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Tuning the Production of Variable Length, Fluorescent Polyisoprenoids Using Surfactant-Controlled Enzymatic Synthesis

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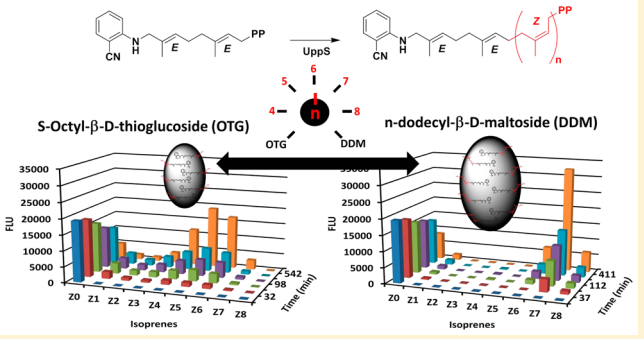
Supporting Information

ABSTRACT: Bactoprenyl diphosphate (BPP), a two-*E* eight-*Z* configuration C_{55} isoprenoid, serves as a critical anchor for the biosynthesis of complex glycans central to bacterial survival and pathogenesis. BPP is formed by the polymerase undecaprenyl pyrophosphate synthase (UppS), which catalyzes the elongation of a single farnesyl diphosphate (FPP) with eight *Z*-configuration isoprene units from eight isopentenyl diphosphates. *In vitro* analysis of UppS and other polyprenyl diphosphate synthases requires the addition of a surfactant such as Triton X-100 to stimulate the release of the hydrophobic product from the enzyme for effective and efficient turnover. Here using a fluorescent 2-nitrileanilinogeranyl diphosphate analogue of FPP, we have found that a wide range of surfactants can stimulate release of product from UppS and that the structure of the surfactant has a major impact on the lengths of products produced by the protein. Of particular importance, shorter chain surfactants promote the release of isoprenoids with four to six *Z*-configuration isoprene additions, while larger chain surfactants promote the formation of natural isoprenoid lengths (8*Z*) and larger. We have found that the product chain lengths can be readily controlled and coarsely tuned by adjusting surfactant identity, concentration, and reaction time. We have also found that binary mixtures of just two surfactants can be used to fine-tune isoprenoid lengths. The surfactant effects discovered do not appear to be significantly altered with an alternative isoprenoid substrate. However, the surfactant effects do appear to be dependent on differences in UppS between bacterial species. This work provides new insights into surfactant effects in enzymology and highlights how these effects can be leveraged for the chemoenzymatic synthesis of otherwise difficult to obtain glycan biosynthesis probes. This work also provides key reagents for the systematic analysis of structure−activity relationships between glycan biosynthesis enzymes and isoprenoid structure.



Bactoprenyl monophosphate (BP), a C_{55}, 11-unit polyisoprene with two-*E* and eight-*Z* configuration isoprene units [2*E*, 8*Z* (Figure 1)], is central to the biosynthesis of critical bacterial glycans, including peptidoglycan, capsules, and glycosylated proteins.[1−4] BP is formed via the dephosphorylation of bactoprenyl diphosphate (BPP),[5,6] which is produced by the polymerase undecaprenyl pyrophosphate synthase (UppS).[7,8] Increasingly, isoprenoid biosynthesis, isoprenoids themselves, and glycan production are becoming attractive targets for the development of novel antimicrobial agents.[9−12] However, little is known about how UppS effectors that alter isoprenoid chain length would affect glycan biosynthesis machinery.

Studies of the contribution of isoprenoid structure to glycan biosynthesis have been limited, in part because of the lack of ready access to systematically varied polyisoprenoid substrates. Most of the polyisoprenols available for assessing these structure−activity relationships are natural products isolated from diverse plant sources.[1,13] To probe how polyisoprene structure affects the activity of glycan biosynthesis enzymes, a method for effectively and rapidly producing systematically varied substrate analogues would be ideal. Chemical syntheses of variable length isoprenoids with controlled configurations have been described previously.[14−16] These methods depend on a series of isoprene building blocks and provide a wide range of options for assembling variable length glycan biosynthesis substrates. However, altering the structure beyond isoprenoid length and double bond configuration is a challenge. In particular, methods for chemically incorporating fluorescent tags into these variable length isoprenoids have not been developed, yet incorporation of such tags greatly simplifies the ability to detect the activity of proteins involved in glycan biosynthesis.[17,18]

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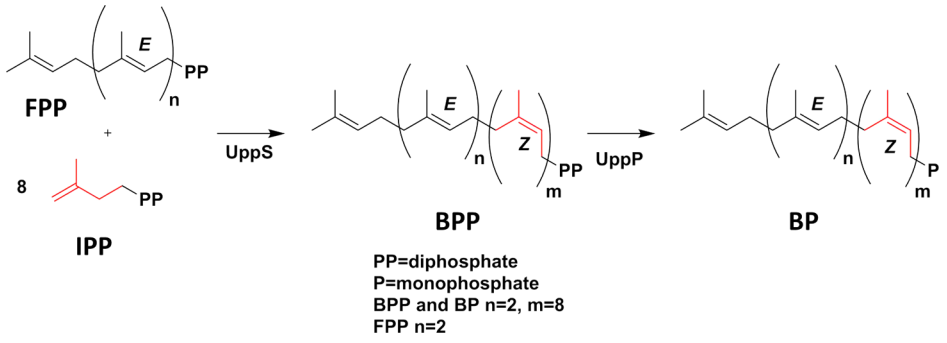

Figure 1. Biosynthesis of bactoprenyl phosphate.

Recently, our group has found that UppS from the gut microbe *Bacteroides fragilis* ($UppS_{Bf}$) can reliably form fluorescently tagged BPP mimics with six to eight *Z*-configuration isoprene units.[18−20] However, the enzyme does not readily form shorter isoprenoids. Transient and steady-state kinetic studies of *Escherichia coli* UppS ($UppS_{Ec}$) have shown that surfactants have a profound influence on BPP formation.[8,21,22] Triton X-100 (TX-100) promotes the release of full length product from the enzyme, increasing the steady-state reaction rate by changing the rate-limiting step from final product release to the rate of an early condensation step.[8] In $UppS_{Ec}$ reactions that do not include surfactant, BPP production occurs very slowly and products larger than the 2*E*, 8*Z* natural product are formed. Furthermore, a recent study describing the effects of surfactants on a series of nine previously uncharacterized, all-*E* polyisoprene diphosphate synthases (PDSs) highlighted the fact that there were major differences in product lengths that were dependent on the specific surfactant present.[23] However, no clear surfactant structure to polyisoprenoid length relationship was uncovered. The wide range of activity discovered with PDSs and surfactants prompted us to consider whether different surfactants influenced UppS product distributions. Here, we report a systematic analysis of how surfactants influence UppS product distributions and take advantage of specific effects to tune *B. fragilis* UppS as a synthetic tool for the controlled production of variable length fluorescent polyisoprenoids.

