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Initiation of rubber biosynthesis: *In vitro* comparisons of benzophenone-modified diphosphate analogues in three rubber-producing species

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ABSTRACT

Natural rubber, *cis*-1,4-polyisoprene, is a vital industrial material synthesized by plants via a side branch of the isoprenoid pathway by the enzyme rubber transferase. While the specific structure of this enzyme is not yet defined, based on activity it is probably a *cis*-prenyl transferase. Photoactive functionalized substrate analogues have been successfully used to identify isoprenoid-utilizing enzymes such as *cis*- and *trans*-prenyltransferases, and initiator binding of an allylic pyrophosphate molecule in rubber transferase has similar features to these systems. In this paper, a series of benzophenone-modified initiator analogues were shown to successfully initiate rubber biosynthesis *in vitro* in enzymatically-active washed rubber particles from *Ficus elastica*, *Hevea brasiliensis* and *Parthenium argentatum*.

Rubber transferases from all three species initiated rubber biosynthesis most efficiently with farnesyl pyrophosphate. However, rubber transferase had a higher affinity for benzophenone geranyl pyrophosphate (Bz-GPP) and dimethylallyl pyrophosphate (Bz–DMAPP) analogues with ether-linkages than the corresponding GPP or DMAPP. In contrast, ester-linked Bz–DMAPP analogues were less efficient initiators than DMAPP. Thus, rubber biosynthesis depends on both the size and the structure of Bz-initiator molecules. Kinetic studies thereby inform selection of specific probes for covalent photolabeling of the initiator binding site of rubber transferase.

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1. Introduction

Natural rubber, *cis*-1,4-polyisoprene, is a strategically important plant-derived material used in thousands of industrial applications. Currently, *Hevea brasiliensis* (Brazilian rubber tree) is the sole source of natural rubber; most countries depend on imports of *H. brasiliensis* rubber to sustain demand. Further, decades of inbreeding have rendered commercial *H. brasiliensis* varieties susceptible to abiotic stress and pathogen attack.

Alternative natural rubber-producing plants capable of growing in temperate climates are actively sought. Guayule, *Parthenium argentatum*, is a natural rubber-producing woody shrub native to the southwestern United States and northern Mexico (Bonner, 1943; Backhaus, 1985; Madhavan et al., 1989; Whitworth and Whitehead, 1991). Recently, guayule rubber has been commercialized as an alternative source of rubber, but the need for natural rubber far outweighs the projected growth of the guayule supply. Genetic engineering holds significant potential for increased rubber yields, thereby enhancing the competitiveness of the US domestic rubber crop. Unfortunately, such efforts in crop improvement have been hampered by a lack of gene sequence knowledge, especially for gene(s) encoding the rubber transferase.

Rubber transferase is localized to the surface of cytosolic vesicles known as rubber particles, and biosynthesis is initiated through the binding of an allylic pyrophosphate (APP) primer. Progressive additions of isopentenyl pyrophosphate (IPP) molecules ultimately result in the formation of high molecular weight *cis*-1,4-polyisoprene (McMullin and McSweeney, 1966; Walsh, 1979; Tanaka, 2001). Enzymatically-active, partially-purified (washed) rubber particles can be isolated such that, when provided with an appropriate APP primer, magnesium ion cofactor, and IPP monomer, rubber is produced *in vitro* (Archer and Audley, 1967; Light and Dennis, 1989; Madhavan et al., 1989). Kinetic studies determined that rubber transferase is highly tolerant of APP primers of differing lengths and stereochemistries, including dimethyl allyl pyrophosphate (DMAPP), geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and others (Archer and Audley, 1987; Cornish et al., 1998). Structural analyses of natural rubber (Tanaka, 1989, 2001; Tanaka et al., 1996) and kinetic analyses of the rubber

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transferase (Cornish et al, 1998) suggest FPP functions as the actual APP primer *in vivo*.

Genetic sequences of rubber transferase remain unknown because it is a membrane-associated enzyme present in relatively low abundance (Cornish, 1993, 2001). Classical biochemical approaches depend upon their ability to follow enzymatic activity throughout protein purification, but activity in rubber particles is rapidly lost upon disruption of their structural integrity. As an alternative, we have chosen an approach of covalent photoaffinity tagging of rubber transferase using benzophenone (Bz)-containing analogs of the rubber biosynthetic initiator, FPP. This approach allows rubber transferase to be followed throughout purification even after enzymatic activity is lost.

