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Oxygen isotope signature of P_i regeneration from organic compounds by phosphomonoesterases and photooxidation

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Abstract

Phosphomonoesters are an important source of dissolved inorganic orthophosphate (PO_4 or P_i), the preferred form of P utilized by microbiota and aquatic plants in marine and freshwater ecosystems. Two enzymes involved in phosphomonoester metabolism and commonly detected in natural waters (alkaline phosphatase and 5′-nucleotidase) have been studied to determine the oxygen isotope signature of P_i-regeneration from phosphomonoesters by enzymatic degradation. Oxygen (O) isotope ratios of water and P_i released from phosphomonoesters during enzyme hydrolysis experiments demonstrate that released P_i incorporates one oxygen atom from water. The isotopic fractionation between this incorporated water O and ambient water O is -30 (± 8)‰ for alkaline phosphatase and -10 (± 1)‰ for 5′-nucleotidase, with very weak dependence on temperature. The result of these enzyme-specific isotopic fractionations at one of the four O sites in PO_4 is that the $\delta^{18}O$ value of P_i regenerated by 5′-nucleotidase is 5‰ higher than P_i regenerated by alkaline phosphatase from the same phosphomonoester substrate. The $\delta^{18}O$ value of regenerated P_i also reflects inheritance of 75% of O from the phosphomonoester substrate, thus making the $\delta^{18}O$ of phosphomonoester-derived P_i a potential tracer of organophosphorous compound sources. Phosphomonoesterase-regenerated P_i has a distinct phosphate oxygen isotope signature that is different and distinguishable from that of biologically recycled and subsequently equilibrated P_i and P_i regenerated from photooxidation of organic matter. The $\delta^{18}O$ value of regenerated P_i will correlate positively with the $\delta^{18}O$ value of bulk water and the fractionation, α, between regenerated P_i and water, $\alpha_{\text{regen Pi-water}}$, should converge toward equilibrium $\alpha_{\text{Pi-water}}$ values with increased biological cycling of P_i.

1. Introduction

Phosphorus (P) is a key nutrient essential for all living organisms and *ortho*phosphate (PO_4 or P_i) is the preferred source of P which is taken up directly by microorganisms and plants in natural aquatic environments. Orthophosphate concentrations in many aquatic systems are too low to meet the P requirements of living organisms, thus organophosphorous compounds (P_{org}) become an important alternative source of P (Ammerman and Azam, 1991; Bjorkman and Karl, 1994; Benitez-Nelson, 2000). Most organic compounds are too large to cross cytoplasmic membranes of cells intact (Lugtenberg, 1987). Therefore, P_{org} metabolism begins as an extracellular process that takes place through the action of freely dissolved or membrane-bound microbial enzymes. Many different types of extracellular enzymes (alkaline phosphatase, 5′-nucleotidase, peptidase, aminopeptidase, cyclic nucleotide phosphodiesterase, chitinase, *etc.*) have been detected in marine sediments, lakes, estuarine and coastal ecosystems and variations in their activities are related to environmental parameters such as nutrient availability and water depth (Perry, 1972; Ammerman and Azam, 1985; Chrost, 1989; Barfield and Francko, 1991; Jacobsen and Rai, 1991; Jones and Lock, 1991; Smucker and Kim, 1991). Results of previous studies indicate that activities of extracellular enzymes are important to inorganic phosphate (P_i) regeneration in aquatic environments.

Among the numerous enzymes involved in regeneration and acquisition of P_i and other nutrients, alkaline phosphatase (APase) and 5′-nuleotidase are the most commonly observed extracellular phosphoenzymes found in a wide

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range of habitats (Wai et al., 1960; Perry, 1972; Taft et al., 1977; Ammerman and Azam, 1985; Siuda and Gude, 1994; Hoppe and Ullrich, 1999). APase, the most widely studied phosphoenzyme in aquatic systems, is a relatively non-specific phosphohydrolase enzyme that releases P_i from a variety of phosphomonoesters (McComb et al., 1979) (Fig. 1a). The location of microbial APase depends on the structure of the cell wall. Bacterial APase is found in the periplasmic space of Gram-negative bacteria while Gram-positive bacteria, which lack a periplasm, tend to release APase extracellularly (Cembella et al., 1984; Wetzel, 1991). Synthesis of APase is regulated by the intracellular P_i pool, which in turn, reflects ambient, extracellular P_i concentrations (Chrost, 1991). The synthesis of APase is induced by P_i starvation or low ambient P_i concentrations in natural waters. APase from different sources may respond to ambient environmental changes differently. For example, it has been observed that algal APase activity is competitively inhibited by high P_i concentration, whereas the P_i concentration affects the activity of bacterial APase only slightly (Chrost, 1991). 5′-Nucleotidase is a membrane-bound enzyme that rapidly hydrolyzes phosphomonoester bonds in nucleotides (Neu, 1967a,b; Zimmerman, 1992) (Fig. 1b). Ammerman and Azam (1991) showed that high 5′-nucleotidase activity was correlated with high growth rates of bacteria in coastal marine waters and not with conditions of P_i starvation and depletion as in the case of APase.

Organic P compounds also contain C and N (e.g., proteins, sugars, nucleotides and nucleic acid) which, like P, are essential for growth. In addition to P_i, organic C and N groups are also products and, in some cases, targets of P_{org} hydrolysis. Thus, living organisms use phosphoenzymes to release and assimilate not only P_i, but also other nutrients contained in P_{org} compounds (Wanner, 1987). Chrost (1991) observed that in response to addition of glucose-6-phosphate, bacteria produced APase in order to utilize glucose as C source. Under carbon-limiting conditions, the C- and N-containing portions of nucleotides are assimilated by cells with the help of 5′-nucleotidase and APase (Wilkins, 1972; Bjorkman and Karl, 1994). The presence of relatively high APase activity in deep ocean water compared to the euphotic zone, suggests the supply of bioavailable organic C to C-limited deep-ocean bacteria *via* P_{org} degradation (Hoppe and Ullrich, 1999). Hence, phosphoenzymes not only influence P cycling but also C and N cycling, which in turn, affects the cycles of atmospheric O_2 and CO_2 and, ultimately, global climate.

Biogeochemical cycling of P is dominated by biologically mediated reactions catalyzed by enzymes. Oxygen isotope effects of phosphoenzymes, thus provide a potential method for identifying specific metabolic/enzymatic pathways operating during biogeochemical cycling and regeneration of P_i in natural systems. The metabolism and biological turnover of P compounds by living organisms has a significant effect on the oxygen isotope composition of ambient dissolved P_i, $\delta^{18}O_P$ (Blake et al., 1997, 1998a,b, 2001, 2005; Paytan et al., 2002). Results of laboratory and field experiments show that living organisms can shift ambient $\delta^{18}O_P$ values rapidly and on large scales (Paytan et al., 2002). Based on laboratory culture studies Blake et al. (1997, 1998a) concluded that metabolism and recycling of P by microbial cells will cause P_i to approach

