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Activation and inhibition of rubber transferases by metal cofactors and pyrophosphate substrates

Deborah J. Scott[a], Bernardo M.T. da Costa[b], Stephanie C. Espy[b], Jay D. Keasling[b], Katrina Cornish[a,*]

[a] *USDA-ARS, Western Regional Research Center, 800 Buchanan Street, Albany, CA 94710, USA*
[b] *Department of Chemical Engineering, University of California, Berkeley, CA 94720, USA*

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Metal cofactors are necessary for the activity of alkylation by prenyl transfer in enzyme-catalyzed reactions. Rubber transferase (RuT, a *cis*-prenyl transferase) associated with purified rubber particles from *Hevea brasiliensis*, *Parthenium argentatum* and *Ficus elastica* can use magnesium and manganese interchangably to achieve maximum velocity. We define the concentration of activator required for maximum velocity as $[A]_{max}$. The $[A]_{max}^{Mg2+}$ in *F. elastica* (100 mM) is 10 times the $[A]_{max}^{Mg2+}$ for either *H. brasiliensis* (10 mM) or *P. argentatum* (8 mM). The $[A]_{max}^{Mn2+}$ in *F. elastica* (11 mM), *H. brasiliensis* (3.8 mM) and *P. argentatum* (6.8 mM) and the $[A]_{max}^{Mg2+}$ in *H. brasiliensis* (10 mM) and *P. argentatum* (8 mM) are similar. The differences in $[A]_{max}^{Mg2+}$ correlate with the actual endogenous Mg^{2+} concentrations in the latex of living plants. Extremely low Mn^{2+} levels in vivo indicate that Mg^{2+} is the RuT cofactor in living *H. brasiliensis* and *F. elastica* trees. Kinetic analyses demonstrate that FPP–Mg^{2+} and FPP–Mn^{2+} are active substrates for rubber molecule initiation, although free FPP and metal cations, Mg^{2+} and Mn^{2+}, can interact independently at the active site with the following relative dissociation constants $K_d^{FPP} < K_d^{FPP-Metal} < K_d^{E-Metal}$. Similarly, IPP–$Mg^{2+}$ and IPP–Mn^{2+} are active substrates for rubber molecule polymerization. Although metal cations can interact independently at the active site with the relative dissociation constant $K_d^{IPP-Metal} < K_d^{E-Metal}$, unlike FPP, IPP alone does not interact independently. All three RuTs have similar characteristics—indeterminate sized products, high K_m^{IPP}, high metal $[A]_{max}$, metal cofactor requirements, and are membrane-bound enzymes.
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Keywords: *Hevea brasiliensis*; *Parthenium argentatum*; *Ficus elastica*; Rubber transferase; Rubber particles; *cis*-Prenyl transferase; Magnesium; Manganese; Zinc; Calcium; FPP; IPP; Activation; Inhibition

1. Introduction

Hevea brasiliensis Müll. Arg., *Parthenium argentatum* Gray and *Ficus elastica* Roxb. are evolutionarily divergent rubber-producing species from three different superorders of dicotyledonous plants. Natural rubber is an enzymatically-produced *cis*-1,4-polyisoprene of various molecular weights, ranging from high molecular weight (>1,000,000) in *H. brasiliensis* and *P. argentatum* to low molecular weight (<50,000) in *F. elastica* (Cornish et al., 1993, 2000; Castillón and Cornish, 1999). Rubber biosynthesis in these species is catalyzed by a membrane-bound *cis*-prenyl transferase, rubber transferase (EC 2.5.1.20), found at the surface of cytoplasmic rubber particles (Archer et al., 1963; D'Auzac et al., 1989; Madhavan et al., 1989; Cornish and Backhaus, 1990; Cornish, 1993; Cornish et al., 1993; Cornish and Siler, 1996). The reaction is an alkylation by prenyl transfer to the initiator allylic pyrophosphate (APP)-electrophile—by the monomer isopentenyl pyrophosphate (IPP)—nucleophile (Walsh, 1979).

A combination of structural and kinetic studies indicate that the substrates for rubber biosynthesis enter the rubber particle at the surface and the product is elongated to the interior of the rubber particle on the far

* Corresponding author. Tel.: +1-510-559-5950; fax: +1-510-559-5663.
E-mail address: kcornish@pw.usda.gov (K. Cornish).

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side of a monolayer biomembrane and that RuT catalyzes the reaction in a hydrophobic column (Cornish et al., 1999; Wood and Cornish, 2000; Cornish, 2001a,b). Extension of the elongating rubber polymer into the hydrophobic rubber interior of the particle is probably essential to the continued polymerization reaction. Without the hydrophobic compartment drawing the polymer from the enzyme the polymer would rapidly block the active site. The aqueous-organic interface provided by the rubber particle monolayer biomembrane has not been reproduced in vitro which may account for the lack of reproducible reports of solubilized RuT activity. The use of purified, enzymatically-active, rubber particles in biochemical investigations of rubber biosynthesis is long established (Archer et al., 1963; D'Auzac et al., 1989; Madhavan et al., 1989; Cornish and Backhaus, 1990; Cornish, 1993; Cornish et al., 1993; Cornish and Siler, 1996). Although an intact system, rather than a purified soluble enzyme or enzyme complex, only one enzyme has been detected on the rubber particle that can use IPP and FPP as substrates (Cornish and Backhaus, 1990). Thus, purified rubber particles present a single enzyme system, positioned on a suitable aqueous-organic interface, valid for biochemical investigation. In addition, the study of biochemical parameters on the native particles is likely to provide kinetic information directly applicable to the in vivo situation, something always questionable in the study of solubilized membrane-bound enzymes.

Other *cis*-prenyl transferases have been studied: the soluble, prokaryotic undecaprenyl pyrophosphate synthase (UPPS) (Baba and Allen, 1978; Muth and Allen, 1984; Shimizu et al., 1998; Apfel et al., 1999; Hemmi et al., 2001; Kharel et al., 2001; Ko et al., 2001) and the membrane-associated, eucaryotic dehydrodolichyl pyrophosphate synthase (DDPPS) (Adair and Cafmeyer, 1987; Matsuoka et al., 1991; Ericsson et al., 1992; Sato et al., 1999; Chang et al., 2001). Unlike RuT, both UPPS and DDPPS have products with defined molecular weights. Soluble *trans*-prenyl transferases also have been studied extensively, including solanesyl pyrophosphate synthase (SPPS) (Ohnuma et al., 1991, 1992) and avian *trans*-prenyl transferase, farnesyl pyrophosphate synthase (FPS), which has been the model for the prenyl alkylation reaction (Reed and Rilling, 1975, 1976; Poulter et al., 1976; Poulter and Rilling, 1976, 1978; King and Rilling, 1977; Laskovics et al., 1979; Saito and Rilling, 1979; Tarshis et al., 1994, 1996; Kellogg and Poulter, 1997). These enzymes also have products with defined molecular weights.

Metals are essential activators for the catalytic activity of prenyl transferases, but no *cis* or *trans* prenyl transferases characterized so far have been true metalloenzymes, i.e. the metal activator in the prenyl transferase is not a structural component of the enzyme. Substrates can bind to the enzyme alone or with the metal (King and Rilling, 1977; Tarshis et al., 1994), but the metal is required for catalysis in all cases. RuT activity also requires divalent cations, Mg^{2+} or Mn^{2+}, for activity (Archer and Audley, 1987; Madhaven et al., 1989; Cornish and Backhaus, 1990; Cornish, 1993; Tanaka et al., 1996), but their precise role has not been elucidated.

