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Yeast Squalene Synthase

A MECHANISM FOR ADDITION OF SUBSTRATES AND ACTIVATION BY NADPH*

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Squalene synthase catalyzes the condensation of two molecules of farnesyl diphosphate (FPP) to give presqualene diphosphate (PSPP) and the subsequent reductive rearrangement of PSPP to squalene. Previous studies of the mechanism of addition of FPP to the enzyme have led to conflicting interpretations of initial velocity measurements (Beytia, E., Qureshi, A. A., and Porter, J. W. (1973) *J. Biol. Chem.* 248, 1856–1867; Agnew, W. S., and Popjak, G. (1978) *J. Biol. Chem.* 253, 4566–4573). Initial velocities for synthesis of PSPP and squalene were measured over a wider range of FPP and NADPH concentrations than previously reported, using a soluble form of recombinant enzyme. In the absence of NADPH, PSPP formation was activated by FPP at concentrations above ~0.5 μM. At fixed levels of NADPH, the dependence of initial rates of PSPP and squalene synthesis on FPP concentrations indicated that the C_{15} substrate added by a sequential mechanism. In addition, NADPH stimulated synthesis of PSPP by 40-fold at saturating levels of the cofactor. This stimulation is, at least in part, by reduction of PSPP to squalene.

Squalene synthase (farnesyl diphosphate:farnesyl diphosphate farnesyl transferase; EC 2.5.1.21) catalyzes the formation of squalene from two molecules of farnesyl diphosphate (FPP)[1] and NADPH. As illustrated in Scheme 1, this transformation occurs in two distinct steps. The first is a prenyl transfer reaction where the farnesyl residue of one FPP is inserted into the C(2)-C(3) double bond of the other to give presqualene diphosphate (PSPP). PSPP is then converted to squalene by a series of carbocationic rearrangements that are terminated by the transfer of hydride from the cofactor (1). These are the first pathway-specific reactions in cholesterol biosynthesis.

In addition to the synthesis of cholesterol, FPP is a precursor for ubiquinones required for cellular redox reactions (2), dolichols needed for glycoprotein biosynthesis (3), heme a for oxygen transport (4), and the C_{15} and C_{20} isoprenoid chains found in post-translationally modified proteins (5). The highly branched nature of the pathway and the central role of FPP in isoprenoid metabolism makes squalene synthase an attractive target for inhibiting cholesterol biosynthesis without disrupting the flow of FPP into non-sterol metabolites (6–10).

Several groups have reported kinetic studies of squalene synthase. Shechter and Bloch (11) proposed a sequential mechanism based on initial velocity measurements for the synthesis of squalene from FPP. However, this work was reported before PSPP was discovered and did not take into account the more complex nature of the transformation. In a later study, Beytia *et al.* (12) measured initial velocities for the formation of PSPP and squalene from FPP. They concluded that PSPP was formed by a ping-pong mechanism and that PSPP was then converted to squalene by a sequential process. The ping-pong mechanism was questioned by Agnew and Popjak (13), who concluded that it was not possible to distinguish between ping-pong and sequential additions of FPP to squalene synthase from their data or those of Beytia *et al.* (12).

There is little information about the catalytic site of squalene synthase. Agnew and Popjak (13) found that the rates of PSPP and squalene formation were stoichiometric at low FPP concentrations when synthesis of PSPP and squalene from FPP were measured simultaneously. However, at higher FPP concentrations conversion of PSPP to squalene was severely inhibited, while PSPP synthesis was not. They proposed that squalene synthase had different, but not completely independent, binding sites for FPP and PSPP. In a later study with purified enzyme, Sasiak and Rilling (14) did not observe the preferential inhibition of squalene synthesis reported by Agnew and Popjak (13). A similar conclusion was reached by Poulter *et al.* (15) from studies with inhibitors designed to mimic putative carbocationic intermediates in the conversion of PSPP to squalene. They found that the compound inhibited both steps in a similar manner and proposed that the enzyme contained a single or two overlapping catalytic sites.

Squalene synthase is a microsomal enzyme that has resisted purification since its discovery in the late 1950s and only recently have small quantities of homogenous wild-type protein become available (14). Much of the discrepancy among various kinetic studies reported to date can be traced to problems encountered with measuring initial velocities with impure microsomal preparations of the enzyme. DNA sequences of the open reading frames for squalene synthase from *Saccharomyces cerevisiae* (16, 17), *Schizosaccharomyces pombe* (18), rats (19), and humans (18, 20) have recently been reported. From the sequence of amino acids deduced for the yeast protein, Jennings *et al.* (16) proposed that the membrane-bound enzyme contained a large catalytic domain that was exposed to the cytoplasm and anchored to the endoplasmic reticulum by a single C-terminal transmembrane helix. This hypothesis was subsequently supported by genetic manipulation of the yeast enzyme (21) and limited proteolysis of the rat protein (22). Zhang *et al.* (21) recently synthesized a soluble form of yeast squalene synthase in *Escherichia coli* by deleting the C-terminal helix. The truncated recombinant protein was purified to homogeneity, and its catalytic properties were found to be similar to those of the microsomal wild-type enzyme. We now report a kinetic