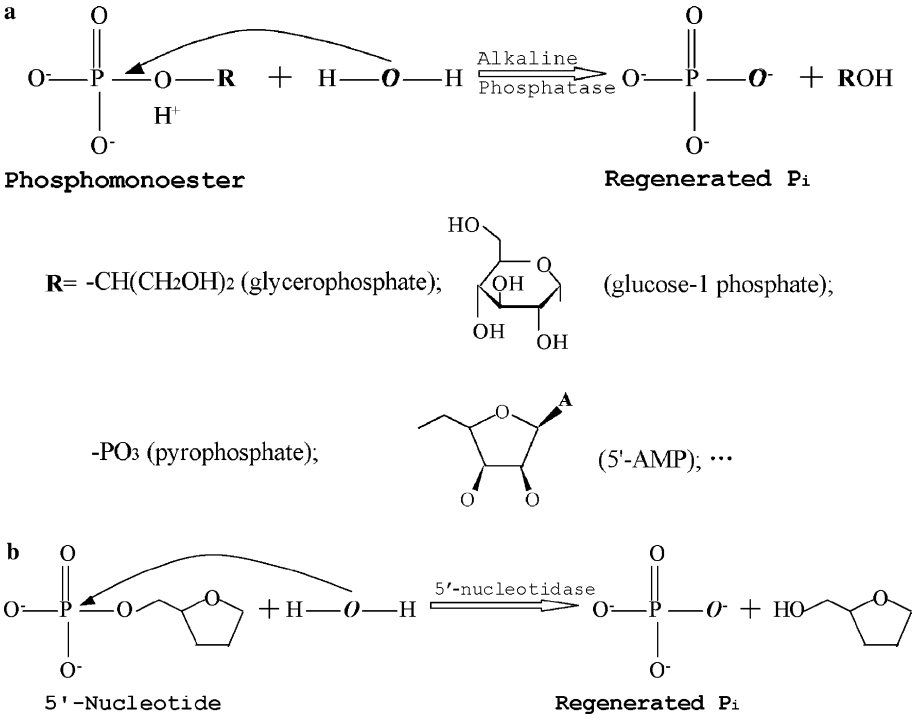

Fig. 1. Phosphomonoesterase reaction mechanisms. (a) Alkaline phosphatase; (b) 5′-nucleotidase.

oxygen isotope equilibrium with ambient water, in general agreement with the PO_4–H_2O paleothermometry equation of Longinelli and Nuti (1973). Subsequent laboratory experiments on isotope effects of cell-free phosphoenzymes indicate that attainment of temperature-dependent isotopic equilibrium is promoted largely by the action of the ubiquitous intracellular enzyme inorganic pyrophosphatase (Blake et al., 1998b, 2005).

The goal of this work was to determine the oxygen isotope effects of key enzymes involved in the metabolism and regeneration of P_i from P_{org} compounds commonly found in nature (e.g., phosphomonoesters). Most importantly, we seek to link specific enzymatic pathways with specific isotope effects, thereby generating a new diagnostic tool for the study of biogeochemical cycling of P in aquatic systems.

2. Materials and methods

To advance our understanding of the reaction processes that occur during P cycling, oxygen isotope effects of P_i regeneration from phosphomonoesters was investigated by measuring the $\delta^{18}O$ of P_i released by phosphomonoesterases (alkaline phosphatase and 5′-nucleotidase). Different phosphomonoester substrates, such as α-D-glucose 1-phosphate, α- and β-glycerophosphate and adenosine 5′-monophosphate (5′-AMP), were used in phosphomonoesterase-catalyzed P_{org} hydrolysis experiments to determine the effects, if any of the substrate on the $\delta^{18}O$ value of the P_i product of hydrolysis. Reactions were carried out in pH-buffered solutions made with waters having a wide range of $\delta^{18}O$ (−19‰ to +99‰) in order to detect the influence of ambient water oxygen on phosphate $\delta^{18}O$ values and to elucidate reaction mechanisms. All experiments were carried out under sterile conditions in temperature-controlled baths and incubators. The $\delta^{18}O$ value of water in reaction mixtures was measured at the start and at the end of each experiment. A typical reaction system comprised enzyme, buffer, metal cation co-factor (usually Mg^{2+}), which binds to the enzyme and facilitates the reaction, and phosphomonoester substrates. Reactions were carried out under conditions (e.g., pH, temperature, buffer) specified as *optimal* for each enzyme by the manufacturer (Sigma or USB).

In the first series of enzyme-catalyzed hydrolysis experiments, three different APases were used to determine if APase extracted from different sources (e.g., eukaryotic, prokaryotic) would have different isotope effects on P_i released from phosphomonoesters. In order to obtain enough P_i for isotopic analysis and to constrain reaction time, high phosphomonoester substrate and enzyme concentrations were employed, however, the concentrations of substrate and enzyme used are not expected to alter the enzymatic reaction mechanism. Two eukaryotic APases were used, including a mammalian APase from calf intestine (calf APase) and an arthropod APase derived from shrimp. Calf APase (Sigma P6271) hydrolysis media comprised 45 mM glycine, 0.45 mM magnesium chloride and 10–30 mM phosphomonoesters. Before adding calf APase to start hydrolysis reactions, buffer solutions with phosphomonoester substrates were adjusted to pH 8.8 and equilibrated at the reaction temperature (37 °C). α-D-Glucose-1-phosphate, α- and β-glycerophosphate and 5′-AMP were used as phosphomonoester substrates in calf APase experiments.

In control experiments designed to test for any back-reaction of hydrolysis products (i.e., free P_i + organic-C compound) calf APase media comprised 45 mM glycine, 0.45 mM magnesium chloride and 10–30 mM P_i (Na_2HPO_4) and glycerol instead of phosphomonoesters. Shrimp APase (USB 70092Z) media contained 150 mM glycine, 1.5 mM magnesium chloride, 1.5 mM zinc chloride and 30 mM β-glycerophosphate. The optimum pH specified for Shrimp APase was pH 10.4.

Bacterial APase (Sigma P5931) derived from *Escherichia coli* (*E. coli* APase) was used as a prokaryotic APase source. *E. coli* APase hydrolysis media consisted of 87 mM glycine, 0.90 mM magnesium chloride, 0.87 mM zinc chloride, and 10–30 mM phosphomonoester substrate. The pH optimum for *E. coli* APase was 10.4. α-D-Glucose-1-phosphate and β-glycerophosphate were the two phosphomonoesters used as substrates in *E. coli* APase catalyzed reactions.

In order to test the effect of temperature on the $\delta^{18}O$ of P_i released from phosphomonoesters by APase, β-glycerophosphate was hydrolyzed by calf APase at 37.0 (±0.2) °C, room temperature 21 (±2) °C and 15.0 (±0.2) °C. In these experiments, the enzyme/P ratio and $\delta^{18}O_W$ (−6.0‰) were the same.

The second series of experiments was designed to determine the isotope effects of P_i-regeneration by 5′-nucleotidase. 5′-Nucleotidase derived from *Crotalus Atrox* venom (Sigma N5880) was used to hydrolyze 5′-AMP, α-D-glucose-1-phosphate and β-glycerophosphate, the same phosphomonoester substrates used in experiments with APase. The 5′-nucleotidase reaction media comprised 150 mM glycine, 10 mM magnesium sulfate and 10–30 mM phosphomonoesters. Before adding the enzyme, the buffer solutions were adjusted to pH 9. APase activity was followed using *p*-nitrophenyl phosphate (PNPP) as an indicator. Two hundred microliters of the reaction solution was pipetted into 500 μl of *p*-nitrophenyl phosphate solution (18 mg PNPP in 10 ml sterile DI water). If the test solution turned yellow—indicating free *p*-nitrophenol, within 20 min, the enzyme in solution was still highly active.

The third series of experiments was designed primarily to test analytical procedures, but also to provide data on the isotope effect associated with the natural process of P_{org} degradation by photooxidation. Photooxidation of organic matter occurs in the uppermost euphotic zone of surface waters and photodecomposition of P_{org} by UV light is a widely used method to release organically bound P_i for accurate determination of dissolved organic phosphorous (Amstrong et al., 1966; Francko and Heath, 1982; Yanagi

et al., 1992). Phosphomonoester substrates were exposed to UV radiation (UVR) to release P_i. The hope was that the photodecomposition would take place without hydrolyzing P—O bonds and incorporating ambient water O into the P_i released from P_{org}, thus permitting determination of $\delta^{18}O$ values of the unaltered PO_4 moiety in pre-hydrolyzed, phosphomonoester substrates (P_{org}-$\delta^{18}O_P$). UVR experiments were carried out in a 3.5 ml quartz cell with 5 mM phosphomonoester under UVR produced by a 500 W mercury lamp. H_2O_2 (0–300 μl) was used to accelerate the rate of P_i release. Reaction temperature was less than 40 °C.