METHODS

General. 2CNA-GPP, 2AA-GPP, IPP synthesis, and UppS expressions were performed as described previously.[18−20] All HPLC analysis was performed on an Agilent 1100 HPLC instrument equipped with an autosampler, a diode array detector, a fluorescence detector, and an inline degasser. The HPLC stationary phase was a reverse phase C18 Agilent Eclipse XDB-C18, 5 μm, 4.6 mm × 150 mm column. The mobile phase consisted of 100 mM ammonium bicarbonate (A) mixed in variable amounts of *n*-propanol (B) as described below. Nitrileaniline-linked analogues were monitored at 340 nm absorbance or fluorescence at an excitation wavelength of 340 nm and an emission wavelength of 390 nm. The anthranilamide-linked analogue (2AA-GPP) was monitored at an excitation wavelength of 350 nm and an emission wavelength of 450 nm.

General Assay Protocol. All UppS reaction mixtures were prepared in the same reaction buffer, which contained a final concentration of 25 mM Bicine (pH 8.35), 0.5 mM $MgCl_2$, and 5 mM KCl unless noted otherwise. Analytical assays were prepared in reaction buffer with 6 μM 2CNA-GPP or 4 μM 2AA-GPP, 0.25 mM IPP, and either 86 nM $UppS_{Bf}$ or 94 nM $UppS_{Ec}$. Assay mixtures, unless noted otherwise, were incubated at 24 °C for 24−26 h, and then 100 μL of each was quenched with 100 μL of *n*-propanol. Aliquots (10 μL) were analyzed using the gradient HPLC method described below. $UppS_{Bf}$ 2CNA-GPP reaction mixtures contained 0.009, 0.027, 0.15, 0.30, 0.60, 1.2, 4.8, and 9.6% DDM. 2AA-GPP and $UppS_{Ec}$ variable DDM assays were prepared with DDM concentrations of 0.027, 0.12, 0.60, 2.4, and 9.6%. Assays for monitoring surfactant identity effects were prepared with 0.2, 2, 20, and 200 times the CMC listed in Table 1.

Table 1. Surfactant Properties and Major Products Formed

surfactant	CMC (%)[a]	monomer MW (g/mol)	200 × CMC
T-20	0.0072	1228	9−10*Z*
B-35	0.011	1198	9−10*Z*
TX-100	0.015	647	7−9*Z*
DDM	0.009	511	6−8*Z*
DM	0.087	483	5−7*Z*
HM	8.9	426	ND[b]
NG	0.20	306	ND[b]
OTG	0.28	308	ND[b]
HG	6.6	264	ND[b]

[a]All CMC values obtained from Anatrace or Pierce for the detergent in pure water. Products given are the major products with each surfactant at 200 × CMC. [b]Not determined.

Mixed Surfactant Assays. Assays were prepared in a final volume of reaction buffer of 200 μL with 59 μM 2CNA-GPP, 86 nM $UppS_{Bf}$, and 250 μM IPP. Control reaction mixtures testing T-20 binary mixtures contained either 5.7 mM T-20 (100 × CMC), 6.5 mM OTG (0.7 × CMC), or 5.9 mM DDM (33 × CMC) alone. Binary mixtures contained 5.7 mM T-20 and either 6.5 mM OTG or 5.9 mM DDM. Control reaction mixtures testing DDM binary mixtures contained 17.6 mM DDM (100 × CMC), 17.4 mM T-20 (300 × CMC), or 19.5 mM OTG (2.2 × CMC). Binary mixtures contained 17.6 mM DDM and either 17.4 mM T-20 or 19.5 mM OTG. Control reaction mixtures testing OTG binary mixtures contained either 9.7 mM OTG (1.1 × CMC), 9.4 mM DDM (53 × CMC), or 8.9 mM T-20 (150 × CMC). Binary mixtures contained 9.7 mM OTG and either 9.4 mM DDM or 8.9 mM T-20. Mixtures were incubated for 2 h at 37 °C and then reactions were quenched by a 100 μL addition of a 1:1 *n*-propanol/water mixture. HPLC analysis was conducted on 5 μL injections of each quenched reaction.

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Fine-Tuning Product with OTG/DDM Binary Mixtures. Reaction mixtures were prepared in a final volume of reaction buffer of 100 μL containing 15 μM 2CNA-GPP, 86 nM $UppS_{Bf}$, 0.25 mM IPP, and 0.18% DDM (3.5 mM, 20 × CMC). Reaction mixtures were also prepared with the same components and OTG at 1.3, 2.6, 5.2, 10.4, 20.8, 41.6, 83.2, or 166 mM (0.04−4.5%). Reaction mixtures were incubated overnight at 16 °C and then reactions were quenched with 50 μL of a 1:1 *n*-propanol/water mixture, and 10 μL injections were analyzed by HPLC.

Rates of Reaction of 2CNA-GPP with $UppS_{Bf}$. Reaction mixtures for monitoring the turnover rate of $UppS_{Bf}$ were prepared in reaction buffer containing 28 μM 2CNA-GPP and 0.25 mM IPP in either 1.8% DDM or 2.8% OTG. Rates were tested with $UppS_{Bf}$ concentrations of 8.6 and 4.3 nM. Upon addition of enzyme, 2 μL of the reaction mixture was sampled within 1 min and analyzed by HPLC. After each HPLC analysis, an additional 2 μL of the reaction mixture was sampled (~38 min intervals for 8.6 nM $UppS_{Bf}$ reactions and 72 min for 4.3 nM $UppS_{Bf}$ reactions in which OTG and DDM reactions were staggered). A minimum of five time points were acquired for each analysis. Peaks representing starting material and each product were integrated. Reaction rates for 2CNA-GPP consumed were calculated on the basis of a percentage of 2CNA-GPP remaining from the first time point (no significant product had been consumed at this time point, which is designated as time zero). Rates are reported as micromolar product formed per micromolar enzyme per second. Rates for product formations were not calculated because fluorescence increases with increasing isoprenoid lengths and propanol concentrations.

Micropreparative Scale UppS Reactions. Reaction mixtures were prepared in 200 μL of standard reaction buffer with 10 mM 2CNA-GPP, 1% DDM, 75 mM IPP, and 17.2 μM $UppS_{Bf}$. The reaction was quenched with *n*-propanol after 6 h. An identical scale reaction was performed in 2.8% OTG instead of DDM and was quenched after 1.5 h. Products from each reaction were purified by HPLC using the conditions described below. Fractions containing each 2CNA-BPP were then treated with 1 μL of alkaline phosphatase-linked anti-mouse secondary antibody (Pierce) diluted 1:100 for 1−3*Z* polyisoprenoids and undiluted for larger isoprenoids. Fraction volumes ranged from 0.5 to 3 mL. After incubation with phosphatase for at least 2 h, solvent was removed on a centrifugal vacuum concentrator. Product was resuspended in 40% *n*-propanol, vigorously vortexed, and placed in a sonication bath for 2 min. The concentration of product was determined on the basis of the extinction coefficient previously reported for the nitrileaniline at 340 nm of 2700 M^{-1} cm^{-1}. Diphosphate, monophosphate, and alcohol were then purified by HPLC, dried, and quantified. Monophosphates were prepared in 10 nmol aliquots, dried, and stored at −80 °C for future use, including ESI-MS analysis.

HPLC Analyses of Variable Component Reactions. Analytical HPLC was performed using a gradient method with a mobile phase of 100 mM ammonium bicarbonate (A) and *n*-propanol (B). For diphosphate and monophosphate analysis, the method started at 15% B and then increased to 80% B over 30 min (gradient of 2.167% B/min). Alcohol was analyzed utilizing a similar method, except the level of B was increased to 95% over 36.9 min. Purification of diphosphates was performed using procedures identical to the analytical method with a lowered fluorescence detector sensitivity from a photomultiplier gain of 18 to 8, and detection by absorbance at 340 nm. Monophosphates were analyzed and purified using a stepped gradient method in which 1ZP was analyzed and purified with a method starting at 22% *n*-propanol with a 2.17% B/min gradient, 2ZP was analyzed with a method starting at 28% *n*-propanol, and the remaining were analyzed and purified with a starting point for the 2.17% B/min gradient at 6% higher per isoprene unit.

