Iron mediates catalysis of nucleic acid processing enzymes: support for Fe(II) as a cofactor before the great oxidation event

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ABSTRACT

Life originated in an anoxic, Fe2+-rich environment. We hypothesize that on early Earth, Fe2+ was a ubiquitous cofactor for nucleic acids, with roles in RNA folding and catalysis as well as in processing of nucleic acids by protein enzymes. In this model, Mg2+ replaced Fe2+ as the primary cofactor for nucleic acids in parallel with known metal substitutions of metalloproteins, driven by the Great Oxidation Event. To test predictions of this model, we assay the ability of nucleic acid processing enzymes, including a DNA polymerase, an RNA polymerase and a DNA ligase, to use Fe2+ in place of Mg2+ as a cofactor during catalysis. Results show that Fe2+ can indeed substitute for Mg2+ in catalytic function of these enzymes. Additionally, we use calculations to unravel differences in energetics, structures and reactivities of relevant Mg2+ and Fe2+ complexes. Computation explains why Fe2+ can be a more potent cofactor than Mg2+ in a variety of folding and catalytic functions. We propose that the rise of O2 on Earth drove a Fe2+ to Mg2+ substitution in proteins and nucleic acids, a hypothesis consistent with a general model in which some modern biochemical systems retain latent abilities to revert to primordial Fe2+-based states when exposed to pre-GOE conditions.

INTRODUCTION

Iron was abundant, benign and soluble when life originated on the ancient earth (1–5). The geological record indicates that for ~2 billion years the oceans contained vast quantities of soluble Fe2+, with concentrations on the order of 10^-4 M. The reducing conditions of the ancient Earth favored Fe2+ over Fe3+ and mitigated destructive iron-mediated processes such as Fenton chemistry (6,7). During the first half of Earth’s history iron became broadly involved in protein-mediated biochemistry (8,9). The wide distribution of iron in extant biological systems (10), despite its harmful effects and low solubility on the surface of the extant Earth, highlights this element’s catalytic utility and deep evolutionary history.

Approximately 2 billion years ago, O2 began accumulating in the atmosphere, triggering global shifts in biochemistry and microbiology. This Great Oxidation Event (GOE) brought the modern condition of iron scarcity (10^-9 M in the oceans) and iron-mediated oxidative damage to biological systems (11). The GOE drove substitution of copper, zinc, manganese and other metals for iron in metabolic enzymes (8,12–18) as well as tight regulation of cellular distributions and concentrations (10).

We hypothesize that on the ancient earth, Fe2+ was a primary divalent cofactor in protein-based nucleic acid processing enzymes as well as in ribozymes (23,24). In this model, the GOE drove biosphere-wide Fe2+ → Mg2+ substitutions in both enzymes and ribozymes. The abilities of these two metals to substitute for each other are consistent with similarities in their coordination chemistries (Table 1). An earth-wide Fe2+ → Mg2+ substitution in nucleic acid processing enzymes and in ribozymes has analogy to well-established substitutions of Cu2+, Zn2+, Mn2+ for Fe2+ in metabolic enzymes (8,12–18). In our Fe2+ → Mg2+ model, the early emergence of polymerases, ligases, nucleases, repair enzymes and ribozymes was facilitated by interactions with Fe2+.

Magnesium seems to be essential for function of extant DNA polymerases, RNA polymerases and DNA ligases. A consensus of data supports a two divalent metal cation mechanism of phosphoryl transfer by these enzymes (25–30). Magnesium is thought to interact with phosphate groups and to accept and donate protons during catalysis. In a generally accepted transition state in polymerases, a hexacoordinated Mg2+ stabilizes the triphosphate of the newly base paired (d)NTP, while a partially hydrated Mg2+ activates the 3′-OH of the primer for nucleophilic attack on the 5′-phosphate of the (d)NTP, thereby completing the
phosphodiester bond. A similar two metal transition state has been proposed for the last step of phosphodiester bond formation by DNA ligase, whereby one metal stabilizes the phosphates of the temporarily adenylated DNA substrate and another activates the hydroxyl group. Probable metal ligands of polymerases and DNA ligase active sites include protein carboxylates, water molecules, and the non-bridging phosphate oxygens and hydroxyls of nucleic acids. Recent work proposes that a third divalent metal ion may also be essential for catalysis (31).

