



The effect of carbonic anhydrase on the kinetics and equilibrium of the oxygen isotope exchange in the CO₂–H₂O system: Implications for δ¹⁸O vital effects in biogenic carbonates

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Abstract

Interpretations of the primary paleoceanographic information recorded in stable oxygen isotope values (δ¹⁸O) of biogenic CaCO₃ can be obscured by disequilibrium effects. CaCO₃ is often depleted in ¹⁸O relative to the δ¹⁸O values expected for precipitation in thermodynamic equilibrium with ambient seawater as a result of vital effects. Vital effects in δ¹⁸O have been explained in terms of the influence of fluid pH on the overall δ¹⁸O of the sum of dissolved inorganic carbon (DIC) species (often referred to as “pH model”) and in terms of ¹⁸O depletion as a result of the kinetic effects associated with CO₂ hydration (CO₂ + H₂O ↔ H₂CO₃ ↔ HCO₃[−] + H⁺) and CO₂ hydroxylation (CO₂ + OH[−] ↔ HCO₃[−]) in the calcification sites (so-called “kinetic model”). This study addresses the potential role of an enzyme, carbonic anhydrase (CA), that catalyzes inter-conversion of CO₂ and HCO₃[−] in relation to the underlying mechanism of vital effects. We performed quantitative inorganic carbonate precipitation experiments in order to examine the changes in ¹⁸O equilibration rate as a function of CA concentration. Experiments were performed at pH 8.3 and 8.9. These pH values are comparable to the average surface ocean pH and elevated pH levels observed in the calcification sites of some coral and foraminiferal species, respectively. The rate of uncatalyzed ¹⁸O exchange in the CO₂–H₂O system is governed by the pH-dependent DIC speciation and the kinetic rate constant for CO₂ hydration and hydroxylation, which can be summarized by a simple mathematical expression. The results from control experiments (no CA addition) are in agreement with this expression. The results from control experiments also suggest that the most recently published kinetic rate constant for CO₂ hydroxylation has been overestimated. When CA is present, the ¹⁸O equilibration process is greatly enhanced at both pH levels due to the catalysis of CO₂ hydration by the enzyme. For example, the time required for ¹⁸O equilibrium is nearly halved by the presence of 3.7 × 10^{−9} M of CA used for the experiments. Despite its significant influence on the oxygen isotope exchange kinetics, the equilibrium oxygen isotope fractionation between individual DIC species and H₂O is unaffected by CA. Because many CaCO₃-secreting organisms possess active CA, our findings imply that ¹⁸O equilibration of the CO₂–H₂O system is possible within realistic timescales of biogenic calcification.

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1. INTRODUCTION

Stable oxygen isotope values (δ¹⁸O) of marine biogenic CaCO₃ such as foraminiferal tests and coral skeletons have

served as a primary tool to reconstruct past ocean temperatures and global ice volumes on various timescales (e.g., Shackleton and Kennett, 1975; Corrège et al., 2000; Tudhope et al., 2001; Zachos et al., 2001). However, one of the major biases in the paleoceanographic utility of this proxy is that δ¹⁸O values of these carbonates often deviate from the expected thermodynamic equilibrium values as a result of vital effects (Keith and Webber, 1965; McConnaughey, 1989a; Spero et al., 1997; Adkins et al., 2003). Based on the fact that individual dissolved inorganic

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carbon (DIC) species have a distinct equilibrium ^{18}O fractionation relative to H_2O (McCrea, 1950; Usdowski et al., 1991; Beck et al., 2005), Zeebe (1999) explained such disequilibrium CaCO_3 precipitation in terms of the role of fluid pH in defining DIC speciation and therefore the overall ^{18}O fractionation between the sum of DIC species and H_2O (see Zeebe, 2007 for revised formulation). Consequently Lea et al. (1999), Zeebe (2001) and Uchikawa and Zeebe (2010) argued that paleotemperature estimates based on foraminiferal $\delta^{18}\text{O}$ can be significantly biased for past time intervals when seawater carbonate chemistry was markedly different from modern condition, such as during the last glacial maximum, mid-Cretaceous and Paleocene–Eocene Thermal Maximum.

Another conceptual model attributes the disequilibrium phenomenon to kinetic effects (McConnaughey, 1989a,b, 2003). This model assumes that CaCO_3 forms from alkaline fluids in the calcification sites that are isolated by CO_2 -permeable but HCO_3^- -impermeable membranes. Calcification is stimulated by active Ca^{2+} acquisition and simultaneous H^+ removal by alkalinity pumps, which establish large pH and CO_2 gradients across the membranes. This drives passive diffusion of CO_2 into the calcification sites, in which hydration and/or hydroxylation reaction cause strong ^{18}O -depletion in the resultant HCO_3^- . Unless HCO_3^- (and CO_3^{2-}) re-equilibrates before calcification, the ^{18}O -depletion will be recorded in CaCO_3 . This kinetic model is popularly referred to as a leading explanation for the ^{18}O depletion in coral skeletons observed *in situ* and from culture experiments (Allison et al., 1996; Felis et al., 2003; Rosenfeld et al., 2003; Maier et al., 2004; Omata et al., 2008).

More recently an innovative technique for simultaneous $\delta^{18}\text{O}$ and $\delta^{11}\text{B}$ measurements on μm -scale resolution has been developed with the use of ion microprobes (Rollion-Bard et al., 2003, 2008, 2010, 2011; Juillet-Leclerc et al., 2009). Rollion-Bard et al. (2003, 2011) found μm -scale $\delta^{18}\text{O}$ fluctuations exceeding 10‰ for a segment of symbiotic coral skeleton as opposed to only $\sim 2\%$ variations resolved by bulk $\delta^{18}\text{O}$ measurements on a mm-scale sampling resolution. Their coupled $\delta^{11}\text{B}$ analyses also revealed concurrent μm -scale fluctuations, which translate to internal pH variations between 7 and 9. They hence argued that internal pH variations modulate carbonate $\delta^{18}\text{O}$ signatures by regulating DIC speciation and the kinetics of the oxygen isotope exchange in the CO_2 – H_2O system. Furthermore Rollion-Bard et al. (2008, 2010) and Juillet-Leclerc et al. (2009) argued that the early phases of calcification in close association with an organic matrix or certain organic molecules may be crucial for $\delta^{18}\text{O}$ heterogeneity in distinct microstructures of foraminiferal tests and coral skeletons. From these findings it appears that the conceptual models based on just inorganic carbonate chemistry are too simplistic to explain intra-test/skeletal $\delta^{18}\text{O}$ variability, and thus biochemical perspectives also need to be considered for a realistic explanation of vital effects. One of the key biochemical aspects is the role of enzymes during calcification. For instance, an enzyme Ca-ATPase seems to be crucial in maintaining high pH and $[\text{Ca}^{2+}]$ for the extracellular calcification fluid in corals and foraminifera (McConnaug-

hey, 1989b; Ip et al., 1991; Adkins et al., 2003; Erez, 2003). Another potentially important enzyme, which will be explored in this study, is carbonic anhydrase (CA).

CA is a zinc-bearing enzyme that catalyzes inter-conversion of CO_2 and HCO_3^- via CO_2 hydration and its reverse reaction (Paneth and O’Leary, 1985). Many marine calcifiers are known to possess some forms of CA (Nimer et al., 1994; Miyamoto et al., 1996; Furla et al., 2000; Al-Horani et al., 2003; Rost et al., 2003; Soto et al., 2006; Tambutté et al., 2006; Yu et al., 2006; Moya et al., 2008; Bertucci et al., 2011), although their exclusive roles in relation to calcification as well as other metabolic processes are still controversial. Also, it is important to note that CO_2 hydration represents the primary pathway in which direct exchange of oxygen isotopes between DIC species and H_2O takes place (Zeebe and Wolf-Gladrow, 2001). In fact the catalytic role of CA on the oxygen isotope exchange during CO_2 hydration was experimentally demonstrated by Silverman (1973). Therefore it is expected that the presence of CA should reduce the time necessary for ^{18}O equilibration in the CO_2 – H_2O system. If this also holds true within the calcification sites, ^{18}O -depletion due to the kinetic effects can be eliminated and the equilibrium $\delta^{18}\text{O}$ values at a given pH can be reestablished before calcification. Obviously, this has important implications for both pH-based (Zeebe, 1999; 2007) and kinetic-based (McConnaughey, 1989a,b; 2003) explanations for $\delta^{18}\text{O}$ vital effects. Yet a critical unknown is the effectiveness of CA, which depends on the form and concentration of CA and possibly the pH of the aqueous medium. Furthermore, to the best of our knowledge, it is still untested whether the presence of CA alters the equilibrium oxygen isotope fractionations between DIC species and H_2O .

In this paper, we report the results from quantitative inorganic carbonate precipitation experiments. The experiments were performed in time-series over the course of ^{18}O equilibration in the CO_2 – H_2O system with variable CA concentrations. The results from control experiments (no CA addition) are used for validation of the mathematical expression for the uncatalyzed rate of oxygen isotope exchange between DIC and H_2O given in Usdowski et al. (1991). Since the derivation was not addressed in the original publication, we comprehensively derive this expression in Appendix A. The results from control experiments also allow assessment of the reliability of the kinetic rate constants for CO_2 hydroxylation. Finally, the results from CA experiments are used to evaluate the effect of CA concentration on the kinetics and equilibrium of the oxygen isotope exchange in the CO_2 – H_2O system. Our experimental results are discussed in the context of $\delta^{18}\text{O}$ vital effects.

2. UNCATALYZED OXYGEN ISOTOPE EXCHANGE KINETICS IN THE CO_2 – H_2O SYSTEM

Unfortunately, the derivation of the mathematical expression for the uncatalyzed rate of ^{18}O exchange in the CO_2 – H_2O system in Usdowski et al. (1991) was not described in the original publication. This may hinder the utility of this expression, which can be very useful for a variety of applications. We derive this expression following the

classical work of Mills and Urey (1940), which is summarized in Appendix A.

Dissolved CO₂ plays a critical role in ¹⁸O equilibration because direct exchange of oxygen isotopes between DIC and H₂O is only possible via the CO₂ hydration and CO₂ hydroxylation reaction (e.g., Zeebe and Wolf-Gladrow, 2001):



and



Hence the rate of uncatalyzed oxygen isotope exchange is essentially governed by the kinetic rate constants for the CO₂ hydration (k_{+2} and k_{-2}) and hydroxylation (k_{+4} and k_{-4}) as well as the DIC speciation, which is a function of pH. An equation by Usdowski et al. (1991) describes this relationship (see also Zeebe and Wolf-Gladrow, 2001):

$$\ln \left(\frac{{}^{18}\text{R}_S - {}^{18}\text{R}_S^{\text{EQ}}}{{}^{18}\text{R}_S^0 - {}^{18}\text{R}_S^{\text{EQ}}} \right) = - \left(\frac{1}{\tau} \right) \cdot t \quad (3)$$

where ${}^{18}\text{R}_S$, ${}^{18}\text{R}_S^0$, and ${}^{18}\text{R}_S^{\text{EQ}}$ represent the oxygen isotope ratio (¹⁸O/¹⁶O) of S at time t , $t=0$, and at equilibrium. Here, S refers to:

$$S = [\text{H}_2\text{CO}_3] + [\text{HCO}_3^-] + [\text{CO}_3^{2-}] \quad (4)$$

The inverse time constant $1/\tau$ in (Eq. (3)) is given by:

$$\tau^{-1} = (0.5) \cdot \{k_{+2} + k_{+4}[\text{OH}^-]\} \cdot \left\{ 1 + \frac{[\text{CO}_2]}{S} - \left[1 + \left(\frac{2}{3} \cdot \frac{[\text{CO}_2]}{S} \right) + \left(\frac{[\text{CO}_2]}{S} \right)^2 \right]^{1/2} \right\} \quad (5)$$

$[\text{OH}^-]$ can be constrained by pH and K_W , the equilibrium H₂O dissociation constant. Furthermore, by re-arranging (Eq. (3)), the time required for 99% ¹⁸O equilibration (denoted as $t_{99\%}$) can be calculated (Zeebe and Wolf-Gladrow, 2001):

$$t_{99\%} = -\ln(0.01) \cdot \tau \quad (6)$$

Table 1

A list of the equilibrium constants and kinetic rate constants used for calculation of the ratio $[\text{CO}_2]/S$ (see Eqs. (3) and (7)). These freshwater constants were originally compiled by Usdowski et al. (1991).