ESI-MS Analysis of 2-Nitrileanilinobactoprenyl Monophosphates. MS analysis was performed on the 2CNA(1−8*Z*)BP and 2CNA-GP analogues with a Thermo VELOS Pro Dual-Pressure Linear Ion Trap using electrospray ionization, where the sample was introduced into the capillary via infusion with a flow rate of 0.100 mL/min of MS grade acetonitrile in a "T" with a Thermo UHPLC system. The BP analogues were diluted with a 50:50 mixture of MS grade acetonitrile and deionized water to 10 μM. ESI-MS was used in negative mode with a heater temperature of 250 °C, a capillary temperature of 200 °C, and a spray voltage of 3.90 kV.

Variable IPP, UppS, and 2CNA-GPP Assays. Assays with varying IPP, UppS, and 2CNA-GPP concentrations were prepared in reaction buffer as described above with 0.6% DDM. When held constant, the 2CNA-GPP concentration was 6 μM, the IPP concentration 0.25 mM, and the $UppS_{Bf}$ concentration 86 nM. The IPP concentration was varied from 5 to 1000 μM, the $UppS_{Bf}$ concentration from 0.01 to 8.6 μM, and the 2CNA-GPP concentration from 1.5 to 300 μM as indicated. Reactions were quenched as described above, and 10 μL was injected for HPLC analysis for varying IPP and $UppS_{Bf}$ concentrations. Injection volumes with varied 2CNA-GPP concentrations were adjusted to 60 pmol of nitrileaniline injected for each.

RESULTS

HPLC Separations of UppS Products. To characterize how surfactants influence UppS-catalyzed reaction product distributions, a new method for detecting a wide range of polyisoprenoid compounds was required. A series of fluorescent 2-nitrileaniline (2CNA) bactoprenyl diphosphates, monophosphates, and alcohols (Figure 2) were prepared as described

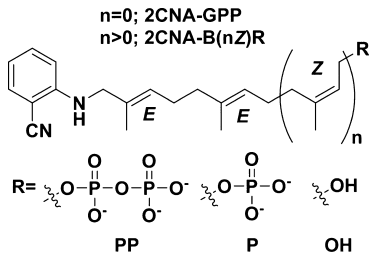

Figure 2. Structure of 2-nitrileaniline analogues.

below, and a single HPLC method was developed for separating and analyzing the variable length forms of each (Table 1 and Figure 1a of the Supporting Information). Under these conditions, the addition of each isoprene unit increased retention time linearly (Figure 1b of the Supporting Information). Importantly, in the analysis of isoprenoids of varying lengths, we found that nearly identical retention times (t_R) were observed when the starting propanol concentration was increased by 6% per isoprene. For example, analysis of a 2-nitrileanilinobactoprenyl monophosphate with one *Z*-configuration isoprene unit [2CNA-B(1*Z*)P] would be performed

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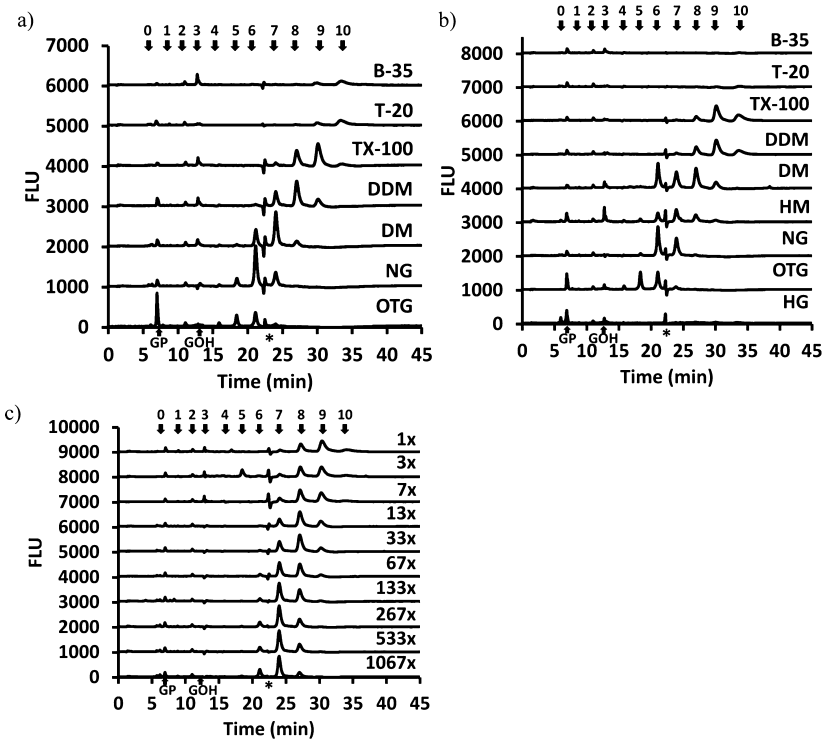

Figure 3. Surfactant identity and concentration influence product distributions. Overnight end point assays were performed with 6 μM 2CNA-GPP and $UppS_{Bf}$ in different detergents at (a) 20 × CMC and (b) 2 × CMC and then were analyzed by HPLC. (c) 2CNA-GPP reactions were prepared with variable DDM concentrations as indicated, and then product distributions were analyzed by HPLC. Each trace has been processed to smooth the baseline by subtracting a chromatogram of a water injection analyzed under identical gradient conditions. Product lengths are indicated above each set. 2CNA-GP (GP) and alcohol (GOH) formed from decomposition of starting material are noted on the time axis of each. The asterisk indicates a fluorescence peak associated with every analysis on multiple columns, including the water blank. The peak size varies slightly from injection to injection and appears to be due to the transition of a mostly aqueous to a mostly organic mobile phase. Note that larger isoprenoid peaks are broadened considerably. The peaks in panel b with B-35 and T-20 are too broad to easily see, and these surfactants may have led to even larger products that were not eluted from the column. Similar results are observed with 3 μM 2CNA-GPP.

starting with 22% *n*-propanol, giving a t_R of 6.4 min. Analysis of the 2CNA-B(2*Z*)P starting at 28% *n*-propanol with an identical gradient would result in a t_R for the 2*Z* material of 6.5 min (data not shown).