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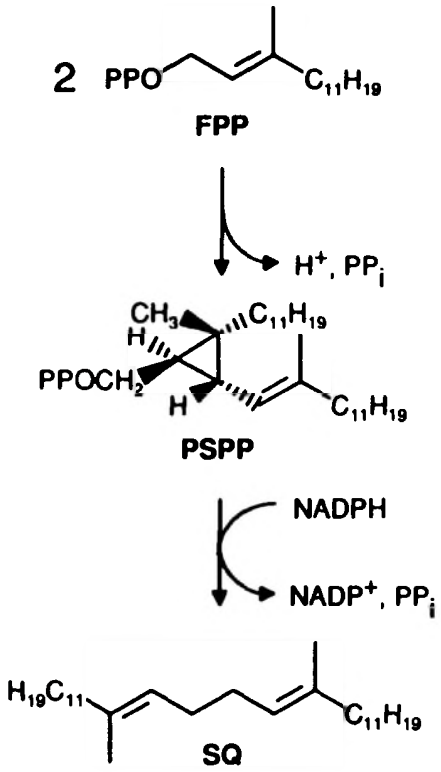
[1] The abbreviations used are: FPP, farnesyl diphosphate; PSPP, presqualene diphosphate; TLC, thin layer chromatography; MOPS, 4-morpholinepropanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)-methylglycine.



SCHEME 1. **Synthesis of squalene from FPP.**

study with the soluble protein that resolves some of the uncertainties in the mechanisms proposed for addition of FPP to the enzyme.

EXPERIMENTAL PROCEDURES

Materials

Silica gel polyester-backed thin layer plates (PE SIL G) were purchased from Whatman. Tween-80 and NADPH were from Sigma; EIA grade gelatin was from Bio-Rad. Opti-Fluor (Packard) was used for liquid scintillation spectrometry. All other reagents and solvents were reagent grade or higher. FPP was synthesized by the procedure of Davisson *et al.* (23). [1-^{3}H]FPP (26.9 Ci/mmol) was purchased from DuPont NEN.

Soluble truncated recombinant *S. cerevisiae* squalene synthase (M_r 48,882) was prepared as described in Zhang *et al.* (21). The enzyme was stored at −70 °C in 200 mM potassium phosphate, pH 7.2, or 50 mM MOPS, pH 7.2, both containing 1 mM dithiothreitol and 10% (v/v) glycerol. Stock solutions of enzyme were thawed and diluted to appropriate concentrations just before use.

The activity of truncated squalene synthase was determined immediately before each set of kinetic measurements by duplicate assays in buffer containing 50 mM Tricine, pH 7.4, 10 mM $MgCl_2$, 2% Tween-80, 1 mM 2-mercaptoethanol, 10% (v/v) methanol, 10% (v/v) glycerol, 0.1 mg/ml gelatin, 0.1 μM FPP, and 1 mM NADPH at 30 °C. This value was used to normalize the data set to a specific activity of 0.097 μmol min^{-1} mg^{-1} obtained with a freshly purified sample under identical conditions.

Methods

Initial Velocity Measurements

Squalene—Assays were carried out at 30 °C in buffer containing 50 mM Tricine, pH 7.4, 10 mM $MgCl_2$, 2% Tween-80, 1 mM 2-mercaptoethanol, 10% (v/v) methanol, 10% (v/v) glycerol, 0.1 mg/ml gelatin, and the indicated concentrations of substrates. Enzyme was diluted in the same buffer just before use. The specific activity of [^{3}H]FPP was adjusted to give a total of 60,000–300,000 disintegrations/min/10 μl of assay mixture. All ingredients except enzyme were combined in a total volume of 9 μl and incubated at 30 °C for 5 min before the reaction was initiated by the addition of 1 μl of a stock solution containing enzyme. The tubes were returned to the water bath; after 2 min the reaction quenched by spotting 2–6 μl on a TLC plate prespotted with 200 mM EDTA, and the spot was air-dried. Unlabeled squalene was then spotted over the sample to facilitate visualization. The plate was developed in $CHCl_3$ and lightly visualized with iodine. The spots corresponding to squalene were cut from the plate, placed in 10 ml of Opti-Fluor, and the amount of [^{3}H]squalene was measured by liquid scintillation spectrometry. Squalene concentrations were calculated assuming that 75% of the tritium in FPP was incorporated into the product (13). All kinetic assays for squalene and PSPP (see below) were run in duplicate, and assays at low substrate or NADPH concentrations where rates were slow were run in triplicate.

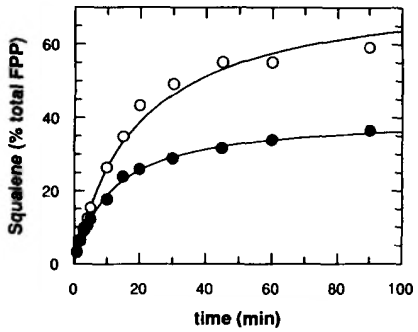


FIG. 1. **Time course for formation of [^{3}H]squalene from 0.1 μM (●) and 5 μM (○) [1-^{3}H]FPP by squalene synthase in buffer containing 1 mM NADPH.** Assays were carried out in duplicate. Data are presented as percent of total FPP converted to squalene.

Presqualene Diphosphate—Formation of presqualene diphosphate was measured by the proton release assay (13, 24). The assay was carried out in the same manner as described for the formation of squalene except that the reaction volume was increased to 30–50 μl, and the reaction quenched by the addition of 0.5 M EDTA to a final concentration of 50 mM. A sample (2–6 μl) was spotted on a silica gel TLC plate, and squalene was assayed as described above. Two milliliters of methanol were added to 30 μl of the quenched sample, the methanol was distilled, and radioactivity in the distillate was determined by liquid scintillation spectrometry. The amount of presqualene diphosphate was estimated by assuming that 25% of the radioactivity in FPP was released as $^3H^+$ upon formation of presqualene diphosphate (13). Controls with no enzyme were analyzed in order to obtain background levels of methanol-distillable tritium.

