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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.

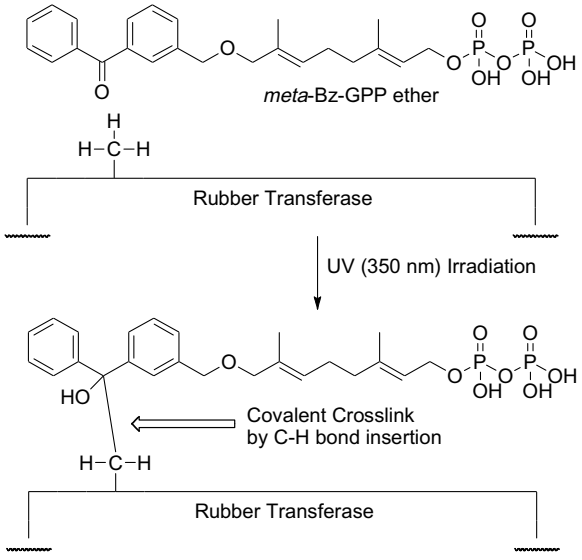

Fig. 1. C–H bond insertion reaction resulting in protein labeling by a benzophenone analogue.

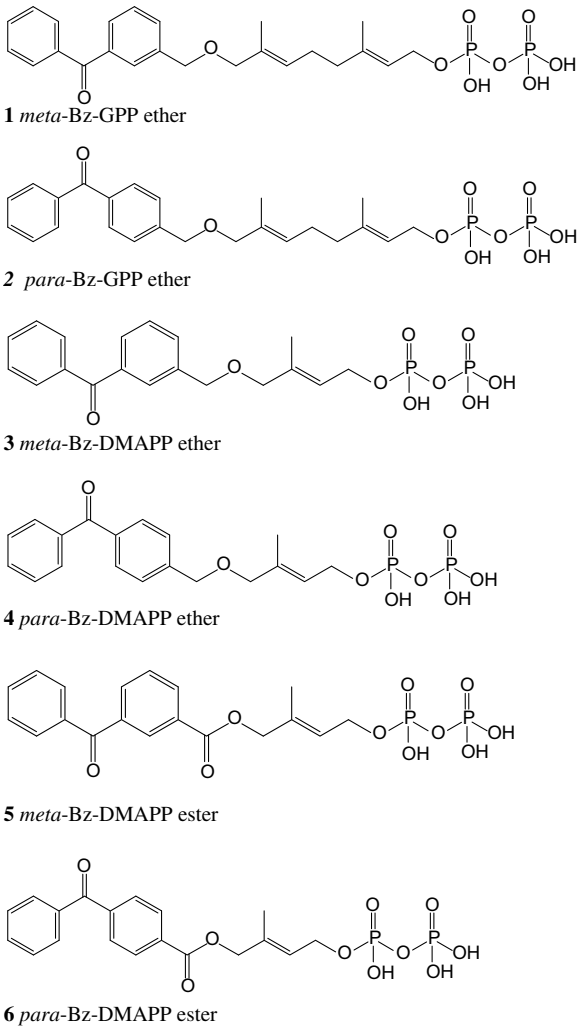

Fig. 2. Structures of benzophenone-modified initiator analogues.

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