Surfactant Identity Affects UppS Product Distributions. Because surfactants have a unique influence on UppS activity, it was possible that altering this additive could allow for the controlled production of variable length 2*E*, *XZ* configuration BPPs, where *X* could be any number of isoprene units. We chose first to test this with UppS derived from *B. fragilis*, because in previous work we observed enhanced activity and broader product distributions relative to those of UppS derived from *E. coli* and *Vibrio vulnificus*.[19] To examine the influence of surfactant on UppS product distributions, we tested the most commonly utilized detergents in the study of UppS activity: TX-100, a relatively large polyethylene glycol (PEG)-based surfactant,[8,22] and the disaccharide-linked hydrocarbon *n*-dodecyl β-D-maltoside (DDM).[18,19] Two additional surfactants, Tween-20 (T-20) and Brij-35 (B-35), were also chosen to test the effects of very high molecular weight PEG-based materials (Figure 2 of the Supporting Information). The activity of a surfactant is typically dependent on the concentration relative to the critical micelle concentration [CMC (Table 1)]. $UppS_{Bf}$ activity was assayed with 2CNA-GPP in the presence of each surfactant below and well above the CMC (0.2 and 200 times, respectively) in overnight end point assays. As expected, when the surfactant concentration was below the CMC, no product was formed except in the presence of TX-100, where some long chain 9*Z* and 10*Z* isoprenoids were detected (Figure 3a of the Supporting Information). The 9−11*Z* isoprenoid identities were inferred on the basis of t_Rs of the smaller isoprenoids, which were characterized as described below. At 200 × CMC, the identity of the surfactant had a major influence on the size of products. With T-20 and B-35, very hydrophobic products that corresponded to 9−10*Z* polyisoprenoids were produced, and all starting material had been consumed (Table 1 and Figure 3b of the Supporting Information). With the monomeric lower-molecular weight TX-100, the major peaks observed corresponded to 7−9*Z* 2CNA-BPP, while with the even smaller DDM, the major peaks corresponded to the 6−8*Z* products. From these product distributions, there appeared to be a clear correlation between the size of the surfactant and the size of the product formed in its presence.

Surfactant Monomer Size Correlates with Product Length. Because there was a substantial difference in products formed with the large PEG-based surfactants, it was best to further examine these effects with materials that had systematically varied structural properties. To do this, five additional surfactants with controlled tail lengths and similar head groups were tested (Figure 2 of the Supporting Information). These were the variable tail length gluco- and maltoside-linked hexyl-β-D-maltoside (HM), hexyl-β-D-glucoside (HG), S-octyl-β-D-thioglucoside (OTG), nonyl-β-D-glucoside (NG), and decyl-β-D-maltoside (DM) (Table 1). Reaction mixtures with each of the maltoside, glucoside, and PEG surfactants were prepared at

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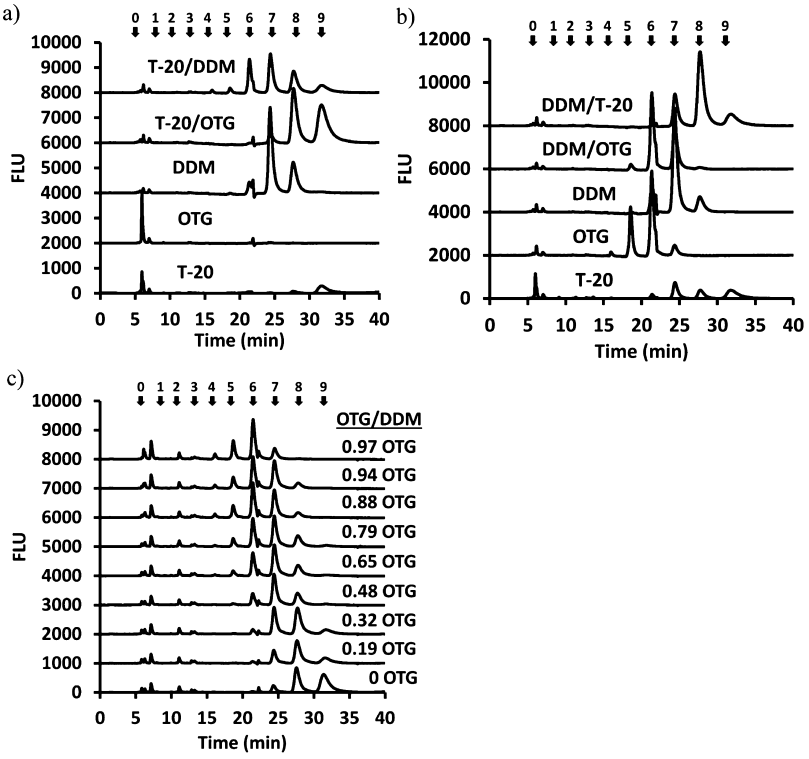

Figure 4. Binary surfactant systems lead to intermediate product lengths. Surfactant mixtures and controls were prepared as described in Methods with 6 μM 2CNA-GPP and $UppS_{Bf}$ and (a) 100 × CMC T-20 with equimolar DDM or OTG and (b) 100 × CMC DDM with equimolar T-20 and OTG. (c) Surfactant mixtures were prepared with 20 × CMC DDM and increasing OTG as indicated. HPLC analysis of each was processed as described in the legend of Figure 3. Similar results were observed with a wide range of surfactant concentrations in the binary mixtures with 3 μM 2CNA-GPP.

0.2, 2, 20, and 200 × CMC and were analyzed for product distributions after overnight reactions. Like the PEG-based materials, none of the glucoside or maltoside surfactants supported UppS-catalyzed turnover below CMC (Figure 3a of the Supporting Information). However, we noticed a peculiar trend with the 200 × CMC (Table 1), 20 × CMC (Figure 3a), and 2 × CMC (Figure 3b) reactions. With each surfactant, as the monomer size decreased, the product lengths produced by UppS also decreased. Interestingly, while the HM tail length was shorter than OTG and NG, the HM product distribution appeared to be similar to that of the next smallest maltoside surfactant DM. HG was unable to support formation of any product with the enzyme. These results suggested that smaller surfactants led to smaller isoprenoid products while larger surfactants resulted in much larger products, including products larger than would be expected *in vivo*.

An Increasing Surfactant Concentration Leads to Decreased Isoprenoid Length. In the data described above, it appeared that as the surfactant concentration increased, the size of products formed decreased. To more precisely investigate how surfactant concentration influences the products formed by the enzyme, overnight end point assays were prepared in the presence of DDM at 1−1067 × CMC (0.009−8.3%). Consistent with the data described above, the surfactant concentration did have a major influence on product distributions (Figure 3c). At 1 × CMC, the three major product peaks observed were 8−10*Z* 2CNA-BPP. As the surfactant concentration was increased, the relative sizes of the products decreased. At ≥33 × CMC, increasing levels of 2CNA-B(6*Z*)PP were formed, and at ≥133 × CMC, the 2CNA-B(9*Z*)PP product was no longer observed.

Surfactant Mixtures Lead to Intermediate Product Distributions. Single-surfactant-containing reaction mixtures led to a wide variety of reaction products. The length of hydrocarbon tails is known to strongly influence head-to-head micelle diameter, where increasing chain length leads to larger micelles.[24] The lengths of isoprenes formed by UppS appeared to correlate with these properties, with a possible exception of the hexyl chain surfactants. Recent work has also shown that binary mixed micelle solutions can form ideal mixtures with intermediate physical properties of the two surfactants making up the solution.[25] If binary surfactant mixtures lead to mixed micelles, then it is therefore possible that intermediate sized products could be produced in the presence of such binary mixtures. To test this possibility, a series of surfactant mixtures were prepared with T-20, DDM, and OTG, and UppS product formation with 2CNA-GPP was analyzed after a 2 h incubation. First, the T-20 concentration was kept at 100 × CMC, and either OTG or DDM was added to give a 0.5 mole fraction of the added surfactant relative to T-20. Each reaction product formed was compared with reaction mixtures containing only one of the surfactants at the same molar concentration (Figure 4a). In reactions with T-20 alone, the major product was a 10*Z* isoprenoid and more than half of the starting material remained. The incomplete reaction could have been due to limiting IPP, which was 8 equiv relative to 2CNA-GPP. OTG reactions alone at the same molar concentration of T-20 gave no product, as was expected because this was below the CMC for the surfactant. DDM provided a product similar to what would be expected on the basis of Figure 3c. Surprisingly, even though OTG was below CMC in the OTG/T-20 mixture, there was still a major influence on products formed relative to T-20