Parameter	Chemical reaction	Temperature dependency	Temp. Range (°C)	Reference
K_W	$\text{H}^+ + \text{OH}^- \leftrightarrow \text{H}_2\text{O}$	$\log_{10}(K_W) = -(6013.79/T) - 23.6521 \cdot \log_{10}(T) + 64.7013$	0–60	Harned and Owen (1958)
k_{+2}	$\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$	$\log_{10}(k_{+2}) = 329.850 - 110.541 \cdot \log_{10}(T) - (17265.4/T)$	0–38	Pinsent et al. (1956)
k_{-2}	$\text{H}_2\text{CO}_3 \rightarrow \text{CO}_2 + \text{H}_2\text{O}$	$\ln(k_{-2}) = -(8417.6955/T) + 31.5294$	0–37	Roughton (1941)
K'	$\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3$	N/A [†]		N/A
k_{+4}	$\text{CO}_2 + \text{OH}^- \rightarrow \text{HCO}_3^-$	$\log_{10}(k_{+4}) = 13.635 - (2895/T)$	0–40	Pinsent et al. (1956)
K''	$\text{HCO}_3^- \leftrightarrow \text{H}^+ + \text{CO}_3^{2-}$	$\log_{10}(K'') = -(2902.39/T) + 6.4980 - (0.02379/T)$	0–50	Harned and Scholes (1941)
$K_{\text{H}_2\text{CO}_3}^*$	$\text{H}_2\text{CO}_3^* \leftrightarrow \text{H}^+ + \text{HCO}_3^-$	$\log_{10}(K_{\text{H}_2\text{CO}_3}^*) = -(3404.71/T) + 14.8435 - 0.032786 \cdot T$	0–50	Harned and Davis (1943)
$K_{\text{H}_2\text{CO}_3}$	$\text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^-$	N/A [‡]		N/A

Remarks:

H₂CO₃ refers to the true carbonic acid, H₂CO₃. On the other hand, H₂CO₃^{*} is defined as H₂CO₃^{*} = CO_{2(aq)} + H₂CO₃.

T refers to the temperature in Kelvin.

[†]The equilibrium constant K' can be calculated as $K' = k_{+2}/k_{-2}$.

[‡]The equilibrium constant $K_{\text{H}_2\text{CO}_3}$ can be calculated as $K_{\text{H}_2\text{CO}_3} = K_{\text{H}_2\text{CO}_3}^* \times \{1 + (1/K')\}$.

The ratio $[\text{CO}_2]/S$ in (Eq. (5)) at a given pH can be calculated from a set of equilibrium constants summarized in Table 1:

$$\frac{[\text{CO}_2]}{S} = \left[K' + \frac{K' \cdot K_{\text{H}_2\text{CO}_3}}{[\text{H}^+]} + \frac{K' \cdot K_{\text{H}_2\text{CO}_3} \cdot K''}{[\text{H}^+]^2} \right]^{-1} \quad (7)$$

Because of our experimental conditions, we used freshwater kinetic and equilibrium constants to model the ¹⁸O exchange kinetics (Table 1). Modeling results with seawater constants can be found in Zeebe and Wolf-Gladrow (2001) and Rollion-Bard et al. (2011).

As shown in Table 1, Usdowski et al. (1991) and Zeebe and Wolf-Gladrow (2001) used the k_{+4} by Pinsent et al. (1956) for their calculations. But we note that recently Wang et al. (2010) reevaluated the temperature dependency of k_{+4} . The Arrhenius fitting of their new k_{+4} data against experimental temperatures gives the following relationship:

$$\ln(k_{+4}) = 35.2319 - (7697.3961/T) \quad (8)$$

where T is temperature in Kelvin. Since the new k_{+4} values are greater than previously published data by a factor of about 2, the selection of the numeric value for k_{+4} by Wang et al. (2010) or by Pinsent et al. (1956) results in a notable offset in the calculation for pH > 8, where CO₂ hydroxylation becomes comparatively more important (Fig. 1). This topic will be further discussed below.

3. METHODS

3.1. Brief overview of the experimental approach

The influence of CA on the kinetics and equilibrium of the oxygen isotope exchange in the CO₂–H₂O system was investigated by quantitative inorganic carbonate precipitation experiments. When a carbonate mineral is quantitatively precipitated from a HCO₃[−] and/or CO₃^{2−} dominated solution (i.e., HCO₃[−] and CO₃^{2−} are quasi-instantaneously and completely transformed into the mineral), the δ¹⁸O value of the resultant mineral reflects the weighted average of the oxygen present in HCO₃[−] and CO₃^{2−} at the time of precipitation and hence records the

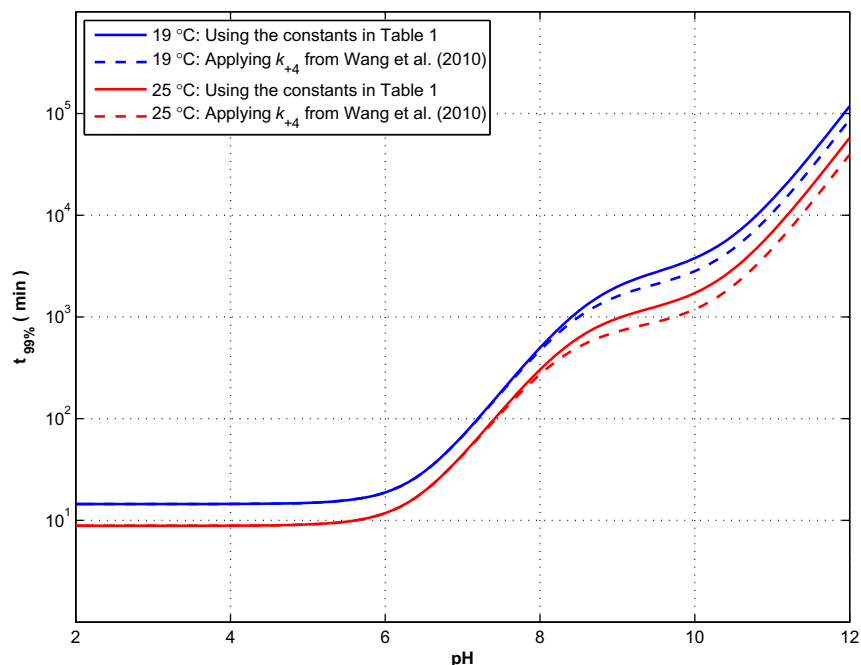


Fig. 1. The time required for 99% completion of uncatalyzed ^{18}O equilibration in the $\text{CO}_2\text{-H}_2\text{O}$ system ($t_{99\%}$, Eqs. (3) and (6)) as a function of pH. Calculations were made at 0 salinity. (Solid blue) Calculation by Zeebe and Wolf-Gladrow (2001) at 19 °C using the constants in Table 1. (Dashed blue) Calculation at 19 °C using the kinetic constant for CO_2 hydroxylation, k_{+4} , by Wang et al. (2010). (Solid red) Calculation at 25 °C using the constants listed in Table 1. (Dashed red) Calculation at 25 °C using the k_{+4} by Wang et al. (2010). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$\delta^{18}\text{O}$ of the sum of DIC species (McCrea, 1950; Usdowski et al., 1991; Usdowski and Hoefs, 1993; Zeebe, 1999; Beck et al., 2005). To examine the effect of CA on the kinetics of the oxygen isotope exchange in the $\text{CO}_2\text{-H}_2\text{O}$ system and on the equilibrium ^{18}O fractionations between DIC species and H_2O , quantitative precipitation experiments were conducted as a time-series. Parent NaHCO_3 solutions with different CA concentrations were quantitatively transformed into BaCO_3 at various times over the course of ^{18}O equilibration in the $\text{CO}_2\text{-H}_2\text{O}$ by reacting with $\text{BaCl}_2\cdot 2\text{H}_2\text{O}$ and concentrated NaOH solution. “Time 0” in our experiments refers to the moment when the NaHCO_3 powder was dissolved in H_2O . The parent NaHCO_3 solutions were adjusted to pH 8.3 and pH 8.9. The first value is similar to the average surface ocean pH today and the latter value is particularly relevant to the elevated pH levels within the calcification sites of certain coral and planktonic as well as benthic foraminiferal species (Jørgensen et al., 1985; Rink et al., 1998; Al-Horani et al., 2003; Rollion-Bard et al., 2003; Köhler-Rink and Köhl, 2005; Bentov et al., 2009; de Nooijer et al., 2009; Rollion-Bard and Erez, 2010). Combinations of the CA concentrations and the pH of the parent NaHCO_3 solutions tested in our experiments are summarized in Table 2.

3.2. Experimental procedures

3.2.1. Preparation and equilibration

Experimental procedures were modified from Beck et al. (2005). The NaHCO_3 solutions (0.015 M) were prepared in serum bottles capped and sealed with rubber stoppers and

aluminum crimps. Care was taken to minimize the headspace in the bottles. Although the NaHCO_3 powder (Certified A.C.S. grade: Fisher Lot #050282) used for the experiments were not isotopically labeled, its $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values were homogeneous ($\delta^{13}\text{C}_{\text{VPDB}} = -2.89 \pm 0.02\text{‰}$, $\delta^{18}\text{O}_{\text{VPDB}} = -15.81 \pm 0.10\text{‰}$, $n = 13$). For the experiments at pH 8.9, the pH of the NaHCO_3 solutions was adjusted with 0.5 M NaOH solution prepared from low-carbonate NaOH pellets (Reagent A.C.S. grade: J.T. Baker #3722). Small amounts of CA solutions (see below) were also added to the NaHCO_3 solutions for desired concentrations as described in Table 2. All of the solutions described above were prepared using CO_2 -free deionized H_2O . The water was continuously bubbled with N_2 gas for a minimum of a week in a large carboy prior to use in order to eliminate dissolved CO_2 . Aliquots of H_2O used to prepare the NaHCO_3 solutions were sampled for $\delta^{18}\text{O}$ measurements. The NaHCO_3 solutions were equilibrated in water baths maintained at 25 ± 0.04 °C using immersion circulators (Thermo Scientific HAAKE C10 and SC100 model).

3.2.2. Quantitative precipitation

The NaHCO_3 solutions were withdrawn from the serum bottles and transferred into serum vials containing excess $\text{BaCl}_2\cdot 2\text{H}_2\text{O}$ powder (Reagent grade: J.T. Baker #0974) under N_2 -atmosphere. Addition of the NaHCO_3 solution resulted in complete dissolution of the $\text{BaCl}_2\cdot 2\text{H}_2\text{O}$ powder within a few seconds. This step was immediately followed by addition of small amounts of 2.5 M NaOH solution to trigger instantaneous BaCO_3 precipitation. Disposable clinical syringes were used for the transfer of the solutions.

Table 2

A summary of the solution chemistry for individual experimental series. All experiments were conducted at 25 °C.

Experimental series	NaHCO ₃ conc. (M: mol/L)	pH	CA conc. (mg/mL)	CA conc. [†] (M: mol/L)	Total CA activity (E.U. [‡])
TS1 (Control)	0.015	8.33 ± 0.01 (<i>n</i> = 15)	0	0	0
TS1-1CA	0.015	8.33 ± 0.02 (<i>n</i> = 15)	2.8 × 10 ⁻⁵	9.3 × 10 ⁻¹⁰	2.4
TS1-2CA	0.015	8.34 ± 0.01 (<i>n</i> = 13)	5.6 × 10 ⁻⁵	1.9 × 10 ⁻⁹	4.7
TS1-intCA	0.015	8.33 ± 0.01 (<i>n</i> = 13)	1.1 × 10 ⁻⁴	3.7 × 10 ⁻⁹	9.4
TS1-4CA	0.015	8.34 ± 0.02 (<i>n</i> = 13)	1.4 × 10 ⁻⁴	4.6 × 10 ⁻⁹	11.8
TS1-5CA	0.015	8.34 ± 0.01 (<i>n</i> = 13)	2.8 × 10 ⁻⁴	9.3 × 10 ⁻⁹	23.6
TS1-6CA	0.015	8.36 ± 0.02 (<i>n</i> = 13)	5.6 × 10 ⁻⁴	1.9 × 10 ⁻⁸	47.2
TS2 (Control)	0.015	8.89 ± 0.02 (<i>n</i> = 18)	0	0	0
TS2-mnCA	0.015	8.90 ± 0.02 (<i>n</i> = 18)	1.1 × 10 ⁻⁴	3.7 × 10 ⁻⁹	9.4
TS2-1CA	0.015	8.90 ± 0.01 (<i>n</i> = 22)	1.4 × 10 ⁻⁴	4.6 × 10 ⁻⁹	11.8
TS2-2CA	0.015	8.90 ± 0.02 (<i>n</i> = 22)	2.8 × 10 ⁻⁴	9.3 × 10 ⁻⁹	23.6
TS2-3CA	0.015	8.91 ± 0.03 (<i>n</i> = 19)	5.6 × 10 ⁻⁴	1.9 × 10 ⁻⁸	47.2

[†] Molarity (mol/L) was calculated from the molecular weight of the CA reported by the manufacturer (30,000 g/mol).