In this paper, we describe the effect of Mg^{2+} and Mn^{2+}, and IPP and FPP on the activity of the RuT in *F. elastica, H. brasiliensis* and *P. argentatum*, their roles in substrate–enzyme interactions and catalysis, and relate kinetic parameters to the in vivo concentration of these metals in the latex of *F. elastica* and *H. brasiliensis*.

2. Results

2.1. Rubber particle-bound enzyme activity

Hill plots of IPP incorporation as a function of [IPP] (Fig. 1, gradients near 1) demonstrate that the rubber particles of all three species possess only one enzyme capable of using IPP as a substrate, confirming an earlier demonstration (linear Eadie-Hofstee plot) in *P. argentatum* (Cornish and Backhaus, 1990). Thus, purified, enzymatically-active particles of the three species may be used to study the kinetic behavior of RuT.

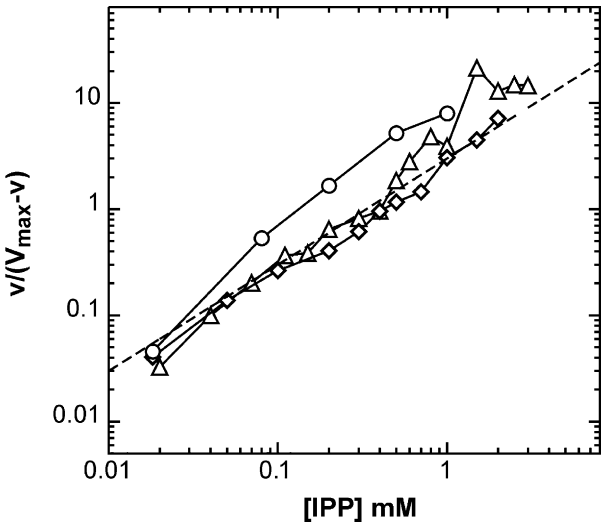

Fig. 1. Hill plot of IPP incorporation rate as a function of IPP concentration by enzymatically active rubber particles of three species. Incorporation of [^{14}C]IPP was determined in the presence of 20 μM FPP, 1 mM $MgSO_4$ for *H. brasiliensis* and *P. argentatum* or 4 mM $MgSO_4$ for *F. elastica*. A standard line is added, generated by the formula $y = 3 * x^1$ and represented by dashed line. [The formulae for best fit are as follow: *H. brasiliensis* $y = 5.03 * x^{1.27}$ ($R = 0.71$ correlation 0.85); *P. argentatum* $y = 2.69 * x^{1.06}$ ($R = 0.94$, correlation 0.99); *F. elastica* $y = 11.15 * x^{1.30}$ ($R = 0.77$, correlation, 0.97).] *Hevea brasiliensis*, Δ; *Parthenium argentatum*, ◇; *Ficus elastica*, ○.

Table 1
Metal concentrations in the latex of *Hevea brasiliensis* and *Ficus elastica* determined by inductively coupled plasma (ICP)

Metal	Wavelengths (nM)	Metal content (Latex)			
		Hevea brasiliensis		*Ficus elastica*	
		mM	S.E.	mM	S.E.
Na	589.592, 588.995	42.00	0.43	3.17	0.14
Mg	279.079, 280.27, 285.213, 279.553	12.04	0.07	53.45	0.32
K	766.491	65.91	0.55	2.81	0.07
Ca	317.933, 396.847, 422.673, 315.887	0.46	0.01	1.29	0.01
		μM	S.E.	μM	S.E.
Mn	257.61, 260.569, 294.92	14.18	1.78	1.63	0.27
Fe	238.204, 239.562	63.21	4.91	32.46	4.44
Cu	259.94, 234.349	25.94	1.69	9.28	1.19
Zn	234.83, 238.863, 273.955	413.82	6.97	153.12	6.89
		mM		mM	
Total		120.93		60.92	

2.2. Metals

2.2.1. Metal content in F. elastica and H. brasiliensis

Metal content was determined for several cations in the latex of *F. elastica* and *H. brasiliensis* (Table 1). We found that the levels of Na (13 times), K (23 times), Mn (9 times), Fe (2 times), Cu and Zn (each 3 times) were all higher in *H. brasiliensis* than *F. elastica*, but that of Mg (4 times) and Ca (3 times) were higher in *F. elastica* than *H. brasiliensis*. The greatest difference was found in both the Na and K concentrations, which contributed to a cation concentration of 121 mM in the latex of *H. brasiliensis*, twice the total metal concentration (61 mM) found in the latex of *F. elastica*.

In vivo cation concentrations were not reported for *P. argentatum* because homogenate concentrations would not reflect the levels found in the cytosol of bark parenchyma cells.

2.2.2. Effects of metals on rubber transferase activity

The effect of Mg^{2+}, Mn^{2+}, Ca^{2+}, and Cu^{2+} on RuT activity was tested on *H. brasiliensis*. Only Mg^{2+} and Mn^{2+} were activating. Cu^{2+} was inhibitory when tested in the presence of Mg^{2+}. Ca^{2+} was neither activating nor inhibitory, even at levels greater than 20 mM. Activating cations, still present in purified rubber particles of *H. brasiliensis*, *P. argentatum* and *F. elastica,* were eliminated with 7 mM EDTA (Fig. 2) and the EDTA concentration dependence was similar for all three species.

The dependence of RuT activity on Mg^{2+} was determined for the three species (Fig. 3). In Fig. 3(a–c), the data are displayed in velocity versus magnesium concentration plots. In all three species, the addition of Mg^{2+} did not follow Michaelis–Menten kinetics and the activity curves were sigmoidal. K_m is not an appropriate parameter for examining the concentration dependence of the metal on enzyme activity since the metal is involved in catalysis and is not consumed. Therefore, we propose the following term, $[A]_{max,}$ the concentration of activator that achieved V_{max}. (The polynomial best fit for the curve v versus v/s was used to determine V_{max} (y intercept).) $[A]_{max}^{Mg2+}$ was much higher in *F. elastica* (100 mM), than in *H. brasiliensis* (10 mM) and *P. argentatum* (8 mM) (Table 2). Above these

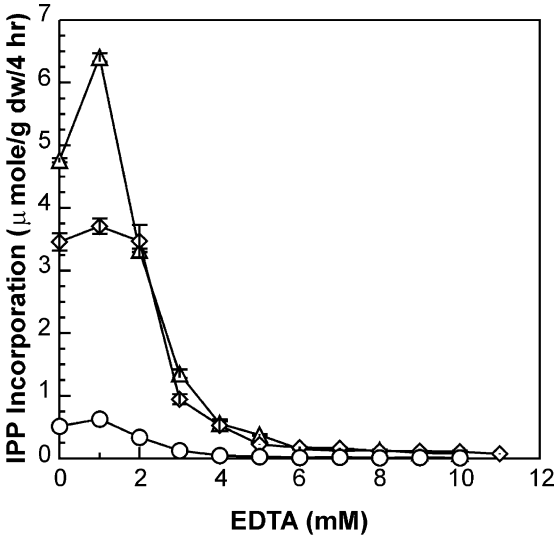

Fig. 2. Reduction of rubber transferase activity by EDTA in rubber particles purified from three species. Incorporation of [^{14}C]IPP was determined in the presence of 20 μM FPP, 1.25 mM $MgSO_4$ and 1 mM IPP for *H. brasiliensis* and *P. argentatum* and 200 μM IPP for *F. elastica*. Data for *H. brasiliensis* and *P. argentatum* are the means of 3±S.E. whereas *Ficus elastica* are single data points. *Hevea brasiliensis*, △; *Parthenium argentatum*, ◇; *Ficus elastica*, ○.

