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Substrate Selectivity of Squalene Synthetase[†]

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ABSTRACT: Six 1-^{3}H-labeled analogues of farnesyl pyrophosphate have been studied as potential substrates for yeast and rat liver squalene synthetases: 2-methylfarnesyl pyrophosphate (4), 3-demethylfarnesyl pyrophosphate (5), 7,11-dimethyl-3-ethyl-2,6,10-dodecatrienyl pyrophosphate (6), 6,7,10,11-tetrahydrofarnesyl pyrophosphate (7), 4-methylthiofarnesyl pyrophosphate (8), and 4-fluorofarnesyl pyrophosphate (9). Analogues 4 and 5 are enzymatically incorporated into 11-methylsqualene (10) and 10-demethylsqualene (11), respectively, even if no farnesyl pyrophosphate is *added* to the incubations. None of the other analogues gives nonpolar products with either the yeast or liver enzymes. No tritium is enzymatically released to the medium from any of the analogues, indicating that they are not accepted at the first (proton exchanging) site. The data rule out formation of dead-end presqualene pyrophosphate products with analogues as first, but not as second, substrates. Implications of these results for the enzyme active-site topology and mechanism are discussed.

The biosynthesis of squalene (3) from two molecules of farnesyl pyrophosphate (1) is a surprisingly complicated process. During the condensation reaction a proton on the pyrophosphate bearing carbon of one of the two identical substrate units is replaced by a hydrogen from NADPH[1] (Popjak et al., 1961, 1962; Childs and Bloch, 1962). This proton exchange, which is not subject to an isotope effect (Popjak et al., 1962), was elegantly shown to occur with a net retention of configuration, while displacement of the pyrophosphate group from the nonexchanging substrate unit occurs with a net inversion of configuration (Cornforth et al., 1966a,b). The complexity implied by asymmetric proton exchange was substantiated on isolation (Rilling, 1966) and structural characterization (Epstein and Rilling, 1970; Popjak et al., 1973; Altman et al., 1971; Coates and Robinson, 1971) of presqualene pyrophosphate (2), a discrete intermediate in the enzymatic process (Muscio et al., 1974). Squalene synthetase, the membrane-bound reaction catalyst, has been isolated in soluble form from yeast (Schechter and Bloch, 1971; Qureshi et al., 1973b), and

[1] Abbreviations used are: NADP+, NADPH, oxidized and reduced nicotinamide adenine dinucleotide phosphate; TLC, thin-layer chromatography; GLC, gas-liquid chromatography.

in a partially purified but still insoluble state from pig liver (Dugan and Porter, 1972). The soluble enzyme has been reported to exist in a fully competent polymeric form which reversibly dissociates to protomeric units only able to synthesize presqualene pyrophosphate (Qureshi et al., 1973b). Kinetic studies with soluble, polymeric enzyme showed that presqualene pyrophosphate is the product of a ping-pong bi-bi binding sequence, consistent with irreversible loss of inorganic pyrophosphate from one substrate unit[2] before binding of the second (Beytia et al., 1973). Independently, it was found that presqualene pyrophosphate is converted to squalene by a sequential ordered bi-ter kinetic sequence, with NADPH binding before 2 while products are released in the order inorganic pyrophosphate, squalene, NADP⁺. The kinetic meshing of the two reactions (1 → 2 → 3) in the intact enzyme, however, is unclear, since the binding sequences are based on a study of the uncoupled reactions with solubilized enzyme.

Limited data on the enzyme active site have been obtained with artificial substrates. Extension of the tail end of farnesyl pyrophosphate by one carbon atom is acceptable to the enzymes from rat (Polito et al., 1972) and pig (Ogura et al., 1972) liver, although extension by two carbons is reported to be unacceptable in the case of pig liver. On the other hand, the coupling of geranylgeranyl pyrophosphate by yeast squalene synthetase has been observed (Qureshi et al., 1973a), implying that chain extensions of more than one carbon are acceptable. It has also been reported that farnesyl triphosphate is not a viable substrate (Schechter, 1973). Nevertheless, the mechanism, active-site topology, and substrate specificity of squalene synthetase remain very poorly defined. As part of a general investigation of the active site of this vital enzyme, we report here a study of squalene synthetase using six critically modified pseudosubstrates as experimental probes. Part of the results with two of the six substrate analogues has been the subject of preliminary communications (Ortiz de Montellano et al., 1976a,b).