Experiments were also carried out to observe the effect of natural sunlight on degradation of dissolved P_{org}. Three phosphomonoesters (α-D-glucose-1-phosphate, β-glycerophosphate and 5′-AMP) were tested. 125 ml of 200 μM phosphomonoester solutions were sealed in 250 ml quartz tubes (2.5 cm o.d.) and exposed to summer sunlight for 24 h. Control experiments were carried out in parallel to exclude factors other than sunlight that may release P_i from phosphomonoesters. In control experiments, quartz tubes containing phosphomonoester solutions were wrapped in aluminum foil. The amount of P_i released from phosphomonoesters by exposure to sunlight was determined by measuring the difference in P_i concentration between exposed versus control tubes.

The concentrations of P_i released from phosphomonoesters were monitored during the course of experiments by extracting a small aliquot of solution and performing a colorimetric analysis adapted from Murphy and Riley (1962). When P_i yield was close to 100% and no additional P_i was released from P_{org} even after addition of fresh enzyme, the reaction was terminated by adjusting the pH to less than 5, which inactivates both APase and 5′-nucleotidase. P_i released during hydrolysis reactions was precipitated using a method modified from Kolodny et al., 1983. Briefly, P_i was first precipitated as ammonium phosphomolybdate (APM), then recrystallized as magnesium ammonium phosphate (MAP). Finally, the MAP was converted to silver phosphate, the compound used for $\delta^{18}O_P$ analysis.

Samples extracted from enzyme experiments were analyzed for $\delta^{18}O_P$ following methods adapted from O'Neil et al. (1994). Briefly, this involves reaction of silver phosphate with graphite at 1450 °C to produce CO_2. O isotope ratios of phosphate were calibrated against values obtained by fluorination using silver phosphate standards and the methods of Vennemann et al. (2002). All oxygen isotope data are reported relative to V-SMOW. Oxygen isotope analyses of phosphates were performed at the Earth System Center for Stable Isotope Studies (ESCSIS) of the Yale Institute for Biospheric Studies. Water samples were analyzed at ESCSIS and at the Stable Isotope Facility of the University of California, Davis. Phosphate derived from UVR experiments was precipitated as silver phosphate and analyzed by TC/EA as described in Vennemann et al. (2002). The precision of these data based on replicate analysis of standards is ±0.2–0.3‰.

3. Results

$\delta^{18}O$ values of water ($\delta^{18}O_W$) and P_i from experiments on phosphomonoester degradation by enzyme-catalyzed hydrolysis and photooxidation are presented in Tables 1–3 and plotted in Figs. 1–5. $\delta^{18}O$ values of water in reaction media were measured before the addition of enzymes and at the end of reactions. Water O isotope compositions did not change significantly over the course of experiments.

3.1. Eukaryotic alkaline phosphatase

Results of experiments on P_i regeneration using a mammalian APase derived from calf intestine (calf APase, EC.3.1.3.1) and α-D-glucose-1-phosphate, β-glycerophosphate and 5′-AMP as substrates, are presented in Table 1. Inorganic phosphate was released from all three substrates in 95–100% yield. $\delta^{18}O_P$ values range from −5‰ to 35‰ and correlate positively with water $\delta^{18}O$ values. Although oxygen isotope ratios of P_i released by calf APase are different for different substrates (due to inheritance effects discussed later), the slopes of plots of $\delta^{18}O$ of released P_i versus $\delta^{18}O$ of water are the same within error. Linear relations were observed for every set of experiments and the slopes derived from regression of all data sets ranged from 0.24 to 0.28 (Fig. 2).

Two control experiments were carried out to test for PO_4–water exchange catalyzed by possible impurities in the commercial APase preparations. In the first experiment, APase was added to a solution of glycerol and inorganic phosphate (NaH_2PO_4) with an initial $\delta^{18}O_P$ value of +14.1‰. In the second control experiment, only NaH_2PO_4 was incubated with APase. After an incubation period of 1 week, the oxygen isotope composition of P_i was identical, within error, to the initial $\delta^{18}O_P$ value in both control experiments (Table 1).

$\delta^{18}O$ values of released P_i catalyzed by calf APase decrease very slightly with increased temperature (Table 2). This small temperature effect is an order of magnitude less sensitive to temperature than fractionations between biogenic phosphates and water determined by Longinelli and Nuti (1973) and dissolved P_i and water determined by Blake et al. (1998b, 2005); Lecuyer et al. (1999), and O'Neil et al. (2003), and thus is considered to be insignificant.

Arthropod APase, another eukaryotic APase derived from shrimp (shrimp Apase, EC.3.1.3.1), was also tested. The $\delta^{18}O$ values of P_i released from hydrolysis of β-glycerophosphate catalyzed by shrimp APase in −6.1‰ water lie on the linear regression line of the $\delta^{18}O_P$ *vs.* $\delta^{18}O_W$ values from calf APase experiments (Fig. 3). This result indicates that P_i regeneration catalyzed by shrimp APase has a similar isotope effect as the reaction catalyzed by calf APase.

3.2. Prokaryotic alkaline phosphatase

The O isotope effects of P_i regeneration by APase derived from a prokaryotic (bacterial) source were compared

Table 1
Phosphomonoesterase-catalyzed phosphomonoester hydrolysis in ^{18}O-labeled waters

Enzyme	Substrates	$\delta^{18}O_W$ (‰)	$\delta^{18}O_P$ (‰)	Enzyme/P ratio
Calf APase	α-D-Glucose-phosphate	−18.83	4.0	0.02 U/7.6 μmol
		44.08	20.7	
		99.84	34.6	
	β-Glycerophosphate	−18.83	−4.7	0.02 U/7.6 μmol
		44.08	11.3	
		99.84	24.4	
	Adenosine 5′-monophosphate	−6.09	−1.9	0.4 U/7.6 μmol
		46.51	12.9	
		98.71	28.1	
	NaH_2PO_4[a]	−6.5	14.0	0.02 U/7.6 μmol
	NaH_2PO_4 and glycerol[a]	−6.5	13.9	0.02 U/7.6 μmol
E. coli APase	α-D-Glucose-1-phosphate	−6.09	9.7	
		19.80	15.7	
		45.57	21.8	
		71.09	27.3	
		97.75	33.5	
	β-Glycerophosphate	−6.08	0.4	
		19.76	6.2	
		44.99	12.9	
		70.37	18.6	
		96.01	25.3	
Shrimp APase	β-Glycerophosphate	−6.5	−1.0	
5′-Nucleotidase	Adenosine 5′-monophosphate	−5.88	4.7	
		20.54	11.5	
		46.66	18.2	
		71.96	24.9	
		98.65	31.5	

[a] Control experiments. The $\delta^{18}O$ value of NaH_2PO_4 is 14.1‰.

Table 2
Calf APase catalyzed β-glycerophosphate hydrolysis at different temperatures

Temperature (°C)	$\delta^{18}O_P$ (‰)	Average $\delta^{18}O_P$ (‰)	δ
37	1.0	1.1	0.1
	1.1		
	1.2		
21	1.4	1.5	0.2
	1.3		
	1.7		
15	1.8	1.6	0.1
	1.6		
	1.5		
	1.5		

with P_i regeneration by eukaryotic APase (calf and shrimp). The $\delta^{18}O$ values of P_i released from α-D-glucose-1-phosphate and β-glycerophosphate by *E. coli* APase are presented in Table 1 and plotted in Fig. 4.

As with eukaryotic APase, the $\delta^{18}O$ values of P_i released from phosphomonoester substrates by *E. coli* APase correlate positively with the $\delta^{18}O$ value of ambient water, indicating that water oxygen atoms are incorporated into released P_i (Fig. 4). The slopes of the linear regression trends on plots of $\delta^{18}O_P$ *vs.* $\delta^{18}O_W$ for *E. coli*. APase are 0.23 and 0.24, also similar to the results for calf APase.