Benzophenone-containing photoaffinity labeling probes undergo C–H bond insertion reactions upon excitation with long wavelength (350 nm) light. Stable adducts between a variety of functional groups present in biomolecules and the carbonyl carbon of the benzophenone group form in these reactions, making them highly useful for identifying active site residues and ligand binding sites in proteins (Dorman and Prestwich, 1994; Turek-Etienne et al., 2003) as exemplified in Fig. 1. Photoaffinity labeling studies have been used to identify binding regions in specific enzymes and to isolate a number of previously unidentified proteins, (Yokoyama et al., 1995; Gaon et al., 1996a; Turek et al., 1997, 2001; Zhang et al., 1988, 2004; Webb et al., 1999) including protein prenyltransferases (Omer et al., 1993; Bukhtiyarov et al., 1995; Edelstein and Distefano, 1997) with remarkable specificity (Dorman and Prestwich, 1994). FPP binding to the rubber transferase active site occurs in a similar manner to the FPP-requiring enzymes mentioned above (Mau et al., 2003). Indeed, we have previously employed a Bz-containing inhibitor of rubber synthesis to label proteins found in enzymatically active rubber particles suggesting that this could be a valuable approach (DeGraw et al., 2007). However, in that case the molecule used was an inhibitor. A better approach would be to use isoprenoid diphosphate analogues that could be *bona fide* initiators of rubber synthesis. Thus, to identify an appropriate Bz-containing initiator, we have tested a series of Bz-modified FPP analogues for their ability to initiate biosynthesis in rubber particles purified from three different rubber-producing species, *Ficus elastica*, *H. brasiliensis* and *P. argentatum*. Initiator analogues varied by alkyl chain length, by linkage between the alkyl chain and the Bz group (ether vs. ester), and by the position of the Bz relative to the alkyl chain (*meta* vs. *para*) (Fig. 2). In studies with farnesyltransferase, all of these analogues (Fig. 2) could inactivate and covalently label the enzyme upon photolysis (Gaon et al., 1996b; Turek et al., 1996; Yokoyama et al., 1995; Turek et al., 2001) suggesting they would be good probes for studying the rubber transferase.

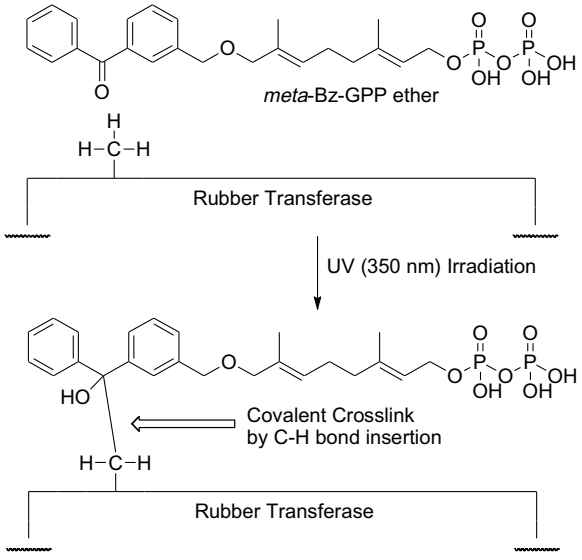

Fig. 1. C–H bond insertion reaction resulting in protein labeling by a benzophenone analogue.

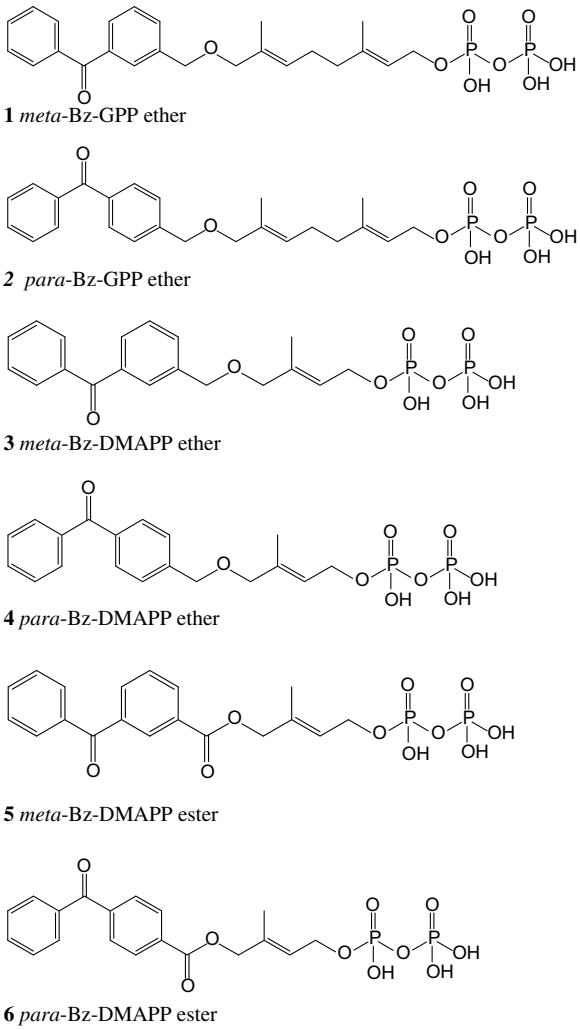

Fig. 2. Structures of benzophenone-modified initiator analogues.

