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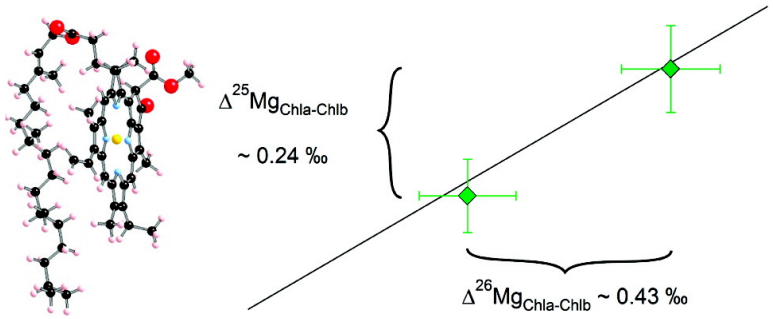

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Magnesium Isotopic Equilibrium in Chlorophylls

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Photosynthesis generates ~100 gigatons of carbon per year and drives many terrestrial geochemical cycles.[1] Magnesium is the metal center of all chlorophylls and is thus central to photosynthesis. Plants and microorganisms synthesize a number of structurally distinct chlorophylls. The two main forms are chlorophyll-a (Chl-a) and chlorophyll-b (Chl-b). The ratio of these chlorophylls varies as the plants adapt to specific environmental conditions.[2] The complexity of the enzymatic pathways involved in effecting this variation leads us to speculate that the chlorophyll cycle may produce a difference in the Mg isotope composition of Chl-a and Chl-b. In this study, we attempt to detect such an isotopic difference, which would put constraints on the lifetime of magnesium in these biomolecules. It is well-known that biosynthetic pathways can cause fractionation of light isotopes; ^{13}C is preferentially distributed to certain sites of Chl-a.[3] A few previous studies[4−7] indicate a potential biogenic fractionation of the three naturally abundant stable isotopes of magnesium ($^{24,25,26}Mg$) in chlorophyll. Here, using multicollector inductively coupled plasma mass spectrometry (MC-ICP-MS), we demonstrate a mass-dependent isotopic fractionation of Mg between Chl-a and Chl-b and also between the porphyrin species and the inorganic Mg complexes in a leaf's reservoir. Density functional (DFT) electronic structure calculations indicate that the measured Chl-a/Chl-b isotope ratio is consistent with an equilibrium fractionation.

Chl-a and Chl-b are structurally similar, with a formyl functional group of Chl-b replacing the methyl functional group on the seventh carbon of the porphyrin ring of Chl-a. Prior to the synthesis of either form of chlorophyll, the Mg is inserted into the center of the protoporphyrin IX molecule. This reaction is mediated by the Mg chelatase enzyme and requires the hydrolysis of adenosine triphosphate (ATP). The hydrolysis of ATP is thought to enhance dehydration of the strongly bound waters on the $Mg(H_2O)_6^{2+}$ ion and to facilitate the formation of the Mg−N bonds in the center of the porphyrin.[8] Once the Mg is inserted into protoporphyrin IX, the Mg−protoporphyrin complex undergoes a series of biosynthetic steps to form chlorophyllide-a, the main precursor to the formation of Chl-a and Chl-b.[9] Formylation of the seventh carbon of chlorophyllide-a is mediated by the enzyme chlorophyllide-a oxygenase (CAO)[2] to form chlorophyllide-b. Chlorophyllide-a or -b undergoes esterification with a phytol group to form Chl-a and Chl-b, respectively.

It is unlikely that the biosynthetic step responsible for converting chlorophyllide-(a,b) to Chl-(a,b) distinguishes between the different Mg isotopes because there is only a slight structural difference in the functional group on the seventh carbon of the porphyrin ring. Any difference in isotopic composition between Chl-a and Chl-b is more likely due to other enzymatic pathways that regulate the ratio of these species in the plant. For example, during the chlorophyll cycle (Figure S1), Chl-b can be converted directly to Chl-a, while the reverse process has not been identified. If biosynthetic pathways fractionate Mg isotopes in Chl-a and Chl-b, the distribution would be preserved.

Chl-a and Chl-b were extracted and purified from the leaves of English Ivy (*Hedera helix* L.) to measure their Mg isotopic composition. In addition, we measured the overall Mg isotopic composition of the leaf, allowing us to determine the isotopic fractionation between the main Mg reservoirs within the plant. Ratios of $^{26}Mg/^{24}Mg$ and $^{25}Mg/^{24}Mg$ were measured using MC-ICP-MS and reported relative to the international standard DSM3 in a δ^xMg notation ($\delta^x Mg = \{(^xMg/^{24}Mg)_{Sample}/(^xMg/^{24}Mg)_{DSM3} - 1\}$, $x = 25, 26$).

