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Unusual subunits are directly involved in binding substrates for natural rubber biosynthesis in multiple plant species

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ABSTRACT

Rubber particles from rubber-producing plant species have many different species-specific proteins bound to their external monolayer biomembranes. To date, identification of those proteins directly involved in enzymatic catalysis of rubber polymerization has not been fully accomplished using solubilization, purification or reconstitution approaches. In an alternative approach, we use several tritiated photoaffinity-labeled benzophenone analogs of the allylic pyrophosphate substrates, required by rubber transferase (RT-ase) to initiate the synthesis of new rubber molecules, to identify the proteins involved in catalysis.

Enzymatically-active rubber particles were purified from three phylogenetically-distant rubber producing species, *Parthenium argentatum* Gray, *Hevea brasiliensis* Muell. Arg, and *Ficus elastica* Roxb., each representing a different Superorder of the Dicotyledonae. Geranyl pyrophosphate with the benzophenone in the *para* position (Bz-GPP(*p*)) was the most active initiator of rubber biosynthesis in all three species. When rubber particles were exposed to ultra-violet radiation, 95% of RT-ase activity was eliminated in the presence of 50 μM Bz-GPP(*p*), compared to only 50% of activity in the absence of this analog. ^{3}H-Bz-GPP(*p*) then was used to label and identify the proteins involved in substrate binding and these proteins were characterized electrophoretically.

In all three species, three distinct proteins were labeled, one very large protein and two very small proteins, as follows: *P. argentatum* 287,000, 3,990, and 1,790 Da; *H. brasiliensis* 241,000, 3,650 and 1,600 Da; *F. elastica* 360,000, 3,900 and 1,800 Da. The isoelectric points of the *P. argentatum* proteins were 7.6 for the 287,000 Da, 10.4 for the 3,990 Da and 3.5 for the 1,790 Da proteins, and of the *F. elastica* proteins were 7.7 for the 360,000 Da, 6,0 for the 3,900 Da, and 11.0 for the 1,800 Da proteins. *H. brasiliensis* protein pI values were not determined.

Additional analysis indicated that the three proteins are components of a membrane-bound complex and that the ratio of each small protein to the large one is 3:1, and the large protein exists as a dimer. Also, the large proteins are membrane bound whereas both small proteins are strongly associated with the large proteins, rather than to the rubber particle proteolipid membrane.

1. Introduction

Rubber transferase (EC 2.5.1.20) is a membrane-bound *cis*-prenyl transferase, which produces polymers of indeterminate length between 50,000 and greater than 1,000,000 g/mol. It is well established that rubber transferase (RT-ase) is bound to cytoplasmic rubber particles (RPs), with an unconventional proteolipid monolayer membrane (Siler et al., 1997; Cornish et al., 1999; Wood and Cornish, 2000). The ability to make rubber is widespread across eudicotyledonous plants and rubber particles have species-specific complements of lipids and proteins complicating the identification of RT-ase (Siler et al., 1997). *H. brasiliensis* rubber particles are especially complex with hundreds of different associated proteins.

Several different proteins have been implicated as playing a role in rubber molecule formation, including a high molecular weight, hydrophobic, integral membrane, dimeric glycoprotein (LPR) in *F. elastica* rubber particles (Siler and Cornish, 1993; Cornish et al., 1994). Large rubber particle bound proteins also were found and characterized in *P.*

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argentatum and *H. brasiliensis* (Cornish et al., 1993; Siler and Cornish, 1994). Other rubber particle-bound proteins which may be involved include the small rubber particle protein (SRPP) and related rubber elongation factor (REF) (Dennis and Light, 1989; Schmidt et al., 2010b; Berthelot et al., 2014; Laibach et al., 2015; Tong et al., 2017; Wadeesirisak et al., 2017). Various soluble *cis*-prenyl transferases also have been proposed (Post et al., 2012; Qu et al., 2015; Light and Dennis, 1989; Light et al., 1989) although definitive proof of a role as RT-ase is lacking. Most recently, RT-ase activity was reconstituted in *H. brasiliensis* RP's which had been inactivated using detergents to partially deproteinize the RPs (Yamashita et al., 2016). However, activity could not be reconstituted in liposomes, suggesting that either structural or other essential proteins remaining on the detergent-treated RPs are essential to RT-ase activity.