2. Results and discussion

2.1. In vitro rubber synthesis by *F. elastica*, *H. brasiliensis* and *P. argentatum* rubber transferases with endogenous initiators

Initial kinetic studies were performed to determine the binding affinities for the naturally-occurring allylic pyrophosphate initiators (i.e. FPP, GPP and DMAPP) using washed rubber particles purified from three different rubber-producing plant species (*F. elastica, H. brasiliensis and P. argentatum*). In all cases, rubber transferase activity was measured as incorporation of [1-^{14}C] IPP into higher molecular weight rubber produced *in vitro* and normalized to the amount of rubber present in WRP preparations. The multiple washing steps used in preparation of WRPs remove non-membrane-bound protein; however, since membrane-bound WRP protein content is highly variable and species dependent, normalizing activity to WRP rubber content, rather than protein content, allows for cross species comparisons of rubber transferase activity

(Cornish and Backhaus, 1990). All assays were performed at previously determined optimal temperatures, over a 4 h incubation period with a saturating concentration of IPP, ensuring that the reaction was not substrate limiting, and that enzyme activity was stable and only limited by the concentration of the initiator.

According to Cornish and Scott (2005), at lower APP concentrations, multiple binding constants can represent a combination of active site-specific binding plus nonspecific hydrophobic interactions by the rubber transferase. A modified Michaelis–Menten single enzyme kinetic model can, however, be applied based on linear v vs. $v/[S]$ and Hill plots. On that basis, apparent kinetic constant (K_m) values for APP binding have been determined (Table 1) from linear portions of Hill plots (not shown).

The results show *F. elastica, H. brasiliensis* and *P. argentatum* rubber transferases have different binding affinities for initiators of rubber biosynthesis, as reflected in K_m values. *H. brasiliensis* had the highest affinity for all three unmodified initiators (FPP, GPP, DMAPP) reflected by the low K_m values, followed by *P. argentatum*, then *F. elastica*. In fact, *F. elastica* rubber transferase was 4–20 times lower in substrate affinity compared to *H. brasiliensis*. Similar results had been observed previously (Espy et al., 2006). FPP initiated rubber synthesis more efficiently than did GPP or DMAPP in all three rubber-producing species, as indicated by a higher rate of IPP incorporation (Fig. 3). The binding affinity (K_m) was a strong function of both the initiator (FPP > GPP > DMAPP) and the source of the WRP. Such dependence is similar to earlier reports (Cornish et al., 2000; Cornish, 2001). This order of preference has been attributed to the length of the carbon chain of the initiator; e.g. *H. brasiliensis* and *F. elastica* rubber transferases showed higher affinity for the even longer geranyl geranyl pyrophosphate (C20) as an initiator (Cornish and Scott, 2005).

K_m values for FPP initiation of 0.01, 1.5, and 0.6 μM have been previously reported for *P. argentatum*, *H. brasiliensis*, and *F. elastica*, respectively (Cornish et al., 2000). In this study, stronger binding affinity was found for FPP in *H. brasiliensis* compared to *P. argentatum*; with K_m values of 0.04 μM and 0.464 μM, respectively. Interestingly, while the *P. argentatum* enzyme appeared to have the lowest affinity for FPP, it possessed a remarkable 2 and 6 fold higher V_{max} value than *H. brasiliensis* and *F. elastica*, respectively (Fig. 3A–C). In *P. argentatum*, the rubber transferase K_m^{FPP} and V_{max} are known to vary depending on the choice of cultivars and collection season (Cornish and Backhaus, 2003). As such, K_m values ranging from 0.01 to 3 μM have been reported (Cornish and Backhaus, 1990; Scott et al., 2003). In this study, washed rubber particles with the highest level of rubber transferase activity were used from our collections throughout an entire seasonal cycle (Xie and McMahan, unpublished data). It should be noted that WRP preparations from *H. brasiliensis* are less variable throughout a season (Xie and McMahan, unpublished data).