3.3. 5′-Nucleotidase

5′-Nucleotidase (E.C. 3.1.3.5) was used to catalyze hydrolysis of α-D-glucose-1-phosphate, β-glycerophosphate and 5′-AMP. P_i was not released in experiments with α-D-glucose-1-phosphate and β-glycerophosphate. $\delta^{18}O$ values of water and P_i released in 5′-nucleotidase-catalyzed hydrolysis of 5′-AMP are presented in Table 1. Once again, there is a strong linear relation between the oxygen isotope composition of water and released P_i indicating that oxygen from water was incorporated into released P_i (Fig. 5). Similar to the APase experiments, the slope of the linear trend of $\delta^{18}O_P$ *vs.* $\delta^{18}O_W$ plots is 0.25.

3.4. Determination of $\delta^{18}O$ of PO_4 in phosphomonoesters by UV radiation

Experiments were designed to test if water was incorporated into P_i released from P_{org} by UV radiation (UVR) and if H_2O_2 affects the $\delta^{18}O$ values of released P_i. α-D-glucose-1-phosphate, β-glycerophosphate and 5′-AMP were exposed to UVR (500 W mercury lamp) in ^{18}O-labeled water with and without H_2O_2. The reaction time required for more than 90% release of P_i from phosphomonoester substrates depended on whether H_2O_2 was added to the reaction solutions and varied from 3 h with H_2O_2, to several weeks without H_2O_2.

Table 3
Phosphate regenerated from phosphomonoesters by UV radiation

Substrates	Natural sunlight	Intensive UV light		
	P_i (μM)	$\delta^{18}O_W$ (‰)	$\delta^{18}O_P$ (‰)	$\delta^{18}O_P$ average
Without H_2O_2				
β-Glycerophosphate	1	99.8	13.8	13.6 (±0.3)
		99.8	13.4	
		−5.5	10.0	10.8 (±0.7)
		−5.5	11.5	
		−5.5	10.8	
With H_2O_2				
β-Glycerophosphate		99.8	12.5	11.8 (±0.9)
		99.8	11.1	
		−5.5	10.6	10.7 (±0.1)
		−5.5	10.8	
α-D-Glucose1-phosphate	2	99.8	21.9	21.1 (±0.6)
		99.8	21.4	
		99.8	20.8	
		99.8	20.4	
		−5.5	18.6	19.4 (±0.5)
		−5.5	20.0	
		−5.5	19.7	
		−5.5	19.3	
		−5.5	19.5	
5′-AMP	1	−5.5	11.5	11.7 (±0.2)
		−5.5	11.8	

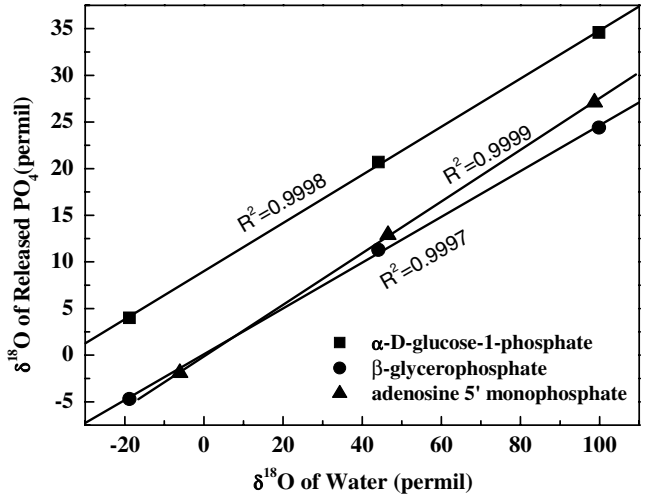

Fig. 2. PO_4–H_2O exchange catalyzed by cell-free alkaline phosphatase from calf intestine (calf APase) at 37 °C. The slopes of linear regressions of α-D-glucose-1-phosphate, β-glycerophosphate and adenosine 5′-monophosphate data are 0.258 (±0.0004), 0.245 (±0.006) and 0.276 (±0.003), respectively.

Comparison of $\delta^{18}O$ values of P_i released from β-glycerophosphate with and without H_2O_2 in the same labeled water shows that the presence of H_2O_2 does not affect $\delta^{18}O$ values of released P_i in −5‰ water, within error (Table 3). In the presence of H_2O_2, there is a maximum 1.8‰ difference between the $\delta^{18}O$ of P_i released in +99‰ and −5‰ water (Table 3). This maximum 2‰

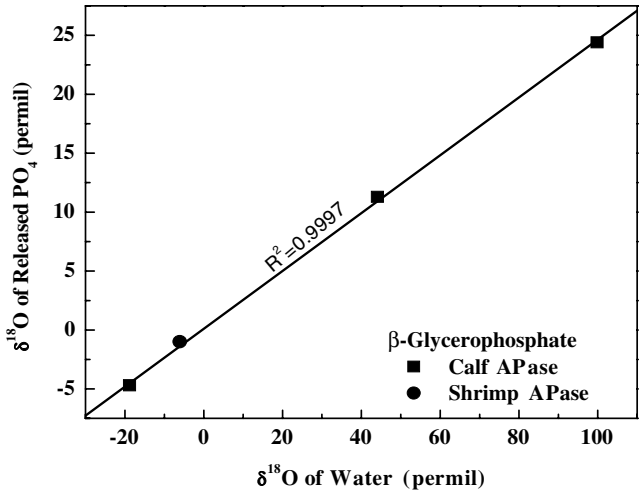

Fig. 3. PO_4–H_2O exchange catalyzed by cell-free alkaline phosphatase from eukaryotic sources (calf and shrimp) at 37 °C. The slope of the linear regression for β-glycerophosphate data is 0.243 (±0.005).

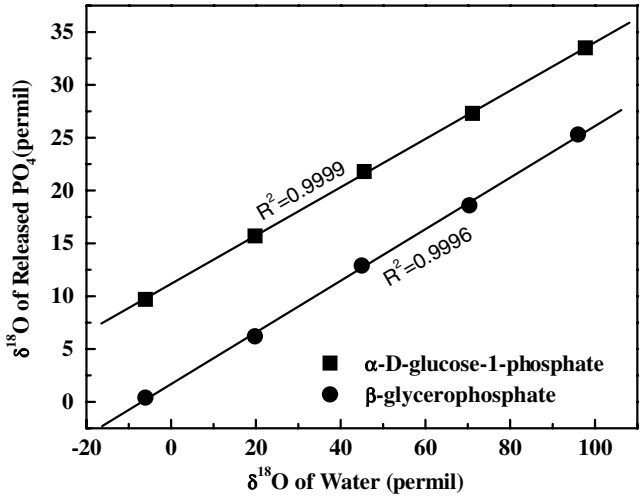

Fig. 4. PO_4–H_2O exchange catalyzed by cell-free alkaline phosphatase from a prokaryotic source (*Escherichia coli*) at 37 °C. The slopes of linear regressions of α-D-glucose-1-phosphate and β-glycerophosphate data are 0.228 (±0.002) and 0.244 (±0.004), respectively.

$\delta^{18}O$ difference is negligible compared with the >100‰ difference in $\delta^{18}O$ values of the water, indicating that all four oxygen atoms in P_i released by UVR digestion were inherited from the original phosphomonoester substrate, without fractionation. Based on UVR digestion experiments, the $\delta^{18}O$ values of the PO_4 moiety groups in α-D-glucose-1-phosphate, β-glycerophosphate and 5′-AMP are +20 (±1)‰, +11 (±1)‰ and +12 (±1)‰, respectively (Table 3).

