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RNA with iron(II) as a cofactor catalyses electron transfer

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Mg^{2+} is essential for RNA folding and catalysis. However, for the first 1.5 billion years of life on Earth RNA inhabited an anoxic Earth with abundant and benign Fe^{2+}. We hypothesize that Fe^{2+} was an RNA cofactor when iron was abundant, and was substantially replaced by Mg^{2+} during a period known as the 'great oxidation', brought on by photosynthesis. Here, we demonstrate that reversing this putative metal substitution in an anoxic environment, by removing Mg^{2+} and replacing it with Fe^{2+}, expands the catalytic repertoire of RNA. Fe^{2+} can confer on some RNAs a previously uncharacterized ability to catalyse single-electron transfer. We propose that RNA function, in analogy with protein function, can be understood fully only in the context of association with a range of possible metals. The catalysis of electron transfer, requisite for metabolic activity, may have been attenuated in RNA by photosynthesis and the rise of O_2.

For around 1.0–1.5 billion years before photosynthesis began to produce substantial free O_2, biological systems inhabited an Earth with abundant soluble iron and essentially no O_2 (refs 1,2). The 'great oxidation' led to Fe^{2+}/O_2-mediated cellular damage[3] and depletion of soluble iron from the biosphere[4]. We hypothesize that Fe^{2+} was an RNA cofactor when iron was benign and abundant and that Fe^{2+} was replaced substantially by Mg^{2+} during the great oxidation. The RNA–Fe^{2+} to RNA–Mg^{2+} hypothesis is in close analogy with known metal substitutions in some metalloproteins[5–10]. An ancestral ribonucleotide reductase (RNR), for example, spawned di-iron, dimanganese and iron–manganese RNRs[11]. Our hypothesis is supported by observations[12] that (1) RNA folding is conserved between complexes with Fe^{2+} and Mg^{2+} and (2) at least some phosphoryl transfer ribozymes are more active in the presence of Fe^{2+} than of Mg^{2+}. Here, we demonstrate that reversing the putative metal substitution in an anoxic environment by removing Mg^{2+} and adding Fe^{2+} expands the catalytic repertoire of RNA. Fe^{2+} can confer on some RNAs a previously uncharacterized ability to catalyse single-electron transfer. Catalysis is specific, in that it is dependent on the type of RNA. The 23S ribosomal RNA (rRNA) and transfer RNA (tRNA), some of the most abundant and ancient RNAs[13], are efficient electron-transfer ribozymes in the presence of Fe^{2+}. Therefore, the catalytic competence of RNAs may have been greater in early Earth conditions than in extant conditions, and the experiments described here may be reviving latent function.

Without cations, RNA is essentially incapable of molecular recognition or catalysis. RNA requires cations in the form of Na^+, K^+ and Mg^{2+} for folding and function[14–16]. Originally, Mg^{2+} was demonstrated to be especially important in the folding of tRNA[17–19] and is now known to be critical for compaction of large RNAs. Mg^{2+} ions neutralize the negative charge of the RNA backbone and bind specifically to complex structural features of RNA[20]. Mg^{2+} is required by many ribozymes to organize RNA or water molecules within active sites, and to stabilize reactants or transition states[21,22].

Using a standard peroxidase assay[23], we investigated the catalytic abilities of a variety of native nucleic acids to determine if they can catalyse single-electron transfer in association with Fe^{2+} instead of

