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pH dependent conformational changes within the iron responsive element

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Abstract

The expression of several mRNAs related to iron importation, storage and utilization within mammalian cells are regulated through interactions of iron regulatory proteins with an iron responsive element, an RNA hairpin with a bulged C. A dimethylsulfate modification interference assay was used to demonstrate that the iron responsive element undergoes significant pH dependent conformational changes. Specifically, it was demonstrated that the phylogenetically conserved A within the hairpin loop and an intra-loop C-G base pair are highly sensitive to changes in pH. The conserved C of the bulged loop does not significantly affect the pH dependent conformational changes of the hairpin loop. These studies have structural implications for an RNA-protein interaction that is critical to mammalian iron regulation.

Abbreviations: IRP – iron regulatory protein; IRE – iron-responsive element; DMS – dimethylsulfate.

Introduction

Interactions of two related iron-regulatory proteins (IRP-1 and IRP-2) with mRNAs containing an iron responsive element (IRE) are central to intracellular mammalian iron homeostasis. Under conditions of iron repletion, the RNA binding site of IRP-1 is obstructed by a 4Fe-4S cluster. This cluster becomes labile as the cytosolic iron concentration decreases and upon disassembly, exposes the RNA binding site (Kaptain *et al.* 1991; Haile *et al.* 1992; Kennedy *et al.* 1992; Hirling *et al.* 1994). In contrast, IRP-2 is not inactivated under iron replete conditions through the formation of a 4Fe-4S cluster (Guo *et al.* 1994; Henderson *et al.* 1993). Instead, it contains a 73 amino acid sequence that directly interacts with aqueous iron leading to oxidation of the protein and targeting for ubiquitin-dependent degradation (Iwai *et al.* 1998). The binding of an IRP to an IRE can alter the expression of several mRNAs encoding proteins involved with iron storage, transport and utilization (Hentze *et al.* 1987; Casey *et al.* 1988; Mullner & Kuhn 1988; Dandekar *et al.* 1991; Gray *et al.* 1996).

The IRE consists of a six nucleotide RNA hairpin loop five base pairs removed from a phylogenetically conserved C that is part of a bulge or bulged loop. The hairpin loop within the natural IREs all contain the sequence CAGWGH where W is A or U and H is A, C or U. This structure has been supported by chemical probing (Schlegl *et al.* 1997; Wang *et al.* 1990), *in vitro* selection (Henderson *et al.* 1994; Butt *et al.* 1996), mutagesesis (Leibold *et al.* 1990; Bettany *et al.* 1992; Jaffrey *et al.* 1993), and NMR (Laing & Hall 1996; Addess *et al.* 1997; Gdaniec *et al.* 1998).

NMR studies indicate that the IRE contains an ionizable group with a pK_a between 5 and 6, but the identity of this group has not been identified (Addess *et al.* 1997; Gdaniec *et al.* 1998). Although unstructured RNA does not contain an ionizable group with a pK_a in this range, adenosines and cytosines in several structured RNAs have been identified that have a pK_a shifted towards neutrality from pH 3.5 and 4.2, respectively. These include the leadzyme (Legault & Pardi 1997), arginine and guanosine binding RNAs (Connell & Yarus 1994), the hepatitis delta virus ribozyme (Nakano *et al.* 2000; Shih & Been, 2001) and the pep-

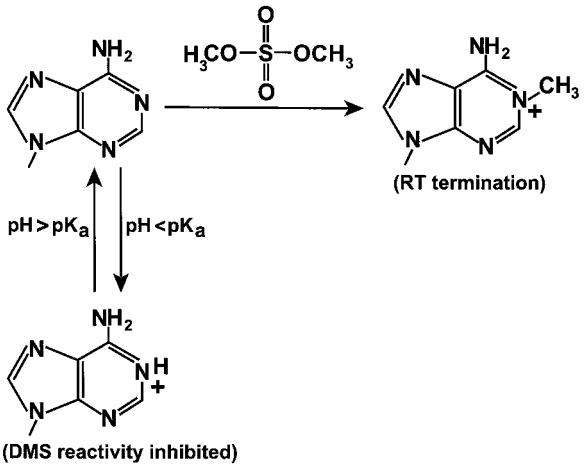

Fig. 1. The DMS modification interference assay used to detect nucleotides with a perturbed pK_a. At a pH below the pK_a the base becomes protonated and is not able to act as a nucleophile in the reaction with DMS. DMS modifications are detected as terminations during reverse transcription (RT) of the RNA.

tidyl transferase center of the ribosome (Muth *et al.* 2000). Ionizable groups with a pK_a shifted toward neutrality extend the catalytic and structural potential of RNA in a manner analogous to the role of histidine in many proteins.