P_i (0.1–0.3 μmol) was released from each phosphomonoester experiment during 24 h exposure to natural summer sunlight, which indicates that natural sunlight can break down 0.5–1% of total phosphomonoesters per day (Table 3).

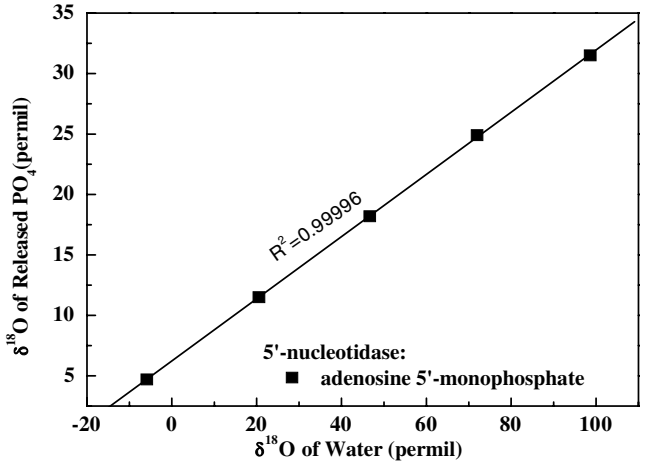

Fig. 5. PO_4–H_2O exchange catalyzed by cell-free 5′-nucleotidase (*Crotalus Atrox* venom) at 37 °C. The slope of the linear regression of adenosine 5′-monophosphate data is 0.257 (±0.002).

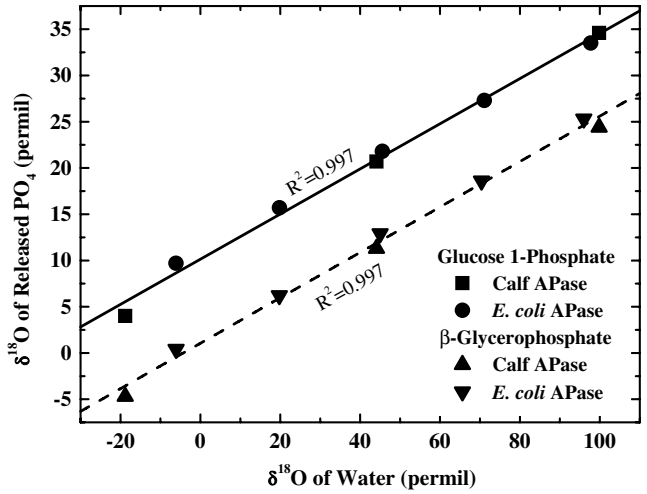

Fig. 6. α-D-Glucose-1-phosphate and β-glycerophosphate hydrolysis catalyzed by different cell-free alkaline phosphatases at 37 °C. The slope of the linear regression of is 0.244 (±0.007) for glucose-1-phosphate data and 0.245 (±0.008) for β-glycerophosphate data.

4. Discussion

4.1. APase

Alkaline phosphatase is produced by a wide range of aquatic organisms and its sources in aqueous environments may vary from bacteria, picoplankton and algae, to animals (McComb et al., 1979; Flynn et al., 1986). Amino acid sequence determination of *E. coli*, yeast and mammalian APase has shown that 25–30% of the entire APase sequence is conserved and the active site of APase, where cleavage of P—O bonds in phosphomonoesters occurs, is completely conserved with only three exceptions (Kim and Wyckoff, 1991; Murphy et al., 1995). The conservation of active site among these APases from different sources suggests that the major reaction mechanisms of APases are the same. Since these three active site differences change the position of the PO_4 group relative to the metal co-factor and affect the initial protonation of the serine hydroxyl residue on the enzyme, which is responsible for nucleophilic attack on phosphorus, this could potentially lead to changes in the rate-determining step and pH-optima of mammalian and *E. coli* APase (Murphy et al., 1995). Therefore, although the major functions and reaction mechanisms of APase from different sources are the same, there are some important differences such as activity optima, heat stability, pH preference and the requirement of different metal co-factors to achieve maximum activity. To interpret the oxygen isotope signature of P_i derived from enzyme-catalyzed P metabolism/cycling in natural waters, it is important to determine the oxygen isotope effects of phosphoenzymes derived from different sources in intact cells as well as cell-free purified enzyme systems.

Alkaline phosphatases studied here, extracted from different organisms (eukaryotic, prokaryotic), produced similar oxygen isotope patterns between released P_i and water, suggesting that the hydrolytic reaction mechanism of each enzyme is the same (Fig. 6). Thus, our isotopic results support previous studies on structures of APase described above which indicate that the catalytic mechanism of different APases should be the same (Kim and Wyckoff, 1989; Coleman, 1992; Murphy et al., 1995).

Under the same reaction conditions (pH, T, cofactor, enzyme/P ratio), higher P_i release rates are related to higher APase activity. Even though APase is a non-specific phosphomonoesterase and can release P_i from a variety of different phosphomonoester substrates, APase enzyme activity is different for each individual phosphomonoester. With the same enzyme/P ratio, P_i release from 5′-AMP as the substrate was much slower than with α-D-glucose-1-phosphate and β-glycerophosphate as substrates. For example, after 7 days of reaction, the yield of P_i in α-D-glucose-1-phosphate and β-glycerophosphate experiments reached 90% and 92%, respectively, whereas the yield of P_i in 5′-AMP experiments barely approached 50%, even after the enzyme/P ratio was later increased by a factor of three. In addition, activities of calf APase in 5′-AMP hydrolysis experiments were much lower than in experiments with other substrates. These observations suggest that calf APase activity is inhibited by the presence of 5′-AMP or adenosine. In order to obtain enough P_i for isotopic analysis in 5′-AMP hydrolysis experiments, a much higher enzyme/P ratio had to be employed (Table 1).

4.2. 5′-Nucleotidase

APase is a relatively non-specific enzyme compared with 5′-nucleotidase. In 5′-nucleotidase experiments, P_i was not released from α-D-glucose-1-phosphate and β-glycerophosphate, indicating that 5′-nucleotidase could not catalyze hydrolysis of these phosphomonoester substrates and is specific for its nucleotide substrates. This finding is consistent with the known active site configuration of 5′-nucleo-

tidase which recognizes and interacts with the nucleoside base portions (e.g., adenosine) of nucleotide molecules in addition to PO_4 groups (Knofel and Strater, 2001). By contrast, the APase active site interacts with the PO_4 group of phosphomonoesters as well as inorganic phosphorus compounds such as pyrophosphate.

Hydrolysis of P_{org} catalyzed by three different APases and 5′-nucleotidase acting on four different substrates resulted in similar isotope systematics of P_i regeneration. The slopes of linear trends on plots of $\delta^{18}O_P$ *vs.* $\delta^{18}O_W$ for all experiments with phosphomonoesterases were close to 0.25 which indicates that one of four oxygen atoms in P_i released from phosphomonoesters by APase and 5′-nucleotidase is derived from ambient water. These results also indicate that P_i released from phosphomonoesters contains 75%, or three O atoms, that are inherited from the original phosphomonoester substrate. The contribution of this inherited oxygen component to the $\delta^{18}O$ of P_i released *via* enzymatic hydrolysis of phosphomonoesters is responsible for the offset of linear trends and different *y*-intercepts observed for the different substrates (Figs. 2, 4 and 6).