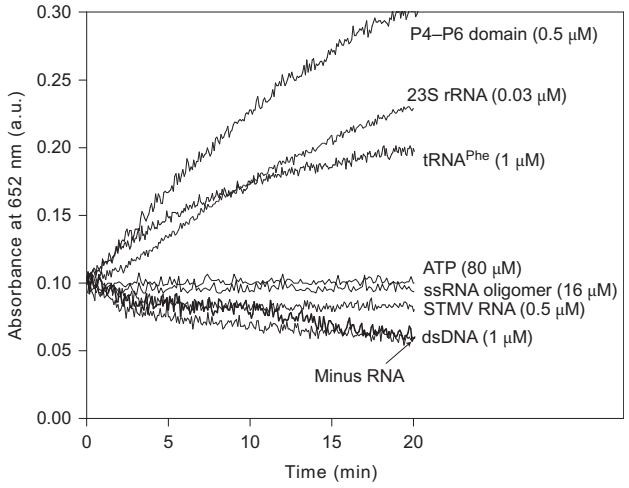

Figure 1 | Some RNAs in combination with Fe^{2+} catalyse single-electron transfer. The 23S rRNA from *Thermus thermophilus*, the P4–P6 domain of the *Tetrahymena thermophila* Group 1 intron and yeast $tRNA^{phe}$ catalyse single-electron transfer. Other nucleic acids, including ATP, a short RNA oligomer, double-stranded DNA (dsDNA) and the genome of STMV, are inefficient catalysts. All the reactions were performed in the absence of O_2 and Mg^{2+}, and in the presence of Fe^{2+} and H_2O_2. RNA concentrations are given in units of molecules (single strands). The concentration per nucleotide is roughly the same for ATP, the ssRNA oligomer, P4–P6 domain RNA, 23S rRNA, tRNA and DNA (that is, ~80 μM), and is greater for the STMV genome (~500 μM). The 23S rRNA gives the most-efficient catalysis of electron transfer, at a lower concentration of RNA strands than that of the other RNAs.

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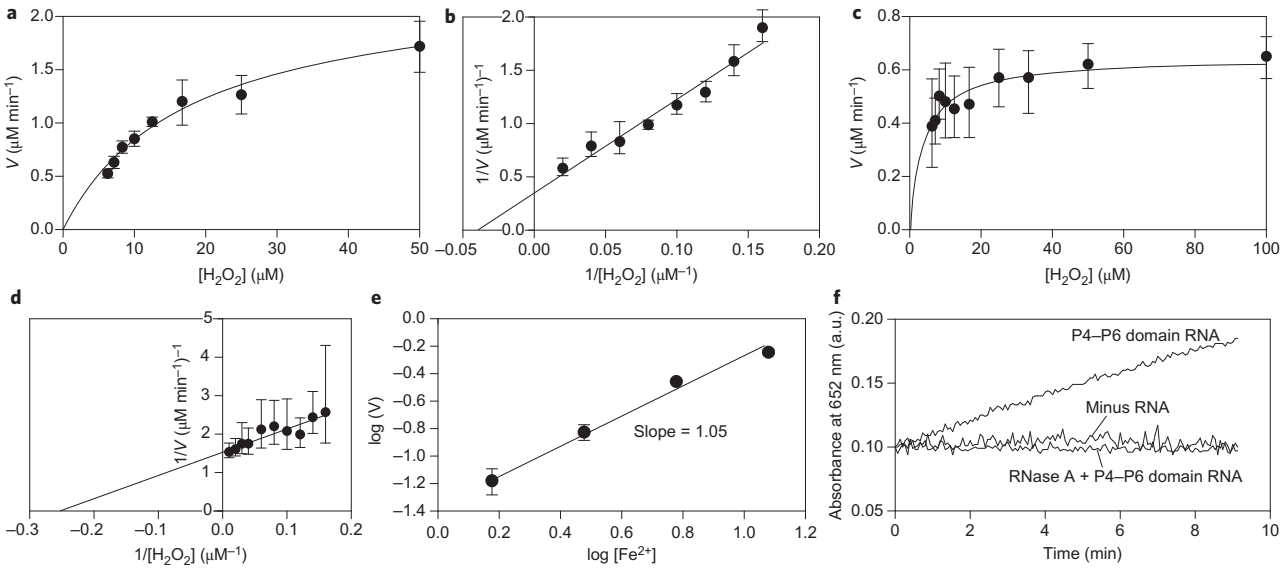

Figure 2 | Fe^{2+}-RNA catalysed single-electron transfer reactions follow enzyme kinetics. a, The electron-transfer reaction catalysed by 23S rRNA of *T. thermophilus* is saturable, reaching a plateau at high [H_2O_2]. **b**, The kinetics of the 23S rRNA reaction can be fitted to the Michaelis–Menten model. **c**, The electron-transfer reaction catalysed by the P4–P6 domain of the *T. thermophila* Group 1 intron is also saturable. **d**, The kinetics of the P4–P6 domain RNA reaction can be fitted to the Michaelis–Menten model. **e**, The Fe^{2+} dependence of the reaction rates catalysed by P4–P6 domain RNA suggests that one Fe^{2+} is involved in the catalysis. **f**, RNase A digestion of RNA prevents electron-transfer catalysis. For error analysis, a bootstrapping method (MATLAB R2011b) was employed to generate 1,000 bootstrap samples in each velocity increment. The initial rate of each reaction is the mean of the bootstrap samples. The 95% confidence interval for each initial rate was determined from the 2.5 percentile and the 97.5 percentile of the bootstrap samples. The reactions shown in **a** and **b** were performed with 0.03 μM 23S rRNA, 6 μM Fe^{2+}, pH 6.5 and 500 μM TMB. The reactions in **c**–**f** were performed with 0.5 μM P4-P6 domain RNA, 6 μM Fe^{2+}, pH 6.5 and 500 μM TMB, except for **e** where the [Fe^{2+}] was varied. O_2 was excluded from all the reactions. Concentration units are RNA strands. The lines in **b**, **d** and **e** were obtained by linear regression. The kinetics of these reactions are well-behaved and are consistent with catalysis by Fe^{2+}-RNA complexes. *V* is the reaction velocity in μM min^{-1}.