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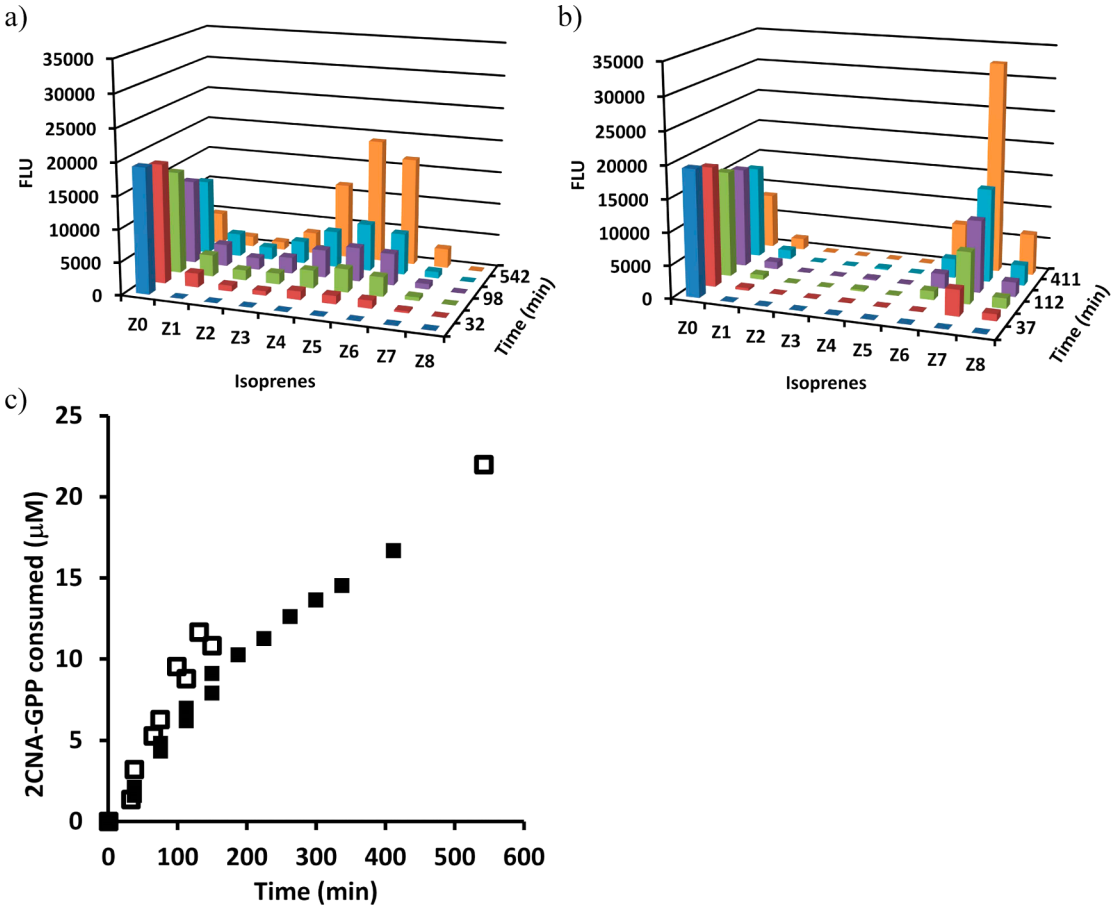

Figure 5. UppS releases short chain products. UppS reactions were analyzed by HPLC at intervals of 32−33 min in the presence of 8.4 nM enzyme and (a) OTG or (b) DDM. Product distributions were largely consistent throughout the reaction, although with OTG smaller isoprenoid products began to be consumed during later stages of the reaction. Peaks associated with each isoprenoid size were integrated and plotted as integral FLU vs isoprene number incorporated vs reaction time. Note that larger isoprenoids at higher propanol concentrations have increased fluorescence associated with them. Therefore, product peak integrals are larger than input substrate integrals. (c) Integrals from 2CNA-GPP peaks of replicate 8.4 nM $UppS_{Bf}$ reactions in either DDM (■) or OTG (□) were used to determine the substrate consumed over time. Another reaction (data not shown) was performed with 4.2 nM $UppS_{Bf}$ with each surfactant that was the expected half of the rate of the 8.4 nM reactions. Different colors are used to ease visualization of product at different time points for panels a and b.

alone. Rather than the 10*Z* product being formed, there was a clear distribution of the 7−9*Z* products in the presence of equimolar OTG. In the presence of DDM, there was also an intermediate product distribution relative to T-20 or DDM. In the presence of OTG or DDM, the T-20 reaction went to completion rather than stopping, again most likely because of the number of equivalents of IPP available for the shorter chain products. The products formed between T-20 and OTG were larger than those formed with T-20 and DDM. However, when the DDM concentration was held constant at 100 × CMC and T-20 or OTG was added to give a 0.5 mole fraction of each, the products differed substantially from those of the 100 × CMC T-20 reactions (Figure 4b). In this analysis, the T-20/DDM mixture led to an intermediate product between the two that was larger than the OTG/DDM mixture. In this case, the OTG concentration was above the CMC, and we noticed that while in OTG alone, substrate was not completely consumed by the enzyme. However, in the presence of DDM, the OTG/DDM mixture did lead to complete substrate consumption and to a product size intermediate between those of DDM and OTG. A similar experiment was performed with OTG at 1 × CMC (Figure 4 of the Supporting Information), which led to results similar to those shown in Figure 4b.

Tuning Polyisoprene Length Using Binary Surfactant Mixtures. Because the binary mixtures of surfactants led to intermediate product lengths, it was possible that with the right combination of surfactants the output of UppS could be tuned to a particular set of products. Binary mixtures of DDM and OTG were prepared in which DDM was held constant (0.18%, 20 × CMC), and OTG concentrations were increased from 0.04 to 4.5% (0.14−16 × CMC). As the mole fraction of OTG increased relative to that of DDM, there was a clear stepwise decrease in the length of isoprenoid products formed (Figure 4c). The UppS products under these conditions ranged from those that would be expected from DDM (7−9*Z*) alone to products that would be expected from OTG alone (4−7*Z*).

