysis of FRP binding to RNA transcripts

Repression of ferritin mRNA translation in vitro by FRP is believed to result from the specific interaction of FRP with the IRE (Brown et al. 1989; Walden et al. 1989). In order to study this interaction in more detail, we employed a nitrocellulose filter binding assay. Purified FRP was incubated with various in vitro transcripts and the mixture was passed through a nitrocellulose filter under conditions in which only protein or protein/nucleic acid complexes would be retained. Figure 1 shows the results of protein saturation experiments using an IRE-containing transcript, M1, and a transcript lacking the IRE, NS2 (Fig. 1A). Consistent with previous results (Walden et al. 1989), FRP binds to the M1 transcript, showing typical saturation kinetics. Half-maximal binding is attained at approximately 100 pM FRP, suggesting that the K_d of the M1/FRP interaction is of this magnitude. In contrast, the NS2 transcript showed no binding up to 10 nM FRP. This shows that interaction of FRP with non-IRE-containing sequences is very poor, having a K_d much greater than 10 nM. These results are consistent with previous findings which showed that binding of FRP to M1 transcripts is not competed by non-IRE transcripts (Walden et al. 1989).

IRE-dependent repression of CAT mRNA translation by FRP

It has been shown that the IRE is sufficient to confer iron regulation to a mRNA in vivo (Caughman et al. 1988). The results presented in Fig. 1 suggest that this should be true for repression by FRP in vitro as well. To investigate this further, we appended the M1 sequence onto a complete CAT open reading frame (ORF), giving the transcript M1/CAT (Fig. 2A). This transcript was translated in the wheat germ extract in the presence of varying amounts of FRP. Translation of 18R/CAT, a mRNA which lacks the IRE (Fig. 2A), is unaffected by the addition of FRP to the wheat germ extract (Fig. 2B, compare lanes 3 and 4 with lane 2). In sharp contrast, addition of FRP to a wehat germ extract programmed with M1/CAT resulted in inhibition of CAT synthesis (Fig. 2B, compare lanes 6 and 7 with lane 5). These results demonstrate that the IRE is both necessary and sufficient for repression of translation by FRP in vitro. Furthermore, they are consistent with findings which show that the IRE is necessary and sufficient to confer iron responsiveness to a mRNA in vivo (Caughman et al. 1988).