Purification of enzymatic activity away from the rubber particle membrane has been difficult because of loss of structural integrity and the need for an aqueous-organic interface to maintain the reaction. Solubilization attempts often entrap proteins as particles collapse and coagulate. Two proteins solubilized from the *P. argentatum* RP membrane (52,000 and 130,000 Da) may be involved in rubber biosynthesis in *P. argentatum* (Benedict et al., 1990; Madhavan et al., 1989) but the solubilized activity reported has not been repeated. Early reports of solubilized activity in *H. brasiliensis* appear to have been the result of inadvertently adding an initiator system rather than RT-ase enzyme to the assay system (see reviews in (Cornish, 1993; Cornish and Siler, 1996)).

Due to the difficulty of purifying enzymatically-active RT-ase, a functional labeling approach was used, in this report, as a prelude to protein identification. Benzophenone photophore biochemical probes (Dorman and Prestwich, 2000), have been extensively used in chemistry and biology over the past two decades and comprehensively reviewed (Dorman et al., 2016). Benzophenone derivatives of allylic pyrophosphates (Dorman and Prestwich, 1994), were successfully used in two distinct isoprenoid enzyme groups, the bacterial prenyltransferases (Marecak et al., 1997; Zhang et al., 1998) and the protein prenyltransferases from yeast (Gaon et al., 1996a,b; Turek-Etienne et al., 2003) and mammals (Ying et al., 1994). The allylic pyrophosphate initiator binding site of the RT-ases of both *H. brasiliensis* and *P. argentatum* share similar chemical preferences to the FPP binding site of protein farnesyltransferases, and inhibitors of the protein farnesyltransferase also inhibit the RT-ases of *P. argentatum* and *H. brasiliensis* (Mau et al., 2003). This similarity means benzophenone APPs, which act as substrates in protein farnesyltransferase, will likely also act as substrates for RT-ase.

In this paper, we describe the use of APPs covalently attached to benzophenones by ether linkage (Marecak et al., 1997) as photo affinity labeled substrates of RT-ase, and, when tritiated, as biochemical probes to identify proteins directly involved in the catalytic reaction of rubber biosynthesis. These experiments are performed using enzymatically-active rubber particles purified from three phylogenetically divergent species, because we reason that their kinetically similar RT-ases (Cornish, 2001a,b) will also be structurally similar. We report on the characterization of the labeled proteins and propose a preliminary architecture of the RT-ase enzyme complex, which appears to be largely conserved across the Eudicot clade of the plant kingdom.

2. Results

2.1. Benzophenones as allylic pyrophosphate initiators of rubber polymerization

When the benzophenone (Bz) allylic pyrophosphate (APP) substrate analogs (Fig. 1) were tested for their effectiveness as initiators of rubber biosynthesis, RT-ases from all three species were able to incorporate IPP into new rubber (Fig. 2). The IPP incorporation rates initiated by Bz-GPP(*p*) and Bz-FPP(*m*) were at least as high as the IPP incorporation rate initiated by *E,E*-FPP, the natural initiator *in vivo*. Within each species, the V_{max}'s for Bz-GPP(*p*), Bz-FPP(*m*) and *E,E*-FPP were similar (Table 1), except that the $V_{max}{}^{Bz-GPP(p)}$ for *H. brasiliensis* was 155% of the $V_{max}{}^{E,E\text{-}FPP}$. The *H. brasiliensis* RT-ase was able to use Bz-DMAPP (*m*) and (*p*) to initiate IPP incorporation at a very similar rate to *E,E*-FPP, as well. However, for *F. elastica* and *P. argentatum*, the RT-ase velocities in Bz-DMAPP(*m*) and Bz-DMAPP(*p*) were substantially lower than those with *E,E*-FPP (Table 1).