The similar patterns of water incorporation during phosphomonoester hydrolysis by eukaryotic APase, prokaryotic APase and 5′-nucleotidase suggests that the isotope effects and PO_4–H_2O O isotope fractionations imposed by this class of enzymes (i.e., phosphomonoesterases) are the same. Further detailed investigations of APase and 5′-nucleotidase isotope effects show that this is not the case. Comparison of experiments in which 5′-AMP was used as a substrate for both 5′-nucleotidase and calf APase show that P_i released from 5′-AMP by 5′-nucleotidase is consistently ~5‰ heavier than P_i released from 5′-AMP by APase (Fig. 7). This indicates that the water oxygen atom incorporated into P_i during hydrolysis may have been fractionated relative to ambient water versus incorporated directly. Furthermore, this result suggests that the incorporated water oxygen atom was fractionated differently by the two different enzymes. Recall that all three APases (mammalian, arthropod, bacterial) gave similar released P_i $\delta^{18}O$ values for the same phosphomonoester substrate (Table 1, Fig. 2). From the standpoint of interpretation of P cycling pathways in natural systems, a long range goal of these studies, this result suggests that APase P_i-regeneration pathways may be distinguishable from 5′-nucleotidase P_i-regeneration pathways, and thus, may provide new insights into the details of P-cycling processes.

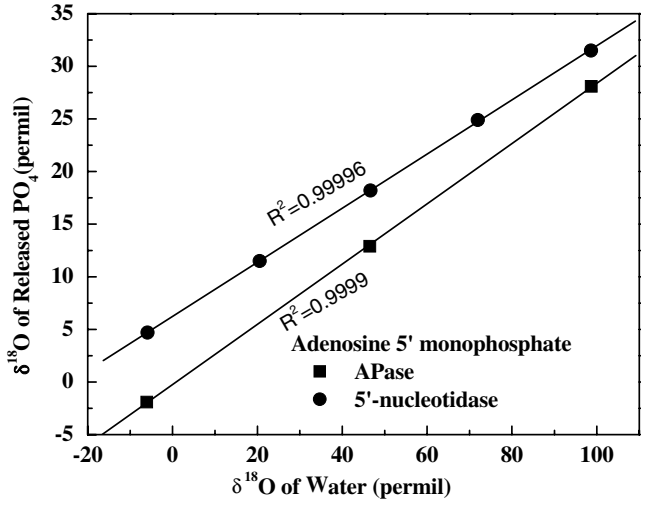

Fig. 7. Cell-free 5′-nucleotidase- and calf APase-catalyzed adenosine 5′-monophosphate hydrolysis at 37 °C. The slopes of linear regressions of APase and 5′-nucleotidase data are 0.276 (±0.003), and 0.257 (±0.002), respectively.

4.3. Enzyme-specific isotopic fractionations

In order to determine the fractionation accompanying the incorporation of ambient (bulk) water oxygen into 1–4 oxygen sites in PO_4 released during hydrolysis of phosphomonoesters, it is necessary to know the $\delta^{18}O$ value of the organically bound PO_4 moiety in the phosphomonoester substrate before hydrolysis (P_{org}-$\delta^{18}O_P$). A conventional mass-balance approach cannot be used to determine this fractionation because it requires oxygen isotope exchange between the oxygen reservoirs (i.e., water and PO_4) and knowledge of the $\delta^{18}O$ values of these reservoirs before and after exchange takes place. Hydrolysis of phosphomonoesters by APase and 5′-nucleotidase is a nucleophilic substitution (S_N2) reaction with ambient water acting as the nucleophile that attacks the electrophilic phosphorus atom in phosphomonoesters. This is a unidirectional reaction that is not accompanied by oxygen isotope exchange. During hydrolysis, the P—O bond in the P—O—C ester is cleaved and an alcohol group HO—C is formed. The reaction products are free PO_4 that contains one oxygen atom from ambient water and a moiety containing an alcohol group. That is, one oxygen atom in the PO_4 of P_{org} is replaced by one oxygen atom from the water and the remaining original oxygen atom becomes part of the organic product. Phosphate oxygen in the ester becomes incorporated in the HO—C product and there is no oxygen isotope *exchange* between P_{org} and water. Thus, the conventional mass-balance approach which requires exchange between the PO_4 and water oxygen reservoirs (as well as foreknowledge of the fractionation factor associated with this exchange) cannot be used to determine the fractionation accompanying incorporation of ambient water oxygen into P_i during enzymatic hydrolysis.

If the incorporation of water oxygen during P_{org} hydrolysis is direct (i.e., with no fractionation) then a mass-balance approach may be used to determine the oxygen isotope composition of PO_4 in un-hydrolyzed P_{org} (P_{org}-$\delta^{18}O_P$). The calculations are made on the basis of measured $\delta^{18}O$ of P_i released by hydrolysis of P_{org} in different waters and the $\delta^{18}O$ of water before and after hydrolysis. As pointed out above, however, the comparison of results from APase and 5′-nucleotidase hydrolysis of the same substrate and the expected kinetic control of phosphomonoesterase reactions indicate that there *is* a

fractionation accompanying incorporation of oxygen from water into P_i during P_{org} hydrolysis by APase and 5′-nucleotidase.

An additional factor precluding a mass-balance approach to determination of P_{org}-$\delta^{18}O_P$ values and fractionations during phosphomonoesterase hydrolysis is that in the chosen reaction system, the number of oxygen atoms in the water is overwhelmingly greater than the number of oxygen atoms in the PO_4 such that the change in water $\delta^{18}O$ during hydrolysis is negligible compared to measurement error.

We chose to test the suitability of UV digestion of P_{org} to determine the original P_{org}-$\delta^{18}O_P$ values of phosphomonoester substrates. Digestion of P_{org} compounds by UVR has been shown to convert organic C in P_{org} to CO_2 quantitatively (Armstrong et al., 1966). Such specific attack on C may leave O atoms bound to P in PO_4 moieties unaltered. This hypothesis was tested by exposing phosphomonoesters to UVR in different ^{18}O-labeled waters and determining the oxygen isotope effect on released free P_i. Results of these experiments demonstrated that P_i released from phosphomonoesters by UVR did not incorporate water O and thus retained original P_{org}-$\delta^{18}O_P$ values (Table 3).

The original P_{org}-$\delta^{18}O_P$ values determined from UVR experiments can be compared with $\delta^{18}O$ of P_i released by hydrolysis of phosphomonoesters by APase and 5′-nucleotidatse, to determine the isotopic fractionation factors (F) associated with enzyme-specific hydrolysis. P_i released from phosphomonoesters (i.e., P_{org}) by APase and 5′-nucleotidase has three O atoms derived from the PO_4 moiety group of original P_{org} and one oxygen atom is derived from ambient water (Fig. 1), such that:

$$\delta^{18}O(P_i) = \tfrac{1}{4}\delta^{18}O(IW) + \tfrac{3}{4}\delta^{18}O(P_{org}) \quad (1)$$

where, $\delta^{18}O(P_i)$ is the oxygen isotope composition of P_i released from P_{org}, $\delta^{18}O(IW)$ is the isotopic composition of the water O atom incorporated into released P_i, and $\delta^{18}O(P_{org})$ is the $\delta^{18}O$ value of the PO_4 moiety group of original unhydrolyzed P_{org}.

With knowledge of both the $\delta^{18}O$ value of released P_i and the $\delta^{18}O$ value of the PO_4 moiety group in P_{org} (from UVR experiments), the $\delta^{18}O$ value of incorporated water O, $\delta^{18}O(IW)$, can be calculated from:

$$\delta^{18}O(IW) = 4 \times [\delta^{18}O(P_i) - 0.75 \times \delta^{18}O(P_{org})] \quad (2)$$

The isotopic fractionation between incorporated water O and ambient water O, $\alpha_{(Incorporated\text{-}bulk\ water)}$ or F, is described by

$$F = \delta^{18}O(IW) - \delta^{18}O_W \quad (3)$$

where, $\delta^{18}O_W$ is the $\delta^{18}O$ value of bulk ambient water. Combining Eq. (2) with Eq. (3) will give:

$$F = 4 \times [\delta^{18}O(P_i) - 0.75 \times \delta^{18}O(P_{org})] - \delta^{18}O_W \quad (4)$$

Using Eq. (4), fractionation factors for each type of phosphomonoesterase have been calculated and are presented in Table 4.