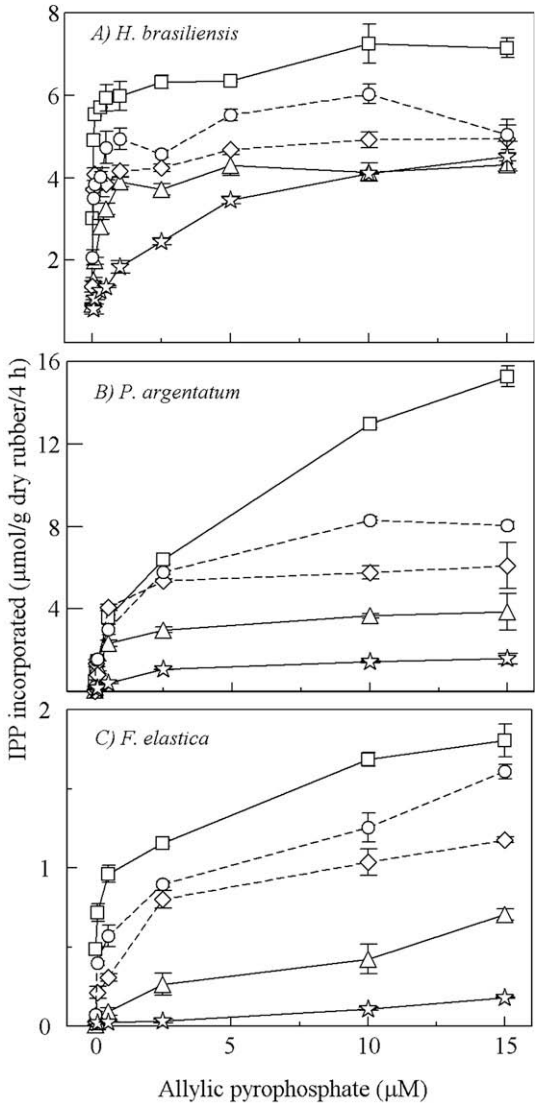

Fig. 3. Effect of Bz-GPP initiator structure on rubber transferase activity. Incorporation by purified washed rubber particles from (A) *H. brasiliensis*, (B) *P. argentatum*, (C) *F. elastica* in the presence of Bz-GPP analogues, FPP, □; GPP, △; *meta*-Bz-GPP ether (**1**), ◇; *para*-Bz-GPP ether (**2**), ○; DMAPP, ☆.

Table 1
Apparent K_m and V_{max} values for initiation of rubber synthesis by benzophenone allylic pyrophosphates in *F. elastica*, *H. brasiliensis* and *P. argentatum*[a]

Initiators/analogues	*F. elastica*		*H. brasiliensis*		*P. argentatum*	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
FPP	0.17	1.81	0.04	7.25	0.46	15.27
GPP	1.82	0.71	0.09	4.31	0.63	3.86
meta-Bz-GPP ether	0.53	1.18	0.05	4.94	0.23	6.1
para-Bz-GPP ether	0.40	1.61	0.03	6.03	0.39	8.3
DMAPP	18.4	0.25	1.20	4.52	5.41	1.58
meta-Bz-DMAPP ether	0.56	0.15	0.31	5.27	0.65	2.29
para-Bz-DMAPP ether	0.97	0.36	0.24	5.47	0.61	4.48
meta-Bz-DMAPP ester	1.20	0.04	0.69	2.89	ND	0.55
para-Bz-DMAPP ester	ND	0.04	2.10	1.85	ND	0.17

[a] K_m (μM) was calculated from log plots of $V/(V_{max} - V)$ against $[S]$. Assays were performed for the allylic pyrophosphates in the presence of 1 mM unlabeled IPP. The reaction was for 4 h at 16 °C for *P. argentatum* or 25 °C for *F. elastica* and *H. brasiliensis*. ND – not determined. V_{max} was determined from IPP incorporation in μmol/g dw/4 h.

2.2. In vitro rubber synthesis by F. elastica, H. brasiliensis and P. argentatum rubber transferases with benzophenone-modified (Bz) GPP initiators (1, 2)

This series of experiments was conducted to determine if Bz-modified initiators can be used by rubber transferases, and whether the orientation of the Bz moiety relative to the isoprenoid chain (*meta* vs. *para*) impacts the rate of biosynthesis. All three plant species readily used Bz-GPP initiators to synthesize rubber (Fig. 3A–C). Both *meta* (**1**) and *para* (**2**) Bz-GPP are more efficient initiators *in vitro* than GPP (higher V_{max} and lower K_m) but less so than FPP in all cases (Table 1). The fact that the Bz group did not prevent IPP incorporation substantiates the previous observation that rubber transferases of all three species are tolerant of variety in both the size and structures of the allylic pyrophosphate (Cornish, 2001). As noted in earlier work with natural isoprenoid

diphosphates (Cornish and Siler, 1995; Cornish et al., 1998) our results with Bz-modified initiators demonstrate that biosynthetic rate increases with increasing length of allylic pyrophosphate molecule. This has been shown to be true even for protein farnesyltransferase inhibitors (Mau et al., 2003) where lengthening the hydrophobic moiety of analogues increased the binding affinity of the inhibitor for the substrate binding site.

We demonstrate that additional structural or stereochemical factors also play a role in recognition by rubber transferases. For example, all three rubber transferases incorporated more IPP with the *para*-Bz-GPP ether analogue (**2**) as an initiator than with the *meta*-Bz-GPP ether analogue (**1**). The preference of rubber transferase for *para*-linked Bz initiators may be due to the Bz moiety in the *para* form acting as a pseudo-extension of the allylic carbon chain, effectively mimicking the structure of FPP. It appears this molecule may be better able to span both the specific allylic-APP binding site and the hydrophobic region immediately proximal to that site (Cornish, 2001) than the *meta* form allows (Fig. 2).

2.3. In vitro rubber synthesis by F. elastica, H. brasiliensis and P. argentatum rubber transferases with benzophenone-modified (Bz) DMAPP initiators (*3*, *4*, *5*, *6*)

The Bz-DMAPP analogue series of experiments systematically compares kinetics of rubber biosynthesis initiation for analogs of similar size but varying by the linkage between the alkyl chain and the Bz group (ether vs. ester), and by the position of the Bz relative to the alkyl chain (*meta* vs. *para*). Initiator analogues of *meta* (**3**) and *para* Bz-DMAPP (**4**) containing an ether-linked benzophenone group promoted faster IPP incorporation than unmodified DMAPP for all three rubber-producing species (Fig. 4A–C). In addition, the *para*-Bz-DMAPP ether analogue (**4**) allowed incorporation of substrate at more than twice the rate of the *meta*-Bz-DMAPP ether analogue (**3**) in *F. elastica* and *P. argentatum* rubber transferases. Similar to the case of *para*-Bz-GPP (**2**), the *para*-linked DMAPP (**4**) analogue may create an extension of the carbon chain in a conformation similar to GPP, a preferred initiator. Although it is a flexible molecule, the *meta*-linked DMAPP (**3**) can only adopt a conformation similar to that of DMAPP and hence has a similar IPP incorporation rate.