Short Reaction Times Lead to the Release of Smaller Isoprenoids. From the assays described above, it was clear that isoprenoids with 4−8 *Z*-units could be readily formed, but obtaining smaller isoprenoids remained problematic. We considered that smaller products, especially in the presence of smaller surfactants, may be released early in the reaction and then are taken back up by the protein and converted to larger products. In simple end point assays, as described above, it was not possible to determine whether the UppS product distributions were the same throughout the reaction. Previous

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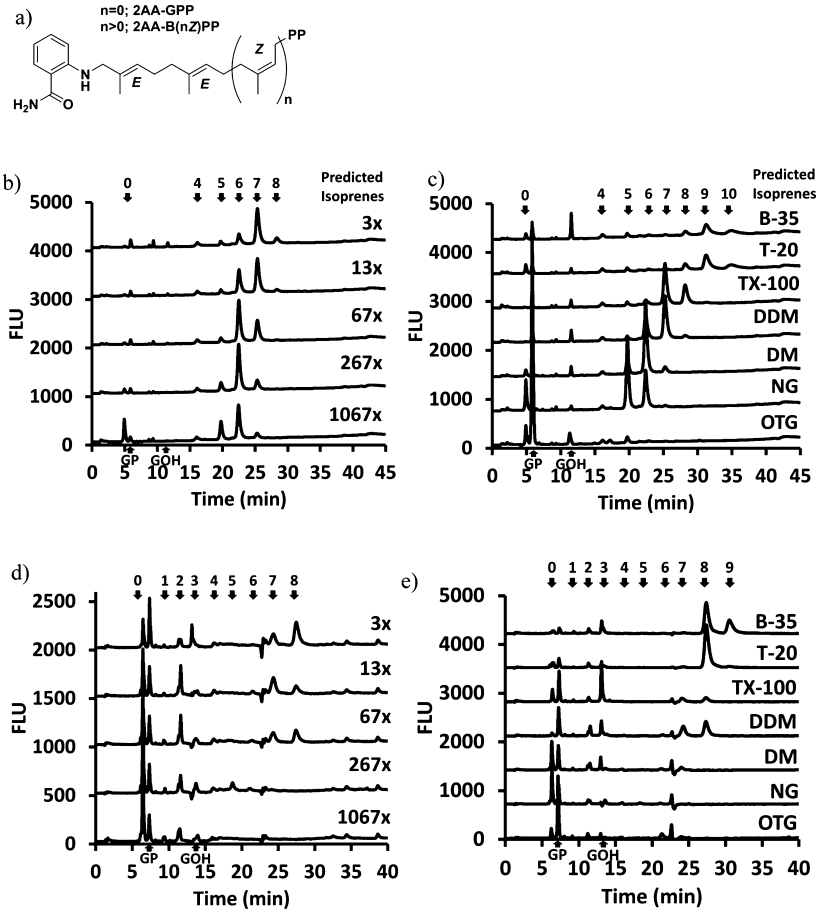

Figure 6. Surfactant effects are dependent on UppS species and independent of isoprenoid. The (a) 2AA-GPP analogue was tested for (b) surfactant concentration effects in DDM and (c) surfactant identity effects in the given surfactants at 20 × CMC. Products were analyzed by HPLC, and isoprene length is shown as predicted values. Chromatograms are of 4 μM 2AA-GPP reaction mixtures and are representative of at least two reaction sets. The 2CNA-GPP analogue was tested with UppS from *E. coli* as described above with 2AA-GPP in (c) varying concentrations of DDM and (d) different surfactants at 20 × CMC. Chromatograms represent reactions with 6 μM 2CNA-GPP and are similar to those observed with 3 μM substrate. A water blank was not subtracted from 2AA-GPP reaction chromatograms.

work from our group described a fluorescence plate reader assay for monitoring UppS product formation. However, that assay provides little information about product length.[19] In addition, changing the concentrations of surfactants in these assays leads to differences in fluorescence baselines that make kinetic comparisons between reaction conditions difficult to interpret (data not shown). To avoid complications associated with the plate-based method, we instead monitored the turnover of all products using a discontinuous HPLC method. 2CNA-GPP reactions were prepared with $UppS_{Bf}$ in DDM (100 × CMC) and OTG (10 × CMC). Rates were adjusted with a decreasing $UppS_{Bf}$ concentration to allow for removal of an aliquot that was immediately analyzed by HPLC, and then after each analysis, another aliquot was removed and analyzed. Each peak representing a product was integrated, and the products formed over time were compared (Figure 5a,b). From this analysis, it was clear that with both surfactants there was a linear decrease in 2CNA-GPP concentration over time in which the starting material was consumed at rates of 0.13 ± 0.05 and 0.13 ± 0.03 μM 2CNA-GPP (μM UppS)$^{-1}$ s^{-1} with OTG and DDM, respectively (Figure 5c). This indicated that the smaller product formation with OTG in the end point assays was not due to the enzyme functioning slower and denaturing before forming a product similar to DDM. Instead, these data suggested that OTG did promote the release of smaller product more readily than the larger surfactant. It was also clear from this analysis that the 1*Z* product could be released in the reaction regardless of whether the surfactant was OTG or DDM (Figure 5a,b), yet 2−3*Z* isoprenoids were released in significant steady-state quantities only with OTG. Lastly, in the presence of OTG, the nunber of 1−3 *Z*-unit products increased over the first few hours of the reaction, but by the time of the last analysis, these products were partially consumed. These data indicated that the UppS reaction could be further tuned to provide smaller isoprenoid chains by altering the timing of the reaction in the presence of OTG.

Preparation of Variable Chain Length Isoprenoids. Using the information from the assays described above, we attempted to prepare each isoprenoid length in micro-preparative quantities (>100 nmol, where 0.5 nmol would be required for downstream use with glycosyltransferases).[17−19,26] Reaction mixtures were prepared in a volume of 200 μL, which contained 17.2 μM $UppS_{Bf}$, 10 mM 2CNA-GPP, and 75 mM IPP in OTG at 20 × CMC or DDM at 100 × CMC. The OTG reaction was quenched after 1.5 h with *n*-propanol, while the DDM reaction was allowed to continue for several hours. We chose the 1.5 h time point with OTG because it provided large quantities of smaller isoprenoids that could have been consumed if the reaction mixture had been incubated for a longer period of time. Under these conditions with OTG,

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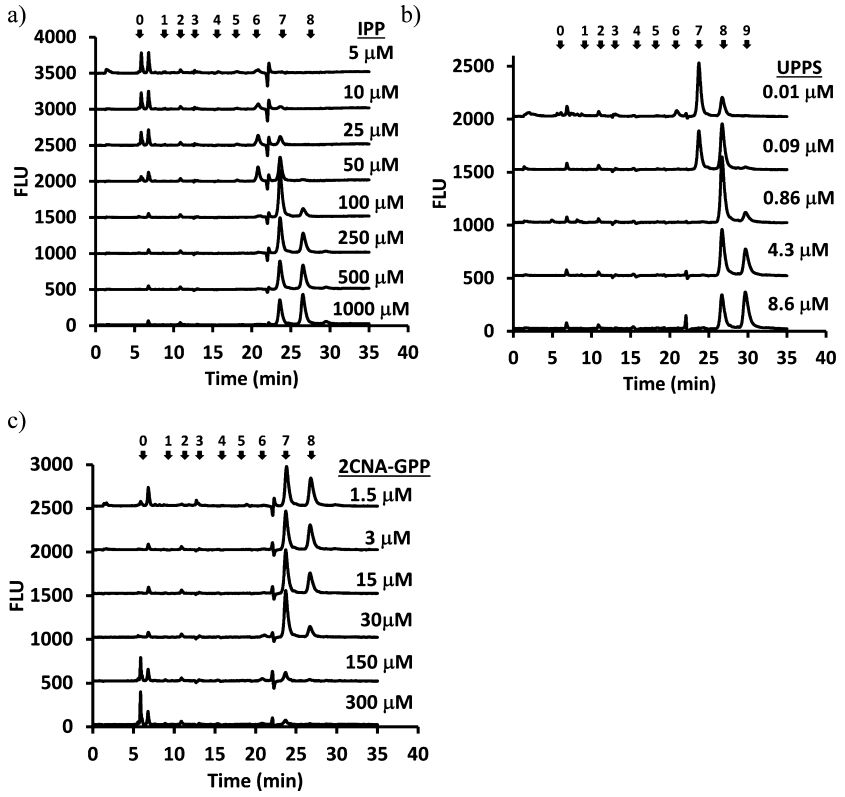

Figure 7. Isoprene donor and enzyme concentration influence product distributions. Overnight end point assays with 2CNA-GPP and $UppS_{Bf}$ were prepared with varying (a) IPP, (b) $UppS_{Bf}$, or (c) 2CNA-GPP concentrations. Chromatograms have a water blank injection subtracted from each and are representative of at least two trials.