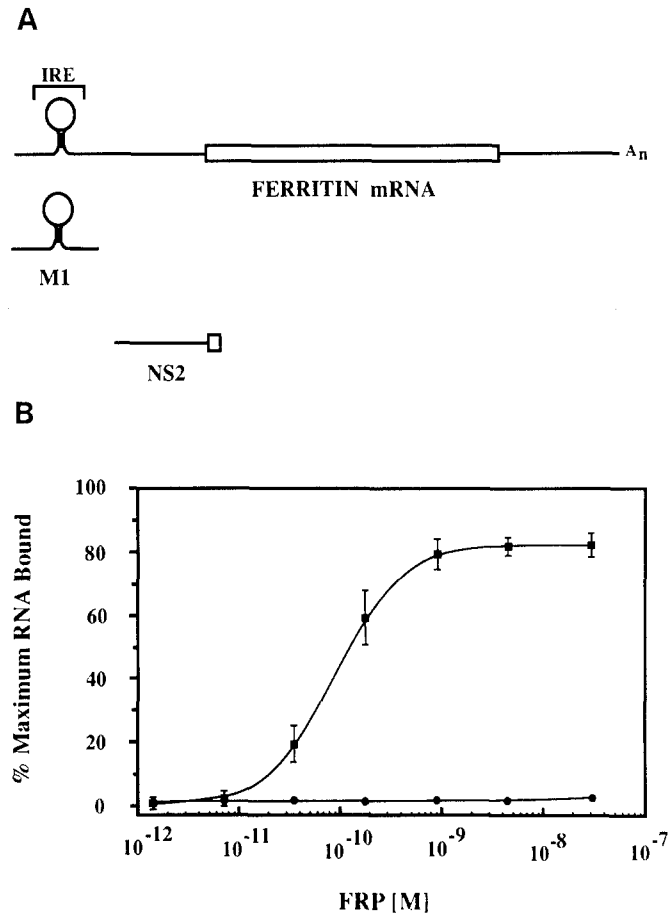

Fig. 1. Analysis of FRP/RNA interaction by nitrocellulose filter binding assay. (**A**) Diagrammatic representation of transcripts used in binding studies in relation to ferritin mRNA. M1 is a transcript of the first 92 nucleotides from the 5′ untranslated region of the human L-ferritin cDNA which includes the conserved IRE sequence. NS2 is a transcript of the rabbit L-ferritin cDNA starting 100 nucleotides upstream of the AUG and including the first three codons of the ferritin ORF (Daniels-McQueen et al. 1988). Solid line, untranslated region, open box, ferritin ORF. The IRE denoted by the bracket is not to scale. (**B**) An increasing amount of purified FRP was added to a constant amount of ^{32}P-labeled transcript and binding was detected as described in Materials and methods. M1 (■) NS2 (●)

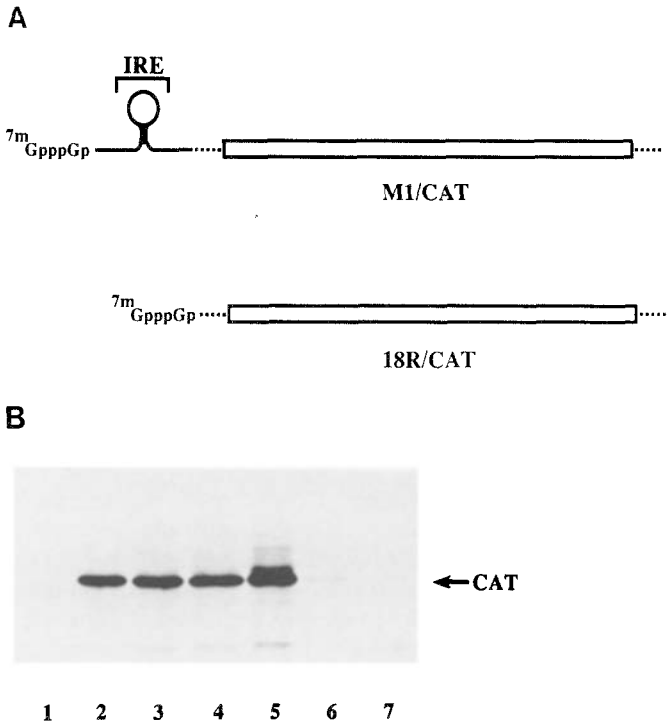

Fig. 2. Effect of FRP on translation of a chimeric IRE/CAT mRNA. (**A**) Diagrammatic representation of capped transcripts used for in vitro translation experiments. M1/CAT, chimeric transcript of the first 92 nucleotides of the human L-ferritin cDNA joined to the CAT mRNA; 18R/CAT, transcript of the CAT mRNA lacking ferritin sequences. Solid line, sequences from human L-ferritin mRNA UTR; open box, CAT ORF; dashed line, untranslated region from CAT mRNA and vector. (**B**) In vitro translations were performed in wheat germ extracts programmed with 10 fmol 18R/CAT (lanes 2–4) or 10 fmol M1/CAT (lanes 5–7). FRP was added to the reactions shown in lanes 3 and 6 (0.1 μg); lanes 4 and 7 (0.2 μg). No RNA was added to the reaction shown in lane 1.

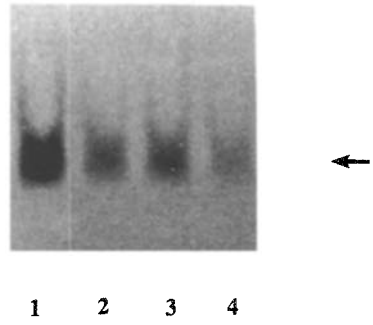

Fig. 3. Effect of iron on IRE binding activity in RK-1 cell extracts. RK-1 cells were grown to 90% confluence at which time ferric ammonium citrate was added to a final concentration of 100 μM. Extracts were prepared at 0 h (lane 1), 2 h (lane 2), 4 h (lane 3), and 6 h (lane 4) following addition of the ferric ammonium citrate. Aliquots containing equal amounts of protein were incubated with ^{32}P-labeled M1 transcript and analyzed by RNA-band shift as described in Materials and methods. The arrow indicates the positon of the FRP/M1 complex.