We use experiments to characterize Fe\textsuperscript{2+}-mediated biochemistry in simulated ancient Earth conditions. We have recreated anoxic, Fe\textsuperscript{2+}-rich conditions (pre-GOE conditions) in the laboratory. We test predictions of the Fe\textsuperscript{2+} → Mg\textsuperscript{2+} model by removing Mg\textsuperscript{2+} from three nucleic acid processing proteins, and replacing it with Fe\textsuperscript{2+} in the absence of O\textsubscript{2}. We test abilities of a DNA polymerase, an RNA polymerase and a DNA ligase to function using Fe\textsuperscript{2+} as a cofactor in place of Mg\textsuperscript{2+}. Specifically, we substituted Fe\textsuperscript{2+} for Mg\textsuperscript{2+} in a thermostable DNA polymerase (Deep Vent exo-) (32), in T7 RNA polymerase (33) and in T4 DNA ligase (34). The results show that Fe\textsuperscript{2+} can substitute for Mg\textsuperscript{2+} in initiation and elongation by the DNA polymerase, in RNA synthesis from a DNA template by the RNA polymerase, and in the joining of DNA oligonucleotides by the ligase.

In addition, we use calculations to reveal differences in energetics, structures and reactivities of Mg\textsuperscript{2+} and Fe\textsuperscript{2+} complexes. We observe that conformations and geometries of hexa aquo or first shell phosphodiester-complexes are conserved when Fe\textsuperscript{2+} is replaced by Mg\textsuperscript{2+}. Compared to Mg\textsuperscript{2+}, Fe\textsuperscript{2+} more effectively withdraws electrons from first shell ligands, causing increases in the electrophilicity of phosphorus atoms of first shell phosphodiester-complexes. By the same mechanism, compared to Mg\textsuperscript{2+}, Fe\textsuperscript{2+} increases the acidity of first shell water molecules. Therefore, Fe\textsuperscript{2+} is expected to be a more effective cofactor than Mg\textsuperscript{2+}. The combined experimental and computational results are consistent with the Fe\textsuperscript{2+} → Mg\textsuperscript{2+} model and suggest that some modern nucleic acid processing enzymes retain latent abilities to revert to primordial Fe\textsuperscript{2+}-based states when exposed to pre-GOE conditions.

## MATERIALS AND METHODS

### Polymerase chain reactions

Polymerase chain reactions (PCR) was performed in a Coy anaerobic chamber in 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 0.1% Triton\textsuperscript{®} X-100, 100 μg/ml nuclease-free bovine serum albumin, 1 mM dNTPs, 500 nM Cy3-labeled reverse primer, 500 nM forward primer, 0.5 nM template, 1 U DeepVent\textsuperscript{®} (exo-) DNA polymerase (New England Biolabs). Reaction solutions contained either 2 mM MgSO\textsubscript{4}, 2 mM FeCl\textsubscript{3}, 2 mM MnCl\textsubscript{2} or in negative controls, no divalent cations. Master mix solutions were prepared with all components except the polymerase and divalent cation, and lyophilized to dryness. We have previously demonstrated that our protocols remove oxygen from reaction mixtures (23,24). Using these methods there is no observable Fenton degradation of RNA in the presence of Fe\textsuperscript{2+} and Fe\textsuperscript{2+} substitutes for Mg\textsuperscript{2+} in RNA folding and ribozyme catalysis.

The DNA polymerase was added to the dry master mix, which was then transferred into the anaerobic chamber. Divalent cation solutions and nuclease-free H\textsubscript{2}O\textsubscript{2}, pre-equilibrated in the anoxic atmosphere, were added to produce four PCR mixtures, one with 2 mM Mg\textsuperscript{2+}, one with 2 mM Fe\textsuperscript{2+}, one with 2 mM Mn\textsuperscript{2+} and one lacking divalent cations. Ten microliter of each PCR solution was aliquoted to eight PCR tubes for a total of 32 reactions. One of these was kept at room temperature and served as a negative PCR control (Reaction 0). Tubes were heated to 95 °C for 2 min in a thermal cycler and cycled through 2, 4, 8, 12, 16, 20 or 24 PCR cycles. A cycle consisted of (i) denaturation at 95 °C for 30 s, (ii) annealing at 52 °C for 30 s and (iii) extension at 72 °C for 30 s. Divalent cations were removed from the reaction mixtures by incubation with Bio-Rad Chelex 100 Resin. The resin was removed with 0.22 μm centrifugal filters, rendering the samples stable in the presence O\textsubscript{2}.