Mg^{2+} concentrations RuT activity was inhibited in all three species [Fig. 3(a–c) and (d–f)].

Velocity and Hill plots for Mn^{2+} are shown in Fig. 4(a–c) and (d–f), respectively. The addition of Mn^{2+} generated similar sigmoidal curves to Mg^{2+} (Fig. 3) as well as similar maximum activation and inhibition by additional metal. The $[A]_{max}^{Mn2+}$ in *H. brasiliensis* (3.8 mM), *P. argentatum* (6.8 mM) and *F. elastica* (11 mM) are very similar to each other under these conditions (Table 2). The $[A]_{max}^{Mn2+}$ was similar to the

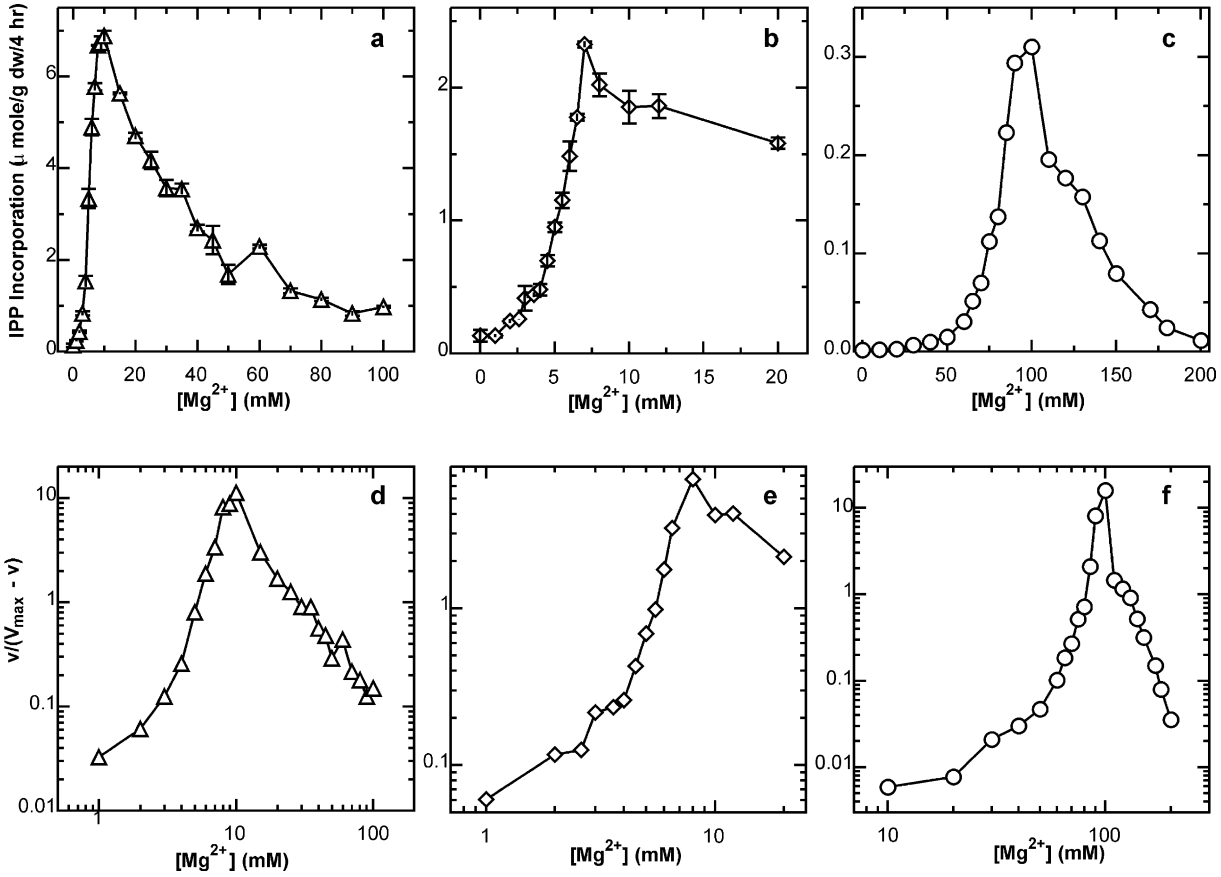

Fig. 3. Magnesium concentration dependence of [^{14}C]IPP-incorporation by rubber transferase in rubber particles purified from three species: (a–c) Velocity versus [Mg^{2+}]. (d–f) Hill plots. Incorporation rate of [^{14}C]IPP was measured in the presence of 20 μM FPP, 1 mM IPP and 7 mM EDTA for *H. brasiliensis* and *P. argentatum* and in the presence of 20 μM FPP, 200 μM IPP and 20 mM EDTA for *F. elastica*. Data for *H. brasiliensis* and *P. argentatum* are the means of 3±S.E. whereas *F. elastica* are single data points. *H. brasiliensis*, Δ, (a, d); *P. argentatum*, ◇, (b, e); *F. elastica*, ○, (c, f).

Table 2
Kinetic constants for rubber transferase in purified rubber particles

Substrate of activator	Species					
	H. brasiliensis		*P. argentatum*		*F. elastica*	
Activator	$[A]_{max}$ (mM)	V_{max}[a]	$[A]_{max}$ (mM)	V_{max}[a]	$[A]_{max}$ (mM)	V_{max}[a]
Mg^{2+}	10	7.5	8.0	2.5	100	0.35
Mn^{2+}	3.8	10	6.8	2.8	11	0.16
Initiator	K_m (mM)	V_{max}[a]	K_m (mM)	V_{max}[a]	K_m (mM)	V_{max}[a]
FPP (1 mM IPP)	2.2	1.6	0.04	1.6	0.8	0.9
Monomer	K_m (mM)	V_{max}[a]	K_m (mM)	V_{max}[a]	K_m (mM)	V_{max}[a]
IPP (1/10 K_m^{FPP})	0.6	0.09	0.4	0.12	2.2	0.3
IPP (K_m^{FPP})	0.5	0.14	0.3	0.2	0.3	0.3
IPP (20 μM FPP)	0.4	6.7	0.4	1.9	0.1	1.4

[a] IPP incorporation in μmol/g dw/4 h.

$[A]_{max}^{Mg2+}$ in *H. brasiliensis* and *P. argentatum*. However, the $[A]_{max}^{Mn2+}$ for *F. elastica* was less than 6 times lower than $[A]_{max}^{Mg2+}$ (Table 2).

The data shown in Fig. 4 may be interpreted in two ways. Since EDTA has a stronger affinity for Mn^{2+} than Mg^{2+} [stability constant (log K_1) for EDTA–Mn^{2+} is 13.56 and for EDTA–Mg^{2+} is 8.69 (Furia, 1972)], more Mn^{2+} is required to chelate any excess EDTA and allow RuT access to free cations. The data shown has not used any correction. A correction to a true zero $[Mn^{2+}]$ does seem indicated for *F. elastica*, which would alter the $[A]_{max}^{Mn2+}$ from 11 to 6 mM. However, it is not possible in the same way to determine a true zero $[Mn^{2+}]$ in the other two species as IPP incorporation rates gradually increase across the lowest concentrations.

2.3. Pyrophosphates

2.3.1. Effects of allylic pyrophosphate

The velocity plots in all three species were hyperbolic for [FPP]s below those needed for V_{max} (Fig. 5). Above 50 μM FPP, RuT activity was inhibited although to a different degree among the three species. In Fig. 5(d–f), the data is displayed in Hill plots, where K_m is the concentration of FPP when $y = 1$ ($v = 1/2\ V_{max}$). The K_m^{FPP}'s differed substantially among species with *P. argentatum* having by far the lowest (Table 2).