Effect of iron on FRP activity in vivo

As stated earlier, exposure of cells to excess iron results in a rapid increase in ferritin mRNA translation. In a variety of cultured cells, induction of ferritin synthesis is near maximum by 2 h after iron addition (unpublished observations). To determine the effects on FRP during iron induction of ferritin synthesis, RK-1 cells were incubated for 0 h, 2 h, 4 h or 6 h in media containing excess iron. Extracts were prepared and FRP was assayed by RNA-band shift. FRP activity is inhibited in extracts of cells which have been exposed to elevated levels of iron (Fig. 3). Reduction in activity is detected by 2 h after adding iron to cells. Extracts from cells exposed to excess iron for 6 h showed little difference in FRP activity when compared to 2 h extracts. This suggests that near-maximal inactivation of FRP is attained in cells by at least 2 h after iron addition, which correlates well with the pattern of induction of ferritin synthesis in these cells (data not shown). It should also be noted that the IRE binding activity detected in extracts of the RK-1 cells can be completely inhibited by antisera raised against purified rabbit liver FRP (data not shown). Taken together, these data support the notion that FRP is the iron-sensitive repressor of ferritin mRNA translation.

Scatchard analysis of FRP binding to the IRE

In order to obtain a more quantitative measure of the affinity of FRP for the IRE, Scatchard analysis of the interaction was performed using the nitrocellulose filter binding assay. An increasing concentration of ^{32}P-labeled M1 transcript was added to a constant amount of FRP, and incubation and filtration through nitrocellulose membranes was performed as described in Materials and methods. Figure 4 shows a representative Scatchard plot resulting from analysis using the standard binding conditions (see Materials and methods). Analysis of the Scatchard results by curve fitting predicts a single high-affinity binding site for M1 on FRP. The K_d

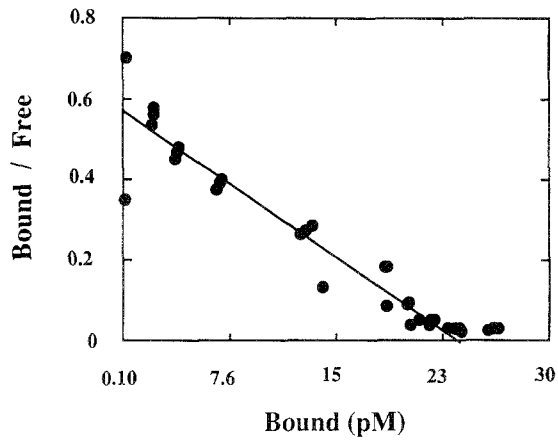

Fig. 4. Scatchard analysis of FRP binding to the IRE. Purified FRP was incubated with ^{32}P-labeled M1 transcript at concentrations of 0.19 pM–9.65 nM. Reactions were passed through nitrocellulose and quantified as described in Materials and methods.

Table 1. Results for Scatchard analysis of the FRP/IRE interaction

Conditions	K_d (pM)	FRP binding (pM)	Binding cf. initial (%)
Standard	49.5± 5.7	21.3	100
Translation	48.9± 7.7	26.5	124
Hemin-treated:			
5 μM	28.9±17.6	20.5	96
25 μM	43.7± 5.07	6.45	30
100 μM	75.9±13.3	3.29	15

calculated for this interaction is 49.5±5.7 pM. This value for the K_d is in close agreement with that reported for the interaction of the IRE-binding protein with the IRE (Haile et al. 1989; Barton et al. 1990). Scatchard analysis of the FRP/M1 interaction performed under conditions normally used for translations shows an interaction of a single, high-affinity binding site with a similar K_d to that obtained under standard conditions (Table 1). Therefore the high-affinity interaction of FRP with the IRE does occur in translation reactions and helps to explain the high efficiency of repression observed with this protein (Fig. 2B).