In contrast, *H. brasiliensis* rubber transferase did not show a preference for *meta* (**3**) or *para* (**4**) analogue structures (Fig. 4A). Both ether-linked analogues allowed IPP incorporation at a similar rate, although faster than unmodified DMAPP.

Ester-linked Bz-DMAPP analogues (**5**,**6**) resulted in very low IPP incorporation rates in all cases, with a slight preference for *meta* (**5**) over *para* DMAPP (**6**) in *H. brasiliensis* and *P. argentatum,* but not in *F. elastica*. This suggests subtle differences in all three rubber transferases with respect to initiator binding. However, the remarkable difference in binding between ether and ester-linked initiator analogues, in all cases, suggests the binding site on the enzyme is indeed able to distinguish the less polar, more flexible ether-linked Bz analogues (**3**,**4**) (vs. ester-linked **5**,**6**) as initiators of rubber biosynthesis. This appears to be a general characteristic of prenyltransferases; ether-containing FPP analogues bound to protein farnesyltransferase (PFTase) with higher affinity when compared to similar compounds with ester or amide linkages (Turek et al., 2001). Indeed, in this study the binding affinities of all Bz-DMAPP analogues was at least an order of magnitude better than DMAPP alone, in all species studied (Table 1).

2.4. Effect of different allylic pyrophosphate analogues of GPP and DMAPP on rubber transferase activity: addition experiments

In *P. argentatum*, initiation by the *para* Bz ether-linked DMAPP (**4**) reached saturation at ~10 μM (Fig. 4B). Addition of GPP, up to 15 μM, did not result in further IPP incorporation (Fig. 5A) indicating compound (**4**) is as effective as GPP for initiation. However, addition of GPP to saturated *meta*-Bz ether-linked DMAPP (**3**) allowed additional IPP incorporation (Fig. 5B), probably as a consequence of the lower incorporation rate (at low concentrations) of the *meta* analogue relative to that of GPP. Binding affinity of the three analogs was, in fact, quite similar, further evidence that incorporation is rate (V_{max}) controlled (Fig. 4A–B). Ester-linked DMAPP (**5**,**6**) analogues gave similar results upon addition of GPP (Fig. 6A–B) or DMAPP (results not shown). Both *meta* (**5**) and *para* (**6**) ester-linked analogs, slower to incorporate than GPP, are less effective (lower V_{max}) initiators than GPP, and consequently reduce the overall IPP incorporation rate compared to GPP alone Therefore, for the DMAPP series, *para* Bz ether-linked DMAPP (**4**) is clearly the most effective initiator analogue for *P. argentatum*.

As described earlier, in *H. brasiliensis, para* (**4**) and *meta* (**3**) ether-linked Bz-DMAPP analogues bind at similar rates (Fig. 4A); however the type of linkage (ether ≫ ester) had a major impact on V_{max}. At saturating concentrations of the analogues, addition of GPP to ether-linked Bz-DMAPP (**3**,**4**) analogues only modestly increased rate of IPP incorporation (Fig. 7A–B); reflecting small