2.3.2. Effects of isopentenyl pyrophosphate

The velocity plots in all three species were hyperbolic for [IPP]'s lower than the concentration needed for V_{max} (Fig. 6). The maximum velocity was dependent completely on the [FPP] at levels of IPP greater than 1 mM. RuT was not inhibited even by 3 mM IPP. In *H. brasiliensis* and *P. argentatum*, the K_m^{IPP}s were not affected by [FPP] and were very similar, 0.4 mM and 0.6 mM, respectively. However, in *F. elastica* the K_m^{IPP} decreased from 2.2 mM to 0.1 mM as the concentration of FPP increased from 0.02 μM to 20 μM (Table 2).

3. Discussion

3.1. Kinetics

Enzymes that utilize metals may be either metalloenzymes or metal activated enzymes. If an enzyme is a metalloenzyme, the metal is tightly bound with high

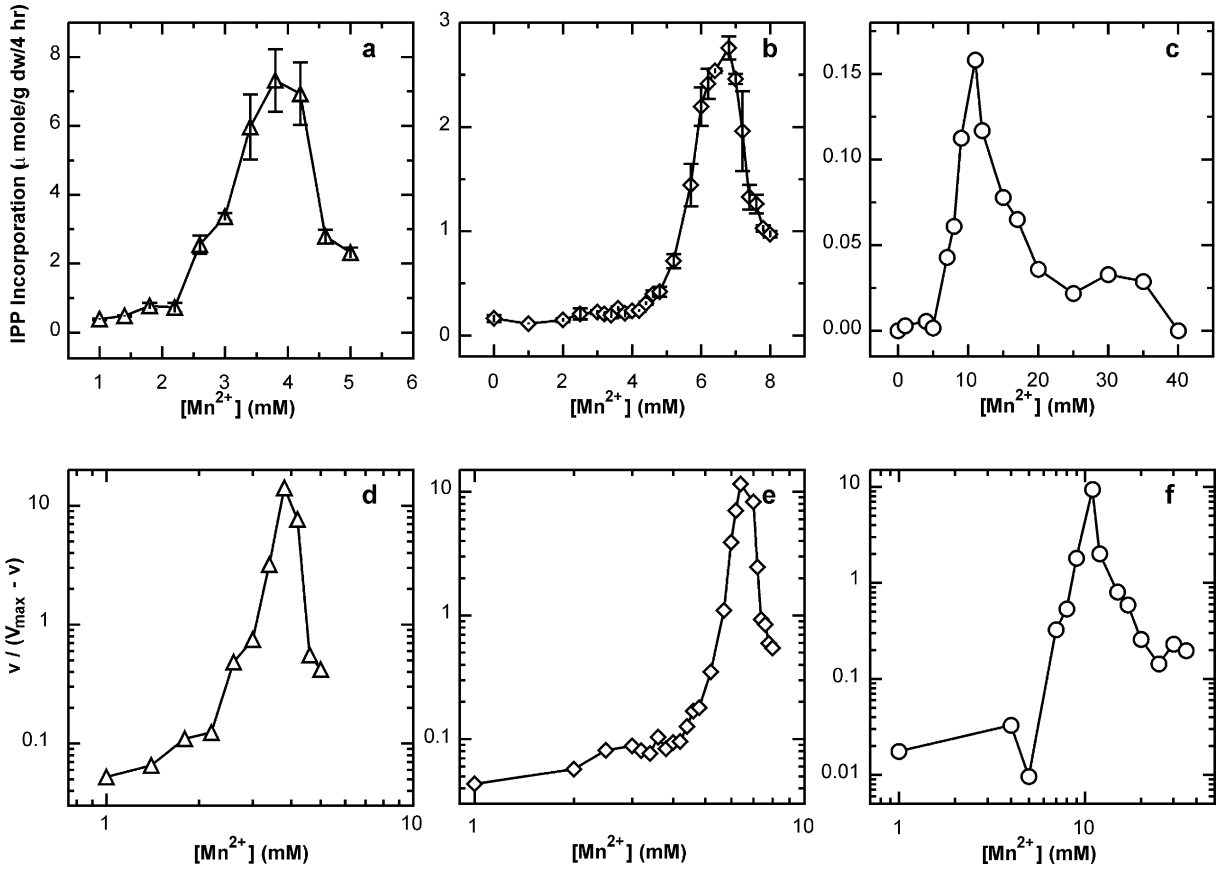

Fig. 4. Manganese concentration dependence of [^{14}C]IPP-incorporation by rubber transferase in rubber particles purified from three species: (a–c) Velocity versus $[Mn^{2+}]$. (d–f) Hill plots. Incorporation rate of [^{14}C]IPP was measured in the presence of 20 μM FPP, 1 mM IPP and 7 mM EDTA for *H. brasiliensis* and *P. argentatum* and in the presence of 20 μM FPP, 200 μM IPP and 20 mM EDTA for *F. elastica*. Data for *H. brasiliensis* and *P. argentatum* are the means of 3 ± S.E. whereas *F. elastica* are single data points. *H. brasiliensis*, Δ, (a, d); *P. argentatum*, ◇, (b, e); *F. elastica*, ○, (c, f).

affinity and is an integral component of the enzyme. If the metal is not always associated with the enzyme, but effects activity, the enzyme is classified as using metals as activators (King, 2003). It is well established that RuT activity can be readily eliminated by EDTA and the actual [EDTA] required (Fig. 2) was similar for all three species purified rubber particles and agreed with an earlier report for *Ficus carica* (Kang et al., 2000). When the RuT activity was restored by the addition of either Mg^{2+} (Fig. 3) or Mn^{2+} (Fig. 4), the response indicated that RuT is acting as a metal activated enzyme. Also, neither FPS (*trans*-prenyl transferase) nor UPPS (*cis*-prenyl transferase) are metalloenzymes, which may or may not be true for prenyl transferases in general.

As described by Segel (1993), when a metal is essential for enzyme activity, the true substrate is considered to be the complex of metal–substrate. The metal (A), the substrate (S) and the metal–substrate complex (SA) may bind to the enzyme independently and the dissociation constant (K_d) of the enzyme for each can be different. Because RuT is activated by the addition of a metal and because the velocity increases with increasing metal concentration, metals are essential activators for RuT (Figs. 3 and 4). RuT has two substrates S_1 (FPP) and S_2 (IPP) and a metal activator A (Mg^{2+} or Mn^{2+}), defining its metal–substrate complex as S_1A and S_2A (FPP–Metal, IPP–Metal).

The sigmoidal nature of the velocity versus [Mg^{2+}] and [Mn^{2+}] plots [Figs. 3(a–c) and 4(a–c)] in all three species may be interpreted as follows. As [Metal] increases the [FPP–Metal] increases. FPP–Metal competes with FPP for the active site of the enzyme, displacing the FPP (IPP does not interact alone with the enzyme as described below), permitting initiation of rubber biosynthesis. This effect is called deinhibition. [A complete analysis of the subject can be found in Segel (1993, Chapter 5): substrate activator complex is the true substrate.] Up to a certain level, V_{max}, the metal continues to activate RuT activity. By adding yet more metal, the activity of RuT is inhibited. The metal displaces FPP–Metal from the active site, stopping the initiation reaction, and causing the inhibition. The additional Metal may also displace the IPP–Metal complex preventing condensation (the polymerization reaction) from occurring.