Effect of hemin on FRP binding to the IRE

It has recently been shown that hemin can derepress translation of ferritin mRNA in vitro (Lin et al. 1990a). Pretreatment of FRP with hemin prior to its addition to the translation assay prevents subsequent repression of ferritin mRNA translation. The effect of hemin on the ability of FRP to interact with the IRE was investigated. Purified FRP was preincubated with various concentrations of hemin, after which it was tested for binding to the M1 transcript using the filter binding assay. The resulting data were subjected to Scatchard analysis and are summarized in Table 1. It is interesting that hemin pretreatment has no significant effect on the measured K_d of the FRP/M1 interaction, nor does this pretreatment result in the appearance of a low-affinity form of FRP (Haile et al. 1989). However, hemin pretreatment reduces the total binding activity in the FRP preparation (Table 1). The effect of hemin on FRP activity is dependent on hemin concentration. Pretreatment of FRP with 5 μM hemin inactivates only about 4% of the protein, whereas pretreatment with 100 μM hemin inactivates 85%. This is consistent with earlier observations which showed that translational repression of ferritin mRNA in vitro is relieved by hemin concentrations between 30–200 μM (Lin et al. 1990a).

Effect of hemin and diamide on FRP/IRE complex stability

It is likely that induction of ferritin synthesis by iron involves the disruption of pre-existing FRP/mRNA complexes, which then allows the translation of ferritin mRNA. Therefore, it was of interest to determine if hemin could cause breakdown of preformed FRP/M1 complex. Radiolabeled M1 transcript was incubated with purified FRP in order to form the proten/RNA complex. Hemin was then added and the amount of M1 transcript remaining bound to FRP at various times after hemin addition was determined using the nitrocellulose filter binding assay. When buffer alone is added to the FRP/M1 complex it remains stable for as long as 150 min at 37° C (Fig. 5). Addition of hemin results in an initial rapid decay of complex which occurs during the first 15 min after hemin addition. The rate of this initial decay appears to be independent of hemin concentration since the rate of complex breakdown caused by 5 μM hemin is essentially equivalent to that observed with 100 μM hemin. However, the magnitude of complex breakdown is hemin-concentration dependent since only 35% of the FRP/M1 complex is disrupted by 5 μM hemin, whereas 70% of the complex is disrupted by 100 μM hemin. Interestingly, the level of complex remaining after 20 min is stable for at least an additional 2 h showing that the major portion of complex breakdown resulting from hemin occurs very rapidly, within the first 15 min of hemin addition. Taken together, these results suggest that the effects of hemin are due to a specific binding of hemin to FRP. In support of this notion, it has been found that hemin can be crosslinked to a specific site on FRP (J.-J. Lin and R. E. Thach; personal communication).

The effect of the sulfhydryl reagent, diamide, on the stability of the FRP/M1 complex was also investigated. Addition of 25 mM diamide to the preformed complex resulted in rapid breakdown which appears to continue