Data Analysis

Kinetic data were fitted to the appropriate equations using GraFit (Erithacus Software). The regression analyses were performed using a statistical variation where σ_i^2 (σ_i is the variance in v_i) varies in proportion to v_i and the weighting function is $(y_i)^{-1}$.

RESULTS

Assays

A series of experiments was conducted with soluble squalene synthase to determine optimum conditions for measuring initial velocities over a wide range of FPP concentrations. The best reproducibility was obtained for short reaction times in buffer containing methanol, glycerol, gelatin, and Tween-80. Under these conditions, the rates of formation of PSPP and squalene were linear up to 10% conversion of FPP. PSPP synthesis was followed by release of tritium from the *S*-enantiomer of [1-^{3}H]FPP during the condensation to form the cyclopropane ring in PSPP, according to the protocol originally developed by Kuswik-Rabiega and Rilling (24). The released tritium was recovered as a methanol distillate and represented 25% of the total radioactivity available in two molecules of racemic [1-^{3}H]FPP. Squalene synthesis was followed by monitoring incorporation of tritium into material purified by TLC. In this case, the recovered radioactivity represented 75% of that originally present in two molecules of the racemic substrate. The rates of PSPP and squalene synthesis did not depend on the specific activity of the substrate.

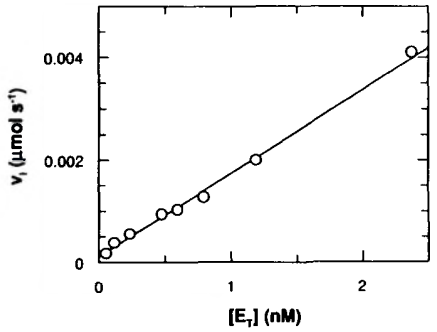


FIG. 2. **Variations of initial velocity for squalene synthesis with enzyme concentration in buffer containing 30 μM FPP and 1 mM NADPH.**

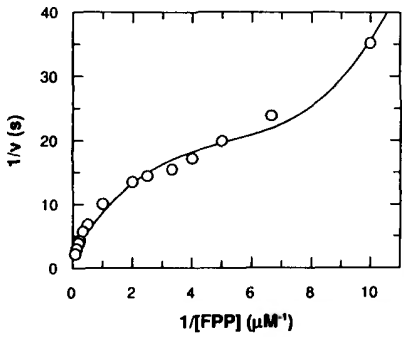


FIG. 3. **A double-reciprocal analysis of the variation in initial velocity for PSPP formation with FPP concentration in the absence of NADPH.**

Fig. 1 shows a time course for formation of squalene from 0.1 and 5 μM [1-^{3}H]FPP in the presence of 1 mM NADPH. The progress curves were linear during the first 10% of the reaction. At higher conversions, the rate of squalene synthesis decreased sharply. All kinetic runs were conducted for 2 min under conditions where the reaction was in the linear region of the progress curve. Initial velocities were linear with the concentration of squalene synthase over the 20-fold range used in the study (see Fig. 2). The pH dependence of initial velocities for the enzyme in buffer containing 1 mM NADPH was studied from 0.1 to 10 μM FPP. The results were similar at all FPP concentrations. A broad plateau was seen between pH 6.5 and 7.5, with a sharp drop at higher pH corresponding to deprotonation of an essential residue with a pK_a ~ 8 or denaturation of the enzyme (data not shown).

Aggregation of FPP

In order to measure the ability of FPP to aggregate in our buffer system (50 mM Tricine, 10 mM $MgCl_2$, 10% (v/v) methanol, 10% (v/v) glycerol, and 2% (v/v) Tween-80, pH 7.4) at concentrations between 1 μM and 1 mM, [1-^{3}H]FPP was incubated in assay buffer at room temperature for 2 h. The mixture was applied to a 1-ml Sephadex G-15 spun column equilibrated with the same buffer, and the radioactivity of FPP eluting in the void volume was used to estimate the percentage of the molecule in higher molecular weight aggregates (data not shown). Less than 10% of the total radioactivity eluted in the void volume at FPP concentrations below 50 μM. Above this concentration, there was an increase in the void volume radioactivity that rose sharply to 60% of the total. These results indicate that there was no substantial change in the state of aggregation of the substrate over the range of concentrations used in our kinetic studies and that most of the FPP was not localized in detergent micelles under these conditions.

Initial Velocity Studies

PSPP Synthesis in the Absence of NADPH—The initial velocity (v) for PSPP synthesis was measured at different FPP concentrations in the absence of NADPH by the tritium release assay. A plot of v^{-1} *versus* [FPP]$^{-1}$ is shown in Fig. 3. Substrate activation of the enzyme reaction is clearly apparent at FPP concentrations greater than 0.5 μM, while below this concentration, the double-reciprocal plot appears to be parabolic. Such behavior is consistent with the observations that PSPP is a tight binding substrate for yeast squalene synthase (12), and the rate of its formation from FPP in the absence of NADPH may be limited by its release from the enzyme. The increase in velocity observed here at higher FPP concentrations might result from an increase in competition for the enzyme by FPP.