Mg^{2+}. In this assay, an organic dye (a reducing agent) was oxidized by hydrogen peroxide (an oxidizing agent) via electron transfer. Specifically, one electron was transferred from 3,3′,5,5′-tetramethylbenzidine (TMB) to hydrogen peroxide to form a radical cation ($TMB^{\bullet+}$) according to the equation:

$$\tfrac{1}{2}(HOOH) + TMB + H^+ \rightarrow H_2O + TMB^{\bullet+}$$

Broadly, this reaction was used to detect electron transfer. Absorbances at 370 nm and 652 nm were used to monitor the course of the reaction.

Results and discussion

Catalysis of single-electron transfer was observed for a subset of RNAs tested here (Fig. 1). Fe^{2+}, RNA and the exclusion of O_2 were required. In solutions that contain Mg^{2+} instead of Fe^{2+}, catalysis of electron transfer was not detected. Nor was catalysis detected in solutions that lack RNA or in solutions with degraded RNA. Some RNAs catalysed electron transfer much more efficiently than other RNAs at lower concentrations of RNA (0.03–1.0 μM strand, 75–100 μM in nucleotide). For the inefficient RNAs, electron transfer was observable only at high concentrations (>500 μM in nucleotide). Relative rates of electron transfer were inferred from the initial slopes of graphs such as those represented in Fig. 1. Additional information on materials, methods, reaction rates, RNA stability and control reactions is given in the Supplementary Information.

The 23S rRNA gave efficient catalysis of electron transfer at a low concentration of RNA strands (Fig. 1), with a greater k_{cat} (Fig. 2) than that of other RNAs. Per nucleotide, the P4–P6 domain RNA and yeast $tRNA^{phe}$ are the most efficient, catalysing electron transfer with roughly similar efficiencies. The appropriate choice of concentration units, strand or nucleotide is dependent on whether the Fe^{2+} binds at a few highly specific sites, or less specifically to many sites on the RNAs.

We examined a diverse set of nucleic acids in an attempt to isolate properties responsible for catalysis of electron transfer in the presence of Fe^{2+} (Fig. 1). Closely spaced phosphate groups, as in adenosine triphosphate (ATP), were not sufficient to induce catalysis. The regular array of phosphate groups and other structural features of B-form DNA were insufficient for efficient catalysis. The combination of B-DNA and Fe^{2+} did not catalyse electron transfer. We investigated a double-stranded 32 base pair DNA duplex composed of a mixture of C–G and A–T base pairs. To investigate whether a short unstructured RNA catalyses electron transfer in the presence of Fe^{2+}, we used a small single-stranded RNA (ssRNA) oligomer (5′-GCACU-3′). This short RNA oligomer did not catalyse electron transfer. We investigated the satellite tobacco mosaic virus (STMV) genomic RNA[24] to determine if a large, partially unstructured, RNA is competent to catalyse electron transfer in the presence of Fe^{2+}. This 1,058 nucleotide RNA genome did not catalyse electron transfer at 0.5 μM strand, which, in terms of nucleotide concentration, greatly exceeds the concentration at which P4–P6 domain RNA, 23S rRNA and tRNA are catalytic.

The differential catalysis of single-electron transfer by various RNAs supports a model in which the potential to form a well-defined three-dimensional RNA structure is important to catalysis. The ability of RNA to coordinate divalent cations may be significant. In the large ribosomal subunit, a subset of associated divalent cations is chelated by up to three phosphate groups of the 23S rRNA[25,26]. The P4–P6 domain RNA chelates fewer divalent cations, but with analogous coordination geometry[27]. tRNA also interacts strongly with divalent cations[17–19]. In contrast, RNAs

such as nucleotides, small ssRNAs, B-DNA and the STMV RNA genome, which lack the potential to form well-defined structures are much less efficient electron-transfer catalysts.