When the K_m's (Table 1) of the Bz-APP initiators were compared to *E,E*-FPP, species-dependent variations became clear. In *F. elastica*, the maximum variation in K_m was only a factor of three, while in *H. brasiliensis* and *P. argentatum*, the variation in K_m was much greater, factors of 50 and 15, respectively. The K_m's of all the Bz-APP initiators in *H. brasiliensis* were considerably lower than the K_m for *E,E*-FPP. In fact, the K_m's for both Bz-DMAPP(*p*) and Bz-GPP(*p*) were as low as those found for *E,E*-FPP in *P. argentatum*, indicating a very high affinity of the RT-ase for these substrates. In addition, the relative binding affinities of the RT-ases for the different APP initiators were different among the three species. The affinity orders were as follows:

F. elastica

$$K_m^{Bz-DMAPP(m)} = K_m^{Bz-DMAPP(p)} < K_m^{Bz-GPP(p)} = K_m^{E,E-FPP} < K_m^{Bz-FPP(m)}.$$

H. brasiliensis

$$K_m^{Bz-DMAPP(p)} < K_m^{Bz-GPP(p)} < K_m^{Bz-FPP(m)} < K_m^{Bz-DMAPP(m)} < K_m^{E,E-FPP}.$$

P. argentatum

$$K_m^{E,E-FPP} < K_m^{Bz-DMAPP(m)} < K_m^{Bz-DMAPP(p)} = K_m^{Bz-FPP(m)} < K_m^{Bz-GPP(p)}.$$

2.2. Inhibition of activity by UV

2.2.1. Time

The UV light exposure necessary to inactivate RT-ase activity by photo affinity labeling was determined for the Bz-APP initiator, Bz-GPP (*p*) in all three species. Bz-DMAPP(*p*) also was tested but only in *H. brasiliensis*. The percentage of remaining RT-ase activity in washed (purified) (WRP) after UV light exposure time (Fig. 3) indicated that 70% of activity was lost from all three RT-ases after 5 min of UV light exposure in Bz-GPP(*p*) compared to the non-illuminated controls. During the same exposure time, RT-ase activity, assayed in the presence of *E,E*-FPP, was not inhibited, and 5 min of UV exposure seemed to slightly enhance activity of the *F. elastica* and *H. brasiliensis* RT-ases.

Maximum inhibition levels were reached in *P. argentatum* after 15 min (Fig. 3c), but 30 min were required to fully inhibit RT-ase activity in *F. elastica* (Fig. 3a) and *H. brasiliensis* (Fig. 3b). Bz-DMAPP(*p*) required approximately double the UV exposure time to inhibit the *H. brasiliensis* RT-ase to the same degree as Bz-GPP(*p*) (Fig. 3b).

It is also clear from the UV exposure of the *E,E*-FPP controls that prolonged exposure to UV light inhibited RT-ase activity. The *P. argentatum* RT-ase was the most sensitive to UV of the three enzymes, whereas the *H. brasiliensis* enzyme was the most resistant to UV light-induced inactivation. This is expected because the *P. argentatum* RT-ase is also the most temperature sensitive enzyme of the three (Cornish and Backhaus, 1990) due to its purification from tissue homogenates rather than tapped latex. Homogenization causes extensive release of degradative enzymes. UV light absorption, itself, damages proteins (and nucleic acids) and will eventually cause enzyme denaturation. Additional damage in the presence of the Bz-analogs is caused by reaction of the pi-pi star excited benzophenones' ketyl radicals non-specifically damaging proteins and lipids, and when the analogs covalently link to the proximal amino acids in the RT-ase active site they block access by other substrates (Dorman et al., 2016).

2.2.2. Concentration of Bz-GPP(p)

The three RT-ases selected to probe have similar but not identical