[‡] Wilbur and Anderson (1948) enzyme unit. The Wilbur–Anderson enzyme unit (E.U) is defined as $E.U = (t_{\text{Blank}} - t_{\text{CA}})/t_{\text{CA}}$ (Eq. (9)).

The precipitates were quickly vacuum-filtered onto 0.2 μm cellulose–nitrate membrane filters and thoroughly rinsed with CO₂-free deionized H₂O. The BaCO₃ samples were oven dried at 65 °C overnight, weighed and stored in airtight glass vials until stable isotope analyses. For each NaHCO₃ solution, quantitative precipitation was performed in duplicate. The remainders of the NaHCO₃ solutions were used for pH measurements. We used either a benchtop pH meter (Thermo Scientific Orion 3-Star Plus model) or the auto-titrator system of Zeebe and Sanyal (2002) for the measurements. These instruments were equipped with an Orion triode combination pH electrode (Thermo Scientific #9157BNMD) and an Orion sure-flow pH electrode (Thermo Scientific #8272BN), respectively. The electrodes were calibrated daily using Orion pH buffers (pH 4.01, 7.00, 10.01) that are traceable to NIST standard reference materials.

Precipitation of BaCO₃ was selected over CaCO₃ in our experiments. This is because BaCO₃ only forms a single crystal structure with orthorhombic orientation (as witherite) unlike CaCO₃, which can form different types of polymorphs (see Mackenzie and Lerman, 2006). During the method development, our test samples precipitated as CaCO₃ indeed resulted in co-precipitation of calcite and variable amounts of vaterite (roughly from 30 to 50% of total CaCO₃). Precipitation of a single crystal form is crucial for accurate δ¹⁸O measurements based on the conventional H₃PO₄ digestion approach, as described in Kim et al. (2007). X-ray diffraction analyses on select samples confirmed that the precipitated minerals are witherite, regardless of the CA concentration in the parent NaHCO₃ solutions.

3.3. CA assay

The CA used in this study is of bovine erythrocyte origin. The enzyme was purchased from MP Biomedicals (#153879). Molecular weight of the product reported by the manufacturer is 30,000 g/mol, which is consistent with a published value of 31,000 ± 1000 g/mol for bovine erythrocyte CA by Lindskog (1960). The protocols for CA assay for activity measurements were modified from Worthington

(1993), which is based on the electrometric approach originally developed by Wilbur and Anderson (1948). A round-bottom flask containing 12 mL of ice-cold 0.02 M Tris–HCl buffer was buried in crushed ice. After thermal equilibration, the reaction was initiated by adding 8 mL of ice-cold CO₂ saturated deionized H₂O while gently stirred and the time interval required for the pH decline from 8.3 to 6.3 was measured. The activity was reported in Wilbur–Anderson enzyme unit (E.U) per mg of CA used for assays (Wilbur and Anderson, 1948). The E.U is defined as:

$$E.U = \{(t_{\text{Blank}} - t_{\text{CA}})/t_{\text{CA}}\} \quad (9)$$

where t_{CA} and t_{Blank} are the measured time interval for the pH decline with and without CA in the medium, respectively. For CA assays, 0.04 mL of 0.1 mg/mL CA solution was added to the Tris–HCl buffer. The pH changes during the assay were recorded by the auto-titrator system of Zeebe and Sanyal (2002) described above.

Concentrations of the CA solutions used for the experiments were 0.1 mg/mL and 0.01 mg/mL. These solutions were left at room temperature. Earlier studies demonstrate notable instability of CA and gradual loss of its activity in dilute solutions (e.g., Clark and Perrin, 1951; Thode et al., 1965). As a stability test, we performed daily assays on these CA solutions for 5 consecutive days. No evidence of weakening of the catalytic ability in these CA solutions was observed for the duration (see Section 4.1). Nonetheless, we limited the use of these CA solutions for the experiments within 3 days after preparation in order to ensure the integrity of the CA solutions. After 3 days the CA solutions were discarded and a new solution was freshly prepared. Each experiment series shown in Table 2 were completed within 3 days. In other words, individual NaHCO₃ solutions for a given experimental series were prepared from the same CA solution.

3.4. Stable isotope analyses

The BaCO₃ and H₂O samples were analyzed at University of California Santa Cruz (UCSC) or University of California Davis (UCD) stable isotope laboratory for δ¹⁸O and

$\delta^{13}\text{C}$ measurements depending on the instrument accessibility for the analyses at both institutions.

Isotopic analyses of the BaCO_3 samples were conducted by the conventional acid digestion approach where samples were reacted with supersaturated H_3PO_4 (specific gravity = 1.93 g/cm^3) to liberate CO_2 gas. At UCSC, ThermoFisher Mat 253 dual-inlet isotope ratio mass spectrometer (IRMS) interfaced to a Kiel IV carbonate device was used. Samples were reacted with H_3PO_4 at 75°C . An in-house working standard and NBS-19 standard were used during the analyses for a drift correction and for the monitoring of the operating condition. Typical precisions were better than $\pm 0.07\text{‰}$ for $\delta^{18}\text{O}$ and $\pm 0.05\text{‰}$ for $\delta^{13}\text{C}$ ($\pm 1\sigma$). At UCD, the BaCO_3 samples were digested at 90°C using an ISOCARB common acid bath autocarbonate device. The resultant CO_2 gas was analyzed by a Micromass Optima IRMS. Instrument precisions for carbonate $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ analyses based on repeat analyses of an in-house standard were $\pm 0.05\text{‰}$ and $\pm 0.04\text{‰}$ ($\pm 1\sigma$), respectively.

The $\delta^{18}\text{O}$ values of the H_2O samples were determined by the conventional CO_2 equilibration technique. At UCSC, a ThermoFinnigan Delta Plus XP IRMS interfaced to a GasBench II device in continuous flow mode was used. Typical precision from replicate analyses of an in-house standard was better than $\pm 0.07\text{‰}$. At UCD, an automated equilibrator attached to a Finnigan MAT 251 IRMS was used for the analyses. The analytical precision was better than $\pm 0.03\text{‰}$.

The isotopic results are reported in δ notation:

$$\delta = \left(\frac{R_{\text{Sample}}}{R_{\text{Standard}}} - 1 \right) \times 1000 \quad (10)$$

where R is isotope ratio $^{18}\text{O}/^{16}\text{O}$ or $^{13}\text{C}/^{12}\text{C}$. The carbonate $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ data are reported relative to the VPDB standard, whereas the water $\delta^{18}\text{O}$ data are relative to the VSMOW standard. To calculate the isotope fractionation factor between BaCO_3 and H_2O , the $\delta^{18}\text{O}$ values of the BaCO_3 samples are also re-scaled to VSMOW following the expression by Coplen et al. (1983):

$$\delta^{18}\text{O}_{\text{VSMOW}} = 1.0391 \times \delta^{18}\text{O}_{\text{VPDB}} + 30.91\text{‰} \quad (11)$$

The oxygen isotope fractionation factor α between BaCO_3 and H_2O is defined as:

$$\alpha_{\text{BaCO}_3\text{-H}_2\text{O}} = \frac{^{18}\text{R}_{\text{BaCO}_3}}{^{18}\text{R}_{\text{H}_2\text{O}}} = \frac{\delta^{18}\text{O}_{\text{BaCO}_3} + 1000}{\delta^{18}\text{O}_{\text{H}_2\text{O}} + 1000} \quad (12)$$

4. RESULTS

4.1. CA assay and stability test

Based on 6 runs of blanks and CA assays (Fig. 2), t_{Blank} and t_{CA} were 156.8 ± 3.0 and 27.4 ± 1.8 s, respectively. Since 4×10^{-3} mg of CA was used for CA assays, the specific activity of the CA used for this study is calculated to be 1179.4 E.U per mg of CA.

As a stability test, we performed daily assays using 0.1 mg/mL and 0.01 mg/mL CA solutions left at room temperatures for 5 consecutive days. Significant slowdown of the pH decline from 8.3 to 6.3 can be considered as the evidence for a weakening of the catalytic ability. Such trend was not observed for the duration of the stability test (Fig. 3). This suggests that both 0.1 mg/mL and 0.01 mg/

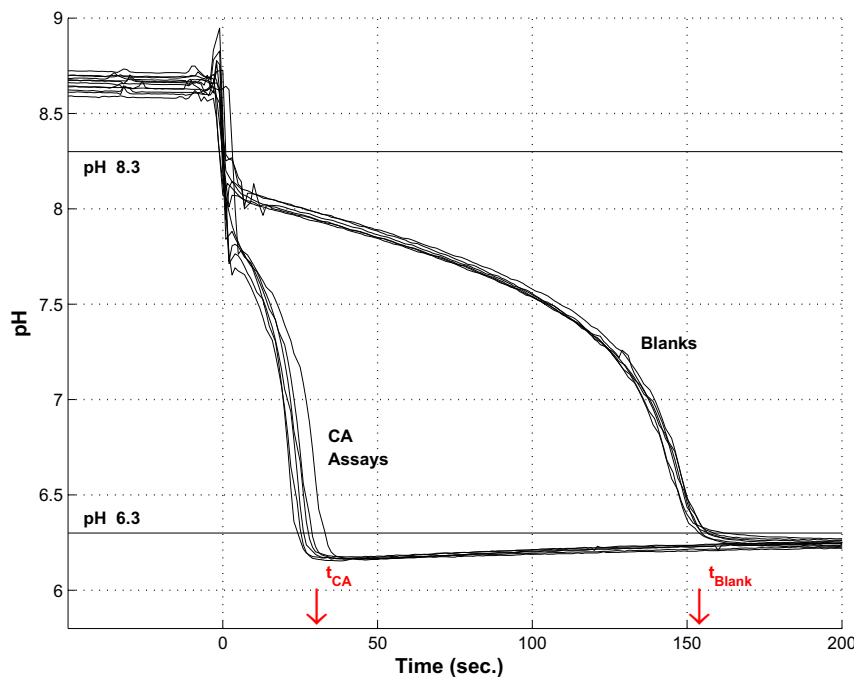


Fig. 2. Determination of the CA activity based on 6 replicate blanks (buffer only) and assays (buffer + CA). The substrate (CO_2 -saturated H_2O) was added to the medium after 3 min of thermal equilibration. The time course of pH decline from 8.3 to 6.3 was recorded to determine t_{Blank} and t_{CA} (Eq. (9)).

mL CA solutions retained their original catalytic ability for well beyond 3 days without refrigeration.

4.2. BaCO₃ data

The data from quantitative BaCO₃ precipitation experiments are presented in the [supplementary data](#) (Tables S1–S12). See [Appendix B](#) for the online access of the [supplementary data](#).

4.2.1. Data screening

For the purpose of this study, a fundamental requirement is that the BaCO₃ samples were indeed *quantitatively* precipitated from the parent NaHCO₃ solutions. We assigned two criteria to evaluate the validity of the BaCO₃ samples. The first is sample yield in % relative to the theoretical yield, which can be obtained by stoichiometric calculations using the concentration and the volume of the parent NaHCO₃ solution used for precipitation. The average yield is $99.4 \pm 1.2\%$ ($n = 380$) when 3 samples with yield below 95% are ignored. The lower yields associated with these 3 samples are all due to mishandling during the filtration and not necessarily reflect incomplete BaCO₃ precipitation. The second and more important criterion is the $\delta^{13}\text{C}$ values of the BaCO₃ samples. Because the NaHCO₃ powder used to prepare the parent solutions represents the only carbon source for BaCO₃, the $\delta^{13}\text{C}$ values of the NaHCO₃ ($\delta^{13}\text{C}_{\text{VPDB}} = -2.89 \pm 0.02\%$, $n = 13$) and BaCO₃ samples should be identical if the samples are quantitatively precipitated. Significant offset in the $\delta^{13}\text{C}$ values would indicate (1) contamination by absorption of atmospheric CO₂ during the solution transfers and/or filtration, (2) incomplete removal of dissolved CO₂ in the deionized H₂O used for

preparation of various solutions for the experiments, (3) incomplete BaCO₃ precipitation or (4) any combinations of the above. The isotopic data for samples whose $\delta^{13}\text{C}$ values deviate from that of the source NaHCO₃ by more than $\pm 0.5\%$ were rejected for further interpretations. After removing these particular samples (8 samples in total), the $\delta^{13}\text{C}$ values of the remaining BaCO₃ samples range from -3.46% to -2.60% with an average of $-3.19 \pm 0.11\%$ ($n = 375$). Note that the data from 3 particular samples with yields below 95% due to mishandling during filtration mentioned above were not rejected because their $\delta^{13}\text{C}$ values are within the $\pm 0.5\%$ threshold. In total 375 samples were considered to be successfully precipitated quantitatively based on both stoichiometric and $\delta^{13}\text{C}$ constraints. Accordingly, the $\delta^{18}\text{O}_{\text{BaCO}_3}$ values obtained from these BaCO₃ samples should reflect the overall $\delta^{18}\text{O}$ values of the sum of DIC species at the time of precipitation (e.g., Zeebe, 1999; 2007).