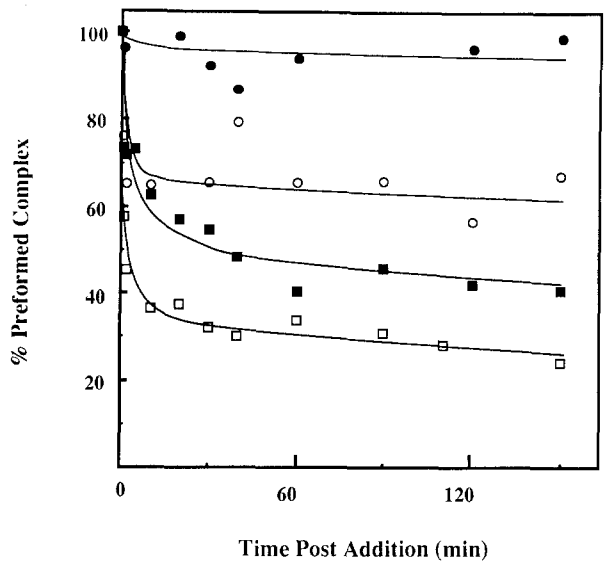

Fig. 5. Effect of hemin on preformed FRP/IRE complex. FRP was incubated with ^{32}P-labeled M1 transcript to allow complex formation, following which either buffer alone (●), or buffer containing 5 μM (○), 25 μM (■) or 100 μM hemin (□) was added. Aliquots were taken at timed intervals after hemin addition, passed through nitrocellulose and analyzed for bound radioactivity as described in Materials and methods.

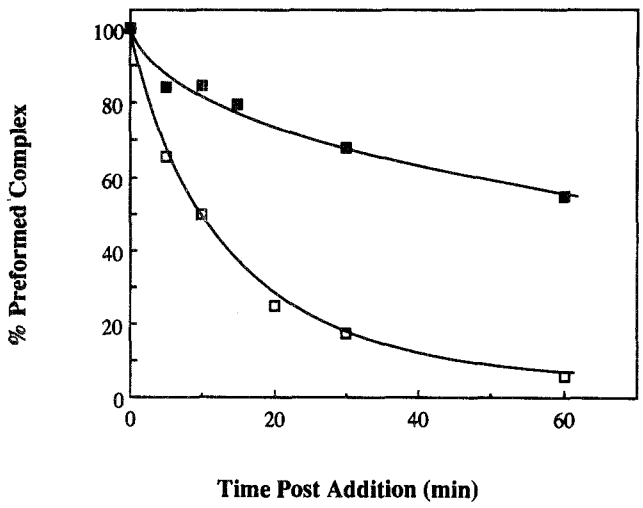

Fig. 6. Effect of diamide on preformed FRP/IRE complex. Incubations and analysis were performed as described in the legend to Fig. 5 and the Materials and methods. 5 mM diamide (■); 25 mM diamide (□)

towards complete complex breakdown (Fig. 6). Addition of 5 mM diamide to the complex also causes breakdown but at a rate significantly slower than that observed with 25 mM diamide. The FRP/M1 complex is stable for at least 1 h in the presence of 1 mM diamide (data not shown). These results are unlike those observed in the presence of hemin, where the initial rate of FRP/M1 complex breakdown was independent of hemin concentration, whereas the magnitude of complex breakdown was concentration dependent (Fig. 5). Nonetheless, it is clear that both hemin and diamide are capable of causing breakdown of the FRP/M1 complex.

Discussion

The data presented in this communication describe some interesting features of the interaction of the ferritin repressor protein with the iron-responsive element. Binding of FRP to the IRE is unusually tight. The K_d measured for the interaction ranges over 20–50 pM which is at least two orders of magnitude lower than other known RNA/protein interactions that regulate translation (Carey et al. 1983; Vartikar and Draper 1989). Similar K_d values have been reported for the interaction of the IRE binding protein with the IRE as measured by quantitative RNA-band shift assay (Haile et al. 1989; Barton et al. 1990). This is to be expected since recent analysis of cDNA clones of the IRE binding protein and FRP show that these proteins are identical (Rouault et al. 1990; M. M. Patino and W. E. Walden, unpublished results). It is not altogether clear why such a high-affinity RNA/protein interaction has evolved to mediate this regulatory system. Certainly, high-affinity interaction would be beneficial to the efficiency and specificity of translational repression. It has been proposed that repressors such as FRP repress translation by blocking access of mRNA-binding translation initiation factors to the target mRNA (Walden and Thach 1986). In such a scheme FRP would have to compete with initiation factors for access to ferritin mRNAs. Kinetic studies predict that a high-affinity repressor/mRNA interaction would be necessary for efficient repression of translation in vivo (Walden and Thach 1986).