The metal-free solutions were taken out of the anaerobic chamber and diluted 1:10 with nuclease-free water. One microliter of each diluted PCR solution was mixed with 9 μl of 10% glycerol and loaded onto a 12%:1:91 polyacrylamide gel buffered in Tris–Borate–Ethylenediaminetetraacetic acid (EDTA), pH 8.4. The reverse primer was also diluted in 10% glycerol and loaded onto the same gel. Each of the polyacrylamide gels were run at 100 V for 70 min at ambient temperature. Gels were imaged using a General Electric Typhoon Trio+ Imager. PCR template and primer sequences are provided in the Supplementary Information.

### In vitro transcription

For in vitro transcription reactions, DNA template was generated by digestion of intact plasmid pUC19 containing the *Thermus thermophilus* Domain III RNA gene (35) with HindIII, overnight, in NEB CutSmart buffer. The enzyme was heat inactivated at 80 °C for 20 min and linearized DNA purified with an IBI Scientific Gel/PCR DNA Fragment Extraction kit.

The in vitro transcription reactions were performed in a Coy anaerobic chamber. Master mix solutions were pre-

### Table 1. Mg\textsuperscript{2+} versus Fe\textsuperscript{2+}

<table>
<thead>
<tr>
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<th>r (Å)\textsuperscript{(a)}</th>
<th>AOCN\textsuperscript{(b)}</th>
<th>-ΔH\textsubscript{hydr}\textsuperscript{(c)}</th>
<th>pK\textsubscript{a}\textsuperscript{(d)}</th>
<th>ΔH\textsuperscript{(b)}</th>
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</thead>
<tbody>
<tr>
<td>Mg\textsuperscript{2+}</td>
<td>0.65</td>
<td>6</td>
<td>458\textsuperscript{(e)}</td>
<td>11.4</td>
<td>-1.3</td>
</tr>
<tr>
<td>Fe\textsuperscript{2+}</td>
<td>0.74</td>
<td>6</td>
<td>464\textsuperscript{(f)}</td>
<td>9.5</td>
<td></td>
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(a) Ionic radius (19); (b) Average Observed Coordination Number (19); (c) Hydration enthalpy (kcal mol\textsuperscript{-1}); (d) pKa of M\textsuperscript{2+}(H\textsubscript{2}O\textsubscript{6}) where M\textsuperscript{2+} = Fe\textsuperscript{2+} or Mg\textsuperscript{2+} (20); (e) From Rashina and Honig (21); (f) High spin; (g) From Uudsemaa and Tamm (22); (h) Relative interaction enthalpy (kcal mol\textsuperscript{-1}) for RNA clamp formation (23).
pared with all components except the T7 RNA polymerase and divalent cation, lyophilized to dryness, then introduced to the anaerobic chamber. In the chamber, the master mix was re-suspended in water that had been pre-equilibrated to the chamber atmosphere. Final reaction conditions were 1 × RNA polymerase reaction buffer (40 mM Tris–HCl, pH 7.9, 2 mM spermidine, 1 mM dithiothreitol), 0.375 mM each nucleoside triphosphate, 5.4 mM spermidine, 1 mM dithiothreitol, 36 U of T7 RNA Polymerase (cat no. M0251S) per microliter of reaction volume, in addition to variable and 5–6 U NEB T7 RNA Polymerase (cat no. M0251S) per microliter of reaction volume, in addition to variable components except the T7 RNA polymerase and divalent cation, lyophilized to dryness, transferred to the anaerobic chamber. The master mix was divided into three aliquots. Mg^{2+}, Fe^{2+} or nuclease-free water was added to each of the tubes. The reactions were incubated at room temperature for 1 h. Reactions were terminated by incubation with Bio-Rad Chelex 100 Resin. The resin was removed with 0.22 µm centrifugal filters transferred out of the chamber for analysis. Ten microliter of the filtrate was added to 10 µl of loading dye (47.5% formamide, 0.01% SDS, 0.01% bromophenol blue, 0.005% Xylene Cyanol, 0.5 mM EDTA), followed by heating to 90°C for 5 min and quick cooling on ice for 10 min. Samples were loaded onto a 6% denaturing polyacrylamide gel buffered in tris–borate EDTA, pH 8.4. Gels were run at 100 V for 30 min. at ambient temperature, stained with SYBR Green I and imaged on a General Electric Typhoon Trio+ Imager.