We found that the two substrates, IPP and FPP, interacted differently with the enzyme in all three RuTs. FPP, FPP–Metal and Metal all interact independently with the enzyme. In contrast, IPP–Metal and Metal

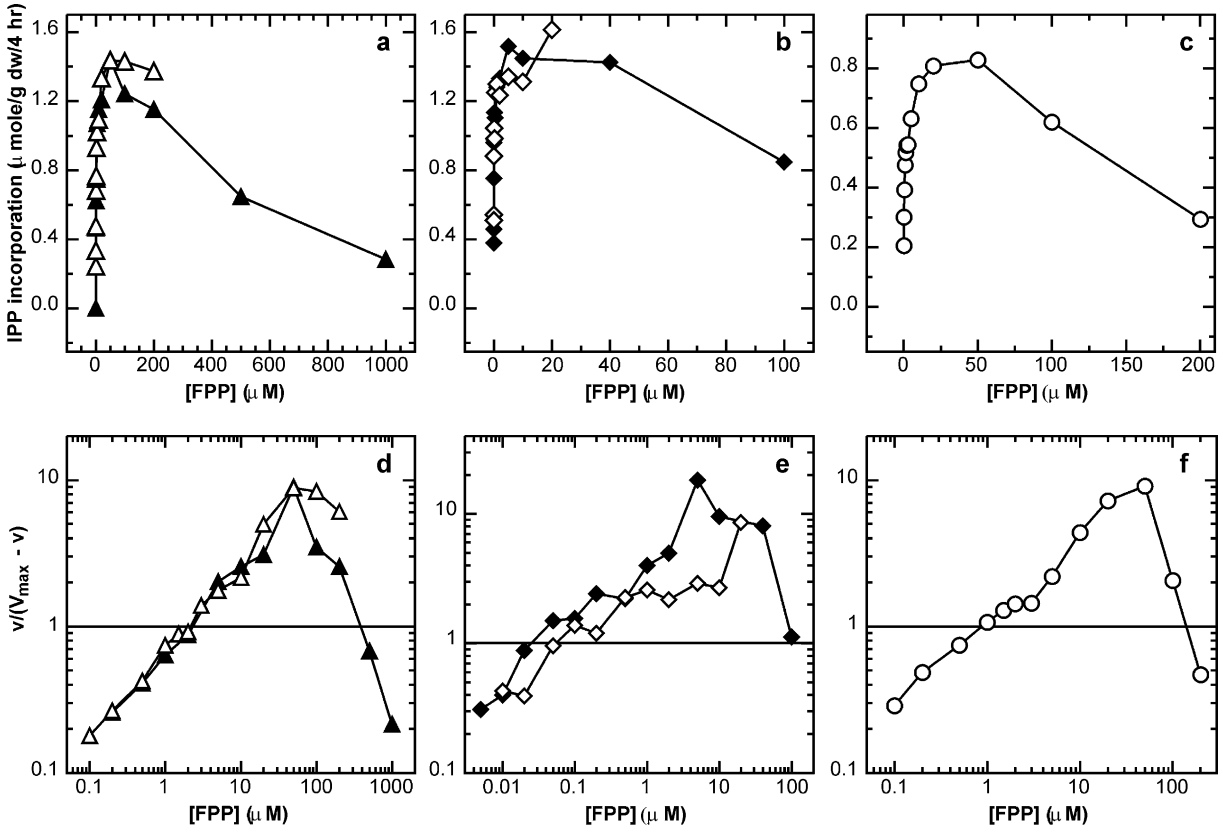

Fig. 5. FPP concentration dependence of [^{14}C]IPP-incorporation by rubber transferase in rubber particles purified from three species: (a–c) Velocity versus [FPP]. (d–f) Hill plots. Incorporation rate of [^{14}C]IPP was measured in the presence of either 1 mM IPP (open symbols) or 80 μM IPP (black symbols) and 1 mM Mg^{2+} for *H. brasiliensis* and *P. argentatum* or 1 mM IPP (open symbols) and 4 mM Mg^{2+} for *F. elastica*. *H. brasiliensis*, Δ, (a, d); *P. argentatum*, ◇, (b, e); *F. elastica*, ○, (c, f).

could interact with RuT but not IPP alone. The shape of the velocity versus substrate at fixed activator concentration shows the strength of the association of the enzyme for the substrate and the metal-substrate (Segel, 1993). The hyperbolic nature of the RuT plot of velocity vs [FPP] indicates that the FPP–Metal does not displace the FPP, and that $K_d^{FPP} < K_d^{FPP-Metal}$ (Fig. 5). Similarly, the inhibition of RuT activity with excess FPP (Fig. 5) indicates that the K_d of the enzyme for FPP is less than for FPP-Metal or $K_d^{FPP} < K_d^{FPP-Metal}$. The lack of inhibition with excess IPP (Fig. 6) indicates that IPP does not associate independently with the enzyme and $K_d^{IPP} = \infty$. The hyperbolic shape of the velocity vs IPP (Fig. 6) indicates that IPP-Metal has a greater association with the enzyme than the Metal has with the enzyme and $K_d^{IPP-Metal} < K_d^{E-Metal}$. Since $[A]_{max}$, 3.8–100 mM depending upon the species, is greater than the total concentration of pyrophosphate substrates required, 1.02 mM (sum of 1 mM IPP and 20 μM FPP) for *H. brasiliensis* and *P. argentatum* and 220 μM (sum of 200 μM IPP and 20 μM FPP) for *F. elastica*, the association of the enzyme with FPP–Metal or IPP–Metal is stronger than the association of the enzyme with Metal and $K_d^{FPP-Metal} < K_d^{E-Metal}$ and $K_d^{IPP-Metal} < K_d^{E-Metal}$. (Exact K_d values cannot be calculated because the exact amount of the enzyme per rubber particle is not known.)

The above discussion does not address the issue of the association constants, of the IPP with Metal and FPP with Metal, K_a (defined by Segel as K_o). Since IPP requires Metal to interact with RuT, the complex IPP–Metal must first be formed in solution. The $K_a^{IPP-Mg2+}$ is 2.2×10^{-3} μM^{-1} (Gotoh, 1989). As indicated by this association constant, the ratio of [IPP]×[Mg^{2+}]/[IPP–Mg^{2+}] is 454 μM. An excess of IPP and/or Mg is needed to produce the IPP–Mg^{2+} complex. If [Mg^{2+}] remained constant, increases in [IPP] would increase the amount of IPP–Mg^{2+} available to the RuT. Similarly, in a constant [IPP], increasing [Mg^{2+}] would also increase [IPP–Mg^{2+}]. In RuT, we found that both IPP and Mg^{2+} affinities reflect the lack of association of the enzyme with IPP, indicated by the high K_m^{IPP}s and high $[A]_{max}^{Mg2+}$s. This ensures that IPP is not used to make rubber when [IPP]'s are low and needed for a higher priority biochemical pathway. In contrast to IPP, FPP alone can associate with RuT that then facilitates the association of Metal with FPP within the active site. Thus the formation of E–FPP–Metal is not dependent