Table 4
O isotope fractionation between water incorporated into released P_i and ambient water

Enzyme	Substrates	$\delta^{18}O_W$ (‰)	$\delta^{18}O_P$ (‰)	$\delta^{18}O$ (P_{org})	F (‰)	Average of F (‰)	Error (‰)
Calf APase	α-D-Glucose-1-phosphate	−18.83	4.0	20	−25	−23	2
		44.08	20.7	20	−21		
		99.84	34.6	20	−21		
	β-Glycerophosphate	−18.83	−4.7	11	−33	−33	2
		44.08	11.3	11	−32		
		99.84	24.4	11	−35		
	Adenosine 5′-monophosphate	−6.09	−1.9	12	−38	−30	8
		46.51	12.9	12	−31		
		98.71	28.1	12	−22		
E. coli APase	α-D-Glucose-1-phosphate	−6.09	9.7	20	−15	−19	2
		19.80	15.7	20	−17		
		45.57	21.8	20	−18		
		71.09	27.3	20	−22		
		97.75	33.5	20	−24		
	β-Glycerophosphate	−6.08	0.4	11	−25	−27	1
		19.76	6.2	11	−28		
		44.99	12.9	11	−26		
		70.37	18.6	11	−29		
		96.01	25.3	11	−28		
Shrimp APase	β-Glycerophosphate	−6.5	−1.0	11	−31		
5′-Nucleotidase	Adenosine 5′-monophosphate	−5.88	4.7	12	−11	−10	1
		20.54	11.5	12	−11		
		46.66	18.2	12	−10		
		71.96	24.9	12	−8		
		98.65	31.5	12	−9		

On the basis of UVR determinations of P_{org}-$\delta^{18}O_P$, the calculated fractionation factors show that ^{16}O is preferentially incorporated into released PO_4 during APase catalyzed hydrolysis reactions with a fractionation of −30 (±8)‰. This kinetic fractionation is consistent with the APase-catalyzed reaction being controlled by diffusion (Simopoulus and Jencks, 1994). Data presented in Table 4 also show that during 5′-nucleotidase catalyzed 5′-AMP hydrolysis, oxygen from water is incorporated into released P_i with a fractionation of −10 (±1)‰. As noted above, when the same P_{org} substrate is hydrolyzed in the same labeled water by different APases (e.g., calf *vs. E. coli*), the same $\delta^{18}O$ value is obtained for released P_i, due to the similarity in reaction mechanism and isotopic fractionation. Conversely, $\delta^{18}O$ values of P_i released from 5′-AMP by 5′ -nucleotidase were consistently ∼5‰ heavier than P_i released from 5′-AMP by calf APase under the same conditions (Fig. 7). The calculated fractionation factors for APase and 5′-nucleotidase are consistent with this observation.

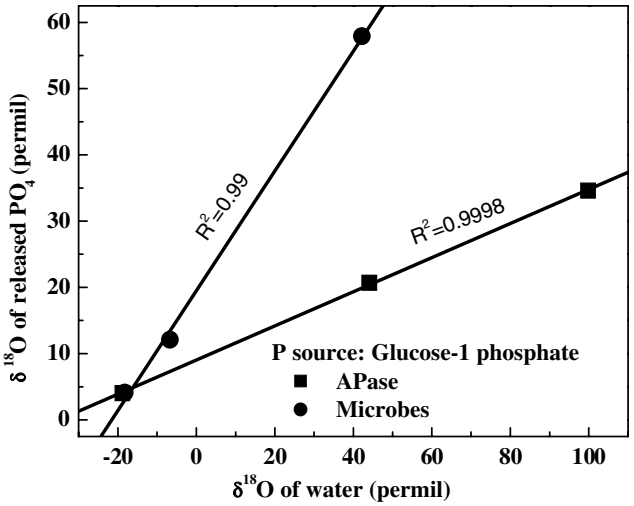

Fig. 8. Oxygen isotope effects of cell-free APase-catalyzed P_i regeneration *vs.* microbial turnover of P (Blake et al., 2005). Glucose-1-phosphate is used as the only P source in both experiments. The slope of the APase data set is 0.257 and that of the microbial experiment data set is 0.90.

4.4. Isotope effects of different P cycling pathways

The water incorporation patterns of APase and 5′-nucleotidase catalyzed 5′-AMP hydrolysis reactions are the same with a slope of 0.25 (±0.02), however, as the above example demonstrates, the isotopic fractionations imposed by these two enzymes are different. In both cases, one water oxygen atom is incorporated into released P_i, but the water oxygen incorporated during 5′-nucleotidase catalyzed hydrolysis is isotopically heavier than that in the APase catalyzed reaction, leading to the consistent overall ∼+5‰ offset of P_i in the 5′-nucleotidase experiments. Previous studies show that APase is induced by conditions of P_i starvation and P_i-limitation whereas 5′-nucleotidase is present at non-limiting high P_i concentrations. APase and 5′-nucleotidase are associated with different P_i status conditions, and as demonstrated herein, should have distinguishably different isotope effects. Thus, absolute and relative $\delta^{18}O$ values of dissolved P_i measured in natural waters may be a useful indicator of P_i-status, and correlate with the availability of P_i and related nutrients in aquatic systems. For example, for large bodies of water with constant $\delta^{18}O$ values such as the open ocean or large lakes, changing P_i degradation pathways could be indicated by shifts in $P_i\delta^{18}O$ values, with lower values indicating the APase degradation pathway which also indicates P_i-limiting conditions.

The oxygen isotope imprint left by extracellular phosphomonoesterase-catalyzed P_i regeneration is significantly different from the oxygen isotope signature of other pathways controlling the biogeochemical cycling of P (Blake et al., 2005) most importantly, microbial uptake, and cellular turnover of P_i and the reaction catalyzed by cytoplasmic inorganic pyrophosphatase (Fig. 8). Experiments on microbial metabolism and turnover of P_i performed by Blake et al. (1998a, 2005) show that a positive correlation exists between oxygen isotope ratios of DIP and ambient water in microbial growth media with a slope approaching 1. This is significantly different from the slope of 0.25 for $\delta^{18}O_P$ *vs.* $\delta^{18}O_W$ observed for phosphomonoesterase-catalyzed P_i regeneration reported here. It has also been recently shown that cycling of P_i in microbial cultures (Blake et al., 2005) and P_i in natural waters (Colman, 2002; Colman et al., 2005) leads to temperature-dependent equilibrium oxygen isotope exchange between P_i and ambient water. In contrast, phosphomonoesterase-catalyzed P_i regeneration drives the O isotope composition of P_i toward values consistent with incorporation of O from ambient water, which is accompanied by large kinetic fractionations on the order of −10‰ to −30‰, and away from equilibrium values.

In addition to phosphoeznymes, UVR also impacts the bioavailability and biogeochemical cycling of nutrient elements such as C, N and P in shallow water environments and high latitude lakes (Mopper and Kieber, 2002). Photochemical reactions promote dissolved organic compound degradation/mineralization. In the water column, UVR can reach depths of 10 m and its biological effect goes deeper to 20 and 30 m (Karentz and Lutze, 1990). Wetzel et al. (1995) showed that UVR might influence P cycling in aquatic environments by increasing P_i availability through the breakdown of P_{org} compounds. Thus, UVR provides another P_i regeneration pathway in aquatic systems. Based on the results of this study, P_i released by UVR should retain the original O isotope composition of phosphomonoester sources. Without water O incorporation, the phosphate O isotope signature of P_i derived from phosphomonoester degradation *via* UVR should differ significantly from enzyme-catalyzed degradation or biological turnover of P_i. The $\delta^{18}O_P$ of all possible original (unhydrolyzed) P_{org} sources (e.g., marine phytoplankton, phosphonates, terrestrial organic matter, municipal waste, fertilizers) would be required for absolute vs. relative interpretations.