Ligase reactions

Ligase reactions were performed in the Coy anaerobic chamber in 50 mM Tris–HCl (pH 7.4), 1 mM adenosine triphosphate and 10 mM dithiothreitol. Two semi-complementary DNA 12-mers, one with 5′-phosphate, were used in the ligation reaction. The sliding half-complementary oligonucleotide sequences were 5′-pGAATGGGTAGAC-3′ and 5′-CCATCGCTCTA-3′. Seven µM of each oligonucleotide was incubated with 200 U of T4 DNA ligase (New England Biolabs) and 10 mM MgCl₂ or 10 mM FeCl₂. Negative control experiments lacked divalent cations.

DNA oligonucleotides and a ligase master mix containing all reaction components except divalent cations and ligase were lyophilized to dryness, transferred to the anaerobic chamber, equilibrated with the anoxic atmosphere and dissolved in degassed nuclease-free water. T4 DNA ligase was brought into the chamber and equilibrated with the anoxic atmosphere. Once equilibrated, the ligase was then added to the master mix. The solution was divided into three aliquots. Mg^{2+}, Fe^{2+} or nuclease-free water was added to each of the tubes. The reactions were incubated at room temperature for 1 h. Reactions were terminated by incubation with Bio-Rad Chelex 100 Resin. The resin was removed with 0.22 µm centrifugal filters transferred out of the chamber for analysis. Ten microliter of the filtrate was added to 10 µl of loading dye (47.5% formamide, 0.01% SDS, 0.01% bromophenol blue, 0.005% Xylene Cyanol, 0.5 mM EDTA), followed by heating to 90°C for 5 min and quick cooling on ice for 10 min. Samples were loaded onto a 6% denaturing polyacrylamide gel buffered in tris–borate EDTA, pH 8.4. Gels were run at 100 V for 30 min. at ambient temperature, stained with SYBR Green I and imaged on a General Electric Typhoon Trio+ Imager.

Computation

Hexa aquo complexes [M^{2+}(H₂O)₆] and M^{2+}-RNA clamps (where M = Fe^{2+} or Mg^{2+}) were optimized at the unrestricted B3LYP/6–31G(d,p) level of theory. For Fe^{2+} the spin was two and multiplicity was five. For Mg^{2+}, the spin was zero and the multiplicity was one. Single point energies for these complexes were further obtained at the (U)B3LYP/6–311++G(d,p) level of theory using SCF options DIIS, NOVARACC, VTL and MaxCyc = 1000.

The coordinates of the Mg^{2+}-RNA clamp were extracted from the x-ray structure of the Haloarcula marismortui large ribosomal subunit (PDB entry: 1JJ2) (36) as previously described (37). The free 5′ and 3′ termini of the phosphate groups were capped with methyl groups in lieu of the remainder of the RNA polymer, and hydrogen atoms were added where appropriate. An Fe^{2+}-RNA clamp was constructed from the Mg^{2+} clamp by converting Mg^{2+} to Fe^{2+} as described (23). The binding of an Mg^{2+} or Fe^{2+} ion to an RNA fragment is described by the following reaction:

$$\text{RNA}_2^- + \text{M}^{2+}(\text{H}_2\text{O})_6 \rightarrow \text{RNA}_2^- - \text{M}^{2+}(\text{H}_2\text{O})_4 + 2\text{H}_2\text{O},$$

where, $$\text{M}^{2+} = \text{Mg}^{2+} \text{or Fe}^{2+}$$.