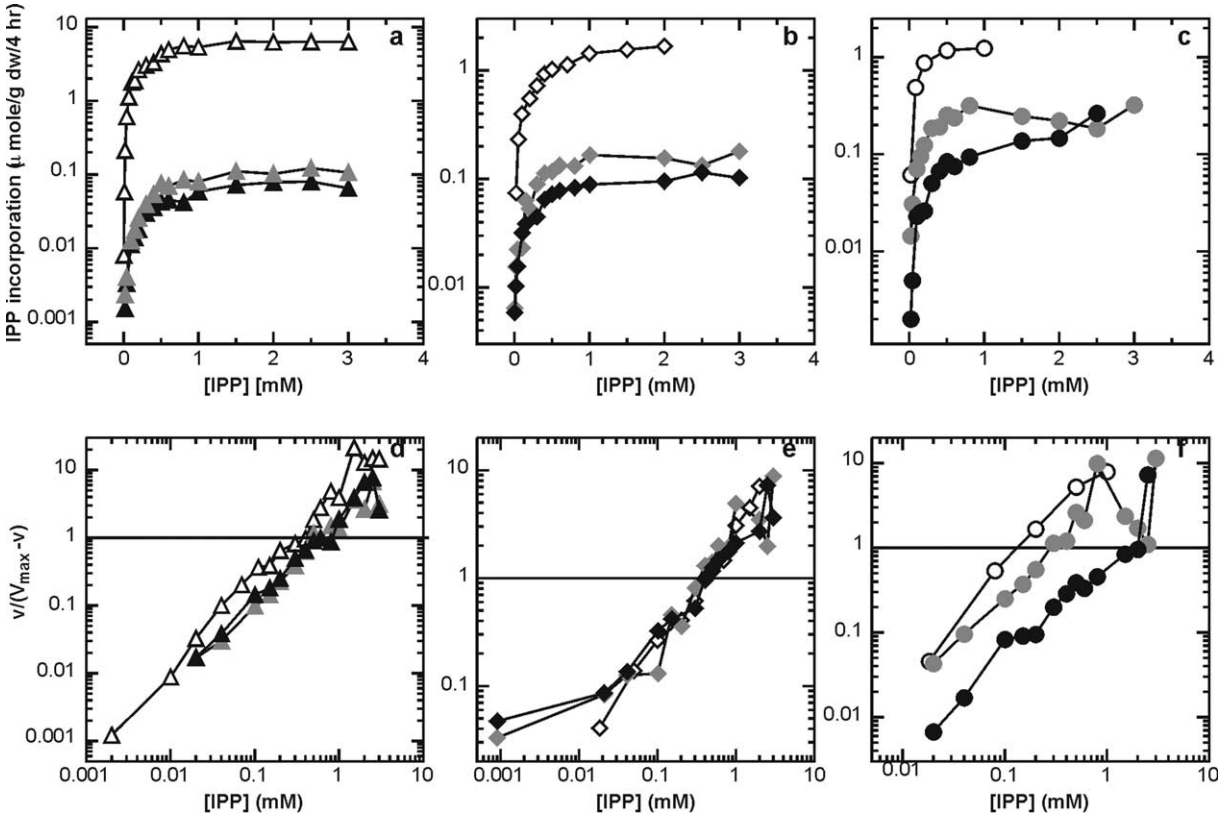

Fig. 6. IPP concentration dependence of [^{14}C]IPP-incorporation by rubber transferase in rubber particles purified from three species: (a–c) Velocity versus [IPP]. (d–f) Hill plots. Incorporation rate of [^{14}C]IPP was measured in the presence of 1 mM Mg^{2+} (4 mM Mg^{2+} for *F. elastica*) and either 20 μM FPP—(saturating concentration of FPP) (open symbols), [FPP] at K_m^{FPP} (grey symbols) or [FPP] at 1/10 K_m^{FPP} (black symbols). [FPP]s were 20 μM, 1 μM, and 0.1 μM FPP for *H. brasiliensis*, 20 μM, 0.02 μM, and 0.002 μM FPP for *P. argentatum*, and 20 μM, 0.2 μM, and 0.02 μM FPP for *F. elastica*. *H. brasiliensis*, Δ, (a, d); *P. argentatum*, ◇, (b, e); *F. elastica*, ○, (c, f).

upon the formation of [FPP–Metal] in solution. This also means that $K_a^{FPP–Mg2+}$ has very little impact on the formation of E–FPP–Mg^{2+} whereas the $K_a^{IPP–Mg2+}$ is critical to the formation of E–IPP–Mg^{2+}.

3.2. Inter-species comparison

Investigation of rubber biosynthesis in our three evolutionary-divergent species permits identification of features that are required for successful rubber production as well as highlighting how far apart these species have diverged in unconserved aspects of rubber production. For example, rubber particles of *F. elastica* (Heinrich, 1970) and *H. brasiliensis* (D'Auzac et al., 1989) are found in laticifers, while in *P. argentatum* they are found in bark parenchyma cells (Whitworth and Whitehead, 1991). Although gross particle architecture has been conserved, each particle consisting of a rubber core surrounded by a monolayer biomembrane (Cornish et al., 2000; Wood and Cornish, 2000), the biochemical components of the membrane are species-specific (Siler et al., 1997), the rubber molecular weight varies considerably (Cornish et al., 1993; Siler et al., 1997), and the particle size is very different (Cornish et al., 1993; Siler et al., 1997; Wood and Cornish, 2000).

Kinetically, there is a great deal of similarity in the effects Mg^{2+} and Mn^{2+} have on RuT activity in *H. brasiliensis*, *P. argentatum*, and *F. elastica*. All three RuTs use Mg^{2+} and Mn^{2+} as enzyme activators; all are deinhibited at concentrations of metals below V_{max}; all are inhibited by the addition of excess metals. However, the extent of deinhibition by Mg^{2+} varies among the species, being strongest in *F. elastica* and weakest in *H. brasiliensis* (Fig. 3). Mn^{2+} causes very little deinhibition in *F. elastica* and *H. brasiliensis* only deinhibiting *P. argentatum* (Fig. 4). The three species have unique protective structural mechanisms for their RuT initiation sites that prevent high metal concentrations, either in the latex of *H. brasiliensis* or of *F. elastica* or in the parenchymal bark cytosol of *P. argentatum*, from inhibiting initiation which allows FPP to interact with RuT without Mg^{2+}. Because of this, the $K_a^{FPP–Mg2+}$ has no effect on the available concentration of FPP–Mg^{2+} at the active site.

Also, we found similarities and differences in the way RuTs utilize the allylic and nonallylic pyrophosphate substrates. As has previously been shown, V_{max} is dependent upon the [FPP] and [IPP] in all three species (Archer and Audley, 1987; Madhavan et al., 1989; Cornish and Siler, 1995, 1996; Cornish et al., 1998). For the *H. brasiliensis* and *P. argentatum* RuTs, the K_m^{FPP}, as well as the K_m^{IPP}, are largely unaffected by changing [IPP] and [FPP]. However, for the *F. elastica* RuT, we found the K_m^{IPP} varied from 2.2 mM to 0.1 mM depending upon [FPP] (Table 2). The decreased affinity for IPP in low [FPP] may divert limiting amounts of FPP away from rubber towards the synthesis of important sesquiterpenes. Another striking interspecies difference is the very high affinity of the *P. argentatum* RuT for FPP (Table 2) which is much greater than that found in either *H. brasiliensis* or *F. elastica* (or in soluble FPP-using enzymes). In addition, the *P. argentatum* RuT exhibits strong negative cooperativity for FPP from 0.05 to 8 μM, not seen in *H. brasiliensis* or *F. elastica* (Cornish et al., 2000). Thus, the *P. argentatum* RuT is most likely to make high molecular weight rubber in vivo over a wide range of [FPP]'s. These kinetic differences indicate that significant structural differences have arisen in RuTs during the divergence of these three species making their conservation of rubber biosynthesis requirements even more remarkable.