>80% of the starting material had been consumed on the basis of integration of the UV−absorbance trace of the HPLC chromatogram (Table 2 of the Supporting Information). All of the starting material was consumed in the DDM reaction, as expected. The isolated product yields based on input 2CNA-GPP were 44 and 47% in the OTG and DDM reactions, respectively (Table 2 of the Supporting Information). In our experience, we have had difficulty obtaining electrospray ionization mass spectrometry (ESI-MS) data on the isoprenoid-linked diphosphates but not the monophosphate. Fractions collected from the HPLC isolations were treated with alkaline phosphatase to hydrolyze the diphosphate to a monophosphate, using a slight variation of a previously described protocol.[18,20] After incubation over 2 h, solvent was removed and product isolated by HPLC (Table 3 of the Supporting Information). Monophosphates (1−8*Z*) were characterized by ESI-MS (Figure 5 of the Supporting Information). It is important to note that the polyisoprenyl diphosphates could be cleaved by the alkaline phosphatase to give monophosphate in the highest yields, but some alcohol product was also formed. Interestingly, smaller isoprenoids appeared to be more sensitive to overdigestion than the larger isoprenoids on the basis of an experiment in which 10 μM solutions of each were treated with alkaline phosphatase and then assayed by HPLC (Figure 1a of the Supporting Information). We found that diluting the alkaline phosphatase 1:100 greatly reduced the rate of overdigestion (data not shown).

Surfactant-Influenced Product Distributions Are Not Unique to 2CNA-GPP. It was possible that the product distributions observed with 2CNA-GPP could be specific to the use of that particular substrate analogue. To determine whether this effect was specific to 2CNA-GPP, the anthranilamide analogue 2AA-GPP (Figure 6a) was tested with $UppS_{Bf}$. The response to varying surfactant concentrations with this analogue was very similar to the response with 2CNA-GPP (Figure 6b), where an increasing surfactant concentration led to smaller isoprenoids produced, as indicated by a decreased t_R. In addition, an assay was performed with 2AA-GPP with OTG, NG, DM, DDM, TX-100, T-20, and B35 at 20 × CMC. It was found that a trend similar to that of 2CNA-GPP was again observed, where much larger surfactants led to larger products (Figure 6c). One noticeable difference was that OTG was not an effective surfactant for the 2AA-GPP reaction, as very little product was formed. However, this was consistent with the decreased rate of turnover with 2CNA-GPP (Figure 3a,b) and the known lower activity of 2AA-GPP with $UppS_{Bf}$.[19] We attempted to use a binary mixture of OTG with DDM to improve the yields of smaller isoprenoids with 2AA-GPP, but this was unsuccessful (data not shown).

Surfactant-Influenced Product Distributions Are Not Universal. The surfactant effects observed above did not appear to be dependent on the isoprenoid used, but it was not clear whether UppS from other organisms would be affected in the same way. Using 2CNA-GPP with UppS from *E. coli*, the response to varying DDM concentrations was dramatically different from that of the *B. fragilis* protein (Figure 6d). Products primarily with 7−8*Z* isoprene units were formed using $UppS_{Ec}$ with DDM from 3 to 67 × CMC, while at ≥267 × CMC, very little, if any, product was observed. In all cases, there was some decomposition of starting material to monophosphate and alcohol. Lastly, an assay with 2CNA-

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GPP was prepared with $UppS_{Ec}$ and the same surfactants that were tested with 2AA-GPP. It was found that no larger isoprenoid products were formed in the presence of surfactants smaller than DDM (Figure 6e). Some 7−8*Z* products were formed with DDM and TX-100, yet considerable 2CNA-GPP remained. However, in the presence of the longer chain T-20 and B-35, all 2CNA-GPP appeared to be consumed with very little decomposition to monophosphate or alcohol. Of particular interest was the fact that only 2CNA-B(8*Z*)PP appeared to form with T-20, and in the presence of B-35, both 8*Z* and 9*Z* 2CNA-BPP products were formed. It is important to note that nearly all other studies of UppS have detected product on the basis of either the release of diphosphate or the incorporation of radiolabel from isopentenyl diphosphate.[8,12,22] The decreased rate of turnover of starting acceptor isoprenoid (FPP or analogues of FPP) is rarely analyzed. Together, these data suggested that the *B. fragilis* UppS was sensitive to surfactant concentration in a way that did not lead to the failure of the enzyme to function and that this sensitivity was predominantly independent of the isoprenoid supplied to the protein. However, the *E. coli* protein appeared to form only 2CNA-B(7−9*Z*)PP products and potentially some low levels of 2*Z* and 3*Z* products regardless of surfactant concentration, except in cases where the surfactant concentration was too high to support significant turnover. The inability to support turnover in higher concentrations of surfactant or varying surfactants could relate to the stability of the protein in these mixtures, although this was not directly probed.

The Isoprene Donor Influences the Product Distribution. Previous work with $UppS_{Ec}$ has demonstrated that altering the relative concentrations of FPP to the concentration of IPP or enzyme leads to altered product distributions.[8] Overnight $UppS_{Bf}$ reaction mixtures with 6 μM 2CNA-GPP were prepared with IPP concentrations ranging from 5 to 1000 μM. HPLC analysis of these mixtures demonstrated that, with an increasing IPP concentration, clear increases in the total amount of product formed and in the size of product were apparent (Figure 7a). As the number of IPP equivalents was decreased, the distribution of products trended slightly toward smaller products. However, when ≤8 equiv of IPP, relative to analogue, was used, some 2CNA-GPP remained or had decomposed to monophosphate. While smaller isoprenoids were formed using lower concentrations of IPP, the amount of consumed 2CNA-GPP was clearly not appropriate for making this an effective strategy for preparing short chain materials on a usable scale. UppS concentrations were varied from 0.010 to 8.6 μM with 0.25 mM IPP and 6 μM 2CNA-GPP. Near stoichiometric concentrations of enzyme (relative to 2CNA-GPP) provided primarily 8−9*Z* products, while lower concentrations led to 7−8*Z* products (Figure 7b). Lastly, product distributions were similar when the 2CNA-GPP concentration was increased from 1.5 to 30 μM in the presence of 0.25 mM IPP (Figure 7c). Increasing the concentrations of 2CNA-GPP to near stoichiometric amounts (relative to IPP) led to results similar to those observed with decreasing IPP concentrations. This suggested that alterations in product by changing 2CNA-GPP concentrations were primarily an effect of the number of IPP equivalents present.