The reactants and products of this reaction were fully optimized using the density functional theory with the hybrid B3LYP functional, which combines the generalized gradient approximation (GGA) exchange three-parameter hybrid functional of Becke (38) and the correlation functional of Lee-Yang-Parr (39), with the 6–311++G(d,p) basis set as implemented in Gaussian 09 (40).

Natural Bond Order (NBO) (41) and natural energy decomposition analysis (NEDA) (41,42) were performed on the optimized complexes at the (U)B3LYP/6–31G(d,p) level of theory using the GAMESS package (43) and the NBO 5.0 routine. For NEDA calculations, metal phosphate clamps were treated as products and the free sugar-phosphate backbones and hexa aquo metals were reactants.

RESULTS

We have investigated the ability of a DNA polymerase, an RNA polymerase and a DNA ligase to function using Fe^{2+} instead of Mg^{2+} as a cofactor under simulated ancient earth conditions. All reactions were performed in a Coy anaerobic chamber in an atmosphere of 95% argon and 5% hydro-
Fe\textsuperscript{2+} or Mn\textsuperscript{2+} can replace Mg\textsuperscript{2+} as a cofactor for Deep Vent (exo-) DNA polymerase. We have investigated polymerization using Deep Vent (exo-) DNA polymerase. We used PCR in the presence of Mg\textsuperscript{2+} or Fe\textsuperscript{2+} or Mn\textsuperscript{2+} or in the absence of divalent cations to amplify a 72 nucleotide DNA fragment. Mg\textsuperscript{2+}, Fe\textsuperscript{2+} and Mn\textsuperscript{2+} each facilitate formation of polymerization product. We determined the amount of reactants (primers) and product in the reaction mixture at every other PCR cycle (Figure 1). For divalent cations Mg\textsuperscript{2+} or Fe\textsuperscript{2+} or Mn\textsuperscript{2+}, reaction product is first visible on the gel at cycle eight. The primer is fully consumed in all reactions by cycle twenty. It can be seen that these three divalent cations produce identical yields under the conditions of the reaction within the error of the experiment. If the yields were not similar for each divalent cation at each cycle, the cycle numbers at which primer disappears and at which product appears would vary between the different divalent cations. The absence of product in the reaction lacking divalent cations demonstrates that Mg\textsuperscript{2+} extraction methods are efficient and that the product observed in the Fe\textsuperscript{2+} reaction is not attributable to contaminating Mg\textsuperscript{2+}.

RNA polymerase

Fe\textsuperscript{2+} substitutes for Mg\textsuperscript{2+} as a cofactor for T7 RNA polymerase. Digested plasmid encoding a template for a 376 nucleotide fragment of Domain III rRNA (44) was used with varying concentrations of Mg\textsuperscript{2+} or Fe\textsuperscript{2+}. Full length RNA product is observed at all concentrations of Mg\textsuperscript{2+} investigated here, and at concentrations of Fe\textsuperscript{2+} up to 6 mM (Figure 2). No RNA product is observed in control reactions without added divalent metals, confirming that the RNA products in the Fe\textsuperscript{2+} reactions do not result from Mg\textsuperscript{2+} contamination. The divalent cation concentration that gives a maximum RNA yield is less for Fe\textsuperscript{2+} than for Mg\textsuperscript{2+}. At 0.75 mM of either divalent cation, the product yield is greater for Fe\textsuperscript{2+} than for Mg\textsuperscript{2+}. Therefore, at low divalent metal concentrations T7 RNA polymerase appears more active in the presence of Fe\textsuperscript{2+} than in Mg\textsuperscript{2+}. These experiments were independently replicated, giving consistent results in each replica. The identities of other bands on the gels are discussed in 'Materials and Methods' section and Supplementary Figure S1.