PSPP and Squalene Synthesis with 1 mM NADPH—Initial velocities (v) for synthesis of PSPP and squalene were measured simultaneously using the proton-release (PSPP) and TLC (squalene) assays. Plots of v *versus* FPP concentration for PSPP and squalene are shown in Fig. 4*a*. The rate of squalene synthesis was slightly less than for PSPP synthesis. However, this difference could result from a systematic error in correlating the assays for PSPP and squalene. Within the error of our experiments, both compounds were formed at essentially identical rates in buffer containing 1 mM NADPH where 0.1 μM ≤ [FPP] ≤ 10 μM. Similar behavior was reported by Agnew and Popjak (13) for FPP concentrations below 27 μM. These results indicated that synthesis of PSPP was rate-limiting.

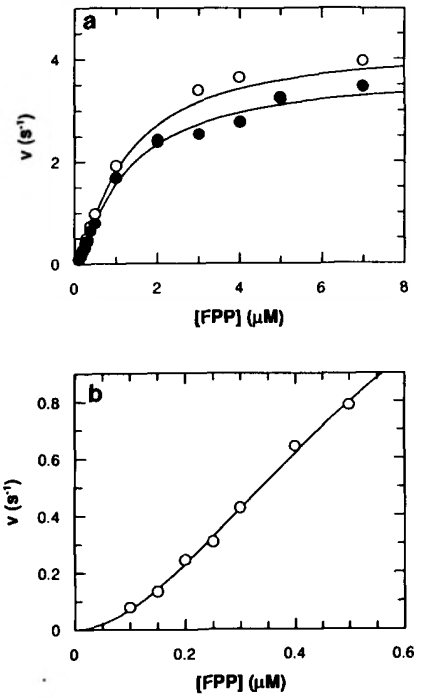
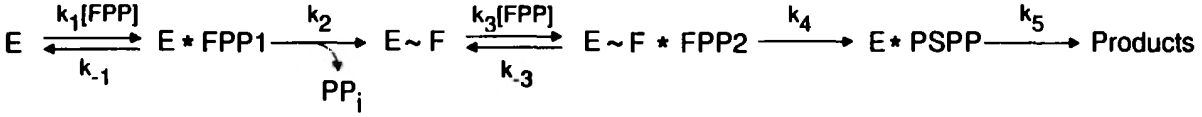


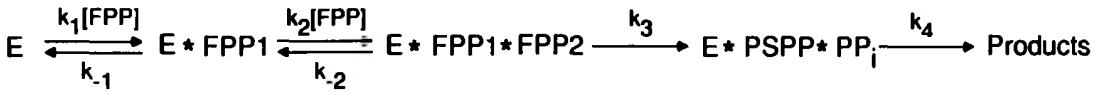
FIG. 4. **Initial velocities for formation of PSPP (○) and squalene (●) *versus* FPP concentration in buffer containing 1 mM NADPH (*a*); replot of data for squalene at low FPP concentrations (*b*).** Assays were carried out in duplicate.

In an earlier kinetic study, Beytia *et al.* (12) reported linear double-reciprocal plots of v_i for PSPP synthesis *versus* [FPP] for 1 μM ≤ [FPP] ≤ 4 μM at different fixed concentrations of NADPH and concluded that PSPP was formed by a ping-pong mechanism of the form shown in Scheme 2. The rate expression for the ping-pong mechanism is given in Equation 1, where $B = K_M^{FPP1} + K_M^{FPP2}$.

$$v = \frac{V_{max}[FPP]}{B + [FPP]} \quad \text{(Eq. 1)}$$



SCHEME 2. **Ping-pong mechanism for addition of FPP.**



SCHEME 3. **Ordered sequential mechanism for addition of FPP.**

K_M^{FPP1} and K_M^{FPP2} are Michaelis constants for addition of the first and second molecules of FPP, respectively, and V_{max} is the maximal velocity. Equation 1 is hyperbolic, and a double-reciprocal plot of v *versus* $[FPP]^{-1}$ is predicted to be linear, as illustrated by Equation 2.

$$v^{-1} = \left(\frac{B}{[FPP]} + 1\right)V_{max}^{-1} \qquad \text{(Eq. 2)}$$

Subsequently, Agnew and Popjak (13) extended the range of FPP concentrations to 0.8 μM ≤ [FPP] ≤ 12 μM and also found linear double-reciprocal plots for synthesis of both PSPP and squalene at different fixed concentrations of NADPH. However, they questioned the ping-pong mechanism proposed by Beytia *et al.* (12) and pointed out that double-reciprocal plots of initial velocities *versus* FPP concentrations may appear to be linear for a sequential mechanism for certain combinations of substrate concentrations and kinetic constants.

If one assumes an ordered addition of FPP as outlined in Scheme 3, the initial velocity equation for synthesis of PSPP is

$$v = \frac{V_{max}[FPP]^2}{A + B[FPP] + [FPP]^2} \qquad \text{(Eq. 3)}$$

where $V_{max} = k_3k_4[E_T]/(k_3 + k_4)$, $A = k_{-1}(k_{-2} + k_3)/k_1k_2k_3$, and $B = (1/k_1 + (k_{-2} + k_3)/k_2k_3)$. $A = K_D^{FPP1}K_M^{FPP2}$ and $B = K_M^{FPP1} + K_M^{FPP2}$, where K_D^{FPP1} is the dissociation constant for the $E{\cdot}$FPP complex and K_M^{FPP1} and K_M^{FPP2} are Michaelis constants. A similar dependence on FPP concentration is predicted for a random sequential mechanism, except that the term for K_D^{FPP1} is more complex. The double-reciprocal form of Equation 3 describes a parabola.