Although NMR has been used successfully to identify bases with a shifted pK_a, unambiguous identification of the base with an altered pK_a has not always been possible. A complementary strategy to identify cytosines and adenosines with an altered pK_a exploits a dimethylsulfate (DMS) modification interference assay(Connell & Yarus 1994; Muth *et al.* 2000). DMS methylates both N1 of adenosine and N3 of cytosine, and the modifications can be detected as a termination during subsequent reverse transcription of the RNA (Figure 1). At a pH below their pK_a, N1 of adenosine and N3 of cytosine are protonated and as a result are unreactive toward DMS and do not cause termination during the reverse transcription. In addition to detecting bases with an elevated pK_a, the DMS modification interference assay would also detect pH dependent conformational changes that result in the strengthening or weakening of interactions involving these functional groups.

We demonstrate here that the conserved A within the hairpin loop of the IRE has a pH dependent DMS reactivity consistent with the pK_a having been shifted upward several pH units. The strength of the previously reported intra-loop C-G base pair is also very pH dependent, and the base pair is not evident at pH 7. The bulged C of the ferritin IRE is not required for the pH dependent conformational changes within the hairpin loop.

Materials and methods

RNA preparation

RNAs were synthesized by T7 transcription from the corresponding oligodeoxynucleotides as previously described (Milligan & Uhlenbeck 1989). The ferritin H chain IRE has an artificial 3′ extension that was used as a primer binding site during the extension reaction. It had previously been determined that this 3' extension has less than a 2 fold affect on the K_d for binding to IRP-1 (Meehan & Connell 2001).

DMS reaction conditions

The RNAs were heated at 65 °C for 5 min in dH_2O at a concentration of 1.5 pmol/μl. Three pmol of the RNA were then added to 198 μl of a solution containing 10 mM NaCl, 0.2 mM EDTA and 100 mM of the buffer at the indicated pH. The RNA was left at 37 °C for 10 min to facilitate proper folding. The pH 7 solution was buffered with HEPES and the pH 5 and 6 solutions with MES. The pH of each buffer was adjusted at 22 °C using a temperature coefficient (ΔpK_a/°C) of -0.014 for HEPES and -0.011 for MES so that the indicated pH values would be obtained at 37 °C. The stock DMS (10.5 M) was diluted 6 fold with 95% ethanol and 3 μl added to the RNA solutions at 37 °C and reacted for 10 minutes. Reactions were terminated by the addition of 7 μl 14 M 2-mercaptoethanol, 25 μl 3M NaOAC, 10 μg glycogen and 1 ml ethanol. Reactions were precipitated for 1 h on ice, pelleted at 14,000 *g* for 30 min, rinsed with 70% ethanol, air-dried and re-suspended in 10 μl of dH_2O. Reverse transcription of 2 μl of the RNA was performed as previously described (Inoue & Cech 1985) using a ^{32}P-labeled deoxynucleotide primer complementary to the site indicated in Figure 2A.

Results

The DMS modification interference assay indicates that nucleotide C18 becomes less reactive toward DMS as the pH is decreased from 7 to 6 (Figure 2). However, the NMR spectra of the IRE at pH 5.8 and