The Mg\textsuperscript{2+} optimum for T7 DNA polymerase is around 10 mM, depending on the presence of other cations (45). The concentration optimum is an order of magnitude lower for Fe\textsuperscript{2+} than for Mg\textsuperscript{2+}. Under the conditions of these reac-

Figure 1. Fe\textsuperscript{2+} or Mn\textsuperscript{2+} can replace Mg\textsuperscript{2+} as a cofactor for Deep Vent (exo-) DNA polymerase. Reaction mixtures with Fe\textsuperscript{2+} or Mg\textsuperscript{2+} or Mn\textsuperscript{2+} or no divalent cation were analyzed for amount of reactants and products at every other PCR cycle for 24 cycles. Reactant consumption and product yields are identical within the uncertainty of this experiment among (A) Fe\textsuperscript{2+}, (B) Mg\textsuperscript{2+} or (C) Mn\textsuperscript{2+}. (D) The divalent-minus controls, which lack divalent cations, did not generate product. Neither did the ‘minus template’ negative control reactions (far left lanes of each gel), taken through 24 cycles, generate product.
The calculations show why hexa aquo Fe\(^{2+}\) is a stronger acid than hexa aquo Mg\(^{2+}\). Fe\(^{2+}\) withdraws 0.142 more electrons from its first shell water molecules (Supplementary Tables S4 and S5). The net charge on Fe\(^{2+}\) in a hexa aquo complex is +1.631 while the net charge on Mg\(^{2+}\) in a hexa aquo complex is +1.773. The calculations show that 0.369 electrons are transferred from the six first shell water molecules to Fe\(^{2+}\), while only 0.227 electrons are transferred to Mg\(^{2+}\). The net number of electrons transferred from the average first shell water of hexa aquo Fe\(^{2+}\) is 0.060 electrons per water molecule. The net number of electrons transferred from the average first shell water of hexa aquo Mg\(^{2+}\) is 0.037 electrons per water molecule.

**DISCUSSION**

Here we recapitulate the reductive potential of the Archean atmosphere (46–48). Kasting’s high CO\(_2\) model of the Archean atmosphere (46) has been re-evaluated (48); geological data is considered to be incompatible with this model (47,49). Although the specific chemical composition of the Archean atmosphere remains unresolved, it is accepted that it was reductive and that the biosphere was iron-rich and was lacking O\(_2\). Life originated and first proliferated in a reductive iron-rich environment, which persisted until around 2 billion years ago, when the GOE began depleting iron from the biosphere (1–4,23) and fostering Fe\(^{2+}/O_2\) mediated cellular damage (7).

The environment of the ancient Earth is consistent with a Fe\(^{2+}/Mg\(^{2+}\) model in which Fe\(^{2+}\) was an important cofactor for both nucleic acids and proteins during early evolution. Extant protein enzymes process nucleic acids using Mg\(^{2+}\) as a cofactor but might have used Fe\(^{2+}\) on the early earth. It is possible that extant biopolymers retain intrinsic adaptation to Fe\(^{2+}\). Indeed, previous experimental substitution of Fe\(^{2+}\) for Mg\(^{2+}\) in association with RNA demonstrated that Fe\(^{2+}\) can facilitate RNA folding and expand RNA catalytic breadth (23,24).

To determine if the Fe\(^{2+}/Mg\(^{2+}\) model is plausible, and if ancient nucleic acid processing enzymes might have used Fe\(^{2+}\) instead of Mg\(^{2+}\) as a primary cofactor, here we substituted Fe\(^{2+}\) for Mg\(^{2+}\) in a DNA polymerase, a RNA polymerase and a DNA ligase. Polynucleotide polymerases are ubiquitous enzymes that perform some of the most critical and universal enzymatic activities in the biological world. Polymerases synthesize polynucleotides from (d)NTPs by covalently joining nucleotides as directed by a template. Polymerases use di-metal centers with metals coordinated by the protein and during catalysis, by phosphate groups of the substrate (25). The di-metal centers in DNA and RNA polymerases are thought to (i) stabilize a pentacovalent transition state, (ii) facilitate the leaving of pyrophosphate and (iii) lower the pK\(_a\) of the 3'-hydroxyl of the terminus. Mg\(^{2+}\) is the thought to be the preferred divalent ion in vivo for polymerases. The results here demonstrate that Fe\(^{2+}\), in a reductive environment, can substitute for Mg\(^{2+}\) in function of both DNA and RNA polymerases. It was shown previously that Mn\(^{2+}\) (50,51), or with reduced functionality, Ca\(^{2+}\) (52), can substitute for Mg\(^{2+}\) in some polymerases in vitro.