Experimental Section

Substrates. The synthesis of the following unlabeled compounds has been described (Ortiz de Montellano et al., 1977): 2-methylfarnesyl pyrophosphate (4), 3-demethylfarnesyl pyrophosphate (5), 7,11-dimethyl-3-ethyl-2,6,10-dodecatrienyl pyrophosphate ("3-ethylfarnesyl pyrophosphate", 6), 6,7,10,11-tetrahydrofarnesyl pyrophosphate (7), and 4-methylthiofarnesyl pyrophosphate (8). Analogue 9, 4-fluorofarnesyl pyrophosphate, was prepared by phosphorylation of (2E,6E)-4-fluorofarnesol exactly as described for the other pyrophosphates. The fluorinated alcohol was obtained by LiAlH₄ reduction of ethyl 4-fluorofarnesoate (Machleidt and Grell, 1965; Ortiz de Montellano and Vinson, 1977). Since the alcohol precursors of all the pyrophosphate analogues except 5 were obtained by metal hydride reduction of the corresponding ethyl esters, the C-1 tritium-labeled compounds were made by reduction of the esters with [³H]LiAlH₄. Oxidation of farnesol and 3-demethylfarnesol to the corresponding aldehydes with activated manganese dioxide (Fieser and Fieser, 1967), followed by reduction with [³H]LiAlH₄, provided the C-1 labeled alcohol precursors of 1 and 5. [1,1-²H₂]-1 was prepared by phosphorylation of the alcohol obtained by reduction of ethyl farnesoate with LiAlD₄. The specific activities of the tritium-labeled pyrophosphates are given in Table I.

R = geranyl =
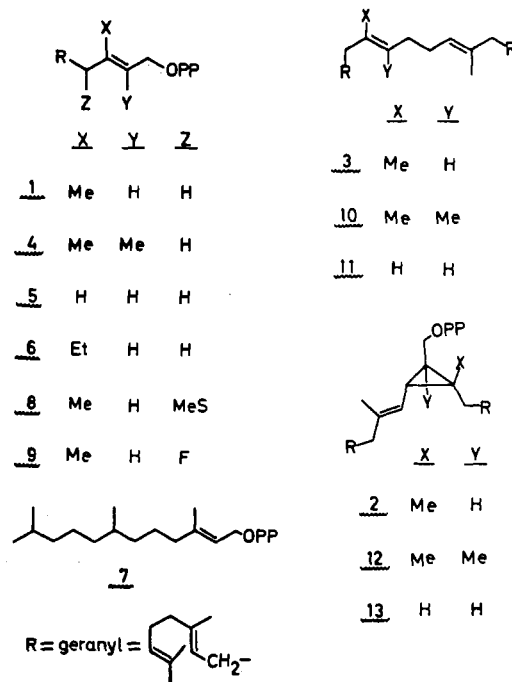
Their structures were confirmed by thin-layer chromatographic analysis on silica gel plates (1-propanol–NH₄OH–H₂O, 6:3:1), by quantitative phosphorus analysis, and, except for 9, by regeneration of the starting alcohols on treatment with bacterial alkaline phosphatase (Ortiz de Montellano et al., 1977). Analogue 9, a poor substrate for the phosphatase, appeared to isomerize during hydrolysis.

Enzymes. The particulate but partially purified preparation of squalene synthetase from baker's yeast used in this investigation was obtained as previously described (Ortiz de Montellano et al., 1977). Rat liver squalene synthetase was prepared by an exactly analogous sequence of steps except that disruption of cells from male rat liver was accomplished with a Potter-Elvejhem homogenizer rather than by sonication. Both enzymes were stored in small aliquots at −20 °C until used. Protein concentrations were determined by the procedure of Sutherland et al. (1949).

Incubations and Product Isolation. Standard incubations contained the following components at the given concentrations: labeled substrate, 20 μM; MgCl₂, 10 mM; NADPH, 1.6 mM; NH₄OH, 0.8 mM (due to storage of substrates in this medium); protein, 1.34 mg/mL; and potassium phosphate buffer (pH 7.5), 50 mM. When indicated, unlabeled farnesyl pyrophosphate (20 μM) was also added. Individual components of the standard assay mixture varied in specific experiments as indicated in the text. Incubations, initiated by addition of the enzyme to the other components prewarmed to 37 °C, were terminated by addition of ethanol or methanol (2 mL per mL of incubation mixture). Incubations were carried out at 37 °C for 10 min unless otherwise indicated. Inactivation of the enzyme in control experiments was usually accomplished by adding the ethanol to the enzyme *before* the incubation, although in some cases the enzyme was denatured by immersion in boiling water.