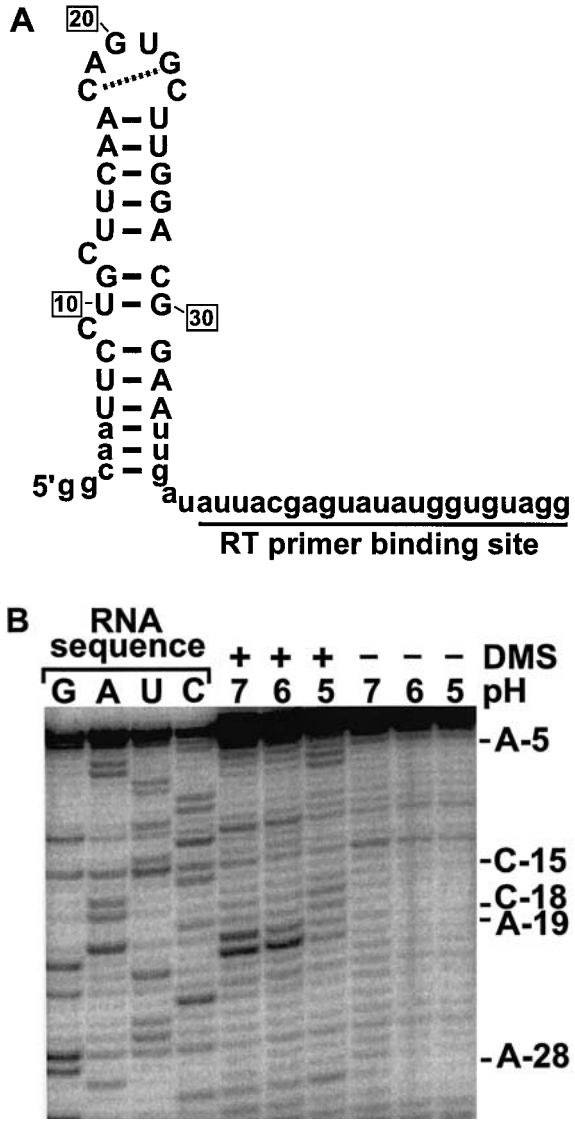

Fig. 2. Detection of pH dependent conformational changes within the IRE. A) Secondary structure of the ferritin H-chain IRE. Lowercase letters are not part of the natural IRE sequence and have little effect on binding affinity to IRP-1 (Meehan & Connell 2001). The location of the primer binding site used for the reverse transcription (RT) reactions is underlined and the intra-loop C-G base pair is indicated. B) Reverse transcription of the IRE that was treated (+) or not-treated (−) with DMS at the indicated pH. Terminations resulting from base modification are one nucleotide shorter than those resulting from dideoxynucleotide incorporation during the sequencing reaction. The gel is representative of four sets of reactions.

5.5 both indicate that C18 forms a Watson-Crick base pair with G22 (Laing & Hall 1996; Addess *et al.* 1997). This suggests that the loss of DMS reactivity is most likely caused by a pH dependent change that strengthens the C-G interaction rather than being attributable to the N3 position of the cytosine being protonated. The degree to which the strength of the intra-loop C-G base pair is pH dependent has not been fully appreciated although it has previously been observed that a 10.7 ppm resonance assigned to the imino proton of the G sharpens at pH 5.5 (Addess *et al.* 1997).

Nucleotide A19 also becomes less reactive toward DMS at pH 5. NMR data collected at pH 5.5 indicates that A19 base stacks on the C-G pair and such a structure predicts that the N-1 position should be accessible to DMS modification (Addess *et al.* 1997). The diminished DMS reactivity of this position at pH 5 therefore is consistent with an elevated pK_a.

The A and C nucleotides of both the bottom and top helices of the IRE become more reactive toward DMS at pH 5 (A4, A5, C15, A16, A17, A28) suggesting that both the top and bottom helices become less stable at the lower pH. Although it is clear from the NMR structures that these helices are still forming at least at pH 5.5 (Addess *et al.* 1997), there are also some indications from the pH 5.5 NMR spectra that the helices may not be very stable: the U imino resonance was not observed in the 1D spectrum for the A17-U24 base pair and the U imino resonance could not be detected in a 2D NOESY spectrum above 1 °C for the U13-A28 base pair.

The pH induced changes would be expected to be highly dependent upon the secondary and tertiary structure of the IRE since some type of structure would be required to perturb the pK_a of the functional group(s). Mutations to the IRE that significantly disrupt the global IRE structure would, therefore, be expected to alter the pH induced changes. Although the bulged C (C12 in Figure 2A) does not appear to have a perturbed pK_a (Figure 2B), we were interested in knowing whether this position affects the pH dependent changes observed within the hairpin loop of the IRE. Mutation of C12 to a U has previously been demonstrated to decrease IRP binding affinity (Jaffrey *et al.* 1993; Henderson *et al.* 1994). However, since the pH profile of DMS reactivity of this mutation is similar to the wild-type sequence (Figure 3), it is probable that the bulge position is not strongly interacting with the hairpin loop. This is also consistent with NMR spectroscopic data indicating that the conformation of

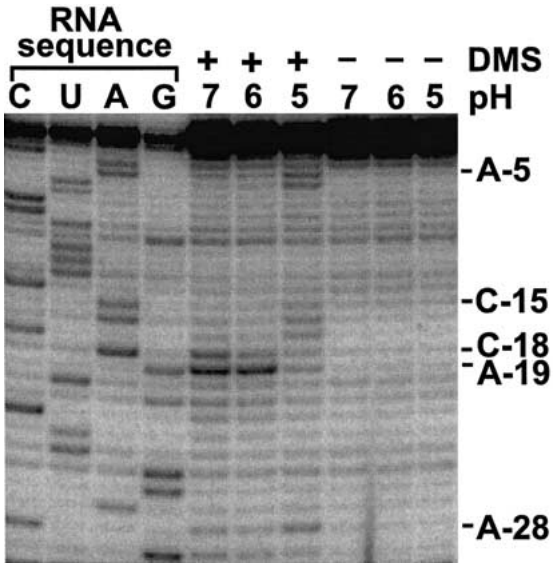

Fig. 3. Mutation of the bulged C to a U has little effect on the pH dependent conformational changes. Conditions were the same as for Figure 2B. The gel is representative of four sets of reactions.

the bulged C is not well defined (Addess *et al.* 1997; Gdaniec *et al.* 1998).