4.2.2. Control experiments

Fig. 4 displays the time evolution of the $\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}$ values obtained from TS1 and TS2 control experimental series, where no CA was added to the parent NaHCO₃ solutions. Note that well-defined plateaus in the profile indicate ¹⁸O equilibrium in the CO₂–H₂O system. For TS1 control series (average pH of 8.3) the system reaches isotopic equilibrium sometime between 500 and 600 min. In case of TS2 control series (average pH of 8.9), ¹⁸O equilibration was complete around 1080 min (18 h). Theoretical calculations using the set of constants listed in [Table 1](#) suggest that the CO₂–H₂O system comes to 99% completion of ¹⁸O equilibrium ($t_{99\%}$) in roughly 500 min at pH 8.3 and 900 min at pH 8.9 at 25 °C.

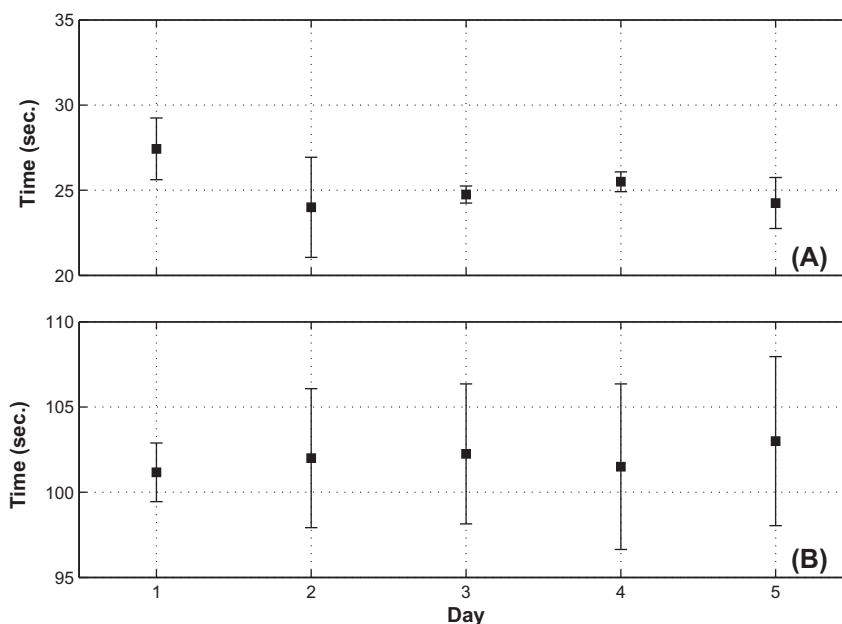


Fig. 3. Stability test for 0.1 mg/mL (Panel A) and 0.01 mg/mL CA solutions (Panel B). Both CA solutions were kept at room temperatures for the duration. Daily results are based on 4 repeated assays, except for the first day when 6 assays were performed. The y -axis represents the time interval for pH decline of the assay medium from 8.3 to 6.3.

In the plateaus, the equilibrium $10^3 \cdot \ln \alpha_{\text{BaCO}_3\text{-H}_2\text{O}}$ value averages 30.6 ± 0.2 for TS1 control experiments ($n = 8$) and 30.4 ± 0.2 for TS2 control experiments ($n = 10$). Our equilibrium $10^3 \cdot \ln \alpha_{\text{BaCO}_3\text{-H}_2\text{O}}$ value for TS2 control experiments at pH ~ 8.9 is consistent with Beck et al. (2005), who found a value of 30.46 from a BaCO_3 sample quantitatively precipitated from a NaHCO_3 solution at pH 8.93 fully equilibrated at 25 °C. Based on Zeebe (2007), the equilibrium $10^3 \cdot \ln \alpha_{\text{BaCO}_3\text{-H}_2\text{O}}$ value is expected to be 31.0 at pH 8.3 and 30.8 at pH 8.9. These are in reasonable agreement with the experimental data. General agreement between empirical data and theoretical calculations observed here demonstrate that our experimental approach is capable of reflecting the kinetics of the oxygen isotope exchange and equilibrium ^{18}O fractionations in the $\text{CO}_2\text{-H}_2\text{O}$ system.

4.2.3. CA experiments

Fig. 5 compares the results from control experiments and CA experiments at pH ~ 8.3 and ~ 8.9 . At both pH levels, CA addition to the parent NaHCO_3 solutions accelerated the ^{18}O equilibration process. Furthermore, the time necessary for ^{18}O equilibration decreases with CA concentration. For example, the ^{18}O equilibration time is nearly halved when CA is present in the NaHCO_3 solution at concentration of 3.7×10^{-9} M. These results exemplify the effect of CA on the ^{18}O exchange in the $\text{CO}_2\text{-H}_2\text{O}$ system via catalysis of CO_2 hydration. And importantly, the catalytic ability of CA was preserved even at pH ~ 8.9 which is comparable to the typical pH values within the calcification sites.

Fig. 6 compares the average equilibrium $\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}$ values that are calculated from the data from the well-defined

plateaus revealed in Fig. 5 and expected equilibrium $\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}$ values at pH 8.3 and 8.9 from Zeebe (2007). The figure shows that the average equilibrium $\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}$ values are indistinguishable at both pH levels, regardless of the presence or concentrations of CA in the parent NaHCO_3 solutions. Moreover, the equilibrium $\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}$ values from control and CA experiments are in reasonable agreement with the expected values.

5. DISCUSSION

5.1. Kinetic rate constant for CO_2 hydroxylation reaction (k_{+4})

The rate of ^{18}O equilibration is controlled by kinetic rate constants for CO_2 hydration (k_{+2}) and hydroxylation reaction (k_{+4}) and the pH-dependent DIC speciation (Eqs. (1)–(5)). For their study, Usdowski et al. (1991) used the temperature dependency of k_{+4} by Pinsent et al. (1956). Recently Wang et al. (2010) published a new set of k_{+4} data for a range of temperatures from 6.6 to 42.8 °C using the spectrophotometric stopped-flow method. The temperature dependency based on their new experimental results is given in Eq. (8). But their k_{+4} measurements are greater than previously established values (including the ones by Pinsent et al., 1956) by a factor of about 2. Wang et al. (2010) did not thoroughly investigate the potential cause(s) for the offset between their k_{+4} measurements and previous values mainly from 1950s and 1960s (original references therein), yet they commented that “considering the difficulties in the determination of this fast rate constant and the lack of available instrumentation some 50 years ago when

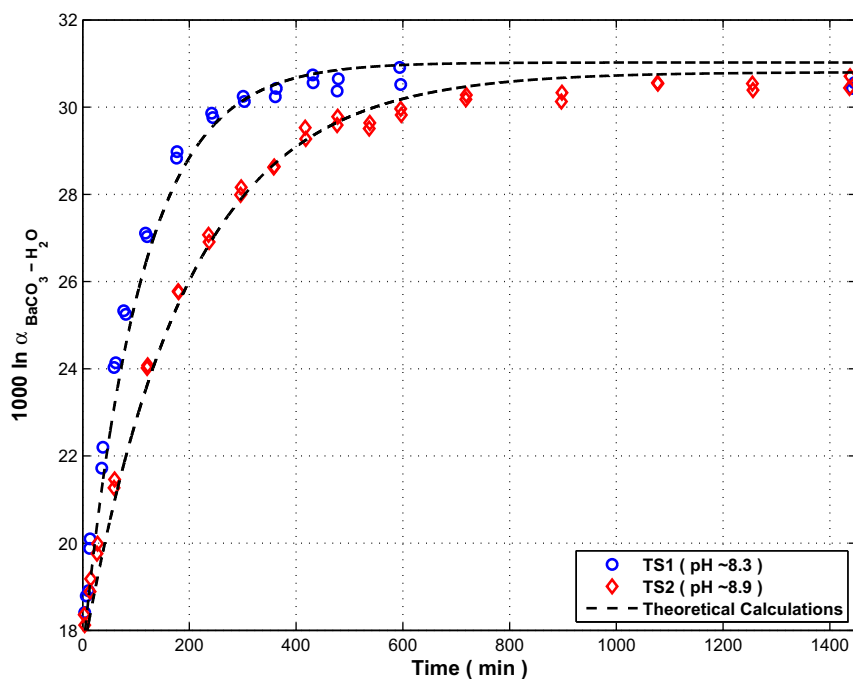


Fig. 4. Comparison of the results from control experiments without CA addition (TS1 series at pH 8.3 and TS2 series at pH 8.9) and theoretical time evolution of $\alpha_{\text{S-H}_2\text{O}}$ at respective pH levels (see Eq. (14)). Estimated time for 99% completion of ^{18}O equilibration ($t_{99\%}$) is about 480 and 900 min at pH 8.3 and 8.9, respectively.

most of the values were published, the analysis presented here is more likely the most reliable.”

The results from our TS2 control experimental series can be used to indirectly evaluate the fidelity of the conflicting k_{+4} values by Pinsent et al. (1956) and Wang et al. (2010). Since CO₂ hydroxylation is comparatively more important at higher pH, the results from our TS2 control experiments are better suited for the purpose. From the def-

inition of the oxygen isotope fractionation factor α between BaCO₃ (and hence the sum of DIC species) and H₂O (Eq. (12)), the rate expression for the ¹⁸O exchange between DIC and H₂O (Eq. (1)) can be rearranged to:

$$\ln \left(\frac{\alpha_{\text{BaCO}_3\text{-H}_2\text{O}} - \alpha_{\text{BaCO}_3\text{-H}_2\text{O}}^{EQ}}{\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}^0 - \alpha_{\text{BaCO}_3\text{-H}_2\text{O}}^{EQ}} \right) = - \left(\frac{1}{\tau} \right) \cdot t \quad (13)$$

which can be subsequently solved for $\alpha_{\text{S-H}_2\text{O}}$:

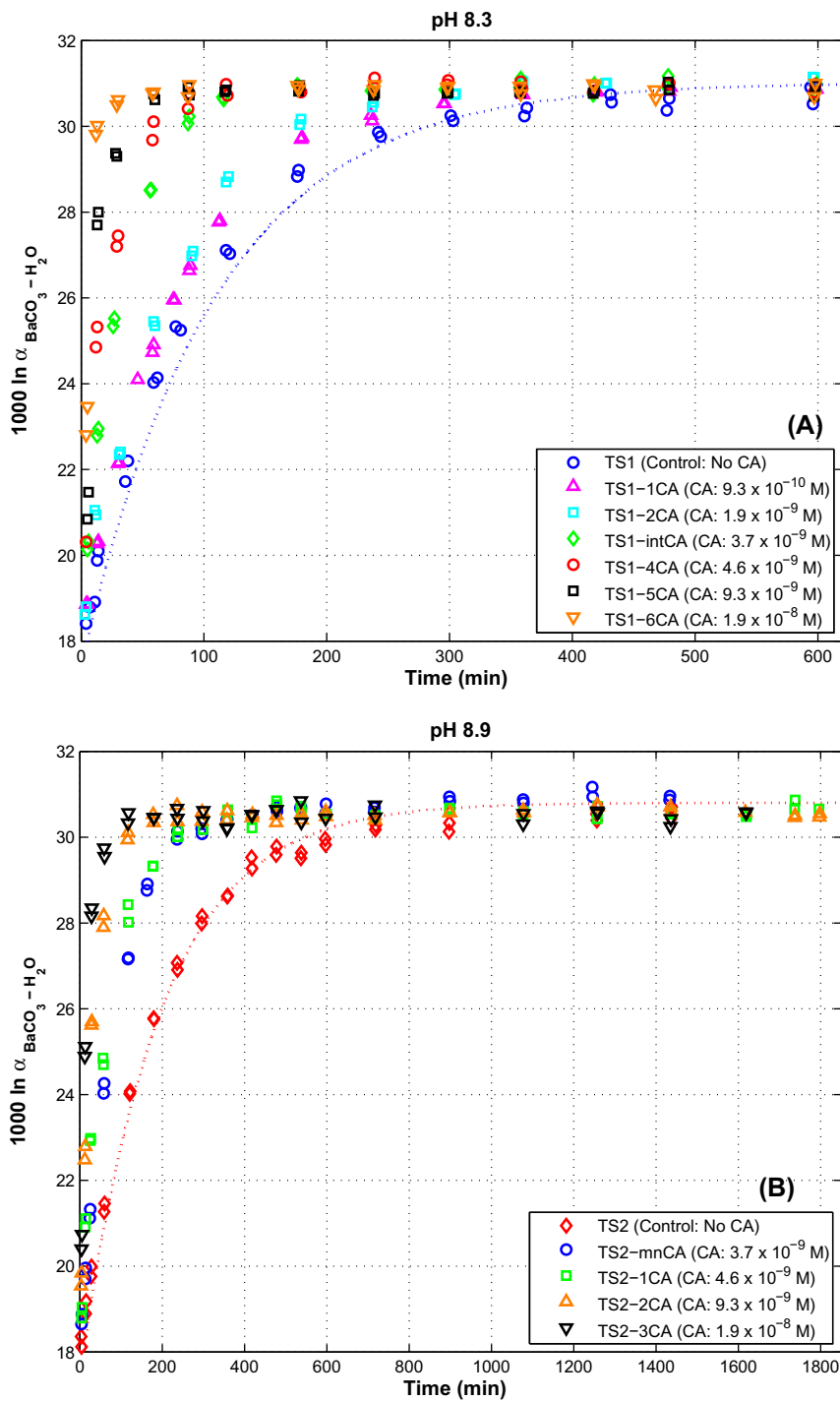


Fig. 5. Summary of the results from control experiments and CA experiments at pH 8.3 and 8.9. At both pH levels, the time required for ¹⁸O equilibration in the CO₂-H₂O system decreased with CA concentration.