Small (1 mL) incubations were employed, except as indicated, to quantitatively evaluate the incorporation of labeled substrate analogues into nonpolar, squalene-like, products. The nonpolar products, after extraction with 30–60 °C petroleum ether, were filtered through silica gel minicolumns and were assayed by liquid scintillation counting with internal standardization as previously described for farnesyl pyrophosphate

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[2] Throughout this paper, the farnesyl pyrophosphate unit which undergoes proton exchange is referred to as the first substrate, and its binding site as the first substrate site.

TABLE I: Enzymatic Hydrocarbon Synthesis and Tritium Release.[a]

Substrate	Sp act. (mCi/mmol)	Yeast enzyme	Nonpolar products (dpm)	Net nonpolar products (dpm)	Distilled[b] tritium (dpm)	Net distilled[b,d] tritium (dpm)
1	2.1	Active	82 400	82 300	5 100	4870[c]
		Inactive	80		230	
4	0.52	Active	570	510	500	0
		Inactive	60		500	
5	17.2	Active	2 100	1 750	11 300	0
		Inactive	350		11 400	
6	4.6	Active	70	0	500	0
		Inactive	70		600	
7	16.2	Active	190	0	330	0
		Inactive	190		370	
8	2.8	Active	590	40	1 300	0
		Inactive	550		1 400	
9	0.80	Active	70	0	120	0
		Inactive	70		140	

[a] Measured in standard, 2 mL, incubations containing 20 μM unlabeled 1 and 4.75 mg/mL enzyme protein. [b] Radioactivity in the 2-mL methanol distillate. The approximate total tritium (as H^+) in the medium can be obtained by multiplying the given value by 6.48, the calculated mole ratio of exchangeable protons in 6 mL of 2:1 methanol-water to those in 2 mL of methanol (Rilling, 1970). [c] This tritium release (4870 $\times$ 6.48 = 31 600 dpm) is consistent with that required by the squalene formed (82 300/3 = 27 400 dpm). [d] A zero value is indicated when more tritium was observed in the control than in the active enzyme incubation.

itself (Ortiz de Montellano et al., 1977). Control experiments showed that polar materials, such as the free alcohols of the analogues, did not interfere in the assay.

Large scale incubations of approximately 100 mL were employed in isolation of products for structural elucidation studies. Only the yeast enzyme was used in these cases. Unlabeled 1 was routinely added to large scale incubations once it was established it did not alter the product distribution. Because preparative incubations were continued for 90 min, the mixtures were flushed with nitrogen prior to addition of the enzyme and were maintained under nitrogen during the incubation period. Nonpolar products, extracted with freshly distilled hexane (3 $\times$ 300 mL), were purified either by preparative layer chromatography on commercial glass-backed silica gel plates which had been precleaned by developing twice with freshly distilled $CHCl_3$, or by chromatography on a 0.6 $\times$ 5 cm column of 100-200 mesh neutral (activity I) alumina, using 5% diethyl ether in hexane as eluting solvent. Control experiments with analytical plates established that essentially all of the radioactivity due to enzymatic processes was located in a band surrounding endogenous squalene, although approximately 25% of the hexane-extractable radioactivity remained near the origin. This labeled polar fraction was observed, however, even in incubations with inactive enzyme.

Product Characterization. Purified nonpolar products, indistinguishable from squalene by TLC, were submitted to gas chromatographic analysis on a flame ionization instrument fitted with a 4:1 ratio stream splitter (collection outlet favored) and 6 ft $\times$ 2 mm i.d. glass columns packed with 3% OV-225 on 100-200 mesh Varaport 30. Sample collection from the splitter was achieved by passing the effluent through U-shaped tubes cooled in dry ice-acetone. The condensate was subsequently eluted with hexane and its radioactivity measured by liquid scintillation counting. Comparison of the injected and collected radioactivity demonstrated that about 75% of the label was recovered. Retention time comparisons between radioactive peaks and authentic compounds were made by coinjection of mixed samples.