$$v^{-1} = \left(\frac{A}{[FPP]^2} + \frac{B}{[FPP]} + 1\right)V_{max}^{-1} \qquad \text{(Eq. 4)}$$

We extended the range of previously reported FPP concentrations to 0.1 μM ≤ [FPP] ≤ 10 μM for the initial velocity measurements shown in Fig. 4*a*. The data gave an excellent fit to Equation 3, including at low concentrations of FPP where a sigmoidal dependence was seen for squalene synthesis (see Fig. 4*b*). Similar behavior was seen for PSPP synthesis (data not shown). A double-reciprocal plot of the data for PSPP synthesis had substantial curvature (Fig. 5) as predicted by Equation 4 for a sequential addition of FPP.

PSPP and Squalene Synthesis at Different Concentrations of NADPH—Normal and double-reciprocal plots of v *versus* [FPP] for synthesis of PSPP and squalene at different fixed concentrations of NADPH are given in Figs. 6 and 7. Although the appearance of the plots was similar to those obtained at 1 mM NADPH, v increased with increasing concentrations of NADPH. The dependence of v for PSPP synthesis on NADPH concentration suggested that the cofactor enhanced v without changing the mechanism for addition of FPP. Plots of v^{-1} *versus* $[NADPH]^{-1}$ at different fixed FPP concentrations (see Fig. 8) gave a family of lines with similar slopes and different y intercepts, suggesting that the cofactor added after an irreversible

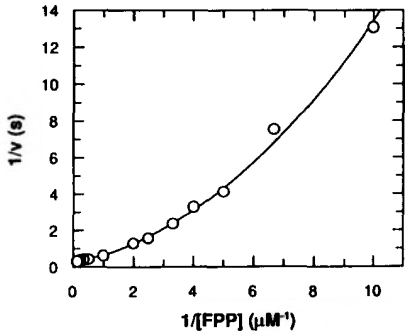


FIG. 5. **A double-reciprocal analysis of the data shown in Fig. 4 for synthesis of PSPP.**

step. This behavior is predicted by the mechanism shown in Scheme 4, where NADPH activates the reaction by generating a branch for regeneration of free enzyme that is faster than the dissociation of PSPP. The normal and reciprocal forms of the rate equation are given by Equations 5 and 6.

$$v = \frac{V'_{max}[FPP]^2}{A' + B'[FPP] + [FPP]^2} \qquad \text{(Eq. 5)}$$

$$v^{-1} = \left(\frac{A'}{[FPP]^2} + \frac{B'}{[FPP]} + 1\right)(V'_{max})^{-1} \qquad \text{(Eq. 6)}$$

V'_{max}, A', and B' are apparent constants that vary with the concentration of NADPH, where

$$C = \frac{k_4(k_{-5} + k_6) + k_5k_6[NADPH]}{(k_{-5} + k_6) + k_5[NADPH]} \qquad \text{(Eq. 7)}$$

and

$$A' = A\left(\frac{k_3C}{k_3 + C}\right) \qquad \text{(Eq. 8)}$$

$$B' = B\left(\frac{k_3C}{k_3 + C}\right) \qquad \text{(Eq. 9)}$$

$$V'_{max} = [E_T]\left(\frac{k_3C}{k_3 + C}\right) \qquad \text{(Eq. 10)}$$

Thus, Equation 6 becomes

$$\frac{[E_T]}{v} = \frac{A}{[FPP]^2} + \frac{B}{[FPP]} + \frac{k_3 + C}{k_3C} \qquad \text{(Eq. 11)}$$

For varied [FPP] at different fixed [NADPH], Equation 11 predicts a family of parallel parabolas, as seen in Fig. 7. The apparent maximal velocity is given by $k_3C/(k_3 + C)$, where

$$V'_{max} = \frac{k_3(k_4(k_{-5} + k_6) + k_5k_6[NADPH])}{(k_3 + k_4)(k_{-5} + k_6) + k_5(k_3 + k_6)[NADPH]} \qquad \text{(Eq. 12)}$$

For the case where (*a*) product release is rate limiting, (*b*)

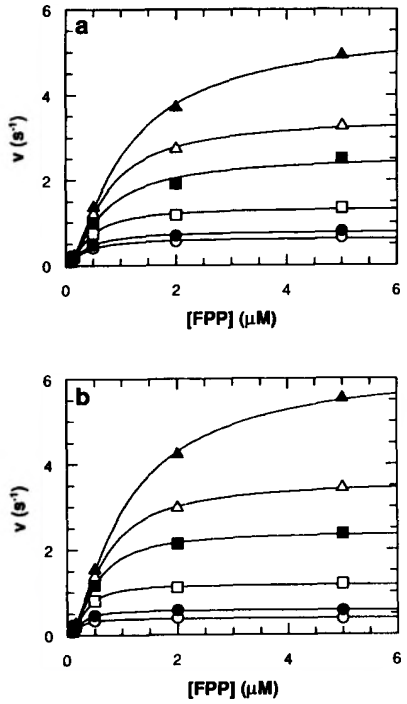


FIG. 6. **Variation of initial velocity for the synthesis of PSPP (*a*) and squalene (*b*) with FPP concentration at different fixed concentrations of NADPH: ○, 7 μM; ●, 10 μM; □, 20 μM; ■, 50 μM; △, 100 μM; ▲, 300 μM.**