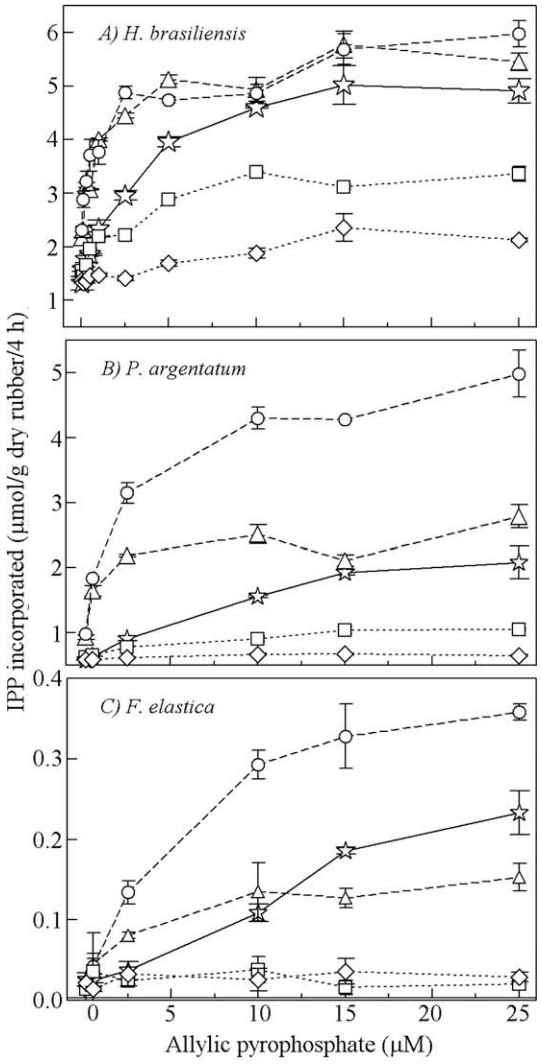

Fig. 4. Effect of Bz-DMAPP initiator structure on rubber transferase activity. incorporation by purified washed rubber particles from: (A) *H. brasiliensis*, (B) *P. argentatum*, (C) *F. elastica* in the presence of Bz-DMAPP analogues, DMAPP, ☆; *meta*-Bz-DMAPP ether (**3**), △; *para*-Bz-DMAPP ether (**4**), ○; *meta*-Bz-DMAPP ester (**5**), □; *para*-Bz-DMAPP ester (**6**), ◇.

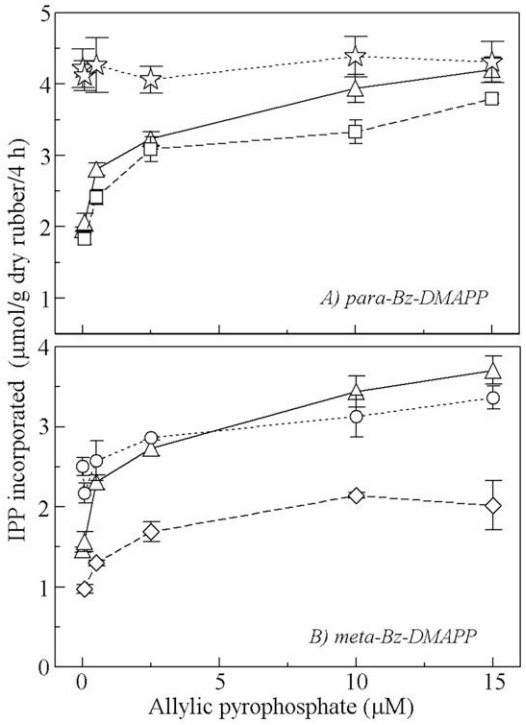

Fig. 5. Effect of GPP addition on rubber transferase activity in the presence of Bz-DMAPP ether analogues. IPP incorporation by purified *P. argentatum* washed rubber particles initiated by either GPP, △; *para*-Bz-DMAPP (**4**), □; *meta*-Bz-DMAPP (**3**), ◇; or in combinations of: (A) 15 μM *para*-Bz-DMAPP ether analogue (**4**) plus GPP, ☆; or (B) 15 μM *meta*-Bz-DMAPP ether analogue (**3**) plus GPP, ○.

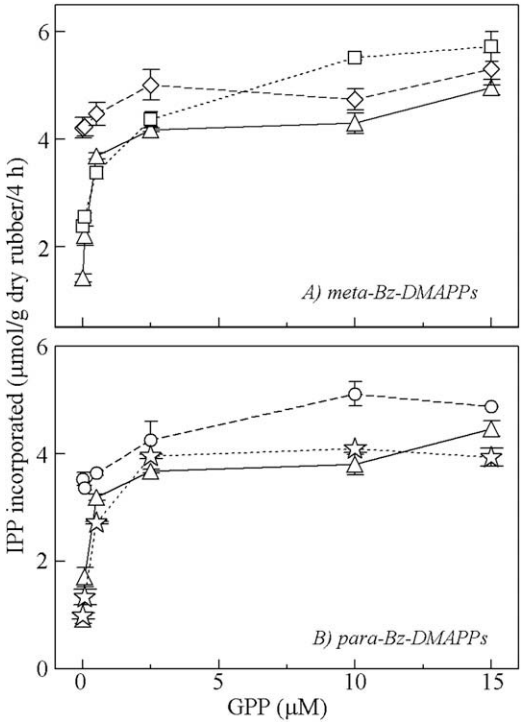

Fig. 7. Effect of GPP addition on rubber transferase activity in the presence of Bz-DMAPP analogues. IPP incorporation by purified *H. brasiliensis* washed rubber particles initiated by either GPP, △; or a combination of: (A) 15 μM *meta*-Bz - DMAPP ether (**3**) plus GPP, ◇; and 15 μM *meta*-Bz-DMAPP ester (**5**) plus GPP, □; or (B) 15 μM *para*-Bz-DMAPP ether (**4**) plus GPP, ○ and 15 μM *para*-Bz-DMAPP ester (**6**) plus GPP ☆.