All gas chromatography-mass spectrometry was performed with previously described equipment (Gruenke et al., 1974),

using 2% Dexsil 300 on 80-100 Chromosorb GHP as the gas chromatographic column packing. The synthesis of the following gas chromatographic and mass spectrometric standards has been described: 11-methylsqualene (10), 11,14-dimethylsqualene, 10-demethylsqualene (11), and 10,15-didemethylsqualene (Ortiz de Montellano et al., 1976a,b; Corey et al., 1968).

Enzymatic Proton Exchange. Hydrogen exchange during enzymatic condensation was determined by measurement of tritium in the medium after enzymatic incubation of C-1 tritium-labeled farnesyl pyrophosphate analogues (Epstein and Rilling, 1970; Rilling, 1970). Standard incubations (2 mL each), including unlabeled farnesyl pyrophosphate, were terminated with methanol. After petroleum ether extraction and normal radioassay of the hydrocarbons, 2 mL of methanol was distilled from the remaining (about 6 mL) aqueous methanol mixture, and its radioactivity was measured by liquid scintillation counting. Tritium release was measured at several protein concentrations to permit a more reliable differentiation between enzymatic and nonenzymatic processes.

Results

The formation of radioactive nonpolar products on incubation of labeled analogues 4-9 with a yeast enzyme preparation is compared in Table I with the synthesis of squalene from [1-^{3}H]farnesyl pyrophosphate. The data in the table are from incubations to which unlabeled 1 has been added, although the pattern of results is similar when this cosubstrate is omitted (see Table II). Control experiments, however, suggest that low levels of farnesyl pyrophosphate are present in the enzyme preparation itself. The increase in nonpolar product radioactivity due to incubation of 4 or 5 with active vs. inactive enzyme clearly suggests enzymatic catalysis, while the absence of such an increase in incubations of 6-9 argues that extractable radioactivity in these cases is due to nonenzymatic processes. In order to identify enzymatic reactions with greater precision, the formation of nonpolar products was measured for each analogue as a function of protein concentration. The resulting linear increase in nonpolar product formation from 4 or 5 (Figure 1) confirms the intervention of
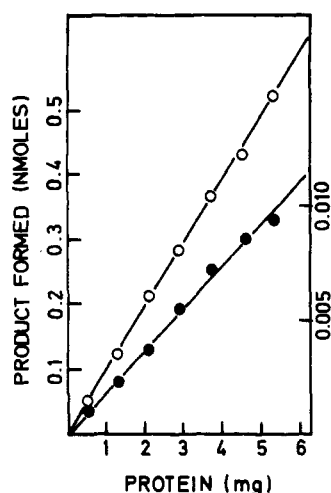
FIGURE 1: Incorporation of 4 and 5 into hydrocarbons as a function of protein concentration. Open circles are data points for 4 and refer to the y axis on the left side; closed circles are data points for 5 and refer to the y axis on the right side.
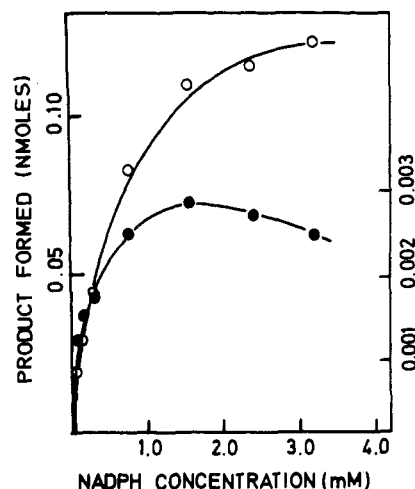

FIGURE 2: Hydrocarbon synthesis from 4 and 5 as a function of NADPH concentration. Open circles are data points for 4 and refer to the y axis on the left side; closed circles are data points for 5 and refer to the y axis on the right side.