3.3. Metal content implications in vivo

Our finding that *F. elastica* latex contained 4.4 times more Mg than *H. brasiliensis* latex (Table 1) is similar to an earlier report (Kang et al., 2000) that *Ficus carica* had 7 times more latex Mg than *H. brasiliensis*. Since *H. brasiliensis* produces larger molecular weight rubber (>1,000,000) in vivo than *F. elastica* (<50,000) and since the [Mg] in latex of *H. brasiliensis* is 1/4 the concentration in *F. elastica,* there may be a correlation between these latex ions and the molecular weight of the rubber in vivo with higher [Mg] leading to lower molecular weight. The $[A]_{max}^{Mg2+}$ for *H. brasiliensis* under these conditions closely matches the in vivo physiological concentrations for *H. brasiliensis*, but $[A]_{max}^{Mg2+}$ for *F. elastica* is twice the concentration we found in vivo. The discrepancy between $[A]_{max}^{Mg2+}$ for *F. elastica* and the in vivo concentration of Mg^{2+} may also be related to the lower molecular weight rubber produced by this species. The nine times larger [Mn^{2+}] in *H. brasiliensis* than in *F. elastica* is probably not significant in vivo since the $[A]_{max}^{Mn2+}$ for both RuTs (cf. Tables 1 and 2) is 270–6500 times higher, respectively. However, the levels of total metal, especially due to the Na and K content are substantially greater in *H. brasiliensis* than in *F. elastica*. The [K] in *H. brasiliensis* has previously been reported (D'Auzac et al., 1989) to be 44 mM in latex and is dependent upon the height of extraction from the tree although no details were given. The somewhat higher [K] in our sample (66 mM) may be caused by the [Na] in our latex sample (42 mM) (Table 1). We are unaware of any previous reports of latex [Na]. *H. brasiliensis* is not particularly salt tolerant or drought tolerant [International Rubber Research and Development Board (http://www.irrdb.com)]. However, at least in *H. brasiliensis* and *P. argentatum*, K and Na do not inhibit RuT activity at these concentrations, and even 500 mM only causes partial inhibition (Cornish, unpublished results). Also since we found that [Ca^{2+}] up to 20 mM had no effect on the in vitro activity of RuT in *H. brasiliensis*,

the [Ca] in the latex of *H. brasiliensis* (0.46 mM) should have no effect on RuT in vivo. A proposed purpose for Ca in latex has recently been described. Rippel et al. (2002) found a parallel distribution of Ca and carbon in rubber latex films of *H. brasiliensis*. Other metals, including Na and Mg, were found in small clusters surrounded by rubber polymer. They concluded that Ca plays an important role in forming ionic bridges that stabilize natural rubber.

It is known that Zn increases the activity of some prenyl transferases and may be essential for others. Sagami et al. (1984) found that, although Zn^{2+} was not a true cofactor, 0.2 mM Zn^{2+} enhanced the activity of both avian and pig livers FPS (*trans*-prenyl transferase) assayed in the presence of the metal cofactors Mg^{2+} or Mn^{2+}. Zn had no effect on the activity of either IPP isomerase or squalene synthetase. In contrast, Kharel et al. (2001) found that UPPS, a *cis*-prenyl transferase, could utilize Zn as a cofactor. Although we found that the [Zn] was 3 times higher in the latex of *H. brasiliensis* than in *F. elastica*, there is no evidence that Zn^{2+} affects RuT activity.

3.4. Other prenyl transferases

Like RuT, the activity of avian farnesyl pyrophosphate synthase, FPS, a *trans*-prenyl transferase, is inhibited by the APPs, GPP and FPP, at concentrations $> K_m$ (Reed and Rilling, 1976; Laskovics et al., 1979). Additionally, FPS activity is inhibited by excess metal cofactors, either Mn^{2+} or Mg^{2+} (King and Rilling, 1977) as we saw for RuT. However, unlike RuT, [IPP]s $> K_m^{IPP}$ also inhibit FPS activity (Reed and Rilling, 1976; Laskovics et al., 1979). Studies examining the binding of IPP and DMAPP to FPS showed that both interact with the enzyme without Mg^{2+} (King and Rilling, 1977). This is distinctly different from RuT, for IPP must complex with Mg^{2+} before it can interact with the active site. The effect of increased APP, IPP and metal cofactor concentration on activity have not yet been described for the *trans*-prenyl transferase, SPPS, although increasing [Mg^{2+}] and [IPP] increased product length up to, but not exceeding, the C_{45} polymer. Thus, although superficially similar in substrate and cofactor requirements, RuTs are kinetically distinct—interaction of IPP–Mg^{2+} with the enzyme only, no IPP alone, no IPP inhibition, no product inhibition and no predetermined product size.

The effects of APP and IPP on activity, on inhibition or on the role of Mg^{2+} in substrate–enzyme interactions with two other *cis*-prenyl transferases, UPPS and DDPPS, have not been reported. However, UPPSs from several sources have different metal cofactor preferences as well as differing from RuT. For activation of UPPS, the [Mg^{2+}] ranges from 240 nM to 2 mM, much less than the 8–100 mM for RuT. UPPS from *Micrococcus luteus* B-P 26, utilized Mg and Zn equally [240–600 nM for 60 nM enzyme (Kharel et al., 2001)], Mn^{2+} was a weak activator, [Zn^{2+}] >600 nM did inhibit activity, but excess Mg^{2+} was not inhibitory. Similarly, the UPPS from *Sulfolobus acidocaldarius* utilized Mg^{2+} as a cofactor (0.5–2.0 mM) but could not use Mn^{2+} (Hemmi et al., 2001). Also, no inhibitory effect was mentioned for excess Mg^{2+}. However, in contrast, and like RuT, the UPPS activity from *Escherichia coli* was inhibited by [Mg^{2+}] > 1 mM (Apfel et al., 1999). As shown for SPPS, increasing [Mg^{2+}] in vitro for UPPS increases product size up to, but not exceeding, the predetermined C_{75}–C_{100} for the enzyme (Pan et al., 2000). The UPPS from *Lactobacillus plantarum* utilizes Mg^{2+} (0.2 mM) as a cofactor (Baba and Allen, 1978) but other metals were not investigated. Thus, the metal cofactor preferences for UPPSs are species specific, which is not the case for the three divergent RuT species studied here.

The DDPPSs studied all used Mg^{2+} as metal cofactors in in vitro assays but no information was given regarding inhibition. Like RuT, *Saccharomyces cerevisiae* DDPPS could also use Mn^{2+}, and 1 mM Mn^{2+} or 1 mM Mg^{2+} equally activated the enzyme (Adair and Cafmeyer, 1987), while Ca^{2+} had little effect. For the DDPPS from *Ratus ratus* liver, activity required Mg^{2+} (0.3 mM) and lipid for activation, and the molecular weight of the product depended on both concentrations (Matsuoka et al., 1991; Ericsson et al., 1992). No other metals were described as activating. Only 4 mM Mg^{2+} was used to study the DDPPS activity from *Arabidopsis thaliana* (Cunillera et al., 2000). Since we found that the activity of RuTs can be altered by small changes in metal concentration (Fig. 3), this is likely the case for UPPS and DDPPS and their metal concentration dependencies warrant investigation.

In conclusion, we found many kinetic and structural commonalities among RuTs from three evolutionary-divergent dicotyledonous plant species. The true substrates for RuTs are FPP–Metal and IPP–Metal complexes. The RuTs do not interact with IPP without the metal cofactor, but can strongly interact with FPP alone. All RuTs have very high K_m^{IPP}s, low but different K_m^{FPP}s, similar preference for Mg^{2+} and Mn^{2+}, and produce indeterminate-sized polymers. All RuTs are membrane-bound enzymes, possibly complexes, with hydrophilic substrates entering from the cytosolic side and hydrophobic products elongated into and deposited in the rubber core of a rubber particle.

4. Experimental

4.1. Materials

Mature, field-grown *P. argentatum* (line 11591) plants were grown at the US Water Conservation Laboratory,

Phoenix, AZ. *F. elastica* plants were purchased from a local nursery and grown in a greenhouse in Albany, CA. *H. brasiliensis* line PB260 latex was donated by the Rubber Research Institute of India. Unlabelled IPP, *E,E*-FPP and [^{14}C]IPP (55 mCi/mm) were obtained from American Radiolabeled Chemical Inc., St. Louis, MO.