DNA ligase, another ancient protein enzyme, is required for DNA replication and repair (34,53). DNA ligase catalyzes the joining of terminal 5'-phosphoryl and 3'-hydroxyl groups of DNA fragments. DNA ligase joins Okazaki fragments produced by lagging strand DNA synthesis and seals nicks after DNA excision repair. Ligation uses three sequential nucleotidyl transfers, each of which requires a divalent
Figure 2. Fe$^{2+}$ can replace Mg$^{2+}$ as a cofactor in transcription by T7 RNA polymerase. Full length RNA transcript is observed with either Mg$^{2+}$ or Fe$^{2+}$. No product is observed in the no-divalent negative control. The far left lane contains ssRNA size markers. The top of the gel contains large and/or aggregated nucleic acids that remain in the wells during electrophoresis.

Figure 3. Fe$^{2+}$ can replace Mg$^{2+}$ as a cofactor for T4 DNA ligase. Ligation products of sliding-half complementary oligonucleotides are observed with both Mg$^{2+}$ and Fe$^{2+}$. No product is observed in the divalent-minus negative control. See ‘Materials and Methods’ section for oligonucleotide sequences.

Metal cofactor. Mg$^{2+}$ is thought to be the preferred ion in vivo. A ferric iron-containing ligase with an exceptionally low pH optimum has been isolated from an acidophilic ferrous iron-oxidizing archaeon (34). Structural and possibly catalytic roles for the ferric iron are possible. Unlike the systems we investigate here, the ferric ligase is not active in the presence of Mg$^{2+}$.

Like DNA and RNA polymerases, DNA ligases are believed to contain di-metal centers within their active sites. Metals are coordinated by the protein and, during catalysis, by substrate phosphate groups (34,53). The di-metal center activates hydroxyl groups for nucleophilic attack and stabilizes leaving groups. The results here indicate that Fe$^{2+}$ can substitute for Mg$^{2+}$ in a ligase di-metal center. In the presence of Fe$^{2+}$, the ligase is functional but may be less active than in the presence of Mg$^{2+}$.

Mechanisms of phosphoryl transfer and geometries of di-metal centers are highly conserved and thought to be evolutionarily ancient (25). Formation of di-metal centers appears to be required for activity of the enzymes investigated here. Therefore, it appears functional di-iron centers form in DNA and RNA polymerases and in DNA ligases under our experimental conditions. The facility of substitution of Fe$^{2+}$ for Mg$^{2+}$ demonstrated here suggests that di-metal centers, including di-iron centers in enzymes such as ribonuclease reductase (15) and di-magnesium centers in polymerases (25,55) and in large RNAs (56), may be related by common ancestry or by origins in a common chemical environment. Observed activity of polynucleotide polymerases and a DNA ligase in the presence of Fe$^{2+}$ is consistent with similar coordination geometries and chemistries between Mg$^{2+}$ and Fe$^{2+}$ (Table 1). However, decreased activity of these enzymes in the presence of Fe$^{2+}$ instead of Mg$^{2+}$ might be expected after nearly 2 billion years of evolution that would have optimized the use of Mg$^{2+}$ as a cofactor, rather than Fe$^{2+}$.

Although Mg$^{2+}$ and Fe$^{2+}$ are characterized by similar coordination geometries and chemistries (Table 1), and can substitute for each other in di-metal centers, they are distinguished by important differences. We have observed that Fe$^{2+}$ can be a more potent cofactor than Mg$^{2+}$ in RNA folding. RNA folds at lower concentrations of Fe$^{2+}$ than Mg$^{2+}$, and that at least a subset of ribozymes are more active in Fe$^{2+}$ than in Mg$^{2+}$ (23). In addition, we observe here that at low concentrations of divalent cations, T7 RNA polymerase is more active in the presence of Fe$^{2+}$ than Mg$^{2+}$.