Fe^{2+} can substitute for Mg^{2+} in RNA compaction, as demonstrated previously for the P4–P6 domain RNA, which folds to the same state with either Mg^{2+} or Fe^{2+} ions[12], with common binding sites for either metal[28]. However, the low salt conditions that yielded the best catalysis here suggest that the RNAs are not fully folded to their native states in our experiments and, therefore, the structures of RNA–Fe^{2+} complexes that yield catalysis remain to be characterized fully.

The catalytic nature of these electron-transfer reactions is supported by standard enzyme assays. Since the amount of enzyme in a reaction mixture can be limiting, enzyme-catalysed reaction rates plateau rather than increase monotonically with increasing substrate concentration. For both 23S rRNA and the P4–P6 domain RNA, the electron-transfer reaction does, indeed, saturate (Fig. 2a,c).

Kinetic parameters for single-electron transfer by 23S rRNA and the P4–P6 domain RNA with Fe^{2+} were determined by fitting data to a Michaelis–Menten model (Fig. 2). Nonlinear regression analysis of the experimental data shows that for P4–P6 domain RNA, $k_{cat} = 2.2 \times 10^{-2}$ s^{-1}, $K_m = 4.15 \times 10^{-6}$ M and $k_{cat}/K_m = 5.3 \times 10^{3}$ $M^{-1}\,s^{-1}$. For the 23S rRNA, k_{cat} =1.28 s^{-1}, $K_m = 1.75 \times 10^{-5}$ M and $k_{cat}/K_m = 7.3 \times 10^{4}$ $M^{-1}\,s^{-1}$. However, if the RNA is not saturated with Fe^{2+}, the data underestimate the true k_{cat}.

Previously, Breaker and co-workers isolated a series of hammerhead ribozymes that cleave RNA upon binding to divalent metal cations[29]. These phosphoryl-transfer ribozymes show a linear relationship of log rate versus log [cation] for RNA cleavage. Slopes of either one or two in the log–log graphs were interpreted as indicating that either one or two cations were required for catalysis. Using this approach here, for the P4–P6 domain RNA the log of the rate varies linearly with log [Fe^{2+}] (Fig. 2e). The slope is approximately one. Although more-complex models cannot be excluded, these observations are consistent with a mechanism in which occupancy of a single Fe^{2+} site confers catalytic activity to the RNA. When RNA is degraded with RNase (Fig. 2f) or with heat (Supplementary Information), the catalytic activity is lost.

We observe that replacement of Mg^{2+} by Fe^{2+} *in vitro* alters and expands the functional capabilities of some biological RNAs to include redox activity. Our results suggest the possibility that RNA collaborates with iron and a variety of other metals in various functional roles *in vivo*. This interpretation is consistent with previous observations of direct Fe^{2+} interactions with RNA *in vivo*[30,31]. We propose that RNA function, in analogy with protein function, can be understood fully only in the context of association with a range of possible metals. It may be that, as for metalloproteins, metallo-RNAs in contemporary biological systems are controlled by metal receptors, chaperons and compartmentalization.

Previously, Suga and co-workers used *in vitro* selection to obtain redox-active RNA[32]. Suga's activity requires covalent attachment of a substrate to RNA[32]. Sen and Poon showed that RNA can enhance the redox activity of iron protoporphyrin IX[33]. We extended this previous work to observe that some of the most abundant and evolutionarily conserved RNAs have an intrinsic redox functionality that is activated simply by interaction with Fe^{2+}. Our results show that there are more potential functions for RNA sequences, that is, that RNA sequence space is more densely populated with function, than previously expected. This expansion of the apparent catalytic power of RNA adds a new dimension to the RNA world hypothesis and suggests that sophisticated biochemical transformations, such as reduction of ribonucleotides to deoxyribonucleotides, were possible in an RNA world.

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Author contributions

C.H., N.V.H, R.M.W., S.C.H. and L.D.W. conceived and designed the experiments and co-wrote the manuscript. C.H., C.D.O. and I-C.C. performed the experiments and analysed the data. J.C.B., E.B.O'N, S.S.A. and A.P. contributed materials and analysis tools. All the authors discussed the results and contributed to writing and editing the manuscript.

Additional information

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Competing financial interests

The authors declare no competing financial interests.