4.5. $\delta^{18}O_P$ values of enzyme-regenerated P_i in natural systems

The optimal conditions applied in laboratory studies often differ from those of natural aquatic systems, especially pH and temperature. Temperature did not significantly affect the APase reactions studied here. The optimal pH for different enzymes used in our experiments ranged from 8.8 to 10.4 for APases and was 9 for 5′-nucleotidase. From experiments at pH close to that of sea water (calf APase, pH 8.8) to experiments at pH 10.4, the phosphate-water O isotope effects were similar: a positive correlation existed between $\delta^{18}O_P$ *vs.* $\delta^{18}O_w$ and the slopes of these correlations were 0.25 (±0.02). These observations suggest that even though the differences in reaction conditions between artificial and natural systems may affect enzyme activity, they should not alter the enzymatic reaction mechanism in the pH 8–10 range which includes seawater. Therefore, our laboratory results should be relevant to natural marine environments where it has also been found that phosphomonoesters are the dominant component of the dissolved organophosphorus compound pool (Clark et al., 1999; Benitez-Nelson, 2000).

Here we present an example calculation for a system with typical marine characteristics. In the marine environment, water $\delta^{18}O$ values are constant and uniform at around 0‰. For open ocean regions far from terrestrial P_{org} input, we assume that P_{org}-$\delta^{18}O_P$ value s will be constant due to production of P_{org} by *in situ* biomass under constant water $\delta^{18}O$ conditions. Very few values of natural marine P_{org}-$\delta^{18}O_P$ have been reported (Longinelli et al., 1976). Although it has been demonstrated that absolute values of these previously reported data were likely affected by analytical artifacts (Blake et al., 1997; Colman et al., 2005; Liang, 2005), the range of values is small (1‰ for Atlantic ocean samples; Longinelli et al., 1976). If it is assumed that water temperature = 20 °C, $\delta^{18}O_w = 0.0$‰, and the original phosphomonoester substrate P_{org}-$\delta^{18}O_P = 20.0$‰, then applying Eq. (1) and using the fractionations determined above for the APase and 5′-nucleotidase mechanisms, we calculate that the $\delta^{18}O$ value of enzyme-regenerated P_i from this phosphomonoester will be 12.5‰ for the APase pathway and 7.5‰ for the 5′-nucleotidase pathway. In both cases, the regenerated $P_i\delta^{18}O$ values reflect 75% inherited oxygen from the original phosphomonoester substrate (i.e., +20‰). If biological turnover of P_i is significant, then P_i will be further shifted toward the equilibrium $\delta^{18}O_P$ value of 21.3‰ at 20 °C. Biological turnover would not produce fractionations similar to either enzyme-catalyzed P_i-regeneration pathway, because an equilibrium P_i-water fractionation of 12.5‰ or 7.5‰ would indicate unreasonably high seawater temperatures of 58 to 79 °C. Photooxidation of P_{org}, although not expected to play an important role in P-cycling in the open ocean, could also be distinguished in this system as P_i released from phosphomonoesters by UVR would retain the $\delta^{18}O_P$ value of the substrate, 20.0‰.

Thus, for a phosphomonoester source with a P_{org}-$\delta^{18}O_P$ value of 20.0‰, the expected O isotopic fractionation between regenerated P_i and water is 7.5‰ for the APase pathway and 12.5‰ for the 5′-nucleotidase pathway. These fractionations are significantly different from the equilibrium value expected for biologically cycled P_i, 21.3‰. New data on open ocean DIP $\delta^{18}O_P$ values shows that there is a shift from equilibrium $\delta^{18}O_P$ values toward lower $\delta^{18}O_P$ values of DIP in the zone of P_i regeneration in both the Pacific and Atlantic oceans (Colman et al., 2005). This shift toward lower than equilibrium $\delta^{18}O_P$ values is consistent with the above scenario of incorporation of O from ambient water during enzymatic hydrolysis of sinking P_{org} derived from warmer surface waters. The magnitude of the shift, however, is only 1–2‰, indicating biological turnover and equilibration of P_i subsequent to release from P_{org}, relatively heavy P_{org}-$\delta^{18}O_P$ values for un-hydrolyzed source P_{org}, or that natural phosphoesterase enzymes in these systems produce similar, but smaller oxygen isotope effects on P_i regenerated from P_{org}.

The APase and 5′-nucleotidase fractionations, which are not temperature sensitive, will be constant under conditions of constant water $\delta^{18}O_P$ and P_{org}-$\delta^{18}O_P$. For systems with P_{org}-sources having variable P_{org}-$\delta^{18}O_P$ or variable water $\delta^{18}O$ values, such as estuaries, coastal or terrestrial environments, the enzyme-specific fractionations would reflect these variations. The experimental studies presented here are but the first of many required to fully characterize the oxygen isotope systematics of biogeochemical cycling of P in natural systems.

Because 75% of the $P_i\delta^{18}O_P$ signature is derived from the P_{org} source, resolution of these questions and a clear interpretation of DIP $\delta^{18}O_P$ values depend upon determination of P_{org}-$\delta^{18}O_P$ values of un-hydrolyzed P_{org} sources. Only two published studies report attempts to obtain P_{org}-$\delta^{18}O_P$ values (Longinelli et al., 1976; Paytan et al., 2002). However, strong acid hydrolysis was used to extract P_i from P_{org} in these studies and P_{org} was not separated from intracellular P_i prior to hydrolysis (Longinelli et al., 1976; Paytan et al., 2002). Despite artifacts produced by these experimental procedures, a relation between oxygen in P_{org} and ambient water was observed, but with no apparent effect of ambient water temperature. Full understanding of the relationship between P_{org}, water, temperature and ambient P_i is needed and experiments are underway to fill this remaining gap in our knowledge of the O isotope systematics of phosphate.

5. Conclusions

Alkaline phosphatase (APase) and 5′-nucleotidase are the two most commonly observed phosphoenzymes in aquatic environments and produce similar oxygen isotope relations between regenerated inorganic phosphate (P_i or PO_4) and ambient water, consistent with one of four oxygen atoms in PO_4 derived from water. The same class of phosphomonoesterase (e.g., APases) derived from different

sources (eukaryotic, prokaryotic) was found to produce the same oxygen isotope effect on regenerated P_i. Different classes of phosphomonoesterase enzymes (i.e., APase *vs.* 5′-nucleotidase) were found to produce widely different oxygen isotope fractionations between bulk water O and O incorporated into one of four oxygen sites in PO_4 regenerated *via* hydrolysis of phosphomonoesters ($-30‰$ *vs.* $-10‰$), leading to clearly distinguishable $\delta^{18}O$ values (on the order of 5‰ differences) of free P_i released as a final product of hydrolysis *via* the APase vs. the 5′-nucleotidase pathway. Furthermore, the isotopic signatures of both enzymatic P_i-regeneration pathways were distinguishable from that of microbial turnover and recycling of P_i.

The consistency in isotopic behavior observed for similar enzyme and compound classes is very promising for the application of $\delta^{18}O$ values of P_i in natural waters to studies of specific P-cycling pathways, however, we have only begun to scratch the surface of this topic and studies of additional classes of phosphatases (e.g., acid phosphatases, thermophilic APase's) as well as C-P lyase and phosphonate substrates are currently in-progress.

Results of laboratory experiments using UV radiation to release organically bound PO_4 suggest that the $\delta^{18}O$ value of inorganic phosphate ($\delta^{18}O_P$) regenerated from organic matter by photooxidation in natural aquatic systems should reflect the isotopic composition of the organic matter source with insignificant contribution from ambient water. These results demonstrate the potential for linking $\delta^{18}O$ values of P_i with specific enzymatic and chemical reactions and support the expanded application and development of phosphate oxygen isotope ratios as a tracer of biogeochemical cycling of P.

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