TABLE II: Hydrocarbon Formation by Yeast and Liver Squalene Synthetases.[a]

Labeled substrate	Added unlabeled 1	Labeled substrate incorporated (nmol)	
		Yeast	Liver
1[b]	No	3.73	6.87
	Yes	2.54	5.89
4	No	0.06	0.23
	Yes	0.02	0.11
5	No	0.007	0.02
	Yes	0.010	0.04

[a] Measured in standard incubations containing 15 μM substrate and, when indicated, 15 μM 1. [b] The observed radioactivity was multiplied by 1.33 to correct for loss of one-quarter of the label by enzymatic exchange. Similar corrections are not necessary in the other cases.

an enzyme, while the absence of such a systematic increase with the other four compounds clearly demonstrates that they are not enzymatically incorporated into nonpolar products. This latter experiment specifically demonstrates that the small enhancement in nonpolar products due to incubation of 8 with active enzyme (Table I) is not significant.

The incorporation of 4–9 into nonpolar products has also been investigated with rat liver microsomal preparations. As with yeast, only 4 and 5 are detectable substrates. The incorporation of these two artificial substrates into hydrocarbons by yeast and rat liver squalene synthetases, in the presence and absence of added unlabeled 1, is compared in Table II. Exactly the same pattern of results is obtained with both enzymes, although the liver preparation has a higher (per weight) level of enzymatic activity.

An investigation was made of the formation of nonpolar products from 4 and 5 as a function of the concentration of the labeled pseudosubstrates, of NADPH, and of unlabeled 1, in order to characterize the enzymatic transformation. Kinetic saturation of hydrocarbon synthesis is observed as the concentration of 4 or 5 is increased to 40 μM (not shown), or as the concentration of NADPH is raised to 4 mM (Figure 2). Inhibition of the enzymatic reaction occurs at the higher concentrations of each variable, as expected from the known

substrate and cofactor inhibition of squalene synthetase (Beytia et al., 1973; Dugan and Porter, 1972). The formation of nonpolar products as the concentration of added unlabeled farnesyl pyrophosphate is varied from 0 to 80 μM is stimulated at low concentrations but is depressed at higher values. The difference in the added concentration of 1 which maximizes hydrocarbon synthesis from 4 (2–3 μM) and 5 (about 10 μM) explains why in Table II the addition of farnesyl pyrophosphate reduces labeled product formation from 4 but raises that from 5.

Two approximately equal peaks of radioactivity were found on gas chromatographic analysis of the product from 4, one with a retention time similar to that of endogenous squalene (7.6 min, 232 °C), and the other associated with a higher retention time, flame ionization detectable peak (10.0 min, 232 °C). This latter peak, unlike squalene, is not found in control incubations with inactive enzyme. The radioactivity distribution is approximately the same whether unlabeled 1 is added to the incubations or not, although the squalene peak is greatly enhanced in the presence of 1. Coinjection of authentic all-(E)-11-methylsqualene (10) and 11,14-dimethylsqualene, the most likely products, demonstrated that the radioactive peak at 10.0 min had the same retention time as 10, while no radioactive peak coincided with the dimethyl analogue. The product with the 10.0-min retention time was conclusively identified as 10 by its mass spectrum (Figure 3), which exhibits a fragmentation pattern parallel to that of squalene itself (Polito et al., 1972), and which is nearly identical with that from the synthetic sample of 10. The lower retention time radioactive product has not been identified because its mass spectrum and properties are masked by those of the superimposed squalene, although its TLC and GLC behavior are consistent with those of a C_{31} squalene-like hydrocarbon.

Gas chromatographic analysis of the product from 5 revealed a single peak of radioactivity. Only this one radioactive peak was observed whether unlabeled 1 was added to the incubation or not. Coinjection of synthetic all-(E)-10-demethylsqualene (11) and 10,15-didemethylsqualene established that the radioactive substance had the same retention time as 11 (6.11 min, 230 °C). Firm identification of the radioactive product as 11 was possible on the basis of its mass spectrum (Figure 4), which is, except for minor variations, identical with that of an authentic sample.
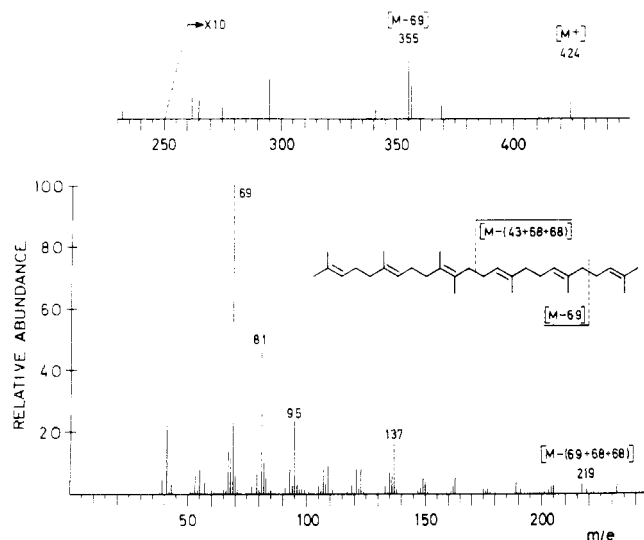
FIGURE 3: Mass spectrum of enzymatically synthesized 11-methyl-squalene (10).