The measured $\delta^{25}Mg$ versus $\delta^{26}Mg$ ratios are plotted in Figure 1 (reported in Table S1). The results are summarized in Table 1 and show that Chl-a is isotopically heavier than Chl-b ($\Delta^{26}Mg_{Chla-Chlb} = 0.434$ ‰ (±0.148) and $\Delta^{25}Mg_{Chla-Chlb} = 0.241$ ‰ (±0.108), where $\Delta^x Mg_{L-L'} = \delta^x Mg_L - \delta^x Mg_{L'}$). Furthermore, both chlorophylls are heavier than the overall isotopic composition of the leaves from which they were extracted. To confirm that no fractionation was induced during the processing of the samples on anion and cation exchange resins, the isotopic composition of the extracts can be calculated using the isotopic ratios measured for the individual samples of Chl-a and Chl-b (IV10−IV13, Table S1) and the relative percentages of Chl-a and Chl-b in the corresponding pigment extracts (IV08−IV09, Table S2). The calculated value is plotted in Figure 1b and falls within the error of the measured Chl-a + Chl-b sample, confirming that our results are not artifacts of sample processing.

We carried out density functional theory (DFT) electronic structure calculations (O3LYP/6-31G*)[12] of the vibrational frequencies of relevant species to estimate the fractionation of Mg isotopes between the Chl-a and Chl-b molecules and inorganic forms of dissolved Mg. We also present results with frequencies obtained using stretch, bend, and torsional scaling factors.[11] In the harmonic approximation, the reduced partition ratio, β, is given by[13]

$$\beta = \left(\frac{Q_h}{Q_l}\right) = \prod_i \frac{u_{hi}}{u_{li}} \frac{e^{-u_{hi}/2}}{1 - e^{-u_{hi}}} \frac{1 - e^{-u_{li}}}{e^{-u_{li}/2}} \qquad (1)$$

where $u_{(h,l)}$ are a function of the frequencies of vibration for the heavy (h, ^{26}Mg or ^{25}Mg) and light (l, ^{24}Mg) isotopes, and its product extends to all frequencies of the molecule. The equilibrium fractionation factor between compounds L and L′ (see Table 1 caption) is then given by

$$\alpha_{L/L'} = \beta_L/\beta_{L'} \qquad (2)$$

The porphyrin species include neutral Mg-centered Chl-a and Chl-b molecules (both histidine-complexed and free), chlorophyllide-a and -b (free and histidine-complexed), and protoporphyrin IX. The inorganic forms include two different conformers of $Mg(H_2O)_{18}^{2+}$, $Mg(H_2O)_{32}^{2+}$ and Mg citrate. Reduced partition function ratios (β, eq 1) were computed for $^{25/24,26/24}Mg$ (Table S3) from

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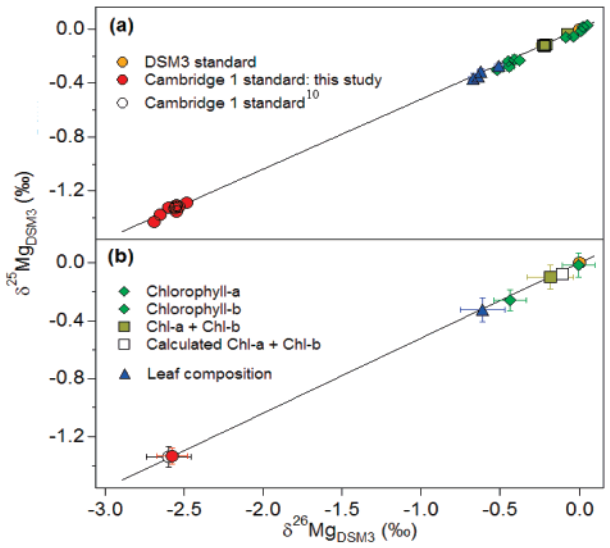

Figure 1. Magnesium isotope ratios expressed (in per mil) relative to the standard DSM3. The error bars correspond to $\pm 2\sigma$. (a) High-precision, individual sample and standard measurements from this study. (b) The average results of sample and standard measurements.