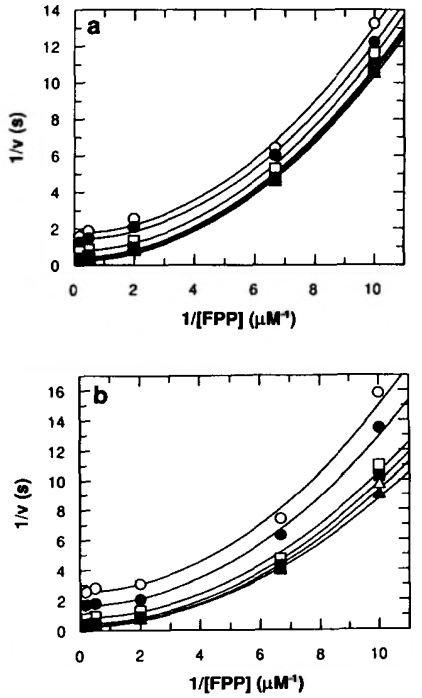


FIG. 7. **A double-reciprocal analysis of the data shown in Fig. 6 for synthesis of PSPP (*a*) and squalene (*b*).**

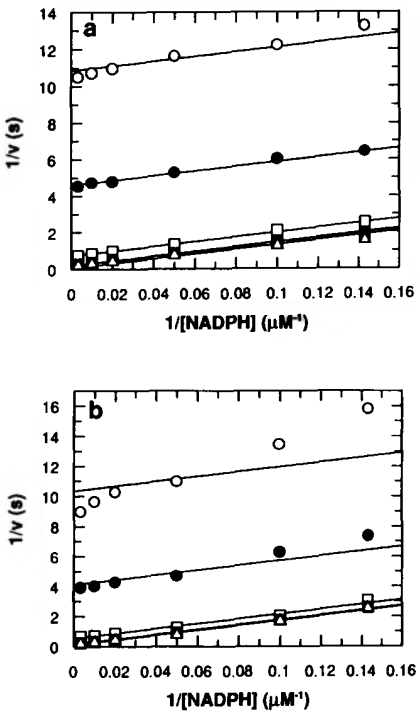


FIG. 8. **A double-reciprocal analysis of initial velocities for synthesis of PSPP (*a*) and squalene (*b*) *versus* NADPH concentration at different fixed concentrations of FPP: ○, 0.1 μM; ●, 0.15 μM; □, 0.5 μM; ■, 2 μM; △, 5 μM.**

NADPH substantially stimulates this step, and (*c*) the dissociation constant of the *E*·PSPP·NADPH complex is small, $k_4 << k_3$ and k_6, and $k_{-5} << k_5$. At NADPH concentrations where k_5[NADPH] >> k_4, Equation 12 becomes

$$V_{max} = \frac{k_3 C}{k_3 + C} = \frac{k_3 k_5 k_6 [\text{NADPH}]}{k_3(k_{-5} + k_6) + k_5(k_3 + k_6)[\text{NADPH}]} \quad \text{(Eq. 13)}$$

and substitution into Equation 11 gives

$$\frac{[E_T]}{v} = \left(\frac{k_{-5} + k_6}{k_5 k_6}\right)\frac{1}{[\text{NADPH}]} + \left(\frac{A}{[\text{FPP}]^2} + \frac{B}{[\text{FPP}]} + \frac{k_3 + k_6}{k_3 k_6}\right) \quad \text{(Eq. 14)}$$

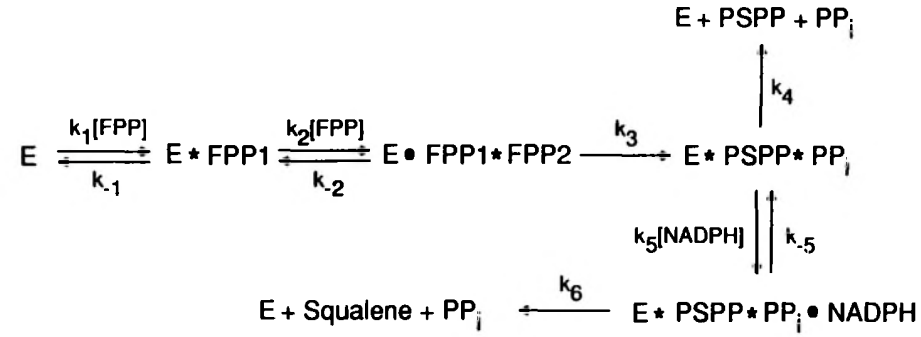
Equation 14 predicts a family of parallel lines for plots of v^{-1} *versus* [NADPH]$^{-1}$.

Replots of *y* intercepts *versus* [NADPH]$^{-1}$ for the theoretical curves in Fig. 7 are linear (see Fig. 9) in accord with Equation 11 with $K_M^{\text{NADPH}} \simeq 180$ μM. In a similar manner, replots of the *y* intercepts in Fig. 8 *versus* [FPP]$^{-1}$ are parabolic (see Fig. 10), as predicted by Equation 14. The magnitude of the stimulation by NADPH was determined from a plot of V'_{max} *versus* [NADPH] for PSPP synthesis. At the limits, when [NADPH] → 0, $V^0_{max} \sim (k_3 k_4/(k_3 + k_4))[E_T]$ and $V^0_{max} \sim 0.2$ s^{-1}. In a similar manner, when [NADPH] → ∞, $V^\infty_{max} \sim (k_3 k_6/(k_3 + k_6))[E_T]$ and $V^\infty_{max} \sim 7.4$ s^{-1}. This difference must reflect the even larger difference between k_4 and k_6, where k_4 is much less than k_6. Thus, squalene synthase is stimulated approximately 40-fold by NADPH.