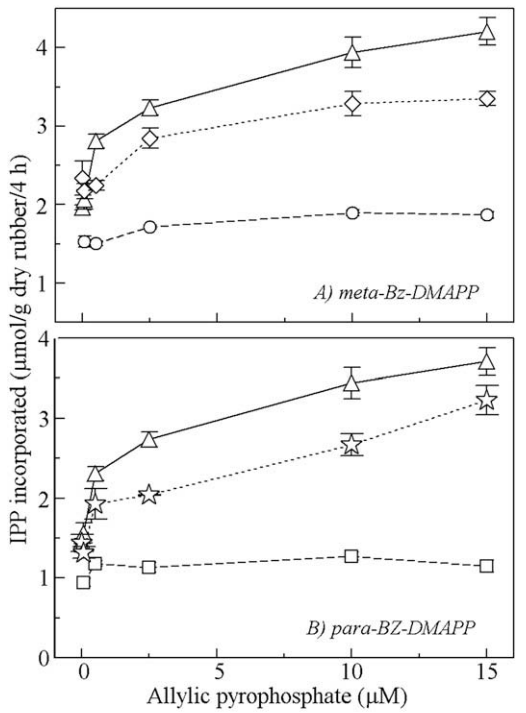

Fig. 6. Effect of GPP addition on rubber transferase activity in the presence of Bz-DMAPP ester analogues. IPP incorporation by purified *P. argentatum* washed rubber particles initiated by either GPP, △; *para*-Bz-DMAPP (**6**), □; *meta*-Bz-DMAPP (**5**), ○; or in combinations of: (A) 15 μM *meta*-Bz-DMAPP ester analogue (**5**) plus GPP, ◇; or (B) 15 μM *para*-Bz-DMAPP ester analogue (**6**) plus GPP, ☆.

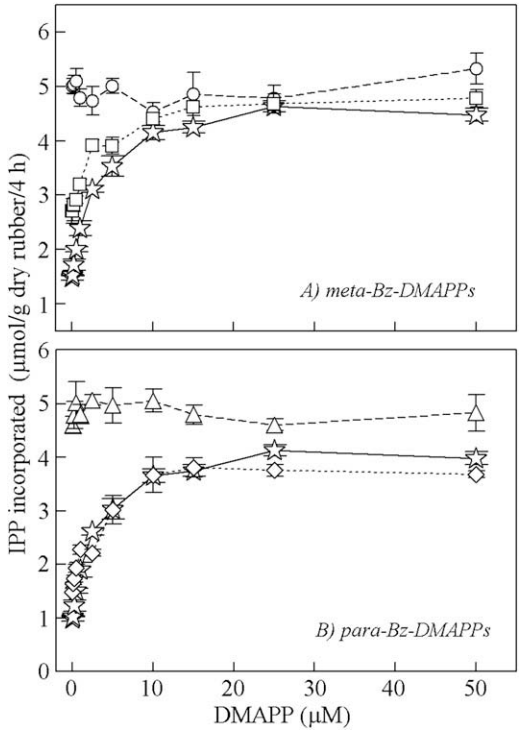

Fig. 8. Effect of DMAPP addition on rubber transferase activity in the presence of Bz-DMAPP analogues. IPP incorporation by purified *H. brasiliensis* washed rubber particles initiated by either DMAPP, ☆; or a combination of: (A) 15 μM *meta*-Bz - DMAPP ether (**3**) plus DMAPP, ○; and 15 μM *meta*-Bz-DMAPP ester (**5**) plus DMAPP, □; or (B) 15 μM *para*-Bz-DMAPP ether (**4**) plus DMAPP, △; and 15 μM *para*-Bz-DMAPP ester (**6**) plus GPP, ◇.

differences in rate and affinity (and no inhibition) whether *meta* (**3**) (Fig. 7A) or *para* (**4**) (Fig. 7B). However, at saturating concentrations of ester-linked Bz-DMAPP (**5**,**6**), addition of GPP increases IPP incorporation rates to levels comparable to GPP alone, as expected from the two-fold higher affinity of the rubber transferase for the GPP compared to the Bz analogues (Table 1). Similar results were found when ether-linked analogues (**3**,**4**) compete with DMAPP for initiation (Fig. 8A–B).

3. Concluding remarks

Benzophenone-modified initiator analogues successfully initiated rubber biosynthesis in the three species studied, reinforcing the observation that the active site of the rubber transferase enzyme is able to accept a wide range of allylic pyrophosphate initiators. The analogs studied, all fairly flexible molecules which can adopt multiple conformations, range in V_{max} only by a factor of 10. Despite a range of structural differences these enzyme–substrate systems all successfully initiated rubber biosynthesis. It is important to note that their commonality – a < C5 isoprenoid carbon chain with an allylic pyrophosphate – meets the requirements for specific binding. The remainder of the molecule then is positioned at the nonspecific binding area per the model of the rubber transferase enzyme proposed by Cornish (2001). However, significant differences in enzyme-substrate affinities mediated by this 'nonspecific binding' have been observed.

The primary factor determining the affinity of a rubber transferase for a given initiator is the size of the allylic hydrocarbon portion of the initiator, as documented previously and confirmed by these results. The relative hydrophobicity and rigidity of the initiator/analogue are also important, and perhaps responsible for the difference in affinity for Bz-APP analogues with ester vs. ether-linkages. Finally, rubber transferase appears to discriminate spatial orientations, even for these flexible molecules, and thereby has higher affinity for *para*-linked Bz-APPs than their *meta*- forms.