FIGURE 4: Mass spectrum of enzymatically synthesized 10-demethylsqualene (11).

The tritium released to the medium on incubation of each analogue with active vs. inactive enzyme in a typical experiment is given in Table I. It is clear from the results that enzymatic tritium release does not occur with any of the analogues, although significant amounts of tritium are released by nonenzymatic processes. In contrast, tritium exchange during enzymatic synthesis of squalene from 1 is in close agreement with the expected value. Because of the high background radioactivity obtained with some analogues, part of which was generated during methanol distillation, the release of tritium from each analogue was measured as a function of protein concentration. Failure to observe a systematic increase in tritium release in these latter experiments reinforces the conclusion that C-1 protons are not enzymatically removed from any of the analogues. These results demonstrate that none of the analogues undergoes catalysis as a first (proton exchanging) substrate, and thus specifically establish that 4 and 5 are incorporated into 10 and 11, respectively, only as second substrates. This was confirmed, in the case of 10, by the mass spectrum of the product obtained on preparative incubation of unlabeled 4 with 1,1-dideuteriofarnesyl pyrophosphate. The mass spectrum exhibits a parent peak and rationalizable fragments primarily attributable to monodeuterio-10, thus implying that dideuterio-1 was utilized primarily or exclusively as the first substrate.

Discussion

The conformational preferences of the six analogues have been studied using Dreiding models in conjunction with computer minimization of the steric interactions between substituents on adjacent atoms, assuming standard bond lengths and "hard sphere" atomic radii.[3] This simple analysis, which neglects electronic effects, suggests that analogues 4–6 and 9 have favored conformations similar to that of 1, while those of 7 and 8, although probably different, are not separated from it by major steric barriers. Although electronic effects may affect the conformations of the heteroatom-substituted compounds 8 and 9, it is unlikely from the steric analysis that any of the

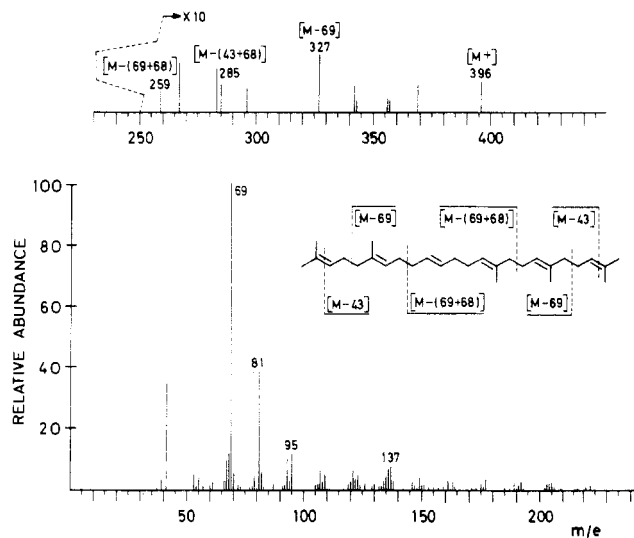analogues will be catalytically frustrated purely because of a conformational prohibition. Experimental support for this conclusion is drawn from the fact that each of the analogues is a competitive, or partially competitive, inhibitor of squalene synthetase (Ortiz de Montellano et al., 1977). The analogues thus exist in conformations suitable for binding at the enzyme active site. The relatively permissive requirements for active-site binding, however, contrast sharply with the stringent demands of the squalene synthetase catalytic mechanism. Of the six analogues, only 4 and 5 are enzymatically incorporated into squalene-like products (10 and 11, respectively), and then only as non-proton-exchanging cosubstrates with farnesyl pyrophosphate. This was true of both yeast and rat liver squalene synthetases, although the hydrocarbon products were completely characterized only in the case of yeast. The absence of enzymatic tritium release from any of the C-1 labeled analogues furthermore demonstrates that none of the compounds was incorporated as a *first* (proton exchanging) substrate into a dead-end presqualene pyrophosphate structure, although the data on 6–9 do not preclude their incorporation as second substrates into such structures. The exclusion of analogues 4 and 5 from one of the two substrate incorporation steps demonstrates that the two farnesyl pyrophosphate precursors of a squalene molecule are bound in topologically distinguishable sites, the first[2] site being more discriminating. These two sites may each include independent pyrophosphate and hydrocarbon binding regions, since both are important for binding (Ortiz de Montellano et al., 1977), or they may consist of a single bifurcated region with one pyrophosphate and two hydrocarbon binding domains. This latter alternative is possible since kinetic studies suggest that the pyrophosphate is displaced from the first substrate unit before binding of the second (Beytia et al., 1973).