Values for *A* and *B* can be estimated by dividing *A'* and *B'* by V'_{max} for a given concentration of NADPH. If one neglects the data at low [NADPH] where the errors are larger for *B'*, $A^{\text{PSPP}} \sim 0.081$ μM^2, $B^{\text{PSPP}} \sim 0.12$ μM, $A^{\text{SQ}} \sim 0.077$ μM^2, and $B^{\text{SQ}} \sim 0.10$ μM (where SQ is squalene). Unfortunately, it was not possible to resolve the complex constants *A* and *B* given in Table I in terms of K_D^{FPP1}, K_M^{FPP1}, and K_M^{FPP2}. However, since $A = K_D^{\text{FPP1}} K_M^{\text{FPP2}}$ and $B = K_M^{\text{FPP1}} + K_M^{\text{FPP2}}$, K_M^{FPP1} and K_M^{FPP2} are less than ~0.1 μM and K_D^{FPP1} is greater than ~0.8 μM.

DISCUSSION

Squalene synthase catalyzes the first pathway-specific steps in the cholesterol biosynthetic pathway, where the synthesis of



SCHEME 4. **A mechanism for activation by NADPH.**

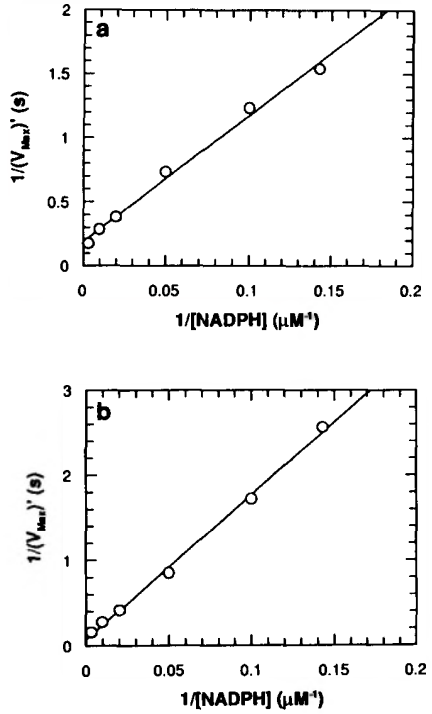


FIG. 9. **A double-reciprocal replot of the *V* intercept from Fig. 7 for synthesis of PSPP (*a*) and squalene (*b*) *versus* NADPH concentration.**

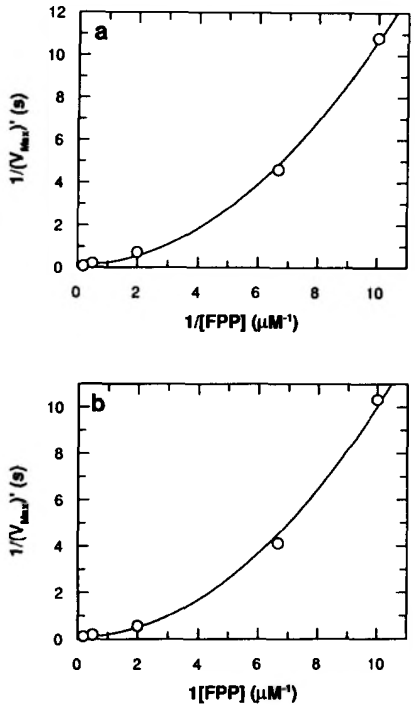


FIG. 10. **A double-reciprocal replot of the *V* intercept from Fig. 8 for synthesis of PSPP (*a*) and squalene (*b*) *versus* FPP concentration.**

squalene from FPP marks a transition from hydrophilic to hydrophobic intermediates. Although the enzyme was discovered over 30 years ago, it is a microsomal protein and progress on its purification and characterization has been painfully slow. Only recently have substantial quantities of a soluble variant of the recombinant protein become available for study (21). Although this enzyme had kinetic properties similar to those of the microsomal wild-type protein, its activity is stimulated in the presence of detergents, which presumably facilitate dissociation of the hydrophobic products from the *E*-product complex (21).

From our kinetic studies, it is unclear if both reactions occur at a single catalytic site or if there are distinct sites for synthesis of PSPP and squalene. However, the parabolic nature of the double-reciprocal plots in Figs. 5 and 7 clearly demonstrates the sequential addition of the two FPP molecules. A linear plot would result for a ping-pong mechanism (Scheme 2) or if the binding of the first FPP molecule were the rate-limiting step. While the latter explanation is possible for a membrane-bound form of the enzyme and water-soluble FPP, the parabolic behavior observed in our studies precludes a ping-pong pathway where the addition of the FPP molecules is separated by an irreversible step, presumably the dissociation of pyrophosphate after the formation of an enzyme-farnesyl intermediate. The reversible formation of a farnesyl cation-pyrophosphate anion ion pair before the addition of the second FPP cannot be rigorously ruled out. However, addition of a second FPP to an enzyme-stabilized ion-pair complex is unlikely given the short lifetime of this highly reactive species (25). Other reasons for not observing parabolic behavior in the earlier studies may be due to the unavailability of highly active soluble enzyme and/or the limited range of FPP concentrations employed. We circumvented these difficulties by using a homogeneous soluble enzyme and by employing FPP concentrations that covered over two orders of magnitude. The parabolic kinetic behavior also suggests that the two molecules of FPP could be bound with substantially different affinities to two distinct regions of the catalytic site, represented by FPP1 and FPP2. This observation is consistent with product studies by Ortiz de Montellano and co-workers (26, 27) using substrate analogs of FPP. They discovered that while the analogs inhibited the formation of squalene from FPP, they only served as alternate substrates when coincubated with FPP. Labeling experiments indicated that these compounds were only incorpo-