To attempt to understand the origins of these differences, we have used computation to determine the effects of substitution of Fe$^{2+}$ for Mg$^{2+}$ in hexa aquo complexes and in complexes with first shell phosphodiester ligands. We have modeled phosphate complexes of Fe$^{2+}$ and Mg$^{2+}$. The results allow us to understand the roles of divalent cations in RNA folding and during important catalytic steps of polymerization and ligation reactions. The forces, energetics and electronic perturbations within phosphate complexes of Fe$^{2+}$ and Mg$^{2+}$ complexes were characterized by density functional methods (57–63). Components of the interaction energy, such as charge transfer, polarization and exchange were investigated with NBO and NEDA (41,42). The computations reveal subtle yet critical differences between Mg$^{2+}$ and Fe$^{2+}$.

Computation helps explain why Fe$^{2+}$ can be a potent cofactor for nucleic acid processing enzymes. Conformations
Figure 4. (A) A three-dimensional representation of a common $M^{2+}$ complex in RNA in which the $M^{2+}$ ion is coordinated directly by two phosphate oxygens in an RNA-$M^{2+}$ clamp. The $M^{2+}$ is six coordinate; the four first shell water molecules are omitted for clarity. (B) Electronic schematic of the $M^{2+}$ complex shown in panel A where $M^{2+} = Mg^{2+}$. The two axial water oxygen atoms are omitted for clarity. (C) Electronic schematic of the $M^{2+}$ complex shown in panel A where $M^{2+} = Fe^{2+}$. The atoms are labeled in all three panels.
and geometries are nearly identical between complexes of Fe²⁺ or Mg²⁺. These similarities are observed in both hexa aquo and phosphate complexes. Important differences between Mg²⁺ and Fe²⁺ follow. (i) Interactions with water. Hexa aquo Fe²⁺ is a stronger acid than hexa aquo Mg²⁺ (Table 1). Our calculations show greater depletion of electrons from water molecules that coordinate Fe²⁺ than those that coordinate Mg²⁺. (ii) Interactions with phosphorus. Because of low lying d orbitals, Fe²⁺ has greater electron withdrawing power than Mg²⁺ from first shell phosphate ligands (Figure 4, Supplementary Tables S2 and 3). In coordination complexes with phosphate groups, the phosphorus atom is a better electrophile when M²⁺ = Fe²⁺ than when M²⁺ = Mg²⁺. MODULATION OF RIBOZYME ACTIVITY 2. Popović and Ditzler performed what was, to our knowledge, the first ever in vitro RNA selection under plausible pre-GOE conditions (64) and demonstrated that ribozymes obtained by selection with Fe²⁺ are active upon substitution of Mg²⁺ for Fe²⁺. Here we begin to test the hypothesis that Fe²⁺ was a divalent cation that actively facilitated processing of nucleic acids by proteins on the ancient Earth. We have investigated ancient Earth biochemistry of proteins that play important roles in nucleic acid processing. Polymerases and ligases are both important in DNA replication, RNA transcription and DNA repair. The ability of Fe²⁺ to substitute for Mg²⁺ in the polymerases and the ligase suggests that Fe²⁺, in the absence of O₂, could substitute for Mg²⁺ in a broad variety of enzymes involved in nucleic acid processing. We have used calculations to show that Fe²⁺ is a viable substitute for Mg²⁺ in nucleic acid-cation interactions. Therefore, Fe²⁺ might have played a role in the early evolution of proteins and nucleic acids.

CONCLUSION
Popović and Ditzler performed what was, to our knowledge, the first ever in vitro RNA selection under plausible pre-GOE conditions (64) and demonstrated that ribozymes obtained by selection with Fe²⁺ are active upon substitution of Mg²⁺ for Fe²⁺. Here we begin to test the hypothesis that Fe²⁺ was a divalent cation that actively facilitated processing of nucleic acids by proteins on the ancient Earth. We have investigated ancient Earth biochemistry of proteins that play important roles in nucleic acid processing. Polymerases and ligases are both important in DNA replication, RNA transcription and DNA repair. The ability of Fe²⁺ to substitute for Mg²⁺ in the polymerases and the ligase suggests that Fe²⁺, in the absence of O₂, could substitute for Mg²⁺ in a broad variety of enzymes involved in nucleic acid processing. We have used calculations to show that Fe²⁺ is a viable substitute for Mg²⁺ in nucleic acid-cation interactions. Therefore, Fe²⁺ might have played a role in the early evolution of proteins and nucleic acids.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.