Available evidence suggests that a mechanism(s) other than direct iron binding regulates the activity of FRP in vivo (Dickey et al. 1988; Lin et al. 1990a). It has been postulated that hemin is a regulator of FRP activity in vivo (Lin et al. 1990a). This proposal is based primarily on the finding that hemin can rescue translation of ferritin mRNA repressed by FRP in vitro. In addition, recent evidence shows that hemin crosslinks to FRP in vitro (J.-J. Lin and R. E. Thach, personal communication). Our results show that hemin pretreatment inactivates FRP for subsequent binding to the IRE (Table 1). Moreover, hemin treatment of preformed FRP/IRE complex results in complex breakdown. This latter effect of hemin is a requirement for regulation in vivo since the mRNA regulated by FRP would already be complexed with it. It is of interest that inactivation of FRP by hemin is dose dependent. Low concentrations of hemin inactivate only a small fraction of FRP while higher concentrations inactivate much more (Table 1). However, the FRP which remains active after hemin treatment displays an affinity for the IRE equal to that of untreated FRP. Therefore exposure of FRP to subsaturating levels of hemin should only give partial derepression of ferritin mRNA translation. In fact, this is seen in vitro (Lin et al. 1990a). Moreover, exposure of cells to less than saturating levels of iron results in only a partial derepression of ferritin mRNA, as judged by polysome distributions, and less than maximal induction of ferritin synthesis (Aziz and Munro 1986; W. E. Walden, unpublished observations).

It has been suggested that the effect of hemin on FRP is non-specific (Haile et al. 1990). The basis for this conclusion is the observation that the activity of several nucleic acid binding proteins can be inhibited by hemin in vitro. We believe that hemin has a specific effect on FRP for the following reasons. (a) The initial study which showed a hemin effect on FRP was performed by adding hemin and FRP to in vitro translation reactions programmed with ferritin and control mRNAs (Lin et al. 1990a). Since hemin had no inhibitory effects on translation of the control message it can be concluded that, at the concentrations which inhibit FRP activity, hemin has no inhibitory effects on the numerous protein/nucleic acid interactions which must occur for successful translation. (b) Hemin has no effect on a variety of restriction endonucleases when these enzymes are treated in a mixture with partially purified FRP (Lin et al. 1990b). However, FRP is inhibited by hemin under these conditions. Therefore, though it can be shown that a number of restriction endonucleases are sensitive to hemin, FRP appears to be much more sensitive (Lin et al. 1990b; Haile et al. 1990; J.-J. Lin and R. E. Thach, personal communication). (c) Exposure of FRP to hemin results in only a fraction of the total protein being inactivated, without an effect on

the affinity with which FRP interacts with the IRE (Table 1 and Fig. 5). The amount of FRP inactivated is dependent on hemin concentration, thus suggesting a stoichiometric interaction of hemin with FRP. Taken together, these results support the notion that FRP has a specific binding site for hemin and that hemin exerts its effect on FRP only after binding at this site. That hemin can be crosslinked to a specific peptide in FRP also supports this notion (J.-J. Lin and R. E. Thach, personal communication).

Other investigators have detected, in cell extract, two forms of FRP which differ in affinity for the IRE (Haile et al. 1989; Barton et al. 1990). One form displays a K_d for the IRE which is equivalent to that seen with purified FRP in this study. The other form has a much lower affinity for the IRE, having a K_d of 2–3 nM. It has been proposed that repression of ferritin mRNA and the stability of the transferrin receptor mRNA is modulated by converting FRP between these two forms (Haile et al. 1989; Haile et al. 1990). It is also postulated that FRP is reversibly converted between these forms via oxidation/reduction of critical sulfhydryls within the protein (Haile et al. 1990); oxidation of FRP converts it to the low-affinity form, whereas reduction would convert FRP to the high-affinity form. In cell extracts, conversion of the low-affinity form of FRP to the high-affinity form can be achieved by treatment with high concentrations of reducing agents (e.g. 2–2.5% 2-mercaptoethanol; Haile et al. 1989; Barton et al. 1990). Interestingly, pretreatment of purified rabbit liver FRP in this way does not increase the total high-affinity binding activity detectable in the preparation (data not shown). This is in spite of the fact that not all of the FRP in the preparation is active. It is possible that the reason for this is that the inactive fraction in the purified protein is inactive for reasons other than oxidation. It is also of interest that hemin treatment of FRP does not generate a low-affinity binding species (Table 1; Haile et al. 1990). This indicates that hemin, most likely, does not exert its effect by oxidation of sulfhydryls. Consistent with this notion, the effect of hemin on FRP cannot be reversed by high 2-mercaptoethanol concentrations (Haile et al. 1990). The data in Fig. 6 clearly show that the FRP/IRE complex can be disrupted by the sulfhydryl reagent diamide. This indicates that oxidation of sulfhydryls is also a possible mechanism by which FRP activity is regulated in vivo. It is an interesting possibility that FRP activity is regulated through multiple mechanisms within the cell. Further work is necessary to fully elucidate these possibilities.