The enzymatic rejection of 4–9 as first substrates provides insight into the active site of squalene synthetase. Although the sensitivity of the enzyme to introduction of a 4-methylthio function on the substrate is not surprising, considering the size of the substituent, its impotence in the face of a 4-fluoro moiety is more unexpected. Since the increase in bulk due to fluorine substitution is minimal, the effect of this highly electronegative moiety is likely to result from an electronic perturbation. The observation that prenyltransferase catalyzed reactions of

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[3] The computer modeling was performed on the PROPHET system sponsored by the National Institutes of Health for manipulation of biomedical data.

(E)-3-trifluoromethyl-2-buten-1-yl pyrophosphate (Poulter et al., 1976) and 2-fluoroisopentenyl pyrophosphate (Poulter and Rilling, 1976) are much slower than those of the normal substrates has been interpreted as evidence for an ion pair enzymatic reaction mechanism, in which fluorine inhibits the reaction by inductive destabilization of the cationic transition state. The close structural similarity of these pyrophosphates and **9**, the obvious evolutionary relationship between prenyl-transferase and squalene synthetase, and the required displacement of the pyrophosphate from the first substrate are all consistent with a similar explanation for the catalytic failure of **9**, although alternative rationalizations for a fluorine electronic effect are possible. The ability of the 2,3-double bond to complex and react with electrophiles, for example, is also compromised by the 4-fluoro substituent.

The incompetence of both the 3-demethyl (**5**) and 3-ethyl (**6**) analogues as first substrates contrasts with the acceptance of **5**, but not **6**, at the second substrate site. If the attenuated activity of **5** at either site were purely a consequence of an electronic difference between the normally trisubstituted 2,3-double bond and the disubstituted bond in **5**, then **6**, which is again trisubstituted, should have been a substrate. The catalytic failure of **6** thus suggests that the 3-methyl is bound in a sterically congested region unable to accommodate an ethyl group. The importance of the 3-methyl is delineated by the fact that **5** is approximately ten times poorer as a cosubstrate than **4**. Few mechanisms can be formulated to explain the required presence of the 3-methyl group if electronic, conformational, and active-site binding rationalizations are excluded. The only straightforward explanations are that the methyl is specifically bound to anchor a favored substrate orientation, that the methyl triggers a conformational change in the protein to a catalytically active form, or both. The acceptance of **5** as a second substrate, albeit poor, suggests an immobilization role for the 3-methyl in that substrate site. The absence of detectable turnover of **5** in the first site, on the other hand, although explainable by an even stricter immobilization requirement, is more consistent with an induced-fit mechanism.

Catalytic rejection of the tetrahydro analogue **7** and its demonstrated weak binding at the active site (Ortiz de Montellano et al., 1977) indicate that for steric or electronic reasons at least one of the removed double bonds must be present for productive binding. If only one is essential, its relative proximity to the reacting center and the greater conformational perturbation associated with its reduction suggest that the 6,7-double bond is likely to be the more critical.

The tritium release experiments rule out formation of dead-end presqualene pyrophosphate analogues, or other polar products, with **4–9** as first substrates. Evidence is not available to rule out incorporation of **6–9** as second substrates into such nonviable polar products, although the synthesis of **10** and **11** from **4** and **5**, respectively, implies that the corresponding presqualene structures **12** and **13** are catalytically acceptable.

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