Table 1. Measured and Calculated Fractionation Factors

compound (L)/compound (L′)[a]	Δ^{26}Mg[b] (‰) ($\pm 2\sigma$)	Δ^{25}Mg[b] (‰) ($\pm 2\sigma$)
Measured:		
Chl-a/Chl-b	0.434(0.148)	0.241(0.108)
Chl-a/Mg reservoir in leaf	0.604(0.175)	0.311(0.117)
Chl-b/Mg reservoir in leaf	0.171(0.174)	0.069(0.109)
Calculated:		
Chl-a/Mg$(H_2O)_6$ flat + 12H_2O	3.08	1.58
Chl-b/Mg$(H_2O)_6$ flat + 12H_2O	2.45	1.25
Chl-a−HIS/Mg$(H_2O)_6$ flat + 12H_2O	1.51	0.77
Chl-a/Mg$(H_2O)_6$ *T*-symm + 12H_2O	4.06	2.03
Chl-a/Mg$(H_2O)_6$ + 26H_2O	5.52	2.84
Chl-a/Mg citrate + 14H_2O	3.03	1.57
Chl-a/Mg−protoporphyrin IX	0.60	0.31
Chl-a/Chl-b	0.63 {0.59}[c]	0.33 {0.30}
Chl-a−HIS/Chl-b−HIS	0.11 {0.10}	0.05 {0.05}
chlorophyllide-a/chlorophyllide-b	0.67 {0.62}	0.35 {0.32}

[a] Fractionation factors are determined between compound (L) over compound (L′) using eq 2 and converted to per mil using: $\Delta^{x}Mg_{Comp(L)-Comp(L')} = (\alpha^{x}_{Comp(L)-Comp(L')} - 1) \times 10^3$. [b] Positive per mil difference means compound (L) is heavier in the corresponding isotope (^{26}Mg or ^{25}Mg) than compound (L′) and vice versa for the negative difference. [c] {Modified Scaled Quantum Mechanical Force Field}.[11] HIS = histidine.

the calculated frequencies, and the fractionation factors are given in Table 1. There is a clear trend showing that Chl-a and Chl-b are isotopically heavier than the bioavailable forms. In addition, Chl-a is consistently heavier than Chl-b, for all classes including chlorophyllide a/b, chlorophyll a/b, and chlorophyll a/b complexed with histidine. The Chl−HIS and Chlide−HIS complexes exhibit very small isotopic fractionations. Chl-(a,b)−HIS complexes with "free" histidine have much shorter Mg−HIS bond distances (2.2 Å) than those found in the protein (2.9 Å). The fractionation factor characteristic of the protein environment should lie somewhere between that of the Chl in the absence of HIS and the Chl−HIS complex with the histidine unconstrained, as indicated by our measurements. The force constant scaling procedure (Table 1, values in {mSQM}) gives negligible changes in the results, indicating that the calculations are robust.

Direct comparison of the differences between Chl-a and Chl-b, from calculations and measurement (Table 1), shows that they are both on the order of $\Delta^{26}Mg = 0.5$ and $\Delta^{25}Mg = 0.25$. This similarity indicates that Mg in Chl-a and Chl-b has reached isotopic equilibrium. The measured fractionation between the isotopically light leaf reservoir and the isotopically heavier porphyrin species is consistent in sign with the calculated fractionation factor of the porphyrin species relative to bioavailable forms of Mg. The difference between the calculated and measured magnitude (calculated: $\Delta^{26}Mg \sim 1.5-5.5$‰; measured: $\Delta^{26}Mg \sim 0.17-0.6$‰) could arise from some isotopic fractionation during metal translocation within a plant. It may also reflect a nonoptimal model for the bioavailable Mg in the calculations or systematic errors in the particular choice of methods for the electronic structure calculations.

If isotopic equilibrium is established between Chl-a and Chl-b, Mg must be labile between these compounds on time scales of the order of the lifetime of the pigment. Had Mg been inert once inserted into protoporphyrin IX, the isotopic composition of Chl-a and Chl-b would have been identical. The lability of Mg from the porphyrin ring of chlorophyll, under conditions of stress (e.g., toxic levels of Cu, Pb, Hg), is well-established by the occurrence of transmetalated chlorophylls.[14] These new results, however, show that the isotopic composition of Mg in chlorophyll undergoes mass-dependent fractionation and the various chlorophylls appear to be in isotopic equilibrium with one another based upon the similarity of the calculated and measured fractionation factors. Thus the magnesium is labile on the time scale of the chlorophyll cycle. Similar analyses of the isotope chemistry of nontraditional stable isotopes[6] in biomolecules have the potential to yield novel insights into the role of these metals in plant physiology.

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Supporting Information Available: Materials and methods; Tables S1−S3; Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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