Acknowledgements. This investigation was supported by grants from the National Science Foundation (DMB-8818203) and the Schweppe Foundation.

References

Aziz N, Munro HN (1986) Both subunits of rat liver ferritin are regulated at a translational level by iron induction. Nucleic Acids Res 14:915–927

Aziz N, Munro HN (1987) Iron regulates ferritin mRNA translation through a segment of its 5′ untranslated region. Proc Natl Acad Sci USA 84:8478–8482

Barton HA, Eisenstein RS, Bomford A, Munro HN (1990) Determinants of the interaction between the iron-responsive element-binding protein and its binding site in rat L-ferritin mRNA. J Biol Chem 265:7000–7008

Brown PH, Daniels-McQueen S, Walden WE, Patino MM, Gaffield L, Bielser D, Thach RE (1989) Requirements for the translational repression of ferritin transcripts in wheat germ extracts by a 90-kDa protein from rabbit liver. J Biol Chem 264:13383–13386

Carey JL, Cameron V, deHaseth PL, Uhlenbeck OC (1983) Sequence-specific interaction of R17 coat protein with its ribonucleic acid binding site. Biochemistry 22:2601–2610

Casey JL, Hentze MW, Koeller DM, Caughman SW, Rouault TA, Klausner RD, Harford JB (1988) Iron-responsive elements: regulatory RNA sequences that control mRNA levels and translation. Science 240:924–928

Casey JL, Koeller DM, Ramin VC, Klausner RD, Harford JB (1989) Iron regulation of transferrin receptor mRNA levels requires iron-responsive elements and a rapid turnover determinant in the 3′ untranslated region of the mRNA. EMBO J 8:3693–3699

Caughman SW, Hentze MW, Rouault TA, Harford JB, Klausner RD (1988) The iron-responsive element is the single element responsible for iron-dependent translational regulation of ferritin biosynthesis: evidence for function as the binding site for a translational repressor. J Biol Chem 263:19048–19052

Daniels-McQueen S, Ray A, Walden WE, Ray BK, Brown PH, Thach RE (1988) Nucleotide sequence of cDNA encoding rabbit ferritin L chain. Nucleic Acids Res 16:7441

Dickey LF, Wang YH, Shull GE, Wortman IA, Theil EC (1988) The importance of the 3′ untranslated region in the translational control of ferritin mRNA. J Biol Chem 263:3071–3074

Haile DJ, Hentze MW, Rouault TA, Harford JB, Klausner RD (1989) Regulation of interaction of the iron-responsive element binding protein with iron-responsive RNA elements. Mol Cell Biol 9:5055–5061

Haile DJ, Rouault TA, Harford JB, Klausner RD (1990) The inhibition of the iron-responsive element RNA-protein interaction by heme does not mimic in vivo iron regulation. J Biol Chem 265:12786–12789

Hentze MW, Caughman SW, Rouault TA, Barriocanal JG, Dancis A, Harford JB, Klausner RD (1987) Identification of the iron-responsive element for the translational regulation of human ferritin mRNA. Science 238:1570–1573

Hentze MW, Rouault TA, Harford JB, Klausner RD (1989) Oxidation-reduction and the molecular mechanism of a regulatory RNA-protein interaction. Science 244:357–359

Klausner RD, Harford JB (1989) *Cis-trans* models for post-transcriptional gene regulation. Science 246:870–872