These Bz-initiator molecules, in photocrosslinking experiments, will provide powerful probes for biochemical characterization of the rubber transferase enzyme(s). If the apparent K_m values reported here represent substrate binding (not initiator dissociation), the best probe is the one with greater affinity, not the one with more efficient processing (or higher rate). The optimal benzophenone probe design would incorporate a long chain length (~C15) with ether-linkages for high rubber transferase affinity. In *P. argentatum*, these probes have a lower K_m but half the rate of FPP, the preferred initiator, thus maximizing their presence in the binding site during the photolysis reaction. This is similar to what has been observed for Bz analogs with farnesyltransferase, i.e., better binding than the natural substrate but slower processing. If dissociation is controlling, the initiator analog molecule would bind to the active site but would be delayed in release, probably due to structural perturbations.

Further, selection of probes should consider whether binding of a Bz-initiator to the active site allows normal elongation, and whether the initiator is in the active site during the photolysis crosslinking reaction. According to the model of rubber biosynthesis proposed by Cornish (2001), once the APP binds specifically to the rubber initiation site, a molecule of IPP is added on the pyrophosphate end of the chain, opposite the benzophenone group. Addition of successive molecules of IPP would effectively displace the Bz moiety towards the interior of the rubber particle. In addition, enzyme activity in terms of IPP incorporation may be impacted by interactions between the Bz and the growing rubber chain. Based on this possibility and the results presented herein, high-affinity inhibitors may be preferred to substrates as probe molecules. Rubber transferases are effectively inhibited by protein farnesyltransferase inhibitors *in vitro* with species-specific differences (Mau et al., 2003). Interspecies differences, observed here as well, suggest that selection of probes for photo-labeling experiments might be tailored to the species for best results.

4. Experimental

4.1. General experimental procedures

[1-^{14}C]IPP (55 mCi/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). MultiScreen HTS DV opaque filter plates and vacuum manifolds were from Millipore Co. (Bedford, MA, USA). ScintiVerse BD Cocktail was from Fisher Scientific (Santa Clara, CA, USA). All other chemicals were obtained from Sigma–Aldrich Chemical Company (St. Louis, MO, USA).

4.2. Preparation of enzymatically-active rubber particles

Mature, whole *P. argentatum* shrubs were freshly harvested, shipped overnight, stored at 4 °C, and processed within 96 h. Bark tissue from stems was homogenized and rubber particles isolated and purified using the method of Siler and Cornish (1993), Cornish and Backhaus (1990) and Cornish and Siler (1995). Latex tapped from *H. brasiliensis* (generous gift of Dr. R. Krishnakumar) and *F. elastica* greenhouse-grown plants was used to prepare washed rubber particles (WRP) using methods previously described (Siler and Cornish, 1993).

4.3. Synthesis of benzophenone-containing analogues

Benzophenone-containing analogues were synthesized as previously described: Bz-GPP ethers (**1**,**2**) (Gaon et al., 1996b; Marecak et al., 1997), Bz-DMAPP ethers (**3**,**4**) (Yokoyama et al., 1995; Turek et al., 2001), and Bz-DMAPP esters (**5**,**6**) (Turek et al., 1996; Marecak et al., 1997). In brief, the DMAPP-based analogues were synthesized by oxidation of a protected form of dimethylallyl alcohol followed by *O*-alkylation (for the ethers) (Turek et al., 2001) or *O*-acylation (for the esters) (Turek et al., 1996) to install the benzophenone unit. The final products were produced by deprotection, alcohol activation to the corresponding bromide and displacement with $[(n\text{-Bu})_4N]_3HP_2O_7$. The GPP-based analogues were prepared in a similar fashion from a protected form of geraniol (Gaon et al., 1996a,b). All compounds were characterized by ^{1}H NMR, ^{31}P NMR, UV spectroscopy and mass spectrometry to confirm their structures and were greater than 90% pure as determined by reversed-phase HPLC analysis. Previous work has shown that these compounds are stable for days at room temperature at neutral pH.

4.4. In vitro assay of rubber synthesis

Rubber transferase activity was measured by IPP incorporation rates using a modification of a previously described method (Mau et al., 2000). The reaction took place in wells of 96-well filter plate. The reaction volume was 40 μl containing 100 mM Tris–HCl, pH 7.5, 1.25 mM $MgSO_4$, 5 mM DTT, 1 mM unlabelled IPP, 0.9 nmol [^{14}C]IPP, and various concentrations of allylic pyrophosphates. Each well also contained 0.5 mg WRPs. The reaction time was 4 h at 16 °C for *P. argentatum* (Cornish and Backhaus, 1990), or 25 °C for *F. elastic* and *H. brasiliensis* (Mau et al., 2000). Reactions were stopped by 40 mM EDTA. The filter plate was then washed two times with 150 μl water and twice with 95% ethanol. The filter plate was oven-dried at 37 °C for 30 min. The filters were removed from the plate and placed into vials with 1.5 ml ScintVerse BD Cocktail. The amount of [^{14}C] IPP was determined by scintillation

counting (Beckman Coulter, Fullerton, CA, USA). Each value is the average of three replicates. The concentration of purified enzymatically-active rubber particles from *F. elastica, H. brasiliensis* and *P. argentatum* was determined by dry weight, and used to normalize IPP incorporation to a per gram dry rubber basis.

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