Multiwell plates used in this study were MultiScreen® R1 plates (MultiScreen—Filter Plates for High Throughput Separations; MultiScreen—R1; 1 mm Hydrophilic PTFE Membrane; Glass-Filled PP Plate; Non-Sterile with Lid, Millipore, Bedford, MA, USA; catalogue number MAR1N1010). Siliconized 1.5 ml tubes were supplied by USA Scientific (Ocala, FL, USA). A vacuum manifold (Millipore catalogue number MAVM0960R) was used for some assays. Filters for metal analysis are from Whatman (Newton or Marlborough, MA, USA) and are as follows: 0.45 μm–25 mm GD/X—Glass Microfiber GMF with polypropylene housing; 0.22 μm—Ultrafree MC—low binding Durapore Membrane; 0.1 μm Acrodisc Syringe Filter—Supor Membrane. An Eppendorf (Brinkmann, Westbury, NJ, USA) centrifuge, model 5415C, was used in preparation of latex samples for metal analysis. Ready Safe scintillation fluid was purchased from Beckman Instruments, Fullerton, CA, USA. Chemicals, unless otherwise noted, were purchased from Sigma (St. Louis, MO, USA).

4.2. Preparation of purified rubber particles

Enzymatically-active rubber particles from *H. brasiliensis* and *P. argentatum* were purified using the method of Siler and Cornish (1993) and Cornish and Backhaus (1990), respectively, and stored as glycerol-stabilized beads in liquid nitrogen until used (Cornish and Bartlett, 1997).

Enzymatically-active rubber particles from *F. elastica* were purified from tapped latex from the stems and petioles, collected on ice in a collection buffer, purified by a centrifugation procedure (Cornish and Siler, 1996), and stored according to the protocol of Cornish and Bartlett (1997).

4.3. Assay of in vitro rubber biosynthesis

4.3.1. Assays on the effect of metals

IPP-incorporation rates were assayed in *H. brasiliensis* and *P. argentatum* purified rubber particles using a modification of the method by Mau et al. (2000). The wells were siliconized with Sigmacote (Sigma-Aldrich, Corp., St Louis, MO, USA, # SL-2) for 2 min, rinsed with ddH_2O and with 95% ethanol, and dried at room temperature overnight. Reaction volume was 50 μl (1 mM IPP; 20 μM FPP; 100,000 dpm of [^{14}C]IPP; 100 mM Tris–HCl, pH 7.5; 5 mM DTT). EDTA (7 mM) was added to chelate preexisting metals. Metals were added as either $MgSO_4$ or $MnSO_4$. The reaction took place in individual wells of a 96-well plate. The reaction was begun by the addition of 0.25 mg purified rubber particles of *H. brasiliensis* or *P. argentatum* into each well and the filter plate was placed on a hollow ceramic plate equipped with a circulating water bath. After 4 h at 25 °C for *H. brasiliensis* and at 16 °C for *P. argentatum*, the reaction was stopped by the addition of 25 μl 500 mM EDTA.

Filters were washed using the Millipore vacuum manifold with 100 μl of 95% ethanol, 3 × 150 μl of 95% ethanol, 2 × 150 μl of ddH_2O, and 2 × 150 μl of 95% ethanol to each well. Filter plates were oven-dried at 37 °C for 20 min; the filters were removed from the plate and placed individually into vials with 2.5 ml of scintillation fluid. The amount of [^{14}C]IPP incorporation was determined by liquid scintillation spectroscopy using Beckman LS6500 (Beckman Coulter, Fullerton, CA, USA).

Because the *F. elastica* particles do not coagulate as tightly as *H. brasiliensis* or *P. argentatum*, we used the assay method described by Cornish and Siler (1996). Reaction volume was 100 μl (200 μM IPP; 20 μM FPP; 200,000 dpm [^{14}C]IPP; 100 mM Tris–HCl, pH 7.5; 5 mM DTT) in a 1.5 ml siliconized microfuge tube. In a separate siliconized tube, 2.5 mg of *F. elastica* purified rubber particles were combined with buffer and EDTA to chelate endogenous cofactors. Aliquots from both tubes were then added to a specified number of siliconized tubes with varying $MgSO_4$ or $MnSO_4$ concentrations. Reactions were incubated at 25 °C in a shaking water bath and stopped after 4 h by adding 50 μl of 500 mM EDTA. The reaction mixtures were pipetted onto separate filters on a vacuum manifold. Each filter was individually rinsed with ddH_2O and dried on the vacuum manifold for 15–20 min. The rubber-bound filters were placed in separate scintillation vials and washed with 5 ml of 1 M HCl and 3 × 4 ml 95% ethanol. The filters were immersed in 5 ml scintillation fluid and [^{14}C]IPP incorporation was determined by liquid scintillation spectroscopy. Replicates were not used in these assays because of the large number of concentrations and expense incurred by the large volumes used.

4.3.2. Assays on the effect of pyrophosphates

IPP-incorporation rates were assayed in *H. brasiliensis* and *P. argentatum* as described by Cornish et al. (1993). Reaction volumes were 500 μl (100,000 dpm of [^{14}C]IPP; 100 mM Tris–HCl, pH 7.5; 5 mM DTT; 1.00 mM $MgSO_4$) and 4–10 mg rubber particles were used per reaction. Rubber particles were filtered, washed and newly synthesized rubber was determined by liquid scintillation spectroscopy.

IPP-incorporation rates were assayed in *F. elastica* as described in the previous section except that the assays were done in the presence of 4 mM $MgSO_4$.

4.4. Metal analysis

Latex was collected from *F. elastica* without buffer. The rubber was coagulated by adding 200 μl of glacial acetic acid to 1 ml of latex in 1.5 ml microfuge tubes; the tubes were spun for 12 min at 16,110 *g*. The coagulated rubber was removed and the liquid was transferred to a new 1.5 ml microfuge tube. The sample was spun for 12 min at 16,110 *g*. The supernatant was filtered through a 0.45 μm syringe filter and placed on a 0.22 μm microfuge filter and spun for 5 min at 8300 *g*. The filtrate was filtered through a 0.1 μm syringe filter and collected in a microfuge tube. The final filtrate was diluted 10× and 100× and the metal content was measured using Inductively Coupled Plasma (ICP) Optima 3000 DV (Perkin Elmer, Shelton, CT, USA).

H. brasiliensis latex diluted with buffer (0.1 M $NaHCO_3$, 5% glycerol, 0.3% sodium azide, and 5 mM cysteine) in a ratio 2:1 (buffer to latex) was frozen at −20 °C until analyzed. The rubber was coagulated by adding 50 μl of glacial acetic acid to 1 ml latex in a 1.5 ml microfuge tube. The sample was spun for 10 min at 16,110 *g*. The coagulated rubber was removed and the liquid was transferred to a new 1.5 ml microfuge tube. The sample was spun twice more for 10 min at 16,110 *g*. The supernatant was filtered through a 0.45 μm syringe filter and placed on a 0.22 μm microfuge filter and spun for 2 min at 8300 *g*. The filtrate was again filtered through a 0.1 μm syringe filter and collected in new 1.5 ml microfuge tube. The final filtrate was diluted 10× and 100× and the metal content was measured using ICP.

A serial dilution of a solution containing the metals to be measured was prepared and used to calibrate the ICP. The metal concentrations of the 10× and 100× samples from *H. brasiliensis* and *F. elastica* were then measured.

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