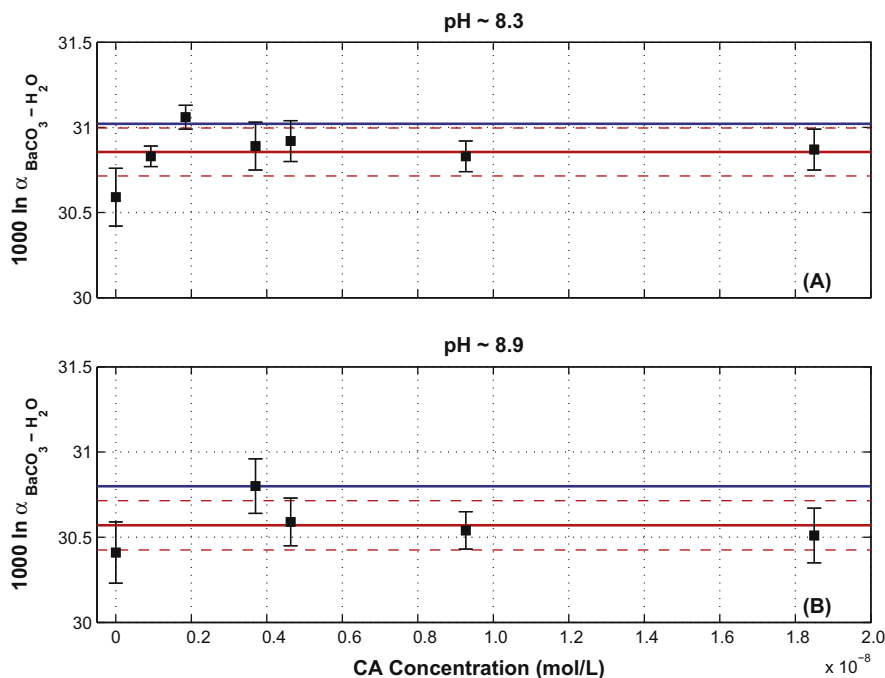


Fig. 6. The average equilibrium $\alpha_{\text{S-H}_2\text{O}}$ values as a function of CA concentration (Black). These values were calculated from the $\delta^{18}\text{O}$ values of the BaCO_3 samples precipitated after the $\text{CO}_2\text{-H}_2\text{O}$ system reached ^{18}O equilibrium, which is represented by the well defined plateaus in Fig. 5. The error bars represent 1σ standard deviations. (Red) The average and the 1σ standard deviations of the averaged equilibrium $\alpha_{\text{S-H}_2\text{O}}$ values shown by the black symbols in the plot. (Blue) Expected equilibrium $\alpha_{\text{S-H}_2\text{O}}$ values at pH 8.3 and 8.9 calculated from Zeebe (2007). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

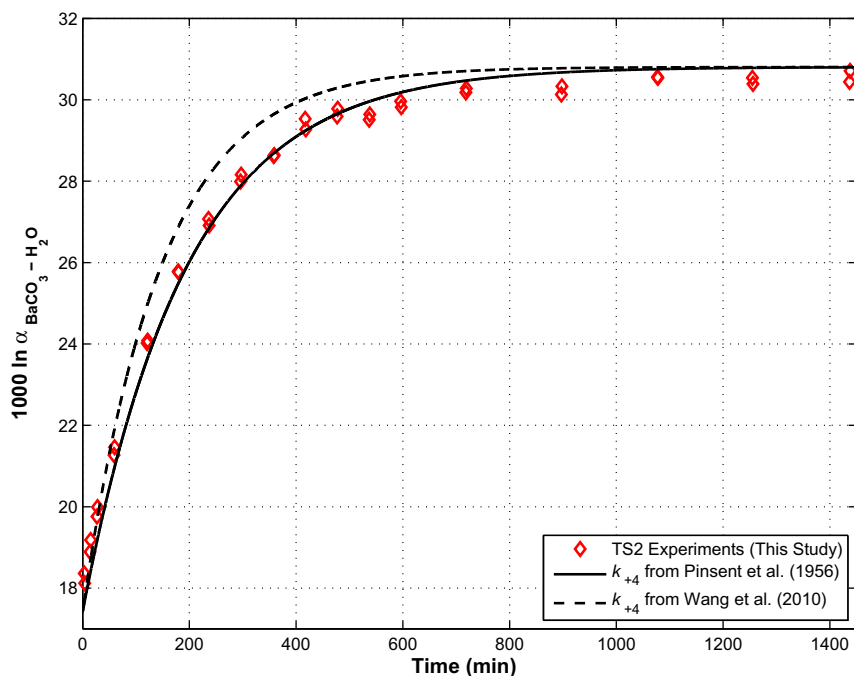


Fig. 7. Comparison of the results from TS2 control experiments performed at pH 8.9 (red symbols) and theoretical time evolution of $\alpha_{\text{S-H}_2\text{O}}$ at pH 8.9 modeled by Eq. (14) using the kinetic rate constant for CO_2 hydroxylation k_{+4} by Pinsent et al. (1956) (solid curve) and by Wang et al. (2010) (dashed curve). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$$\alpha_{\text{BaCO}_3\text{-H}_2\text{O}} = \alpha_{\text{BaCO}_3\text{-H}_2\text{O}}^{\text{EQ}} + (\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}^0 - \alpha_{\text{BaCO}_3\text{-H}_2\text{O}}^{\text{EQ}}) \cdot \exp\left(-\frac{t}{\tau}\right) \quad (14)$$

This equation enables modeling of the time evolution of $\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}$ from the initial condition $\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}^0$ to the equilibrium condition $\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}^{\text{EQ}}$. The $\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}^0$ value can be determined from the $\delta^{18}\text{O}$ of the NaHCO_3 used for the experiments ($\delta^{18}\text{O}_{\text{VSMOW}} = 14.5\text{‰}$) and the average $\delta^{18}\text{O}$ value of H_2O for the TS2 experiments ($\delta^{18}\text{O}_{\text{VSMOW}} = -3.0\text{‰}$). The $\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}^{\text{EQ}}$ values at pH 8.89 (average pH for individual experiments in TS2 series) can be calculated by using the formulation by Zeebe (2007).

Fig. 7 compares the data from the TS2 control experiments and the simulated time evolution of ^{18}O equilibration based on the k_{+4} value at 25 °C from Pinsent et al. (1956) and from Wang et al. (2010). The greater k_{+4} values given by Wang et al. (2010) predict more rapid oxygen isotope exchange via CO_2 hydroxylation and therefore much faster overall ^{18}O equilibration in the $\text{CO}_2\text{-H}_2\text{O}$ system in comparison to the estimate based on the k_{+4} from Pinsent et al. (1956). This trend should be even more pronounced at higher pH levels. However, our TS2 data closely trace the time evolution modeled with the k_{+4} by Pinsent et al. (1956). As mentioned above, Wang et al. (2010) compared their k_{+4} data with previously published results from six experimental studies including Pinsent et al. (1956). The k_{+4} values from these six studies are in good agreement (see Fig. 6 in the supplementary online material by Wang et al., 2010). In fact, the k_{+4} measurements below 16 °C by Wang et al. (2010) are also consistent with these studies. But above 25 °C, their measurements are anomalously higher than the data from previous studies. The evidence from our TS2 data and the consistent k_{+4} measurements in previous experimental studies lead us to conclude that the values given by Wang et al. (2010) are overestimated. This suggests that the work by Pinsent et al. (1956) is more reliable.

5.2. Effect of CA on the equilibrium ^{18}O fractionation in the $\text{CO}_2\text{-H}_2\text{O}$ system

By definition, enzymes accelerate chemical reactions by lowering the activation energy. Hence the catalytic activity of CA is not expected to alter the equilibrium ^{18}O fractionations between DIC species and H_2O . But this might not be the case if CA itself or its derivatives such as amino acids or some type of degraded products form complexes with DIC species. From Fig. 6, however, the average equilibrium $\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}$ values are generally consistent throughout the range of CA concentrations tested in this study. These experimentally-derived equilibrium $\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}$ values are also very similar to the expected values (Zeebe, 2007). These lines of evidence suggest that the presence of CA does not affect the equilibrium ^{18}O fractionation in the range of CA concentration investigated in this study.

5.3. Effect of CA on the kinetics of ^{18}O equilibration

In the discussion above, we demonstrated that the uncatalyzed ^{18}O equilibration in the $\text{CO}_2\text{-H}_2\text{O}$ system can be explained by the mathematical model given in Usdowski et al.

(1991) (Eqs. (3)–(5)). But as shown in Fig. 5, CA accelerates the oxygen isotope exchange by catalyzing CO_2 hydration and the exchange rate increases with CA concentration. In order to apply the model to our results from CA experiments, a new kinetic parameter needs to be implemented to account for the catalyzed CO_2 hydration. Boyer (1959) derived a rate expression for the catalyzed isotope exchange as a function of enzyme concentration, which was applied in Silverman (1973) to describe the influence of CA on the ^{18}O exchange between HCO_3^- and CO_2 . But some of the parameters such as equilibrium dissociation constant for the enzyme–substrate complex included in this expression are specific to the types of CA and these data are rarely reported in the literature. For our experimental conditions, a similar yet simpler parameterization can be employed.

A generalized expression for chemical reactions catalyzed by active enzymes can be written as:



where E, S and P denote the enzyme, substrate and product associated with the reaction, respectively. The notation EX represents the enzyme–substrate complex. The forward and reverse rate constant for the enzyme–substrate binding are denoted as k_+ and k_- , respectively. Finally k_{cat} is the catalytic rate constant, often referred to as the turnover number. In case of the CO_2 hydration catalyzed by CA, the substrate and product for the reaction would be $\text{CO}_{2(\text{aq})}$ and HCO_3^- , respectively. Following the Michaelis–Menten kinetic model, the rate of increase in the product HCO_3^- due to the catalyzed CO_2 hydration can be written as:

$$\frac{d[\text{HCO}_3^-]}{dt} = k_{\text{cat}} \cdot [\text{CA}] \cdot \frac{[\text{CO}_2]}{K_M + [\text{CO}_2]} \quad (16)$$

where K_M is the Michaelis–Menten constant ($K_M = (k_- + k_{\text{cat}})/k_+$). Note that in our approach the contribution of the dehydration reaction ($\text{HCO}_3^- \rightarrow \text{CO}_2$) is automatically taken into account. The system is in chemical equilibrium, although not in isotopic equilibrium (see Appendix A). Between pH 8 and 9, k_{cat} appears to be independent of pH (Steiner et al., 1975). At 25 °C, K_M of CA purified from bovine erythrocytes is also constant at $\sim 12 \times 10^{-3}$ M between pH 7 and 10 (DeVoe and Kistiakowsky, 1960; Kernohan, 1965; Pocker and Bjorkquist, 1977; Dodgson et al., 1990). Based on the algorithm of Zeebe and Wolf-Gladrow (2001), $[\text{CO}_{2(\text{aq})}]$ in the parent NaHCO_3 solutions is 9.0×10^{-5} M at pH 8.3 and 1.9×10^{-5} M at pH 8.9. Hence $K_M \gg [\text{CO}_2]$ in our experimental condition and Eq. (16) can be approximated by:

$$\frac{d[\text{HCO}_3^-]}{dt} = \frac{k_{\text{cat}}}{K_M} \cdot [\text{CA}] \cdot [\text{CO}_2] \quad (17)$$