Leibold EA, Munro HN (1988) Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5′ untranslated region of ferritin heavy- and light-subunit mRNAs. Proc Natl Acad Sci USA 85:2171–2175

Lin J-J, Daniels-McQueen S, Patino MM, Gaffield L, Walden WE, Thach RE (1990a) Depression of ferritin mRNA translation by hemin in vitro. Science 247:74–77

Lin J-J, Daniels-McQueen S, Gaffield L, Patino MM, Walden WE, Thach RE (1990b) Specificity of the induction of ferritin synthesis by hemin. Biochem Biophys Acta 1050:146–150

Mullner EW, Kuhn LC (1988) A stem-lop in the 3′ untranslated region mediates iron-dependent regulation of transferrin receptor mRNA stability in the cytoplasm. Cell 53:815–825

Mullner EW, Neupert B, Kuhn LC (1989) A specific mRNA binding factor regulates the iron-dependent stability of cytoplasmic transferrin receptor mRNA. Cell 58:373–382

Munson PJ, Rodbard D (1980) LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107:220–239

Murray MT, White K, Munro HN (1987) Conservation of ferritin heavy subunit gene structure: implications for the regulation of ferritin gene expression. Proc Natl Acad Sci USA 84:7438–7442

Neupert B, Thompson NA, Meyer C, Kuhn LC (1990) A high yield affinity purification method for specific RNA-binding proteins: isolation of the iron regulatory factor from human placenta. Nucleic Acids Res 18:51–55

Owen D, Kuhn LC (1987) Noncoding 3′ sequences of the transferrin receptor gene are required for mRNA regulation by iron. EMBO J 6:1287–1293

Rao KK, Shapiro D, Mattia E, Bridges K, Klausner R (1985) Effects of alterations in cellular iron on biosynthesis of the transferrin receptor in K562 cells. Mol Cell Biol 4:595–600

Rao KK, Harford JB, Rouault T, McClelland A, Ruddle FH, Klausner RD (1986) Transcriptional regulation by iron of the gene for the transferrin receptor. Mol Cell Biol 6:236–240

Rouault TA, Hentze MW, Haile DJ, Harford JB, Klausner RD (1989) The iron-responsive element binding protein: a method for the affinity purification of a regulatory RNA-binding protein. Proc Natl Acad Sci USA 86:5768–5772

Rouault TA, Tang CK, Kaptain S, Burgess WH, Haile DJ, Samaniego F, McBride OW, Harford JB, Klausner RD (1990) Cloning of the cDNA encoding an RNA regulatory protein – the human iron responsive element binding protein. Proc Natl Acad Sci USA 87:7958–7962

Shull GE, Theil EC (1982) Translational control of ferritin synthesis by iron in embryonic reticulocytes of the bullfrog. J Biol Chem 257:14187–14191

Theil EC (1987) Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. Annu Rev Biochem 56:289–315

Theil EC (1990) Regulation of ferritin and transferrin receptor mRNAs. J Biol Chem 265:4771–4774

Vartikar JV, Draper DE (1989) S4-16S ribosomal RNA complex: binding constant measurements and specific recognition of a 460-nucleotide region. J Mol Biol 209:221–234

Walden WE, Thach RE (1986) Translational control of gene expression in a normal fibroblast. Characterization of a subclass of mRNAs with unusual kinetic properties. Biochemistry 25:2033–2041

Walden WE, Daniels-McQueen S, Brown PH, Gaffield L, Russell DA, Bielser D, Bailey LC, Thach RE (1988) Translational repression in eukaryotes: partial purification and characterization of a repressor of ferritin mRNA translation. Proc Natl Acad Sci USA 85:9503–9507

Walden WE, Patino MM, Gaffield L (1989) Purification of a specific repressor of ferritin mRNA translation from rabbit liver. J Biol Chem 264:13765–13769

Zahringer J, Baliga BS, Munro HN (1976) Novel mechanism for translational control in regulation of ferritin synthesis by iron. Proc Natl Acad Sci USA 73:857–861