TABLE I
Kinetic constants for synthesis of PSPP and squalene

Product	[NADPH]	A'	B'	V'_{max}
	μM	μM^2	μM	s^{-1}
PSPP	7	0.04 ± 0.02	0.2 ± 0.1	0.6 ± 0.04
	10	0.06 ± 0.01	0.2 ± 0.1	0.8 ± 0.03
	20	0.12 ± 0.02	0.2 ± 0.1	1.4 ± 0.05
	50	0.19 ± 0.06	0.4 ± 0.3	2.6 ± 0.03
	100	0.30 ± 0.03	0.4 ± 0.1	3.5 ± 0.1
	300	0.44 ± 0.05	0.8 ± 0.2	5.7 ± 0.3
SQ	7	0.04 ± 0.01	0.05 ± 0.05	0.4 ± 0.01
	10	0.06 ± 0.01	0.05 ± 0.05	0.6 ± 0.01
	20	0.10 ± 0.01	0.1 ± 0.05	1.2 ± 0.04
	50	0.20 ± 0.02	0.14 ± 0.1	2.4 ± 0.07
	100	0.28 ± 0.02	0.3 ± 0.1	3.7 ± 0.1
	300	0.43 ± 0.04	0.8 ± 0.1	6.5 ± 0.2

rated into product when the $E{\cdot}S{\cdot}A$ complex had an analog (A) in the acceptor region and FPP (S) in the donor region of the catalytic site (26). Thus, the transferred residue was selectively provided by FPP.

In the absence of NADPH, the formation of PSPP as a function of FPP concentration shows complex kinetics. At low FPP concentrations, the double-reciprocal plot is parabolic, indicating a sequential mechanism. Thus, the mechanism in the absence and presence of the cofactor is essentially the same. At FPP concentrations greater than ~0.5 μM, there is apparent activation of the reaction. Since PSPP, the product of this reaction, is a substrate for the subsequent enzyme catalyzed rearrangement to squalene, it is likely that its dissociation is slow. A slow dissociation rate is consistent with the earlier (13) and current observation that there is a 1:1 stoichiometry in the rates of PSPP and squalene formation with no net accumulation of the former in the presence of NADPH. The stoichiometry would favor PSPP if either the reduction of PSPP to squalene was slower than the dissociation rate, or if there were a substrate-facilitated dissociation of PSPP. The former mechanism is observed at low NADPH concentrations (<10 μM), where there is a significant excess of PSPP over squalene. The latter mechanism could explain the build up of PSPP observed by Agnew and Popjak (13) at FPP concentrations >27 μM, even in the presence of cofactor. A substrate-facilitated dissociation of PSPP could occur through the formation of an $E{\cdot}PSPP{\cdot}FPP$ complex in which FPP competes for a subsite on the enzyme that also binds a prenyl group of PSPP. A similar mechanism has been reported by Cleland and co-workers (28) for aconitase, in which the dissociation of a tricarboxylate product is facilitated by the binding of the tricarboxylate substrate. Thus, the substrate activation suggests that PSPP and FPP share common interactions with the enzyme, supporting the idea of a single or considerably overlapped active site for the two half-reactions proposed earlier through different approaches (15).

Several groups have reported the stimulation of the rate of PSPP synthesis by NADPH (13, 21). At 1 mM NADPH, our data are consistent with the sequential addition of two FPP molecules before the release of a product. Since the rates of PSPP and squalene formation are essentially identical, the rate-limiting step appears to be before the reduction of the intermediate to the hydrocarbon. We considered several mechanisms for the activation of PSPP synthesis by NADPH. The one shown in Scheme 4, where NADPH adds after the irreversible formation of PSPP, is consistent with our data. Other mechanisms, where NADPH added before the irreversible step, predicted intersecting double-reciprocal plots. If the rate of PSPP dissociation in the absence of NADPH is rate limiting, then the reduction of the intermediate to squalene, which may dissociate more rapidly, would increase the rate of PSPP formation. Thus, alleviation of PSPP inhibition appears to be the mechanism for NADPH activation. This conclusion is in sharp contrast to that drawn by Agnew and Popjak (13), who observed different V_{max} values for PSPP formation in the absence and presence of NADPH. Careful examination of our double-reciprocal plots for PSPP formation at higher FPP concentrations shows that the extrapolated maximum velocities in the presence and absence of NADPH are very similar in agreement with the proposal that activation by NADPH results from reduction of PSPP to squalene.

We are now investigating a number of issues concerning the mechanism of squalene synthase. These include the binding order of the donor and acceptor FPP molecules and the preferred order of addition of PSPP and NADPH in the synthesis of squalene from PSPP. The mode of binding of several potent inhibitors (29, 30) is also being assessed in order to define the FPP-binding sites of the enzyme. The availability of a soluble form of the enzyme and the delineation of the kinetic mechanism should greatly facilitate such studies.

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