Furthermore, the rate of increase in HCO_3^- due to the uncatalyzed CO_2 hydration is:

$$\frac{d[\text{HCO}_3^-]}{dt} = k_{+2} \cdot [\text{CO}_2] \quad (18)$$

Combining the contribution from the catalyzed and uncatalyzed CO_2 hydration, the total rate of increase in HCO_3^- is:

$$\frac{d[\text{HCO}_3^-]}{dt} = \left(k_{+2} + \frac{k_{\text{Cat}}}{K_M} \cdot [\text{CA}] \right) \cdot [\text{CO}_2] \quad (19)$$

Thus, in order to account for the catalyzed and uncatalyzed CO_2 hydration, the time constant τ^{-1} (Eq. (5)) can be modified to:

$$\tau^{-1} = (0.5) \cdot \{ k_{+2}^* + k_{+4}[\text{OH}^-] \} \cdot \left\{ 1 + \frac{[\text{CO}_2]}{S} - \left[1 + \left(\frac{2}{3} \cdot \frac{[\text{CO}_2]}{S} \right) + \left(\frac{[\text{CO}_2]}{S} \right)^2 \right]^{1/2} \right\} \quad (20)$$

where k_{+2}^* is the overall CO_2 hydration constant, which accounts for both uncatalyzed and catalyzed CO_2 hydration:

$$k_{+2}^* = k_{+2} + \frac{k_{\text{Cat}}}{K_M} \cdot [\text{CA}] \quad (21)$$

In Fig. 8 our experimental results are rearranged according to the left-hand side of Eq. (13). Note that the data presented here are limited to the early phase of ^{18}O equilibration (i.e., data from the regions where $\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}$ values are rapidly changing with time in Fig. 7). This is because the $\ln\{(\alpha_{\text{BaCO}_3\text{-H}_2\text{O}} - \alpha_{\text{BaCO}_3\text{-H}_2\text{O}}^{\text{EQ}})/(\alpha_{\text{BaCO}_3\text{-H}_2\text{O}}^0 - \alpha_{\text{BaCO}_3\text{-H}_2\text{O}}^{\text{EQ}})\}$ values calculated from the data in the plateaus are obscured by experimental uncertainties. Then the results can be nicely fit by linear regressions (Table 3). The r^2 values for the regressions were better than 0.95 except for the TS1-6CA experimental series ($r^2 = 0.83$). This is due to scarcity of the data points since the ^{18}O equilibration was very rapid at this experimental condition relative to our experimental resolution. The slopes obtained from the linear regressions are

equivalent to the numerical values of the time constant τ^{-1} , from which k_{+2}^* can be calculated (see Eq. (20)).

Fig. 9 displays the dependency of k_{+2}^* on the CA concentration in the parent NaHCO_3 solution. The relationship between k_{+2}^* and CA concentration is best described by a linearity ($r^2 = 0.99$), as expected from Eq. (21). It should be noted that the intercept and the slope from the linear regression reflect k_{+2} and k_{Cat}/K_M , respectively (Eq. (21)). The value of the intercept is $2.3 (\pm 0.1) \times 10^{-2} \text{ s}^{-1}$, which is in agreement with the uncatalyzed k_{+2} of $2.6 \times 10^{-2} \text{ s}^{-1}$ by Pinsent et al. (1956) at 25°C . Due to considerable variations in the k_{Cat} values of bovine CA reported in the literature (e.g., Kernohan, 1965; Donaldson and Quinn, 1974; Pocker and Bjorkquist, 1977; Dodgson et al., 1990), the k_{Cat}/K_M value for bovine erythrocyte CA at 25°C may vary between 2.3×10^7 and $8.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ based on the K_M value of $12 \times 10^{-3} \text{ M}$. The k_{Cat}/K_M value obtained from our experimental results is $2.7 (\pm 0.1) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, which fits into the expected range. This suggests that our experimental results are consistent with the theoretical constraints based on the fundamental kinetic principles.

Our experiments were performed at 25°C . However, the equilibrium constants and uncatalyzed kinetic rate constants applied in our model are well established for temperature range from 0 to 40°C or so (Table 1). Also the k_{Cat}/K_M of bovine CA appears to be unaffected by temperature from 5 to 30°C (Ghannam et al., 1986). Therefore our experimental results can be extrapolated to the typical range of oceanic temperatures in which majority of biogenic calcification takes place. In addition, the parameterization of the catalyzed CO_2 hydration using the k_{Cat}/K_M in our

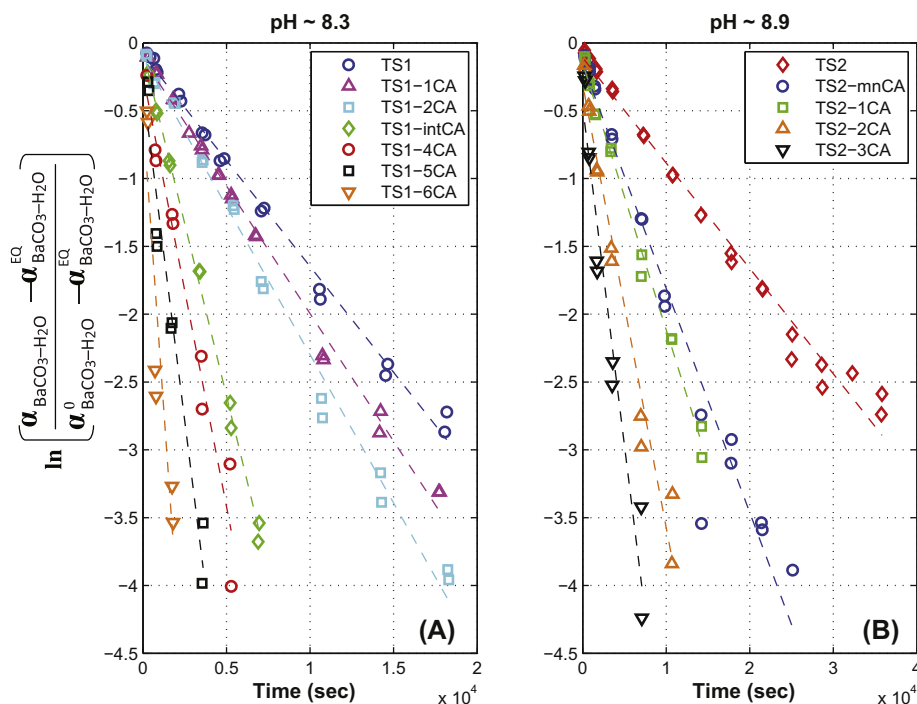


Fig. 8. Linear regressions of the experimental results following Eq. (13). The slopes from the regressions (see Table 3) are equivalent to the numerical values of the time constant $-\tau^{-1}$ (Eq. (20)).

Table 3

The results of linear regressions of the experimental results against time following Eq. (13) (see Fig. 8). The slopes obtained from the regressions are equivalent to the numerical values of the time constant $-\tau^{-1}$ (Eq. (20)).

Experiment series	Slope (s^{-1})	Slope σ^\dagger	Intercept	Intercept σ^\dagger	r^2
TS1 (Control)	-1.56×10^{-4}	2.96×10^{-6}	-0.09	0.03	0.99 ($n = 19$)
TS1-1CA	-1.88×10^{-4}	3.38×10^{-6}	-0.11	0.03	0.99 ($n = 21$)
TS1-2CA	-2.19×10^{-4}	5.28×10^{-6}	-0.11	0.05	0.99 ($n = 18$)
TS1-intCA	-5.08×10^{-4}	8.63×10^{-6}	-0.06	0.03	1.00 ($n = 12$)
TS1-4CA	-6.37×10^{-4}	4.90×10^{-5}	-0.23	0.15	0.96 ($n = 9$)
TS1-5CA	-9.85×10^{-4}	8.97×10^{-5}	-0.32	0.18	0.95 ($n = 8$)
TS1-6CA	-1.75×10^{-3}	3.90×10^{-4}	-0.53	0.44	0.83 ($n = 6$)
TS2 (Control)	-7.77×10^{-5}	2.06×10^{-6}	-0.11	0.04	0.98 ($n = 24$)
TS2-mnCA	-1.65×10^{-4}	8.87×10^{-6}	-0.16	0.11	0.96 ($n = 19$)
TS2-1CA	-1.97×10^{-4}	4.84×10^{-6}	-0.15	0.04	0.99 ($n = 14$)
TS2-2CA	-3.27×10^{-4}	1.80×10^{-6}	-0.30	0.10	0.97 ($n = 12$)
TS2-3CA	-5.00×10^{-4}	4.29×10^{-5}	-0.47	0.16	0.94 ($n = 10$)

† Standard error of the slope and intercept calculated from Higbie (1991).

model is highly practical. Since the k_{Cat}/K_M is a primary measure of the catalytic efficiency of an enzyme, this parameter is routinely determined in enzymatic studies. Hence the kinetics of the ^{18}O exchange in the $\text{CO}_2\text{--H}_2\text{O}$ system catalyzed by different classes of CA other than bovine CA can be readily modeled with the k_{Cat}/K_M data.

5.4. Implications for $\delta^{18}\text{O}$ vital effects in marine biogenic carbonates

Two different models have been proposed as an explanation for ^{18}O depletion in biogenic CaCO_3 . McConnaughey (1989a, 1989b, 2003) argued that ^{18}O -depletion as a result of the kinetic effects associated with CO_2 hydration and hydroxylation could be recorded in CaCO_3 if the precipitation rate is faster than ^{18}O equilibration in the $\text{CO}_2\text{--H}_2\text{O}$ system. On the other hand Zeebe (1999, 2007) argued that $\delta^{18}\text{O}$ vital effects are largely due to the role of fluid pH in defining the DIC speciation and consequently the overall $\delta^{18}\text{O}$ signature of the sum of DIC species. Corals are capable of maintaining elevated pH levels in the extracellular calcifying fluid from which calcification proceeds (Al-Horani et al., 2003; Rollion-Bard et al., 2003, 2011). Foraminifera can also maintain strong pH gradients between intracellular fluid and ambient seawater (Rink et al., 1998; Köhler-Rink and Kühl, 2005; de Nooijer et al., 2009; Bentov et al., 2009; Rollion-Bard and Erez, 2010). Hence the pH control on the overall $\delta^{18}\text{O}$ of the sum of DIC species in such alkaline fluid may be important for ^{18}O depletion in biogenic CaCO_3 with respect to anticipated $\delta^{18}\text{O}$ values that are in thermodynamic equilibrium with ambient seawater (Adkins et al., 2003; Rollion-Bard et al., 2003).

A crucial aspect for both the kinetic-based and pH-based model is the balance between the rate of DIC utilization for calcification and the rate of ^{18}O equilibration in the $\text{CO}_2\text{--H}_2\text{O}$ system. If the equilibration process is sufficiently rapid, the HCO_3^- and CO_3^{2-} ions that are potentially depleted in ^{18}O due to the kinetic effects can re-establish local ^{18}O equilibrium in the calcification sites and thus no kinetic effects would occur. The counter-argument for the pH mod-

el points to the question whether ^{18}O equilibrium in the $\text{CO}_2\text{--H}_2\text{O}$ system can be established quickly enough at elevated pH levels in the calcification sites (recall that equilibration takes considerably longer at higher pH as shown in Fig. 1).

In this study, we experimentally demonstrated that the ^{18}O equilibration process is accelerated by the presence of small amounts of CA due to its ability to catalyze the CO_2 hydration reaction (Fig. 5). In addition, the catalytic ability of the enzyme can be fully expressed even at elevated pH at 8.9 that is typical for the calcification sites. These findings support the notion that the ^{18}O equilibrium in the calcification sites is feasible. However, this idea requires the presence of extracellular CA in the actual calcification sites. Another crucial requirement is that the CA in these organisms needs to be as active and efficient as the purified bovine CA used in our experiments.