DISCUSSION

In this work, we have found that the type of surfactant used in *in vitro* UppS reactions significantly affects the distribution of products formed by the polymerization enzyme in a way that has not been observed before. It has been known for nearly 15 years that the surfactant TX-100 promotes release of product from UppS and has been used in the majority of work in which the protein has been studied.[8,22,27] Monomers of TX-100 have even been noted in X-ray crystal structures of the protein, which suggest the location of a critical hydrophobic tunnel through which the isoprenoid is thought to thread during elongation.[21] The work presented here suggests that surfactants do not just promote the release of the final product of the UppS-catalyzed reaction but in some instances can promote the early release of the isoprenoid from the protein.

The tendency of surfactants to promote premature release or lead to the release of longer chain products than would be expected *in vivo* may have a profound influence on how UppS is screened for potential inhibitors. Here we have shown that $UppS_{Bf}$ was much more sensitive to surfactant effects than $UppS_{Ec}$, and in the presence of TX-100, $UppS_{Bf}$ catalyzed the production of isoprenoids much longer than would be expected. Interestingly, $UppS_{Ec}$ products were of the expected length, but T-20 was much more effective at promoting full consumption of starting material and producing only the 8*Z* BPP product. These results strongly suggest that care should be taken with the surfactant chosen for large screens of UppS activity as one type may not be effective for every UppS from every bacterial species.

Another critical aspect of UppS catalysis that this work highlights is the fact that while different products are produced in the presence of different surfactants, the influence on the overall rate of the reaction does not appear to be substantial. This does not come as a surprise because surfactant-promoted release of product changes the rate-limiting step of the reaction from product release to one of the condensation steps.[8] Importantly, with $UppS_{Ec}$, the smallest single-turnover rate constant is associated with the conversion of the 1*Z* to 2*Z* product where the rate constant is 2 s^{-1} while all other condensations are 2.5−3 s^{-1}.[8] Similarly, in our end point assays, we see an early and significant amount of released 2CNA-B(1*Z*)PP that is later consumed by the enzyme. This buildup of the smaller products is likely due to a similar rate-limiting step in the *B. fragilis* enzyme. This could be extremely important. This suggests that steady-state rates are largely dependent on these first steps in the reaction. Steady-state measurements typically performed in large drug screens depend on primarily measuring the rate of diphosphate released or radiolabel incorporated from IPP, and only compounds that affect these rates are further scrutinized for chain length effects.[22,27,28] A small molecule effector of UppS that induces the early release of product may not be detectable by these screens even though this could be a viable way to affect downstream glycan biosynthesis machinery. Alternatively, screens coupling both the rate and major product produced may prove to be critical in detecting such compounds from potential drug libraries.

How the varying surfactants lead to changes in UppS product distributions has led us to recalibrate how we think about the way that surfactants promote release of product by the enzyme. First, it does not appear that the chain must be fully elongated for effective release from the enzyme. This was especially surprising considering the processive nature of the protein. Instead, it appears that the ability of the isoprenoid to insert into a micelle has a much more critical influence. Recent work from the Columbus group has highlighted the effects of surfactant size and in particular the length of hydrophobic tails on the size of micelles formed by surfactants.[24] On average, the

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length of a micelle from headgroup to headgroup decreases with tail length by ~2.9 Å per carbon for maltosides and ~2.6 Å per carbon for glucosides. Here we have observed UppS products with DDM, DM, NG, and OTG decrease one to two isoprene units per surfactant tail carbon. However, we also observed that HM leads to a product that is the same length as DM, suggesting there may be a lower limit to this effect that could be dependent on the size of the headgroup. Surfactants that would be expected to lead to larger micelles also lead to larger isoprenoid products. Altogether, we propose that the effect on chain length observed may be due to the size of micelles formed by these surfactants, and the product released is the size that is more easily accommodated by a particular sized micelle. However, it is important to be clear that more data need to be obtained with respect to other micelle dimensions to confirm this. It is also not clear how micelle packing may influence product distributions, although on the basis of the surfactant concentration effects observed it may play a significant role. In addition, other surfactants may be more spherical than the maltosides and glucosides or have other shape parameters that affect the ability of the isoprenoids to pack into them. It would be of considerable interest to see how phospholipid-based bicelles affect product distributions and how bicelles that more closely mimic the inner membrane makeup of the particular organism in question influence product formation. It is widely assumed that BPP is a 2*E*, 8*Z* isoprenoid in all bacteria, but this may not always be the case.

While synthetic methods have been developed for making a wide range of polyisoprenoids and are ideal for multigram quantities of these materials,[14,16] the dependence on a large number of synthetic steps to the individual building blocks and additional steps to form the targeted isoprenoid can be challenging. Alternatively, other groups have focused on the utilization of naturally available isoprenoids for the study of effects of isoprenoid structure on glycan biosynthesis machinery.[1] However, short chain isoprenoids are largely not available from known sources. Smaller isoprenoids are typically easier to handle in most assay systems, as there is less potential for aggregation and the formation of micelles by the isoprenoids alone. This isoprenoid micelle formation may contribute to the decreased activity observed for some natural isoprenoids with glycan biosynthesis enzymes.[16,20,29] Here we provide a simple rapid method (two enzymatic steps for the fluorescent analogue of FPP) for the production of a wide range of fluorescent polyisoprenoid substrates to systematically evaluate how isoprenoid length influences the activity of proteins involved in glycan biosynthesis. The methods outlined should also be amenable to the natural substrate, FPP, or a radiolabeled form of that isoprenoid. In addition, access to the smaller isoprenoid substrates will allow for the determination of the minimal substrate for these enzymes and allow for the use of simple smaller isoprenoids for the study of a wide range of glycan biosynthesis proteins.

■ ASSOCIATED CONTENT

Supporting Information

Chromatograms of 2CNA-geranyl and bactoprenyl diphosphates, monophosphates, and alcohols for each length, a plot of isoprenoids incorporated versus retention time, surfactant structures, 0.2 and 200 × CMC reaction chromatograms with each surfactant and 2CNA-GPP, chromatograms of reaction mixtures comparing binary mixtures of surfactants with 1.1 × CMC OTG, ESI-MS of all monophosphates, fluorescence increase over time in the presence of variable amounts of HG, chromatograms of reaction mixtures with variable IPP, UppS, or 2CNA-GPP concentrations, and tables of retention times, product yields, and product characterizations. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00310.

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Notes

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■ ABBREVIATIONS

FPP, farnesyl diphosphate; IPP, isopentenyl diphosphate; t_R, retention time; ESI-MS, electrospray ionization mass spectrometry; HPLC, high-performance liquid chromatography; 2CNA, 2-nitrileaniline; BPP, bactoprenyl diphosphate; BP, bactoprenyl phosphate; BOH, bactoprenol; GPP, geranyl diphosphate; GP, geranyl monophosphate; GOH, geraniol; FPP, farnesyl diphosphate; IPP, isopentenyl diphosphate; CMC, critical micelle concentration; UppS, undecaprenyl pyrophosphate synthase; PDS, polyisoprenyl diphosphate synthase; PEG, polyethylene glycol; TX-100, Triton X-100; DDM, *n*-dodecyl-*β*-D-maltoside; T-20, Tween-20; B-35, Brij-35; HM, hexyl-*β*-D-maltoside; HG, hexyl-*β*-D-glucopyranoside; OTG, *S*-octyl-*β*-D-thioglucoside; NG, nonyl-*β*-D-glucopyranoside; DM, decyl-*β*-D-maltoside; FLU, fluorescence units; Ex, excitation; Em, emission; UV/vis, ultraviolet/visible.

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