Discussion

The DMS modification interference study indicates that there are conformational changes in the IRE that occur between pH 5 and 7, consistent with the earlier NMR spectra (Addess *et al.* 1997; Gdaniec *et al.* 1998). The hairpin loop nucleotides C18 and A19 are both affected. The inhibition of DMS reactivity at C18 is consistent with the strengthening of a Watson-Crick base pair with G22. The inhibition of the reactivity of A19, though, is more consistent with this nucleotide having an elevated pK_a as it is not predicted by the pH 5.5 NMR structure to be hindered from reacting with the DMS.

The underlying cause of the C18-G22 base pair appearing to be stronger at pH 5 than at neutral pH still is not completely understood. The bases with a shifted pK_a identified by NMR in other RNAs are all cytosines or adenosines . However, it is evident that some other functional group is probably responsible for causing the C18-G22 base pair to be stronger at lower pH. One appealing hypothesis is that the imino proton at N1 of G22 is dissociated above pH 6 which would inhibit Watson-Crick pairing with C18. The pK_a for the removal of the N1 proton of guanosine is normally 9.4 (Saenger 1984) which means that the pK_a would have to be shifted down approximately 3 pH units. Although this seems like a large shift, it is less in magnitude than the pK_a perturbation of adenosines and cytosines within several other RNAs (Boulard *et al.* 1992; Leroy *et al.* 1993; Jaishree & Wang 1993; Legault & Pardi 1997; Shih & Been 2001).

Alternatively, it is possible that an ionizable group at a different site within the IRE is responsible for strengthening the C18-G22 interaction at pH 6. This study can not rule out the possibility that the perturbation of the pK_a at the N1 position of any guanosine nor the N3 position of a uridine ($pK_a = 9.4$) is involved. The base pair corresponding to U10-G30 in a frog IRE has previously been shown to be pH dependent (Gdaniec *et al.* 1998). However, this is more likely related to the global structural changes we detected at pH 5 rather than being a trigger for the strengthening of the C18-G22 interaction detected between pH 6 and 7. Since pH induced conformational changes are observed in IREs having different 5′ termini (Figure 2; Addess *et al.* 1997; Gdaniec *et al.* 1998), it is unlikely that the 5′ terminal phosphate with a pK_a of 7.7, even if shifted downward, would be the trigger of the conformational change. The bridging phosphates and the N7 position of guanosine and adenosine, all have a pK_a less than 2 and therefore are also less likely candidates. The remaining position that needs to be considered is the N7 of methyl guanosine that forms during the DMS reaction and has a pK_a of approximately 7. However, this group would not have been present during the NMR studies and therefore is also not a likely candidate.

Although the DMS assay indicates that there are significant conformational changes that occur between pH 5 and 7, the NMR spectra at pH 5.5 and 6.8 do not differ to the same extent (Addess *et al.* 1997; Gdaniec *et al.* 1998). A stronger interaction may be required for a Watson-Crick base pair to be protected from DMS modification than that required for it to be detected by NMR spectroscopy. Alternatively, the lower temperatures at which the NMR studies were performed may also have stabilized the structure. As a result, it is possible that the NMR conditions (pH and temperature) optimized for the exchangeable proton assignments may not necessarily be conditions under which the IRE adopts the most biologically relevant conformation (pH 7 and 37 °C).

The pH dependence for the formation of the intraloop C-G base pair is of special interest. We did not detect evidence of this base pair at physiological pH

by protection from DMS modification, yet the protection at pH 5 and 6 is consistent with its formation. *In vitro* selection studies also suggest that the base pair is important for IRP binding (Henderson *et al.* 1994; Butt *et al.* 1996). It is possible that the base pair is stabilized at physiological pH by a bound IRP and that in the absence of protein, it is not formed or only weakly formed; the low pH could possibly be mimicking an effect of the protein.

Several roles have been proposed for the bulged C of the IRE. We tested whether the bulged C directly interacts with the hairpin loop by looking for perturbation of the pH induced conformational changes when the bulge position was mutated to a U. Since the mutation had no significant effect on the pH induced conformational changes within the hairpin loop (Figure 3), it is unlikely that the bulged C is intimately involved with the formation of a structure that is required for the shifted pK_a. However, we can not rule out the possibility of more subtle interactions between the bulge and hairpin loops.

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