Several studies provide direct evidence for the presence of extracellular CA in the coral calcification sites (Furla et al., 2000; Tambutté et al., 2006; Moya et al., 2008). Particularly Moya et al. (2008) isolated extracellular CA named STPCA in the calcification sites of a symbiotic branching scleractinian coral *Stylophora pistillata* and analyzed the catalytic efficiency of the enzyme. Based on the measurement at 20 °C and pH 7.5, the k_{Cat}/K_M value for STPCA was found to be $4.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. Despite intracellular presence in the oral endoderm and the aboral tissues, notably Bertucci et al. (2011) isolated another type of CA, STPCA-2, in the same coral species *S. pistillata*. It is worthwhile to emphasize that the catalytic efficiency of the STPCA-2 ($k_{\text{Cat}}/K_M = 8.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) is comparable to that of a human cytoplasmic CA (hCA-II type, $k_{\text{Cat}}/K_M = 1.5 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$), which is classified as one of the most efficient and active form of CA (Bertucci et al., 2011). By applying the STPCA and STPCA-2 k_{Cat}/K_M data, it is possible to calculate the time required for $t_{99\%}$ (Eq. (6)) as a function of concentration of these enzymes using Eqs. (6), (20), and (21). Under uncatalyzed condition, $t_{99\%}$ is roughly 900 min at pH 8.9 (Table 4). But with the presence of only as little as $9.3 \times 10^{-10} \text{ M}$ of STPCA and STPCA-2, the $t_{99\%}$ will be reduced to roughly 620 and

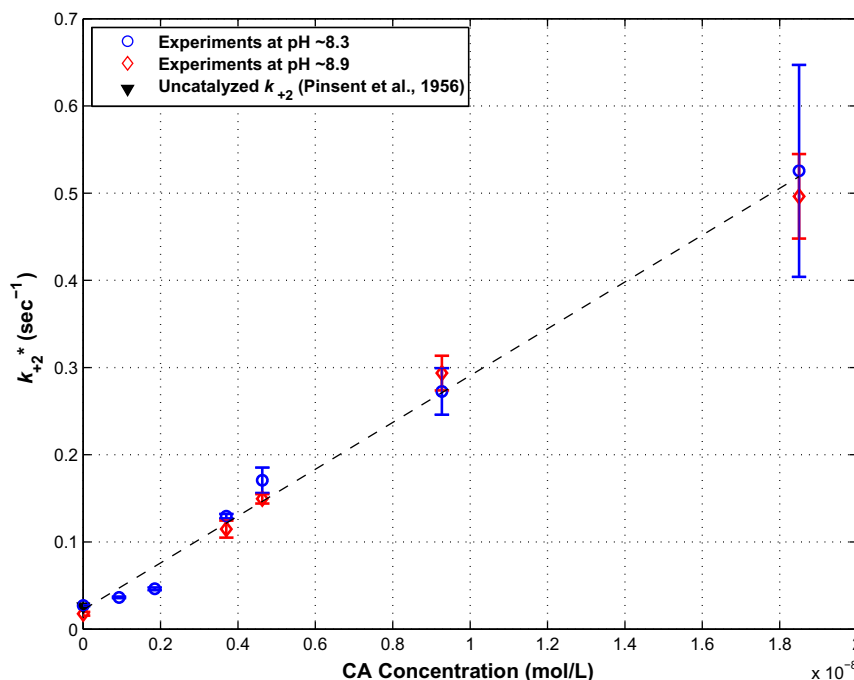


Fig. 9. The effect of CA concentration on the overall CO₂ hydration constant k_{+2}^* (Eqs. (18) and (19)), which accounts for both the uncatalyzed and catalyzed CO₂ hydration. The error bars indicate the propagated errors in the $-\tau^{-1}$ values based on the linear regressions of the experimental results (Table 3). The dashed line represents the linear regression for all of the data points in the figure. The intercept of the regression line is equivalent to the kinetic rate constant for the uncatalyzed CO₂ hydration k_{+2} . The slope reflects the k_{Cat}/K_M of the CA used in this experiment. The k_{+2} from our experimental results are in good agreement with Pinsent et al. (1956).

500 min, respectively. These are approximately 30% and 45% reduction from the uncatalyzed ¹⁸O equilibration time. If these enzymes are present at 1.9×10^{-8} M, which represents the maximum CA concentration tested in this study, the 99% equilibration time will be shortened to about 90 min for STPCA and 50 min for STPCA-2.

Are these estimates of the influence of STPCA and STPCA-2 on ¹⁸O equilibration relevant to the realistic timescales of biogenic calcification? McConnaughey (2003) suggested that the residence time of DIC in the coral calcification site can be estimated by:

$$\text{DIC Residence Time} = [\text{DIC}] \cdot h \cdot R^{-1} \quad (22)$$

where h is the thickness of the calcifying space and R is the calcification rate. By employing $[\text{DIC}] = 2 \times 10^3 \text{ mol/m}^3$, $h = 10^{-6} \text{ m}$ and $R = 10^{-7} \text{ mol/m}^2/\text{s}$ after McConnaughey (2003), the DIC residence time in the coral calcification sites appears to be on the order of 5.5 h. This should be sufficient for the CO₂-H₂O system in calcification site to reach ¹⁸O equilibrium, if the system is catalyzed by only $\sim 3 \times 10^{-9}$ M of CA that is as effective as STPCA and STPCA-2 (see Table 4).

One caveat in the discussion above is the potential influence of possible activators/inhibitors on the kinetic properties (k_{Cat}/K_M) of extracellular CA *in vivo*. For example, Bertucci et al. (2010) demonstrated that certain amino acids and amines can act as activators and increases k_{Cat} of STPCA. On the contrary, STPCA activity can be inhibited by certain inorganic anions (Bertucci et al., 2009). Our calculations of $t_{99\%}$ using the k_{Cat}/K_M values of STPCA and STPCA-2 (Table 4) do not account for these factors, which

could be important in the actual calcification sites. Another critical unknown is the actual concentrations or the total extracellular CA activities in the calcification sites. We note, however, that the study by Tambutté et al. (2006) marks an important step forward in this respect. They performed enzyme assay and measured the extracellular CA activity in the soluble proteins extracted from the skeletal organic matrix of a non-symbiotic coral *Tubastrea aurea*. In case of foraminifera, the presence of CA has not been unequivocally confirmed. ter Kuile et al. (1989) observed reduced calcification and photosynthetic rate in *Amphistegina lobifera* and *Amphisorus hemprichii* cultured with a CA-inhibitor (ethoxzolamide). Although this finding supports the presence of CA in some foraminiferal species, the localities, catalytic properties and concentrations of CA still need to be clarified.

But at this point, the presence and robust activity of extracellular CA and its direct involvement in the calcification are inarguable at least in some corals (e.g., Furla et al., 2000; Moya et al., 2008; Tambutté et al., 2011). Based on these lines of evidence, establishment of ¹⁸O equilibrium in the calcification sites is possible depending on the actual concentrations of CA such as STPCA and STPCA-2. In that case ¹⁸O-depletion in DIC species (namely in HCO₃⁻ and CO₃²⁻) resulting from the kinetic effect associated with CO₂ hydration and hydroxylation can be eliminated, which is crucial for comprehensive understanding of $\delta^{18}\text{O}$ vital effects. Evaluation of this hypothesis requires inputs from future research, which should focus on identification, characterization and quantification of extracellular CA in marine calcifiers.

Table 4

Comparison of the effect of concentration and the type of CA on the time required for 99% completion of ^{18}O equilibration ($t_{99\%}$) in the $\text{CO}_2\text{--H}_2\text{O}$ system at pH 8.9 and at 25 °C. The estimates are based on the mathematical model developed in this study (see Eqs. (6), (20), and (21)).

CA conc. (M: mol/L)	$t_{99\%}$ (min.)		
	Bovine CA*	STPCA [†]	STPCA-2 [‡]
0	904.7	904.7	904.7
9.3×10^{-10}	715.1	621.7	496.7
1.9×10^{-9}	591.6	474.1	342.8
3.7×10^{-9}	439.5	321.2	211.5
4.6×10^{-9}	389.2	276.4	177.3
9.3×10^{-9}	247.7	163.0	98.2
1.9×10^{-8}	143.8	89.7	52.0

* For bovine erythrocyte CA, the k_{cat}/K_M value of $2.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ from our experimental results was used for the estimates (Fig. 9).

^{†,‡} The estimates are based on the k_{cat}/K_M value of $4.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for STPCA (Moya et al., 2008) and $8.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for STPCA-2 (Bertucci et al., 2011). These k_{cat}/K_M values were determined at pH 7.5 and at 20 °C (see the original references for details). No further corrections on the k_{cat}/K_M values were made for the $t_{99\%}$ estimates presented here.

6. CONCLUSIONS

We have performed quantitative BaCO_3 precipitation experiments to quantify the effect of CA on the kinetics and equilibrium of the ^{18}O exchange in the $\text{CO}_2\text{--H}_2\text{O}$ system. The results from our control experiments indicate that the uncatalyzed evolution of ^{18}O equilibrium can be modeled by the mathematical expression given in Usdowski et al. (1991), which is derived in Appendix A. Furthermore, we find that the recently published kinetic rate constant for CO_2 hydroxylation by Wang et al. (2010) is inconsistent with our results as well as previously published rate constants. We argue that the measurements by Wang et al. (2010) are most likely overestimates. When CA is present in the $\text{CO}_2\text{--H}_2\text{O}$ system, the time required for ^{18}O equilibrium is greatly reduced due to the catalysis of CO_2 hydration and its reverse reaction. On the other hand, CA does not alter the equilibrium oxygen isotope fractionation in the $\text{CO}_2\text{--H}_2\text{O}$ system. Under our experimental conditions, the catalysis of the CO_2 hydration by CA can be parameterized by the k_{cat}/K_M and the total enzyme concentration. Given the evidence for the presence of highly active CA such as STPCA and STPCA-2 (Moya et al., 2008; Bertucci et al., 2011), it is possible that ^{18}O equilibration of the $\text{CO}_2\text{--H}_2\text{O}$ system can outpace the DIC utilization in the calcification sites. We therefore argue that the catalytic role of CA may be of critical importance in developing a realistic model of $\delta^{18}\text{O}$ vital effects in biogenic CaCO_3 .

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APPENDIX A

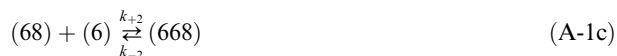
Derivation of the rate law for the oxygen isotope exchange in the $\text{CO}_2\text{--H}_2\text{O}$ system via uncatalyzed CO_2 hydration and hydroxylation.

Usdowski et al. (1991) gave their rate expression without describing the derivation process. In this appendix we show that the expression can be derived by following the scheme presented in Mills and Urey (1940). In this classical paper, an expression for the rate of ^{18}O exchange involved in CO_2 hydration reaction was introduced.

For CO_2 hydration, simple chemical equilibrium can be written as:



where k_{+2} and k_{-2} are the kinetic rate constant for the forward and reverse (dehydration) reaction. By applying a simple numeric notation 6 and 8 for the ^{16}O and ^{18}O isotopes in CO_2 , H_2O and H_2CO_3 (e.g., Gao and Marcus, 2001), the oxygen isotope equilibration for the same reaction can be written as:



where (6) = H_2^{16}O , (68) = $\text{C}^{16}\text{O}^{18}\text{O}$, (668) = $\text{H}_2\text{C}^{16}\text{O}^{16}\text{O}^{18}\text{O}$ and so on. Following Mills and Urey (1940), the rate of change in the ^{18}O content of CO_2 can be expressed as:

$$\begin{aligned} -\frac{d^{18}\text{O}(\text{CO}_2)}{dt} &= k_{+2}[68][6] + k_{+2}[68][8] + 2k_{+2}[88] \\ &\quad \times [6] + 2k_{+2}[88][8] - \frac{2}{3}k_{-2}[668] \\ &\quad - \frac{4}{3}k_{-2}[688] - 2k_{-2}[888], \end{aligned} \quad (\text{A-2a})$$

which can be rearranged to:

$$\begin{aligned} -\frac{d^{18}\text{O}(\text{CO}_2)}{dt} &= k_{+2}\{[6] + [8]\}\{[68] + 2[88]\} \\ &\quad - 2k_{-2}\left\{\frac{1}{3}[668] + \frac{2}{3}[688] + [888]\right\} \end{aligned} \quad (\text{A-2b})$$

However, oxygen isotope exchange associated with CO₂ hydroxylation also needs to be taken into account for the CO₂–H₂O system when pH > 7. By adopting a notation such as (6)' and (888)' to differentiate the ¹⁶O and ¹⁸O isotopes in OH[−] and HCO₃[−] ions from H₂O and H₂CO₃, the chemical equilibrium and associated oxygen isotope equilibration for the CO₂ hydroxylation reaction can be similarly written as:



and:



Following the approach by Mills and Urey (1940), the rate of change in the ¹⁸O content in CO₂ due to CO₂ hydroxylation is:

$$\begin{aligned} -\frac{d^{18}\text{O}(\text{CO}_2)}{dt} &= k_{+4}[68][6]' + k_{+4}[68][8]' + 2k_{+4}[88] \\ &\quad \times [6]' + 2k_{+4}[88][8]' - \frac{2}{3}k_{-4}[668]' \\ &\quad - \frac{4}{3}k_{-4}[688]' - 2k_{-4}[888]', \end{aligned} \quad (\text{A-4a})$$

which can be rearranged to:

$$\begin{aligned} -\frac{d^{18}\text{O}(\text{CO}_2)}{dt} &= k_{+4}\{[6]' + [8]'\}\{[68] + 2[88]\} \\ &\quad - 2k_{-4}\left\{\frac{1}{3}[668]' + \frac{2}{3}[688]' + [888]'\right\} \end{aligned} \quad (\text{A-4b})$$

From Eqs. A-2b and A-4b, the net rate of change in the ¹⁸O content in CO₂ due to both CO₂ hydration and hydroxylation is:

$$\begin{aligned} -\frac{d^{18}\text{O}(\text{CO}_2)}{dt} &= k_{+2}\{[6] + [8]\}\{[68] + 2[88]\} \\ &\quad - 2k_{-2}\left\{\frac{1}{3}[668] + \frac{2}{3}[688] + [888]\right\} \\ &\quad + k_{+4}\{[6]' + [8]'\}\{[68] + 2[88]\} \\ &\quad - 2k_{-4}\left\{\frac{1}{3}[668]' + \frac{2}{3}[688]' + [888]'\right\} \end{aligned} \quad (\text{A-5})$$

Similar rate equations can be set up for H₂CO₃ and HCO₃[−] using the expression for oxygen isotope equilibration via hydration and hydroxylation (Eqs. (A-1a–A-1f), (A-3a–A-3f)):

$$\begin{aligned} -\frac{d^{18}\text{O}(\text{H}_2\text{CO}_3)}{dt} &= k_{-2}[668] + 2k_{-2}[688] + 3k_{-2}[888] \\ &\quad - k_{+2}[66][8] - k_{+2}[68][6] \\ &\quad - 2k_{+2}[68][8] - 2k_{+2}[88][6] \\ &\quad - 3k_{+2}[88][8] \end{aligned} \quad (\text{A-6})$$

$$\begin{aligned} -\frac{d^{18}\text{O}(\text{HCO}_3^-)}{dt} &= 3k_{-4}\left\{\frac{1}{3}[668]' + \frac{2}{3}[688] + [888]'\right\} \\ &\quad - k_{-4}[6]'\{[68] + 2[88]\} \\ &\quad - k_{-4}[8]'\{[66] + 2[68] + 3[88]\} \end{aligned} \quad (\text{A-7})$$

Then we define α , β , γ , ε and θ as the atom fraction of the ¹⁸O isotopes in CO₂, H₂O, H₂CO₃ and HCO₃[−] and OH[−]:

$$\alpha = \frac{2[88] + [68]}{2[\text{CO}_2]} \quad (\text{A-8})$$

$$\beta = \frac{[8]}{[\text{H}_2\text{O}]} \quad (\text{A-9})$$

$$\gamma = \frac{[668] + 2[688] + 3[888]}{3[\text{H}_2\text{CO}_3]} \quad (\text{A-10})$$

$$\varepsilon = \frac{[668]' + 2[688]' + 3[888]'}{3[\text{HCO}_3^-]} \quad (\text{A-11})$$

$$\theta = \frac{[8]'}{[\text{OH}^-]} \quad (\text{A-12})$$

Note that in (Eq. A-5) the term $\{[6] + [8]\}$ is essentially $\{[\text{H}_2^{16}\text{O}] + [\text{H}_2^{18}\text{O}]\}$, which is simply $[\text{H}_2\text{O}]$. Likewise, $\{[66] + 2[68]\}$ and $\{[6]' + [8]'\}$ are equal to $[\text{CO}_2]$ and $[\text{OH}^-]$, respectively. In addition, equilibrium constant (K) and kinetic rate constants (k) for a given chemical reaction are interrelated, such that for CO₂ hydration:

$$\begin{aligned} K_2 &= \frac{[\text{H}_2\text{CO}_3]}{[\text{CO}_2][\text{H}_2\text{O}]} = \frac{k_{+2}}{k_{-2}} \iff k_{-2}[\text{H}_2\text{CO}_3] \\ &= k_{+2}[\text{CO}_2][\text{H}_2\text{O}] \end{aligned} \quad (\text{A-13})$$

and for CO₂ hydroxylation:

$$\begin{aligned} K_4 &= \frac{[\text{HCO}_3^-]}{[\text{CO}_2][\text{OH}^-]} = \frac{k_{+4}}{k_{-4}} \iff k_{-4}[\text{HCO}_3^-] \\ &= k_{+4}[\text{CO}_2][\text{OH}^-] \end{aligned} \quad (\text{A-14})$$

Note that by using these relationships, the contribution of the dehydration reaction (HCO₃[−] → CO₂) is automatically taken into account in our approach (system is in chemical equilibrium, although not in isotopic equilibrium). After algebraic manipulations using the definitions for α , γ and ε (Eqs. A-8–A-12) and the relationships described above, the rate equation for the change in ¹⁸O content in CO₂, H₂CO₃ and HCO₃[−] (Eqs. A-5–A-7) can be rearranged to:

$$2[\text{CO}_2]\frac{d\alpha}{dt} = 2[\text{CO}_2]\{k_{+2}(\gamma - \alpha) + k_{+4}[\text{OH}^-](\varepsilon - \alpha)\} \quad (\text{A-15})$$

$$3[\text{H}_2\text{CO}_3]\frac{d\gamma}{dt} = k_{+2}[\text{CO}_2](2\alpha + \beta - 3\gamma) \quad (\text{A-16})$$

$$3[\text{HCO}_3^-]\frac{d\varepsilon}{dt} = k_{+4}[\text{CO}_2][\text{OH}^-](2\alpha + \theta - 3\varepsilon) \quad (\text{A-17})$$

Note that $[\text{H}_2\text{O}]$ terms in these equations are dropped because the $[\text{H}_2\text{O}]$ contribution is already taken into account in the equilibrium (K) and kinetic constants (k) compiled by Usdowski et al. (1991), which are given in Table 1 of this paper. Also note that similar rate expressions for β and θ can be established. However, the ^{18}O content of H_2O is constant and OH^- is in isotopic equilibrium with H_2O . Hence the rate expressions for these components do not require further considerations.

It is important to note that protonation and deprotonation reaction among H_2CO_3 , HCO_3^- and CO_3^{2-} ions is a very rapid process ($\sim 10^{-7}$ s) relative to CO_2 hydration and hydroxylation reaction (~ 10 s) (Zeebe and Wolf-Gladrow, 2001). Consequently we can assume that the ^{18}O content in these particular DIC species at a given time over the course of ^{18}O equilibration of the CO_2 – H_2O system are inseparable, hence we need to consider total ^{18}O content in the sum of H_2CO_3 , HCO_3^- and CO_3^{2-} . Also note that an independent rate reaction for ^{18}O content in CO_3^{2-} is not necessary. This is because CO_3^{2-} ions do not directly exchange oxygen isotopes with H_2O or OH^- , and CO_3^{2-} and HCO_3^- come to ^{18}O equilibrium very rapidly relative to the timescale of our interest here. Therefore Eqs. (A-16) and (A-17) can be combined to give a new expression for the rate of change in the ^{18}O content of the sum of H_2CO_3 , HCO_3^- and CO_3^{2-} :

$$\begin{aligned} & \frac{d^{18}\text{O}([\text{H}_2\text{CO}_3] + [\text{HCO}_3^-] + [\text{CO}_3^{2-}])}{dt} \\ &= k_{+2}[\text{CO}_2](2\alpha + \beta - 3\gamma) + k_{+4}[\text{CO}_2][\text{OH}^-](2\alpha \\ & \quad + \theta - 3\varepsilon) \end{aligned} \quad (\text{A-18a})$$

By defining μ as the atom fraction of ^{18}O isotopes in CO_3^{2-} , the left-hand side of the equation above can be written as:

$$\begin{aligned} & \frac{d(3[\text{H}_2\text{CO}_3]\gamma + 3[\text{HCO}_3^-]\varepsilon + 3[\text{CO}_3^{2-}]\mu)}{dt} \\ &= k_{+2}[\text{CO}_2](2\alpha + \beta - 3\gamma) + k_{+4}[\text{CO}_2][\text{OH}^-](2\alpha \\ & \quad + \theta - 3\varepsilon) \end{aligned} \quad (\text{A-18b})$$

In order to simplify the following algebraic manipulations, we assume $\gamma = \varepsilon = \mu$. The fidelity of this assumption was tested by numerically solving the following differential equations (see below) by adopting two boundary conditions; $\gamma = \varepsilon = \mu$ and $\gamma \neq \varepsilon \neq \mu$. For the latter case, the numeric values for γ , ε and μ were assigned according the equilibrium oxygen fractionation factors between individual DIC species and H_2O from Beck et al. (2005). Irrespective of the boundary conditions, however, the final outcomes of the computations were essentially identical. Hence assuming $\gamma = \varepsilon = \mu$, the equation above can be transformed into:

$$\begin{aligned} 3S \cdot \frac{d\gamma}{dt} &= k_{+2}[\text{CO}_2](2\alpha + \beta - 3\gamma) + k_{+4}[\text{CO}_2][\text{OH}^-] \\ & \quad \times (2\alpha + \theta - 3\varepsilon) \end{aligned} \quad (\text{A-19})$$

where

$$S = [\text{H}_2\text{CO}_3] + [\text{HCO}_3^-] + [\text{CO}_3^{2-}] \quad (\text{A-20})$$

From Eqs. (A-15) and (A-19), a set of homogeneous differential equations with respect to α and γ can be obtained:

$$\begin{aligned} \frac{d\alpha}{dt} &= P(\gamma - \alpha) \\ \frac{d\gamma}{dt} &= \frac{2}{3}Q\alpha - Q\gamma \end{aligned} \quad (\text{A-21})$$

where

$$\begin{aligned} P &= k_{+2} + k_{+4}[\text{OH}^-] \\ Q &= \frac{k_{+2}[\text{CO}_2] + k_{+4}[\text{CO}_2][\text{OH}^-]}{S} \end{aligned} \quad (\text{A-22})$$

The differential equations (Eq. A-21) have a general solution of the form:

$$\begin{aligned} \alpha &= a_1 e^{-\lambda_1 t} + a_2 e^{-\lambda_2 t} \\ \gamma &= b_1 e^{-\lambda_1 t} + b_2 e^{-\lambda_2 t} \end{aligned} \quad (\text{A-23})$$

where $a_{1,2}$ and $b_{1,2}$ are constants. The differential equations can be solved by arranging a 2 by 2 matrix in this case, such that:

$$\begin{bmatrix} \frac{d\alpha}{dt} \\ \frac{d\gamma}{dt} \end{bmatrix} = \begin{bmatrix} -P & P \\ \frac{2}{3}Q & -Q \end{bmatrix} \begin{bmatrix} \alpha \\ \gamma \end{bmatrix} \quad (\text{A-24})$$

The eigenvalues λ in Eq. (A-23) must satisfy the following relationship:

$$\det \begin{vmatrix} -(P + \lambda) & P \\ \frac{2}{3}Q & -(Q + \lambda) \end{vmatrix} = 0 \quad (\text{A-25})$$

The expression above can be simplified to:

$$\lambda^2 + (P + Q)\lambda + \frac{1}{3}PQ = 0 \quad (\text{A-26a})$$

Thus, the solutions ($\lambda_{1,2}$) to the equation are:

$$\lambda_1 = \frac{-(P + Q) + \sqrt{(P + Q)^2 - \frac{4}{3}PQ}}{2}$$

and

$$\lambda_2 = \frac{-(P + Q) - \sqrt{(P + Q)^2 - \frac{4}{3}PQ}}{2} \quad (\text{A-26b})$$

From the definition of P and Q (Eq. A-22), the final expressions for λ_1 and λ_2 are:

$$\begin{aligned} \lambda_{1,2} &= 0.5(k_{+2} \\ & \quad + k_{+4}[\text{OH}^-]) \left[1 + \frac{\text{CO}_2}{S} \pm \sqrt{1 + \frac{2}{3} \cdot \frac{[\text{CO}_2]}{S} + \left(\frac{[\text{CO}_2]}{S}\right)^2} \right] \end{aligned} \quad (\text{A-27})$$

The contribution of the term containing λ_1 to the overall solution (Eq. A-23) is negligible on the timescale concerned here. In other words, the solution can be solely described by the term containing λ_2 , which is identical to the time constant τ^{-1} in the formulation by Usdowski et al. (1991).

APPENDIX B. SUPPLEMENTARY DATA

Supplementary data (Tables S1–S12) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.gca.2012.07.022>.

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